Douglas T. Carrell · Peter N. Schlegel
Catherine Racowsky · Luca Gianaroli
Editors

Biennial Review of Infertility

Volume 4
2015

Springer

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ISBN 978-3-319-17848-6 ISBN 978-3-319-17849-3 (eBook)
DOI 10.1007/978-3-319-17849-3

Library of Congress Control Number: 2015942509

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

We dedicate this volume of Biennial Review of Infertility to those who receive far too little credit for their essential roles in assisting infertile patients and clinicians, including nurses, medical assistants, embryologists, andrologists, and office personnel. Your compassion, dedication, and skills make all the difference.

Preface

This is the fourth volume of Biennial Review of Infertility (BRI). The objective of this series is to bring to clinicians, embryologists, andrologists, scientists, laboratory technicians, and ancillary healthcare providers of infertile patients a timely collection of topics that are cutting edge and written by thought leaders in the field of infertility care and research. While that goal is daunting, the previous volumes of Biennial Review of Infertility have succeeded and been valuable to the community. We are excited to continue with volume 4 of Biennial Review of Infertility.

Volume 4 continues the tradition of providing reviews and commentaries on the cutting edge of male reproductive medicine, female reproductive medicine, and the field of assisted reproduction technologies (ART). Chapters included in this volume of BRI cover topics such as the use of stem cell technologies in male infertility therapy, molecular mechanisms and causes of reduced oocyte quality associated with aging, the use of time-lapse imaging in the ART laboratory, a critical review of the use of acupuncture during ART, and the use of the Internet in clinical practice to benefit patients and the clinic. That is certainly a diverse range of chapters, which highlights the breadth of topics that the clinician encounters in daily practice. As always, those selected to present the topics are unquestionably leaders in the field and have provided clear and thought-provoking reviews.

While science sometimes moves forward in incremental leaps, often it is the gradual addition of data that provides a slower path forward. For that reason, as well as the complexity of the problems studied, best clinical practice can sometimes be gray and meandering, with thoughtful and honest clinicians disagreeing about the best therapy for a given problem. For that reason, we continue with the "Controversies" section of BRI in this volume. This section is likely the most popular section of the series, since it provides clear and distinctly different conclusions from well-respected thought leaders. In BRI4, Eli Adashi and Dmitry Kissin argue that elective single embryo transfer should be the standard of care for ART patients, while G. David Adamson presents the arguments for the alternative conclusion. Second, Darius Paduch and Peter Schlegel debate the pros and cons of early treatment of the adolescent patient with Klinefelter Syndrome. Lastly, the controversial topic of the use of dietary supplements in the ART patient is critically reviewed.

We are grateful for the willingness of the authors to share their expertise. In order to get fresh and cutting-edge information, Biennial Review of Infertility operates on a tighter deadline schedule than most books. The authors' efforts

are appreciated. We, as co-editors, are also grateful to the readers of BRI4 for your continued desire to understand complex topics and to use our volume to assist in providing the best care possible to patients. We are confident that this volume of Biennial Review of Infertility will also help stimulate new ideas and perspectives for future clinical and basic science studies.

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Part I

Male

The Epidemiology of Male Infertility

1

John R. Gannon and Thomas J. Walsh

1.1 Identifying Infertility

Infertility is defined as the inability for a healthy couple to conceive after 12 months of regular, unprotected intercourse [1]. Infertility affects approximately 20 % of couples and has been recognized as a disease according to the Americans with Disabilities Act of 1998 [2]. This distinction and recognition as a disease may improve awareness and require that infertility be identified, registered, and treated.

Several characteristics make the epidemiology of infertility challenging to study. Male infertility is not a reportable disease and is therefore not identified or captured in databases such as the Surveillance, Epidemiology, and End Results [SEER] Program that tracks new cases of cancer. Male infertility is diagnosed and treated in the outpatient clinical setting; therefore, limited numbers of cases are captured through hospital admission and billing codes. Fertility care is often not covered by health insurance, and out-of-pocket costs are not identified in the claims data of most large insurance consortiums. Additionally male factor infertility is frequently treated empirically with in vitro fertilization. This focus on the female partner further limits the identification of male factor infertility cases.

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The only existing large-scale database with the goal of identification of male factor infertility is provided by the Center for Disease Control and Prevention. The SART database tracks the utilization of assisted reproductive technology [ART]. While useful, such data has historically provided very limited details regarding the causes of male infertility [3].

The National Survey of Family Growth (NSFG) provides some population-based data on male factor infertility. The purpose of the survey is to obtain national estimates of pregnancy, infertility, contraception, marriage, divorce, and further information. This periodic survey initially was limited to women during the 1970s and 1980s, but starting with the sixth cycle in 2002, men were included. The use of infertility services was identified by one question, "Have you been to a doctor to talk about ways to help have a baby together" [4, 5]? A study published in 2013, examining data from the NSFG, revealed that 18–27 % of men in couples experiencing infertility were not evaluated for male factors [4]. An additional study by Hotaling et al. published in 2012 examined data from the NSFG cycle 6; this study found that marital status and education level were associated with those who sought care at a fertility center [5]. These studies suggest that the rate of male infertility may be underestimated, and studies that report data on male infertility from large, tertiary, referral centers may not be generalizable to all infertile men (Table 1.1).

D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_1

Table 1.1 Population-based studies describing infertility

Study title	Author	Year	Population	Couple infertility (%)	Male factor (%)	Female factor (%)
Estimation of the prevalence and causes of infertility in Western Serbia	Philippov et al. [8]	1998	2,000 married women; 186 couples	38.70	6.40	52.70
High prevalence of male infertility in Southeastern Nigeria	Ikechebelu et al. [9]	2003	314 couples	25.80	42.40	20.70
Clinical patterns and major causes of infertility in Mongolia	Bayasgalan et al. [10]	2004	430 couples	18.80	25.60	45.80
Incidence and main causes of infertility in a resident population (1,850,00) of three French regions	Thonneau et al. [6]	1991	1,686 couples	30	20	39

1.2 Incidence and Prevalence of Male Infertility

Male infertility may be caused by numerous factors including genetic causes, poor semen quality, medical disease, hormonal aberrations, or other unknown causes [2].

Several studies have sought to quantify the burden of male factor infertility [6–9]. Findings from these studies are difficult to interpret due to methodological flaws, and their generalizability is limited due to the specificity of the populations studied; however, they provide some information on the epidemiology of male infertility.

A study published in 1998 in Western Siberia, conducted on 2,000 married women using the World Health Organization questionnaire, found the prevalence of couple infertility to be 16.7 %. The prevalence of male infertility was 6.4 %, with an unusually high rate of female factor infertility (nearly 53 %). The rate of female factor infertility was determined to be secondary to high postpartum complications and abortions [8].

A retrospective review conducted on 314 couples evaluated from 1997 to 1998 in Southeastern Nigeria suggested an unusually high rate of male infertility at 42.4 %. High rates of male infertility in this study were attributed to sexually transmitted disease and inadequate treatment for these conditions [8, 9].

A study conducted by Thonneau et al., "Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989)," examined three defined geographic regions in France. This study revealed male factor infertility in 20 % of the 1,686 couples studied in a specific French region in 1991. Despite the title of Thonneau's study suggesting *incidence*, the authors in fact quantified the *prevalence* of male factor infertility in this study population given that this study was conducted at a single point in time (cross-sectional study design) [6].

A retrospective study conducted in Mongolia and published in 2004 found male factor accounted for 25.6 % of all infertility. This study, like the prior study from 2000 in Western Siberia, showed a high prevalence of female factor infertility, 46 %. Within this specific study, this high rate was felt to be secondary to pelvic inflammatory disease [10].

Many of these studies claim to describe prevalence and incidence but instead report case series data, without a firm understanding of the base population from which the cases arise [11]. None of these studies clearly define the incidence of male infertility given that they fail to report on new cases within a specified time period. These studies were conducted in different geographic regions, and the dramatic differences in the statistics they report underscore the importance of geographic variation and the inability to generalize their findings to other populations. To further add to the differences among these studies and their limitations, the urologic disease project in America in 2007 sought to consolidate the available literature in an attempt to understand the burden of disease. The authors of this study searched multiple databases

including SART, the national survey of ambulatory surgery, VA administration, and others. Ultimately they concluded that male factor infertility might be present in 30 % of all infertility cases, accounting for methodology and selection bias in these prior studies [3, 12].

1.3 Secular and Birth Cohort Trends

Two methods used to describe a disease, such as male infertility, are secular and birth cohort trends. Secular trends describe the change in incidence of a disease over time. Secular trends have been used in studies of fertility to inform how changes in environmental exposures have altered the incidence of infertility within a given population [6–9]. Birth cohort trends describe changes in a disease associated with the generation in which an individual is born. Birth cohorts may be used to compare differences in a disease between generations of individuals. Secular and birth cohorts have been associated with differences in birth rates, semen analyses, and fecundity [1, 13, 14].

Several studies have sought to use birth cohort trends as a means of understanding whether or not infertility rates have changed over time in response to the ever-changing environment in which we live [14]. While there is no data that effectively identifies the incidence of male infertility related to the generation in which a patient was born, data does exist pertaining to semen analyses. A semen analysis is an easily replicated test, which some studies have sought to use as a proxy for male infertility. While there is no direct correlation between a normal semen analysis and fertility, this information is valuable in the assessment of the epidemiology of male factor infertility [14].

In 1992 Carlsen et al. made one of the first attempts to quantify changes in semen analyses in men, examining 61 studies, with nearly 15,000 men and their respective semen analyses from 1938 to 1991. Carlsen's linear regression analysis showed a significant decrease in sperm concentration from a mean of 113 million/mL to a mean of 66 million/mL and a decrease in semen volume from 3.4 mL to 2.75 mL [13]. From this, the authors concluded that semen quality was declining over time.

Fisch, when reviewing these studies, suggested significant flaws with Carlsen's initial analysis. Fisch evaluated the same 61 studies; however, they limited their analysis to larger studies with greater than 100 semen samples. Their review found trends related to geographic variations, with lower sperm counts observed in later years in more developed countries, when compared to earlier specimens [15]. Further examination of the initial 61 studies found additional study limitations such as variable methods of semen collection and no controls for confounding variables such as drug use, smoking, or abstinence [16].

In a repeat analysis of Carlsen's data, Swan and other investigators in 1997, controlled for abstinence, age, collection method, and men with proven fertility. These authors' findings demonstrated significant declines in sperm concentration in the USA, Europe, and Australia [17]. Fisch and Braun conducted a systemic review of 35 semen analysis studies. A total of eight studies accounting for 18,109 men suggested a decline in semen quality and quantity. Twenty-one of the studies examined, with 112,386 men, appeared to show no significant change in semen quality. Six studies, 26,007 men, showed results that were not interpretable due to conflicting or ambiguous results [16].

While we may continue to examine semen analyses using our epidemiologic methods, there is no clear evidence a decline in semen quality exists.

1.4 Geographic Variation

While geography alone is unlikely to put men at risk for infertility, it may be a surrogate marker for other exposures or cultural differences that increase risk. As such, geography is considered a key variable to enable better understanding of disease. Capturing geographic variation in the diagnosis and treatment of male infertility is challenging. There are a limited number of fertility centers, which are often clustered in metropolitan areas, thereby limiting access to care for those from rural areas. The geographic constraints

limit our insights by limiting patient's access to identification, evaluation, and treatment.

In spite of these limitations, several studies corroborate the extent of geographic variation in male fertility. The most recent review of the National Survey of Ambulatory Surgery in 2009 revealed that there was an increased prevalence of outpatient infertility evaluations conducted in the Northeast. The South, Midwest, and finally the West followed the Northeast in the prevalence of outpatient evaluations respectively [18]. These differences may be attributed to nationwide insurance trends as well as increased access to infertility clinics in the Northeast.

In a study performed in 2003, Swan and colleagues examined semen analysis performed in four cities representing each geographic region previously mentioned: Northeast (New York, New York), Midwest (Minneapolis, Minn.), West (Los Angeles, Calif.), and South (Columbia, Miss.). Examining 512 samples, sperm parameters (concentration, motility) were reduced in the semirural and agricultural areas of Mississippi and Minnesota [18]. Additional reviews support these findings. Fisch and collaborators described differences in sperm concentrations between numerous countries, both industrialized and not [12, 14, 16, 17]. The lower semen parameters seen in rural areas were attributed to genetic and environmental factors, including sexually transmitted infections, pesticide use, environmental toxins, and other contributing factors.

1.5 Racial Variation

Studies examining racial variation in male factor infertility are sparse. The NSFG was reviewed in 2013, by Eisenberg, and demonstrated that Caucasian men are more likely to undergo infertility evaluation [4]. Conversely, data from the US Veterans administration suggests that Hispanics followed by African Americans and Caucasians have the highest frequency of undergoing treatment for male factor infertility [3]. The VA study however did not offer a clear cause of what appeared to be a discrepancy among those undergoing treatment.

Examining a large, diverse population of 1.5 million men older than 18 in the Kaiser Permanente of North California (KPNC) network in 2008, Walsh and colleagues described 30,000 men who underwent evaluation for infertility by semen analysis. Overall, 36 % of these men evaluated were found to have abnormalities in their semen analysis [19]. Interestingly, 49 % of African American men were found to have abnormalities on their specimen, while 37 % of Caucasian, 38 % of Asian 38 %, and 39 % of Hispanic men were found to abnormalities [19]. While this study does not inform whether or not race is etiologically involved in fertility or poor semen quality, it suggests that there is significant racial difference in the proportion of men with abnormal semen quality.

A 2001 study conducted by Costabile provided further insight into the association between race and male infertility. This study of a single provider, working in a no-cost, military healthcare system, described the age, race, length of subfertile period, medical history, and lab evaluations of men seeking fertility care. This study found no significant racial differences among men undergoing infertility evaluations [11].

1.6 Conclusions

Epidemiology describes the occurrence and impact of disease in a defined population. Understanding the epidemiology of any given disease may help to identify individuals who are at risk and enable or expedite the identification of causes or treatments. A better understanding of the burden of infertility will allow improvements in both the counseling and treatment of male factor and female factor infertility.

The epidemiology of male infertility is difficult to study. Male infertility is not a reportable disease and is not tracked by a dedicated database. The lack of insurance coverage and treatment in an outpatient setting leads to poor identification and tracking of men undergoing fertility care. Empiric treatment related to female infertility additionally may lead to an underestimation of male factor.

The incidence of male factor infertility has not been determined. The prevalence of male infertility has been estimated in heterogeneous studies that are limited and difficult to apply to the population as a whole. Further studies linking a decline in semen parameters and male infertility are inconsistent and often contradictory. Male fertility appears to be influenced by many factors including geography, race, and environment.

In spite of the challenges we face and the present lack of data, the future of male infertility epidemiologic research holds promise. The creation of a longitudinal cohort accruing all men with infertility will be pivotal as such data could include variables such as demographics, socioeconomic status, and quantification of putative environmental factors. The accumulation of such information will improve treatment and outcomes for men with male factor infertility.

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Vasectomy Reversal or IVF: Analysis of Factors for Success

2

Christopher M. Deibert and Jay I. Sandlow

2.1 Introduction

Men undergoing vasectomy should be counseled that it is a permanent method of sterility. However, multiple options remain available for couples that desire children after vasectomy. The man may undergo surgical reconstruction by either vasovasostomy or vasoepididymostomy. In vitro fertilization (IVF) also allows couples to have a biological child. Both techniques offer couples distinct routes to the same outcome, and the desire of the couple must be incorporated into the counseling. The provider should discuss with the couple any coexisting male and female fertility factors, the morbidity of all related procedures, and the direct and indirect costs of each, as well as the likelihood of achieving a live birth in their specific situation. Extensive counseling allows the couple to select the best available choice to them, commensurate with their fertility desires, financial situation, and personal views of the alternatives. Other options to discuss include donor insemination, embryo adoption, and traditional adoption. In this paper, we review these issues and discuss the treatment options currently available.

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2.2 Vasectomy Reversal for Obstructive Azoospermia

Vasectomy is currently the only permanent form of male contraception available. Nearly 500,000 men undergo vasectomy in the USA each year. Interestingly, up to 6 % of men have an element of regret after vasectomy, and 6 % of spouses regret the vasectomy decision [1, 2]. Two to six percent of these men will seek surgical reversal [3, 4].

Vasectomy reversal consists of surgically reanastomosing the vasal ends. Surgeons have several microscopic techniques to utilize, including standard one-layer, modified one-layer, and multilayer vasovasostomy (VV) anastomoses [5]. Outcomes for the techniques are similar, as long as microscopic magnification is utilized, suggesting that surgeons should use the technique with which they have the most comfort and skill [3, 6]. Most series examining just VV outcomes report patency and live birth rates of 85–98 % and 38–84 %, respectively [7].

At times VV cannot be completed secondary to epididymal obstruction. Over time, the increase in pressure from the obstructed testicular vas leads to epididymal rupture and subsequent epididymal closure at this juncture. In these cases, vasoepididymostomy (EV) must be used instead, whereby the epididymal obstruction is bypassed and the more proximal and patent epididymal tubule is connected directly to the vas. This decision is generally made intraoperatively;

D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_2

however, duration of obstruction is one factor that can predict need for the more complex EV [8, 9]. Importantly, compared to VV, patency and live birth rates are both lower following EV, 70–90 % and 32–56 % [10–12]. Overall, for both VV and EV combined, patency and live birth rates are about 86 % and 58 %, respectively.

Several factors are related to the success of vasectomy reversal. Female partner fecundity and age are critical to success [5, 13]. The duration of post-vasectomy obstruction also likely plays a role in success. Longer intervals increase the likelihood of needing an EV. The quality of sperm in the vas fluid, the nature of the fluid itself, and the length of vas segment between epididymis and vasectomy site also determine the type of reconstruction required [3, 8, 9, 14].

2.3 Sperm Retrieval and IVF

Men with obstructive azoospermia can also consider IVF with sperm retrieval (SR/IVF). Because sperm acquisition will not yield sufficient motile sperm for intrauterine insemination, these couples require IVF in conjunction with the sperm retrieval. IVF requires the use of exogenous gonadotropins to induce multiple follicle growth in the female partner. The mature follicles are then retrieved via aspiration using transvaginal ultrasound guidance. The mature oocytes are combined with the surgically retrieved sperm, usually in conjunction with intracytoplasmic sperm injection (ICSI). This is a process in which a single sperm is injected into a mature oocyte. If fertilization occurs, the resulting embryo is then cultured in special media. Subsequently, 1–2 embryos are then transferred back to the woman's uterus 3–5 days later. For these men, IVF with ICSI obtains superior results compared to IVF alone [15, 16].

The source of the sperm may be from the vas, epididymis, or testis, with the goal to retrieve as many viable sperm for either immediate use or cryopreservation for future conception attempts. For men with obstructive azoospermia, the source or method of sperm retrieval does not appear to affect the success of IVF [17, 18]. Using fresh or cryopreserved sperm appears to yield similar IVF outcomes, though this is still debated [18–20]. We therefore recommend that a trained surgeon use the retrieval technique of their choice, as discussed below.

Percutaneous epididymal sperm aspiration (PESA) involves percutaneous aspiration of the dilated epididymis under local anesthesia with a cord block and skin infiltration [21]. Advantages include short procedure time, can be done under local in the office, no need for special equipment or microsurgical training, and minimal recovery. However, disadvantages include limited quantity/quality of sperm retrieved, as well as the potential for creating epididymal obstruction. If inadequate sperm are retrieved, the patient can progress to any other available sperm acquisition technique.

Microsurgical epididymal sperm aspiration (MESA) is more invasive and complex than PESA. It requires microsurgical puncture or incision and then aspiration of an epididymal tubule from the proximal epididymis [22]. To access the tubule, the tunic overlying it must first be incised. Here, a high concentration of sperm is found (1 million/μl); thus only a small volume of aspirate is needed. Live birth rates following MESA range from 21 % to 100 % [23–26]. Advantages include the ability to retrieve high numbers of motile sperm adequate for cryopreservation. Disadvantages include greater cost (typically done in an operating room under general anesthesia), the need for an operating microscope and microsurgical training, and longer recovery time. Though an effective retrieval option, it is far costlier than other acquisition techniques.

Testicular sperm aspiration (TESA) is another less invasive option for sperm retrieval. Though Belker initially used TESA simply to diagnose azoospermia, it has since been used as a well-tolerated and effective means of sperm retrieval for men [27]. Sperm retrieval is optimized when the lab embryologist provides immediate aspirate assessment to determine when sufficient tissue has been aspirated. Pregnancy rates for men with obstructive azoospermia following TESA range from 12 % to 62 % and live births 32 % to 50 % [28–31]. Advantages include short procedure

time, the ability to perform under local anesthesia in the office, no need for special equipment or microsurgical training, and the ability to cryopreserve multiple specimens. The main disadvantage is that the outcome is tied directly to the experience and comfort of the embryologist to work with testicular sperm.

Testicular sperm extraction (TESE) is typically reserved for men with nonobstructive azoospermia. Though it can be utilized for obstructed men, the higher cost and more invasive nature as compared to TESA lead most surgeons to use TESA or PESA instead; however, there are some who utilize this method for obstructed men with good outcomes.

2.4 Evaluation of the Man Seeking Reversal

Elements of both the history and the physical examination determine successful reversal. During the consultation, the provider should determine the man's prior fertility status, how many prior pregnancies he helped create, the duration of vasectomy obstruction, and if the vasectomy was complicated either during the procedure or afterward. If a man seeks reversal for reasons other than fertility potential, this should be fully discussed. For instance, those with post-vasectomy pain syndrome should first be offered more conservative pain management tools. If pain persists, in selected men, reversal can provide effective pain relief [32]. Female factors must also be queried, as discussed in the next section. Though thorough evaluation of the male seeking vasectomy reversal is critical, the surgeon is remiss if female factors affecting her fecundity are not also elicited and incorporated into the individualized counseling.

Physical examination focuses on the testicular size; the location of vasal defects in the proximal, mid, or distal vas; the length of lost segment; and the presence or absence of a sperm granuloma. The epididymides are also palpated for fullness and induration, signs of potential epididymal obstruction that may necessitate vasoepididymostomy at the time of reversal.

Generally, no specific preoperative testing is required [5]. If general anesthesia will be used, consider basic blood work or EKG, though for a healthy male, this is typically unnecessary. Testing for antisperm antibodies is also unnecessary. Although up to 60 % of men may develop these antibodies following vasectomy, their presence does not impact reversal success [33–35].

2.5 Female Factors

As part of the male evaluation, the female partner must also be considered. Her fecundity, as determined by her age and any coexisting female factors, plays a prominent role in the counseling for the couple considering any microsurgical reconstruction or sperm retrieval procedure. If significant female factors exist, this may preclude successful pregnancy and live birth, even if reconstruction is technically successful for the male partner. These couples may consider sperm retrieval and IVF as a more direct means to their goal than male surgical reconstruction.

Female age specifically is strongly related to success after both vasectomy reversal and with IVF. Pregnancy rates for a couple following vasectomy reversal are closely related to the age of the female partner [36]. When the female partner is 39 or younger, pregnancy occurs in 56 %. When over age 40, this drops significantly to 14 % [37]. Reviewing the 2012 society for assisted reproductive technology (SART) IVF data, which does not explicitly describe the post-vasectomy obstructive male population, the impact of female age remains apparent for couples with male factor infertility. Live birth rate for women 40 and younger is 39.6 % and is only 13.5 % for women 41 or older (Table 2.1) [38].

For men seeking vasectomy reversal with a female partner over the age of 35, female evaluation may be considered. Ovarian reserve testing and/or referral to a reproductive endocrinologist can assist in defining female fecundity. Armed with this information, counseling can be more complete and tailored for the individual couple. It may help couples decide between vasectomy reversal, which requires a longer time horizon

Table 2.1 The Society of Assisted Reproductive Technology 2012 data for patients with male factor infertility undergoing assisted reproduction

Fresh embryos from non-donor oocytes					
Age	<35	35–37	38–40	41–42	>42
Number of cycles	9,697	3,802	2,409	784	277
Percentage of cycles resulting in pregnancies	50.5	41.9	34.5	27	10.5
Percentage of cycles resulting in live births	44.6	35.3	26.5	14.5	5.4
Reliability range	43.6–45.6	33.8–36.8	24.7–28.2	12.1–17.0	2.7–8.1
Percentage of retrievals resulting in live births	46.6	37.6	28.5	16.3	6.5
Percentage of transfers resulting in live births	49.7	40.4	31	17.9	7.2
Percentage of cycles with elective single embryo transfer	14.2	8.6	3.2	1.6	0
Percentage of cancellations	4.3	6	7.2	10.6	16.2
Implantation rate	38.9	29.2	20.1	11.1	3.4
Average number of embryos transferred	1.9	2	2.4	3	3.2
Percentage of live births with twins	29.5	26.5	20.1	17.5	0/15
Percentage of live births with triplets or more	1.2	0.4	1.1	1.8	0/15

Source: Reproduced with permission from SART [38]

for return of sufficient sperm to the ejaculate, or assisted reproductive technologies, which frequently provide a shorter timeframe to pregnancy. This may be especially beneficial when the female partner is older than 40.

2.6 Cost-Effectiveness

Fertility discussions and counseling with men and their partners must incorporate many pieces of information. This data includes the previously mentioned male history and physical examination, an evaluation of the fecundity of the female partner, the number of future offspring desired by the couple, and the timeframe desired. Above all, providers must counsel the couple toward the treatment with the highest likely success for the couple. However, the overall cost expenditures must also be part of this comprehensive discussion. A number of other researchers have examined the cost-effectiveness of these various fertility options.

An early cost-effectiveness analysis by Pavlovich and Schlegel included costs and expected results for ICSI/IVF and microsurgical reconstruction for men with post-vasectomy obstruction and a female partner under 40 years old. It also incorporated indirect costs including lost work productivity during the necessary evaluation and treatments and the cost of multiple gestation pregnancies. In their model, the expected live birth rate per ICSI/IVF cycle was 33 % and 47 % after vasectomy reversal. Estimated cost of delivery was $71,896 and $25,475 [39].

At the time of vasectomy reversal, epididymal obstruction is sometimes present. Kolettis demonstrated that vasoepididymostomy remains a cost-effective treatment option for couples with post-vasectomy azoospermia. The delivery rates used here were 36 % and 29 % for reversal and ICSI, respectively. The cost per delivery in their model was calculated to be $31,099 versus $51,024. Indirect costs were also included in this model, concluding that regardless of the obstructive duration, vasectomy reversal was the cost-effective option for couples [11].

In another analysis, decision modeling with associated probabilities of success, utility, and direct costs compared reversal and IVF/ICSI. Sperm retrieval was also used for men where EV was expected. Vasectomy reversal was found to be the more cost-effective option only if the expected post-reversal patency was greater than 79 %. Here, no indirect costs were included [40].

Markov modeling examines health decisions over time and permits sensitivity analyses where individual component features are altered to examine a wide range of possible health situations. Hsieh et al. applied this Markov model and found that female partner age had a significant effect on

the cost-effectiveness decision. IVF yielded a higher pregnancy rate but at a higher cost. At a cutoff for willingness to pay by the couple of up to $65,000, vasectomy reversal was more cost-effective over the entire female age group. If the couple was willing to pay more than this, then sperm retrieval for ICSI/IVF was more cost-effective over a greater female age range [41].

A further study including direct and indirect costs compared vasectomy reversal to TESE or MESA with ICSI/IVF. Outcome probabilities from the Society for Assisted Reproductive Technologies database were used. Cost analysis included procedure, complications, lost productivity, and multiple gestations. Cost data was sampled from high-volume IVF centers and the Medicare Resource-Based Relative Value Scale. In 2005 dollars, the reversal was $25,321 vs. $58,858 and $61,977 for TESE and MESA followed by ICSI/IVF, respectively [42]. The authors conclude that vasectomy reversal is the more cost-effective alternative when all costs to a couple are considered.

Additional considerations of cost include regional variation and insurance coverage for the various procedures necessary. The personal preferences of the couple also play a role in decision-making. Vasectomy reversal allows for a natural conception. Sperm retrieval may be a better decision for couples with significant female factor infertility or advanced female age. For couples desiring multiple children or those hoping to avoid the increased risk of multiple gestations, vasectomy reversal may be the recommended treatment. These final issues have not been studied in the cost-effectiveness analyses detailed herein [43].

2.7 Conclusions

The clinician must consider multiple factors when counseling couples about fertility potential post-vasectomy. These include obstructive interval, female fecundity, cost-effectiveness, and couple preferences. As ART and associated birth rates continue to improve, reevaluation of the cost-effectiveness of these treatments remains important. Future need for contraception after vasectomy reversal must also be discussed. Vasectomy reversal is generally the recommended option and is also cost-effective across a broad range of clinical situations. A counseling session should cover all these aspects of care with a clinical recommendation from the provider, but ultimately allow the couple to determine the best course of action.

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3 Stem Cell Therapies for Male Infertility: Where Are We Now and Where Are We Going?

Hanna Valli, Kathrin Gassei, and Kyle E. Orwig

3.1 Introduction

Spermatogenesis is an extraordinarily productive process that generates more than 100 million sperm each day from the testes of adult men [1]. This level of productivity is dependent on spermatogonial stem cells (SSCs), which maintain continuous sperm production throughout the postpubertal life of men by precisely balancing self-renewing divisions that maintain the stem cell pool with differentiating divisions that ultimately give rise to mature sperm [2–5]. Spermatogenesis has been recognized as a stem cell-based system for more than a century [5, 6], and several lines of evidence demonstrate that spermatogonial stem cells have the potential to regenerate spermatogenesis when it is depleted, for example, in seasonal breeders [7] or by toxic insults such as chemotherapy or radiation [8–15]. These observations suggest that stem cells might be manipulated to preserve and/or restore spermatogenesis in infertile men.

Infertility impacts 10–15 % of couples in the United States, and a male factor is implicated alone or in combination with female factors in about 50 % of those cases [16, 17]. Epidemiological studies indicate that about 12 % of men in the United States are subfertile or infertile [18, 19] and this can have important emotional, financial, social, and health implications [19–23]. Causes of male infertility can include physical/physiological problems (e.g., varicocele, erectile dysfunction), infection, immunological factors (e.g., antisperm antibodies), chromosomal (e.g., Y chromosome deletions, Klinefelter's syndrome) or other genetic anomalies, endocrine disorders (e.g., Kallmann syndrome), diseases (cancer), medical treatments (androgens, chemotherapy, radiation), and others, but are most frequently of unknown origin (idiopathic). Idiopathic infertility is difficult to counsel and treatment options are empirical [16, 17]. For the purposes of this chapter, we will focus primarily on infertility that is caused by chemotherapy or radiation treatments for cancer or other conditions because (1) the cause of infertility is at least partially understood (depletion SSCs), (2) counseling about fertility risks and fertility sparing options is inadequate or incomplete, so there is room for improvement, and (3) there are new stem cell technologies on the horizon that may significantly impact the fertility prospects for these patients.

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_3

3.2 Fertility After Cancer

Whole body radiation, radiation to the gonads, and alkylating chemotherapies are known to be particularly toxic to spermatogenesis and male fertility [24–27]. This is an important human health concern because nearly 25,000 males under the age of 44 will be diagnosed with cancer each year in the United States. Our review of epidemiological data [27–29] indicates that most of these patients will survive their cancer, but over 4,000 will receive treatments that put them at high risk for infertility (reviewed in [30]). Cancer survivors report that parenthood is important to them and distress over infertility has long-term psychological and relationship implications [31]. Therefore, the American Society of Clinical Oncology [32, 33] and the American Society for Reproductive Medicine [34, 35] recommend that patients be educated about the reproductive risks associated with their therapy and about options for preserving fertility.

Adult men have the option to cryopreserve a semen sample before initiating treatment, which can be thawed at a later date to achieve pregnancy by intrauterine insemination [177], in vitro fertilization (IVF) [178], or IVF with intracytoplasmic sperm injection (ICSI) [179]. Unfortunately, only about 24 % of adult men freeze a semen sample before initiating their therapy [36]. For men who did not preserve a semen sample and have persistent azoospermia after cancer therapy, there is the option to retrieve rare sperm directly from the testis by testicular sperm extraction (TESE). This is possible because a few SSCs survive the gonadotoxic therapy and produce focal areas of spermatogenesis in the seminiferous tubules. Hsiao and colleagues recently described their experience with 73 patients with post-chemotherapy azoospermia [37]. They reported that sperm were successfully retrieved from 37 % of patients on initial attempt with an overall success rate of 42.9 %. Fertilization rate with the retrieved sperm was 57 %; the pregnancy rate was 50 % and the live birth rate was 42 %. Success in retrieving sperm was treatment dependent in that study with the lowest sperm recovery success rates (21 %) in patients receiving alkylating chemotherapy [37]. There are currently no options to treat the infertility of adult patients who did not cryopreserve a semen sample and were not successful with the TESE/ICSI procedure. Adoption and third party reproduction are family building options for these patients, but most cancer survivors prefer to have their own biological children [32]. For these patients, there is cutting edge research (detailed in the induced pluripotent stem cell section below) suggesting that it may one day be possible to reprogram skin cells or other somatic cells into spermatogenic cells that can produce fertilization-competent sperm.

There are currently no standard of care options to preserve the fertility of prepubertal boys who are not yet producing sperm. This is an important human health concern because, with improved therapies, the event-free survival rate of children with cancer is nearly 85 % [29] and these patients can look forward to a full and productive life after cure. The American Society of Clinical Oncology advises that the concept of impaired future fertility may be difficult for children to understand, but infertility is potentially traumatic to them as adults [32]. We estimate that each year in the United States, more than 2,000 boys will receive gonadotoxic treatments for cancer or other conditions (e.g., myeloablative conditioning prior to bone marrow transplantation) that put them at high risk for infertility [30]. Although it is not possible to preserve sperm for these prepubertal patients, they do have SSCs in their testes (Adark and Apale spermatogonia) that are poised to initiate sperm production at the time of puberty [38]. There are promising stem cell technologies in the research pipeline (e.g., SSC transplantation, testicular tissue grafting, testicular organ culture, in vitro derivation of haploid gametes) that could provide fertile options for these boys. With this in mind, several centers in the United States and abroad are already cryopreserving testicular tissue or cells for patients in anticipation that new stem cell technologies will be available for them in the future [39–45]. Two centers

reported that the majority of parents consented to fertility preservation procedures on behalf of their children, even though methods for restoring fertility of sons are still experimental [40, 46]. Therefore, it is critically important for the medical and research communities to responsibly develop stem cell technologies to ensure that they are available and can be safely and effectively translated to the clinic.

3.3 Spermatogonial Stem Cell Transplantation

Ralph Brinster and colleagues pioneered the technique of spermatogonial stem cell transplantation in 1994, demonstrating that SSCs could be isolated and transplanted to regenerate spermatogenesis in infertile recipient mice [47, 48]. SSC transplantation has now been reported in mice, rats, pigs, goats, bulls, sheep, dogs, and monkeys including the production of donor-derived progeny in mice, rats, goats, and sheep [49, 53, 55–61]. SSCs from donors of all ages, newborn to adult, are competent to regenerate spermatogenesis [50, 62], and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation [58, 63, 64]. Thus, it appears feasible that a testicular tissue biopsy (containing SSCs) could be obtained from a prepubertal boy prior to gonadotoxic therapy; frozen; thawed at a later date; and transplanted back into his testes to regenerate spermatogenesis. If robust spermatogenesis occurs from transplanted cells, as has been reported in previous animal studies, it may be possible to restore natural fertility, allowing survivors to achieve pregnancy with their partner by natural intercourse and have their own biological children (Fig. 3.1) (reviewed in [176]). We recently established the proof in principle for this approach in a preclinical nonhuman primate (rhesus macaque) model of cancer survivorship in which animals were rendered infertile by treatment with alkylating chemotherapy [10, 58]. We demonstrated that prepubertal and adult rhesus SSCs could be frozen, thawed, and transplanted to regenerate spermatogenesis, producing sperm that fertilized rhesus eggs and gave rise to preimplantation embryos [58, 66]. Considering the progress in several animal models and the fact that testicular tissues have already been cryopreserved for hundreds of human patients worldwide [39–45, 67, 68], it seem reasonable to expect that SSC transplantation or other stem cell technologies will impact the fertility clinic in the next decade.

It is rarely acknowledged and not widely known that Radford and colleagues already reported cryopreserving testicular cells for 11 adult non-Hodgkin's lymphoma patients in 1999 and subsequently reported transplanting autologous frozen and thawed testis cells back into the testes of seven survivors [67, 68]. Perhaps this is because references to these patients were obscured as brief comments with no supporting data. Perhaps these reports were disregarded because the development and understanding of the SSC transplantation technique was in its infancy at the time and translation to humans was considered premature. There are no published reports of SSC transplantation in humans since Radford's follow-up report of his non-Hodgkin's lymphoma patients in 2003 [68]. The fertility outcomes for those patients have not been reported, and even if the men fathered children, it would not be possible to ascertain whether the sperm arose from transplanted stem cells or surviving endogenous stem cells. This uncertainty will always plague the interpretation of human SSC transplant studies where it is not ethically possible to genetically mark the transplanted cells because the genetic modification would be transmitted to progeny. Therefore, large epidemiological datasets generated over decades will be required to prove the fertility benefit of SSC transplantation. Nonetheless, the pioneering studies of Radford and colleagues demonstrated that patients are willing to pursue experimental stem cell-based options to preserve and restore their fertility.

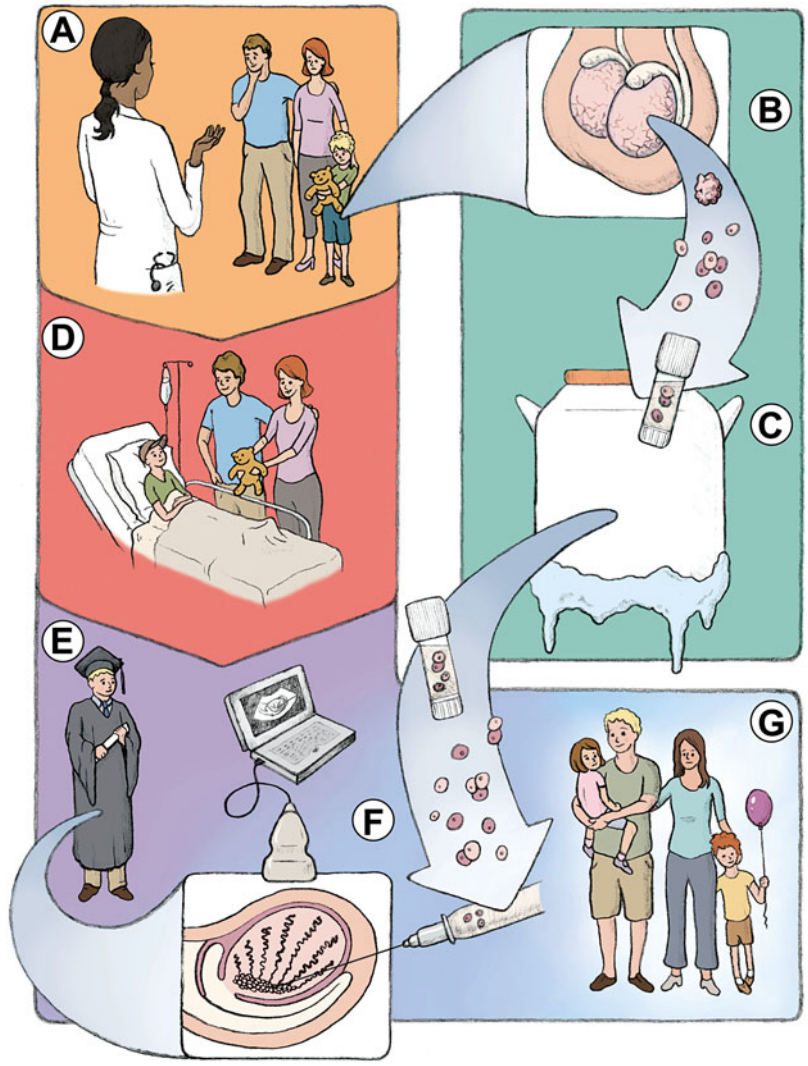

Fig. 3.1 Testicular tissue cryopreservation and spermatogonial stem cell transplantation to preserve and restore the fertility of prepubertal cancer survivors. This flow diagram illustrates the hypothetical scenario of prepubertal patient who is scheduled to undergo a medical treatment that will put him at risk for infertility. The prepubertal patient does not have the option to cryopreserve a semen sample because he is not yet producing sperm, but some centers provide the option to cryopreserve testicular tissue or cells that harbor potentially therapeutic spermatogonial stem cells. (**a**) Testicular tissue cryopreservation is experimental and should only be offered with the appropriate regulatory oversight of an institutional review board and informed consent of the family. Ideally, the testicular biopsy procedure should be performed before the patient is exposed to therapy that could damage the pool of spermatogonial stem cells. (**b** and **c**) Most centers are cryopreserving testicular tissue or cells by controlled rate slow freezing. However, several centers are investigating the efficacy of rapid freezing by vitrification. (**d**) Once the testicular tissue biopsy is taken, the patient can proceed with their medical treatment. The testicular tissue biopsy and cryopreservation procedure is performed in consultation with the oncologist and usually causes minimal delay to treatment. (**e**) The testicular tissue or cells can be thawed at a later date for autologous transplantation back into the testes of the patient by ultrasound-guided rete testis injection (**f**). Other options for use of the preserved testicular tissue/cells reviewed in this chapter include testicular tissue/cell grafting or xenografting and testicular tissue organ culture. (**g**) If transplanted spermatogonial stem cells produce robust spermatogenesis, natural fertility may be restored allowing the patient to father his own biological children, perhaps through normal coitus. If spermatogenesis from transplanted cells is less robust, sperm can be recovered by TESE and used for IVF with ICSI. Artwork by Molly Feuer (http://feuerillustration.com) (Reprinted from Hermann and Orwig [176] with permission from Springer Science + Business Media)

3.4 Challenges and Opportunities for Translating Spermatogonial Stem Cell Transplantation to the Clinic

3.4.1 Challenge: Insufficient Counseling

Why are there no reports of SSC transplantation in humans since 2003? Initially this may have been due to a dearth of eligible patients. Stem cell therapies are not being actively contemplated for adult patients who have the potential to preserve sperm obtained from ejaculated semen or TESE. Prepubertal patients are rarely presented with the option to cryopreserve testicular tissue because few physicians are aware of this option and few institutions have the experience, infrastructure, or regulatory approval to process and preserve testicular tissue/cells for future fertility applications.

3.4.2 Opportunity: Education and Outreach

Increased awareness about the reproductive consequences of medical treatments and options (standard and experimental) for preserving fertility can be attributed in part to the outreach, leadership, and education efforts of the Oncofertility Consortium (http://oncofertility.northwestern.edu/), the Childhood Cancer Survivor Study (https://ccss.stjude.org/), the International Society for Fertility Preservation (http://www.isfp-fertility.org/), the American Society for Reproductive Medicine (http://www.asrm.org/) [34, 35], the American Society of Clinical Oncology (http://www.asco.org/) [32, 33], and others [69–72]. These groups foster valuable cross disciplinary discussions among patients, oncologists, reproductive specialists, ethicists, psychosocial professionals, and researchers around the topic of fertility preservation. Awareness of fertility preservation options for prepubertal patients is improving as centers are beginning to publish their experiences with testicular tissue cryopreservation [40, 41, 43–45, 67, 68, 73]. Based on these published reports and personal communications with the authors, we estimate that there are now several hundred patients worldwide who have preserved their testicular tissue in anticipation that the tissue can be used in the future for reproductive purposes.

3.4.3 Challenge: Prepubertal Boys, Small Testes, Small Biopsies, and Few SSCs

Our experiences at the Fertility Preservation Program in Pittsburgh [39] and published reports [41, 44] indicate that it may be reasonable to expect that 50–200 mg of testicular tissue can be obtained by open biopsy or needle biopsy from a single testis of a prepubertal boy. As a point of reference, a typical adult human testis weighs 11–26 g [74]. The number of stem cells contained in those small biopsies is not known, but is likely to be quite small. Therefore, it is uncertain whether a testicular biopsy obtained from a prepubertal male cancer patient will contain a sufficient number of stem cells to produce robust spermatogenesis and fertility as has been described in animal studies in which the number of donor cells was not limited. For this reason, several groups are investigating methods to expand human SSCs in culture prior to transplant or produce haploid sperm in testicular tissue grafts or organ culture that can be used for in vitro fertilization (IVF) with ICSI (see below).

3.4.4 Opportunity: Spermatogonial Stem Cell Culture

After years of trial, error, and incremental progress [75–79], success in maintaining mouse SSCs in long-term culture with significant expansion in numbers was finally reported in 2003/2004 [80, 81]. Success in culturing mouse SSCs depended on: (1) development of sorting methods to isolate and enrich SSCs while removing testicular somatic cells that can rapidly over-proliferate and

overwhelm the cultures [80–82]; (2) determination that glial cell line-derived neurotrophic factor (GDNF) was critical for maintaining mouse SSCs in long-term culture [78, 80, 83]; (3) mouse SSC cultures are frequently established from the testes of 1-week-old mouse pups because SSCs are enriched at that stage of development [50] and plated on STO (SIM mouse embryo-derived thioguanine and ouabain-resistant) fibroblasts or mouse embryonic fibroblast feeder cells [80, 81]. However, SSC cultures can be established from all ages, neonate to adult [80, 81], and under feeder-free conditions [84, 85], which may facilitate clinical translation. Methods for maintaining SSCs in long-term culture were readily translated to the rat and hamster where the requirement for GDNF was conserved [86–88]. Transplantation and breeding experiments have provided the definitive evidence that rodent SSCs maintained in long-term culture are competent to regenerate spermatogenesis, produce functional sperm, and restore fertility (Fig. 3.2) [80, 81, 86–89].

3.4.5 Challenge: Human Spermatogonial Stem Cell Culture

If cultured human SSCs function similar to cultured rodent SSCs (Fig. 3.2), it should be feasible to expand a few stem cells obtained from the testis biopsy of a prepubertal boy to a number sufficient to produce robust spermatogenesis upon transplantation back into his testes when he is an adult. Several groups have reported culturing SSCs from large animal species [54, 90–94] and humans [42, 43, 95–102], including two studies in which cultures were established from the testes of prepubertal patients [43, 95]. Human SSC cultures have been established from fetal, prepubertal, and adult stages of testis development; all studies employed some method to isolate and enrich the putative human SSCs prior to culture; most studies used GDNF among other growth factors and all used feeder cells or coated plates (Table 3.1). To date, human SSC cultures have been evaluated by quantitative PCR or immunocytochemistry for spermatogonial markers or xenotransplantation into mouse testes. These results are promising, but challenged by the lack of a functional assay to test the full spermatogenic and fertile potential of the cultured human cells, which is the gold standard for evaluation of animal studies. In addition, no reported method for culturing human SSCs has been replicated in another laboratory, and this is necessary to validate methods and move the field forward. The lack of replication probably reflects the early stage of technology development and the lack of consensus about best methods for establishing cultures and the best endpoints for analysis. Based on our own experience and review of the literature, we propose that UTF1, OCT2, EXOSC10, GFRa1, GFR125, UCHL1, ZBTB16, ENO2, SALL4, LIN28, NANOS2, and FGFR3 are excellent markers that are relatively restricted to undifferentiated spermatogonia (including SSC) in human testes [5, 7, 30, 74, 96, 103–107]. Human to mouse xenotransplantation has emerged as a robust bioassay for human spermatogonia [42, 43, 74, 95, 107–109] and important complement to marker expression data. Human spermatogonia do not produce complete spermatogenesis in mouse testes, probably due to evolutionary distance between these species. However, the method does assay the ability of cells to migrate to the basement membrane of seminiferous tubules and proliferate to produce characteristic chains of spermatogonia that persist for a long period of time. Development of in vitro or in vivo methods to assay the full spermatogenic potential of human stem cells would significantly advance the field. Assaying the full spermatogenic potential of human cells by autologous or homologous transplantation in humans is not possible, but it may one day be possible to reconstruct human spermatogenesis by grafting human cells or tissue into mice or by infusing human cells into the scaffold of a decellularized human testis (see discussions in the testicular tissue grafting and building a testis sections below).

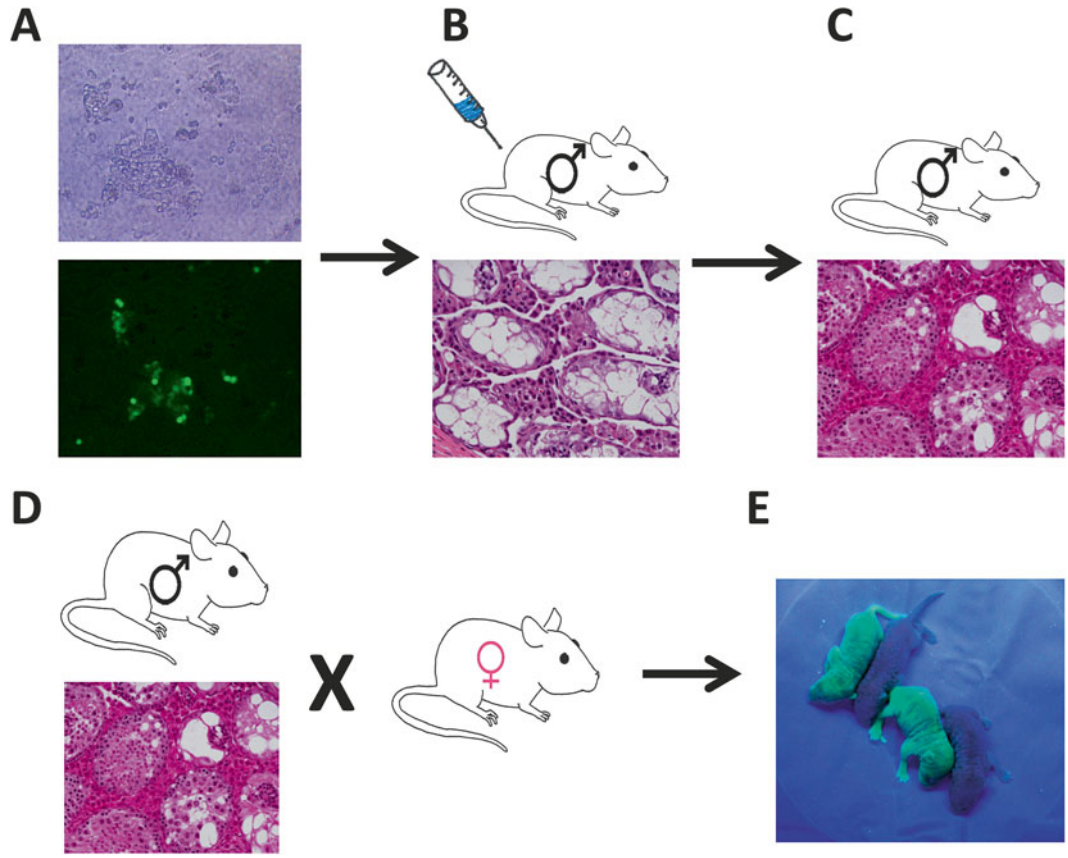

Fig. 3.2 Mouse spermatogonial stem cell culture. THY-1+ testis cells from 1-week-old DBA/2 mice expressing a chicken β-actin (CAGG)-EGFP transgene were placed in culture on STO feeder cells with mouse serum-free medium containing GDNF (20 ng/ml) and FGF2 (1 ng/ml), as described previously [82]. (**a**) Cultures contain characteristic clusters of spermatogonia (**a**, *Top*), including EGFP positive cells (**a**, *Bottom*), and can be maintained through numerous passages with significant expansion in the number of SSCs. (**b**) To confirm that cultured SSCs retained spermatogenic potential, cells from passage 10 were transplanted into the testes of W recipient mice that are infertile due to a mutation in the cKIT receptor tyrosine kinase and exhibit a Sertoli cell-only (SCO) phenotype (**b**, *Bottom*). (**c**) Transplanted cultured SSCs regenerated spermatogenesis in some seminiferous tubules of the recipient animals. (**d**) Recipient males were bred to wild-type females and produced healthy, fertile offspring. All mouse pups came from the cultured SSCs because the recipient male was genetically infertile and incapable of producing sperm. (**e**) Half of the pups were green because the donor animals were hemizygous for the CAGG-EGFP transgene (Peters, Valli, and Orwig, unpublished)

3.4.6 Challenge: Malignant Contamination in Testicular Biopsy

A testicular biopsy obtained from the testis of a cancer patient could harbor occult malignant cells, especially for patients with leukemia. Indeed, Kim and colleagues [110] reported that 20 % of boys with acute lymphocytic anemia had malignant cells in their testicular tissue prior to the initiation of oncologic treatment. Jahnukainen and colleagues [111] reported the transmission of leukemia after transplantation of testis cells from terminally ill leukemic rats into the testes of non-leukemic recipients. The same group further demonstrated that transplantation of as few as 20 leukemic cells was sufficient for disease transmission, leading to terminal leukemia within 3 weeks. Because infertility is not life threatening and fertility treatments are elective, it is essential that risk of cancer recurrence after transplant be reduced to zero.

Table 3.1 Human spermatogonial stem cell culture

Tissue	SSC enrichment	Growth factors	Feeders/substrate	Endpoint	Ref.
Adult	MACS for SSEA4	EGF, FGF, GDNF, and LIF	Matrigel	qRT-PCR—EPCAM, GPR125, SSEA4, ITGA6	[99]
Prepubertal	Differential plating	EGF, GDNF, LIF	Laminin	Xenotransplants and RT-PCR—ZBTB16, ITGA6, ITGB1, CD9, GFRA1, GPR125, UCHL1	[43]
Adult	Differential plating	EGF, GDNF, LIF	Laminin	Xenotransplants; ICC—ZBTB16; RT-PCR—ZBTB16, ITGA1, ITGB1	[42]
Adult	Differential plating	None	Sertoli cells	ICC—OCT4, vimentin; alkaline phosphatase staining	[102]
Fetal	Percoll separation and differential plating	None	Sertoli cells	ICC and Fc—OCT4, SSEA4	[100]
Adult	Differential plating and MACS for GFR125	GDNF, GFRA1-Fc, NUDT6, LIF, EGF, TGFB, nodal	0.1 % gelatin	ICC—GPR125, ITGA6, GFRA1, THY1	[96]
Prepubertal	Isolated by micromanipulator	GDNF and FGF	C166	ICC—UCHL1	[95]
Fetal	MACS for ITGA	FGF, GDNF, LIF	hESCdFs	ICC—OCT4, SSEA1, ITGA6; RT-PCR—OCT4, STRA8, DAZL, NOTCH1, NGN3, SOX3, KIT	[98]
Adult	Differential plating	FGF and LIF	Sertoli cells	Xenotransplants; alkaline phosphatase staining; ICC—OCT4, vimentin; RT-PCR—OCT4, NANOG, STRA8, PIWIl2, VASA	[97]
Adult	FACS—CD45 neg, THY1 neg, SSEA4 pos	EGF, GDNF, LIF	Human THY1+ testicular somatic cells	ICC—SSEA4, VASA	[101]

RT-PCR, reverse transcription-polymerase chain reaction; *qRT-PCR*, quantitative *RT-PCR; ICC*, immunocytochemistry; *Fc*, flow cytometry

3.4.7 Opportunity: Sorting Strategies to Isolate and Enrich Therapeutic Spermatogonia While Removing Malignant Contamination

Recognizing concerns about the risks of reintroducing malignant cells into the testes of cancer survivors, several groups have evaluated fluorescent-activated cells sorting (FACS)- or magnetic-activated cell sorting (MACS)-based methods to remove malignant contamination, with mixed results. To enable these studies, it was necessary to identify markers expressed by cancer cells that are not expressed by SSCs and/or markers that are expressed by SSCs, but not by cancer cells. Fujita and coworkers isolated cells from the testes of leukemic mice in the forward scatter high and side scatter low fraction that has been shown to contain SSCs [112, 113]. This fraction was further divided into cells that expressed the leukemic markers CD45 and the MHC class I antigens ($H\text{-}2K^b/H\text{-}2D^b$) and putative germ cells that did not express the leukemic markers. All recipient males injected with the leukemic cell fraction ($CD45^+$/MHC class I^+) developed terminal leukemia within 40 days. All mice injected with the putative germ cell fraction ($CD45^-$/MHC class I^-) survived for 300 days without onset of leukemia and produced donor-derived offspring [113]. In a subsequent study, the same group reported that seven out of eight human leukemic cell lines expressed the cell surface antigens CD45 and MHC class I [114]. To replicate the prepubertal cancer patient scenario, Hermann and colleagues [115] contaminated prepubertal nonhuman primate testis cells with leukemic cells. Almost all germ cells were recovered by FACS in the $CD45^-$/$THY\text{-}1^+$ fraction of the contaminated cell suspension, and this fraction did not produce tumors in recipient mice. Using a similar approach, Dovey and colleagues [107] contaminated human testis cells with MOLT-4 acute lymphoblastic leukemia cells and demonstrated by xenotransplantation that the $Ep\text{-}CAM^{lo}$/$HLA\text{-}ABC^-$/$CD49e^-$ fraction was enriched 12-fold for transplantable human SSCs and was devoid of malignant contamination (55 total mice transplanted). In addition to the $Ep\text{-}CAM^{lo}$ fraction, transplantable human spermatogonia have been recovered in the $THY\text{-}1^{lo}$, $CD49f^+$, $SSEA4^+$, $GPR125^+$, and $CD9^+$ fractions of human testis cells [74, 96, 99, 108, 109].

3.4.8 Challenge: How to Assay and Interpret Malignant Contamination Results

The in vivo transplant studies described above provide the most convincing evidence that it may be feasible to isolate therapeutic SSCs and remove malignant contamination from human testis cell suspensions. However, other studies have failed to remove malignant contamination using immune-based sorting strategies [116, 117]. In a rat model of Roser's T-cell leukemia, Hou and colleagues concluded that single parameter selection using either leukemic (CD4 and MHC class I) or SSC (Ep-CAM) markers was not sufficient to eliminate malignant contamination [116] and recipient animals developed terminal leukemia. However, these authors successfully removed malignant contamination using a combination of leukemia and SSC markers (plus/minus selection), similar to the results of the nonhuman primate and human studies cited in the previous paragraph [107, 115]. In contrast, using EL-4 lymphoma-contaminated mouse and human testis cell suspensions, Geens and colleagues concluded that FACS- and MACS-based methods were insufficient to remove malignant contamination [117]. Malignant contamination was detected in sorted samples by PCR for the B-cell receptor, culture for tumor cells, and the development of a tumor in 1 out of 20 recipient animals.

It will not be possible to perform comprehensive in vivo testing on patient samples because this would limit the amount of sample available for fertility therapy. More sensitive PCR-based methods have been proposed for detection of minimal residual disease (MRD), and indeed this approached has identified malignant contamination in many ovarian tissue samples that were preserved for leukemia patients, even after negative histology and immunocytochemistry exami-

nation [118, 119]. In one study, cryopreserved ovarian tissues from six chronic myeloid leukemia and 12 acute lymphocytic leukemia patients were evaluated by histology, qRT-PCR, and xenografting for malignant contamination. Histological assessment did not identify malignant cells in any of the samples. However, qRT-PCR revealed the possibility of malignant contamination in nine of the 16 samples in which the leukemic markers were known. Five of the nine samples with positive MRD had evidence of leukemic cells in the ovarian tissue grafts 3 months after transplantation. Four of the nine ovarian tissue xenografts with positive MRD appeared normal with no evidence of leukemia [118]. Were the MRD results in those four cases nefarious or were they accurate and the leukemic cells simply failed to survive freezing, thawing, and grafting? How does the physician decide?

Despite our own successful efforts to demonstrate that it is feasible to isolate and enrich therapeutic SSCs while removing malignant contamination from a testis cell suspension [107, 115], the authors of this chapter (HV and KEO) conclude that current methods are not adequate to provide 100 % certainty that a patient's sample is free of cancer cells. We reached this conclusion based on our observation that even a pure population of MOLT4 acute lymphocytic leukemia cells only produced tumors in 72 % of animals when cells were transplanted into the permissive environment of the testicular interstitium [107]. Therefore, even a negative in vivo tumor burden assessment cannot be interpreted to indicate an absence of malignant cells. Based on these uncertainties, it is necessary to develop alternatives to autologous transplantation where there is concern about the potential for malignant contamination, such as with hematogenous cancers. Experimental xenologous and ex vivo approaches for producing fertilization-competent sperm are detailed below.

3.5 Testicular Tissue Grafting

Testicular tissue grafting may provide an alternative approach for generating fertilization-competent sperm from small testicular biopsies. In contrast to the SSC transplantation method in which SSCs are removed from their cognate niches and transplanted into recipient seminiferous tubules, grafting involves transplantation of the intact SSC/niche unit in the context of the seminiferous tubules in pieces of testicular tissue. Honaramooz and colleagues reported that grafted testicular tissue from newborn mice, rats, pigs, and goats, in which spermatogenesis was not yet established, could mature and produce complete spermatogenesis when xenografted into nude mice [120]. The same group later reported the production of live offspring from sperm obtained from mouse testicular tissue grafts transplanted under the skin of recipient mice [121]. Fertilization-competent sperm was also produced from xenografts of prepubertal nonhuman primate testicular tissue transplanted into mice [122]. These results suggest that it may be possible to obtain fertilization-competent sperm by xenografting small pieces of testicular tissue from a prepubertal cancer patient under the skin of mice or other animal recipients such as pigs that are already an established source for human food consumption, replacement heart valves [123, 124], and potentially other organs [125]. Xenografting would also circumvent the issue of malignant contamination. However, the xenografting approach raises concerns about xenobiotics because viruses from mice, pigs, and other species can be transmitted to human cells [126, 127]. Also, there is no evidence to date that xenografted human testicular tissue can produce spermatogenesis or sperm in mice [128–133]. However, there is reason for optimism because Sato and colleagues observed primary spermatocytes 1 year after xenografting testicular tissue from a 3-month-old boy that clearly did not have spermatocytes at the time of transplantation [132]. The failure of human testicular tissue to produce complete spermatogenesis in mice is probably not due to evolutionary distance between these species since prepubertal nonhuman primate testicular tissue produced complete spermatogenesis and fertilization-competent sperm after xenotransplantation into mice [122]. If human to mouse evolutionary distance is a problem, autologous testicular tissue grafting may be an option.

Leutjens and colleagues demonstrated that fresh autologous testicular tissue grafts from prepubertal marmosets could produce complete spermatogenesis when transplanted into the scrotum, but not under the skin [134]. Frozen and thawed grafts did not produce complete spermatogenesis in that study, but those grafts were only transplanted under the skin. Therefore, additional experimentation is merited. Autologous testicular tissue grafting may circumvent the issue of xenobiotics, but the potential for malignant contamination will still have to be considered before testicular tissue is transplanted back into a cancer survivor.

3.6 Cryopreserving Testicular Tissue or Cells

Several groups have investigated methods for cryopreserving testicular tissue or cells using methods such as controlled rate slow freezing, uncontrolled slow freezing, and vitrification (summarized in Table 3.2). For fertility preservation, most centers are freezing intact pieces of testicular tissue for patients because this preserves the option for both tissue-based and cell-based therapies in the future [40, 41, 44, 45]. However, in all previous animal and human studies where samples were frozen, thawed, and transplanted into recipient seminiferous tubules, samples were digested enzymatically and frozen as a cell suspension, not an intact tissue [10, 58, 63, 64, 66, 74, 107, 108, 145, 146]. Is the recovery and viability of SSCs from frozen and thawed testicular tissue similar to that obtained from a frozen and thawed cell suspension? Two studies tested this question directly on human samples. Yango and colleagues reported that recovery of SSEA4$^+$ cells from cryopreserved fetal testicular tissue was similar to cryopreserved testicular cells, but recovery of SSEA4$^+$ cells from cryopreserved adult testicular cells was greater than cryopreserved testicular tissue [142]. Pacchiarotti and coworkers reported that cryopreservation of testicular tissue was comparable in most aspects to cryopreservation of a cell suspension, except the viability of cells from the cryopreserved tissue was higher. The recovery of SSEA4$^+$ and VASA$^+$ germ cells from cryopreserved tissue sections tended to be greater than cryopreserved cell suspensions, but these differences were not significant [135]. Similar to Pacchiarotti et al., we found that recovery of UTF1$^+$ cells and colonizing activity in the human to nude mouse xenotransplantation assay were greater from cryopreserved human testicular tissue pieces (3–5 mm^3) than from cryopreserved cells (Valli and Orwig, in preparation). This was surprising to us because we hypothesized that uneven penetration of the cryopreservative into three-dimensional tissue pieces would result in decreased viability and decreased recovery of human spermatogonia. We immediately translated this new knowledge to the clinic and now routinely freeze intact testicular tissue pieces for patients and believe this will maximize their fertility options in the future.

3.7 Building a Testis

Even after disaggregation into single cell suspensions, testicular cells (including germ cells, Sertoli cells, peritubular myoid cells, and Leydig cells) have the remarkable ability to reorganize to form normal-looking seminiferous tubules when grafted under the skin of recipient mice [147–151]. Dufour and colleagues demonstrated that isolated Sertoli cells and peritubular myoid cells (minimal contamination with Leydig and germ cells) from the testes of neonatal pigs could reorganize to produce normal-looking seminiferous tubules when grafted under the kidney capsule of immune-deficient SCID mice [147]. Gassei and coworkers subsequently used a combination of in vitro culture (laminin or Matrigel) followed by in vivo grafting to demonstrate that testicular cells from 1-week-old rat pup testes could undergo de novo morphogenesis to produce seminiferous tubules with a properly oriented basement membrane, Sertoli cells, and some putative germ cells [148]. Cytochrome P450 staining indicated the presence of Leydig cells in the interstitial space, but complete spermatogenesis was not observed in this study. However, two subsequent studies from Dobrinski's laboratory demonstrated de

Table 3.2 Cryopreservation of human testicular tissue and cells

Tissue and cells	Freezing method	Freezing conditions	Endpoints	Ref.
Cells	Controlled slow freezing	10 % HSA, 10 % DMSO, 1 % dextran	Viability, Fc—SSEA4, LHR, VASA	[135]
Tissue				
Tissue	Controlled slow freezing	0.7 M DMSO, 0.1 M sucrose	Xenografting, IHC—MAGE-A4, Ki67, 3β-HSD	[136]
	Vitrification	Eq. sol.—7.5 % EG, 7.5 % DMSO, 0.25 M sucrose. vitr. Sol. 15 % EG, 15 % DMSO, 0.5 M sucrose		
Tissue	Controlled slow freezing	0.7 M DMSO, 5 % HSA	IHC—MAGE-A4, TEM, organ culture	[137]
Tissue	Controlled slow freezing	0.7 M DMSO, 0.1 M sucrose, 10 mg/ml HSA	IHC—MAGE-A4 and Ki67	[138]
	Vitrification	Not found		
Tissue	Controlled slow freezing	1.5 M EG, 0.1 M sucrose, 10 % HSA	Morphology, IHC—KIT	[139]
Tissue	Controlled slow freezing	0–2.5 M DMSO or EG or glycerol with 0.1 % ITS and 20 % FBS	Viability, seminiferous tubule culture	[140]
Tissue	Controlled slow freezing	5 % DMSO, 5 % HSA	IHC—MAGE-A4, vimentin, CD34; TEM; tissue culture	[44]
Tissue	Controlled slow freezing	(1) 0.7 M or 1.5 M DMSO and 5 % HSA (2) 0.7 M DMSO, 0.1 M sucrose, 10 % HSA	IHC—TUNEL, PCNA, UCHL1; TEM	[141]
	Uncontrolled slow freezing	0.7 M or 1.5 M DMSO, 0.15 M sucrose, 10 % HSA		
	Solid-surface vitrification	Eq. sol.—1.35 M EG, 1.05 M DMSO. Vitr. Sol. 2.7 M EG, 2.1 M DMSO, 20 % HSA		
	Direct cover vitrification			
Cells	Controlled slow freezing	1.28 M DMSO, 25 % FBS	Fc—CD45, THY1, SSEA4	[142]
Tissue				
Cells	Controlled slow freezing	2 % HSA, 1.4 M DMSO	Cell recovery, viability	[143]
	Uncontrolled slow freezing	2 % HSA, 0.7 M DMSO		
	Semen freezing	Sperm-freezing media		
	Vitrification	Eq. sol.—2 % HSA, 1.1 M DMSO, 1.34 M EG. Vitr. Sol.—2 % HSA, 0.67 M sucrose, 2.3 M DMSO, 1.34 M EG		
Cells	Controlled slow freezing	4 % FBS, 1.5 M DMSO or EG or glycerol or 1,2-propanediol	Viability	[144]

Fc, flow cytometry; *IHC*, immunohistochemistry

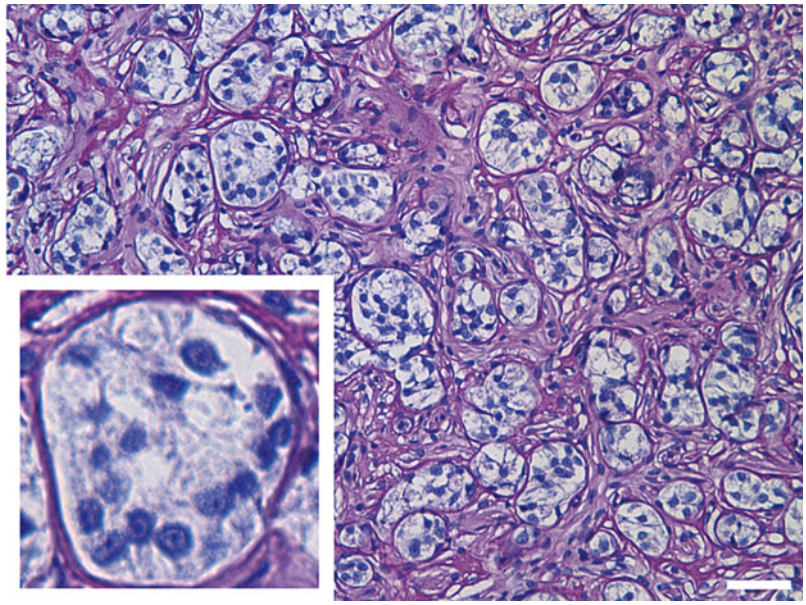

Fig. 3.3 Building a testis. Testis cells from prepubertal rhesus macaques were transplanted with Matrigel under the backskin of immune-deficient nude mice. Grafts were recovered 8 weeks after transplant, fixed in Buoin's solution, embedded in paraffin, sectioned (5 μm), and stained with PAS/hematoxylin. Inset: higher magnification of a single tubule. Scale bar = 50 μm

novo morphogenesis of seminiferous tubules with complete spermatogenesis from neonatal pig and sheep testis cells when grafts were recovered between 16 and 41 weeks after transplant [150, 151]. In these studies, cells were not cultured, but rather pelleted and then grafted directly under the skin of nude or SCID recipient mice. In a slight modification of this approach, Kita and colleagues [149] mixed fetal or neonatal testis cells from mice rats and pigs with growth factor-reduced Matrigel and demonstrated that these cells could reform seminiferous tubules when injected under the skin of nude or SCID mice. Most tubules were characterized as Sertoli cell only, but a few tubules contained spermatogonia and occasionally meiotic cells were observed. In this study, grafts were recovered 7–10 weeks after transplant, and this may explain the limited spermatogenic development. In a subsequent experiment, the same group infused the mouse and rat testis cell suspensions with cultured mouse germline stem cells. Seven to ten weeks after grafting, seminiferous tubules with complete spermatogenesis originating from both intrinsic germ cells and cultured (GFP^+) germ cells were observed. Tubules were dissected and GFP^+ round spermatids were recovered. Spermatids were injected into mouse oocytes which were then transferred to recipient females, resulting in the production of ten mouse pups.

These results in several animal models suggest that it may be feasible to build a human testis even from a disaggregated suspension of testis cells and produce fertilization-competent haploid germ cells. However, the human experiment has not been reported to our knowledge, and progress may be limited by the availability of neonatal or prepubertal human testis cells. It does not appear that anyone has tried to "build a testis" from disaggregated adult testis cells for any species. In a pilot experiment, we transplanted testis cells from prepubertal rhesus macaques with Matrigel under the skin of nude mice and found that those cells reorganized to produce seminiferous tubules when grafts were recovered 8 weeks later (Fig. 3.3; Gassei and Orwig, unpublished). More detailed analyses of these grafts are under way, but in future studies, it will be interesting to determine

whether spermatogenesis in those grafts can be enhanced by increased development time in vivo and/or by infusion of an enriched population of spermatogonial stem cells. One day it may be possible to "build a testis," in vitro or in vivo, on the scaffold of a decellularized human testis [152].

3.8 Testicular Tissue Organ Culture

Sato and colleagues reported that intact testicular tissues from newborn mice (2.5–3.5 days old) could be maintained in organ culture and mature to produce spermatogenesis, including the production of fertilization-competent haploid germ cells [153, 154]. Testicular tissues from neonatal mice were minced into pieces (1–3 mm^3) and placed in culture at the gas/liquid interface on a slab of agarose that was soaked in medium. In this system that was originally developed to maintain differentiated organs in culture [155], the tissue is exposed to air and absorbs nutrients from the medium through the agarose. Haploid round spermatids and sperm were recovered from the tissue after 3–6 weeks in culture and used to fertilize mouse eggs by ICSI. The resulting embryos were transferred to pseudopregnant females and gave rise to healthy offspring that matured to adulthood and were fertile. If testicular tissue organ culture can be translated to humans, it will provide an alternative to autologous SSC transplantation and xenografting in cases where there is concern about malignant contamination of the testicular tissue. The same authors were also successful to produce haploid germ cells in organ culture of frozen and thawed testicular tissues, which is particularly relevant to the cancer survivor paradigm. However, the fertilization potential of those sperm was not tested [153].

3.9 Induced Pluripotent Stem Cells

Several groups have now reported that it is possible to produce transplantable germ cells or haploid germ cells from pluripotent embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Remarkably, Hayashi and colleagues in Mitinori Saitou's group reported that it is possible to differentiate ESCs or iPSCs into epiblast-like cells that then give rise to primordial germ cells when cultured in the presence of BMP4 [156]. The resulting germ cells were transplanted into the seminiferous tubules of infertile recipient mice where they regenerated spermatogenesis and produced haploid gametes that were used to fertilize mouse oocytes by ICSI. The embryos were transferred to recipient females and gave rise to live offspring. These exciting results suggest that it may be possible for a man diagnosed with azoospermia and who did not cryopreserve semen or testicular tissue to produce sperm from his own skin or other somatic cell type and have biological children. However, it must be noted that some of the offspring in the Hayashi study developed tumors in the neck area and died prematurely, indicating that additional studies are needed to demonstrate the safety and feasibility of this method [156]. Nonhuman primate and human pluripotent stem cells have also been differentiated to the germ lineage, producing putative transplantable germ cells and even rare cells that appear to be haploid [157–167]. Investigation of germ lineage development in human fetal gonads provides a blueprint to help interpret the germ cell phenotypes produced in culture from pluripotent stem cells [168]. The challenge with the human studies is that it is not possible to test the function of the putative germ cells by transplantation or fertilization, which are the gold standards in animal studies. Spermatogenic lineage development and testicular anatomy in nonhuman primates is similar to humans [5], and this may serve as a platform for safety and feasibility studies in which putative germ cells can be tested by transplantation and the resulting gametes and be tested by fertilization [58], embryo transfer, and production of live offspring. Perhaps one day it will be possible to build a human testis in vitro or in vivo, and this will provide the ultimate platform to test the spermatogenic potential of putative human therapeutic stem cells.

Table 3.3 Stem cell therapies for male infertility

Source		Stem cell method		ART method	Ref.
Semen	Sperm			IUI	[177]
				IVF	[178]
				ICSI	[179]
Testicular	Tissue	Xenografting		ICSI	[120, 122, 170–172]
		Autologous graft		ICSI	[121, 134, 173]
		Organ culture		ICSI	[153, 154]
	Cells	SSCs		Transplant	[47–53, 55–61, 66, 80, 89]
		Culture	SSCs	Transplant	[54, 80, 81, 87, 89, 94]
			Differentiated germ cells	ICSI	[65, 174, 175]
		Build a testis/tubules		ICSI	[147–151]
Somatic	iPSC	Germline stem cells		Transplant	[156]
		Haploid cells		ICSI	[157–160, 162–167]

3.10 Conclusions

Assisted reproductive technologies that helped an infertile couple in Great Britain have the world's first test tube baby, Louise Brown (born July 25, 1978) [169], have now produced over five million children worldwide and led to the 2010 Nobel Prize in Medicine for Drs. Patrick Steptoe (physician) and Robert Edwards (researcher). Despite this progress treating infertile couples, many remain beyond the reach of current assisted reproductive technologies because they are not able to produce mature sperm or eggs. This chapter reviews stem cell technologies that are in the research pipeline and may provide new options for those couples (Table 3.3).

The example of the prepubertal cancer patient who is at risk for infertility due to a medical treatment and has no options to preserve his fertility was exploited throughout this chapter. Testicular tissues have been cryopreserved for several hundred patients worldwide, and that number increases every year with increased experience and improved patient and physician education. Therefore, it is incumbent on the medical and research communities to responsibly develop technologies that will allow patients to use those tissues for their reproductive purposes.

Spermatogonial stem cell transplantation is well established in several animal models, including nonhuman primates, and therefore appears to be on the brink of translation to the human fertility clinic. It may seem reasonable to wait for the boys preserving tissue today to mature to adulthood and confirm that they are azoospermic before pursuing experimental stem cell therapies. However, evidence in rodents suggests that engraftment and spermatogenesis from transplanted stem cells are more robust in young recipients [50]. This may be because testicular growth and Sertoli cell proliferation during development create new niches for engraftment by transplanted cells. Due to the nature of scientific investigation, we do not know whether the testicular environment will remain functional and hospitable to stem cell engraftment after years or decades of dormancy. Therefore, we propose that it may be reasonable to pilot spermatogonial stem cell transplantation now in patients who are at the highest risk of infertility due to their medical treatment and who have no risk of malignant contamination (e.g., patients receiving bone marrow transplants for nonmalignant hematopoietic deficits such as β-thalassemia).

Testicular tissue grafting, xenografting, and organ culture still need to be validated using human tissues, but will provide important

alternative reproductive options when autologous transplantation of cells or tissue is contraindicated. Pluripotent stem cell-based methods may be furthest from the clinic, requiring years of safety and feasibility testing. However, these methods will dramatically change the management of fertility preservation patients. It will no longer be necessary to retrieve and cryopreserve reproductive tissues or cells prior to treatment. A cancer survivor who desires to start a family and discovers that he is infertile will theoretically be able to produce fertilization-competent sperm from his own somatic cells and have biological children using established reproductive technologies, IVF/ICSI.

The greatest challenge in the development of stem cell technologies for treatment of human male infertility is the lack of experimental tools for testing the spermatogenic and fertile potential of human cells. This means that human studies cannot be held to the same standard for burden of proof that is required of animal studies. While it is not realistic or possible to demonstrate the fertilization potential of human stem cell-derived gametes, it may be possible to develop systems to test the spermatogenic potential of human cells, such as de novo testicular morphogenesis or engraftment of a decellularized testis. Progress along these lines will provide powerful tools to ensure responsible development and validation of stem cell technologies before they are translated to the male fertility clinic.

Acknowledgments The authors would like to thank the Scaife Foundation, the Richard King Mellon Foundation, the Magee-Womens Research Institute and Foundation, and the University of Pittsburgh Departments of Obstetrics, Gynecology and Reproductive Sciences and Urology who have generously provided funds to support the Fertility Preservation Program in Pittsburgh. It is in this context that we have had the opportunity to meet the infertile patients that fuel our passion for fertility research. The Orwig Lab is supported by the Magee-Womens Research Institute and Foundation, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development grants HD075795 and HD076412, the US-Israel Binational Science Foundation, and gift funds from Montana State University, Sylvia Bernassoli, and Julie and Michael McMullen.

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Part II

Female

Insights into Mechanisms Causing the Maternal Age-Induced Decrease in Oocyte Quality

4

Anna L. Boudoures and Kelle H. Moley

4.1 Overview and Introduction

Female fertility sharply declines with increasing age. One prominent cause of this decline in fertility is that females are born with a fixed number of primordial oocytes that are gradually lost. Although recent evidence suggests that a small pool of oogonial stem cells produces mature oocytes, the contribution of this slowly renewing stem cell pool to the ovarian reserve is not sufficient to prevent menopause [1, 2]. The fate of most oocytes is atresia, which occurs in two phases. First, oocyte number decreases linearly from birth until approximately 35 years of age in humans [3] (equivalent to about 12 months in mice [4]). This loss of primordial follicles can also be observed in mice between the time of birth and sexual maturity [5]. In the second phase, follicle loss increases with each menstrual cycle until menopause at around age 50 in humans [3].

Evidence from in vitro fertilization (IVF) indicates that another contributor to declining fertility is loss of oocyte quality. Women over the age of 50 who used their own oocytes for IVF had significantly lower pregnancy rates than women who used oocytes donated by younger women. In fact, older women receiving young donor oocytes experienced pregnancy rates similar to those of young women undergoing IVF [6]. This suggests that oocyte quality and not endometrial receptivity is the main cause for the age-related decline in fertility.

Here, we will review the recent literature indicating that oocyte quality declines with maternal age because of an accumulation of reactive oxygen species (ROS), which can cause mitochondrial, spindle, and DNA damage. We will also summarize potential avenues of treatment to improve oocyte quality for older women.

4.2 Process of Oocyte Maturation

The development of a mature oocyte capable of fertilization takes place in a series of steps occurring over many years in humans. At birth, all oocytes are arrested at diplotene of prophase I and remain at this developmental stage until puberty (Fig. 4.1a). When oocytes are selected to resume maturation, they contain a prominent nucleus, or germinal vesicle. These immature oocytes are referred to as GV oocytes due to the prominent nucleus (Fig. 4.1b). Oocyte maturation is induced by changes in hormone levels in response to the estrous cycle in most mammals

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_4

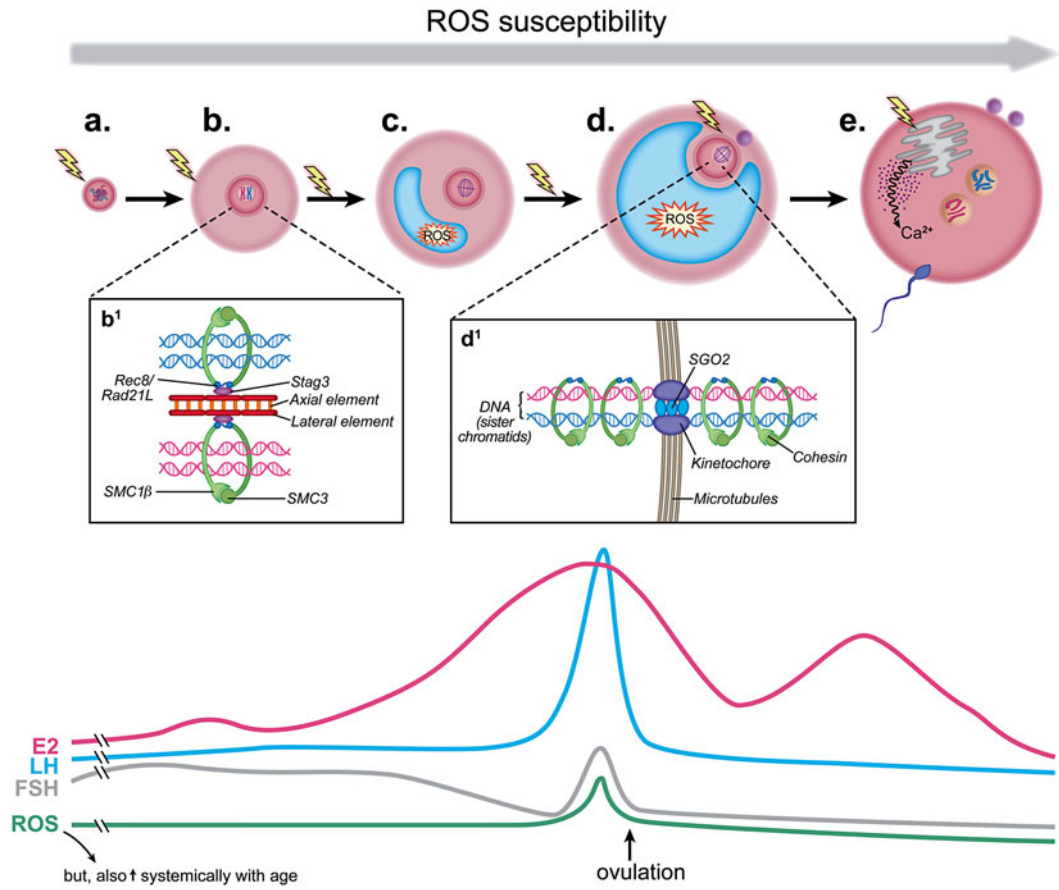

Fig. 4.1 The cytoplasmic and nuclear maturation of an oocyte. All lightning bolts indicate points at which oxidative damage can occur to the oocyte. Along the *bottom* of the figure, the relative levels of hormones are indicated (*FSH* follicle stimulating hormone, *LH* luteinizing hormone, *E2* estradiol) as well as the fluctuation of ROS that are produced during each estrus or menstrual cycle. (**a**) The primordial oocyte is characterized by one layer of surrounding follicular cells. Mammalian ovaries have hundreds of thousands of primordial oocytes to recruit from at the beginning of oocyte maturation. At this time, the nucleus and cytoplasm are susceptible to systemic ROS and ovarian ROS. (**b**) The primary, or germinal vesicle (GV), oocyte has undergone cytoplasmic maturation. The oocyte has increased in size, and many more layers of follicular cells surround and support the oocyte. It is ready for nuclear maturation. Chromosomes begin to condense and the synaptonemal complex forms (shown in detail in **b**^{1}). Crossover events occur at this stage. (**b**^{1}) The synaptonemal complex consists of a cohesin ring with two arms of SMC1β and SMC3 (*green*) held together by Rec8 (*blue*) and joined to the axial and lateral elements (*orange* and *red*, respectively) by Stag3 (*purple*). (**c**) The secondary oocyte has responded to the rise in estrogen levels by resuming meiosis. A spindle forms at this time, and a reductional division of homologous chromosomes occurs at this point. Half of the chromosomes will be extruded in a polar body. A fluid-filled antrum begins to develop within the follicular cells. ROS can accumulate in the antrum, which can damage the oocyte and cumulus cells. (**d**) Just prior to ovulation, a large, fluid-filled antrum has formed within the follicle and the oocyte is arrested in metaphase II. ROS accumulation occurs within the follicular fluid and can be damaging to the oocyte and cumulus cells. The spindle and proteins that hold the sister chromatids together (detailed in **d**^{1}) are susceptible to oxidative damage (indicated by lightning bolt). (**d**^{1}) The synaptonemal complex helps hold sister chromatids together similar to (**b**^{1}), and Sgo2 (*blue*) holds kinetochores together during MII arrest. (**e**) Ovulation is induced by a surge in luteinizing hormone (LH). Cumulus cells have disassociated from the oocyte. If a sperm binds, Ca^{2+} oscillations (*black arrow*) are triggered from the ER (*gray*). These oscillations are altered by oxidative damage (lightning bolt). Meiosis completes and a second polar body is extruded (*purple*). The sperm will release its genetic material into the oocyte as a pronucleus (*blue*) and a female pronucleus will also be visible (*pink*)

and the menstrual cycle in humans (Fig. 4.1). During this process, the ovarian follicles surrounding and supporting each oocyte, and the oocytes themselves, grow in size. The resumption of meiosis is triggered by a surge of luteinizing hormone immediately prior to ovulation, which signals breakdown of the nuclear envelope in a step known as germinal vesicle breakdown (GVBD) [7]. During GVBD, chromatin condenses, the chromosomes align along the metaphase I plate, and a transient but well-defined spindle forms (Fig. 4.1c) [8].

The oocyte cytoplasm divides unevenly at the end of meiosis I, resulting in a formation of a small polar body with half of the chromosomes. The spindle reforms, the chromosomes realign on the metaphase II plate, and the oocyte arrests again until fertilization [9–11] (Fig. 4.1d). At fertilization, meiosis II (MII) resumes leading to the extrusion of a second polar body and a haploid pronucleus in the oocyte [12] (Fig. 4.1e).

The process of oocyte maturation is energy intensive, requiring high levels of nutrient consumption and ATP production to fuel transcription as well as the increases in follicle and oocyte size [13, 14]. The main source of this energy is glucose, which is utilized only by the cumulus cells surrounding each oocyte [15]. The cumulus cells supply the oocyte with pyruvate, which the oocyte uses to generate ATP via oxidative phosphorylation and the electron transport chain (ETC) [15, 16]. Underscoring the importance of sufficient ATP production in oocyte maturation, the ATP content of human oocytes at MII arrest is positively correlated with successful fertilization and IVF outcome [17]. ATP production occurs at three distinct times during oocyte maturation: at GVBD, during spindle migration in MI, and during polar body extrusion at the MI to MII transition (Fig. 4.1b–d) [18]. At each of these times, mitochondria cluster around the nucleus. These observations suggest that ATP produced by mitochondria plays an important role in faithful meiosis and gene expression during nuclear maturation in the oocyte.

4.2.1 Oocyte Reactive Oxygen Species Production

The generation of ATP also results in the production of reactive oxygen species (ROS) as a by-product of the mitochondrial electron transport chain (ETC). In order to generate the necessary proton gradient for ATP production, electrons are passed across the mitochondrial membrane. Inefficiencies in transport generate free radicals, especially at complexes I and III of the ETC, where the free electrons generate superoxide (O_2^-) and hydrogen peroxide (H_2O_2) from water instead of being transferred to their normal intermediates of succinate or $FADH_2$. While this is not the only cellular process that generates ROS, it is a major source of ROS in aerobic cells, including the oocyte. ROS damage DNA and inappropriately modify proteins and unsaturated fatty acids in cell and organelle membranes [19].

ROS cause mitochondrial DNA (mtDNA) damage. mtDNA encodes the ETC proteins, which are necessary for cellular metabolism. ROS-induced mutations in mtDNA create inefficient ETC proteins that increase production of ROS, creating a vicious cycle [20]. Therefore, a major cause of spindle abnormalities and aneuploidy in oocytes may be due to the adverse effects of oxidative stress on mtDNA and ETC proteins.

To study the role of mitochondria and the ETC in oocytes, the mitochondrial membrane potential can be disrupted with FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) [21]. FCCP exposure during in vitro oocyte meiotic maturation delays the completion of meiosis I and meiosis II. FCCP exposure in culture also causes an increased proportion of abnormal spindles and abnormal chromosome alignment in oocytes [22]. Therefore, ETC inhibition causes an increase in spindle abnormalities in oocytes.

Another mechanism to induce oxidative damage specifically to oocyte mitochondria is to use the mitochondrial dye CMXRos combined with photosensitization. Oxidative damage using this technique caused a significant decrease in

meiotic progression and fertilization. Therefore, mitochondria are critical for successful oocyte maturation and fertilization. In the same study, when investigators transplanted the GV nucleus from a damaged oocyte to an undamaged, enucleated oocyte, investigators observed a significant increase in successful meiosis, fertilization, and development to blastocyst [23]. This indicates that oxidative damage specifically to mitochondria leads to decreased fertility.

However, not all effects of ROS on cells, including oocytes, are negative. In oocytes, the initiation of nuclear maturation has been linked to ROS concentration. Depleting ROS and H_2O_2 inhibited ovulation, indicating that ROS are needed to induce oocyte maturation in response to hormone signals (Fig. 4.1). Similarly, it has also been shown that ROS are likely required to activate gene transcription during ovulation [24]. Thus, levels of ROS that are either too high or two low may have detrimental effects on oocyte development.

4.2.2 Compensatory Mechanisms

Because the oocyte relies on ROS to activate gene transcription and oocyte maturation and because oxidative stress is unavoidable, the oocyte has endogenous mechanisms in place to minimize oxidative damage. Oocytes are able to utilize pyruvate for more than just ATP production [25]. Pyruvate is also critical for maintaining the appropriate REDOX potential in the oocyte. Metabolism of pyruvate in the cytoplasm by lactate dehydrogenase (LDH) and by pyruvate dehydrogenase (PDH) in the mitochondria ensures that the appropriate levels of the antioxidants NADPH and NADH are produced to compensate for ROS production during the production of ATP by the electron transport chain [26].

Oocytes also transcribe endogenous antioxidant enzymes. Evidence from hamster oocytes demonstrates a rise in glutathione (GSH), an endogenous antioxidant, occurs concomitantly with chromosome condensation and spindle formation as the oocytes mature. There is also a GSH increase in cumulus cells of maturing oocytes [27]. Therefore, the oocyte has mechanisms in place to combat oxidative damage that is continuously occurring during the growth and maturation processes.

In addition to endogenous antioxidants, the oocyte also has mechanisms to remove damaged mtDNA molecules. Recently, an elegant study in *Drosophila* oocytes demonstrated that mitochondrial function also plays a critical role in mtDNA replication and removal of damaged mtDNA. Investigators showed that ETC protein mutations, such as a mutation to COSVa, impaired mtDNA replication. While quantitation was not possible, observations suggested the oocyte has mechanisms to reduce heteroplasmy by preferentially transcribing copies of mtDNA molecules that did not carry a mutation to the necessary COSVa. Over time, this resulted in the elimination of mutated mtDNA. Furthermore, the elimination of mutated mtDNA continued to occur not only in females' reproductive lifespans but also in her offsprings' lifespan [28]. Therefore, the oocyte has mechanisms in place to reduce heteroplasmy and selectively remove damaged copies of mtDNA. Moreover, mitochondrial function is intimately linked to and dependent on healthy mtDNA.

4.3 Effects of Age on Oocytes

Increased oxidative stress occurs in all tissues with age and is a direct result of ROS produced by the mitochondria. A mouse model with increased expression of the endogenous antioxidant enzyme catalase targeted exclusively in mitochondria significantly lengthens their lifespan by direct reduction of oxidative damage. Importantly, parallel mouse models that targeted catalase expression to either the peroxisome or the nucleus did not have the same effect [29]. Because only catalase expression in mitochondria increased lifespan, mitochondrial ROS production is a main source of oxidative damage that increases with age. While this experiment was in somatic tissues, it is relevant to oocytes because there is an age-related increase in oxidative stress in the oocytes [4, 30–32] that is likely due to

increased production of ROS by inefficient mitochondria.

ROS accumulation has been studied in a variety of ways in fixed oocytes. However, a noninvasive technique to study live oocytes remained elusive until recently. Now, Raman spectra imaging can be applied to oocytes [33]. Raman spectra imaging uses lasers to create vibrational energy within chemical bonds in a single live cell. This vibration energy causes photons to scatter in predictable, unique patterns for different biological molecules. This spectrum can be analyzed by principal component analysis to reveal the unique molecular profiles for individual cells, including oocytes [34]. When CD-1 mouse oocytes were analyzed using Raman spectroscopy, oocytes exposed to oxidizing agents showed a significant change in their lipid profile. Importantly, analysis of live oocytes collected from aged females and young females showed significant differences in between the Raman spectra for multiple molecules. These differences were similar, though not as extreme, as the changes induced by ROS in vitro [33]. This indicates that increased ROS are one mechanism impacting oocyte quality in females of advanced maternal age.

Mammalian oocyte mitochondria are unique in structure and function. Structurally, they are round and may have large, clear vacuoles within the matrix. Cristae either traverse the matrix or are arch shaped and outline its periphery [11]. Functionally, the role of the mitochondria is also unique, as oocyte microinjection with stem cell mitochondria failed to rescue damaged oocytes. However, microinjection of healthy oocyte mitochondria into oocytes from females of advanced reproductive age did improve metabolic parameters [35]. While this technique is not suitable for the clinic, it highlights the unique properties of oocyte mitochondria and the importance of healthy mitochondria in fertilization and development.

The increase in oocyte ROS production with age has been linked to notable, significant changes to mitochondria. Both human and mouse oocytes from females of advanced maternal age have increased mitochondrial aggregates and ROS [4, 36]. Previously, it was shown that aggregated mitochondria are correlated with poor-quality oocytes and decreased fertilization [37]. This would suggest that ROS increase with age and cause a decrease in mitochondrial quality. Supporting ROS-induced mitochondrial damage, the mitochondria in oocytes of aged females also appear abnormal with large vacuoles present [38]. Additionally, ATP content and mtDNA copy number are decreased in both mouse and hamster oocytes from females of advanced maternal age [38]. The decrease in mtDNA copy number indicates decreased mitochondrial numbers, which is supported by lower levels of ATP production.

mtDNA copy number can be used to estimate the total number of mitochondria present in a cell [39]. Oocytes from women of advanced maternal age have decreased mtDNA copy numbers and thus decreased numbers of mitochondria. This decrease in oocyte mtDNA correlated with an increase in the proportion of unfertilized oocytes after IVF. Additionally, fertilized oocytes that developed to cleavage stage embryos also had significantly more mtDNA copies than unfertilized oocytes or zygotes that did not progress. These data indicate that sufficient copies of mtDNA are necessary for fertilization and embryo development [40] and suggest that an age-related reduction in oocyte mitochondria adversely affects fertility and development.

If DNA repair enzyme genes transcribed from nuclear DNA are mutated by oxidative damage, ROS-induced double-strand breaks cannot be repaired in the oocyte. The DNA repair enzymes Brca1, ATM, Mre11, and Rad51 have decreased expression in oocytes analyzed from both mice and humans of advanced maternal age. All of the enzymes listed are important in repairing ROS-induced DNA damage [41]. Whether the decreased expression was due to mutations to DNA by ROS or other changes in the oocyte (such as rates of transcription) was not tested. But, decreased expression of DNA repair enzymes makes the oocytes from older females more susceptible to DNA damage by ROS.

Human oocytes also demonstrate higher levels of oxidative damage. Oocytes from aged women showed increased accumulation of protein markers of oxidative stress as well as markers of protein degradation and apoptosis [42]. Additionally,

when compared to MII oocytes from younger patients, those from women over 38 years old had upregulation of proapoptotic genes and downregulation of antiapoptotic genes [43]. Likely, oxidative damage is initiating apoptosis in aging oocytes.

4.4 Compensation Mechanisms

Oocytes also have mechanisms to reduce oxidative damage. One such mechanism could involve sirtuin-1 (SIRT1), a master regulator of gene expression within cells. SIRT1 gene expression is elevated in oocytes from aged mice [30]. In addition, oocytes from young mice upregulated SIRT1 mRNA if exposed to increased ROS levels, inducing a subsequent increase in the transcription of the ROS scavenger MnSOD. SIRT1 expression in oocytes from aged mice was not as high as in ROS exposed oocytes from young mice. Despite increased SIRT1 transcript, there were significantly lower levels of SIRT1 protein in the oocytes of aged females as compared to young controls. Therefore, aged females were unable to activate the proper SIRT1-induced stress response seen in oocytes from younger females. Unlike young oocytes, oocytes from aged females also demonstrated elevated basal levels of MnSOD expression independent of SIRT1 expression [30]. This data suggests that oocytes from older females are unable to properly respond to the age-induced increase in oxidative damage.

Human oocytes are extremely difficult to obtain for research purposes; thus, to gain insight into oocyte physiology, many studies focus on the cumulus cells which have a close relationship with the oocyte throughout folliculogenesis. Cumulus cells from IVF patients demonstrated abundant expression of two ROS-scavenging enzymes, MnSOD and CuZnSOD, which are negatively correlated with increasing age [44]. A decrease in these enzymes could make the entire follicle more susceptible to oxidative stress and lead to decreased fertility.

Further studies conducted in IVF patients demonstrated that high levels of H_2O_2 in follicular fluid were positively correlated with poor embryo quality, while low levels were positively correlated with empty follicles. Investigators found that intermediate levels of follicular fluid H_2O_2 correlated with good-quality embryos [45]. Therefore, ROS are both necessary for successful oocyte retrieval and embryo development, but if the concentration in the follicular fluid is too high, then the oocytes derived from these follicles will give rise to poor-quality embryos with decreased developmental competence.

4.5 Clinical Effects of Aging

4.5.1 Fertilization

Damage to oocyte mitochondria and low levels of ATP production also lead to decreased fertilization rates. Sperm binding triggers calcium (Ca^{2+}) oscillations within the oocyte, resulting in the completion of meiosis II (Fig. 4.1e). Inhibiting ATP production causes Ca^{2+} levels to drop in the oocyte cytosol. To compensate, the endoplasmic reticulum (ER) releases its Ca^{2+} stores prior to fertilization. Therefore, there is no longer sufficient Ca^{2+} remaining in the ER to trigger the appropriate response in the oocyte when fertilization does occur [46]. A similar premature Ca^{2+} release could be occurring in oocytes as females age, since these oocytes are ATP deficient [38].

Recently, Wakai et al. showed that the fertilization-induced Ca^{2+} oscillations are dependent on both ER Ca^{2+} stores and extracellular Ca^{2+} influx via two ATP-dependent channels, the plasma membrane Ca^{2+}-ATPase (PMCA) and SERCA (sarco-/endoplasmic reticulum Ca^{2+}-ATPase), an ER-specific, ATP-dependent Ca^{2+} transporter. In order to maintain the appropriate concentrations of Ca^{2+} necessary for Ca^{2+} oscillations and successful fertilization, ATP produced by the mitochondria is utilized. Disruption of mitochondrial function in oocytes rapidly depleted Ca^{2+} concentrations within the cytoplasm and the ER. Mitochondrial disruption also severely attenuated and rapidly eliminated oscillations prematurely [47]. This further supports a critical role of healthy, functional mitochondria for successful fertilization. Because oocytes from

aged females have insufficient ATP levels and damaged mitochondria, the ability of the oocytes to trigger the appropriate Ca^{2+} oscillations after sperm binding may be attenuated, preventing fertilization (Fig. 4.1).

After fertilization is achieved, maintaining a proper redox balance is necessary to ensure embryo viability. Inducing oxidative damage to mitochondria during oocyte maturation in vitro caused increased apoptosis prior to fertilization and decreased blastocyst formation after fertilization. This was likely caused by the uncoupling of mitochondrial respiration and a subsequent decrease in ATP content [48]. Increased levels of ROS were positively correlated with increased frequency of embryo fragmentation and apoptosis in human embryos after IVF [49]. If embryos with damaged mitochondria progress past initial cell divisions, development does not occur normally. When two-cell mouse embryos were cultured briefly with agents to damage mitochondria, the embryos developed more slowly and had decreased cell numbers at the blastocyst stage. After being transferred into recipient females, fetuses were smaller at embryonic day 18 [50]. Culturing oocytes with the dye rhodamine-123 and irradiating them induces mitochondrial damage. Applying this technique to mouse oocytes adversely affected the resulting embryos which demonstrated significantly fewer cells in the trophectoderm and decreased implantation rates [51]. These data support the notion that oxidative damage to oocyte mitochondria prior to ovulation and fertilization has negative effects on embryo development.

4.5.2 Spindle Structure and Cohesin Proteins

Sufficient ATP production is critical for appropriate spindle assembly. When oocytes were exposed to increased levels of H_2O_2 to induce oxidative stress, there was a subsequent decrease in ATP production and a corresponding increase in the proportion of spindle and chromosome segregation abnormalities [52]. In a study using oocyte-cumulus complexes taken from a diabetic female mouse model, cumulus cells exhibited decreased glucose uptake. The decrease in glucose availability within cumulus cells correlated with decreased ATP production and an increase in spindle abnormalities in the oocytes of the diabetic females [53]. Similarly, there is a decrease in ATP production in oocytes after oxidative damage. Induced oxidative damage in culture also caused abnormal meiotic spindles and misaligned chromosomes [52]. While these studies use two very different models, the striking similarity is a decrease in ATP production in the oocytes paired with an increase in abnormal spindles. Therefore, sufficient energy production is critical for normal spindle structure.

Chromosome alignment at the spindle equator requires coordinated localization of the cohesin protein complex (Fig. 4.1b). If chromosomes are misaligned, spindles cannot attach and meiosis is delayed [54]. This suggests that not only spindle formation but also chromosome alignment and therefore cohesin proteins are important in meiotic maturation and allowing the oocyte to be ovulated in a fertilizable state.

Cohesin proteins create a ringlike complex around chromosomes in all dividing cells. The cohesin proteins have roles in DNA repair and holding sister chromatids together during mitosis and meiosis (Fig. 4.1b). During meiosis, cohesins also facilitate homologous recombination and resolve the DNA breaks that occur as a result of crossovers. Because cohesins have unique functions in germ cells, oocytes and sperm have specific meiotic homologs of all of the cohesin molecules. These cohesin molecules make up a complex known as the synaptonemal complex (SC) that is present in meiosis I and meiosis II. The SC forms a ringlike structure around chromosomes and is made of four subunits (Fig. 4.1b). In meiosis, the two structural maintenance of chromosome (SMC) subunits, SMC1β and SMC3, form the "arms" of the ring. The non-SMC proteins REC8 and STAG3 hold the SMC subunits together. SMC3 is the only cohesin protein involved in both meiosis and mitosis [55]. During prophase I of meiosis, SC assembly begins along the length of the chromosomes and forms the axial element of the SC. The SC aids in

creating the synapses of crossover events during homologous recombination. In addition to the role in crossover events, the SC also holds sister chromatids together during meiosis I to prevent premature pre-division of sister chromatids [55] (Fig. 4.1).

Due to the critical role of cohesin proteins in ensuring faithful chromosome segregation as well as the increased frequency of aneuploidy with age, researchers have investigated the roles of cohesin proteins during aging. One mechanism involved in the etiology of increased aneuploidy with advanced maternal age is decreased levels of the cohesin proteins [31]. Therefore, investigators created genetic mouse models to mimic this decrease and answer specific questions about the roles of cohesin proteins during meiosis. Deletion of *Rec8* causes sterility in mice. Additionally, oocytes are not able to mature past the primary stage and form a follicle, indicating REC8 is important even early in oocyte cytoplasmic maturation. Finally, *Rec8* knockouts (KO) did not have crossover events, preventing homologous recombination and genetic diversity [56].

To overcome the sterility of *Rec8* KO mice and study the role of REC8 in oocytes, Tachibana-Knowalski et al. [57] created mice with a TEV protease site in the meiotic cohesin REC8. This allowed mice to be fertile and have oocytes that underwent meiotic maturation. To investigate the role of REC8 in meiosis, investigators arrested oocytes at either MI or MII and injected the oocytes with mRNA for TEV protease to induce cleavage of Rec8. Oocyte injection at MI induced premature separation of sister chromatids into bivalents. Similarly, oocyte injection at MII resulted in a premature separation of centromeres. Therefore, Rec8 is necessary for appropriate chromosome segregation during MI and MII [57].

Corroborating this finding, when female mice were generated to be heterozygous for *Rec8*, the number of synaptic errors during prophase I in oocytes was significantly increased as compared to controls. *Rec8* heterozygotes also had significantly fewer crossover sites, indicating *Rec8* has a role in holding sister chromatids together and initiating crossovers [58]. When sections of human ovaries were analyzed for REC8 and SMC1β expression, accumulation of these cohesin proteins decreased significantly in aged ovaries as compared to young ovaries [59]. This suggests that age-associated increases in aneuploidy may be due in part to reduced chromosome cohesion during meiosis.

To assess how frequently MI and MII errors occur with increasing maternal age, the polar body DNA content can be analyzed for hyperploidy (an inappropriate increase in chromosome number). Analysis of polar bodies taken from the oocytes of women undergoing IVF for infertility related to advanced maternal age was analyzed and compared to reproductive outcomes from the same oocytes. Analysis revealed a high percentage of oocytes from older women did not progress through MI or MII faithfully. Additionally, faithful segregation of chromosomes at MI did not guarantee MII segregation would occur without error. One of the most frequently involved chromosomes in aneuploidy was chromosome 21 [60]. Corroborating evidence from mouse models for advanced maternal age shows aneuploidy rates due to nondisjunction at MI increased significantly at 12 months of age and are even higher in 15 months. These events were due mainly to nondisjunction during MI, not premature sister chromatid division during MII [61]. In women, MI errors and trisomy 21 positively correlate with increasing age [62].

Meiosis II errors also increase with maternal age and may be in part due to changes to expression of proteins involved in centromere cohesion. One such protein is SGO2 (Shugoshin-2). A decrease in SGO2 levels in murine oocytes was correlated with increased interkinetochore (iKT) distance and premature sister chromatid segregation during MII arrest. Increased iKT caused oscillation of chromatid pairs at the spindle equator, which would increase the likelihood for aneuploidy at fertilization [63]. Data from this work led the authors to postulate that a decrease in cohesins changes chromosome dynamics during MII due to prolonged MII arrest. Oscillation of chromosomes can also occur if microtubule reattachment to spindles cannot be maintained, which requires the correct expression of cohesin proteins [63]. In a more recent publication, increased iKT

distance was positively correlated with attachment of both kinetochores to the same spindle pole [64], which would lead to aneuploidy.

Changes in iKT distance also occur in women of advanced maternal age. In a study of human oocytes received without ovarian hyperstimulation, there was an increase in iKT distance and hyperploidy with increasing age [65]. This suggests not only that the increased iKT distance is a maternal aging phenotype but also that it is occurring independently of exposure to high levels of exogenous gonadotropins used for infertility treatment. Supporting evidence from aged mice shows that increased iKT distance is positively correlated with age [64, 66]. Additionally, absolute iKT distance in all oocytes from aged mice was on average similar to iKT distance in aneuploid oocytes at all other ages [66], predisposing oocytes to erroneous segregation events and aneuploidy. Furthermore, the significant increase in aneuploidy of oocytes with increased iKT distance suggests some mechanism of cohesion protein loss is leading to aneuploidy.

4.6 Possible Therapies

ROS impact oocyte quality by damaging the mitochondria, DNA, and spindles. However, understanding these mechanisms can guide potential therapeutic strategies using readily available antioxidants and vitamins. Currently, an active area of research uses antioxidant compounds to lower levels of oxidative damage in oocytes, which is promising for women over 35 attempting to conceive. For example, investigators showed that administration of 0.1 mM *N*-acetyl-L-cysteine (NAC, an antioxidant) to mice for 1 year was able to improve fertility and increase trophoblast size in aged mice. There was an also slight improvement to oocyte spindle structure and a decrease in oocyte apoptosis in NAC-treated females. But, the number of oocytes retrieved at MII was not increased in aged females by NAC [67]. This highlights that age-induced oxidative damage to oocytes, not decreased ovarian reserve, is one cause of decreased fertility.

Vitamins E and C also have antioxidant activity in aging females. Dietary supplementation of female mice with vitamins E and C both long term (from weaning) and short term (from 32 months old to sacrifice; 10–15 weeks) increased the number of cytologically normal, retrievable GV and MII oocytes. These vitamins also decreased the percentage of oocytes that were degraded or undergoing apoptosis at older ages [32]. Corroborating evidence from a retrospective clinical study recorded increased intake of vitamin E in women over 35 years old undergoing IVF decreased time to pregnancy as compared to women of the same age who did not conceive [68]. Together, these studies suggest that the antioxidant actions of vitamins E and C are able to improve oocyte quality, and thereby fertility, in both mice and humans.

In addition to antioxidants, enhancing mitochondrial metabolism with specific substrates is also beneficial to oocyte quality. Fatty acids are metabolized in the oocyte mitochondria by β-oxidation, a biochemical process that converts fatty acids to acetyl-CoA in order to be used as a substrate for the TCA cycle and generate ATP. Transport of fatty acids into the mitochondria to be utilized for energy production is dependent on L-carnitine [69]. In oocytes from aged female mice, microinjection of oocytes with L-carnitine and the signaling molecule ceramide reversed age-induced mitochondrial damage and decreased the percentage of oocytes that underwent apoptosis [35]. L-Carnitine supplementation to the culture medium during murine IVF reduced apoptosis and increased the percentage of fertilized embryos that develop to blastocysts. Importantly, L-carnitine was protective against H_2O_2-induced apoptosis [70]. Therefore, L-carnitine may play a dual role during oocyte maturation: one in protecting oocytes and oocyte mitochondria against oxidative damage and one in stimulating fatty acid metabolism in the oocytes.

Dietary supplementation with antioxidants is a feasible treatment. However, care must be taken to avoid overcompensation of antioxidant supplementation. Evidence from mice showed the antioxidant alpha lipoic acid (ALA) exposure in high concentrations was detrimental to follicular

development, oocyte maturation, and preimplantation development despite benefits at lower concentrations [71]. This study demonstrates that antioxidant supplementation can be beneficial, but also that ROS are a necessary component of oocyte maturation and conception, so dosage must be monitored and controlled.

4.7 Conclusions

Maternal age causes an increase in oxidative damage in oocytes. Oxidative damage is linked to spindle abnormalities, decreased ATP content, increased nuclear DNA and mtDNA damage, and inabilities to repair DNA damage efficiently. While an association between oxidative damage and changes to cohesin proteins has not been shown, a feasible hypothesis is that ROS-induced damage to meiotic cohesins causes decreased levels of these proteins. Cohesin decreases cause an inability to maintain chromosome cohesion [58], leading to an increase in aneuploidy as females age.

Accumulation in mitochondrial damage also occurs with aging. This increased damage is likely the root of many issues in oocytes. The oocytes may have mechanisms to counteract this damage [28], but whether or not the mechanisms are sufficient to overcome the rate of damage has not been addressed. Mechanisms to overcome mitochondrial damage include removal of damaged mtDNA and mitophagy (removal of damaged mitochondria) [72]. Whether these processes are highly active in aged oocytes has not been studied.

Finally, treatments to improve oocyte quality may help older women maintain fertility as they age. In particular, targeted treatments to regain the appropriate oxidative balance should be tested. Restoring homeostasis to oocytes would be ideal in preventing age-induced damage. Currently, investigation into antioxidant supplementation is ongoing. However, understanding the underlying mechanism of action of antioxidants within the oocytes is needed. A basic understanding of antioxidants in oocytes will improve two types of therapies: dietary supplementation to women as preventative measure and as a component of oocyte and embryo culture media during IVF to improve outcomes for all patients. In recent years, advances have been made in understanding the cell biology of mammalian oocytes, but large gaps remain. Until these gaps are filled at the basic science level, oocyte quality and a woman's reproductive potential will continue to decline with age.

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Endometriosis and Cancer: Is There an Association?

5

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5.1 Introduction

Endometriosis is an estrogen-dependent disorder that is characterized by the presence of endometrial glands and stroma at sites other than the endometrium. The most common implantation areas are the pelvic organs including the pelvic peritoneum, uterus, ovaries, fallopian tubes, and round and uterosacral ligaments. The presence of extraperitoneal endometriosis has been reported in almost every major organ of the body. The prevalence of endometriosis is difficult to define but is estimated to be as high as 10 % in reproductive-aged women, 30–47 % in infertile women, and 45 % in women with chronic pelvic pain (CPP) [1–5].

Clinically, the most common symptom is pain. In a large national study, 73 % of patients reported having abdominopelvic pain [6]. Endometriosis may present with dysmenorrhea, non-cyclical pelvic pain, deep dyspareunia, infertility, dysuria, dyschezia, hematuria, rectal bleeding, or fatigue [7]. Several studies using self-reported questionnaires have reported a detrimental impact on a woman's physical, social, and psychological functioning, as well as altered pain perception [8–12].

Many differences exist between the endometriotic tissue and normal endometrium, including a higher production of prostaglandins, cytokines, estrogen, and metalloproteinases—combined with an altered immune response, angiogenesis, and apoptosis—which may lead to the proliferation of the diseased tissue [13–17].

5.2 Endometriosis and General Malignancies

The association of endometriosis with an increased risk of developing cancer at sites other than the ovary remains controversial. Heaps et al. [18] reported that malignant transformation of endometriosis can occur in 1 % of cases. Given the strict criteria used to define endometriosis-associated cancer and the underreporting of endometriosis in cases of cancer, the actual percentage may be higher.

The first epidemiological study establishing an association between endometriosis and malignancies was completed by Brinton et al. [19]. Based on a hospital diagnosis of endometriosis at

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_5

discharge and using the National Swedish Patient Register (NSPR), 20,686 women with a mean follow-up of 11.4 years were evaluated. The authors concluded that the overall cancer risk was 1.2 % (95 % CI [confidence interval], 1.1–1.3). A standardized incidence ratio (SIR) was used to estimate the association and found an increased risk for breast cancer (SIR 1.3), ovarian cancer (SIR 1.9), and, interestingly, non-Hodgkin's lymphoma (SIR 1.8). In a subsequent study with a longer follow-up (mean 12.7 years), Melin et al. [20] evaluated 64,492 women in the largest cohort published. The authors found no increased overall risk of cancer (SIR 1.04, 95 % CI 1–1.07), but an increased risk for ovarian cancer (SIR 1.43), endocrine tumors (SIR 1.36), non-Hodgkin's lymphoma (SIR 1.24), and brain tumors (SIR 1.22) was found. The Iowa Women's Health Study used a self-reporting model on 37,434 patients with a mean follow-up of 13 years [21]. A total of 3.8 % reported a history of endometriosis with no increased overall risk of cancer. Specifically, no association was found with ovarian or breast cancer. However, an association to non-Hodgkin's lymphoma was reported (RR 1.8). The relationship between endometriosis and the elevated risk of non-Hodgkin's lymphoma appears to originate from a defective immune system, but further studies are warranted [20].

There appears to be a strong relationship between a positive history of breast cancer and endometriosis (odds ratio (OR) 6.9) [22]. Although, subtypes of breast cancer are not specified in the epidemiologic studies, the relationship may lie within abnormalities in estrogen and progesterone receptors. [23–25]. Women with endometriosis should be carefully counseled regarding screening for breast cancer [26].

Women with laparoscopically confirmed endometriosis were found to have an increased risk for a family history of cancer compared to women without endometriosis (OR 7.7, 95 % CI 3.8–15.7), most notably ovarian cancer (OR 10.5; 95 % CI 2.5–44.2), colon cancer (OR 7.5; 95 % CI 2.7–21.2), and prostate cancer (OR 4.5; 95 % CI 1.4–15.3) [27]. Possible similar genetic and molecular mutations between both diseases could play a role in the pathogenesis, and the authors suggest further analysis [22, 28–30].

Several case reports and case series have been published on endometriosis-associated cancers. More than 20 % of all endometriosis-associated cancers will be extra-ovarian [18]. Patients with extra-ovarian endometriosis-associated cancer are more likely to be postmenopausal and have used unopposed estrogen hormone replacement therapy (HRT) [31, 32]. Although one study reported that up to 25 % of extra-ovarian endometriosis-associated cancers are uterine sarcomas, this association has not been observed in large epidemiologic studies [18, 20]. In extrauterine malignancies associated with endometriosis, up to 32 % of patients may have endometrial abnormalities, including hyperplasia and cancer [33]. More than 50 % of the extra-ovarian malignancies associated with endometriosis have been reported in the rectovaginal septum, colon, and vagina [34, 35]. Other intestinal [36–38] and cervical cancers [39] have been reported.

5.3 Endometriosis-Associated Ovarian Cancer

In 2014, it is estimated that 14,572 women will die from ovarian cancer, the fifth leading cause of death among all cancers in women and the most common cause of death from gynecologic malignancies [40]. Ovarian cancer has a poor prognosis largely because 75 % of cases are diagnosed in advanced stages, which lowers the 5-year survival rate to an estimated 44 % [40, 41].

The association between endometriosis and ovarian cancer has been well documented [42]. The criteria to identify the malignant transformation of endometriosis, initially proposed by Sampson in 1952, are still in use today: (a) evidence of endometriosis close to the tumor, (b) the carcinoma must be seen to arise in endometriosis and not to be invading other sources, and (c) the presence of tissue resembling endometrial stroma surrounding characteristic glands [42]. A fourth criteria, proposed by Scott in 1953, included histopathology to confirm the presence of benign endometriosis adjacent to the malignant tissue,

but this has been reported to be difficult to assess due to the often malignant transformation of initially benign lesions [43, 44]. Although these criteria are rarely met, they serve as evidence of the long-standing recognition by clinicians and pathologists of an association between endometriosis and ovarian cancer.

In their initial epidemiologic report, Brinton et al. [19] reported an increased risk for developing ovarian cancer in women with a history of endometriosis (SIR 1.9; 95 % CI 1.3–2.8). Since then, others have confirmed these associations using case-control studies and self-reported questionnaires, though the results are limited by unreported confounding factors, such as oral contraceptives. Only one prospective cohort study did not find an association between ovarian cancer and endometriosis [20, 21, 45–53]. A systematic review suggested that the effect size was modest (SIR, OR, and RR 1.32–1.9; 95 % CI) [54]. The risk is further elevated in endometriosis patients with primary infertility versus infertile patients without endometriosis (RR 2.77; 95 % CI 1.1–6.7) [45]. This risk appears to increase with a long-standing diagnosis of endometriosis [19].

A recent retrospective cohort study of subfertile women with a median follow-up of 15.2 years included 3,657 women with endometriosis and 5,247 without [55]. For the 78 % of the patients with endometriosis confirmed by pathology, the hazard ratio (HR) for ovarian cancer was 12.4 (95 % CI 2.8–54.2), compared with a HR of 4.3 (95 % CI 1.6–11.2) in those without a histologic diagnosis of endometriosis. These findings suggest that relying on operative reports might underestimate the association. Another interesting finding in this study was that both extraovarian and ovarian endometriosis had similar risks of ovarian cancer.

Endometriosis-associated ovarian cancer (EAOC) represents the majority of endometriosis-associated cancers [33]. Several differences between epithelial ovarian cancer (EOC) and EAOC exist. In Western countries, endometrioid and ovarian clear-cell carcinomas represent 10–20 % and 5–10 % of all EOCs, while high-grade or type II cancers represent 70 % [56]. This is in contrast with EAOC, where endometrioid and ovarian clear-cell carcinomas represent 19 % and 35.9 % of all ovarian cancers [57]. A recent study found an OR of 3.05 (CI 2.43–3.84) for developing clear-cell subtype and an OR of 2.04 (CI 1.67–2.48) for endometrioid subtype [48].

Other differences between both ovarian cancers include a lower mean age of presentation 48.3 (standard deviation ±10.8 years) in EAOC when compared to OC 53.8 (standard deviation ±11.4, $p=0.003$) [49]. It has been argued that the lower mean age of diagnosis may to be related to the overall earlier presentations of clear-cell and endometrioid ovarian cancers as compared to type II ovarian cancers.

5.4 Precursor Lesions

Due to the high concurrency of endometrioid and clear-cell subtypes with endometriosis and after noticing a direct continuity from benign lesions to malignant transformation, endometriosis has been proposed as a precursor lesion that may undergo neoplastic transformation [44, 58–63]. A model of progression has been proposed where benign endometriotic lesions undergo neoplastic changes initially through atypia and finally to malignancy [59].

Initially described by Czernobilsky and Morris [64], mild endometriosis atypia is considered when the epithelial layer has eosinophilic cuboidal or flattened cells with hyperchromatic and pleomorphic nuclei. The criteria for severe endometriosis atypia were revisited in 2000 by Thomas and Campbell [65], who proposed a reduced cytoplasm/nucleus ratio, cellular stratification, and an abnormal large pleomorphic nucleus with either hyperchromatic or hypochromatic characteristics.

Atypical endometriosis has been found in 61–100 % of patients with EAOC [60–62], with a prevalence of 8 %, and can be found in 1–3 % of endometriotic cysts [44, 57, 66–68]. The malignant potential of atypical endometriotic lesions is not well understood, but closer surveillance in these patients is recommended.

Several genetic alterations including ARID1 mutations and hepatocyte nuclear factor-1b

(HNF-1b) upregulation have been found in atypical endometriosis. ARID1 mutations were found in lesions surrounding ovarian clear-cell carcinomas but not on distant lesions, thus suggesting a direct linkage [69]. HNF-1b has been found to be upregulated in up to 40 % of atypical endometriosis and endometriotic cysts [70].

Borderline ovarian tumors (BOTs) have been proposed to be a steppingstone to the development of EAOC. In a retrospective study, 13 % of BOT had concurrent endometriosis [71]. A recent study showed an elevated risk of BOT with endometriosis (HR 5.5; 95 % CI 1.5–20.2), and several case reports exist [33, 46, 48, 52, 55, 72]. The association still remains controversial and authors have failed to find an association in some instances [48, 73]. These findings support a possible progression [74]. Although endometriotic BOTs are uncommon, recent data suggests that the more common mucinous and serous BOTs share several similar histogenetic links and could arise from atypical endometriosis in some cases [75]. This progression is yet to be confirmed; one large pooled analysis of case-control studies did not find an association [48]. No large studies on atypical endometriosis and BOTs have been reported, but an association would be expected. Currently, only case reports of BOTs and atypical endometriosis have been reported [76].

5.5 Pathways to Ovarian Cancer

Several pathways appear viable in the association of endometriosis and ovarian cancer. The most widely accepted theory is that a combination of epigenetic, hormonal, inflammatory, and immunological factors plays a role in cancer development [14, 77].

5.5.1 Immunology

With the growing evidence of an altered immune response, several authors have proposed endometriosis as an autoimmune disorder [78, 79]. Abnormalities of $CD4^+$, $CD25^+$, and $FOXP3^+$ regulatory T cells (Treg cells) have been implicated in a variety of autoimmune diseases and ovarian cancer [80]. Treg cells promote immunologic tolerance and suppress the immune system through macrophages, natural killer (NK) cells, dendritic cells, and cytotoxic T cells and have been studied as immunotherapy in several autoimmune disorders [81, 82]. The presence of Treg cells has also been correlated with poor prognosis in ovarian cancers [83].

The presence of Treg cells in eutopic and ectopic endometrium in endometriosis has been documented [84]. One study found increased Treg cells in the peritoneal fluid and decreased levels in the peripheral blood of women with endometriosis compared to controls without endometriosis. The authors suggest a possible shift from the peripheral blood to the affected tissue as a compensatory method to modulate inflammation [85]. Treg cell abnormalities, such as polymorphisms of FOXP3 gene and increased levels of transcriptional factors for FOXP3, have been found in endometriotic lesions [86, 87]. Alterations in the response and cytolytic action of NK cells have also been linked to endometriosis [88]. Both the increased number of Treg cells with the recent genetic alterations reported in the FOXP3 gene and the alterations in NK cells might play a role in the reduced response to inflammation found in patients with endometriosis and the poor clearance of lesions.

Several factors and cytokines—including interleukin (IL)-1B, IL-6, IL-8, transforming growth factor (TGF), epidermal growth factor (EGF), insulin growth factors (IGFs), platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF), and vascular endothelial growth factor (VEGF)—have been identified in patients with endometriosis and may promote the development, implantation, and survivability of endometriosis by promoting angiogenesis [89, 90].

5.6 Genetic Alterations

Recent data suggests a genetic difference between high-grade and low-grade serous ovarian cancers. Low-grade cancers will generally exhibit

KRAS, BRAF, ERBB2, PTEN, CTNNB1, and/or PIK3CA7 mutations. High-grade cancers will usually exhibit a TP53 (>80 % of cases) and/or CCNE1 mutation [91, 92]. Given the differences in the genetic profile, Shih et al. [74] proposed that ovarian cancer should be divided into type I and type II. Type I will usually be less aggressive and low grade, progressing from a benign cystic or a borderline tumor, and comprised of micropapillary serous carcinoma, mucinous, endometrioid, and clear-cell carcinomas. Type II will usually be high grade and aggressive, usually being diagnosed at an advanced stage and comprised of high-grade serous, malignant mixed mesodermal (carcinosarcomas), and undifferentiated carcinomas.

Several genetic alterations have been described in EAOC. Loss of heterozygosity (LOH) and genetic instability, especially in ovarian endometriotic cysts, have been detected in chromosomes 1p, 9p, 11q, 17p, and 22p [93]. Mutations of PTEN, a tumor suppressor gene, have been documented in ~75 % of EAOC and 15 % of endometriosis cases [94]. More than 50 % of mucinous carcinomas versus 29 % of EAOCs will exhibit KRAS mutations [95]. KRAS alterations, such as polymorphisms, have been found to increase the risk of ovarian cancer [96, 97].

HNF-1b is a homeobox transcription factor that has been found to be abnormally upregulated in clear-cell ovarian cancers. It has a central role in controlling cell proliferation and anti-apoptosis [98]. Through their findings on HNF-1b, Kato et al. [70] suggested that endometriosis, specifically ovarian endometriosis, undergoes differentiation into clear-cell cancer likely through inflammation and regeneration. They found that at least 40 % of endometriotic cysts, endometriosis with reactive atypia (inflammation), and atypical endometriosis have HNF-1b presence and are rarely expressed in other types of cancer [99].

Mutations of ARID1A, a tumor suppressor gene that participates in chromatin remodeling, have been associated with ovarian clear-cell carcinoma (present in 46–57 % of cases) and endometrioid cancers (present in 30 % of the cases). PPP2R1A, a proto-oncogene, has also been associated to ovarian clear-cell carcinoma [69, 100]. Loss of ARID1A has been found in the majority of endometriotic lesions and in cases with clear-cell EAOC, suggesting its loss as an early insult in the progression to cancer [101].

5.7 Reactive Oxygen Species

The role of reactive oxygen species (ROS) in different types of cancer is well documented. Free iron is a known stimulator of ROS and has been found in greater concentrations in endometriotic versus other ovarian cysts [102]. After phagocytizing red blood cells from chronic menstruation and bleeding, macrophages get overwhelmed—leading to high concentrations of free iron in the cyst fluid and increased levels of ROS. Oxidative stress promotes DNA alterations through strand breakage, DNA mutations, cell membrane damage, and lipid peroxidation that could all affect proto-oncogenes and tumor suppressor genes. Among 54 genes to be found upregulated in ovarian clear-cell carcinomas, 87 % are associated with a reduction-oxidation (redox) state of cells; this suggests that impairment of the redox balance may contribute directly to the development of EAOC, especially in the clear-cell subtype [103–105].

5.7.1 Inflammation

Endometriosis as an inflammatory disease has been studied thoroughly. Peritoneal fluid in women with endometriosis has been found to have an elevated number of macrophages and dendritic cells, producing cytokines activating cyclooxygenase-2 (COX-2) and stimulating excess prostaglandin E2 (PGE2) production [90, 106–109]. PGE2 has been found to regulate tumor proliferation [110]. As discussed before, several cytokines promote angiogenesis and cellular survivability in endometriosis. Elevated TNF has been found in EAOC and OC and is linked with tumor promotion. Higher levels of TNF-α correlate with a higher grade of ovarian cancer [111].

5.7.2 Estrogen

Prostaglandin E2 has also been found to be a major player in the stimulation of local estrogen production in endometriosis. Compared to the normal endometrium, endometriosis lesions have the nuclear receptor steroidogenic factor 1 (SF1). SF1 mediates the steroidogenic acute regulatory protein (STAR) and CYP19A1, which encodes the aromatase enzyme, giving endometriosis lesions the ability to locally convert estradiol from cholesterol [112, 113].

A high estrogenic environment serves as a stimulator of cytokine production—specifically IL-8, regulated on activation normal T cells expressed and secreted (RANTES), and prostaglandin E2 through the activation of COX-2—which in turns stimulates estrogen production, creating a vicious cycle allowing for DNA alterations [114–116].

Progesterone resistance and low progesterone receptor levels have been found in endometriosis lesions [117, 118]. Endometriosis cells do not express 17β-hydroxysteroid-dehydrogenase (17β-HSD), which is induced by progesterone in eutopic endometrial cells and acts by converting E2 to the milder estrone [119]. Mainly composed of stromal cells, endometriosis and EAOC will likely not benefit from progesterone therapy as much as endometrial cancer, where there is epithelial cell proliferation [13].

Apart from local estradiol production, the risk factors for EAOC may include high estrogenic states [120], tamoxifen use [121, 122], increased exposure to menstruation (menometrorrhagia or long-lasting short cycles, nulligravidity), and increased body fat [123].

5.8 Surgical and Hormonal Treatment and Risk Reduction

The protective effect of oral contraceptive pills (OCPs) in ovarian cancer overall is well known [124]. Limited data is available on the effects of OCPs for endometriosis treatment and the overall risk reduction. Hormonal treatment with OCPs has been found to decrease the risk of EAOC in two studies. One of them found an 80 % decrease with >10 years of use [125, 126]. This association was not observed in other studies, possibly because the database included patients as far back as the 1960s, when OCP use was not as common [47].

Other hormonal agents commonly used for endometriosis have been examined in two studies [47, 125]. Lupron failed to demonstrate a protective effect for EAOC (OR 1.0; 95 % CI 0.4–2.4) and 0.85 (0.66–1.09). Danazol was actually found to be associated with an increased risk of developing ovarian cancer in both studies: OR 3.2, 95 % CI 1.2–8.5, and OR 1.06, 95 % CI 1.00–1.12. Hormone replacement therapy (HRT) has also been found to be associated with an increased risk of EAOC and, in general, ovarian cancer [31, 127–129].

Although the protective effect of hysterectomy, tubal ligation, and salpingectomy has been well documented on EOC, little is known about the effect of surgery on EAOC [53, 126, 130–132]. Through a population-based, case-controlled study, Rossing et al. first suggested a protective effect of surgery in preventing EAOC. They reported that women with a history of endometriosis who had undergone unilateral oophorectomy had a reduction in ovarian cancer (OR 0.8; 95 % CI 0.3–2.1) compared to women with a lesser extent of ovarian surgery (OR 3.3; 95 % CI 0.7–15.3) [50]. Using the National Swedish Patient Register (NSPR), Melin et al. described a significant risk reduction in EAOC for patients with a history of endometriosis who underwent either unilateral oophorectomy (OR 0.19; 95 % CI 0.08–0.46) or removal of all visible endometriosis (OR 0.30; 95 % CI 0.12–0.74). Interestingly, no risk reduction was observed after hysterectomy, tubal ligation, or salpingectomy. They concluded that if the affected ovary was removed, there was an 81 % risk reduction with the number needed to treat (NNT) of 62 and a 30 % risk reduction if all visible endometriosis was removed with a NNT of 95 [47].

The effect of ovarian cystectomy for endometriomas on subsequent cancer risk reduction has not yet been evaluated in the literature. Although removal of these cysts may be tempting and seem logical, caution must be exercised due to the

possibility of causing diminished ovarian reserve [133]. Surgical removal of ovarian endometriomas has been found to have lower cyst recurrence rates and better subsequent pain relief than ablative techniques [134, 135]. Excisional surgery should be preferred over ablation while being mindful to avoid excess coagulation. The use of hemostatic agents may help in this regard, although their effect on ovarian function has not been addressed in the literature.

5.9 Management of Malignancies

Clinical outcome reports on endometriosis-associated ovarian cancer are rare.

A report by Leiserowitz et al. [32] noted that the ovaries were predominantly the primary site of cancer (62 %) with endometrioid (66.7 %) and clear-cell (14.8 %) tumors being the most common histologic types. They concluded that surgical staging, similar to staging for ovarian cancer, is appropriate for determining the subsequent treatment.

Despite that, EAOC has more favorable characteristics than non-EAOCs, including earlier stage and lower-grade lesions that are likely secondary to their associated subtypes (clear-cell and endometrioid). Mixed outcomes have been reported [31, 32, 136, 137]. A recent meta-analysis failed to find improved survival rates on EAOC compared to the same stage non-EAOC [138]. Treatment options are the same as non-EAOC, including primary cytoreductive surgery with adjuvant taxane, platinum-based chemotherapy, and/or radiation.

5.9.1 Early Detection of EAOC

Early detection and treatment of ovarian cancer substantially improves survival rates [139, 140]. Despite considerable efforts to develop a clinically useful and cost-effective screening test utilizing genes, biomarkers, and ultrasound characteristics, results have been unsuccessful so far, but recent studies appear promising.

The Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, a randomized controlled trial (RCT), failed to reduce mortality rates using a combined screening of transvaginal ultrasound (TVUS) and CA-125 (cut-off $\geq$ 35 kU/L) [141, 142]. In 2012, the United States Preventive Services Task Force (USPSTF) recommended against screening for ovarian cancer in the general population [143]. The United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), a large RCT consisting of 202,638 women, used the risk of ovarian cancer algorithm (ROCA), followed by TVUS (multimodal screening [MMS]), and showed promising sensitivity (89.5 %) and positive predictive value (PPV) (35.1 %) [144]. The mortality rates from this study are not yet available. Using ROCA, patients would undergo TVUS depending on the CA-125 level, and those findings are correlated to the incidence of ovarian cancer based on age [145]. A recent prospective study validated the high PPV (40 %) and specificity (99.9 %) using ROCA. Additionally, early stage cancers were detected (stage I or II) [146].

CA-125 appears to be lower in EAOC than in women with non-EAOC (122.9 vs. 1,377.5) and is more likely to be normal (47.1 % vs. 10 %) [147]. Despite the limitations of screening methods, the majority (67 %) of EAOCs are found in early stages, likely due to having a large percentage of slow-growing clear-cell or endometrioid subtypes [148].

The true value in prevention and early detection may lie in the identification of atypical endometriosis. Atypical endometriosis has a prevalence of up to 8 %. Recently, a protein, IMP3, has been proposed as an immunochemical biomarker. When correlated to histological diagnosis, a wide difference was observed in the sensitivity reported—88.9 % vs. 33.3 % [68]. The role of this new biomarker is yet to be defined.

Plasma microRNAs (miRNAs) have been reported in a variety of cancers, including lung, gastric, breast, pancreatic, and ovarian cancers. A recent study has shown differences in plasma miRNA signatures between serous ovarian carcinomas, endometriosis, EAOC, and healthy individuals. Overexpression of a combination of miR-16, miR-21, and miR-191 may represent an exclusive finding for EAOC. These findings are

yet to be validated, but interestingly, miRNA signatures varied between plasma and tissue which could represent a systemic response to the disease [149]. The future direction of early detection in ovarian cancer, including EAOC, will focus in large part in miRNA as well as in other plasma/serum biomarkers.

5.10 Conclusions

Endometriosis has the potential to give rise to various types of cancers, mainly the ovarian endometrioid and clear-cell subtypes. Epigenetic susceptibility, along with alterations in the oxidative stress, inflammation, and estrogen pathways, most likely in combination with a defective immune system, may mediate the conversion of endometriosis to endometrial cancer. There appears to be a risk reduction with the removal of all visible endometriosis lesions and/or oophorectomy, but more studies are warranted given the high morbidity associated with treating advanced stage endometriosis cases and the reduction in ovarian reserve associated with the removal of ovarian endometriomas. Recent data suggests a stronger association with ovarian cancer than previously attributed to endometriosis. It is questionable whether aggressive surgery and/or treatment with COX-2 inhibitors, aromatase inhibitors, or OCPs should be recommended to diminish the risk of malignant transformation.

Due to the low risk and small number of patients, current data is mostly observational and further studies are needed to attempt to differentiate endometriotic lesions at risk of transformation. Future studies are likely to focus on novel serum markers for early detection and histologic biomarkers for risk stratification.

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Ovarian Tissue Cryopreservation: Where Are We Now?

6

Giuliano Bedoschi and Kutluk Oktay

6.1 Introduction

Ovarian tissue cryopreservation is an experimental technique for fertility preservation. Harvesting of ovarian tissue for cryopreservation is typically performed by laparoscopy except in cases where the patient needs to undergo abdominal surgery for the treatment of cancer. In which case, the ovarian tissue can be harvested during the same surgical procedure. The overall approach involves cryopreservation, followed by thawing and orthotopic or heterotopic tissue transplantation of ovarian tissue.

Advantages of this approach include the lack of a need to significantly delay cancer treatments since it does not require ovarian stimulation, the lack of a need for a partner, and, when successfully transplanted, the ability to restore ovarian endocrine function and spontaneous fertility. Ovarian cryopreservation and transplantation may be offered to sexually immature girls or post-pubertal females, those with an urgent need to start cytotoxic treatment, as well as those who are diagnosed with estrogen-sensitive cancer and do not wish to undergo ovarian stimulation.

Ideally, ovarian tissue harvesting for the purpose of cryopreservation should be carried out before the initiation of cytotoxic treatment since each round of chemotherapy will diminish ovarian reserve in an accumulating manner. However, it may still be feasible to harvest ovarian tissue after initiation of the first courses of chemotherapy, especially in younger patients with large ovarian reserve, as there does not appear to be residual damage on survival primordial follicles.

6.2 Ovarian Tissue Cryopreservation Methods

Cryopreservation of ovarian tissue combined with orthotopic transplantation into an irradiated ovary restored fertility in rodents over 50 years ago [1]. It took more than 30 years, however, to improve the cryopreservation protocols and transplantation procedures in larger animals and, in particular, in species that could be considered applicable to humans. In fact, the only available early cryoprotectant was glycerol. Although sufficient for freezing sperm, it was highly ineffective for cryopreserving oocytes and ovarian tissue [2]. In the 1970s, more effective cryoprotectants emerged, such as propanediol, ethylene glycol, and dimethyl sulfoxide (DMSO). The exhaustive studies of ovarian cryopreservation and

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_6

transplantation conducted in animals, and particularly those in the lamb model [3], finally led to significant improvements in cryopreservation and transplantation techniques and the establishment of a model for applying these investigational procedures to humans. In this model, the ovarian tissue was cryopreserved after improving the current slow-freezing protocols using DMSO as a cryoprotectant. The ovarian tissue was frozen in slices and the thawed slices were later transplanted orthotopically. In the lamb, recovery of cyclic ovarian activity was demonstrated, and pregnancies and live-born lambs as well as long-term ovarian function were achieved [4].

A method for cryopreservation of human ovarian tissue was first reported in 1996 [5]. Several cryoprotectants such as glycerol, ethylene glycol, DMSO, and propanediol were investigated for slow cryopreservation of human ovarian tissues. The highest follicle survival rates were found with ethylene glycol though statistically similar to propanediol or DMSO, whereas glycerol has been associated with the poorest results [2].

Studies investigating the most favorable cooling rates and dehydration times have also been conducted. After evaluating the tissue by electron microscopic morphology, slow programmed freezing with a relatively long dehydration time became a standard cryopreservation method for human ovarian tissue from the end of the 1990s [6]. However, consensus is lacking about how much ovarian tissue should be harvested for cryopreservation, and some centers for fertility preservation propose unilateral oophorectomy as the standard procedure [7, 8].

A concern with slow freezing has been the relatively poor survival of the ovarian stroma [9], which has been demonstrated by transmission electron microscopy studies [10]. The introduction of vitrification techniques for cryopreservation of ovarian tissue has been claimed by some experts to improve the viability of all compartments of the tissue, with a survival rate of follicles similar to that of after slow freezing and retention of much improved integrity of ovarian stroma and no damage to the morphology of the blood vessels [10].

Ovarian tissue vitrification in combination with orthotopic autotransplantation has been successful in rodents [11] and sheep [12]. Human studies comparing slow-freezing protocols with vitrification of ovarian tissue have produced conflicting results, which may be explained by differences in the protocols and the media used [13, 14] as well as the larger size and more fibrous nature of the human ovary compared to the laboratory animal counterparts. While vitrification may be easier to achieve in a single cell like the oocyte, thick and multicellular ovarian tissue may not lend itself to an idealized vitrification protocol as the success of vitrification depends on the rapid and uniform penetration of cryoprotectants.

6.3 Transplantation of Cryopreserved Ovarian Tissue for Restoring Ovarian Function and Fertility in Humans

6.3.1 Orthotopic Transplantation

The successful development of cryopreservation techniques for human ovarian tissue stimulated the initiation of ovarian tissue freezing as an option to preserve female fertility from the late 1990s. In the year 2000, the first case of successful orthotopic autotransplantation of cryopreserved and thawed ovarian tissue was reported [15]. The patient had undergone oophorectomy at the age of 28 years owing to intractable dysfunctional bleeding, and her ovarian tissue was frozen by using a slow-freezing protocol with propanediol as the cryoprotectant. Six pieces of cortex were cultured in vitro for 6 days in the presence of gonadotropins, and increased amounts of estradiol, progesterone, and testosterone could be measured. The ovarian tissue was transplanted into peritoneal pockets created in the pelvic sidewall (ovarian fossa) posterior to the broad ligament by a laparoscopic technique [16]. Ovulation induction with gonadotropins 15 weeks after transplantation demonstrated follicular development and ovulation, which was corroborated by

rising estradiol and subsequently progesterone levels. A corpus luteum in the transplant confirmed ovulation by ultrasonography. The endometrium also showed follicular- and luteal-phase echogenic changes, and the patient menstruated 2 weeks after ovulation.

Since 2004, several centers have reported cases of orthotopic transplantation of cryopreserved ovarian tissue in cancer survivors worldwide, some resulting in live births [17–20]. A recent review of the first 10 cases of successful orthotopic transplantation, which has resulted in the birth of 13 children, indicates that age at cryopreservation may be an important prognostic factor, as all except one woman in that report were younger than 30 years and six of the women were younger than 25 years [21]. As noted by the American Society of Clinical Oncology recommendations on fertility preservation, the benefit of ovarian cryopreservation for women older than 40 years of age is uncertain because of their age-reduced ovarian reserve of primordial follicles [22].

In reports of successful ovarian transplantation, restoration of ovarian function has been shown by the rise of estrogen levels and decrease of gonadotropin levels between 3.5 and 6.5 months after ovarian grafting [21]. Ovarian transplants in those cases have been shown to become functional after grafting in a peritoneal window close to the ovarian hilus and on the ovarian medulla, as proved by follicle development.

In all cases, slow programmed freezing was used to cryopreserve the ovarian tissue, and both large strips of 8–10 mm × 5 mm and small pieces of 2 × 2 mm of tissue were shown to restore the ovarian endocrine function effectively. More than 50 % of the women in that report conceived naturally; only a few women required IVF to become pregnant [21]. It is worth mentioning that a recent report of preparation of thin ovarian fragments for transplantation (less than 350 μm thickness) led to a successful pregnancy after failure of ovarian strip transplantation on two previous occasions [23].

A recently published study summarized the success rates of orthotopic ovarian transplantation from 60 cases and found the success rates to be inconsistent [24]. In that report, authors found that among the 60 patients, 11 conceived with 6 having delivered 12 healthy babies at the time of writing. The authors concluded that improvements in pregnancy rates required further research: (1) to improve freezing techniques and (2) to enhance the "vascular bed" before transplantation to increase pregnancy rates in the future.

6.3.2 Heterotopic Transplantation

The location for heterotopic ovarian transplantation may include the forearm [25] or lower abdominal subcutaneous tissue [26]. Both techniques have resulted in the restoration of hormonal function, follicle development, and oocyte retrieval. In addition, we have succeeded in the embryo generation after IVF of oocytes recovered from heterotopic transplants [26, 27]. Recently, researchers from Australia have reported an ongoing pregnancy from an ovarian tissue transplanted in the abdominal wall, though not subcutaneously [28]. The scarcity of pregnancies from heterotopic ovarian transplants is partly due to the very few attempts being made and possibly secondarily to the differences between the heterotopic and orthotopic microenvironment.

Interestingly, after one case of heterotopic ovarian transplantation, spontaneous pregnancies have occurred [29]. Four pregnancies and three live births resulted within the time span of 5 years after the subcutaneous transplantation of the previously frozen ovarian tissue to the lower abdominal wall. The patient had become menopausal after ovarian cryopreservation and receiving preconditioning chemotherapy for HSCT for relapsing Hodgkin's lymphoma [30]. Several case reports have described spontaneous pregnancies resulting in women who have undergone ovarian graft transplants simultaneously at orthotopic and heterotopic locations [31–33]. These findings raise the question regarding the true success rates of ovarian transplantation as some women may still have a remaining functioning ovary in some cases. However, it is not known how previously menopausal women can spontaneously conceive after heterotopic transplantation as in the case described above. It is hypothesized that the transplantation of ovarian tissue containing a healthy niche may provide endocrine or paracrine signals,

which could activate the remaining chemotherapy-damaged ovary by mechanisms still unknown [30, 34].

In a recent experimental study in baboons [35], four different heterotopic sites for ovarian transplantation were compared. Ovarian biopsies taken after a second-look laparoscopy 3–6 months after transplantation revealed that an omental location was associated with better follicle survival and development of large antral follicles compared with grafts transplanted in the abdominal wall or into the pouch of Douglas; these did not result in follicle development [35].

6.4 Whole-Ovary Cryopreservation and Transplantation

Whole-ovary cryopreservation has also been investigated, with the aim of immediate vascular anastomoses and organ function after transplantation. The method has been shown to restore fertility in rats [36] and sheep [37], but a high rate of follicle loss is still a concern. Directional cryopreservation combined with microvascular anastomosis has improved outcomes, and long-lasting ovarian function has thus been obtained in sheep [38]. In humans, research of whole-ovary freezing and transplantation is still at the initial stages and presents challenges.

6.5 Safety Concerns with Ovarian Tissue Transplantation

Autotransplantation of frozen and thawed ovarian tissue is only feasible if the absence of cancer cells in the graft is confirmed. Though most early stage neoplasms that occur in young females do not specifically spread to the ovaries, it is legitimate to be concerned about the reseeding of malignant cells when carrying out ovarian transplantation. In a mouse model, the potential for a reintroduction of malignant cells has been illustrated by grafting fresh and frozen ovarian tissue from mice with an aggressive type of lymphoma [39]. The transplant of human ovarian tissue, however, from women with Hodgkin's lymphoma in SCID mice did not transfer the disease to the recipients [40].

Methods for detecting cancer cells in the ovarian tissue of women having suffered from hematological malignancies are under development, including immunohistochemistry or the polymerase chain reaction applied to the tissue [41]. A future strategy for the investigation of residual malignant cells in the ovarian tissue could be via xenotransplantation to an immunodeficient SCID mouse before transplant, but the feasibility of this approach has not been demonstrated. Autotransplantation of ovarian tissue to women who have suffered from systemic hematological malignancies is not condoned because of the high theoretical risk of retransmission of malignancy. Only women with cancer diagnosis associated with a negligible or no risk of ovarian metastasis should be considered for future autotransplantation [42].

For those whom the transplantation of the previously frozen ovarian tissue is deemed unsafe, in vitro growth of primordial follicles may be an option in the future. Although many improvements have been reported on the in vitro culture of early stage follicles with the aim of developing them into competent mature follicles, no success has yet been achieved in patients [43–45].

Another theoretical concern is the genomic consequences of ovarian tissue cryopreservation and orthotopic or heterotopic transplantation. Ovarian tissue cryopreservation and transplantation have been shown to not interfere with proper genomic imprinting in mice pups [46]. However, additional studies in larger animal models may be helpful.

6.6 Use of Experimental Human Ovarian Transplantation in the Xenotransplantation Model

As investigation of the transplantation of human ovarian tissue in humans is not feasible, the xenotransplantation of human ovarian tissue

into immune-incompetent severe combined immunodeficiency (SCID) mice was initially proposed [47]. The SCID mice are T- and B-cell immune deficient, owing to a genetic mutation [48]. Taking advantage of this immune tolerance, the SCID model has been used for the investigation of various xenografts, which revascularize and survive without being rejected. Experimental human ovarian xenografts into SCID mice have also enabled the in vivo investigation of transplant function and survival and the experimental induction of follicle development by gonadotropin stimulation [49, 50]. In this model, thawed tissue transplanted under the kidney capsule has also allowed the determination of the relative efficacy of different cryopreservation protocols [51] and investigation of the optimal size of cortical pieces [52] for cryopreservation. In addition, xenograft models have been used to investigate the molecular mechanisms of the chemotherapy-induced ovarian damage [53].

6.7 Studies in the Xenograft Model Aimed at Improving Ovarian Transplant Survival

It has been shown that the survival of an ovarian transplant is greatly dependent on the revascularization process, as the ischemic phase that follows immediately after transplanting is associated with massive follicle loss [54, 55]. In the SCID mouse model, experiments allow investigation of pharmacologically active substances in xenografts. Sphingosine-1-phosphate, a ceramide-induced death pathway inhibitor, has been shown to block cytotoxic-treatment-induced oocyte death in rodents [56] and in humans [57]. Furthermore, sphingosine-1-phosphate also preserved vascular density after transplantation in the human ovarian graft, accelerated neoangiogenesis, and reduced stromal necrosis and tissue hypoxia, resulting in a significant improvement in primordial follicle survival compared with vehicle-treated controls [58].

Another possible approach to improve ovarian transplantation efficiency is the use of robotic surgery. Because of the precision and ability to operate under high magnification, robotic ovarian transplants may result in better outcomes. The use of robotic surgery for ovarian transplantation has resulted in restoration of ovarian endocrine function and embryo development in two patients who had become menopausal post-hematopoietic stem cell transplantation [59]. Further research will be needed to determine if ovarian transplant outcomes can be improved with this approach.

6.8 Conclusions

As a result of the increasing emphasis on preservation of fertility in young female survivors, a number of fertility preservation techniques have been developed. The options for fertility preservation vary depending upon the patient's age, type of treatment, diagnosis, whether she has a partner, and the time available between diagnosis and treatment of disease. While embryo or oocyte freezing can be offered to those individuals who have sufficient time for ovarian stimulation before chemotherapy, a sufficient time period is not available to all cancer patients. Moreover, especially in pre-pubertal children, ovarian stimulation is unlikely to be efficacious. When such limitations exist and when there is also the desire for the preservation of endocrine function, ovarian cryopreservation and transplantation stands out among the other techniques.

Because of the ischemia encountered after ovarian transplantation, a significant loss of follicles can occur. As a result, it is advisable to harvest an entire ovary until such time those ovarian transplantation techniques are improved.

Slow freezing with a relatively long dehydration time is the current method used for cryopreservation of ovarian tissue. In the 15 years that followed the publication of the first successful case of ovarian transplantation with frozen-thawed tissue, restoration of endocrine and follicular function being the end point, many centers reported live births. All live births associated with ovarian transplantation are thus far with slow-frozen tissue.

There are two broad approaches to ovarian transplantation: orthotopic and heterotopic. Natural pregnancy may be achieved after orthotopic ovarian tissue transplantation if the fallopian tubes remain intact. The sites for orthotopic transplantation may include the retroperitoneum in the ovarian fossa area or the remaining ovary if there is one remaining. All live births associated with ovarian transplantation are thus far with orthotopic ovarian transplantation. Heterotopic location may include the forearm or lower abdominal subcutaneous tissue. The advantages of this technique are being able to closely monitor the graft if there is a need, its feasibility when the pelvis has been scarred from previous radiation, or the potential to inject agents directly into the ovarian grafts to enhance graft survival. It is also less invasive and can be done under local anesthesia in an office setting.

The restoration of ovarian function typically takes approximately 2–5 months after transplantation, as shown by the rise of estrogen levels and decrease of gonadotropin levels as well as follicular activity. The lifespan of the transplanted ovarian grafts may be limited, mainly owing to massive loss of ovarian reserve during the hypoxic period after transplantation. A second transplantation can be performed if necessary and if there is sufficient amount of frozen-banked tissue remaining.

The risk of cancer cell transmission is a concern when implanting ovarian tissue. Ovarian metastasis is relatively rare in young females, and its risks depend on the tumor type, grade, and stage. The chance of an ovarian metastasis arising from Hodgkin's lymphoma or Wilms' tumor is small but is a strong theoretical concern in leukemia patients. Even though there have been no reports of cancer recurrence after ovarian transplantation, when there is a significant concern about ovarian involvement, ovarian cryopreservation should not be performed for the purpose of autotransplantation. In addition to histological methods, it has been suggested that ovarian tissue screening should be performed using molecular markers to detect malignant cells prior to performing ovarian transplantation, when available.

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Part III

ART

Animal Models for Developing Clinical Lab Procedures

7

Margo L. Hennet and Catherine M.H. Combelles

7.1 Introduction

The use of assisted reproductive technologies (ART) in the United States has doubled in the last decade, with 65,160 live infants born in 2012 alone (www.cdc.gov/art). Currently, over 1 % of all infants born in the United States are conceived using ART. ART techniques routinely performed in clinical settings include in vitro fertilization (IVF), cryopreservation of gametes and embryos, intracytoplasmic sperm injection (ICSI), embryo culture, embryo selection, and preimplantation genetic diagnosis (PGD). In vitro maturation (IVM) is a promising technology but remains an experimental procedure. ART is unique because its application in human medicine carries with it a great responsibility toward the quality of newly created life.

Each ART procedure has a history of development and research behind it that preceded its clinical application in human medicine. Most procedures are also constantly modified to optimize their outcomes based on new research findings regarding the cellular and molecular dynamics of gamete development, fertilization, and embryo culture. In this way, animal models play an absolutely essential role in the history and progress of human ART. Most ART procedures were inspired, invented, or first tested using animals. Animal models provide researchers with materials (such as gametes) that are not readily available from humans for a variety of reasons, including ethical concerns, scarcity or value, and logistics.

A wide variety of animal species have been employed in the development of ART procedures. Livestock and laboratory animals are common models, in part because there are uses for ART in agriculture and laboratory science that coincide with the need for knowledge in human ART. Although mammalian species share many reproductive similarities, there are differences that become significant when translating research findings from one species to clinical applications in another. Thus, while the use of animal models has been integral to human ART, understanding how relevant physiologic characteristics compare between a model species and humans is equally important to the field.

In this chapter, we present the major procedures and fields of interest in the human clinical laboratory, through the lens of the animal models used to develop them. Special attention is paid to how well or ill suited various species of animal models are to research regarding each procedure

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_7

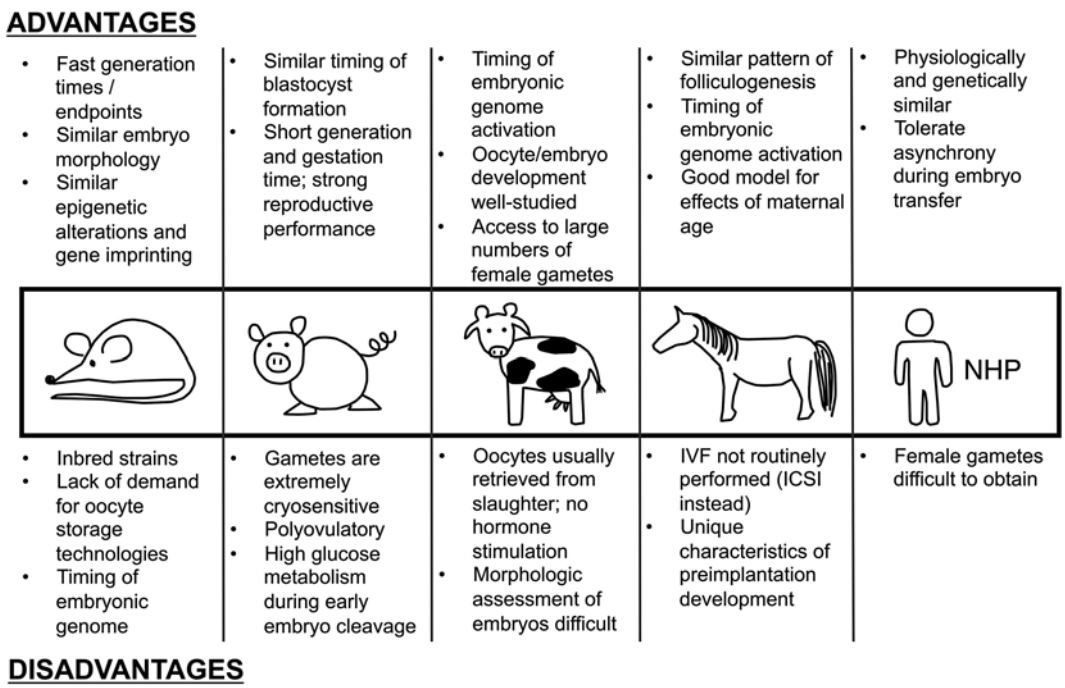

Fig. 7.1 The main advantages and disadvantages for several popular animal models in ART are presented here (this is not intended as an exhaustive list; nonhuman primates are abbreviated as NHP). Advantages include relevant similarities with human characteristics, or species characteristics that make them well suited to research studies. Disadvantages include dissimilarities with humans and disincentives for use as research animals. In all domestic species, large-scale assessments of live births of healthy offspring are difficult, and this remains a significant drawback for these models (not listed above). There is no single "perfect" animal model for ART studies, and thus human ART will continue to benefit most from research utilizing a variety of animal models

(Fig. 7.1), as well as what role animal models currently play in the development of ART. Furthermore, the history of ART procedures is also addressed with regard to the animal models that they were built upon.

7.2 Preparation and Handling of Sperm

The goal of the preparation and handling of spermatozoa is to produce male gametes capable of fertilizing an oocyte. This requires that spermatozoa are not damaged during handling, that they undergo the appropriate physiological modifications (e.g., capacitation) between collection and fertilization, and that any selection process isolates spermatozoa of good quality. In addition to methods for preparation and handling, methods for detecting DNA damage in spermatozoa have also become important in ART.

The capacitation of spermatozoa is essential for successful fertilization both in vitro and in vivo, and in this regard some animal models for human ART research require special consideration. As Yanagimachi [1] points out, "there is no single medium that supports sperm capacitation and fertilization in all species of mammals…[if IVF is difficult in a species] it is we who do not know the tricks." Even between closely related animals, the requirements for spermatozoa during capacitation and fertilization can differ significantly. For instance, while human spermatozoa spontaneously capacitate, macaque spermatozoa do not and require cAMP and caffeine to activate in vitro [2]. Conversely, while progesterone reportedly induces the acrosome reaction in human sperm [3], the same treatment does not

appear to work in macaque sperm (unpublished results [4]). Spermatozoa in the golden hamster require the addition of select chemicals such as taurine, hypotaurine, and epinephrine to IVF media for their survival and capacitation, according to an IVF protocol reported by Bavister [5]. Human and bovine spermatozoa do not require the presence of these molecules in IVF media [6], although the addition of heparin to bull spermatozoa preparations significantly increases the percentage of acrosome-reacted sperm [7]. Thus, most IVF protocols require species-specific adjustments to suit the needs of the spermatozoa in in vitro culture.

Most methods for preparing spermatozoa for ART were developed using human samples, including the commonly used swim-up technique [8], Percoll density gradient centrifugation [9], glass wool column procedure [10], and Sephadex bead filtration [11]. Animal models are consequently of more use in studies seeking to understand the molecular or cellular details of sperm development and quality in vitro, as most of these characteristics are shared (to varying extents) between mammalian species.

Handling and preparation procedures that cause mechanical or chemical stress can lead to physical damage, reactive oxygen species generation, and DNA damage. No single procedure is immune to all stressors, and animal models have served to help investigate the vulnerabilities of certain protocols, or to present potential adjustments of existing ones. For example, boar spermatozoa were used to investigate the impact of sex-sorting sperm by flow cytometry—which involves a variety of mechanical and chemical stresses—on the distribution of heat shock proteins 60, 70, and 80 [12]. These proteins are key factors for fertilizing ability in mammalian sperm and also play a protective role against stressors. Other animal models that have been used to study molecular aspects of sperm handling and preparation include the bull, rabbit, and mouse [13].

Sperm quality assessment primarily depends on evaluations of motility, as this is an indirect measurement of metabolic activity and viability, and is necessary for fertilization (except in ICSI). Computer-assisted semen analysis (CASA) is one way to objectively quantify sperm motility based on a variety of parameters, and its correlation with in vivo fertility has been reported in several species, including horses, boars, and bulls [14–16].

Evaluation of DNA integrity is another method for sperm quality assessment—an approach that needs additional evidence to support its clinical utility [17]. In one 2009 study, goat spermatozoa were used to evaluate, among other fertility predictors, DNA integrity in frozen/thawed spermatozoa by single-gel electrophoresis [18]. Bull and mouse spermatozoa, in addition to human spermatozoa, have also been used to evaluate the capacity of single-gel electrophoresis to detect chromatin integrity in a comparative study [19]. Such comparative studies are useful to highlight species differences—in this study, the authors found that human spermatozoa were more sensitive to DNAse I treatment (an enzyme that preferentially attacks chromatin that is in an open conformation) than bull or mouse spermatozoa. This suggests that human spermatozoa chromatin is packaged in such a way that is easier for the enzyme to access than in the other two species. Indeed, the ratio between histones and protamines (and their characteristics) differs between species [20, 21], with 15 % of human spermatozoa DNA associated with histones (and thus more susceptible to DNAse I [22, 23]) compared to just 1 % in the mouse and bull [20]. Other methods to assess DNA damage include in situ nick translation [24], terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) [25], and sperm chromatin structure assay [26]—all of which were initially developed using human spermatozoa.

Despite significant differences that can make aspects of spermatozoa preparation and handling species specific, animal models remain important as they provide resources that are not available from human samples, whether for ethical or logistical reasons. Murine oocytes, for instance, are used for heterologous tests to elucidate the etiology of infertility cases. Human spermatozoa from patients for whom ICSI was not successful are injected into murine oocytes to test their activation ability [27]—such tests can help to clarify whether fertilization failures originate in the

male or female gamete of couples using ART. These tests have also been proposed as a method with which to "screen" patient sperm for quality before attempting ICSI with valuable human oocytes. Similarly, although many methods for DNA damage detection in spermatozoa were initially explored in human samples, these techniques are used in conjunction with other animal models to improve human ART. For example, sperm chromatin structure assays, TUNEL assays, and single-gel electrophoresis were used to assess the impact of scrotal heat stress on spermatozoa DNA integrity in mice [28, 29]. The results of such animal studies, in turn, have direct clinical implications for humans undergoing ART procedures, including IVF and ICSI. Thus, there is a constant flow of progress and information between human and animal ART.

7.3 Cryopreservation of Spermatozoa

The history of cryopreservation in the field of reproduction begins with amphibian spermatozoa in the eighteenth century, when Italian priest and physiologist Lazzaro Spallanzani used snow to examine the effects of cooling on motility [30]. Early interest in spermatozoa cryopreservation in the United States came from the bovine dairy industry in the late 1930s. Studies of bull sperm morphology, motility, short-term storage, and semen extenders during artificial insemination procedures provided critical information for the onset of cryopreservation as a storage technique. Chicken egg yolk, for instance, was used to protect bull sperm from cooling injury—this led to an understanding of the importance of lipids in cryoprotection [31]. A landmark discovery came in 1949 when Polge et al. demonstrated that chicken sperm treated with a glycerol solution were protected from freezing [32]. These discoveries marked the advent of cryoprotectants, and the success, as well as the utility, of sperm cryopreservation in human and veterinary medicine became widespread. In fact, glycerol remains one of the most widely used cryoprotectants in human sperm cryopreservation. Progress in the field of spermatozoa cryopreservation has been achieved in three main fields of application: laboratory animals (primarily mice), livestock (primarily cattle and horses), and human reproduction.

As for oocyte and embryos, cellular characteristics that influence sperm cryosensitivity are important to study, understand, and manipulate. Spermatozoa from some endangered species provide natural examples of morphologically abnormal spermatozoa, with which characteristics important to cryosurvival (such as the integrity or shape of the acrosome) can be studied and compared (species include cheetahs, black-footed ferrets, and clouded leopards) [33]. It seems, according to these studies and those in Iberian Red Deer [34], that the shape and size of the sperm head influence its susceptibility to cryoinjury, most likely by impacting heat exchange, water and ion movement, and cryoprotectant diffusion. Interestingly, spermatozoa of the giant panda are relatively large sized and round headed and appear to be exceptionally cryoresistant [35]. The morphology of the sperm tail is relevant in this regard, as well. Although rodents are common models in many aspects of human health, including this one, their sperm morphology is markedly different from that of human sperm. Mouse and rat sperm have a tail that is more than twice the length of other common animal model species and humans (~95–110 μm vs. 35–45 μm). These tails therefore contribute to an increase in overall cell volume and cell surface area (2–4 times greater than sperm from most other mammalian species, including humans) [31]. Consequently, protocols for cryopreservation must make species-specific adjustments to accommodate for different cryoprotectant diffusion times.

Glycerol is a common component of human and animal sperm cryopreservation protocols. Ruminant and primate sperm tolerate 4–8 % glycerol during cryopreservation, while other species do not. For instance, boar sperm cannot survive concentrations above 3 % glycerol, and mouse sperm are damaged when the concentration exceeds 1.75 %. Optimal glycerol concentrations for sperm cryopreservation have also been

investigated for a number of other nondomestic species, including marsupials. These differences in glycerol sensitivity suggest important differences in membrane structure and function between species [36].

Furthermore, the composition of plasma membranes (namely, the cholesterol/phospholipid ratio, lipid type, hydrocarbon chain saturation levels, and protein/phospholipid ratio) impacts sperm susceptibility to cold shock. Specifically, low sperm membrane sterol concentrations and high polyunsaturated fatty acid concentrations contribute to the severity of cold shock. Overall, boar sperm are most sensitive to this phenomenon. Bull, ram, and stallion sperm are more sensitive than that of dogs and cats, while rabbit, human, and rooster sperm appear to be the least sensitive [37].

Osmotic effects of cryoprotectants can lead to lysis or damage if not controlled carefully. The extent to which spermatozoa can tolerate shrinking and swelling (their osmotic tolerance limit) varies between species [31]. Mouse sperm are less sensitive to changes in osmolality than boar or rat sperm, but are more sensitive than human and nonhuman primates. Osmotic tolerance limit is an important predictor of sperm response to conditions induced by the addition or removal of cryoprotectants; therefore species differences must be considered when using animal models.

Interindividual differences in sperm tolerance for, and viability after, cryopreservation are generally recognized to exist in a range of species, including humans. Two series of experiments with bull sperm and mouse sperm demonstrated this principle [36], and this concept is currently recognized in humans. Studies exploring new methods for cryopreservation, or modifications of older protocols, often use several species' sperm in their research. This provides for internal comparisons between species and is a convenient way to screen for significant species differences.

Despite its utility for long-term storage, cryopreservation generally causes a decline in sperm quality, namely in terms of DNA damage [38]. Other methods for sperm storage, including evaporative drying, storage in salts or sugars, and storage in electrolyte-free medium, have been explored in the mouse and other mammalian models before testing in the human [39–42]. Obtaining live and healthy offspring is the ultimate test of ART success and is much more easily and quickly achieved under experimental settings in the mouse. For instance, after producing live mouse offspring with sperm stored in electrolyte-free medium in 2007, Riel et al. proceeded to test this storage method against cryopreservation using human spermatozoa (murine oocytes were used to confirm oocyte activation after ICSI with these spermatozoa) [43].

Sperm vitrification was first explored in frogs in 1938 [44] but attempts with mammalian sperm did not meet with success. It is viewed as an attractive alternative to conventional freezing protocols because it avoids ice crystallization (although sperm cells are sufficiently small that intracellular crystal growth during cooling is improbable). However, high concentrations of cryoprotectants are usually used in vitrification protocols and spermatozoa have low tolerances for these substances. Early attempts, but no successes, at sperm vitrification occurred in 1942, when the tolerable limit of cryoprotectants required cooling rates higher than what was achievable at the time [45]. Attempts with "conventional" vitrification—using high cryoprotectant concentrations—have consistently yielded low or no survival. Thus far, human and nonhuman primate spermatozoa have been successfully vitrified without cryoprotectants (a feat that is not possible thus far in the much larger oocytes and embryos) [46, 47]; however studies using rabbit and kangaroo semen reported low or no survival [48, 49]. The ability of sperm cells to survive vitrification without cryoprotectants is likely due to species differences and variations in the vitrification procedures used (speed of freezing and warming, sample volume, sample handling). Some insight into the mechanics of this process comes from animal models. For example, Isachenko [50] postulates that intracellular components such as osmotically inactive water bound to macromolecular structures in sperm cells may act as natural cryoprotectants, based on results of studies in cryopreservation of mouse spermatozoa without the use of permeable cryoprotectants.

Rosato et al. [48] suggest that human spermatozoa, being one of the smallest mammalian germ cells with very few residual histones and very compact DNA, may be less vulnerable to ultrarapid freezing damage than other mammalian spermatozoa.

From the beginning, the study of spermatozoa in reproduction has used animal models to understand human biology. The shared characteristics of mammalian spermatozoa, such as their general structure, differentiation, and fertilization events, allow studies in animals to contribute to human ART. On the other hand, comparing differences between species yields new information regarding the mechanical and molecular dynamics of spermatozoa during the cryopreservation process as well as their subsequent integrity and function.

7.4 Cryopreservation of Oocytes and Embryos

Controlled-rate freezing and vitrification have been explored as options for the cryopreservation of oocytes and embryos. Controlled-rate freezing uses low concentrations of cryoprotectants and slow cooling rates to gradually dehydrate cells. This method was the first one established for both embryos and oocytes, and its main challenge is the avoidance of ice crystal formation. Vitrification is the second, more recently developed, method for storing oocytes and embryos. Vitrification avoids ice crystal formation altogether through the use of high concentrations of cryoprotectants to create a viscous solution, which will then solidify during extremely rapid cooling. Although vitrification avoids the threat of ice crystal formation, the high concentrations of cryoprotectants can be toxic to cells if exposed too long before or after vitrification. Cryoprotectants increase the total concentration of solutes inside or outside the cells [51] and thus help to reduce the amount of ice crystals formed from controlled-rate freezing, reduce the risk of osmotic shock upon thawing, and increase viscosity of solutions (particularly important for vitrification). For the development and continual evolution of both protocols, animal models are used to elucidate which aspects of oocyte and embryo physiology are most important to cryosurvival.

Although cryopreservation of sperm has been an established technique in human ART for several decades now, its female counterpart, oocyte cryopreservation, has been more challenging to develop [52]. Embryo cryopreservation, on the other hand, was developed for human use more quickly and is currently a mainstay clinical ART lab procedure. A variety of animal models, particularly domestic animals, have contributed to the development of oocyte and embryo cryopreservation protocols. In addition to the physiologic suitability of an animal model, demand for oocyte and embryo storage protocols in agriculture and laboratory science has a significant influence on which species predominate as models in the field. As of 2011, embryo cryopreservation has been reported in a total of 40 species [53], including humans and wild/zoo species.

The mouse and cow are common models in this field and constitute much of the early history of cryopreservation protocols. Controlled-rate freezing protocols for embryos emerged in the early 1970s, with the first live births occurring in mice [54, 55]. In the bovine model, live births from frozen embryos followed close behind [56]. Similar protocols for oocytes surfaced soon after, and the first human pregnancy from cryopreserved oocytes in 1986 [57] followed on the heels of pregnancies in the mouse and rat in 1977 and 1979, respectively [58, 59]. However, oocyte cryopreservation was burdened with several challenges that slowed its progress, including damage to important cytoskeletal structures due to ice crystal formation, hindered post-thaw fertilization, and concerns about chromosomal abnormalities.

Vitrification of mouse embryos was first reported in 1985 [60]. For the past two decades, extensive experimentation with cooling rates and carrier systems in the cow has led to further optimization of these protocols both in animal models and in human ART [61, 62]. For instance, the introduction of open-pulled straws and small nylon loops as carrier systems in vitrification protocols was a result of experimentation with

bovine embryos in 1990s [63, 64]. Despite initial interest, relatively few studies of mouse oocyte vitrification have been pursued since the 1990s, due in part to the lack of demand for mouse oocyte storage in fields of laboratory science [65]. However, much of the experimental phase for oocyte vitrification was performed directly in the human, and the first human pregnancy from vitrified oocytes was reported in 1999 [66].

The susceptibility to cryoinjury of oocytes or embryos is influenced by a number of factors in addition to protocol, most notably species type and developmental stage. For human embryo cryopreservation protocols to benefit from research in other animal models, the results of animal studies must be interpreted with physiological and developmental comparisons between species in mind. However, species differences notwithstanding, animal models remain an important resource for the improvement of ART protocols, particularly those that manipulate valuable oocytes and embryos. The pace of progress in ART cryopreservation techniques would not be possible without these models.

Sufficient permeation and diffusion of cryoprotectants through a cell is essential for cryoprotection in both controlled-rate freezing and vitrification. This is more difficult to accomplish in large cells, because they have low surface to volume ratios. Oocytes in particular, being very large cells with cryosensitive cytoskeletal structures, present challenges in this regard. Human, rabbit, and bovine oocytes are similar in diameter, at 130 μm, 120 μm, and 130 μm, respectively. However, mouse oocytes are significantly smaller (60 μm). In this respect, the diffusion time of cryoprotectants during a given protocol differs significantly for mouse oocytes and human oocytes. This was one suggested reason for why oocyte vitrification protocols that were successful in the mouse failed in the human (did not produce blastocysts post-thaw) [67, 68]. Permeability to cryoprotectants also differs between mouse and human oocytes. Human oocytes are more permeable to PrOH (1,2-propanediol) than to DMSO, while mouse oocyte permeability to these two molecules is almost identical and relatively lower than in the human [69–71]. Furthermore, it is significant to note that mouse protocols typically use DMSO as a cryoprotectant for embryo cryopreservation, and although early success with freezing human embryos was achieved by using DMSO [72], the majority of human embryos in the past several decades have been cryopreserved using PrOH and sucrose [72, 73]. Interestingly, this PrOH and sucrose controlled-rate freezing method was developed first in the mouse and then adapted for human use [73].

Cryoprotectant permeability and diffusion is also a concern in vitrification protocols, where high cryoprotectant concentrations present the risk of cytotoxicity. The most common cryoprotectant for vitrification procedures is ethylene glycol (EG). It is reported to have a low toxic effect on mouse embryos and blastocysts and rapidly diffuses into the cells [62]. Studies in animal models such as the mouse seek to understand cryoprotectant cytotoxicity, but the authors caution against extrapolation to the human model [74].

The most common embryonic stage used in cryopreservation in human ART is the zygote or early cleavage-stage embryo [75]. Mature oocytes are more commonly cryopreserved than immature oocytes. However, all stages of early embryonic development and oocyte development are of interest to cryopreservation research, and this interest is reflected in the variety of embryonic and oocyte stages examined in the literature. Physiologic distinctions between embryo and oocyte stages can impact their cryosensitivity, and thus each stage requires special considerations during cryopreservation. Blastocysts, for instance, contain a large volume of water in the blastocoele, which is at significant risk for ice crystal formation during controlled-rate freezing (similar to the oocyte). Additionally, equine expanded blastocysts have been shown to allow limited cryoprotectant influx, demonstrating another mechanism for increased cryosensitivity in an animal model [76]. Furthermore, early cleavage-stage ruminant and pig embryos are more susceptible to damages associated with cryopreservation than blastocysts or hatched blastocysts [77]. Permeability of rat oocytes to

water has been found to decrease throughout maturation [78]. To our knowledge the only study comparing human developmental stages is one between single-cell zygotes and oocytes, in which it was demonstrated that zygotes show greater resistance to chilling injury than oocytes, despite their similar sizes [79]. It is clear that cell size is one among many factors determining cryosurvival for oocytes and embryos, and currently animal models appear to be the main source of information on this topic.

Cryosensitivity differs between species. Porcine oocytes and embryos are famously cryosensitive [55], for instance. Physical changes to lipids that occur when subjected to freezing temperatures are a major source of cryodamage for cells, particularly during controlled-rate freezing. Susceptibility to damage increases with increasing size and/or quantity of lipid droplets. Early stage mouse and human embryos have less intracellular lipid than do ruminant and pig embryos and are more tolerant of cooling in general [80]. Cow, pig, and sheep oocytes differ in the quantity and type of lipids they contain [81], and therefore it is reasonable to assume that they also differ in their cryosensitivity levels.

In clinical settings, insemination of cryopreserved human oocytes is achieved via intracytoplasmic sperm injection (ICSI), rather than by in vitro fertilization (IVF). This is due to the poor efficiency of IVF rates in thawed oocytes, largely attributed to the phenomenon of "zona pellucida hardening" in these cells. Although this problem is circumvented clinically with ICSI, animal models are used to study and understand what features of the cryopreservation process induce this change. It is possible that PrOH triggers the activation of oocytes by causing an increase in intracellular calcium, according to studies in the mouse [69]. Whether premature cortical granule release is involved in zona hardening remains debated and studied in animal and human models [82, 83]. These mechanisms may be valuable to understand for two reasons. First, if cryoprotectants cause intracellular ion concentrations to change significantly, other cellular functions may also be affected—a change that may be relevant to other questions of embryo development following cryopreservation. Second, although ICSI is an adequate solution to this in vitro phenomenon, it does not address what amounts to a departure from in vivo behavior—something that ART protocols seek to minimize as much as possible.

Cryopreservation of oocytes and embryos is a massive field of research and a growing procedure in clinical practice. Its experimental nature, and the intrinsically high value of female gametes, makes animal models a very important resource for this field. Although significant species differences do exist, knowledge gained from animal studies can be applied to human ART as long as those differences are recognized and understood. Therefore, research seeking to identify and explain species differences during cryopreservation of gametes lays an essential foundation for the future development of cryopreservation in human ART.

7.5 In Vitro Fertilization

In vitro fertilization (IVF) entails the acquisition of oocytes, fertilization of mature oocytes in vitro, culture of preimplantation embryos, and transfer of embryos to the uterus. Early research used animal models—primarily the rabbit at first—to investigate each of these aspects independently. Later, these threads coalesced into the single field of IVF, which eventually led to the birth of the first human IVF baby in 1978.

Rabbits and guinea pigs were the animal models with which IVF was first attempted in 1887. Another attempt was made in 1893, but neither were convincing to the scientific community. Around this time, several groups investigated the fundamental features of fertilization using rabbits, sea urchins, and starfish. Hormonal control of ovulation was first investigated in the guinea pig (1917) and rat (1922) and opened the field to studies of female reproductive endocrinology [84].

Rabbits were the mainstay of research leading to IVF in the nineteenth and early twentieth centuries. In 1891, experiments in the rabbit reported successful transfers of embryos from one doe to

another [85]. Furthermore, the first successes in preimplantation embryo culture happened just two decades later [86]. Further, in 1936 the same research group successfully activated rabbit oocytes [87].

Although several claims of in vitro fertilization existed for the rabbit and human models prior to the 1950s, the discovery of sperm capacitation in 1952 introduced a new phenomenon to IVF that had not been considered before. The first undisputed demonstration of IVF occurred in the rabbit in 1959 [88]. IVF using a chemically defined culture medium was later achieved in 1968 in the mouse model [89]. Since then, IVF has been accomplished in the rat, hamster, sheep, cow, pig, monkey, and human as well [84].

Important events in fertilization include the binding and penetration of the zona pellucida by a spermatozoon, decondensation of the sperm nucleus (formation of male pronucleus), the assembly of the zygotic centrosome, and the positioning of centrosomal proteins and sperm aster microtubules around the sperm centriole. These events are necessary for genomic union. Abnormalities in (or, in ART, poor understanding of) any of these events interfere with normal fertilization and often result in fertilization failure. Therefore species differences and similarities in fertilization physiology are important to recognize if animal models are to further clinical progress in human IVF.

The primate zygotic centrosome is primarily paternally derived, as it is in most mammals. In contrast, the zygotic centrosome of the rat, mouse, and hamster is maternally inherited, and therefore it results from a distinctly different assembly process [90]. Rabbits and ruminants mirror humans in their pattern of centrosome inheritance and in this regard are well-suited models for fertilization studies. However, information regarding postfertilization development in the rabbit is relatively scarce, while ruminant fertilization and development have been extensively studied. Furthermore, ruminants offer the additional advantage to IVF studies of having large numbers of gametes accessible through abattoir materials.

Because of its role in spermatozoon binding and the acrosome reaction, the zona pellucida represents a significant landmark in fertilization. To date, most studies of the zona pellucida glycoproteins during fertilization have used the mouse model. However, studies in other models have shown that observations in the mouse model may not be accurate in the chicken, rat, bonnet monkey, and human due to different types and/or numbers of zona pellucida proteins [91–94]. These differences become particularly relevant when using animal models to investigate potential causes of IVF failure, as the biological roles and associated signaling pathways of each zona pellucida protein may differ between species.

Gametes must be handled and processed carefully in IVF procedures, so as to prepare them for fertilization and to avoid damage under in vitro conditions. Spermatozoa handling and preparation are integral to this process and are addressed in a separate section. Media for human IVF were initially either those used for somatic cell incubation (such as Ham's F10 or Earle's) or those used for IVF in laboratory animals (such as Tyrode's T6 or WM1). Experimentation in animal models (particularly the hamster) also revealed the importance of pH in ensuring consistency in IVF protocol success rates [95]. The first human-specific IVF medium was formulated according to the composition of human tubal fluid, using experience from mouse IVF to fill any knowledge gaps—lactate and pyruvate levels, for example, were estimated this way [96]. This same approach was used by Tervit et al. [97] to develop the synthetic oviductal fluid (SOF) medium for sheep and cow IVF and in vitro embryo production. Embryo culture, which follows IVF, is a technique that must address the changing metabolic needs of a growing embryo in vitro prior to implantation in the uterus. Its development and future progress also involve learning from animal models and are detailed in a separate section.

In human IVF, embryos are most commonly transferred back into the uterus during early cleavage at day 2 or 3 of development. This practice introduces a component of asynchrony into

the embryo's development, as an embryo would not normally reach the uterus until between day 3.5 and 4. Primate embryos tolerate this asynchrony well enough to continue development, as was demonstrated with pronucleate rhesus monkey embryos in 1977 [98]. In contrast, this asynchrony reportedly leads to embryo rejection in rodents and domestic animals [99]. The reason for this difference is that while the uterotubal junction in other animals is sufficiently long and circuitous such that uterine and oviduct fluids do not mix, the luminal fluids of primate fallopian tubes and uterus have significant overlap [99, 100]. This same physiological feature in the primate reproductive tract has been proposed as a reason for why human zona pellucida hardening remains unchanged by oviductal fluid exposure, while in the pig, cow, rodent, and rabbit, oviductal fluid does induce zona pellucida hardening [101]. Thus, it appears that adaptations of oocytes to their "expected" route to the uterus may influence their reactions to certain solutions or, conversely, their requirements during culture.

7.6 Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) is the injection of a single spermatozoon into an oocyte. It has a significant role in ART, including male infertility treatments and fertilization of cryopreserved oocytes. The first step on the road that led to ICSI becoming a clinical tool in human ART occurred in starfish at the beginning of the twentieth century. In 1914, Lillie [102] cites the unpublished work of GL Kite, who injected live starfish spermatozoa into eggs and was surprised to find that nothing happened. Similar experiments, with similar results, followed in 1962 with sea urchin gametes [103]. However, Hiramoto [103] did observe that when injected sea urchin eggs were then inseminated "naturally," all activated and commenced polyspermic cleavage, leading him to hypothesize that egg cytoplasm must be activated by a fertilizing spermatozoon. The first live offspring produced by sperm injection were frogs in 1974—although only 4 out of 562 oocytes developed into normal adults [104].

The first attempt at mammalian ICSI occurred in 1976 using the hamster model [105]. However, live hamster offspring were only achieved in 2002 [106]. The first live mammalian offspring to result from ICSI were rabbits [107], but the efficiency of ICSI in this species remains relatively low. Live offspring were also produced in cattle around this time [108]. In all of these studies, investigators experimented extensively with methods of spermatozoon injection and preparation. Several studies found that spermatozoa of some species (such as the mouse, rat, baboon, rabbit, bovine, and human) could develop pronuclei when injected into the oocytes of the same or different species [109]. Human ICSI was first reported in 1988 when Lanzendorf et al. [110] documented pronuclear egg formation after sperm injection. However, the first live human births from ICSI were reported in 1992 [111], using the same protocol still in use today. It is worth noting that the first attempts at human ICSI occurred before live births in most animal species, including the hamster, rabbit, bovine, and mouse [109, 112, 113].

Human ICSI is currently far more successful than animal ICSI (particularly in species other than the mouse), due at least in part to some physiological differences between humans and other species (for a detailed review, see Yanagimachi [109]). Consequently, animal models are not heavily relied upon in efforts to further the clinical success of human ICSI.

An injected spermatozoon introduces materials into the ooplasm that are not normally encountered by the oocyte during natural fertilization, including sperm membrane and acrosome. The stability of the sperm plasma membrane varies between species—higher stability can lead to delayed or failed membrane disintegration within the ooplasm after ICSI. Human spermatozoa have relatively unstable membranes, while many other animals appear to have more stable membranes [109]. This delay of disintegration in animal models can lead to organizational or structural defects in the fertilized oocyte. Similarly, injected

spermatozoa bring their acrosomes with them, which are not well tolerated in some species such as the hamster. In fact, for ICSI to succeed in the hamster, the acrosome must be removed [106]. Even in the rhesus monkey, the presence of the acrosome inside the oocyte delays nuclear decondensation [114], although this is not a problem in humans or mice [109].

Other differences between human and animal models demand adjustments of ICSI technique if working with multiple species. Palermo, in a 2012 review, notes anecdotally that injection in the mouse was a "nightmare" because of the length of the sperm flagellum relative to the size of the oocyte [115]. This is a feature of mouse, rat, and hamster models [109]. Because the centrosome is maternally inherited in these species, one can inject isolated sperm heads to simplify the process. Conversely, since the sperm centrosome is essential for fertilization in most other mammals, the whole spermatozoon (or at least the head and proximal tail) must be injected [109].

The first explorations of ICSI occurred in a variety of invertebrate and mammalian species; however, the success of ICSI in humans has since then surpassed that achieved in animal models. Despite this, animal models serve this field of ART by providing comparative information about fertilization that can benefit other topics of research in ART. Furthermore, in a reversal of roles, human ICSI protocols lay the foundation from which ICSI protocols in other species can be developed.

7.7 Embryo Culture

7.7.1 Culture Systems

Several animal models have proved instrumental to the progress of human embryo culture. Studies in the experimentally tractable rodent species (with a very large number of embryos available) have allowed the optimization of media (semi-defined or defined) for the culture of mouse zygotes to the blastocyst stage. Landmark efforts include the ones undertaken by Brinster, Biggers, and colleagues—namely, with the systematic multifactorial testing and the sequential simplex optimization strategy that led to the formulation of KSOM media [116, 117]. Such prowess not only permitted the reliable culture of mouse embryos (and in turn a wealth of new knowledge on mammalian early development) but also a foundational basis for modifications of human culture systems [118]. Many pioneering studies using embryos from golden hamsters, livestock species, and humans in the late 1960s and 1970s preceded the creation of improved defined media [119]. Adjustments and improvements for human use have continued ever since.

Agricultural needs have driven great advances in the in vitro production (IVP) of embryos from domestic species (notably since the 1980s), leading to the wide use of ART experimentally and commercially and in turn benefiting the field of human embryo culture [120]. Importantly, the developmental similarities between bovine and human embryos are significant; for instance, the timing of embryonic genome activation (EGA) during preimplantation is comparable between human and bovine embryos, while it differs between human and mouse embryos [121, 122]. Additionally, the critical period of EGA is particularly sensitive to environmental conditions [123]; for studies on the requirements of human embryo in vitro, it thus becomes essential to use an animal model with a comparable timeline of developmental milestones during early embryogenesis. The growth, physiology, and cellular characteristics (e.g., protein content) of embryos display dynamics often unique to some species [124, 125], in turn impacting the exact requirements and success of in vitro culture across species. Animal IVP studies, and in vivo comparisons that are possible in animal models, already demonstrate the complex and dynamic nature of embryo needs, notably with respect to metabolism [126, 127]. From studies in domestic species, considerable insight has also been gained from the biochemical characterization of the oviductal fluid, the in vivo milieu that normally sustains early embryonic development [128]. The embryo itself alters its environment in several ways, including the varying depletion of amino acids in the medium during in vitro culture in

both the cow and human [129, 130]. Such basic knowledge directly informs improvements in defining optimal factors and conditions for the culture of human embryos. Animal studies have also indicated the plasticity (or ability to adapt to conditions) of preimplantation embryos, a developmental phenomenon of relevance when modifying in vitro conditions and culturing cells of varying quality (or with varying adaptive potential). Importantly, embryos from large domestic species and humans differ from mouse embryos in some of their metabolic responses to environmental changes [99, 127]. A complete understanding of the cellular responses of embryos to in vitro stress is needed, and comparative investigations in multiple animal models can provide unique insight. Taken together, a mastery of metabolic needs and pathways in early mammalian embryos promises further advances in the formulation of the best possible in vitro conditions that will support the development of human embryos at high efficiency. Examples of areas of continued focus in both animal and human embryos include glucose demands and consumption, oxygen tension, amino acid metabolism, and lipid utilization [119]. Human ART still struggles with identifying the best embryo culture medium, notably one that is defined, reliable, efficient, and safe.

Rapid and remarkable advances characterize animal and human ART, but challenges remain in improving the efficiency of producing and selecting high-quality embryos that result in a healthy adult. Experimental studies have pinpointed the significant influence of the culture environment on the quality of blastocysts in large domestic species [123, 131–133]. Exhaustive comparisons of bovine embryos cultured under various conditions reveal features that result in embryos of inferior quality (e.g., serum-free, oil-free versus serum-supplemented or coculture) [134]. These comparisons also demonstrate a persisting decrease in developmental competence of in vitro produced when compared to in vivo derived embryos. Animal studies have yielded mechanistic insights into underlying differences between in vivo and in vitro produced embryos. Parameters that are influenced by culture conditions include kinetics of embryo development, cell allocation to the inner cell mass and trophectoderm, gene expression, and metabolism [135–138]. Further improvements in culture systems may close, or at least narrow, the gap in embryo quality between in vitro and in vivo environments, but the intrinsic potential of the gametes cannot be neglected. Indeed, animal studies (mostly in cows) convincingly demonstrate that while modifications in embryo culture can influence the formation of blastocysts, it is the developmental conditions experienced by the oocyte that determine the quality of resulting blastocysts [139]. This is an essential lesson for human ART to learn, highlighting the necessity for future improvements in oocyte quality. Increased attention ought to be placed on eliminating conditions or factors (e.g., chemical exposures, stress, obesity, etc.) that may impair the complex and protracted development of the oocyte within the environment of the follicle in vivo. Furthermore, protocols preceding and following oocyte retrieval may also help maximize the developmental potential of the oocyte. Although not within the scope of this chapter focusing on the ART laboratory, research and breeding efforts in cows also prove particularly relevant such as with respect to the optimization of stimulation protocols and the follicular milieu for the retrieval of superior quality oocytes [140].

Despite the continued impact and future promises of animal studies, caution must prevail when extrapolating findings to human ART. For instance, the vast majority of the work in bovine IVP is based on in vitro matured oocytes obtained from abattoir animals without stimulation with exogenous hormones. The source of material thus differs from human ART material in potentially significant ways. Animal studies for breeding and/or research purposes also stem from fertile animals, a distinctly different population of subjects when compared to the infertile patients most commonly seen in human ART. Nonetheless, experience with animal models, and careful extrapolation from those experiences, remains essential prior to testing in humans. To draw meaningful insight from cross-species findings, differences in

experimental design and sample sources must be systematically considered. Experiments undertaken in animal models also permit reliance on informative endpoints that are not always achievable in human studies.

7.7.2 Safety of Embryo Culture

Studies in animal models are instrumental in evaluating the long-term safety of ART lab procedures, including IVF and extended embryo culture—a priority identified in a 2013 ASRM Committee Opinion on blastocyst culture and transfer [141]. Specifically, animal experimentation helps direct clinical efforts towards assessment of outcome measures that are relevant in animals. Two examples include the identification of large offspring syndrome (LOS) and epigenetic disorders that are associated with embryo culture in animal models. LOS includes a range of developmental abnormalities first identified in domestic species, following the transfer of embryos produced in vitro in the presence of serum and/or in coculture systems [135, 142, 143]. Cattle and sheep studies led to testable mechanistic models on the origin of later developmental impairments, specifically following the manipulation, handling, and culture of embryos in vitro [135]. Studies in animal models demonstrate that modifications in culture medium (absence of coculture and serum supplementations) can solve the health hazards of LOS, but with a compromise in embryo formation. Experience in animals has thus made evident the need to aim not only for embryo yield but also for embryo quality (including long-term outcomes) every time any modification in embryo protocol is introduced. Also, the aforementioned differences in gene expression between in vivo and in vitro derived embryos may stem from epigenetic mechanisms, a clinically pertinent hypothesis with accumulating support in animal models [144]. Parallels have been drawn between LOS in the cow and Beckwith-Wiedemann syndrome (BWS) in humans [145]; further, mouse and bovine embryos display changes in the DNA methylation of the maternally imprinted SNRPN gene that may hold clues to the causes of Prader-Willi and Angelman syndromes, two other imprinting disorders of concern in human ART [146, 147]. The use of animal models permits comparison of epigenetic changes with "gold standard" embryos that are created and developed in vivo. There are also significant similarities in epigenetic alterations and gene imprinting between humans, mouse, and cattle [148]. Animal models present great opportunities for discoveries that will be important to human ART. Future studies promise to determine the exact epigenetic risks of practices in the ART laboratory, as well as to unravel best practices for preventing or mitigating epigenetic impairments in the developing human embryo. Animal studies in the Developmental Origins of Health and Diseases (DOHaD) also reveal the sensitivity of the preimplantation period (including culture conditions and other manipulations) on long-term outcomes for the offspring [149]. It is relevant to note that techniques other than embryo culture can also influence the epigenetics of embryos, such as in vitro maturation (IVM), cryopreservation, and sperm handling [144, 150, 151]. The range, invasiveness, and nonphysiological nature of many human ART lab procedures call for comparative evaluations in animal models. Generally, research in animals helps raise red flags, ensuring a complete and hypothesis-driven evaluation of ART safety.

Animal studies that focus on aspects other than medium formulation (e.g., osmolarity, pH buffering, temperature, microfluidic, embryo density, volume of medium, etc.) are also relevant since multiple components of an IVP system influence the developing embryo. For instance, the exciting promises of microfluidic technologies await advances and clinical implementation, but in the meantime studies in animal models (murine, porcine, bovine) can provide innovative strategies and useful experience [152–154]. The availability of animal models also facilitates the exploration of radically new approaches to existing challenges, a path that may be essential for future breakthroughs in embryo culture.

7.7.3 Embryo Assessment

In human ART, there is a critical need for the embryologist to be able to assess embryo quality reliably and noninvasively. The selection and transfer of a single embryo with full developmental potential would then become a reality and standard practice. Historically, and still to date, morphological assessment prevails to estimate the quality or developmental potential of human embryos. In this realm, limited insights are gleaned from animal models, largely due to significant variability in the morphological characteristics and visibility of certain structures in embryos from various species. The darkness and high lipid content of ruminant embryos preclude the detection and evaluation of pronuclei and nuclei. Fragmentation occurs at high frequency in human cleavage-stage embryos, while it is typically not detected until the morula stage in bovine and porcine embryos [155]. Species differences notwithstanding, mouse embryos, with a translucency comparable to human embryos and the availability of powerful experimental endpoints, may still prove useful material in the preclinical testing of novel imaging technologies [156]. Findings in both human and animals demonstrate that embryo morphology and kinetics of preimplantation development vary [157–159], thus paving the way for much-awaited and robust clinical evidence in support of the utility of time-lapse monitoring in human ART [160].

A large number of animal studies continue to test novel noninvasive strategies to identify the embryo with the highest developmental potential. Prospects include oxygen consumption, amino acid profiling, and factor secretion. To date in both animal and human embryos, there is still a lack of clinically useful biomarkers that target secreted factors. Basic studies across multiple animal species and strains support the differential expression of proteins between oocytes of varying quality or developmental history [161]. Focusing on the secretome and metabolome, there is thus a need to continue exploring and testing the utility of new profiling technologies, notably taking advantage of samples that can be more readily obtained from animals than from human patients. With the use of the bovine model, evidence is accumulating for the utility of not only respiration rates [162] but also, most recently, the monitoring of metabolic activity using a COnsumption and RElease (CORE) approach based on many years of research on the metabolism of bovine embryos [163].

Although not clinically applicable, invasive approaches that are only possible using animal models also add novel information to the field. Many transcriptomic analyses of bovine embryos have revealed candidate genes and pathways that reflect developmental potential [138, 164, 165]. Toward the identification of markers of developmental competence, El-Sayed et al. [166] related patterns of gene expression from biopsies of bovine blastocysts with pregnancy outcomes; a similar approach was later applied to human embryos [167]. Translation into clinical practice is still awaiting a deeper understanding and further experimentation. A mastery of characteristics that prove unique to high-quality mammalian embryos will set the stage for future avenues of research into yet-unexplored noninvasive approaches.

7.8 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) is used in select clinical ART cycles to diagnose genetic characteristics of early embryos. The first human birth from PGD was in 1990 for the screening of a sex-linked genetic disease [168]. This clinical pregnancy was preceded, however, by a 1968 foundational study with the successful sexing and birth of rabbit offspring from biopsied blastocysts [169]. The lag of 22 years between this first experimental demonstration and the first clinical application in humans largely reflects bottlenecks in the availability of IVF-derived research embryos (animal and human) and technological limitations with various aspects of PGD (e.g., contamination-free biopsy and the genetic diagnosis of limited cellular material). Experimental studies proceeded in the 1970s and early 1980s in animal models such as the cow,

sheep, and mouse, with the pivotal demonstration in cattle that PGD could be performed using a small amount of cellular material and with methods other than PCR. In the late 1980s, the pace of research accelerated, notably in the mouse model with an eye toward application in human—collectively, these efforts led to the first human birth with PGD in 1990. Theodosiou and Johnson [170] published a historical account of the early contributions, interests, motivations, and players in the development of PGD in human.

In domestic species, notably cattle, there exists a rather long history of PGD for use in embryo sexing (since the 1980s). There is currently renewed interest in PGD in these species due to progress in genomics as well as economic pressures to improve the genetics of animals [171–178]. Animal breeding programs thus provide significant motivation for technologies to control the sex and genetic characteristics of commercially valuable species. The sexing of bovine embryos yields satisfactory pregnancy rates, providing a useful animal model for efforts on improving and testing human PGD. In contrast, PGD studies in animal models other than large domestic species (such as in mice and nonhuman primates) are not motivated by commercial applications but instead are conducted to support and inform practices in agricultural and human ART.

The genetic aspects and clinical effectiveness of human PGD will continue to be best perfected in the human (DNA markers, amplification methods, healthy birth, etc.). However, some of the technical challenges of PGD may be addressed in animal models. For instance, several embryo biopsy techniques (needle, aspiration, and microblade techniques) were evaluated within a single study, with comparisons of pregnancy rates following cryopreservation and embryo transfer to recipient cows [179]. Interestingly, the biopsy method significantly affected the outcome. Animal models may also provide a platform for the early exploration of innovative technologies prior to their potential application in human ART. Given the complexity of the PGD process and, importantly, variations in practices (biopsy procedure, embryo stage, number of cells removed, culture conditions, gamete sources, etc.), careful attention must be taken when comparing results across studies. Animal studies also contribute basic science knowledge that may prove informative to clinical PGD practices; for instance, an early developmental bias (in terms of cell lineage) in mouse cleavage-stage blastomeres may have practical repercussions for PGD [180]. Other practically useful information can come from animal studies, such as the varying reliability of PGD to determine mtDNA heteroplasmy depending on the type of biopsy and the stage of development that is analyzed [181].

Chromosomal mosaicism (defined as blastomeres of a single embryo with distinct chromosomal composition) exists in human embryos [182]. Little is known about the practical repercussions of mosaicism on PGD (notably its diagnostic accuracy) but concerns remain and warrant investigations in whole embryos, embryo cohorts, and embryos from various sources, with or without ovarian simulation, fertile and infertile diagnosis, and different culture conditions. Analyzing each cell of individual embryos is another area where experimentation in animal embryos can provide unique insight, thanks to research material that is not available in human. Importantly, the evaluation of bovine and nonhuman primate embryos validates the utility of certain animal models; indeed, chromosomal mosaicism exists at a high incidence in morphologically normal embryos of fertile cows and rhesus monkeys, whether produced in vivo or in vitro [183–185]. In a mouse model, the incidence of chromosomal mosaicism was increased in in vitro when compared to in vivo produced embryos [186]. The existence of mosaicism raises many developmental questions, including the etiology, ability for self-correction, and fate of mosaic embryos, and basic studies in animal embryos may help provide answers. Beyond striving toward a refined and thorough understanding of early embryonic development, animal models can also afford human ART laboratories with cellular material to train young professionals in procedures with high technical and outcome demands such as PGD.

Almodin et al. [187] reported on the potential benefits of a human PGD training program using bovine embryos.

When experimenting with any new PGD techniques, the bovine provides a model with potential promises. Indeed, a large number of embryos may be available for testing (from abattoir material), and resulting blastocysts can be analyzed for cell numbers or other invasive parameters [188, 189]. Such evaluation is not routinely possible in human embryos. The developmental impact of PGD can also be considered in animal studies. Long-term outcome evaluations such as the live birth of healthy offspring are achievable in the mouse, permitting high sample size at reasonable costs; these are notable advantages of rodent models over domestic species.

Most recently, mouse studies point to concerns about a range of abnormalities in the offspring resulting after blastomere biopsy, including, but not limited to, alterations in steroid metabolism during pregnancy (in both fetus and placenta), decrease in fertility, impaired neural development and function with age, and postnatal growth and behavior in males [190–193]. Even though caution must be exercised when extrapolating risks from animals to humans, findings in animal studies still provide invaluable hints and recommendations about what to address in human clinical practices. Animal models may also help tease apart the individual effects of a single ART procedure when compared to the influences of a gamut of technologies used together. This is relevant to address in light of the multiple insults that exist in vitro and may cause detriment to embryo development. For instance, developmental consequences specific to PGD could be distinguished from those of in vitro cultures by conducting PGD on in vivo derived animal embryos—an opportunity not possible in humans.

7.9 Sex-Sorting Sperm

The world's first human pregnancy resulting from sex-sorted spermatozoa was reported in 1995. Since 1995, at least 924 babies have been born from sex-sorted spermatozoa, using a variety of ART fertilization techniques including IVF and ICSI [194, 195].

As with most ART techniques, sex-sorting spermatozoa were developed first in animal models. After Lawrence Livermore National Laboratory, a weapons laboratory, used flow cytometry to orient mouse spermatozoa for DNA content measurements in their radiation research, flow cytometry was applied in the livestock industry for bull semen analysis in 1981 [196]. In 1982, Pinkel et al. separated the X- and O-spermatozoa of the vole, *Microtus oregoni* [197]. Garner et al. [198] demonstrated shortly thereafter that flow cytometry could differentiate DNA content (and therefore the sex chromosome content) in spermatozoa of cattle, sheep, pigs, and rabbits. At this point, however, the process used a dye that required the removal of spermatozoon membranes, and thus no viable spermatozoa could be recovered after sex chromosome identification. Johnson et al. overcame this obstacle in 1987 by using the dye Hoechst 33342 to stain spermatozoa without removing membranes, thereby preserving their viability [199]. This was first applied on living mammalian sperm in the bull and rabbit, with the first live births in the rabbit in 1989 [200].

The basis for this flow cytometry technique (also called the Beltsville Sperm Sexing Technology) is the difference in DNA content between X- and Y-chromosome bearing spermatozoa. The greater the difference in DNA content between those spermatozoa, the more efficiently they can be separated. Human spermatozoa, for instance, have a 2.8 % difference in total DNA content, while spermatozoa of the vole *Microtus oregoni*, used by Pinkel et al. in 1982, have a 9 % difference [195]. Most domestic livestock reportedly have a DNA difference between 3.6 and 4.2 % [201, 202]. In chimpanzees the difference is 3.3 % and in the hamadryas baboon it is 4.2 % [203]. Not only does the DNA difference between X- and Y-chromosome bearing spermatozoa influence how effectively spermatozoa can be separated, it also is a determining factor for which methods can be used to check the purity of X- or Y-enriched samples. In species with a DNA

content difference greater than 3.0 %, flow cytometric DNA analysis can be used [204]. For species with DNA differences less than 3.0 %, such as humans, polymerase chain reaction (PCR) or fluorescence in situ hybridization (FISH) is more appropriate [205].

Using flow cytometry to sex-sort spermatozoa has great utility in veterinary medicine for breeding livestock and in human medicine for preventing the inheritance of sex-linked diseases. Although humans have a relatively small difference in DNA content between X- and Y-chromosome bearing spermatozoa, the principles of sex-sorting are valid across all mammalian species. Therefore, progress in any animal model has value to human ART.

7.10 In Vitro Maturation

ASRM recently recognized the utility of in vitro maturation (IVM) in a clinical setting, but with the efficacy and safety of this technique not yet demonstrated in humans, it thus remains experimental [206]. Many standing questions currently preclude the routine use of IVM in human ART, including uncertainties with respect to patient preparation, oocyte retrieval and culture, fertilization, and clinical outcome. For instance, no consensus exists on the stimulation protocol prior to aspiration, the criteria for selecting which follicles to aspirate, the method of aspiration, medium composition, culture conditions, when ICSI is indicated over IVF, implantation rates, and the developmental outcomes of IVM children.

Milestones in IVM include the discovery in 1935 that rabbit oocytes spontaneously resumed meiosis when released from the follicle [87] and the ability of in vitro matured oocytes to result in live offspring in the mouse [207]. In the bovine, the use of IVM dates back to the 1980s [208], with extensive application ever since [209, 210]. Indeed, the in vitro production of embryos from livestock species relies heavily on IVM, using oocytes retrieved from abattoir ovaries or ovum pickup. IVM constitutes an important tool in not only animal breeding but also research, as it provides the field with a chance to study the complex and essential process of oocyte maturation in vitro.

Despite its utility in agriculture and research, findings indicate that the developmental competence of in vitro matured oocytes is compromised. The resumption of meiosis (i.e., nuclear maturation) is not typically a hurdle in IVM, with 80 % to >90 % of oocytes from most animal species progressing to metaphase II. Rather, it is cytoplasmic maturation that is often incomplete with IVM. While 30–40 % of bovine oocytes consistently reach the blastocyst stage after IVM, blastocyst yield as high as 70 % can be attained after in vivo maturation [123, 133, 209]. There is thus room for further improvement, even in an animal model with honorable IVM successes. Culture conditions can ameliorate the developmental potential of IVM oocytes but not yet to satisfactory and consistent levels. Ultimately, the intrinsic quality of immature oocytes plays a major role in determining the developmental competence of the oocyte. For the competence of the immature oocyte to be maximized, it becomes essential to recognize the follicle as the in vivo milieu within which the oocyte completes a developmental program that affords its full competence. In the last decade, by targeting the follicular microenvironment in a bovine model (which shares critical similarities in the dynamics and control of folliculogenesis with the human), Sirard and his group have made significant progress in optimizing protocols that yield immature oocytes of superior developmental competence [140]. Basic animal studies continue to decipher the cellular and molecular events required for an oocyte to reach full developmental competence [211–213], as well as the roles of various factors during oocyte maturation, in turn helping ensure that in vitro matured oocytes can achieve all of these requirements. Many of the parameters under investigation in the field of embryo culture are also relevant during IVM, such as medium supplementation, oxygen tension, pH, and physical environment. Yet relative to the wealth of studies on embryo culture, only few aspects of the culture system have thus far been considered for oocytes. Importantly, novel approaches can

more readily be tested in animal models than in humans. For instance, delaying meiotic resumption was first explored in animal models as a potential way to afford the immature oocyte additional time and appropriate conditions to complete its developmental program [214, 215]—to date, this approach has met limited success and merits further advancements in animals. The metabolic needs, as well as the intimate and bidirectional support between the cumulus cells and the oocyte (making up the unique biological unit of the cumulus-oocyte complex), represent other targets for IVM improvements and specific areas of study in animal models [214, 216–218]. It is evident that much research is still in store for the field of IVM, with animal models providing excellent resources for experimentation and validation prior to the preclinical and clinical testing in humans.

Human and animal IVM studies vary greatly in the type of IVM that is performed. For instance, clinical human IVM entails the retrieval of cumulus-intact immature oocytes from non- or minimally stimulated ovaries. In contrast, mouse IVM may be performed on immature oocytes obtained from stimulated or naturally cycling animals (often using inbred strains with low genetic variability), while bovine IVM is routinely conducted on abattoir ovaries from unstimulated animals (typically with high genetic variability) at random times in the estrous cycle and from follicles varying in size and atretic status. Other variables (gestational status, animal age, etc.) can further contribute to the heterogeneity of oocytes used in IVM. Some experimental IVM studies in human are even based on intrinsically limiting systems, such as the culture of cumulus-denuded oocytes following failure to mature in vivo in spite of ovarian stimulation. The range of oocyte material used in animal and human IVM thus warrants careful consideration when comparing and translating results. Animal experience with IVM also provides the field with important notes of caution with respect to the safety of the procedures, such as with epigenetic, genetic, and chromosomal alterations [150]. Taken together, findings in animal models promise to impact the field of human IVM in several aspects, including the support of high-quality oocytes prior to retrieval, the identification of high-quality oocytes following retrieval, and the support of high-quality oocytes during culture.

Significant cross-species variations in IVM studies (with respect to oocyte source, culture conditions, and experimental design) complicate the ability to draw conclusions on shared characteristics. Similarities are apparent in cell cycle signals and other factors that control oocyte maturation, even if the timing of oocyte maturation varies from about 12 h (mouse), 24 h (cow), to 36 h (human). A crucial task thus lies before investigators to identify the similarities and differences that are biologically and/or technically relevant across animal models. Using a range of species and study designs that permit comparison, future attention must be paid to the gamut of events taking place during oocyte maturation (e.g., storage and regulation of maternal mRNA and protein stores, interactions between oocyte and cumulus cells, epigenetic modifications, metabolism, energy stores, signaling).

7.11 Conclusions

To conclude, there is value to understanding similarities and differences in ART lab procedures among a range of species. We may not currently grasp the full impact of such knowledge, as even more lessons remain to be learned. For now, we have summarized several cases when animal models prove useful, or not useful, to the development, improvement, and/or safety testing of human clinical ART procedures. We have also recounted the role of animal models in the development of various ART procedures. This chapter focused on the main mammalian species in which ART is employed (namely rodent and domestic species), but increased insight may be gained from studies in a broader range of species, including ones with unique developmental features. Every effort to master the normal development and function of animal, and in turn human, embryos will be of practical significance to human ART.

Significant benefits result from comparative studies across many mammalian species that include a range of genotypic and phenotypic variations. The field of clinical human ART must never lose sight of the principle set forth by the Nobel Laureate in Physiology and Medicine, August Krogh: "For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied" [219]. Recently, two federal funding agencies (NIH and USDA) established together a program that identifies ART as a research priority. The program entitled "Dual Purpose with Dual Benefit: Research in Biomedicine and Agriculture Using Agriculturally Important Domestic Animal Species" results from a revived recognition of the mutual interests and great promises held by parallel and collaborative efforts in human and domestic species. After all, formidable advances in the laboratory will continue to be made by scientists that bridge animal and human ART, as was the case for the founding fathers of the field.

It may be argued that clinical ART is at an important juncture. It now assists many patients, offering a range of technologies as a direct result of increased knowledge and advances from several decades of research in animal models. Yet, there is so much left to understand about gamete and embryo development, with the ultimate goal to improve the efficacy and safety of ART. Animal studies must be relied upon heavily prior to cautious application in human medicine—preclinically, using human gametes and embryos followed by systematic and rigorous testing in the clinic [220, 221]. Commercial and clinical needs have propelled the fields of livestock and human ART, although perhaps at too precipitous of a pace. To identify and mitigate potential concerns, basic research studies must also continue in a variety of models [135].

The selection of appropriate animal models is important to consider and reevaluate as new insight is gathered. Historically, some models may have been chosen for reasons other than known developmental similarities to humans. But now that our understanding expands, careful attention should be placed on employing the most biologically appropriate species for the task at hand. Animal and human research provides a growing list of cross-species similarities and differences in developmental processes, needs, and responses (Fig. 7.1). It is most often the case that even when developmental similarities are invoked between two species, these remain mere similarities—it is conceivable that any variants, albeit minor, may impair translation to clinical practices. There are no ART models identical to human on all counts. Each species possesses varying relevancies to human biology that depend on the exact developmental event or technical challenge. Importantly, there are many examples of divergences in gene functions and genetic systems between mouse and human, including for mammalian gametogenesis and fertilization [222]. Nonetheless, even if some findings do not faithfully extrapolate to the human, they still provide general mechanisms and critical insight for future directions.

Properly conducted and interpreted animal studies hold the potential to lead to formidable advances in human ART. We must be cautious not to hasten conclusions from animals to humans. Biases in the interpretation of results from animal studies may lead to erroneous conclusions and wasted time, funds, and resources in clinical settings. To maximize the applicability of animal research, studies must be held to the same highest levels of quality standards as human studies, such as with respect to experimental design, randomization, sample size, statistical analysis, blinding, replicability, a multicenter approach, and data reporting [223–226]. This is not always the case, thus potentially jeopardizing the clinical utility of translational efforts. Several reasons underlie existing difficulties with translating research from bench to bedside [225, 227]. Improvements in the effectiveness of translational research will thus ensure that the promises of animal experimentation can be fulfilled toward the success and safety of human ART.

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Current Status of Time-Lapse Microscopy for Embryo Selection

8

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8.1 Introduction

The first successful in vitro fertilization (IVF) pregnancy 36 years ago was a significant milestone for the treatment of infertile patients. Since that first success, which was achieved through a true tour de force, numerous improvements have been made to increase the success rate of IVF. One of the major limitations of IVF over the years has been difficulty in identifying the most developmentally competent embryo(s) in any given cohort. The selection and subsequent transfer of multiple embryos in the hope of at least one leading to delivery of a live-born infant resulted in unacceptably high rates of multiple pregnancies. With these multi-gestation pregnancies came the associated risks to fetal and maternal health [1]. Pregnancy-related maternal complications, including preeclampsia, gestational diabetes, and preterm labor, occur 2–10 times more often in pregnancies with multiple fetuses than singletons. Similarly, perinatal morbidity is significantly increased in multiple pregnancies, resulting in complications such as low (<2,500 g) and very low (<1,500 g) birth weight, preterm birth, and their dangerous consequences (e.g. cerebral palsy, retinopathy, bronchopulmonary dysplasia, polycythemia, hypoglycemia, necrotizing enterocolitis) [1]. Even more sobering is the rate of perinatal mortality, which is fourfold higher for twin gestations and sixfold higher for triplets compared with singleton pregnancies [2].

To avoid these complications, a variety of approaches have been taken in an attempt to identify the most viable embryo(s), the ones most likely to implant and progress to a live birth. These approaches have mostly revolved around morphologic assessment of embryos, although newer -omic technologies, such as proteomics, metabolomics, and genomics, have also been actively researched.

8.1.1 Morphologic Assessment

The human embryo typically develops along a predictable timeline during preimplantation development (Fig. 8.1). Accordingly, morphologic assessment of embryos has been used to appraise viability since the inception of IVF, and it continues to be the first-line approach for the evaluation and identification of the most viable embryos. Two light microscopy approaches to morphologic measures of embryonic development include

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_8

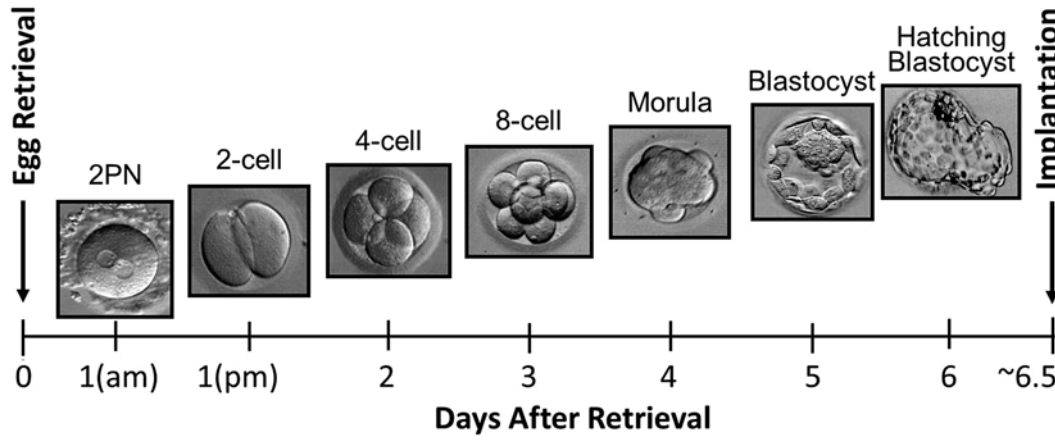

Fig. 8.1 Micrographs of the stages of preimplantation human embryo development as seen in the first 6 days after egg retrieval and fertilization in vitro

developmental rate (i.e., determining if certain milestones are reached within a particular time frame) and morphological characteristics assessed at particular times after insemination. A variety of scoring systems have been described for each of the initial stages of embryonic development, including zygotes at the pronuclear stage, cleavage stage, and blastocyst stage [3]. Within these approaches, evaluation can be done either at a single time point (one-step evaluation) or at several stages during development (multistep evaluation). Existing studies suggest that one or more morphological parameters on any single day of culture can independently predict viable embryo selection. Notably, multiday scoring may provide a predictive value which is no better than single-day morphological evaluation on either day 2 or day 3 of culture [4].

While embryo morphologic assessment is currently the gold standard for embryo evaluation, it has significant limitations with >70 % of embryos failing to implant [5]. This suggests that embryo morphology is not always reflective of embryo viability, and additional methods need to be developed to complement morphologic assessment. Several new techniques, or rather new applications of existing technologies, have attempted to solve this problem. Two general approaches, proteomics and metabolomics, are noninvasive and so are low risk for the developing embryos.

8.1.2 Proteomics

In the context of noninvasive embryo assessment, proteomics more specifically refers to the analysis of the protein makeup of the embryonic secretome, proteins produced and secreted by a developing embryo into the surrounding culture medium. Multiple studies have focused on identifying the composition of the human embryonic secretome in an attempt to differentiate between viable and nonviable embryos. Protein profiling of the embryonic secretome involves the use of mass spectrometry (MS), tandem mass spectrometry, protein microarrays, liquid chromatography, two-dimensional gel electrophoresis, and various combinations of these technologies [5].

The first successful analysis of the protein secretome profile of individual human embryos occurred in 2006 using MS technology [6]. In this study, Katz-Jaffe et al. demonstrated an association between protein expression profiles as identified in spent culture media, the stage of development, and embryo morphology on each day of preimplantation development. Since then, other groups have explored the secretome using other techniques. In 2008, Dominguez et al. used protein microarrays to analyze the human blastocyst secretome and identified two proteins which had significantly decreased expression in pooled spent media of blastocysts that went on to implant versus those that did not [7]. Secretome analyses

were also performed to attempt to differentiate, in a noninvasive manner, between euploid and aneuploid blastocysts. Notably, several potential biomarkers had significant differences in expression in the secretomes of aneuploid versus euploid blastocysts [8].

While these results are promising, significant challenges remain for the field of proteomic analysis of developing blastocysts, including limited knowledge of the constituency of the embryonic secretome and limited technology to allow for these analyses to be performed accurately in individual IVF centers.

8.1.3 Metabolomics

Another noninvasive approach to differentiate viable from nonviable embryos involves analyzing the metabolites, rather than the proteins, in spent embryo culture media. This approach arose from the knowledge that certain nutrients are required for normal in vitro embryo development and that the metabolomic profile of cells is dynamic, changing markedly depending on cell-cycle stage and in response to disease processes or external forces [9]. Thus, changes in the levels of these nutrients or their metabolites could be used to screen out abnormally developing embryos. Similar to proteomic analysis, metabolomic analyses have been carried out using a variety of techniques, including MS, nuclear magnetic resonance spectroscopy (NMR), and vibrational spectroscopy (including Raman and infrared spectroscopy) [9]. Furthermore, these studies have progressed along two different approaches: targeted analyses or profiling studies. The former targets predefined metabolites, while the latter involves analyzing a wide range of metabolites with the objective of identifying one or more that are associated with a specific outcome [3].

The first report of a correlation between embryo viability and the spent culture media metabolome occurred in 2007, 1 year after the first human embryo secretome analysis was published. Seli et al. identified a differential mean spectrum of metabolomes between embryos that failed to implant versus those that resulted in a live birth [10]. The algorithm developed from that study was then tested in a prospective pilot study by Scott et al., who showed a diagnostic accuracy of 80.5 % to predict delivery or failed implantation [11]. Several follow-up randomized control studies (RCTs) had variable results, with two of them showing no improvement in pregnancy rates [12] or live-birth rates [13] when using metabolomic profile with morphology versus morphology alone, and one RCT demonstrating significantly improved implantation rates when morphologic analysis was complemented with metabolomics [14].

However, the field of embryologic metabolomics is still in its infancy, and a commercial version of the near-infrared spectroscopy unit used in these latter studies was withdrawn from the market due to wide variability in performance and inconsistent results [9].

8.1.4 Genomics

While proteomic and metabolomic analyses delved into noninvasive approaches to differentiate viable from nonviable embryos, significant improvements in genomics have also made their mark on preimplantation embryo analysis. Unlike the analysis of spent culture medium, genomic analysis of embryos necessitates invasive procedures to obtain genetic material via biopsy of the polar body, the blastomere, or the trophectoderm. Initially, this genetic material was analyzed using fluorescence in situ hybridization (FISH) technology [15]. Although sound in theory, the promise of increasing implantation and live-birth rates using preimplantation genetic screening (PGS) for aneuploidy has not panned out. Early observational studies showed that use of PGS is associated with higher embryo implantation rates but not higher rates of ongoing pregnancies [16]. Even more notably, a multicenter, double-blind RCT comparing IVF with and without PGS using FISH demonstrated detriment when using PGS, with significantly lower ongoing-pregnancy and live-birth rates in women who were assigned to PGS [16]. Other RCTs have confirmed these

results, and both the *American Society for Reproductive Medicine (ASRM)* [17] and the *European Society for Human Reproduction and Embryology (ESHRE)* [18] issued policy statements stating that PGS with FISH is ineffective in improving clinical pregnancy rates.

Since then, however, new technological advances in genomic screening have brought PGS back to the forefront. Collectively referred to as comprehensive chromosome screening (CCS), these specifically use array comparative genomic hybridization (aCGH), single-nucleotide polymorphism (SNP) microarrays or rapid quantitative real-time polymerase chain reaction (qPCR), and next-generation sequencing (NGS). Two recent RCTs using CCS technologies after blastocyst biopsy demonstrated statistically higher sustained implantation rates and live-birth delivery rates in women who had PGS in comparison to those who did not [19, 20]. Both of these trials were performed on good prognosis patients, thereby limiting the generalizability of the trial to other patient groups. Also, these trials did not include results from subsequent cryopreservation cycles and therefore were unable to address the impact that CCS has on cumulative pregnancy rate (i.e., the pregnancy rate per started cycle including fresh and cryo-transfers). Furthermore, genomic analysis remains an invasive procedure and the long-term consequences of embryonic manipulation on offspring health are still unknown.

8.1.5 Time-Lapse (Dynamic) Imaging

Thus, embryologists are still searching for noninvasive techniques that will help them identify the most viable embryos. A relatively recent entrant into this field is time-lapse microscopy (TLM) and morphokinetics. Interestingly, time-lapse photography of embryo development is not completely novel. In 1929, Lewis and Gregory recorded and analyzed the timing of events in the development of rabbit embryos [21]. Stemming from those early experiments, the goal of TLM is to observe differences in development between viable and nonviable embryos and to use algorithms developed from those differences to predict which embryos are most viable.

8.2 Time-Lapse Microscopy Technology

The basic concept of TLM is the use of a digital camera to take magnified images of embryos at set time intervals; these images can then be played back as a time-lapse sequence to observe embryo development. One of the major new benefits of these systems is that they allow for morphological analysis without removing embryos from the incubator for manual analysis. This decreases the total light exposure and abrupt changes in temperature, oxygen concentration, and pH, as well as the physical manipulation of the embryos, all of which have detrimental effects on embryo development and implantation potential [22]. Moreover, imaging the embryos constantly over time provides a dynamic picture of embryo development.

A number of TLM systems have emerged in the IVF field during the past 5 years, including the EmbryoScope® (Fertilitech, Inc.), the Primo Vision system (Vitrolife), and Eeva™ (Auxogyn, Inc.). These systems differ in several ways. Some are installed into existing embryo incubators (Primo Vision and Eeva™), while others are stand-alone combined TLM incubators (EmbryoScope®). Furthermore, the TLM systems use different light sources to photograph the embryos, bright field versus dark field (Fig. 8.2). Bright-field technology takes advantage of the embryo absorbing light to create contrast, while dark-field technology creates contrast by taking advantage of light scattering [23]. These technological differences have significant implications.

First, the total amount of light to which embryos are exposed differs in each of these systems. Eeva™, a dark-field microscope, uses a light-emitting diode (LED) at a wavelength of 625 nm. Over the course of 3 days of capturing images every 5 min, Eeva™ exposes embryos to a total of 0.32 J/cm^2 of energy, which is equivalent to approximately 21 s of exposure from a

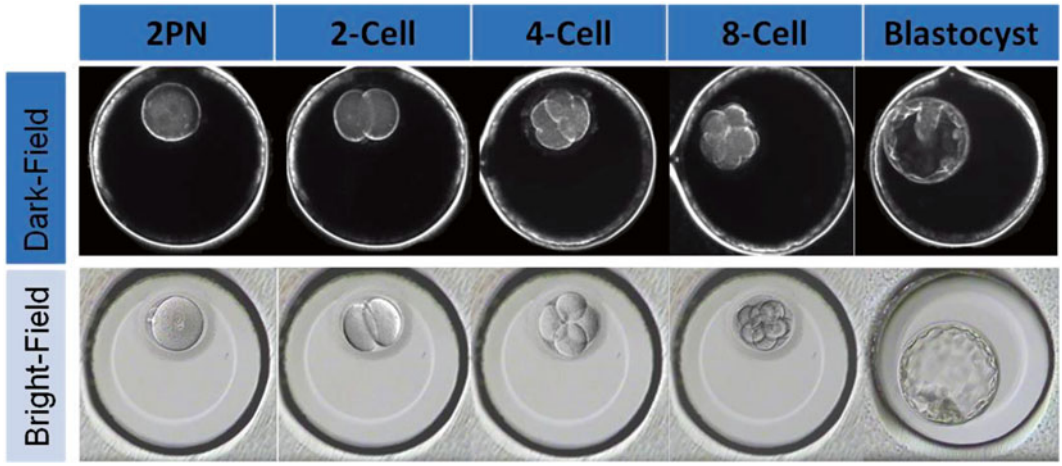

Fig. 8.2 Time-lapse microscopy images of normal embryos at different stages of development as seen using dark-field (*top row*) or bright-field (*bottom row*) technology

traditional IVF bright-field microscope [24]. In contrast, EmbryoScope® uses a bright-field system, although it differs from traditional IVF bright-field microscopy. Similarly to Eeva™, EmbryoScope® uses a single LED at a wavelength of 635 nm, thus eliminating the short wavelengths of light below 550 nm that are known to be damaging to embryo development [25, 26]. Capturing images in five focal planes every 15 min over the course of 3 days, the EmbryoScope® exposes embryos to a total of 20 J/m^2 [27], compared to 394 J/m^2 during traditional IVF microscopy [28]. Thus, both bright-field and dark-field time-lapse imaging systems expose embryos to less light than conventional IVF techniques. Multiple studies of time-lapse imaged human embryos have subsequently demonstrated no negative impact of the imaging technique on fertilization rate, embryo development, blastocyst formation, or implantation [29–32], and dark-field time-lapse imaging had no demonstrable effect on the gene expression in developing mouse embryos [24].

Second, the two types of techniques differ as to what they can detect. Dark-field TLM can solely detect cytokinesis. Bright-field TLM, in contrast, can show both cytokinesis and the presence of nuclei, allowing the embryologist to distinguish large fragments and blastomeres within the developing embryo [33]. Given these differences, Azzarello et al. (2014) prospectively evaluated the potential of nucleus observation vs. kinetic analysis to predict live-birth potential. They concluded that in bright-field time-lapse, the observation of nuclei alone can identify embryos of poor quality and abnormal cytokinesis, and embryo kinetics does not provide added value. Dark-field TLM, although able to identify some of the abnormal embryos, was less sensitive than light-field TLM in recognizing embryos with no live-birth potential [33]. Nevertheless, as discussed below, algorithms using dark-field illumination and based on kinetic markers have potential in predicting outcomes.

8.2.1 Technological Capabilities and Measured Parameters

Given these technical specifications, what parameters can embryologists investigate using TLM? The primary applications of this technology today are in analyzing the morphology and the morphokinetics of developing embryos. The former is an extension of traditional morphologic analysis of embryos at static time points, adding the possibility of identifying developmental milestones normally missed by traditional approaches without disturbing the system. The latter refers to the ability to determine precisely

the timing of specific morphological occurrences, including the length of specific stages of embryonic development and the elapsed time between certain milestones.

8.2.1.1 Morphologic Analysis

8.2.1.1.1 Pronuclear Stage Embryo

One of the first morphologic events assessed during IVF is the presence of two pronuclei (PNs) at the fertilization check. Typically, this assessment is done at approximately 16–18 h after insemination. However, using a single static time point can miss the appearance and disassembly of one or both PNs. Because of continuous recording, TLM can help identify these transient PNs, thereby avoiding discard of diploid zygotes that have undergone early PN disappearance or fusion of the 2PNs to form only 1PN [34]. Furthermore, TLM allows monitoring of PN position. In normal development, the PNs migrate into apposition in the center of cytoplasm. Failure of the PNs to migrate into apposition has been shown to be associated with lower blastocyst developmental potential [35].

8.2.1.1.2 Cleavage-Stage Embryo

Day 2/3 morphology is commonly used in embryo assessment prior to transfer. Morphological assessment on these days includes evaluating the number of cells in the embryo, the extent of fragmentation, blastomeric symmetry, multinucleation, and the presence of compaction [3]. The limitation of static assessment at single time points on days 2 and/or 3 relates to the fact that cell number and extent of fragmentation can change rapidly during dynamic embryonic development. This opens up the possibility for using TLM to observe and track the appearance and disappearance of fragments and better identify embryonic fragmentation [34].

Blastomere multinucleation is a key morphologic observation because it may serve as a possible marker of aneuploidy and implantation potential. Compared to non-multinucleated embryos, embryos with evidence of multinucleation at the 2-cell stage have significantly lower implantation rates (43.6 % vs. 23.3 %, respectively, $p<0.001$) [36]. While traditional morphologic analysis may identify multinucleation, because it is a transient occurrence, TLM can identify this marker with greater sensitivity. Indeed, of embryos found to have multinucleation using TLM, only 27.6 % (44 of 159 embryos) could be detected within the time frames proposed by ESHRE/ALPHA consensus [22–24 h, 25–27 h, or 44–45 h post-intracytoplasmic sperm injection (ICSI)] [36, 37]. As previously mentioned, though, only bright-field TLM has the ability to visualize nuclei, while dark-field TLM is limited in this regard.

8.2.1.2 Morphokinetic Analysis

While morphologic analysis using TLM is an extension of static observations of embryos during classic morphological evaluation, morphokinetic analysis is unique to TLM. Time-lapse technology and continuous monitoring of embryos allow for identifying the precise timing of various milestones in embryonic development. Reported milestones include second polar body extrusion; the appearance of the 2PN and the subsequent fading of the 2PNs/syngamy; cleavage to 2, 3, 4, 5, 6, 7, and 8 cells; morula formation; start and completion of compaction; start of blastocyst cavitation/blastulation/blastocoel formation; full blastocyst formation; expanded blastocyst formation; and blastocoel cavity contractions [38].

Based on these time points, a variety of calculated parameters have been defined to describe the time an embryo spends in each phase of development and how long it takes to go from one phase to another. These calculated time-lapse parameters include various cell-cycle durations, time to complete synchronous division (i.e., blastomere synchrony), duration to compaction, and others. Unfortunately the nomenclature used by various studies to describe these parameters is inconsistent. This is most evident in authors' definitions of "cc" and "synchronicity." Some define "cc" as the time required for a doubling of cell number (cc1 yields 2-cell embryo, cc2 yields 4-cell embryo, cc3 yields 8-cell embryo, etc.) [39], while others define "cc" as the duration of a particular cell stage (i.e., cc2 is duration of 2-cell stage, and cc3 is duration of 4-cell stage) [31] or

as a round of cleavage (cc3 = time to 5-cell stage minus time to 3-cell stage) [40]. The same inconsistency is evident for "synchronicity" with one author [41] defining synchronicity 3 (s3) as the time from the 4-cell to 8-cell stage and another author [40] defining s3 as the time from the 5-cell to the 8-cell stage.

These inconsistencies in the current nomenclature for time-lapse markers make it difficult to compare different studies and draw sound conclusions. Standardizing the language describing various milestones in early embryologic development will allow investigators to define more clearly the underlying biology of developing embryos in the analysis of future TLM studies. Two groups have recently proposed uniform, but distinct, sets of nomenclatures for currently identified TLM markers (Table 8.1). Some of the proposed definitions are the same or similar, while others are unique.

Table 8.1 Proposed standardized nomenclatures for time-lapse morphokinetic markers

Proposed nomenclature		Milestone/developmental measure	
Kaser and Racowsky	Ciray et al.	Kaser and Racowsky	Ciray et al.
t_0	*t*0*	Time of injection (ICSI) or time of sperm head binding to oolemma (IVF)	Mid-time of injection (ICSI) or time of IVF*
t_{2pb}	*t*PB2	Time that the second polar body is first encircled by a complete membrane (completely detached from oolemma)	
–	*t*PN	–	Time at which fertilization status is confirmed
–	*t*PN1a, *t*PN2a, *t*PN3a…	–	Time at which the first, second, third, etc., pronuclei first become visible
t_{2pn}	–	Time that two pronuclei are first visualized	–
$t_{2pn.a}$	–	Time that two pronuclei first remain in contact before onset of dissolution (pronuclear abuttal)	–
t_1	*t*PNf	Time that both pronuclei are no longer visible	
t_{cf1}**, t_{cf2}, t_{cf3}…	–	Time at which the first, second, third, etc., cytokinesis (cleavage) furrow is clearly distinguishable**	–
t_2, t_3, t_4…t_{16}	*t*2, *t*3, *t*4…*t*9	Formation of 2-cell stage, 3-cell stage, 4-cell stage, etc., through the 9- or 16-cell stage. Recorded as the time at which newly formed cells are completely separated by confluent membranes	
t_c	*t*SC	Start of compaction: time at which membranes of adjacent blastomeres start to become indistinguishable	
t_m	*t*Mf/p	Formation of morula—the end of the compaction process. Ciray et al. also defines morula as fully (f) or partially (p) compacted	
t_{cav}	*t*SB	Start of cavitation/blastulation—time at which a pocket of fluid is first identified	
$t_{b.e}$	–	Early blastocyst formation—time at which the blastocoelic cavity first occupies less than half the volume of the embryo	–
$t_{b.xg}$	–	Expanding blastocyst formation—time at which the blastocoelic cavity first occupies more than half the volume of the embryo	–

(continued)

Table 8.1 (continued)

Proposed nomenclature		Milestone/developmental measure	
Kaser and Racowsky	Ciray et al.	Kaser and Racowsky	Ciray et al.
$t_{b.f}$	*t*Byz***	Formation of full blastocyst—time at which the blastocoelic cavity first occupies the entire volume of the embryo	Formation of full blastocyst—last frame before zona pellucida starts to thin
–	*t*Eyz***	–	Initiation of expansion—first frame when zona pellucida starts to thin
$t_{b.xd}$	–	Formation of expanded blastocyst formation	–
$t_{b.hg}$	*t*HNyz***	Initiation of the hatching process—time at which the trophectoderm starts to herniate through the zona pellucida	
$t_{b.hd}$	*t*HDyz***	Formation of hatched blastocyst—time at which the blastocyst completes escapement from the zona pellucida	
$t_{b.c1}$, $t_{b.c2}$, $t_{b.c3}$…	–	Time at which the first, second, third, etc., contraction of the blastocyst occurs (i.e., time of maximum shrinkage during one contraction event)	–

Source: Data from Kaser and Racowsky (Table IV) [42] and Ciray et al. (Table I) [43]
*Ciray et al. suggest using *t*0 (time of IVF or mid-time of injection [ICSI]) as the standard referent for all cycles
**Kaser and Racowsky suggest using *t*cf1 (identification of the first cytokinesis furrow) as the standard referent for all cycles because it is the first clearly identifiable time point in embryologic development that is clearly identifiable regardless of the time-lapse imaging system used or the method of insemination (IVF or ICSI)
***Ciray et al. recommend grading the morphology of the inner cell mass (y) and trophectoderm (z) using static parameters within time frame described

Notably, the proposed nomenclatures both consist of several general principles. First, they both suggest defining a standard referent for all cycles, although what that referent is differs. Kaser and Racowsky suggested using the time that the first cytokinesis furrow is identified as the referent time point, because it is the first time point in embryologic development which is clearly identifiable regardless of the type of time-lapse imaging system used (bright field or dark field) or the method of insemination (ICSI or IVF). The latter avoids the issue of not definitively knowing the exact time of insemination in IVF. Ciray et al., on the other hand, suggested that the main referent time point, *t*0, should be set at the time of sperm injection for ICSI or as the mid-time point from when injection begins and ends for a specific patient's oocytes.

Second, both groups provide guidelines for defining all the other stages. Kaser and Racowksy recommended that stages be defined as the "time of first definitive identification." For example, an embryo reaching the 2-cell stage would be defined as the time at which both the cells are first identified to be completely separated by confluent membranes. Ciray et al. provided a similar guideline, setting the time recorded for all events at the first or last frame (image generated by TLM) at which an event is identified.

Finally, both groups provide suggested annotation for the derivation of time intervals and duration of any specific stage of embryo development. Kaser and Racowsky suggested that the relationship between any two stages in development should be defined by the following general formula: $t_i = t_y - t_x$, where y is a more advanced developmental stage, x is an earlier developmental stage (such as the predefined referent), and t_i is the time interval it takes for an embryo to develop from stage x to stage y. Ciray et al., in turn, suggested an identical formula with a different notation: $d(\text{event}) = t(\text{event})(\text{end}) - t(\text{event})(i)$, where d is duration of the stage and i is the initiation of an event. Both of these formulas and definitions

will help define stage durations, even ones not yet reported in the literature.

No matter which of these proposed nomenclature guidelines is chosen, it is imperative for the success of this field that a uniform nomenclature is accepted and implemented. Without it, future comparisons among studies will become increasingly more difficult as new events in embryo development are defined and measured using TLM.

8.3 Evaluation of the Literature

Given the types of analyses possible with TLM and the morphologic and morphokinetic parameters that have been identified, what does the literature actually say about the utility of TLM in selecting the most viable embryos?

8.3.1 Safety

Before we explore this question, though, it is imperative to discuss the safety profile of TLM with its new culture conditions which differ from conventional practice. A recent review suggested that the optimal study design to assess the safety of culture conditions in TLM would have two arms. In one arm, embryos would be cultured in conventional incubators; in the other arm, embryos would be cultured in a TLM system [42]. So far, three studies have explored this question directly.

The first randomized sibling zygotes to either time-lapse observation using an incubator with an integrated microscope (SANYO In vitro Live Cell Imaging Incubation System using a white LED) or to conventional observation using the same incubator without the microscope [32]. Embryos in the time-lapse group ($n=146$) were continuously evaluated for 72 h after ICSI, while the embryos in the conventional group ($n=146$) were removed from the incubator daily for evaluation. In this study, the authors showed no difference between groups in the quality of the embryos (36 % were of good or excellent quality by modified Veeck's morphological classification in each group, $p=0.872$) or the proportion of fertilized embryos that had at least four blastomeres on day 2 evaluation ($p=0.750$). This suggested that TLM systems may be no worse for embryo quality and development than conventional culture with serial bright-field microscopy evaluation. However, this study was limited by potential confounding associated with daily removal from the incubator of only the non-TLM embryos.

The other two studies were RCTs [29, 43], both of which used the EmbryoScope® to evaluate the safety of TLM. In one of the studies, the authors found no significant difference between the TLM system and the standard incubator in the proportion of embryos that developed into blastocysts or the proportions of embryos that were frozen, transferred, or discarded. The authors also found no difference in the ongoing-pregnancy rate between embryos incubated in the EmbryoScope®, in the standard incubator, or with mixed transfers [29]. Similarly, the second trial demonstrated no significant difference between the proportion of 4-cell embryos on day 2, 7–8-cell embryos on day 3, blastocysts on day 5, or implantation pregnancy rates as documented by the presence of fetal heart activity (FH) by ultrasound at 8 weeks post-transfer [43]. However, Armstrong et al. (2015) argue that both of these studies have flaws in design and interpretation, meaning that neither of the studies can rule out the inferiority of culturing embryos in a TLM system, especially if the desired outcome measurement is ongoing pregnancy and live birth [42].

In the future, additional studies are required that truly validate the safety of TLM. Moreover, as Armstrong et al. (2015) point out, TLM systems are much more expensive than conventional incubators. Therefore, to justify the additional cost of the technology, studies will have to prove that TLM is not only safe but that it also provides substantial clinical benefit [42].

8.3.2 Blastocyst Development

The ability to predict the potential of an embryo to develop into a blastocyst is another attractive possibility. While blastocyst transfers may have

slightly higher live-birth rates than day 2/3 transfers, the cumulative pregnancy rate may be significantly higher for embryos transferred at early cleavage stages rather than as blastocysts, at least with slow-freezing protocols [44, 45]. Moreover, it appears that extended culture is associated with an increased risk of both monozygotic [44, 46] and monochorionic twinning [47]. Thus, there are possible detriments of extended culture. For example, animal studies have shown that in vitro culture has a demonstrable epigenetic effect, with genome-wide disturbances of methylation reprogramming and alteration of parent-specific imprinting. In humans, ART has been associated with an increased prevalence of imprinting defects that cause Beckwith-Wiedemann and Angelman syndromes, although it is unknown whether this increased risk is due to inheritance, ovarian stimulation, or in vitro manipulations [48]. Nevertheless, the ability to predict which cleavage-stage embryos will successfully develop into blastocysts will help increase the proportion of embryos that are transferred on day 2/3 and thus subject embryos to less in vitro culture and manipulation.

The association between blastocyst development and various morphokinetic markers has been evaluated in a number of studies (Fig. 8.3). The first study to show that TLM-monitored morphokinetic parameters can accurately predict the successful formation of a blastocyst was published in 2010 [49]. In this study, the authors analyzed 242 IVF-derived supernumerary thawed embryos. Of 100 embryos that were cultured to day 5 or 6 in a TLM system, 33–53 % formed blastocysts, while the rest arrested at an earlier stage of development. The authors then identified three morphokinetic parameters which collectively could predict blastocyst formation—the duration of the first cytokinesis, the duration of the 2-cell stage, and the time interval between the appearance of cleavage furrows of the second and third mitoses (yielding a 4-cell embryo). By setting certain limits on the duration of these three parameters, the authors were able to predict which embryos went on to reach the blastocyst stage with a sensitivity of 94 % and a specificity of 93 % [49]. A major limitation of this study, however, is that none of the embryos were subsequently transferred for implantation. Thus, it is unknown whether the embryos that meet the criteria set forward by this model would subsequently implant and progress to a live birth more efficiently than the embryos that do not meet the criteria.

Since that initial study, several other groups have published studies that support the possibility of predicting high-quality blastocyst formation using morphokinetic parameters from the first 3 days of embryo development. Hashimoto et al. [50] found that embryos developing into high-scoring blastocysts took significantly less time ($p<0.05$) to develop from the 2-cell stage to the 7-cell and 8-cell stages (30.2 ± 0.8 h and 33.0 ± 1.1 h, respectively) than those that developed into low-scoring blastocysts (37.5 ± 2.8 h and 42.4 ± 2.8 h, respectively). Furthermore, the high-quality embryos also spent significantly less time in the second cleavage (3- to 4-cell, 0.68 ± 0.17 h) and third cleavage (5- to 8-cell, 5.74 ± 1.25 h) stages than the low-quality embryos (3.69 ± 1.0 h and 16.87 ± 2.13 h, $p<0.05$ and $p<0.01$, respectively). On the other hand, they found no difference in the amount of time it took embryos that became high-scoring vs. low-scoring blastocysts to go from pronuclear disappearance to the first cell division and to develop from the 2-cell stage to the 3-, 4-, 5-, or 6-cell stages [50].

In comparison, Cruz et al. [45] showed that good-morphology blastocysts progressed through the cleavage cycles faster than poor-morphology blastocysts, spending less time as 3-cell embryos ($p=0.006$) and reaching the 4-cell ($p<0.05$), 5-cell ($p<0.002$), and morula ($p=0.001$) stages faster. This study also demonstrated that embryos forming blastocysts progressed through the early cleavage cycles significantly faster than embryos that arrested early. Competent embryos took significantly less time to reach the following cell-cycle stages: cleavage to 2 cells (26.8 ± 0.2 h vs. 27.9 ± 0.5 h), 3 cells (39.2 ± 0.4 h vs. 40.8 ± 0.8 h), and 4 cells (39.9 ± 0.4 h vs. 42.4 ± 0.9 h), and morula formation (90.6 ± 0.6 h vs. 93.7 ± 1.6 h) (all $p<0.001$). They also spent significantly less time at the 2-cell (12.4 ± 0.3 h vs. 13.0 ± 0.6 h,

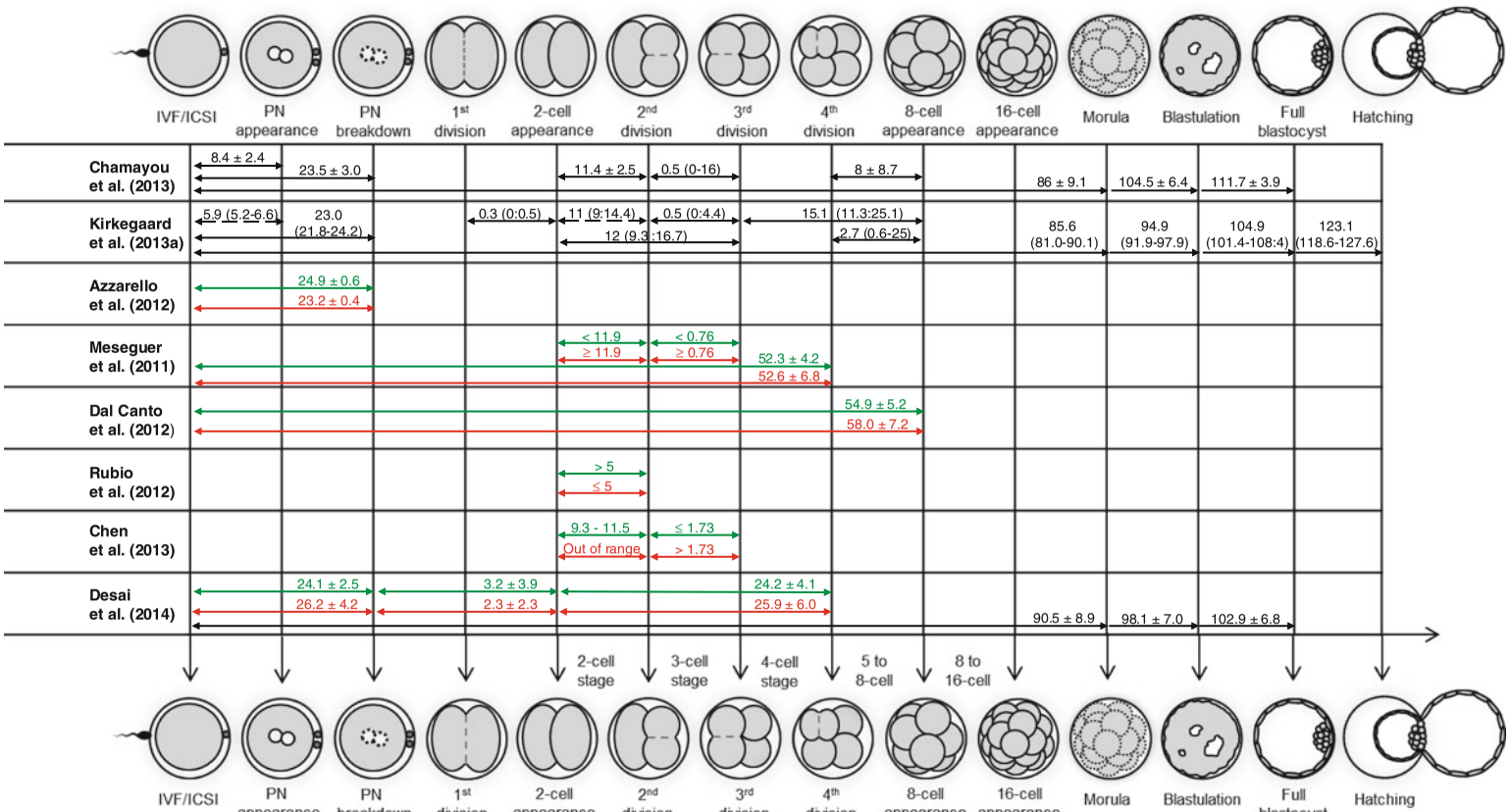

Fig. 8.3 Schematic of preimplantation embryo development with corresponding time-lapse markers from eight of the studies with time values reported. When there was no significant difference observed between "implanters" and "non-implanters," only the value for the implanted embryos is shown (in *black*). When significant differences were reported, the "implanter" values are shown in *green*, and "non-implanters" are in *red*. All values are expressed in hours, as mean ± standard deviation or mean (95 % confidence interval) for normally distributed values and median (minimum/maximum) for non-normally distributed variables. *PN* pronuclei. Originally modified from [63] (Modified from Kaser and Racowsky [56], by permission of Oxford University Press)

$p = 0.006$) and the 3-cell (0.6 ± 0.2 h vs. 1.0 ± 0.5 h, $p = 0.031$) stages [45]. Thus, Cruz et al. showed that faster development to the blastocyst stage was on a continuum, with good-morphology blastocysts developing faster than poor-morphology blastocysts, which in turn progressed through development faster than embryos that arrested prior to reaching the blastocyst stage.

In contrast to these findings, Dal Canto et al. [51] did not find significant differences in the amount of time spent in the 2-cell or 3-cell stages or the amount of time it took to reach the 2-, 3-, 4-, 5-, or 6-cell stages between embryos that arrested after the 8-cell stage versus those that successfully developed into blastocysts. However, this study did show that embryos that successfully developed into blastocysts took significantly less time to reach 7 or 8 cells and subsequently spent less time in the interval between 4 and 8 cells and 5 and 8 cells. Furthermore, they found that of the embryos that reached the blastocyst stage, those that subsequently expanded had progressed significantly faster through the cleavage cycles to reach the 8-cell stage than those that did not expand [51]. This last result thus supports the conclusions of Cruz et al. in showing that blastocysts with better developmental potential develop faster than those with worse development.

Based on observing the development of 180 pronuclear embryos using TLM, Hlinka et al. determined cleavage timeliness by normalization of the resulting data from blastocysts that implanted. Stratifying embryos into those which reached all cleavage milestones in a timely fashion versus those that had at least one untimely time point, they demonstrated that embryos that divide in an untimely fashion are more likely to yield abnormal morphology and much less likely to develop into blastocysts than embryos that are timely [52].

Desai et al. [53] also undertook an analysis of morphokinetic parameters that may predict high-quality blastocyst development. In a sample of 648 embryos, they found significant differences in most early morphokinetic markers between embryos that formed high-quality blastocysts for transfer or freezing and those that formed poor-quality blastocysts or embryos that arrested prior to blastulation. Notably, time to pronuclear fading; times to 2, 4, 8, and 9+ blastomeres; and times to morula, start of blastulation, blastocyst formation, and expanded blastocyst formation were all significantly shorter in the high-quality vs. low-quality embryos. Subsequently, the derived duration of the first cell cleavage, the 2-cell and 3-cell embryo, and time intervals from 4- to 5- and 2- to 5-cell stages were all significantly shorter in the high-quality blastocysts [53].

Kirkegaard et al. [39] then used parameters that had been previously identified in the literature as potential predictors of development (time point of PN breakdown, duration of the first cytokinesis, division to 2-, 3-, and 4-cell embryos, duration of 2- and 3-cell stages, direct cleavage to 3 cells (<5 h from 2 to 3 cells), and multinucleation at 2-cell stage) to create models for the potential of an embryo to develop into a high-quality blastocyst vs. low-quality blastocyst/arrested development. Based on the 571 2PNs in their cohort, the authors found that duration of the first cytokinesis, duration of the 3-cell stage, and absence of direct cleavage to 3 cells can predict the development of high-quality blastocysts. Furthermore, they found that combining the three variables could better predict the development of high-quality blastocysts (area under the curve (AUC) of 0.69 vs. 0.63, 0.63, and 0.58) [39].

Taking a similar approach that combining multiple parameters may predict blastocyst formation and quality better than individual parameters, Cetinkaya et al. [54] analyzed 17 morphokinetic parameters, some of which were absolute timings, while others were derived intervals and ratios, in a cohort of 3,354 embryos. Notably, the authors found that all but one of the measured parameters (time to 3-cell stage) were significantly different (all $p < 0.0001$) between embryos of top and good quality versus embryos of bad quality and arrested embryos (as defined by Gardner's classification). Given these findings, the authors individually tested each of their variables by building receiver-operating curves and calculating the AUCs. Notably, they found that the three highest AUCs were from derived time variables, specifically the duration of time

from 5 to 8 cells (AUC 0.778, sensitivity 77.35 %, specificity 67.65 %), the cleavage synchronicity from 4 to 8 cells[1] (AUC 0.776, sensitivity 82.47 %, specificity 61.92 %), and the cleavage synchronicity from 2 to 8 cells[2] (AUC 0.786, sensitivity 83.43 %, specificity 62.46 %) [54].

Two studies then took morphokinetic analysis from TLM systems one step further by prospectively applying a blastocyst prediction model to cohorts of patients [24, 55]. Both studies employed the Eeva™ TLM system, in which they recorded early embryonic development and determined the time intervals it took embryos to progress through the first three cytokinetic stages, from one to two blastomeres, two to three blastomeres, and three to four blastomeres.

The first study [24] had two phases, a development phase and a test phase. In the development phase, the researchers recorded the defined cytokinetic stages, built a classification tree model to determine optimal timing windows for embryo development to predict blastocyst formation, and created an automated image software that allowed the system to automatically track the embryos and provide a score. This Eeva™ Test categorized embryos into two groups—high and low probability of blastocyst formation—based on two of the three parameters: time between the first and second mitosis and time between the second and third mitosis [24].

Subsequently, in the test phase, embryologists at five different fertility centers predicted blastocyst formation using either day 3 morphology alone or using both day 3 morphology assessment and Eeva™ Test results. The authors found that having the adjunct information from the Eeva™ Test significantly improved both the specificity (84.7 % vs. 52.1 %, $p<0.0001$) and the positive predictive value (54.7 % vs. 34.5 %, $p<0.0001$) of predicting on day 3 the development of usable blastocysts, when compared with morphologic evaluation alone [24].

A subsequent study by a separate group of researchers provided similar results [55]. In this study, the researchers separated embryos into two groups. In the first group, five embryologists independently predicted blastocyst formation using day 3 morphology alone. In the second group, the same embryologists predicted blastocyst formation using both day 3 morphology assessment and Eeva™ Test results. The authors found that having the adjunct information from the Eeva™ Test increased the odds ratio from 1.68 (95 % CI = 1.29–2.19) to 2.57 (95 % CI = 1.88–3.51) of predicting blastocyst formation among embryos graded good or fair [55]. Taken together, these two studies provide the first evidence to prospectively show that TLM may be useful in improving the selection of embryos which will progress to blastocysts in a clinical setting.

One new morphokinetic parameter uses TLM to measure and analyze expansion of blastocoel cavities. The study that first described this parameter used sequential hourly 2D measurements of the cross-sectional area and demonstrated two distinguishing characteristics of expansion—a pulsatile, oscillatory pattern of accelerations and decelerations with a periodicity of 2–3 h in continuous blastocyst expansion and an occasional, acute contraction of the cavity (Fig. 8.4). The clinical significance of these findings is not yet clear [38].

8.3.3 Implantation Potential

While it is useful to predict blastocyst potential early in embryo culture, showing that TLM can help predict implantation or live-birth rates would be much more indicative of the technology's potential. A recent review delved in depth into exploring the clinical outcomes following selection of embryos using TLM based on the parameters measured [56]. As described below, this review concluded that no single parameter consistently correlated with clinical outcome.

The first study to show that early embryo morphokinetics differ between implanting and

[1] Cleavage synchronicity from 4 to 8 cells—defined as the ratio of time an embryo spends developing from 5 to 8 cells in relation to the time it takes to develop from 4 to 8 cells

[2] Cleavage synchronicity from 2 to 8 cells—defined as the ratio of time an embryo spends at the 2-cell and 4-cell stages in relation to the time it takes to develop from 2 to 8 cells

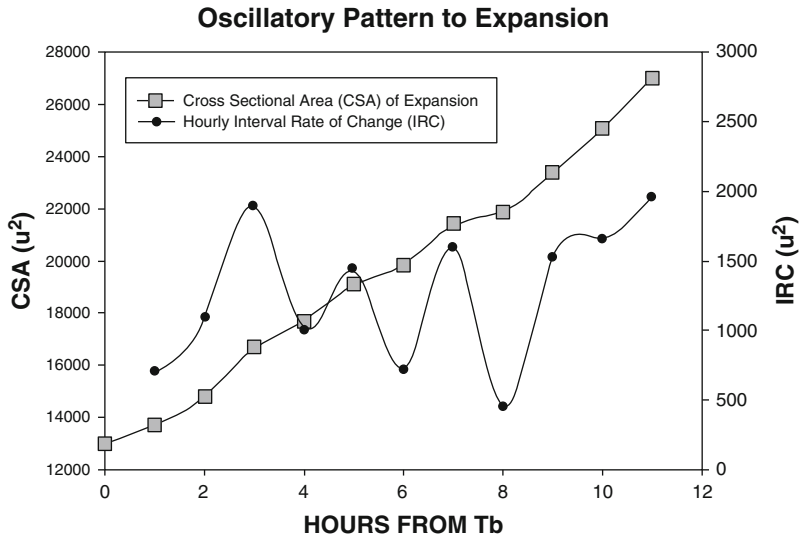

Fig. 8.4 A representative oscillatory pattern from an expanding blastocyst that resulted in a sustained clinical pregnancy. The cross-sectional area (CSA) was measured hourly beginning from the time of blastocyst formation (Tb) measuring ~13,000 square microns until the embryo was prepared for transfer between 11 h later. The hourly interval rate of change in the CSA (IRC) was calculated as the difference (in square microns) of sequential hourly CSA measurements represented on the secondary y-axis. This blastocyst demonstrates a relatively even 2–3 h periodicity and even amplitude but does not show any collapses (negative values in IRC). Thus, expansion is always continuously positive. (Figure courtesy of Dr. Thomas Huang, Pacific IVF Institute, Honolulu, HI)

non-implanting embryos tracked the development of 102 2PN zygotes in a TLM system, 29 of which were subsequently transferred. Of the 19 single-embryo transfers involving 4-cell embryos, 6 implanted. Notably, the embryos that implanted trended toward a faster appearance of nuclei in the first blastomere after the first cleavage than embryos that did not implant, and they had a significantly faster time to synchrony (time from nuclear appearance in the first blastomere to nuclear appearance in the second blastomere after cleavage, $p<0.05$) [57].

Three years later, Meseguer et al. took the capability of TLM further, tracking the morphokinetics of embryos for at least 64 h [27]. Of the 247 embryos with known implantation data (i.e., either implantation of each embryo transferred or no implantation), 61 successfully implanted. Of note, the authors found statistically significant differences in the timing of early cleavage stages; embryos that implanted reached the 2- through 5-cell stages significantly faster than embryos that did not successfully implant, and they spent significantly less time as 2-cell and 3-cell embryos ($p=0.006$ and $p=0.016$, respectively). The authors then identified the exact timing of these first cleavage cycles by quartiles and established optimal ranges reflecting the highest implantation probabilities (which were not necessarily found in the fastest-dividing embryos). Taking this one step further, the authors created a hierarchical model of embryo grading based on the morphokinetic data (time to 5-cell stage, duration of 2-cell stage, and duration of 3-cell stage) and morphologic exclusion criteria. The embryo categories ranged from A through E, with implantation potentials ranging from 52 % down to 8 %, respectively [27]. However, it is critically important to remember that these data were derived retrospectively and may not apply in all situations.

Following up on these results, the same group retrospectively compared implantation rates of embryos cultured in a TLM system (EmbryoScope®) and then selected for transfer using their hierarchical embryo grading system [27] to embryos cultured in a standard incubator and selected using conventional morphologic grading. The authors

noted that after adjusting for oocyte source (autologous versus donor), patient age, day of embryo transfer (day 3 vs. day 5), and number of oocytes, embryos cultured in and selected using the TLM system had significantly higher implantation rates than embryos from the conventional incubator (adjusted odds ratio 1.201, 95 % CI 1.059–1.363, $p=0.0043$) [31].

To further explore the potential benefit of TLM, the same group designed an RCT with two arms identical to the ones described for the retrospective study. The primary study end point was ongoing pregnancy as confirmed by a viable fetus at 12 weeks of gestation. The authors found that when taking into account all cycles, embryos in the TLM group were significantly more likely to result in an ongoing pregnancy than embryos from the control group (RR 1.23, 95 % CI 1.06–1.43, $p=0.005$). Similarly, results per embryo transfer showed improved ongoing-pregnancy rate in the TLM group (RR 1.20, 95 % CI 1.04–1.69), $p=0.01$), and the implantation rate of transferred embryos was also greater in the TLM group (RR 1.43, 95 % CI 1.05–1.39, $p=0.02$). Furthermore, the rates of early pregnancy loss were significantly lower in the TLM group (RR 0.64, 95 % CI 0.45–0.91, $p=0.01$) [58]. Unfortunately, because the embryos were cultured in different incubator systems, neither the above-described retrospective study nor this RCT was able to distinguish what played the greater role in improving the pregnancy outcomes, the stable TLM system culture environment, or the hierarchical selection algorithm based on morphokinetic parameters.

Using the same study data, but published separately [59], the authors also prospectively validated the hierarchical embryo grading system described previously [27], showing that the embryo implantation rate has a direct relationship with the morphokinetic category. The implantation rate decreased from 52.9 % in category A embryos down to 13.7 % in category E embryos.

Dal Canto et al. tracked cleavage development for a slightly longer duration, through the 8-cell stage [51]. Although they did not find a significant difference in the time to develop to the 5-cell stage for those embryos that implanted ($n=19$) versus those that did not ($n=115$) (49.4 ± 4.9 h and 50.7 ± 7.2 h, respectively), they noted that the implanted embryos developed to the 8-cell stage 3.1 h faster than embryos that did not implant (54.9 ± 5.2 h and 58.0 ± 7.2 h, respectively, $p=0.035$) [51].

Several other studies explored very specific TLM parameters and their relationship to implantation potential. Rubio et al. explored the effect of direct cleavage from 2 to 3 cells (i.e., the second cell-cycle duration less than 5 h). In a retrospective cohort, the authors found a significant reduction in implantation rate between those embryos that displayed direct cleavage and those that did not [1/85 (1.2 %) vs. 203/1,007 (20.2 %); $p<0.0001$] [60]. Notably, this finding would seem to be in agreement with results from Kirkegaard et al. (see Sect. 8.3.2), who demonstrated that the absence of direct cleavage to 3 cells was a predictive marker for the development of high-quality blastocysts. However, neither the absence of direct cleavage to 3 cells nor either of the other two morphokinetic parameters predictive of high-quality blastocysts (duration of the first cytokinesis and duration of the 3-cell stage) or any of the other morphokinetic parameters that the authors had recorded could be demonstrated to have significant differences in timing between embryos that implanted versus those that did not [39].

Another study explored several atypical embryo phenotypes, including abnormal syngamy (disordered movement of pronuclei and delayed dispersion of the nuclear envelope), abnormal first cytokinesis (presence of oolemma ruffling before completion of the first cytokinesis), abnormal cleavage (origination of more than 2 cells from a single cell division event), and chaotic cleavage (disordered cleavage behavior up to the 4-cell stage) (Fig. 8.5). Although all of these abnormal phenotypes were significantly associated with decreased blastocyst formation rate, only the presence of abnormal cleavage showed a trend toward a decreased rate of implantation (18 % vs. 3.7 %, $p=0.05$) [61].

In turn, Azzarello et al. explored whether PN morphology and time of PN breakdown can help predict embryo implantation. In a prospective

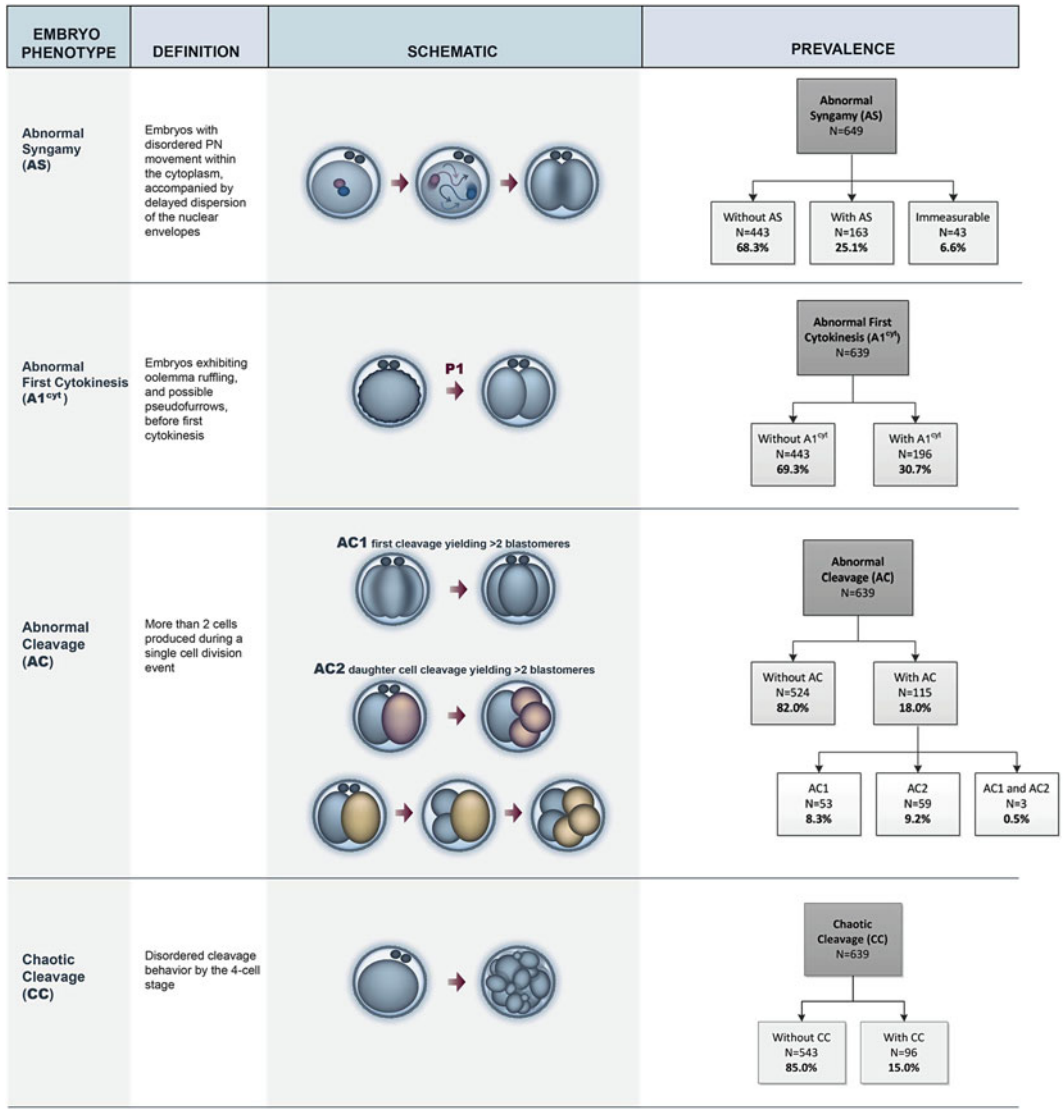

Fig. 8.5 Atypical embryo phenotypes: definition, schematic illustration, and prevalence of abnormal syngamy (AS), abnormal first cytokinesis ($A1^{cyt}$), abnormal cleavage (AC), and chaotic cleavage (CC). (Reprinted from Fertility and Sterility, Vol. 101, Athayde Wirka K, Chen AA, Conaghan J, Ivani K, Gvakharia M, Behr B, Suraj V, Tan L, Shen S, Atypical embryo phenotypes identified by time-lapse microscopy: high prevalence and association with embryo development, pp. 1637–48.e1–5, Copyright (2014) [61], with permission from Elsevier)

cohort of 159 embryos, PN breakdown occurred significantly sooner in unsuccessful embryos compared with those resulting in live birth (23 h 10 min ± 23 min vs. 24 h 52 min ± 35 min, respectively; $p = 0.022$). Furthermore, 0 % (0/17) of embryos in which PN breakdown occurred less than 20 h 45 m after ICSI resulted in a live birth, while 32.4 % (46/142) of embryos in which PN breakdown occurred beyond that cutoff had a successful outcome ($p = 0.003$) [62]. However, none of the six models used to assess pronuclear morphology showed a significant difference in scores between the embryos that resulted in a live birth versus those that did not [62].

In addition to showing that morphokinetic parameters differed significantly between

embryos of high and low quality, Desai et al. assessed utility of the parameters in predicting implantation potential. Notably, they found that embryos that implanted reached several developmental milestones faster than embryos that did not implant, including pronuclear fading and the 2-, 4-, 5-, and 8-cell stages (all $p \leq 0.02$). Furthermore, embryos that went on to implant took less time to progress through the first mitosis and to go from the 2- to the 5-cell stage ($p = 0.04$ and $p = 0.03$, respectively). Thus, some of the parameters that correlated with high-quality embryo development also correlated with successful implantation potential [53].

Chamayou et al. used TLM to evaluate a multitude of morphokinetic parameters as predictors of implantation [40]. Notably, they found no difference between implanted and non-implanted embryos for any parameter, including timing of appearance and disappearance of PNs, 1-cell to 9-cell stages, morula compaction, blastocyst formation, and expanded blastocyst formation. However, a significant difference was observed for implanted vs. non-implanted embryos to develop from the 3- to the 5-cell stage ($p < 0.05$). Specifically, embryos for which the duration of this third round of cleavage was between 9.7 and 21 h were significantly more likely to implant than those embryos which were outside that range (72/128 vs. 0/6, $p < 0.009$) [40].

In a secondary analysis of the morphokinetic parameters in the cohort of embryos described above [24], Chen et al. demonstrated that embryos which had both time markers evaluated using the Eeva™ Test (see Sect. 8.3.2) within range (i.e., Eeva™ High) were statistically more likely to implant than embryos with one of the two parameters out of range (i.e., Eeva™ Low) (49.4 % vs. 21.2 %, $p < 0.001$) [63].

8.3.4 Other Variables

Although some of the studies described above controlled for other variables, such as maternal age, only a few explicitly investigated these variables as independent factors that may affect the developmental timeline of the embryos. In this section, we will specifically explore a variety of these factors to see if any of them change the morphokinetics of developing embryos.

8.3.4.1 Embryonic Factors

8.3.4.1.1 Ploidy Status

Aneuploidy is an extremely common occurrence in human embryos, with estimates ranging between 50 and 80 % of all embryos [34]. Not surprisingly, the ability to identify these embryos using a noninvasive technology such as TLM and then exclude them from transfer would be most beneficial. As discussed above, the current method of identifying aneuploid embryos involves invasive embryo biopsy with genetic screening.

Over the past 3 years, a number of studies have explored the possibility of using TLM to discriminate between euploid and aneuploid embryos. One of the early studies did not identify any difference between euploid ($n = 5$) and aneuploid ($n = 4$) embryos when analyzed by TLM for the timing of a variety of morphokinetic events, including time to syngamy, duration of the first cytokinesis and first cleavage, duration of 2-cell stage, timing of 8-cell stage, or the start of cavitation. The only noted difference was that aneuploid embryos began compaction significantly earlier than euploid embryos (84.0 h vs. 93.6 h $p = 0.025$) [64]. Similarly, another retrospective study of 76 biopsied blastocysts (40 euploid and 36 aneuploid) also showed no significant difference between euploid and aneuploid embryos for the following morphokinetic parameters: the time to 2, 3, and 4 cells, time to the third mitotic division, and the duration of the second and third cell cycles [65]. More recently, Dogan et al. reinforced these conclusions, retrospectively finding no morphokinetic differences between 46 euploid and 106 aneuploid embryos. Interestingly, but not surprisingly, the only parameter that was significantly different between the two groups was maternal age (34.1 ± 4.2 years vs. 37.5 ± 4.6 years, respectively, $p = 0.000$) [66].

However, some published studies do support the utility of TLM annotations for aneuploidy screening. Friedman et al. first reported in 2012

that embryos with abnormal cell-cycle parameters have a higher chance of being aneuploid than embryos with normal cell-cycle parameters [67]. In a follow-up publication, Chavez et al. expanded on these results [68]. Using donated supernumerary human embryos cryopreserved at the 2PN stage, the authors thawed the zygotes and cultured them for 2 days in TLM conditions, tracking three morphokinetic parameters—duration of the first cytokinesis, time from two to three blastomeres, and time from 3 to 4 cells. Of 75 embryos, 53 progressed passed the zygote stage and were subsequently disaggregated at the 4-cell stage for analysis of ploidy in each blastomere. Using the chromosome number results, this study went a step further and identified whether the aneuploidy in each abnormal chromosome was a result of a meiotic or a mitotic error. Looking at the TLM results, the authors identified that in contrast to narrow time windows in which euploid embryos completed the first cytokinesis and the second and third mitosis, aneuploid embryos with meiotic errors exhibited a greater spread of times in all three morphokinetic parameters and aneuploid embryos with mitotic errors had a greater variation in the time intervals from 2 to 3 and 3 to 4 cells [68].

In an extensive study with embryos from 25 couples undergoing infertility treatment and who used PGS, Campbell et al. analyzed time-lapse images from 98 blastocysts grown in an EmbryoScope® and then retrospectively compared them based on embryo ploidy [69]. Similar to other studies, more than 50 % of the embryos were aneuploid (60/98), with 30 embryos displaying single aneuploidy and 30 with multiple aneuploidy. Of the measured parameters, several showed significant differences between euploid and aneuploid embryos. Notably, the start of blastulation was significantly delayed for embryos having both single (median 103.4 h, $p=0.004$) or multiple aneuploidy (median 107.3 h, $p=0.006$) compared with that for euploid embryos (median 95.1 h). Furthermore, multiple aneuploid embryos took longer than euploid embryos to reach the start of compaction (median 85.1 h vs. 79.7 h, $p=0.02$) and to form full blastocysts (median 110.9 h vs. 105.9 h, $p=0.01$). None of the other measured morphokinetic parameters (time to fading of the pronuclei, completion of division to 2–8 cells, morula formation, expanded blastocyst formation, blastocyst hatching) or calculated parameters (time from 2 to 3, 2 to 4, 3 to 5, and 4 to 8 cells and time of blastulation) were significantly different between groups. Finally, the authors saw no significant difference in multinucleation at the 2-cell stage or in the proportion of cells that underwent direct cleavage (i.e., 1–3 cells or 2–5 cells). Using the significant differences in the time to reach the start of blastulation and the formation of full blastocyst, the authors created a classification model with three risk classes of aneuploidy (low, medium, and high). The authors subsequently proposed that the model could be used to rank unscreened blastocysts as having low (probability 0.37), medium (probability 0.69), or high risk (probability 0.97) of aneuploidy.

In a follow-up study, the same research group retrospectively applied this model to 69 couples with known data on implantation rates (presence of a fetal heart beat at 6–8 weeks gestation) and/or live birth; those embryos in the medium- and low-risk classes for aneuploidy were significantly different from each other in terms of implantation rate (72.7 % vs. 25.5 %, $p<0.0001$) and live-birth rate (61.1 % vs. 19.2 %, $p=0.01$). None of the embryos that were retrospectively classified as high risk had implanted. Although this was a retrospective study, this was the first study to show that morphokinetic data from TLM could be used to classify blastocyst-stage embryos by aneuploid risk and correlate this information with a clinical outcome [70]. In contrast, however, when a separate group of researchers retrospectively applied Campbell et al.'s algorithm [69] to a cohort of 106 blastocysts with known ploidy status, they found that the observed aneuploidy frequencies were significantly different from the frequencies predicted by the algorithm for all three risk categories ($p<0.02$) [71]. This result suggests that a universal predictive morphokinetic model may not be feasible due to the variation in practice in ART laboratories around the world.

In the past year, another group has also attempted to devise an algorithm that would

increase the chances of selecting a euploid embryo. Similarly to the study by Campbell et al., but on a larger scale, Basile et al. evaluated 504 embryos (71.7 % aneuploid rate) from 125 patients undergoing PGS [72]. While these authors also found no difference in the time, it took euploid and aneuploid embryos to reach pronuclear fading and the 2- through 4-cell stages; aneuploid embryos reached the 5-cell stage significantly faster than euploid embryos (49.4 h vs. 51.8 h, $p=0.001$). Furthermore, the aneuploid embryos spent significantly less time in the 2-cell and 3-cell stages ($p=0.004$ and $p=0.002$, respectively) and less time to go from the 2- to the 5-cell stage (22.6 h vs. 25.5 h, $p=0.000$). These results contrast with the results from Campbell et al. [69] because they suggest that aneuploid embryos divide faster rather than slower than euploid embryos. Nevertheless, the authors performed a logistic regression analysis using the three derived parameters to define optimal ranges for each of those parameters, which were subsequently used to create a hierarchical model which subdivided the embryos into four categories (A–D). Based on this model, 35.90 % of the embryos meeting category A criteria are likely to be euploid, with the likelihood of normal chromosome content decreasing serially to only 9.80 % of embryos in category D [72]. Given that only about 1/3 of embryos demonstrating optimal morphokinetic milestones are euploid, it is unlikely that this specific model could be used exclusively to avoid PGS.

In a prospective randomized trial of 1,163 sibling oocytes, Yang et al. evaluated whether adding TLM to PGS can improve implantation and pregnancy outcomes [73]. After culture in either a TLM (study group) or a conventional incubator (control group), PGS was done after a trophectoderm biopsy on day 5. Euploid blastocysts with the most predictive morphokinetic parameters as defined for embryos of unknown ploidy in [27] and [31] (study group, $n=285$ blastocysts) or the best morphological grade (control group, $n=278$ blastocysts) were chosen for transfer on day 6. Notably, the TLM group had significantly higher clinical pregnancy (71.1 % vs. 45.9 %, $p=0.037$), implantation (66.2 % vs. 42.4 %, $p=0.011$), and ongoing-pregnancy (68.9 % vs. 40.5 %, $p=0.019$) rates than the study group. No difference in pregnancy loss rate was observed (3.1 % vs. 11.8 %, $p=0.273$). While these results are extremely encouraging, it is critical to note that in the TLM group, none of the evaluated morphokinetic parameters (from early cleavage to expanded blastocyst formation) were significantly different between euploid and aneuploid embryos. Furthermore, the embryos in the two groups were cultured under different conditions (stable TLM system vs. conventional incubator), and it is not possible to differentiate whether the culture conditions or the use of TLM parameters vs. conventional morphologic grading contributed more to the improved clinical outcomes.

8.3.4.1.2 Embryo Gender

None of the studies described above have taken the gender of the embryo into account. However, two studies [74, 75] presented at the most recent annual conference of ASRM (2014) specifically explored whether embryo gender has an impact on embryo morphokinetics. The first study retrospectively analyzed the morphokinetic parameters of 176 male embryos and 161 female embryos (gender identified using PGS). Notably, the authors identified that the length of the 3-cell stage and the time from insemination to morula formation could be used to predict the likelihood that an embryo is female, with four categories ranging from 71 % to 42 % likelihood [74].

The other study explored whether gender and ploidy status of an embryo would affect morphokinetic parameters. Notably, in a sample of 41 male and 51 female embryos which were subsequently subdivided into euploid and aneuploid groups, the authors found no significant differences by gender in the aneuploid embryos. However, male euploid embryos reached several milestones, including syngamy, the start of cavitation, and full blastocyst stages, faster than female euploid embryos [75].

Together, the results of these two studies suggest that use of embryo kinetics to predict the most viable embryos could potentially affect the sex ratio of ART pregnancies in the future.

8.3.4.2 Laboratory/Clinical Factors

8.3.4.2.1 Oxygen Concentration

Although it is well established in animal models that atmospheric oxygen (20 % O_2) is detrimental to embryo development, specifically the pre-compaction stages [76], it was not possible to study this in detail on human embryos without the use of TLM. In a retrospective study of embryos obtained from three different studies, Kirkegaard et al. [76] showed that embryos cultured in 20 % O_2 took longer to progress through the third cleavage stage (from 4 to 8 cells) than embryos cultured partly or exclusively in 5 % O_2. Significant differences were not seen in the time it took embryos to reach the full blastocyst stage, confirming the conclusion from mouse studies that atmospheric oxygen has a stage-specific effect prior to compaction [77].

8.3.4.2.2 Culture Media

Three studies [78–80] have evaluated whether different culture media affect early human embryo development. The first study [78], which compared single (Single Step Media, Irvine Scientific) and sequential media (Early Cleavage Media until day 3 and MultiBlast Media until day 6, Irvine Scientific), showed that all of the developing sibling embryos showed significantly faster progression from the time of fertilization to the time of pronuclear fading and the times to the 2- through 5-cell stages in single media than in sequential media. This trend held true for embryos that went on to implant, although not all parameters reached statistical significance and overall differences did not predict clinical outcomes; the authors observed no difference in the cumulative implantation or pregnancy rates between the two culture types.

The second and third study simply compared two different types of media. One of these studies compared global® medium (LifeGlobal®) and Quinn's Advantage® Cleavage Medium (SAGE®); no differences were found in the morphokinetic parameters of embryos grown in one medium versus the other [79]. The other study compared two sequential culture media, Vitrolife G5 series and MediCult; similarly, this study also found no significant differences in the recorded morphokinetic parameters or clinical outcomes [80]. Therefore, based on these limited data, it would seem that the type of media in which embryos are cultured does not impact the morphokinetics of early development.

8.3.4.2.3 Insemination Technique

While early studies showed that ICSI-derived embryos progress through the first several stages of development faster than IVF-fertilized oocytes, the exact morphokinetics of the difference were not known [81]. Thus, without data from continuous TLM monitoring, it would be nearly impossible to adjust precisely for insemination technique when comparing developmental kinetics between the two fertilization procedures.

Cruz et al. designed a study to solve this dilemma [81]. Embryos inseminated by IVF ($n=622$) or ICSI ($n=581$) were cultured in a TLM system, either immediately after insemination (ICSI) or starting on day 1 after confirmation of fertilization (IVF). When the authors set the time of reference at the time of insemination, they found that ICSI-derived embryos reached the stages of pronuclear fading, 2 cells, 5 cells, 7 cells, and 9 cells, significantly faster than IVF-derived embryos. However, when the authors set the time of reference to a standard clearly identifiable on TLM images, in this case, time of pronuclear fading, all differences in embryo kinetics disappeared [81].

These findings are in agreement with Hashimoto et al. (2012) who showed no difference in two derived time parameters, the time required for the second cleavage (3–4 cells) and third cleavage (5–8 cells), between conventional IVF insemination and ICSI [50]. Similarly, dal Canto et al. showed that although embryos that were generated by IVF took longer to reach the 2-cell stage and the 3-cell stage than embryos generated by ICSI, the IVF-derived embryos spent significantly less time as 2-cell embryos than embryos generated by ICSI, realigning the cleavage kinetics of the two groups for the rest of development, from the 4-cell stage up to the 8-cell stage [51].

Based on these collective results, it seems that embryos created by IVF vs. ICSI likely do not

develop at different rates when the insemination procedure is taken into account. This supports the proposed nomenclature of Kaser and Racowsky [56] which sets the standard of reference at the time of a clearly identifiable stage in development which, in their case, is the formation of the first cleavage furrow [56].

8.3.4.2.4 Cryopreserved Embryos/Oocytes

Hashimoto et al. (2012) showed no difference in time required for the second cleavage (3 to 4 cells) and third cleavage (5 to 8 cells) between embryos thawed after being frozen by different methods (slow freezing vs. vitrification) [50].

However, Aragones et al. found that embryos derived from fresh oocytes reached the 2- through 4-blastomere stages significantly faster than embryos from previously vitrified oocytes. No differences were observed in the time to 5 cells or in the duration of the 2- and 3-cell stages. When the authors subsequently applied the hierarchical model of embryo grading based on these last three parameters (see Sect. 8.3.3; [27]), not surprisingly, they found no differences between oocyte source in the percentage of embryos in each category of embryo quality ($p = 0.270$) [82].

8.3.4.2.5 Blastomere Biopsy

As discussed above, blastomere biopsy has been shown to be detrimental to sustained embryo implantation and live-birth rates. Furthermore, other studies have shown that removing 2 cells versus 1 cell from a cleavage-stage embryo results in a lower blastocyst rate, indicating that cell removal is detrimental to embryo development [83, 84].

To further explore the effect of blastomere biopsy on embryo development, Kirkegaard et al. undertook a study using TLM analysis [30]. Embryos that were morphologically similar at 68 h after fertilization were either un-biopsied controls ($n = 53$) or embryos that were biopsied for PGD ($n = 56$). Not surprisingly, there were no significant differences between the two groups regarding the time it took to reach the 3- through 8-cell stages. However, after blastomere biopsy, embryos spent significantly more time in the stage at which they were biopsied than the equivalent stage for the controls ($p < 0.001$). Thus, biopsied embryos then took significantly longer to reach subsequent stages of development, including compaction, morula formation, early blastocyst formation, and full blastocyst formation. Interestingly, due to a different mechanism of hatching, the biopsied embryos spent significantly less time as blastocysts and thus hatched at about the same time after fertilization as did the controls [30].

Another recent study confirmed some of these results. Comparing 234 embryos biopsied for PGD to 71 embryos from standard ICSI cycles, Ben-Yosef et al. found that blastomere biopsy significantly delayed the timing of compaction (by 4–5 h) and the start of blastulation (by 5–10 h) ($p < 0.01$). Interestingly, they found that the timing of blastomere biopsy may affect early embryo kinetics as well, with embryos biopsied at the 8-cell stage taking longer to reach subsequent developmental stages than embryos biopsied at stages with less than or greater than 8 cells [85].

8.3.4.2.6 Stimulation Cycle Medications and Hormones

Given that the maternal hormone milieu may affect oocyte quality and thus embryo development [as discussed above for polycystic ovary syndrome (PCOS)], an interesting question arises. Do the hormones administered to women during ART cycles affect embryo development? Munoz et al. explored this particular question in two studies [45, 86]. In the first [45], the type and dose of gonadotropin or the serum estradiol and progesterone levels on the day of hCG administration were analyzed with respect to morphokinetics. Based on the analysis of over 2,100 embryos derived from oocyte donors, no difference was found for any morphokinetic parameter between embryos obtained from stimulation cycles using FSH, HMG, or both. Interestingly, though, the total dose of recombinant FSH (rFSH) affected embryo development kinetics. Specifically, as total rFSH dose increased, the longer it took for embryos to reach the 2-cell and the 6- through 9+-cell stages, to begin blastulation, and to complete maximal blastocyst expansion [45].

Furthermore, developmental kinetics were also affected by the serum estradiol concentration, with all milestones through morula formation and blastulation, with the exception of two (time to 5 and 9+ cells), showing significant differences. In contrast, serum progesterone levels seemed to have a significant effect only on the first 4-cell division cycles. Notably, none of the differences for any of these variables (type or dose of gonadotropin, serum estradiol, and serum progesterone) correlated with implantation potential or clinical pregnancy rate [45].

In a follow-up study [86], the same group retrospectively explored whether the hormones used for a controlled ovarian stimulation cycle (GnRH agonist + hCG trigger versus GnRH antagonist + GnRH agonist trigger) affected early embryonic development kinetics. Interestingly, while embryos derived from oocytes obtained in a GnRH agonist + hCG trigger cycle took longer to reach the 2- through 5-cell stages, these differences disappeared as the embryos progressed further through development. Furthermore, no significant differences were observed regarding the quality, implantation rate, or clinical pregnancy rate of embryos derived from the two stimulation types [86].

These two studies thus suggest that the clinical manipulation of maternal hormones is associated with the embryo kinetic parameters. However, the relevance of this association with clinical outcomes remains unknown.

8.3.4.3 Parental Factors

8.3.4.3.1 Age

Increasing maternal age is well known to be associated with oocyte aneuploidy [87], so the question arises as to whether maternal age is independently associated with difference in embryo kinetics. Hashimoto et al. (2012) showed no significant differences based on donor age and the time required for the second cleavage (3 to 4 cells) and third cleavage (5 to 8 cells) [50]. Similarly, Watcharaseranee et al. also showed no difference in any absolute timings or derived durations between embryos that successfully implanted from women <35, 35–37, or ≥38 years old [88]. However, this study was limited by the fact that the ploidy status of the embryos was not known. Thus, from this limited available data, it would appear that maternal age is not associated with differences in kinetics of at least the markers investigated.

8.3.4.3.2 Body Mass Index

Independent of the presence of ovulatory disorders, obesity has been shown to be correlated with lower implantation and pregnancy rates following ART [89]. In a recent study, Bellver et al. analyzed in a TLM system embryos derived from obese [body mass index (BMI) ≥ 30 kg/m^2] women with infertility, normal-weight (BMI = 20–24.9 kg/m^2) women with infertility, and normal-weight oocyte donors. Notably, they excluded all women whose partners (i.e., sperm source) were obese. Significant kinetic differences were observed between obese and normal-weight infertile women. However, embryos from the fertile oocyte donors reached the 2- through 5-cell stages significantly faster than either of the two groups of infertile women (no differences were seen in duration at the 2-cell stage or the time to divide from a 2- to 4-cell or from a 3- to 5-cell embryo). The authors then used the morphokinetic data to categorize the embryos in each group based on the hierarchical classification tree model proposed by Meseguer et al. [27]; they found no significant difference between groups in the proportion of embryos in each category (A–E). Notably, this study found no significant difference in implantation, pregnancy, or miscarriage rates between the three groups.

Another recent study supported the conclusions drawn by Bellver et al. Although the study unconventionally divided the 21 patients into those of normal BMI (18–23 kg/m^2) and high BMI (>23 kg/m^2), the authors identified no significant differences in any of the morphokinetic parameters measured, including time to reach the 2- through 8-cell stages and time to start and complete compaction, cavitation, and full blastocyst formation [90].

8.3.4.3.3 Polycystic Ovary Syndrome

A common co-occurrence with obesity in women with infertility is PCOS. Differences between oocytes from women with PCOS and healthy

women are well documented, including gene expression profiles and defects in oocyte maturation [91]. However, prior to the advent of TLM, any information regarding the relationship between PCOS and embryo kinetics was lacking.

Wissing et al. used TLM to explore embryo morphokinetics in three groups of women: 20 normo-ovulatory controls, 25 women with hyperandrogenic PCOS, and 26 women with normoandrogenic PCOS [91]. In their analysis, the authors found significant differences in some parameters but not others. Notably, times to 2PN breakdown, 2 cells, 3 cells, and 7 cells were significantly shorter for embryos from healthy controls than for those from hyperandrogenic PCOS. Time to 4 cells was significantly longer for embryos from the hyperandrogenic PCOS group than for either of the other two groups. No significant differences were found between groups by the morula stage of development. Furthermore, similar to the study on obesity by Bellver et al. [89], there were no differences in implantation, clinical pregnancy, or live-birth rate between groups (although the study was likely underpowered to detect these differences). Given these results, it remains unclear whether maternal metabolic and hormonal derangements have a significant effect on early embryo development and viability.

8.3.4.3.4 Smoking

In addition to physiological factors, maternal habits also have the possibility of altering IVF success. Smoking, with all its known risks to health, ovarian response, and pregnancy, has not been definitively shown to affect embryo development in human studies [41]. In an attempt to further explore this dynamic process using the flexibility of TLM, Freour et al. [41] carried out a study exploring the morphokinetics of embryos from 23 active smokers (139 oocytes) and 112 nonsmoking patients (729 oocytes). Significant differences were found in the duration of the 2-cell stage, as well as the time to 3, 5, 6, and 8 cells after insemination/ICSI. Notably, the embryos from active smokers took longer to reach each of those stages than embryos from nonsmokers. Furthermore, implantation rates were lower in smokers than nonsmokers (13.8 % vs. 21.2 %, respectively; no *p*-value). To date, this is the only study to document a detrimental effect of maternal smoking on early embryo morphokinetics.

8.4 Conclusions

Time-lapse imaging of preimplantation embryos offers unique opportunities for the noninvasive collection of numerous morphokinetic data points that may be beneficial in selecting or deselecting embryos for transfer. Since the introduction of TLM 5 years ago, more than 20 unique morphokinetic variables for normal embryo development have been described. In retrospective studies, some of these markers have been shown to be highly predictive of blastocyst development or implantation, while others have been identified as atypical phenotypic markers that can identify embryos with lower developmental potential. These newly described predictors of developmental competence, some of which are very subtle and can only be identified using TLM, are promising and offer significantly more detailed and quantifiable information for each observable developmental milestone than can be achieved by manually grading embryos at single static time points. Thus, these parameters in conjunction with our standard morphology grades hold great potential to enhance the selection of the most competent embryo for transfer and the deselection of morphologically normal appearing embryos that have abnormal phenotypes.

Despite the numerous morphokinetic variables identified using TLM, few appropriately designed trials have been conducted to examine whether this additional information enhances our ability to identify the most competent embryo for transfer. Many of the markers have not been thoroughly validated and assigned positive or negative predictive values. A recent meta-analysis on the use of time-lapse imaging for improving reproductive outcomes initially identified 714 publications that used TLM to monitor embryos. Of these publications, however, only two RCTs were considered eligible for the meta-analysis.

Results from these two studies suggest that time-lapse embryo imaging does not improve or reduce the chance of achieving a clinical pregnancy when transferring blastocyst-stage embryos [92]. Moreover, while it appears that such variables as ovarian stimulation regimen, obesity, and smoking may each impact the developmental kinetics of embryos through various milestones, additional and more robust studies are required to explore these relationships further.

The lack of RCTs available to conduct the meta-analysis underscores the need for more prospective trials to determine whether or not TLM actually improves clinical outcomes. A common nomenclature should be adopted in all future TLM studies, and stringent RCTs are required which control for potential confounding of the type of incubator used. An ideal trial that needs to be conducted would measure clinical outcomes after patients are randomized to single-embryo transfer following (a) embryo culture in time-lapse system with selection made based on morphology alone or (b) embryo culture in a time-lapse system with selection based on both morphokinetic parameters and conventional morphology grades. Also, since there is an additional cost associated with TLM (ranging between 10 and 20 % of the cost of an IVF cycle), an economic evaluation should be undertaken as part of this trial. Only after such trials are performed will we be positioned to weigh the true benefit of using TLM for embryo selection. Until sufficient high-quality evidence exists, we do not feel it is ethical for fertility clinics to charge patients for the use of TLM, and we believe this technology should continue to be considered experimental and subject to institutional review and approval.

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The Ideal Spermatozoon for ART

9

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9.1 Envisaging WHO 2010

Since the initial observation of the tiny cells identified as the male gamete [1], there have been considerable advances in elucidating elements essential for sperm production, composition, and function. Thus, the most relevant tenet is that sperm numbers, quality of motion, and shape are diagnostically significant for assessing the fertility status of the male. These sperm cell attributes are collectively analyzed in the semen analysis. Analysis of the ejaculate in its most basic element evaluates the seminiferous tubule sperm production and the fluid vehicle contributed by the accessory glands. The production and packaging of the male genome from spermatogonia through spermatozoa in the testis is critically relevant because without that functional process, there are no cells and therefore no fertility. What remains indeterminate is what number and spermatozoa characteristics are required for a man to be deemed fertile, subfertile, or indeed infertile when matched with a presumably fertile female partner [2]. To address this issue, the World Health Organization (WHO) has published its guidelines aiming at standardizing the examination of human sperm and sperm-cervical mucus interaction [3–6]. The task however is daunting and far-reaching; in fact the WHO guidelines, especially the most recent ones [7], represent an attempt to adopt current ART laboratory evaluation. It stood in the past as an entity seeking standardization of a reference range capable of predicting fertility performance of men regardless of their geo-social background. The extremely low threshold for concentration, motility, and morphology are those of the most relevant papers on semen parameters produced in the last three decades [8].

As a recent graduate in gynecology of the University of Bari, I became a postdoc at the Brussels Free University, and in spite of my limited knowledge in embryology laboratory practices, I was carrying out experiments in assisted fertilization. I was often asked to screen infertile men and questioned about which patients should be candidate for micromanipulation of the gametes versus other more conventional ART procedures. Invariably my answer was in the need to screen men's semen parameters and adopt a threshold that would predict an impaired performance of their specimen when used with standard in vitro insemination. I learned to inseminate individual oocytes with 2,500/3,000 spermatozoa in 25 μl drops under oil. This inspired me to adopt a threshold of 20×10^6 per ml for concentration that I took from the WHO 1987, a motility of 40 % [9], and I embraced as normal sperm morphology the threshold of 14 % from a very fashionable paper [10] at the time [11].

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_9

When I joined Cornell in 1993, I realized that the diagnostic andrology laboratory was exclusively composed of individuals that appeared to be very strict with the male gamete and in fact, rarely reported a morphology that was higher than 6 % even when an ideal anonymous donor sample got into their hands. This made the 1992 WHO Manual with a published morphology threshold of 50 % still clearly out of reach for my new laboratory, and therefore, to maintain the link with the most credited reference, I decided to adopt the 4 % strict morphology considered in the same papers [10] as the threshold with poor prognosis [12].

The WHO Manual contained reference range values for the traditional semen parameters since its initial publication [3] based on limited even if reputable information [7, 13–17]. The latest WHO Laboratory Manual [7], however, presents for the first time statistically derived lower fifth percentile reference limits from several prospective semen analysis studies generated in several laboratories worldwide using standard procedures [8]. This for me was an extraordinary pleasant surprise seeing validated, by the most reputable publication in the field, the values in accordance to my criteria empirically adopted 22 years earlier. The new WHO data more than attempting to portray a cutoff value for diagnosing subfertility reflects the reproductive probabilities based on results from a fertile population.

The semen parameters proposed by the WHO 2010, however, have been criticized for having questionable diagnostic values reinforcing the concept that the cutoffs are mythical and probably unrealistic when attempting to use data to characterize such a dynamic biological system. Indeed, just last year (October 2013) at the American society for reproductive medicine (ASRM) meeting in Boston during a session of the society for male reproductive urology (SMRU), attended by well-respected names in male reproductive urology, a senior colleague stood up to complain about the recently introduced WHO criteria stating …*so you are telling me that just because I do have more than 4 % normal spermatozoa I am normal?*…

Use of standardized procedures for semen analysis yields clinically meaningful data as a part in the attempt to assess male subfertility. In addition to semen analysis, other critical testing, e.g., genetic, hormonal, and structural, etc. contributes greatly to overall power for diagnosing male fertility [18]. The spermatozoon is a very unique cell in that it is comprised of separate yet interrelated components, each of which plays a crucial role during a conception attempt [19]. The head must contain DNA that can be correctly assembled during spermatogenetic meiosis, disassembled for tight packing during spermiogenesis, and then reassembled with histones to partner with female DNA to ultimately form the new conceptus' genome. The midpiece must contain mitochondria to generate energy, and there must be a flagellum to transfer this energy into motion.

9.2 Components

9.2.1 Acrosome

The acrosome develops over the anterior half of the spermatozoon head. It is a cap-like structure derived from the Golgi apparatus that develops with germ cell maturation during spermiogenesis.

Ejaculated mammalian spermatozoa, in fact, are not immediately able to fertilize an oocyte and must undergo a process of maturation known as capacitation in order to implement their function in the female tract [20, 21]. Capacitation involves modifications in the sperm plasma membrane that lead to kinetic hyperactivation and permit the acrosome reaction. This phenomenon involves multiple fusions between the outer acrosome membrane and the overlying sperm plasma membrane, enabling the soluble contents of the acrosome to leak out through the so generated fenestrated membranes [22], simultaneously preparing the surface over the equatorial segment for its cardinal fusogenic role [23].

The relevance of monitoring the process of capacitation and the ability of sperm populations to undergo through this dynamic process in a timely fashion has been the focus of investigation [24, 25]. A recently proposed assay now under clinical testing attempts to measure the ability of a biomarker, G_{M1} [26, 27] (Fig. 9.1a), to diagnose

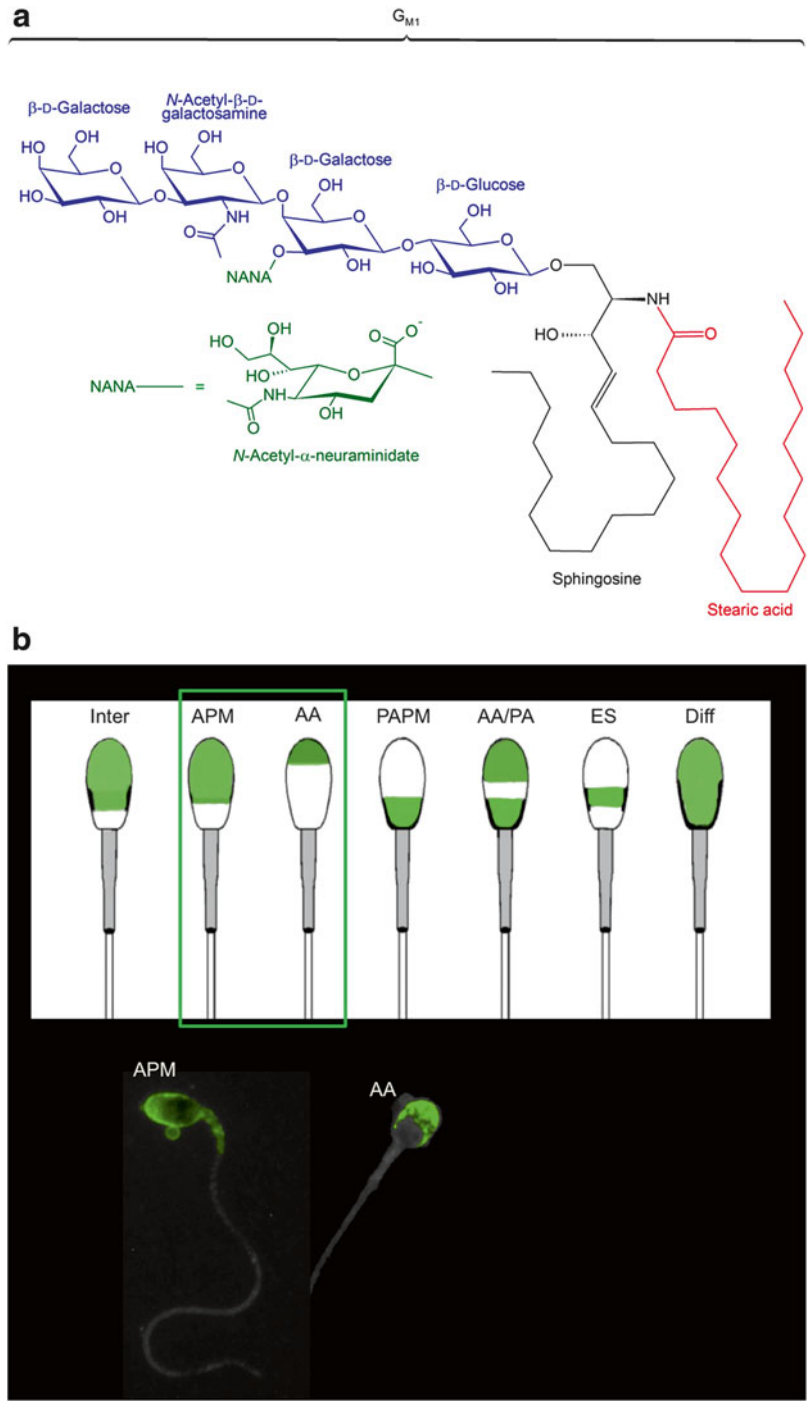

Fig. 9.1 (**a**) Monosialotetrahexosylganglioside, G_{M1}, a ganglioside composed of a glycosphingolipid (ceramide and oligosaccharide) with sialic acid linked on a sugar chain. (**b**) G_{M1} patterns where the two most predictive expressions are APM and AA. *Inter* intermediate, *APM* acrosomal plasma membrane, *AA* apical acrosome, *PAPM* post-acrosomal plasma membrane, *AA/PA* AA and PAPM, *ES* equatorial segment, *Diff* diffused

the sperm's ability to undergo capacitation and fertilize. Currently, there are no sensitive or simple markers for capacitation that can be used in a clinical setting capable of providing such information during the maturational steps that bring the spermatozoon from a motile cell to a fertilizing male gamete. The G_{M1} gangliosides are mostly over the sperm head membrane and display specific patterns (Fig. 9.1b) that can be interpreted in a subpopulation of spermatozoa incubated in standard and in response to stimuli conditions while reaching their fertilizing competence [28].

To validate this assay, we identified 15 men with multiple IUI failures with overall semen characteristics, namely, concentration, motility, and morphology, comparable to a control ($n=20$). The spontaneous appearance of the G_{M1} in standard incubation conditions was 13.5 % at 1 h, 18.2 % at 2 h, and increased to 25.0 % at 3 h. In a specifically designed capacitation-enhancing medium, G_{M1} expression started at 21.7 % at 1 h, 26.9 % at 2 h, and reached 34.5 % at 3 h. When we looked at the specimen of men that had recurrent failed IUI, the spermatozoa following incubation were at 9.1 %–13.4 %–19.2 % and they became 14.2 %–19.5 %–23.9 % in the presence of a capacitation enhancer. Men with recurrent IUI failure had an impaired G_{M1} expression in the presence of this enhancer with a delta at 3 h of 10.6 % in comparison to control men [28].

We postulated that the dynamic profiling of membrane changes occurring during sperm capacitation in standard and particularly following capacitating stimuli would be able to predict gamete competence to fertilize. Moreover, the impaired G_{M1} expression in both testing conditions was comparable in men with normal and compromised semen parameters. Because of the assay's ability to provide indication of sperm function, irrespective of semen parameters, it may also aid physicians toward the appropriate ART method to inseminate infertile couples and would serve as an add-on to conventional semen assays.

In a later analysis, the G_{M1} localization was measured in basal and capacitating media, on semen samples of consenting men ($n=19$) with apparently normal semen parameters undergoing IUI treatment. In a selected group of men serving as controls ($n=22$), the baseline G_{M1} patterns were 14 %–19 %–26 % in standard medium and 23 %–28 %–36 % in capacitating medium, at 1, 2, and 3 h, respectively. In this control population, the IUI clinical pregnancy rate was 31.8 % (7/22). When we looked at the specimens of men in the study group, the G_{M1} expression was 12 %–16 %–21 % in standard condition and 17 %–22 %–26 % with a capacitation enhancer, at 1, 2, and 3 h, respectively. In the study group, regardless of the comparable semen parameters to the control, G_{M1} patterns in both incubation conditions were lower than the control with a delta of 9.1 % and in fact achieved a pregnancy rate of only 5.3 % (1/19) ($P=0.02$). These impaired group of men ($n=7$), however, generated three pregnancies (42.9 %) once treated by ICSI.

ICSI bypasses the events involved in physiological sperm penetration of the oocyte and requires no specific pretreatment of sperm other than immobilization [12, 29, 30]. However, this aggressive compression of the tail prior to injection significantly improves ICSI fertilization rates [31–34]. Although the mechanism of this beneficial effect is not immediately clear, there is indirect evidence that such immobilization triggers changes in the sperm's permeability [35] and that it may expedite changes leading to sperm plasma membrane destabilization culminating in acrosomal disruption [31, 33]. The utility of sperm flagellar damage was supported by the observation that epididymal spermatozoa, characterized by high lipid content in the plasma membrane [36], required more intense flagellar damage to trigger membrane destabilization. This phenomenon is also modulated by the type and concentration of proteins present in capacitation media that during incubation progressively replace membrane lipid components, thus rendering spermiolemma more hydrophilic, responsive to mechanical disruption, and, therefore, ultimately more prone to acrosome reaction. In fact, the introduction of sequential media, formulated with limited glucose and proteins, aimed at supporting the pre-genomic embryo cleavage steps

in an extended culture system, has resulted in complications in the execution of the ICSI procedure [37]. In fact, this minimized protein content has required tweaking of the ICSI injection during treatment of the spermatozoon's flagellum and in the technical withdrawal of the injection tool to minimize oolemma eversion.

9.2.2 Chromosomes

Aneuploidy is the main cause of the high fetal wastage in humans. Most aneuploid pregnancies do not survive in utero, with the majority of losses occurring during the first few weeks of uterine life. Chromosome instability is a hallmark of early life, with whole-chromosome aneuploidy, mosaicism, and segmental aneuploidy being detected in 50 % [38] to 80 % [39] of very early embryos. In clinically recognized spontaneous abortions, trisomies of all chromosomes have been reported, while monosomies are rarely encountered with the exception of 45,X fetuses [40]. Aneuploid conceptions that survive constitute 0.8–1 % of all live births [41]. These offspring are mostly affected by trisomies 13, 18, and 21 and various sex chromosome aneuploidies; these represent the majority of congenital abnormalities, developmental disabilities, mental retardation, and infertility in humans. In general, autosomal trisomies (93 % of trisomy 18, 95 % of trisomy 21, and 100 % of trisomy 16) originate in the maternal line [42], whereas sex chromosomal aneuploidies are more frequently of paternal origin (50 % of 47,XXY, 100 % of 47,XYY, and 70–80 % of 45,X) [43].

While gametic meiotic errors that lead to fetal aneuploidy occur in both the male and the female lines, the frequency of these errors appears lower in spermatozoa at about 9 % in sperm karyotypes [44] and in oocytes mostly at 20 % but as high as 60 % [42, 45, 46].

Nevertheless, the assessment of the chromosomal status of the male gamete retains an important position in pre-fertilization genetic diagnosis. With this in mind, we screened 44 patients who underwent 118 ICSI cycles by FISH [47]. Fixed spermatozoa were decondensed and hybridized with three sets of probe mixtures containing locus-specific probes for chromosomes X, Y, 18, 21, 13, 15, 16, 17, and 22. Semen characteristics were comparable to those commonly seen in our fertility practice.

After sperm scoring, men with abnormalities in $\geqslant$1.6 % of spermatozoa were considered to have a high rate of aneuploidy, while those below the threshold were considered normal (controls). Of those 44 men, 21 (mean age 39.1 ± 6 years) with high aneuploidy rates were treated in 56 ICSI cycles, while 23 men (mean age 39.5 ± 6 years, $n = 62$ cycles) served as controls. Autosomal disomy was the most recurrent abnormality. While compromised motility was seen only in the aneuploidy group ($P < 0.01$), that group's fertilization rate was comparable with the aneuploidy at 70.4 % (462/656) vs. 68.1 % (372/546) in the control. The clinical pregnancy rate in the study group was 21.4 % (12/56), with a 12.5 % (7/56) delivery rate, while in the reference group, it was 29.0 % (18/62) and 21 % (13/62), respectively. Significantly, however, the pregnancy loss rate was 41.7 % (5/12) in couples with men with chromosomally abnormal spermatozoa versus 27.8 % (5/18) in the control ($P = 0.03$) [47]. In a larger series of patients ($n = 55$), we were able to confirm a lower implantation rate along with a significant increase in pregnancy losses ($P < 0.001$) where a predominance of disomy 18 characterized the autosomal aneuploidy in their spermatozoa [48]. Interestingly, even in this series, chromosomal abnormalities of the male gamete had a clear effect on embryo implantation. Performing 24 chromosome FISH on spermatozoa may increase even further our ability to determine the relevance of aneuploidy in a given sample [47]. At our center, we assessed sperm aneuploidy when a couple has a recurrent ART failure or when there is a history of recurrent pregnancy loss. This is particularly stressed in azoospermic men undergoing epididymal and testicular retrieval.

Preconception gender selection, however, has increasingly been sought after by couples looking to minimize the possibility of passing on sex-linked genetic diseases. Gender selection entails

the identification of spermatozoa by different methods with flow cytometric sperm sorting widely regarded as the most effective [49]. This process requires a high number of spermatozoa that are subjected to fluorescent staining, and it implies additional charges. A less popular and controversial technique is the Ericsson method, which utilizes layers of human serum albumin in a test tube [50]. A sperm sample diluted with media is then layered over the albumin and allowed to stand for 1 h. This allows for the separation to occur, as the Ericsson method is based on the assumption that Y-bearing spermatozoa swim faster and are able to reach the bottom layer before X-bearing spermatozoa [51]. We have devised an inexpensive and reliable method of sex selection by utilizing multilayer density gradients to isolate and enrich the population of X- or Y-bearing spermatozoa.

A total of six samples with a concentration of $48.3 \pm 17 \times 10^6$/ml, a motility of 50.9 ± 6 %, and a morphology of 2.4 ± 1 % were included. The overall proportion of gender specific spermatozoa in unselected samples was 50.1 % for X and 49.9 % for Y. When selecting for X, a double layer gave 63.2 %, a triple 75.5 %, and a quad 80.3 %. This provided a direct correlation with the increasing number of layers ($P = 0.0001$). When assessing for Y, a double layer yielded 62.7 %, triple 75.5 %, and quad 78.5 %. As the gradient became less dense, the proportion of Y-bearing spermatozoa increased ($P = 0.0001$) [48]. Couples who are seeking family balancing or reduced risks of passing on sex-linked genetic diseases would benefit from our method that is inexpensive, safe, and easily reproducible for enriching gender-specific spermatozoa and with results comparable to the Ericsson albumin method as well as MicroSort® (Table 9.1).

Table 9.1 Proportion of X- and Y-bearing spermatozoa according to the gender selection method

%	MicroSort®	Ericsson albumin method	Cornell
Y	85	83	75
X	90	78	80

9.2.3 Centrosome

During fertilization, restoration of diploidy to support a normal embryonic development requires that each gamete contributes one half of the chromosomal complement. In humans, the mature oocyte possesses all of the elements necessary for embryonic development except an active division center, which must originate from the spermatozoal centrosome. Boveri [52] first defined the term "centrosome" as a polar corpuscle containing centrioles. Later it was defined more functionally as a microtubule-organizing center (MTOC) [53]. The centrosome in somatic cells is considered to be responsible for two basic events: the nucleation of microtubules and the formulation of an efficient mitotic spindle [54].

In most cells, the MTOC consists of two morphologically distinct centrioles and the pericentriolar material (PCM). Centrioles do not seem to be present in the meiotic spindle of maturing gametes but are present at the spindle poles during the first mitotic division of the zygote of various species [55], including humans [56]. Oogonia and fetal oocytes display normal centrioles until the pachytene stage. In fact, the mature human oocyte has neither centrioles nor functional centrosomes associated with its meiotic spindle, resulting from several microtubular organizing centers (MOC) generating an anastral, barrel-shaped, with microtubules ending abruptly at the poles. The outer pole, however, is closely bound to the egg cortex.

In contrast to the oocyte, the human spermatozoon has two distinct centrioles allowing the postulate of the paternal inheritance of the human embryonic centrosome [57]. The well-defined proximal centriole, located within the connecting piece next to the basal plate of the sperm head, displays a 9+0 pattern of nine triplet microtubules surrounded by electron-dense material and flanked by nine cross-striated columns. The distal centriole is aligned with the axis of the flagellum almost perpendicular to the proximal centriole and gives rise to the sperm tail axonome during spermiogenesis [56, 58, 59].

The absence of the sperm centrosome could be one of the causes of embryonic failure

[60–63]. The utilization of biochemical and immunological techniques has now made it possible to identify proteins that are integral components of the centrosome [60, 62, 64, 65]. Furthermore, FISH assessment of chromosome distribution has revealed that the sperm centrosome is solely responsible for organizing the first mitotic division in human embryos [57]. Centrosome dysfunction can result in abnormalities ranging from the inability of the zygote to cleave to embryonic aneuploidy or even mosaicism [61].

In cases where no syngamy after ICSI or chaotic chromosomal rearrangements were observed, we assessed for the presence, integrity, and reciprocal axis of the sperm centrosome in infertile men and compare them to fertile donors [65].

Various methods to determine sperm centrosome integrity, functioning, and sperm aster formation have been designed and employed in recent years. Among them are studies that have evaluated the formation of the sperm aster within the fertilized oocyte to predict successful union of sperm and oocyte nuclei and positive developmental potential [66, 67]. Several promising assays have been developed in recent years to assess sperm aster formation using heterologous ICSI systems [68] when human spermatozoa were microinjected in mouse, rabbit, or bovine eggs. Such assays clearly established a relationship between infertility and sperm centrosomal dysfunction [69].

At our center, for couples that present with recurrent arrest at pronuclear stage or chaotic mosaic chromosomal complement of their conceptuses, we offer an assay capable of verifying for the presence and integrity of the sperm centrosome utilizing a monoclonal anticentrin antibody coated with a fluorochrome. The centrin localization at the edge of the functional centriole/centrosome and the proximal end of the flagellar centriole allowed also the measurement of the angle occurring between the diplosomes within the basal body. The centrosome was considered intact when two adjacent signals were observed. The angle generated between the proximal centriole and the flagellum was measured in control (known fertile donors) and infertile men (study group). The assessment of the centrosome in human ciliated fibroblasts indicated that this angle is unique to the spermatozoon. A total of 12 consenting men (average age 34.8 ± 7 years) donated their specimens with an average concentration of $56.0 \pm 36 \times 10^6$ per ml (range 118–0.0021), a motility of 39.8 ± 14 % (range 69–14 %), and a normal morphology of 6.3 ± 4 % (12–0 %). While concentration and motility did not differ between the two groups, the proportion of spermatozoa with normal morphology was lower in the infertile group (9.4 ± 2 % vs. 2.4 ± 2 %, respectively; $P < 0.001$) that proved to be older ($P < 0.01$). The presence of intact centrosomes in the infertile men was lower in comparison to the 93.4 % identified in the controls ($P = 0.0001$). The proposed structural integrity assay involving the study of reciprocal centrosomal angle consistently yielded 30–35° among men with proven fertility while proved to be severely altered, in excess or in deficit, in the infertile spermatozoa cohort characterized by compromised midpieces and flagellar sections often referred to as decapitated heads and stump tails [65, 70–74].

Considering the pivotal sperm centrosome function in granting euploid embryo development in humans, an assay capable of identifying its presence and gauging its integrity is undeniable; therefore, the ability to estimate a sperm centriolar angle may aid in this quantification.

9.2.4 Activating Factor

Another important aspect to be considered in selecting the ideal spermatozoon is the acquired ability to activate an oocyte. This occurrence results in failed fertilization with standard in vitro insemination and even after direct injection of the spermatozoon into the ooplasm. At our center, we have about 2–3 % of unexpected complete fertilization failure in couples treated by ICSI. This, however, can be seldomly ascribed to an extremely low number of oocytes, but more often it occurs following injection of an adequate oocyte cohort. It can often be obviated by inseminating the leftover oocytes once they reach

maturity or tweaking the superovulation protocol in a subsequent cycle. In order to attribute the fertilization failure to a sperm activation defect, the absence of the sperm cytosolic factor can be undoubtfully recognized if there is a consistent fertilization failure in more than one cycle, and when in a specific cycle, the in vitro matured leftover ootids fail fertilization as well. This phenomenon fortunately presents only in less than 0.1 % of the total fertilization failure with ICSI, but it is subtle and needs to be confirmed in order to appropriately advise the couple whether to adopt donor male gamete or use assisted oocyte activation.

To better understand the ability of the human spermatozoon to interact with an oocyte without misuse of precious human material, the use of heterologous ICSI insemination of rodent oocytes has been proposed. While hamster oocytes are not suitable for this purpose because they may be easily activated by the injection procedure itself, mouse oocytes on the other hand can be injected with human sperm cells to test their aptitude to activate [75]. Once the test oocyte is activated, the human sperm nucleus decondenses forming a pronucleus allowing the chromosome to replicate in a xenogenic environment.

To achieve fertilization, spermatozoa must activate the oocyte, triggered by the increasing cytosolic free calcium concentration in the mammalian ooplasm [76, 77]. Our laboratory has been among the many attempting characterization of the soluble oocyte activating factor, one of the most important components of the male gamete, identified in rabbit, hamster, boar, and human spermatozoa [62, 78–81] (Fig. 9.2).

A sperm-specific phospholipase C isoform, PLCζ [82], triggered Ca^{2+} oscillations in the mouse indistinguishable from those at fertilization. Human PLCζ was able to elicit mouse egg activation and early embryonic development up to the blastocyst stage [83].

We postulated that the absence of this sperm-soluble factor in spermatozoa of infertile men is the plausible cause of fertilization failure even with ICSI [58, 84] that represents high emotional and financial toll for infertile couples.

In over 15 years, 11,390 couples were treated by ICSI and about 2.0 % experienced fertilization failure. The lack of oocyte activating factor,

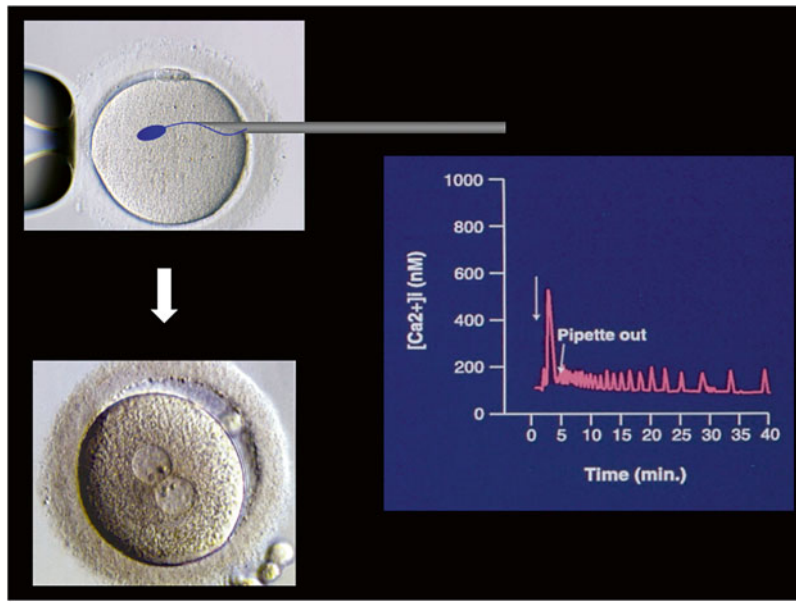

Fig. 9.2 Calcium oscillation generated following injection of spermatozoon into an MII oocyte triggering the process leading to fertilization

however, was suspected in 59 couples that presented with recurrent and complete fertilization failure. Following counseling, only seven couples agreed and consented to undergo assisted oocyte activation. In all instances, the inability of the spermatozoa to induce oocyte activation was confirmed by injecting them into mouse oocytes [84]. In addition, all of the men included in the study had a compromised content of PLCζ in most of their spermatozoa. PLCζ expression in these men ranged from 0 up to 6.4 %, remarkably lower than in fertile individuals with over 80 % presence ($P = 0.0001$).

In cases where we suspect that the sperm cytosolic factor, responsible for jump-starting embryo development, may be reduced or absent, we offer to carry out the PLCζ fluorescence assessment [85, 86]. The ability to recognize those cases versus fertilization failure due to oocyte dysmaturity [37, 86] allows to adopt specific treatment of the spermatozoon [58] and the oocyte [86, 87] to obviate this lack of oocyte activation. Screening for the presence of PLCζ provides the possibility to overcome the dysfunction of these spermatozoa and allows to rescue cycles with recurrent fertilization failure even after ICSI.

9.2.5 DNA

The spermatozoon as a motile cell is not only capable of dynamically relocating to the appropriate site to perform its function but distinguishes itself from other cells for its extraordinary ability to thrive and survive in hostile environments and conditions, such as the acidic vaginal pH and opposing cilia motion encountered within the female genital tract. The spermatozoon's resilience is a product of its fibrous sheath and the high compaction of its nucleic acid [88–91].

The understanding of this unique chromatin packing has important consequences for both the reliability of male infertility screening tests and for the comprehension of the intricate sperm functions, which may also have implications for the outcome of ART [92–98]. It has been postulated that fertile men with normal semen parameters should have an intact chromatin, whereas male infertility presents, especially when compounded by compromised semen parameters, with increased proportion of nicks and breaks in the sperm DNA. To complicate the issues even further, up to 8 % of infertile men will have abnormal sperm DNA integrity not corroborated by impaired semen concentration, motility, or morphology [99, 100]. A systematic observation performed in our laboratory evidenced a correlation between DNA fragmentation level measured by sperm chromatin structure assay (SCSA) or terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) and motility [101]. It appears that the etiology of sperm DNA damage is multifactorial and may be due to intrinsic and/or external factors. Intrinsic defects that may predispose spermatozoa to DNA damage include protamine deficiency, mutations that adversely affect DNA compaction [102], or other "DNA packaging" defects. In addition, advanced male age has been related to a higher occurrence of sperm DNA damage [103–106]. Furthermore, environmental factors ranging from cigarette smoking [107, 108], genital tract inflammation, varicoceles [109], to hormone deficiencies [110] are also associated with an increased production of oxygen-free radicals and consequent rise of DNA damage, as seen in humans and animal models.

Sperm DNA integrity is currently assessed by destructive methods such as TUNEL, comet assay, sperm chromatin dispersion (SCD) test, or SCSA. All of these tests require fixation of the sperm being assessed [98]. The maintenance of DNA integrity is a physiological process needed for the complex packing and intertwining of the typical toroids created during spermiogenesis. Although chromatin fragmentation should be completely repaired in fully developed spermatozoa, the persistence of nicks and breaks in ejaculated spermatozoa that escape the epididymal check point has been linked to poor embryo development and reduced implantation rates [111]. While this correlation is clear in couples attempting natural conception, artificial insemination, and seldom with in vitro insemination, the DNA fragmentation index (DFI) is less predictive of outcome when spermatozoa are inseminated by

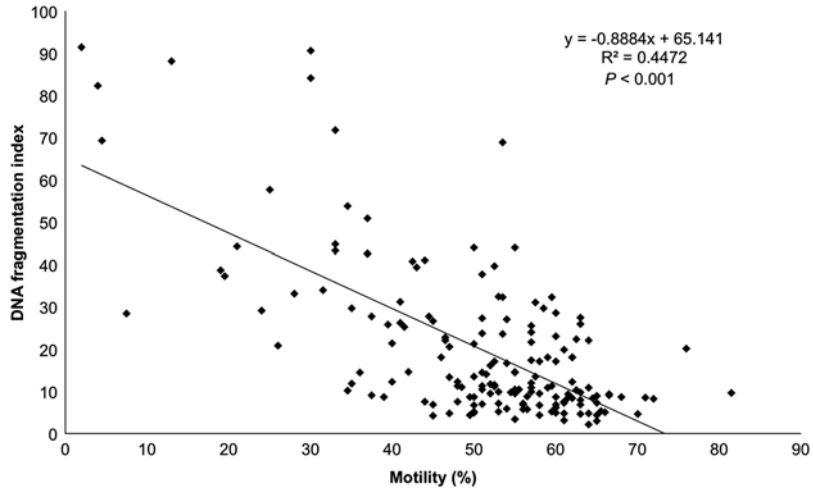

Fig. 9.3 A scatter plot evidencing an inverse relationship between sperm DFI and proportion of motile spermatozoa in semen specimens

ICSI, where only individually selected spermatozoa are used. We postulate that DNA fragmentation rates measured in a particular sample do not take into consideration whether the cells are motile and therefore functionally intact [101].

DFI values obtained by SCSA carried out in 177 men were allocated according to normal (≤25) and abnormal (>25) thresholds. Men with abnormal DFI had lower sperm motility and morphology ($P<0.0001$). DFI values of these patients were plotted against semen characteristics, and a clear inverse relationship between the declining motility and increasing DNA fragmentation was most evident ($P<0.001$) (Fig. 9.3). In fact, lumping the extremes of the spectrum in men with compromised motility at an average of 19.7 ± 3 %, the DNA fragmentation rate reached over 60 %, in contrast to those with normal motility of 48.3 ± 14 % displaying a DFI below 25 % [112]. Interestingly, when these men were inseminated with ICSI and grouped according to their DFI values of ≤25 % and >25 %, the fertilization and pregnancy rates were comparable. The unclear relationship between DNA integrity and pregnancy outcome with ICSI inseminations may again be explained by the fact that the spermatozoa are individually selected for injection according to their preferential appearance and retained motility.

During the later stages of spermiogenesis, DNA breakages are physiologically induced by the integrated painstaking action of DNases and polymerases to allow tight chromatin coiling to achieve adequate compaction and only those spermatozoa with repaired chromatin reach the ejaculate. Throughout the male genital tract, oxygen-free radicals mostly from decaying spermatozoa and other cells are the main cause of DNA damage and responsible for the compromised ART outcome. The observation that lengthening the abstinence period would induce more extensive nuclear damage while spermatozoa are stationed in the epididymis and that DNA fragmentation is more prompt in apoptotic spermatozoa that rapidly lose their motility is in support of this concept. Moreover, recent observations [113] are evidencing that the DFI progressively increases with advancing paternal age [114].

To confirm our hypothesis, we processed ejaculates and enriched their motile cell portion in which we carried out DFI assessment. The selected motile cohort had a DFI of 4.6 %, while the almost exclusively immotile was 40.1 %. This was a clear contrast with the initial raw specimen at 14.3 %. To better investigate our hypothesis, we individually selected spermatozoa by a microinjection tool and separated them according to

the presence or absence of motility. DFI assessment of these individually selected spermatozoa whether motile or immotile evidenced a further decrease in the DFI for the motile cohort in comparison to the DFI of the raw semen. We then decided to retrospectively normalize all DFI in our hand by correcting for the motile sperm cohort, and we developed a formula that we called mDFI. A score was generated by the following formula (mDFI): initial DFI x predetermined constant x motility. In addition, because of the much lower overall values, we arbitrarily select a 3 % threshold for all assays. No correlation was observed between the mDFI below/above threshold with fertilization 73.7 %/73.6 %, clinical pregnancies 32.9 %/29.7 %, or losses 3.9 %/5.4 %. However, when we looked at the implantation ability of embryos generated through ICSI, we saw that the abnormal mDFI had a compromised implantation (15.9 % vs 10.6 %; $P=0.02$) [114].

In spite of the common knowledge that the storage of the produced spermatozoa reside within the epididymis, the site where sperm DNA damage manifest within the entire male genital tract remains puzzling. In our clinical experience, we felt that for couples with recurrent embryo implantation failure and where the male partner's sperm is plagued by an elevated DFI, it would be appropriate to suggest retrieving spermatozoa surgically from the testis or the epididymis.

Men with extremely high DFI in their ejaculates ($n=20$) were counseled to undergo surgical sampling. DFI analysis was carried out on ejaculate, vasal fluid, epididymis, and testis. To determine whether a testicular biopsy would yield spermatozoa with healthier chromatin and superior embryo developmental competence, men underwent ICSI with these specimens. In ejaculated spermatozoa, the average DFI was 43.0 ± 16 % (range 26–96) assessed in 25 occasions. In some of these men, aspiration of the vas deferens ($n=2$) yielded a DFI of 16.5 ± 1 % (range 15.7–17.3), while spermatozoa from the epididymis ($n=8$) had a DFI of 15.8 ± 5 (range 11.7–25.9) and testicular spermatozoa ($n=15$) 11.4 ± 7.9 (range 2–26.2). This topographic representation of the DFI in favor of utilization of testicular spermatozoa encouraged us to utilize these gametes to inseminate oocytes. These couples ($n=8$) obtained 50 % fertilization and an embryo cleavage of 100 % that resulted in a clinical pregnancy of 25.0 %. This finding appears superior to their respective ICSI cycles carried out with ejaculated spermatozoa that resulted in fertilization of 55.9 %, embryo cleavage of 63.6 %, and pregnancy rate of 12.5 % [115]. It is clear that DNA fragmentation has its source within the seminiferous tubules; however, progression through the genital tract toward the ejaculate dynamically increases DFI. This finding may justify offering testicular biopsy to men presenting with very high sperm DNA fragmentation in their ejaculate aiming at better embryo development and implantation.

The topographic assessment of sperm chromatin integrity throughout the male genital tract indicates a disruption of DNA packing during spermiogenesis that does not allow sperm chromatin to withstand even ordinary oxidative stressors, possibly compounded by a compromised total antioxidant capacity in the seminal fluid of these men.

9.3 Morphometrics and Maturational Markers

The ability of ICSI in empowering a single spermatozoon has stimulated a trend toward the identification of the cell that would provide the best chances to generate an embryo capable of sustaining pre- and post-implantation development while at the same time assuring the gain of a healthy offspring. One clear example of this attempt is the selection of a spermatozoon according to its morphometric characteristics while in vivo.

Defined as "motile sperm organelle morphology examination" (MSOME), this approach aims at assessing the living male gamete's phenotype [116]. The procedure referred to as "intracytoplasmic morphologically selected sperm injection" (IMSI) claimed to yield superior clinical outcomes than conventional ICSI [117]. The promised beneficial impact of IMSI has been described in a series of small studies

where the clinical outcome of patients treated by this procedure was compared with that of ICSI [118–121].

The morphological evaluation is carried out using an inverted microscope equipped with a ×100 lens under oil immersion, magnification selector (×1.5), and digital video-coupled magnification ×44 to achieve a final video monitor magnification of over ×6,000. The technical aspect of this approach requires a clarification in relation to the real magnification achievable for the specimen. This has been appropriately coined "empty magnification" that actually occurs at the expense of resolution [122]. This means that the sperm abnormalities evidenced by the IMSI reports can be observed even at the ×400 standard magnification utilizing the best optical lens available on the market (Nikon, MRH68400 CFI S Plan Fluor ELWD NAMC 40XC) and therefore does not necessitate the video blow-out effect of the commercialized expensive MSOME/IMSI setting. The selection is directed toward assessing the overall shape of the spermatozoon with particular attention to the nucleus defined as smooth, symmetric, oval configuration and paying attention to identifying "vacuoles" not exceeding more than 4 % of the nuclear surface area [117]. Most relevant, however, is the role attributed to the putative sperm nuclear vacuole and the meaning of their position on the sperm head. The rationale in identifying these structures and therefore choosing to select spermatozoa void of vacuoles would allow identification of gametes with higher DNA integrity and that are eventually chromosomally normal. This may seem a little far-fetched to consider the morphological assessment as the sole reliable marker with genetic on epigenetic screening capabilities.

Early ultrastructural studies of human sperm in the 1950s and 1960s revealed that vacuoles in the sperm nucleus [123] have been seen in the large majority of human spermatozoa regardless of the fertility potential. Vacuoles in human spermatozoa have in fact been considered as a paraphysiologic finding apparently devoid of consequence on fertility potential [124]. Even the definition of vacuole has been challenged per se; in fact, they can be clearly visualized by transmission electron microscopy (TEM) [123] and are also revealed by confocal [125] and scanning electron microscopy (SEM) [126]. In fact, ultrastructural evaluation reveals them as indentations, craters, dents, or hollows observed on the sperm coat. In such cases, during sperm morphogenesis, the outer acrosomal membrane misforms and generates what appears to be a vacuole [127]. Interestingly, these presumed vacuole structures seem to disappear as the spermatozoon matures in the epididymis following in vitro maturation or at the time of the acrosome reaction [128] (Menezo, personal communications). In other circumstances, however, they seem to increase with temperature (37 °C) and incubation time (>2 h) [129], most probably due to the plication/vacuolization of the rostral spermiolemma during capacitation. In any case, the sperm morphological makeup appears dynamic, and interestingly, it appears that vacuole-like entities are retrievable in over 90 % of spermatozoa, even those of obviously fertile men [125, 126, 130].

In a joint effort to clarify the role of these sperm nuclear features, in consenting couples, we adopted higher magnification screening for sperm surface irregularities and prospectively correlated them to pre- and post-implantation embryonic development. The multicenter effort did not, however, seem to benefit the patients' clinical outcome either for patients with compromised semen parameters and for those undergoing first or repeated ART attempts [130]. Analyses of spermatozoa from different sources, ejaculated or surgically retrieved, also revealed the varying presence and size of sperm nuclear irregularities that develop during the dynamic processes of spermiogenesis and maturation. The surface irregularity did not translate to a higher incidence of DNA fragmentation or aneuploidy, nor to the ability of vacuolated spermatozoa to generate zygotes capable of developing to blastocysts [125, 126, 130].

In addition, we individually selected spermatozoa with and without a vacuole and then processed them for DFI by TUNEL and FISH for aneuploidy. Interestingly, there was no effect on the presence of vacuole on the spermatozoal head, on the incidence of DNA fragmentation and aneuploidy.

In a prospective randomized sibling oocyte study that included 350 ICSI cycles to alleviate

male infertility [131], on the day of treatment, a high-magnification sperm morphology was performed on all sperm and oocytes that were split between IMSI and ICSI. The prevalence of vacuoles in normal-shaped spermatozoa was as low as 27.5 %. The fertilization rate was 79.1 % and 77.3 % after IMSI and ICSI, respectively. Embryo development was similar in both treatment groups up to day 5 of preimplantation development. Clinical pregnancies with fetal heart beat were similar between IMSI (34.4 %) and ICSI (36.7 %). The authors concluded that the routine application of IMSI in unselected artificial reproductive technology patients cannot be advocated. In fact, after a decade from IMSI's introduction, this technique continues to divide reproductive professionals. There is no consensus even on the indication for IMSI, and it appears, even after a systematic review of the literature, that the only plausible utilization would be recurrent implantation failure after ICSI [132]. In fact, it has been suggested that IMSI is not beneficial at enhancing putative "early paternal effects" defined as the sperm contribution to fertilization and early embryo cleavage [133, 134] and hence the comparable embryo quality obtained with typical ICSI. On the other hand, the "late paternal effect" is typified by the contribution of sperm to the later stages of pre- and early-postimplantation development exerted by a compromised DNA chromatin. In this instance, it appears that IMSI is effective in overcoming the latter [134]. A Cochrane study identified that various RCTs do not support the clinical use of IMSI [135]. In addition, there was no evidence of its effect on live birth or miscarriages nor on enhancing clinical pregnancy rates. Moreover, the safety of IMSI, how it is currently performed, needs to be confirmed. In fact, it has been reported that infants born after IMSI have a higher risk of low birth weight (<2,500 g) [136].

In addition, a link between the abnormal phenotype and the chromosomal/chromatinic integrity has also been attempted by the hyaluronic acid (HA) binding assay appearing on the surface of the mature spermatozoa [137–139]. This biochemical marker was used to identify the most viable, mature spermatozoa with intact DNA, limited aneuploidy, restricted residual amount of histones, and increased spermatozoal function [137–139] to be used for ICSI. However, this concept is contradicted by the observation that immature spermatozoa such as those retrieved from epididymis and testis are capable of generating high fertilization and pregnancy rates comparable to their ejaculated counterparts [37]. In our hands, in a total of 15 men, we carried out the selection of spermatozoa that exhibit HA binding sites in which we assessed the chromosomal status and chromatinic competence. We did not find any differences in relation to the morphology, sperm compaction (aniline blue), DNA fragmentation (SCD and TUNEL), and sperm aneuploidy following motility enrichment and HA selection. The selection of HA binding site did not add any further advantage in identifying better spermatozoa than those seen after a simple method of motility enrichment [140].

Also for this assay, as for the IMSI aiming at selecting the best candidate sperm for injection, invariably all studies agree that it is noteworthy that more prospective randomized analyses are required to confirm the superiority of these assays over the standard ICSI selection.

9.4 Conclusions

Today in the developed world, the proportion of children born from assisted reproductive technologies is between 1 and 4 %. Of these infertile couples that benefit from reproductive medicine, about half have a male factor indication, and this has rendered the generation of conceptuses through ICSI very popular. In fact, it can be estimated by the international committee monitoring assisted reproductive technologies (ICMART) data that over two million babies and counting are born from this peculiar insemination method. This has shaken the dogma common in the early days of in vitro insemination that suboptimal or even mildly impaired semen samples were not deemed suitable to participate in normal embryo conception and too easily donor specimens were proposed. The recent accomplishments in the treatment of male infertility have resulted in the empowerment of the male gamete that has regained its status as a capable contributor of the

paternal genome and has shifted the paradigm toward the quest to identify the ideal spermatozoon. This at times appears presumptuous believing that the chosen spermatozoon is the sole responsible for a healthy offspring and almost completely neglecting the contribution of the female gamete, the female genital tract, and the genome of the newly profiled conceptus. The effort to pick out the best gamete, however, is not new and has been attempted since the implementation of ICSI materialized by the proposed ultracentrifugation of semen samples that too swiftly were otherwise considered azoospermic. For the time, these were somewhat extreme techniques; in fact, high speed centrifugation was considered taboo by the purist contemporary andrologists for the presumed effect of generating oxygen-free radicals capable of damaging spermatozoa. Even the injection of suboptimal spermatozoa was disputed, because of their poor appearance according to classical morphological evaluation and too simplistically dismissed as aneuploid. The attempts to enrich sperm incubation media with $CaCl_2$ to enhance spontaneous capacitation, the execution of the flagellar immobilization to expedite the acrosome reaction, and its aggressive modification to address the surgically retrieved spermatozoa were indirect sperm selection attempts. Tedious search for the best-looking sperm and the painstaking relentless observation of the 3D kinetic patterns while swimming in a viscous medium at slow motion were all attempts to identify the ideal spermatozoa to inject. Similarly, the assessment for maturational signs of the spermiolemma presented by the spermatozoon that would stick to the bottom of the petri dish or to the inner lumen of the injection tool aims at the same purpose.

The current approach to diagnostic seminology, as promulgated by the World Health Organization [7], classifies patients according to description analyses of sperm number, motility, and morphology but does not come close to evaluating the full range of properties spermatozoa need to express if they are to establish a normal pregnancy [141]. In fact, a variety of tests to assess spermatozoa competence can and should be performed such as acrosome reaction, antisperm antibodies, PLCζ, centrosome, aneuploidy, and sperm DNA fragmentation. All these assays, however, especially if individually executed still do not have the ability to measure the actual fertilization potential of the spermatozoon.

In our laboratory, we are working on the creation of a new technology in sperm imaging in which we capitalize upon a sperm's innate ability to swim past a fixed camera. This novel technique combines light microscopy with advanced computer vision algorithms to generate a three-dimensional view of the sperm cellular surface. With this new technology, we are able to provide a more accurate spermatozoal assessment given our ability to study structures of the sperm cell that were not easily visible before, but it is possible that in the near future we will have the ability to perform a real-time 3D sperm surface analysis before selecting it for ICSI.

Finally, in spite of all the efforts to tend to the male gamete, it is paramount, from a clinical point of view, to genetically screen couples and inquire about their family history to eventually identify inherited or familiar traits that may functionally, genetically, and epigenetically cause male gamete dysfunction or be responsible for transmitting dys-spermatogenesis such as in Yq-deletion or CFTR dysfunction typical of congenital bilateral absence of the vas deferens or a specific phenotype as in Klinefelter, Kartagener, or globozoospermia, to mention the most known.

Acknowledgments We thank the clinicians, embryologists, andrologists, and scientists of The Ronald O. Perelman & Claudia Cohen Center for Reproductive Medicine and Urology Department, Weill Cornell Medical College. We are grateful to Daniel Ryan and Theodore Paniza for their assistance in the laboratory work and Brian Levine for his attempts at generating a 3D model of the spermatozoon.

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Is Acupuncture Associated with Improved IVF Outcomes?

10

Michelle P. Hay and Alice D. Domar

10.1 Introduction

Infertility is a complicated, ever-changing, and emotionally charged subject. The principles of fertilization, cellular division, hormone signaling, and receptor sites are well researched, but questions remain as to why some women get pregnant while others are not successful. Each patient responds to treatment differently with varying results. Given the financial and emotional costs of unsuccessful treatment and the uncertainty that accompanies every IVF cycle, many fertility patients have turned to acupuncture as a complement to their IVF protocol. There is much debate as to the validity of pursuing that path; the goal of this chapter is to examine this controversy.

10.2 The Paulus Study

The first randomized controlled trial on the impact of acupuncture on IVF outcome was published in 2002 [1]. One hundred sixty patients with good quality embryos were randomized to either receive 25 min of a set protocol of acupuncture before and after embryo transfer or to lay quietly for the same amounts of time. The main outcome measure was clinical pregnancy, as defined by the presence of a fetal sac 6 weeks after embryo transfer. Clinical pregnancies were documented in 42.5 % of the acupuncture patients and 26.3 % of the control subjects. The authors concluded that acupuncture appears to be a useful tool in improving the pregnancy rate during ART.

The clinical use of acupuncture exploded in popularity after the Paulus study was published. Some clinics reported that upward of 80 % of their patients were receiving acupuncture treatment, many centers advertised the on-site availability of acupuncture services, and acupuncturists worldwide were inundated with infertility patients.

However, in the past 12 years, there has been increasing controversy about the efficacy of acupuncture in the IVF population. Numerous RCTs have been performed, meta-analyses have been presented, yet there has been no clear answer to the question if or how acupuncture influences pregnancy rates in IVF patients.

10.3 The Science

There are numerous Western medical explanations about how acupuncture works. The top theories on the potential impact on fertility were put forth in a 2008 article [2]. “Firstly, acupuncture may mediate the release of neurotransmitters,

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_10

which may in turn stimulate secretion of gonadotropin releasing hormone, thereby influencing the menstrual cycle, ovulation, and fertility. Secondly, acupuncture may stimulate blood flow to the uterus by inhibiting uterine central sympathetic nerve activity. Thirdly, acupuncture may stimulate the production of endogenous opioids, which may inhibit the central nervous system outflow and the biological stress response."

Alternatively, from the TCM (Traditional Chinese Medical) perspective, the basic principles in the use of acupuncture are to assess the patient for imbalance, look for excess or deficiency in the body's functions, move blood and energetic potential (Qi) to help stimulate and repair tissue, and calm the Shen (emotional status).

Just from this description alone, the difference between Western and TCM medical terminology poses a problem. How can health-care providers compare strategies and success rates when the basic language is so different?

10.4 The Placebo/Sham Dilemma

Trying to assess the effectiveness of acupuncture in enhancing the pregnancy rates in IVF by doing research that is based on a Western medical model is truly challenging. There are numerous variables in TCM that are not in the Western medical model. The first challenge is the issue of placebo-controlled trials. Typically, pharmacological research is focused around giving an active medication vs. nonactive medication (placebo). The chosen option in most "placebo-controlled" trials with acupuncture has been to use sham needles. A sham trial includes either placing needles in nontherapeutic areas or using needles which do not penetrate the skin.

The Streitberger control is when a noninsertive "sham" needle is used in place of needle through skin acupuncture. The patient feels the needle, but it is not inserted through the skin. It is seen as a noninfluential technique as the "sham needle" is placed over the acupuncture point giving the patient the perception that a needle has been inserted. Much debate resides in the acupuncture community regarding the noninfluential nature of this "needle." For example, some Japanese needle techniques barely break the skin barrier but yet still attain therapeutic effect. If an acupuncture point is being stimulated, but just at the surface of the skin, what is the biological response? How do we address this? Until elimination of any influence regarding "sham" acupuncture on the treatment process can be determined, it cannot be seen as a nonfactor especially when assessing study findings. Clearly, these issues complicate the interpretation of the studies.

There is no data to support the theory that sham acupuncture is in fact a true placebo. It is quite possible that sham acupuncture is not inert. Thus, conducting trials where acupuncture is compared to sham acupuncture may well be pointless. Eric Manheimer [3] argues that with IVF, where the outcome is pregnancy, it is totally objective and not likely to be impacted by a patient's expectation of success, using sham acupuncture as a control condition is unnecessary. He concludes that using sham acupuncture as a control will confuse, rather than clarify, the impact of acupuncture on IVF outcome.

10.5 Brief TCM Background Regarding Fertility

From a fertility perspective, the passage of the reproductive lifespan is one element for which medicine has no control. Similar to the world of Western medicine, a woman's fertility decreases with age. In TCM theory, the pituitary–ovarian–thyroid axis falls under the Chinese character that describes "kidney energy." The Kidney character has less to do with the tangible organ and more to do with the hormonal systems of the body. It heavily influences menstruation and fertility while also including the passing on of genetic information from parent to child. "Kidney" energy peaks at 18 years of age and then decreases every 7 years after that. Very similar to Western medicine, by the time women enter their mid to late 30s and into their 40s, fertility decreases markedly. With that being said, ART has changed

that dynamic. From a biological perspective when a woman's fertility decreases, Western medicine can circumnavigate the traditional pathways by using medication to capitalize on remaining eggs and manipulation of the sperm/egg relationship to boost rates of fertilization. Donor egg is another option that was unforeseen until fairly recently. Even with numerous high-tech options, the rates of take-home baby remain somewhere between 20 and 50 %. This range varies depending on age bracket and clinic statistics.

10.6 The Impact of Acupuncture: Current Research

As mentioned, the Paulus study was the first of many randomized controlled trials to investigate the impact of acupuncture on IVF outcome. The findings of the early trials for the most part replicated Paulus' results with most of the studies indicating higher pregnancy rates in the acupuncture groups. A review and meta-analysis from 2008 [2] concluded that acupuncture was indeed associated with higher pregnancy rates and suggested that "10 patients would need to be treated with acupuncture to bring about one additional clinical pregnancy." Given that the sessions of pre- and post-embryo transfer acupuncture cost in the range of $150–200, compared with the many thousands for the entire IVF cycle, it was not surprising that so many patients chose to add it to their IVF treatment plan.

However, results of acupuncture studies since 2008 have not been as clear. For every study which indicated a positive impact of acupuncture on pregnancy rates, there was one which did not show any impact. The meta-analyses have been equally confusing. Several have shown a positive impact on pregnancy rates. Shen et al. [4] found that acupuncture performed only at the time of embryo transfer did not improve pregnancy rates, but there was a benefit when performed at additional times during the cycle. Zheng and colleagues included 24 trials with 5,807 participants [5]. The analysis compared clinical pregnancy rate (CPR) and live birth rate (LBR). CPR was higher in all of the acupuncture groups than the control but did not reflect the same positive benefits in the LBR. However, an amendment was made in the findings to suggest ignoring the Streitberger control as it "may not be an inactive control." When only studies were included which did not include sham needles, the live birth rates were also significantly higher. Manheimer conducted a meta-analysis and found no pooled benefit of acupuncture other than in trials with low control group rates of pregnancy [6]. Two other meta-analyses did not find any difference in the clinical pregnancy rates of patients who received acupuncture [7, 8].

10.7 Why the Discrepancies in Research Findings?

The Paulus study was unique in one important aspect; it only included women with good quality embryos. Given that most of the research on acupuncture has been on pre- and post-embryo transfer treatment, it is obvious that embryo quality is an important factor. However, there is no intervention in the world, pharmaceutical, surgical, or complementary, which can alter the outcome if a developmentally incompetent embryo is transferred into the uterus.

Many TCM practitioners believe that if treatment is begun earlier, even prior to cycle start, it might have an effect on embryo quality. A recent study supports this theory. Eighty-four patients who had experienced at least two unsuccessful IVF cycles were randomized to receive acupuncture, a sham procedure, or a control group [9]. The acupuncture treatment was delivered on the first and seventh day of ovulation induction, as well as on the day before retrieval, and the day after transfer. It also included the use of moxibustion, a commonly used adjunctive therapy in TCM. The sham patients received needling in eight areas which are not known acupuncture points. The acupuncture patients had a 35.7 % clinical pregnancy rate, compared to 7.1 % in the control group and 10.7 % in the sham group ($p=0.02$).

This study is particularly relevant as it focuses on the most common population of women who

seek acupuncture treatment. Embryo implantation failure is usually defined as having two or more previous failed IVF procedures. Typically patients who have reached this point will look outside of the classical medical realm to seek alternative options and, for some, acupuncture might help bridge that gap of failure.

The other major complicating factor is the acupuncture protocol used. For the Paulus study, one needle protocol was used for all patients. Much of the subsequent research has utilized "the Paulus protocol" so that all patients randomized to the acupuncture group receive the same number of needles in the same places. However, this goes counter to the TCM model of treatment. Prior to an acupuncture treatment, there is an evaluation of an individual patient's history, symptoms, and emotional well-being that results in a differential diagnosis from the acupuncturist. Based on this, the practitioner will take a standard treatment protocol and modify it to address specific needs.

This means, for example, that several patients can present with the diagnosis of PCOS and yet be "diagnosed" with completely different pathologies from the world of TCM. The diagnosis is based on signs, symptoms, the appearance of the tongue, and factors about the pulse, in addition to the patients' constitutional presentation. Analysis also includes physical, mental, and emotional symptoms. The practitioner is looking for clues as to where the patient sits on a spectrum. For example, some PCOS patients are overweight, retain fluid, and rarely ovulate. They tend to fall into a pattern reflecting a predominance of "Spleen deficiency." Conversely, other PCOS patients ovulate (albeit irregularly), are not overweight, and have no fluid retention issues. These patients fall under a different diagnostic pattern of having a predominant "Kidney deficiency." Each patient will look different to the acupuncturist, with a range of different symptoms, i.e., menstrual cycle wise, different tongue, and pulse presentation even though the Western diagnosis of PCOS is the same. Having such a custom-made approach to medicine is wonderful for the patient but difficult for the researcher.

10.8 Is There Cause for Skepticism?

Several articles have been published which discourage the use of acupuncture during the IVF process due to discrepancies in the research findings and/or an assumption that any effects can be attributed to the placebo effect. Interestingly, one concluded with a strong endorsement for a website which sold various nutritional supplements, to be used instead of acupuncture, and one of the coauthors was the owner of that website [10]. There may also be reporting bias. A study which has been widely cited as further proof that any impact of acupuncture is a placebo effect concluded that it is easy to misinterpret the results [11]. The first line of the results section states that "the overall pregnancy rate was significantly higher in the placebo acupuncture group than in the real acupuncture group." However, the authors used a positive urinary pregnancy test as the primary outcome measure. There were actually no significant differences in the rates of ongoing pregnancy or live birth rate.

One can also counter that the placebo effect should not be blithely ignored. If, in fact, acupuncture is associated with an increase in pregnancy rates due to a placebo effect, so what? The argument can be made that any intervention which has the potential to increase pregnancy rates, costs 1 % of a typical IVF cycle, and has no risks or side effects to speak of should be integrated into the treatment plan, whether or not one can explain how it might be working.

10.9 What About the Psyche?

Although the outcome of an IVF cycle is traditionally defined as live birth rate, the psychological health of the patient should not be forgotten. There is strong data to indicate that stress is the most common reason why insured patients terminate treatment, and a patient who drops out of treatment is highly unlikely to conceive on her own. Thus, it is worth exploring the impact that

acupuncture might have on the psychological well-being of the IVF patient.

There have been at least seven studies which have investigated the impact of acupuncture on the psychological status of IVF patients. In an early attempted replication of the Paulus study [12], although there were no differences in pregnancy rates, the acupuncture patients reported significantly less anxiety, more optimism, and enjoyed the intervention more than the controls. In a review of the impact of acupuncture on the emotional health of IVF patients [13], which included 442 women, of the six trials which met the eligibility criteria, five studies reported psychological improvements and one did not report any benefit. Most of the studies included measures of anxiety and/or stress. The authors concluded that acupuncture during IVF treatment is associated with decreases in anxiety, stress, social concerns, and increases in coping. It is also likely that acupuncture can increase resilience to the emotional demands of treatment. Thus, it is possible that patients who receive acupuncture may be more likely to stay in treatment, although well-designed studies are needed to support this hypothesis.

10.10 Acupuncture: Plan for the Future

Because most of the research thus far has been on acupuncture solely pre- and post-embryo transfer, this is the treatment approach that most patients choose. Most acupuncture protocols on the day of transfer focus on influencing blood flow to the uterus and endometrial lining through vasodilation, relaxing uterine muscles, and helping patients feel calmer and more grounded immediately before the transfer. However, this style of one-time treatment does not follow TCM theory. Ideally the acupuncture treatment strategy is slow and steady. Because the patient is not injected or typically treated with any substance or medication, the goal of acupuncture is to allow the body to change by manipulating its own energetic potential through the placement of certain needles. Ideally, the practitioner gets to work with a patient for several weeks or even months before a transfer. The optimum time frame would be a 12-week lead up to help the patient prepare. Acupuncture treatment before, during, and with stimulating medication is seen as an investment in the health of the patient which, theoretically, will lead to a better cycle.

The practitioner–patient relationship may be a significant factor in a cycle's success. Most fertility patients do not get to speak to their physician on a weekly basis regarding changes in emotional and physical symptoms as they are going through a cycle. Yet, this is exactly the type of interaction that an acupuncturist has with their patients. Discussing side effects of medications, trying to mitigate some of those symptoms, and listening to the patient's perspective and feelings help them cope with the stress of IVF. None of these factors may influence live birth rates but they can improve patient compliance and tolerance of cycles. Acupuncturists can help to play a supportive role to the patient as well as to the physician by helping to relay information between the two. It is well known throughout the medical world that patients only tell their doctors what they want them to know.

Another significant concern is the lack of specificity in the research to date. There has been an assumption that if acupuncture is effective in a group of study patients, then it must be effective for all patients. Patients included in research have been highly heterogeneous. There are few treatment modalities in this field which have been shown to be similarly effective for all patient populations. Given that the original research by Paulus only included women with good quality embryos, it is quite possible that pre- and post-embryo transfer acupuncture may only have an impact on patients with normal embryos. Well-designed studies are required to investigate this possibility.

10.11 Research Directions

Clearly, future research regarding acupuncture and fertility needs to correct for the limitations of past studies. Use of traditional research methods

that fail to acknowledge the differences between the Western medical model and the Chinese medical model is like comparing apples and oranges.

The medicine itself is not flawed, but the tools of assessment are. Changing the parameters of design to include more TCM theory, having more defined protocols including choice of points and needle type, eliminating potentially flawed control groups, and stricter guidelines for practitioners would help to clarify results in the future.

Acupuncture has a very low incidence of adverse side effects, is cost-effective, and can easily be implemented as an adjunct therapy to IVF treatment with very low risk of negative interactions. Future studies, with appropriate adjustments to strategy, can have more consistent results to help put the dispute regarding acupuncture's contribution to increased pregnancy rates during IVF to rest.

10.12 Conclusions

Given that the research on the impact of acupuncture on IVF outcome is so contradictory, it is challenging to create specific patient recommendations. It would be unwarranted to recommend that all patients undergo acupuncture treatment as a sure way to increase their odds of conceiving, since the research is not definitive. Although the cost of pre- and post-embryo transfer is minimal, especially when compared to the cost of an ART cycle, it may still pose a burden to some patients. However, can a position not to recommend acupuncture be defended? There are many interventions offered to infertility patients, such as assisted hatching, DHEA treatment, scratch biopsies, aspirin therapy, and others, none of which have been shown to be significantly beneficial to the majority of patients who utilize them, all of which have some risks, and some come at substantial expense. Yet many health-care professionals recommend these approaches to their patients.

Acupuncture is relatively inexpensive and poses few risks, and one cannot ignore the fact that a number of RCTs have indicated higher clinical pregnancy rates for women who receive treatment. Whether or not this is due to a placebo effect is irrelevant; if it aids in conception with minimal downside, why not? The psychological benefits of acupuncture must be taken into consideration as well. Perhaps the bottom line is to let patients decide. Present the current research findings, the pros and cons of adding acupuncture to an ART cycle, and the patient will choose.

Acknowledgments The authors would like to thank Melissa Milstein for her assistance with the literature review needed for this chapter.

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Dr. Web and the New Generation of ART Patients

11

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11.1 Introduction

Over the last two decades, the role of the Internet and of related technologies has become preponderant in several areas of people's lives and healthcare is undoubtedly one of these.

A variety of information previously understandable only to people working in the medical field has become available on the Internet and in other media and so is accessible to a wider user base in a straightforward and simplified way.

When it comes to professional advice, the relationship between doctor and patient has always been crucial in healthcare to provide high-quality medical assistance and treatments. Patients must trust the competence of their physician in order to share the decision-making process in the best possible way while considering the diagnostic pathway to be followed and the treatment options to be chosen.

However, thanks to the tremendous amount of easily understandable information available, this relationship has been changing and, in the eyes of the patients, doctors have been losing some of the "charm" related to their specific knowledge.

On the other hand, the Internet and other media are increasingly being considered a trustworthy source of advice and skills although the information available is not always reliable and accurate.

This phenomenon is so widespread that the US Center for Disease Control and Prevention (CDC) carried out a survey in 2009 to assess it. Results obtained showed that 61 % of adults in the USA have looked for health or medical information on the Internet [1].

The same survey showed that women were more likely than men to use the Internet for health information, regardless of the age group to which they pertained.

Ethnicity and education level also influenced the use of the Internet for health and medical information. White and Asian people used the Internet more frequently when compared to Black and Hispanic people. Similarly, people with higher education and income were more likely to surf the Internet for health information.

This survey also highlighted another interesting aspect: while patients commonly use the Internet to get general information, they showed some concerns regarding confidentiality and security issues when it comes to using this technology to schedule medical appointments or to access medical records on-line.

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_11

Although specific data are not available, it is realistic to assume that similar trends may apply also to the majority of Western countries.

11.2 Pros and Cons of Internet Use in Healthcare Practice

Among all the media, the most frequently used to seek medical and health information is undoubtedly the Internet. This is due to the fact that the web is easily available, cheap and fast and that it allows one to obtain large amounts of information in a very short time.

The use of information technologies (ITs) in medical practice has a huge potential. Patients can use the Internet to interact both with professionals and other individuals in the same situation. Moreover, it provides patients with a variety of easily accessible information, allowing them to increase their awareness regarding health and medical issues and their involvement in the decision-making process as well as in the subsequent treatment.

Contact with patients, patients' organizations and healthcare providers can also offer support and comfort to people suffering from specific conditions. Last but not least, the web can be an extremely effective tool to encourage disease prevention.

On the other hand, the use of the Internet in the medical field can have some associated threats. First of all, the medical information available is not always accurate, as its quality is often difficult to assess [2, 3]. This may mislead patients in their decision-making process, causing them to put at risk their well-being based on unreliable sources of information.

A second threat is the availability of drugs and supplements sold on-line without medical supervision. Their subsequent use without consulting a healthcare provider can be extremely dangerous.

Finally, the security and privacy of medical data exchanged on the Internet can be subject to failures of data-protection systems [4].

11.3 On-Line Behaviour of ART Patients

It has been observed that ART patients are among the most avid users of the Internet to get health and medical information. This is most likely due to the fact that infertile couples are usually relatively young and therefore are familiar with ITs. Moreover, they generally wish to be well informed about issues related to their condition [4].

The fact that infertility often has a negative connotation and it is not always socially accepted can also lead couples to take advantage of the anonymity guaranteed by the Internet to gather information before seeking medical advice.

As the prevalence of infertility and the demand for ART treatments undergo a constant expansion all over the world, the subsequent use of the Internet to get information on these issues is also increasing. As a consequence, a variety of websites related to ART clinics, patients' associations and public institutions and a wide range of independent web sources are currently easily available.

Each patient differs from the other with regard to the amount and the kind of information needed to face a specific condition, and this is specifically true for infertile patients. The Internet allows them to fully control the information supply with beneficial effects on their well-being during a diagnostic and therapeutic pathway that implies a significant emotional distress for both partners [5]. It is interesting to notice that a survey carried out in 2008 in the UK suggested that the information routinely supplied to couples during consultations (either in written or verbal form) is often perceived as insufficient, leading patients to seek further details elsewhere [6].

Provenance of patients also affects their use of Internet. Couples coming from North America and Northern Europe are usually more familiar with these technologies and are more inclined to use the Internet as a source of information. On the other hand, the use of the web for medical purposes is less common among patients from Southern Europe.

Although this difference is still perceivable the gap is becoming less evident in younger generations.

11.4 The Phases of Information Search

The search for information by infertile patients is generally a prerogative of women and it can be divided into four main phases.

The first one can be defined as an interlocutory phase, during which patients seek information regarding the planning of a pregnancy. In case, this goal is not achieved within the amount of time considered as "acceptable", patients usually turn to the web to understand whether there is the possibility that they might have a problem requiring medical advice. This can be done through websites containing generic information and/or through forums and chatrooms where patients share their personal experience (in most cases in an anonymous way).

The second phase has to do with the diagnostic pathway. At this stage, most patients search for information about physicians and clinics in order to choose the ones that they consider the most reliable. Further details on diagnostic procedures are also sought. Finally, some patients might feel the need to share the outcome of consultations and preliminary tests with other couples in the same condition, especially when this is either extremely positive or negative.

This phase is particularly important as the quality and amount of information collected (both from the Internet and from practitioners) can influence the choice of a subsequent treatment, with all its implications, and of the clinic where it shall be performed.

The third step corresponds to the actual ART treatment. During it, patients mostly use the web for access to specific medical information regarding the therapeutic procedure they are undergoing and to find relief from anxiety. The amount of on-line services provided by individual clinics (on-line medical records, on-line consultations, on-line personalized therapies, on-line medical reports, etc.) also influences the use of the Internet by patients. Forums and chatrooms can provide a comforting space to share fears and worries in the attempt to find support by other users.

The fourth phase starts once patients learn about the outcome of the treatment. When positive, the Internet can become a useful source of information for the management of the pregnancy. Moreover, patients who achieve their goal can be more motivated to share their experience to encourage other couples in their same condition. In case of an unsuccessful outcome, patients can use the web to find relief by pouring out their sadness and disappointment, as well as to get further information on potential alternative options.

The above-mentioned search for information related to the planning of a pregnancy, and the management of potential delays in getting it, is especially important because in this phase patients rarely resort to medical advice, which is generally sought later on only in case problems cannot be managed individually.

Keeping this in mind, researchers from Yale University Medical School and Hofstra University School of Medicine carried out an on-line survey among a pool of Internet-using women from the USA aged 18–40 years to assess their knowledge, attitudes and practices regarding selected aspects of reproductive health. The aim of this project was to pursue the optimization of women's health before planning a pregnancy. In fact, a clear understanding of the modalities used by women to gather information about reproductive health can give healthcare providers useful tools to improve communication and information dissemination [7].

Respondents were stratified by age, ethnicity, employment, income, marital status, sexual orientation and previous pregnancies/parity. With reference to prevention of infertility, results obtained showed that a significant amount of women were not aware of the negative effect of painful periods (2/3 of all participants), sexually transmitted diseases (1/3 of all participants) and weight (1/4 of all participants) on fertility. Women aged 25–34 were more aware of the adverse impact of age on the chances of getting pregnant and on the increased risk of chromosomal abnormalities, while the level of knowledge of younger women (18–24 years old) regarding this aspect was significantly lower ($P < .05$) [7].

These data show that, although the so-called new-generation patients have access to a wider range of information compared to older people, this does not necessarily increase their knowledge and awareness on health and medical issues.

11.5 The Use of the Internet for Planning an ART Treatment

Following the preliminary collection of general information on infertility and on the different therapeutic options available, during the second and the third phases described above, the Internet has become a useful tool to assist patients in the choice of their physicians and of the clinic where they wish to be treated. This is especially true when a couple decides to undergo treatments abroad [8].

In Western countries most clinics have their own websites, although the quality of the information they contain can differ significantly.

IVF patients rely heavily on the information they find on-line [9], and when choosing a clinic or a practitioner, patients often refer to their official websites first. After that, before pursuing an actual contact, they usually turn to forums to look for comments by other patients on medical, emotional and financial aspects of treatments.

Although contents discussed in forums are not always accurate, patients tend to rely on this kind of information because it provides practical details shared in a simple and straightforward way. Patients often fear to be judged by physicians when they ask information that might be considered banal, while they feel more comfortable asking questions to their peers. Anonymity is also an incentive, as it allows discussion of personal details without disclosing personal identity.

11.6 The Use of the Internet During an ART Treatment

Once patients start the actual treatment, the use of the Internet varies depending on the amount of on-line services provided by individual clinics.

A vast majority of clinics provide an increasing range of on-line services to their patients. The most common ones are on-line medical records, on-line consultations, on-line personalized therapies and on-line medical reports. When it comes to stressful, costly and time-consuming procedures like ART treatments, these services are appreciated by an increasing number of patients as they reduce the amount of time required for the treatment and they facilitate interaction with the clinic.

A study carried out on infertile patients at Radboud University Nijmegen Medical Center, which offered couples a website allowing them to get general information as well as to interact with other patients and physicians, showed that most patients give a great value to these services. The survey resulted in the identification of the following three different kinds of on-line behaviour:

The first is the so-called individual information style (prevalent in 33.2 % of couples). Patients showing this behaviour were mainly interested in using the website to get specific information related to their personal treatment, and they only used generic pages (e.g. pages containing generic information regarding treatments and the clinic) to interpret a treatment properly and to manage their therapeutic process.

The second on-line behaviour was defined as "generic information style" (prevalent in 29.0 % of couples). In this case, patients were mostly interested in generic information on infertility and treatment options available.

Finally, the last kind of on-line behaviour was called "communication style" (prevalent in 37.8 % of couples). Patients showing this kind of behaviour mainly took advantage of the communication functions of the website (e.g. the forum and the chatroom) to interact with other patients and physicians. It was observed that this behavioural style was more common in anxious patients, as it seemed to offer relief in case of stress.

Patients who did not pertain to any of the above-mentioned groups were defined as "non-users". This subpopulation is not necessarily homogeneous, as it is likely to include both patients who do not have the opportunity to use the website (e.g. due to lack of time) and patients who do not need on-line support before and during treatment [5].

11.7 The Use of ITs to Simplify the Management of ART Treatments: The EasyIVF Approach

Traditionally, assisted reproduction treatments are expensive and time consuming due to the long diagnostic work-up and to the complexity of the techniques used.

Changes occurring in the social structure and in the labour market of Western countries are reducing the spending power of younger people (especially those belonging to the age groups who might need and benefit from ART treatments) as well as the amount of time they can dedicate to medical treatments. This phenomenon worsens the already low fertility rate in these countries, with a variety of social and economical long-term consequences.

In order to solve this problem, a Swiss company called IIARG (International Institutes of Advanced Reproduction and Genetics) recently developed an innovative programme called EasyIVF. This programme takes advantage of the application of innovative technologies in order to minimize the amount of time and the number of visits to the clinic by using a specifically designed website composed of a public area and a restricted area (Fig. 11.1) to manage all the preliminary phases of the treatment. This results in a reduction of treatment costs by almost one half.

To develop the EasyIVF programme, IIARG put together a small working group dedicated exclusively to this project which was composed of professionals in the field of reproductive medicine and of IT experts. The reproductive medicine team included two clinicians, a midwife, an embryologist, a psychologist and a secretary, while the IT team was composed of a biomedical engineer and a software programmer.

The reproductive medicine team provided the medical and laboratory know-how to the IT experts, who translated it into an innovative software capable of performing all the functions required by both patients and professionals.

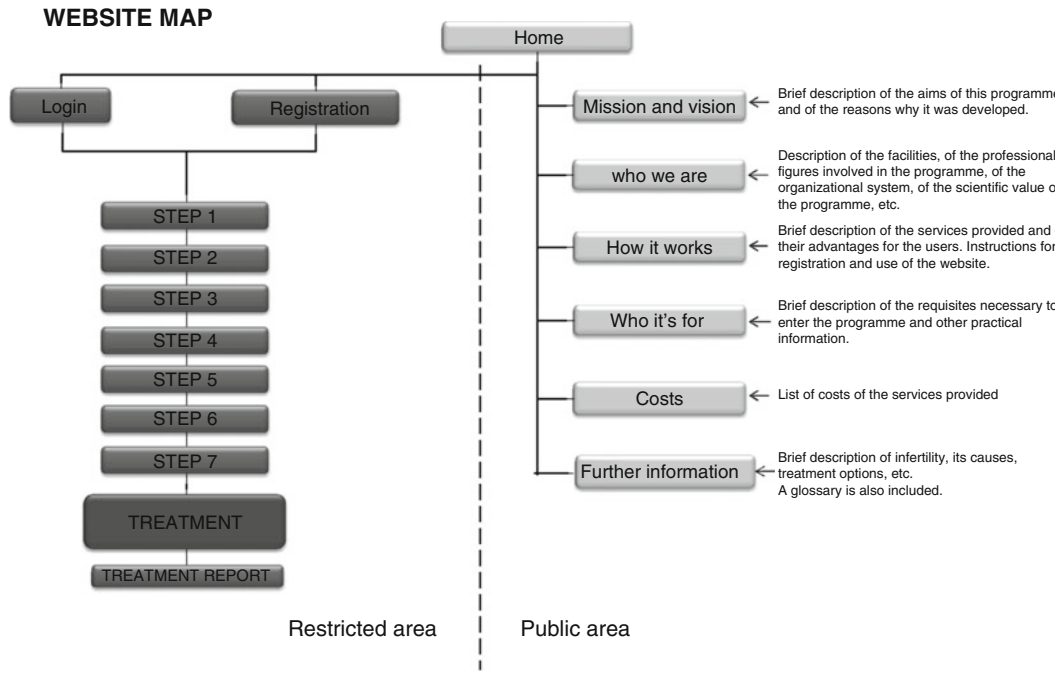

Fig. 11.1 The EasyIVF website—website map

Textual contents were developed by the reproductive medicine team and subsequently uploaded on the website.

During the developmental phase, the software was periodically tested by operators and potential patients, in order to check its performance and to make all modifications necessary to enhance its functioning. This process lasted approximately 1 year and it resulted in the creation of a highly flexible web platform which can be easily adapted to specific needs and contexts (i.e. to specific national regulations). The interface elaborated is extremely user-friendly both for operators and patients, and it allows access to data at any time anywhere by logging in to the restricted area of the website using personal and case-sensitive credentials. It is also compatible with computers and portable devices, such as tablets and smartphones.

From a clinical point of view, EasyIVF is directed to a specific subgroup of so-called good-prognosis patients (age of the female partner below 38, no PCOS, no severe endometriosis, BMI below 30, no infectious diseases, no azoospermia, no abnormal karyotype), and it includes the following treatments: standard fresh IVF cycle, standard fresh ICSI cycle and transfer of cryopreserved embryos.

Patients can complete the preliminary phase of treatments through the restricted area of the website under the constant supervision of a clinician, who checks all medical data and tests submitted in a short time. Psychological counselling is also available through a live messaging service. Patients only come to the clinic on the day of HCG administration, on the day of egg retrieval and on the day of embryo transfer. Risks related to the treatment are minimized by using mild stimulation protocols.

The EasyIVF programme is composed of nine different steps that have to be completed in sequence in order to proceed. Steps 1, 2 and 3 aim to verify that patients comply with the criteria required to access the treatments included in the EasyIVF programme. During these steps, couples are asked to submit personal and clinical information regarding both partners. Once each step is completed, all data submitted by patients are carefully checked by the medical team. If the outcome of these verifications is positive, patients will be allowed to proceed with the following steps. Steps 4, 5, 6 and 7 include preliminary medical tests and provide all the information about the ovarian stimulation protocol, as well as the list of necessary drugs (Fig. 11.2). The clinical and laboratory stages are recorded on the website by the clinician and the embryologist. After completing the cycle, patients receive a report summarizing the treatment and its outcome (Fig. 11.3).

The website where patients upload medical information and where clinicians upload instructions and prescriptions is conceived according to the highest data-protection standards to guarantee safety and privacy.

This programme is currently undergoing an experimental phase in an Italian clinic, and unpublished preliminary data provided by the centre show that the results obtained so far appear to be encouraging. Once this trial is concluded, the programme will become available in other clinics all over the world.

11.8 The Use of the Internet in ART: The Clinician's Perspective

While patients are becoming more and more familiar with ITs for health and medical purposes, clinicians struggle to keep pace with them.

In fact, healthcare professionals do not always master these technologies properly and some of them are often suspicious and sceptical towards their usefulness and reliability.

As mentioned before, the huge amount of information available does not necessarily imply an increased knowledge and awareness of patients. Moreover, information available through the media can be misleading or, in some cases, even wrong, putting patients in danger.

For these reasons, clinicians should master these tools in order to guide patients to reliable sources of information and to warn them to avoid inaccurate ones [10].

Fluency in the use of ITs can also give practitioners the opportunity to interact more easily

The “EasyIVF” programme

The EasyIVF Programme is composed of 9 different steps that have to be completed in sequence. Steps 1,2 and 3 aim to verify that patients comply with the criteria required to access treatments included in the EasyIVF programme. During these steps, couples are asked to submit personal and clinical information regarding both partners. Once each step is completed, all data submitted by patients are carefully checked by the medical team. If the outcome of these verifications is positive, patients will be allowed to proceed with the following steps. Steps 4, 5, 6 and 7 include preliminary medical tests and all information about the ovarian stimulation protocol, as well as the list of drugs necessary. After completing the cycle, patients will receive a report summarizing the treatment and its outcome.

1	Personal data collection and Center selection	completed
2	Medical history	completed
3	Preliminary Medical Tests	in progress
4	Informed consent, contract and payment	pending
5	Drugs prescription and information about treatment	pending
6	First day of menstrual cycle: communication to the clinic	pending
7	Ovarian stimulation + communication of the appointment for admission to the clinic	pending
8	**Treatment cycle**	pending
9	Final report	pending

Fig. 11.2 The EasyIVF website—patient’s advancement status

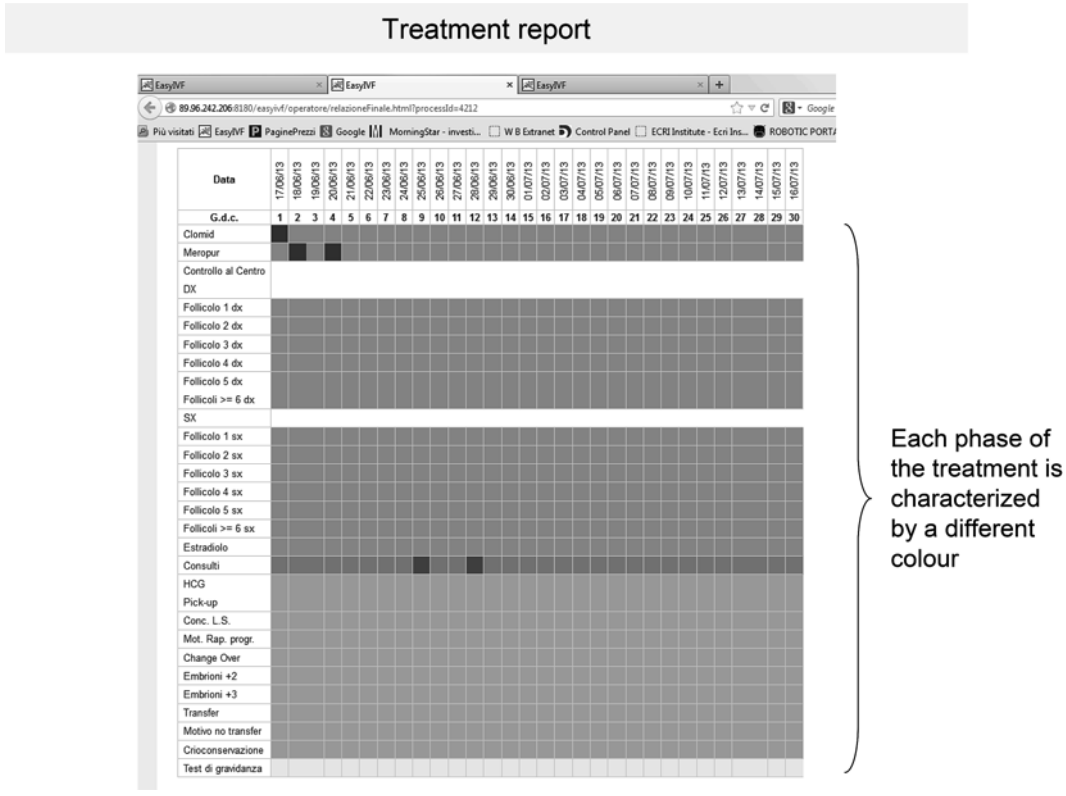

Fig. 11.3 The EasyIVF website—treatment report

and quickly with patients as trustworthy and privileged interlocutors.

Finally, the Internet and other media can be exploited by professionals and institutions to enhance communication and information dissemination by identifying how patients access health and medical materials, as well as to spread in an effective way messages concerning prevention, particularly to those individuals who do not often turn directly to health providers [7].

Aside from communication and information purposes, ITs can support professionals in their everyday work. An increasing number of clinics and practitioners are using these instruments to speed up their workflow and manage patient and treatment data.

This allows clinicians to access medical records anytime anywhere just by using a computer or other devices connected to the Internet. Data can also be available to more than one professional at the same time.

Another advantage is the reduction in the amount of paper documents, which require storage facilities and that are at risk of being damaged or lost.

Finally, the use of a specifically designed software to record every aspect of treatments facilitates data collection and analyses for statistic and scientific purposes.

11.9 The Risks of Information on the World Wide Web

Infertility treatment entails a significant psychological burden: searching for information and sharing experiences with other patients who suffer from the same condition can help relieve stress. However, the high degree of personalization implied in this kind of treatment requires stringent surveillance and control to ensure reliability of information, especially when it is not derived from official medical sources.

Since health information obtained from the Internet can influence treatment choices, it is important to evaluate the quality of materials available to infertile patients.

It has been shown that 91 % of infertile patients use search engines to reach fertility-related sites [11]. For this reason, it is likely that they will visit the first sites listed on the search page before accessing the ones at a lower level.

In spite of that, needless to say, the order of sites does not necessarily reflect the quality of their content [12].

Several surveys were carried out to analyse the quality of infertility information available on the web, evaluating reliability and accuracy of the content, layout and interactivity of websites, currency of information and disclosure of authors and sponsors.

In 2002, Okamura et al. evaluated 197 US sites and reported that fewer than a half of them satisfied one or more of the above-mentioned standards. Only 2 % of websites complied with all standards [3].

A subsequent survey by Abusief et al. [13] showed that the majority of clinics' websites in the USA did not comply with ASRM's advertisement guidelines issued in 2004. In particular, it was reported that success rates were often published in a potentially misleading way.

The lack of regulations related to the posting of information on the Internet leads to a huge variability in the quality and accuracy of information available. Patients are not always capable of discerning reliable sources of information from inaccurate ones, especially because in most cases they use search engines which lead them to commercial websites, rather than those of medical institutions [10].

Since regulation of information published on the web is nearly impossible to achieve, the only tools available to make sure that patients turn to reliable information are the following:

- Clinicians should address patients to only those websites they have personally reviewed to verify their accuracy.
- Authorities should review websites and formally endorse the most accurate ones.
- Authorities should publish institutional websites containing straightforward and easily accessible medical information on specific health issues, also for preventive purposes.

Among websites providing infertility information, forums and chatrooms should be treated separately due to their peculiar characteristics. In fact, forums are generally designed to promote interaction among patients, mostly in an anonymous way. Patients use them to exchange information not only about medical aspects of diagnosis and treatment of infertility but also about practicalities such as costs, reimbursement policies, etc. In addition, they frequently provide opinion and advice regarding specific clinics/practitioners, recommending or advising against them. Finally, forums can be useful tools to relieve stress and anxiety by sharing experiences among peers. As already mentioned, patients tend to rely on information provided in forums, as it is considered to be provided by individuals who are in their same situation [8].

11.9.1 Three Types of Forums Can Be Identified

The first category includes forums related to clinic websites. In most cases, only patients of the clinic are allowed to access the forum using personal credentials provided by the institution itself. The content of discussions is often supervised by clinicians or nurses/midwives who can intervene in case information provided is incorrect, or they can answer questions from the users directly. These forums are considered safer because users are actually and almost exclusively ART patients and their content is checked by professionals. On the other hand, in most cases potential patients (who might require a greater amount of accurate information) are not allowed to enter these forums unless they have had at least one consultation at the clinic.

The second category includes forums related to patients' associations. Users may or may not be members of the association (depending on each association's policy) which gives access through personal credentials. The content of discussions is generally supervised by patients with a good knowledge of fertility issues. Depending on the structure of the association, professionals such as clinicians, psychologists and nurses/midwives might also be involved, thus increasing the quality of information exchanged. These forums can represent a useful and quite reliable source of knowledge both for potential and for actual patients.

The last category is composed of independent forums unrelated to clinics or associations. All the users who log in, regardless of their status, can read and post information. Moderators, if present, are not always experts in the field and information exchanged is not always accurate, or its reliability may be difficult to assess. Obviously, this latter type of forum can potentially be the most dangerous one.

Aside from the presence of inaccurate information, the greatest potential danger of forums is that, without proper supervision from moderators, patients might exchange advice on drugs and their administration without medical control. This poses enormous threat to patient safety, especially considering that ART treatments currently involve a high degree of personalization, with some therapies suitable exclusively for specific categories of patients and not for others. This does not only jeopardize the outcome of the treatment but also the health of the patient.

11.10 Conclusions

The Internet and related media are being increasingly used by patients to search for information about specific medical conditions and about healthcare providers, and this is especially true for infertile patients. To have an in-depth knowledge of their conditions enables patients to make informed decisions regarding their diagnostic and therapeutic options, favouring their compliance to treatments. However, information available on-line is not necessarily accurate or reliable and, in any case, it is not always sufficient.

Considering the importance given by patients to information exchanged through the web, it is very important that healthcare providers have a

good mastery of these tools in order to detect inaccurate information and address patients to reliable sources. ITs can also be useful in their everyday work, as they can facilitate interaction with patients and data management.

Healthcare providers can also benefit of the potential of the web to raise awareness on specific issues and as an effective tool for prevention among specific targets.

Some innovative programmes are showing that use of the Internet for ART treatments can reduce associated costs, stress and time, without affecting the probability of a successful outcome. Some categories of patients showed a particular appreciation for these aspects.

In conclusion, it is realistic to expect that the use of the Internet in the field of infertility will increase further in the future. Denying and opposing this is not only useless but also anachronistic.

ITs will be applied to an increasing number of aspects of infertility diagnosis and treatment. Professionals should make efforts to contribute to the accuracy of the information available on-line by managing reliable and straightforward websites and by providing patients with dedicated spaces to interact among them in a safe way. On the other hand, patients should not replace medical advice with information obtained through the web. During treatments, the privileged interlocutors should remain clinicians, whether they are reached in person or by other means of communication.

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Part IV

Controversies

12 Yes, Elective Single-Embryo Transfer Should Be the Standard of Care

Dmitry M. Kissin, Sheree L. Boulet, and Eli Y. Adashi

12.1 Historical Perspective of Embryo Transfer Practices

In 1978, doctors Steptoe and Edwards documented the first live human birth following in vitro fertilization (IVF) of a single oocyte [1, 2]. In the years following this sentinel event, births of infants conceived through IVF were reported in Australia, the United States, Sweden, and France. Progressive improvements in oocyte retrieval and embryo transfer techniques were quickly and widely adopted [3]. However, during the first years of using IVF, reported pregnancy rates among patients in their early thirties with tubal factor infertility were as low as 6 % [4] due to difficulties in predicting ovulation and the ability to retrieve a single oocyte during an unstimulated cycle [3]. In an effort to increase the number of oocytes available for retrieval, ovarian stimulation protocols using human menopausal gonadotropin were implemented in anovulatory women, resulting in pregnancy rates as high as 30 %, particularly when two or more embryos were transferred [5]. As such, it was suggested that multiple-embryo transfer was advantageous over single-embryo transfer because implantation and pregnancy rates were substantially higher; however, even at this early stage of practice, clinicians recognized that such benefits should be considered in the context of higher than expected multiple birth rates [5, 6].

Since the birth of the first baby conceived through IVF in 1978, the use of assisted reproductive technology (ART) has increased substantially. Advances in technology and treatment procedures continue to improve the likelihood of success with pregnancy rates, which in 2012 ranged from 47 % in women less than 35 years of age to 5 % for women older than 44 among fresh autologous ART cycles [7]. However, while the effectiveness of ART procedures has increased over time, the rate of multiple births following these treatments remains high, despite early warnings from the pioneers of IVF treatment who acknowledged these risks over 30 years ago and recommended prudence in weighing the risks and benefits of multiple-embryo transfer. Today 1.5 % of all US live births in 2010 were conceived using ART; approximately 46 % of those ART births were twin, triplet, or higher-order multiples, accounting for 20 % of all multiple births during that year [8]. It is therefore not surprising that ART births contribute disproportionately to adverse perinatal outcomes and represented

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_12

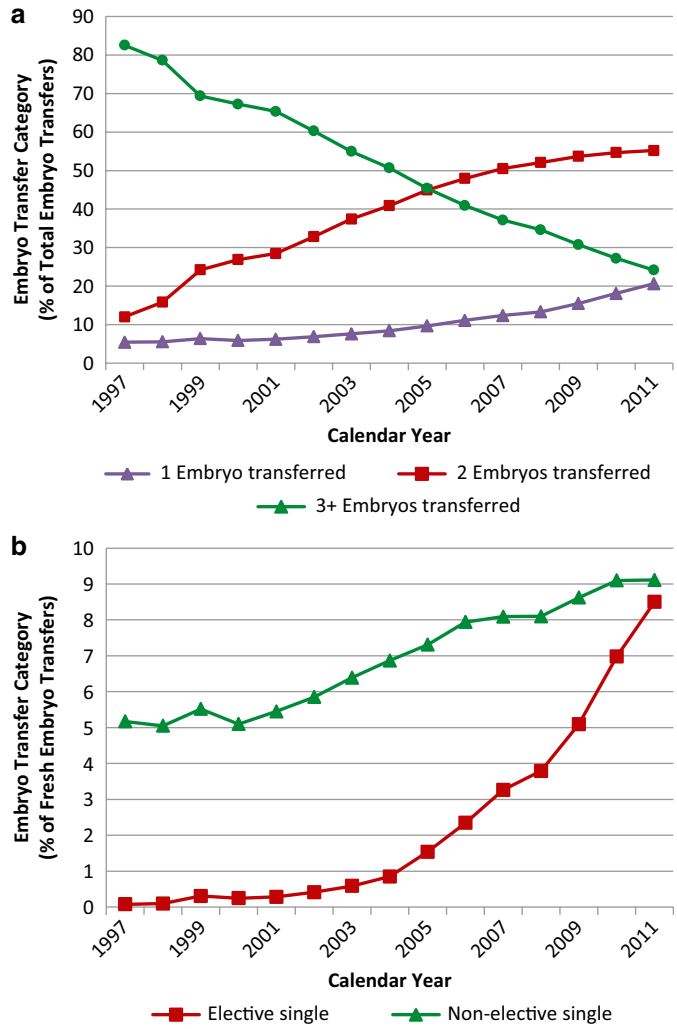

Fig. 12.1 Number of embryos transferred during IVF, United States, 1997–2011 (from New England Journal of Medicine, Kulkarni AD, Jamieson DJ, Jones HW, Jr., Kissin DM, Gallo MF, Macaluso M, Adashi EY, Fertility treatments and multiple births in the United States, Vol. 369, pp. 2218–25 [9] Copyright © 2013 Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society)

approximately 6 % and 4 % of all low birth weight and preterm infants, respectively [8].

While multiple birth rates following ART remain high, considerable progress has been made in reducing the total number of embryos transferred and the resulting rates of triplet and higher-order birth rates. In the United States, the percentage of ART procedures in which three or more embryos were transferred declined from 79 % in 1998 to 24 % in 2011 (Fig. 12.1) [9], due in large part to changes in clinical practice in accordance with practice guidelines developed by the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology [10]. However, during the same time period, the proportion of IVF cycles in which two embryos were transferred increased from 16 % to 55 %, and the proportion of IVF cycles with a single-embryo transfer increased from 6 % to 21 % [9]. The increase of single-embryo transfer was observed in both elective and non-elective single-embryo transfer groups. While such changes in practice resulted in a 79 % decline in the rate of triplet and higher-

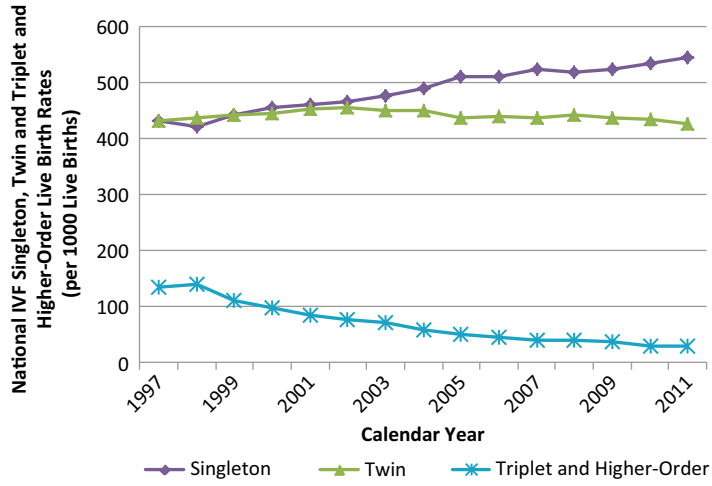

Fig. 12.2 National rates of singleton, twin, triplet, and higher-order live births resulting from IVF, United States, 1997–2011 (from New England Journal of Medicine, Kulkarni AD, Jamieson DJ, Jones HW, Jr., Kissin DM, Gallo MF, Macaluso M, Adashi EY, Fertility treatments and multiple births in the United States, Vol. 369, pp. 2218–25 [9] Copyright © 2013 Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society)

order IVF-related births between 1998 and 2011, there was only a 29 % increase in IVF-related singleton births over the same time period, while IVF-related twin birth rates were stable (Fig. 12.2) [9]. Thus, it appears that temporal reductions in the multiple birth rates following ART can be mostly attributed to the increasing use of double-embryo transfer in place of the transfer of three or more embryos.

12.2 Evidence in Support of Elective Single-Embryo Transfer

Elective single-embryo transfer (eSET), commonly defined as the decision to transfer one embryo even though more than one high-quality embryo is available for transfer, is the most effective method for reducing ART-associated multiple births [11, 12]. Evidence shows that using eSET rather than double-embryo transfer in certain groups of patients can and will reduce perinatal morbidity and mortality, improve the likelihood of having a healthy singleton infant, and reduce healthcare costs.

12.2.1 Elective Single-Embryo Transfer Reduces Perinatal Morbidity and Mortality

The most compelling argument in favor of eSET versus the transfer of two or more embryos among appropriate groups of patients is its ability to reduce multiple births and associated morbidity and mortality among infants and mothers. Although the risk of multiple births with eSET still exists due to monozygotic twinning [13], almost all births (98.3 %) that result from eSET are singleton births, whereas only about half of births that result from transfers involving two or three embryos are singleton births (53.6 % and 54.4 %, respectively) (Fig. 12.3). As a result, single-embryo transfer is associated with a higher percentage of term births (88.1 %) than with double- or triple-embryo transfer (64.1 % and 51.1 %, respectively).

Multiple gestation pregnancies more often result in miscarriage than singleton pregnancies. The most serious adverse consequences of multiple births are prematurity and low birth weight, which are associated with increased risk of neonatal mortality. According to the US

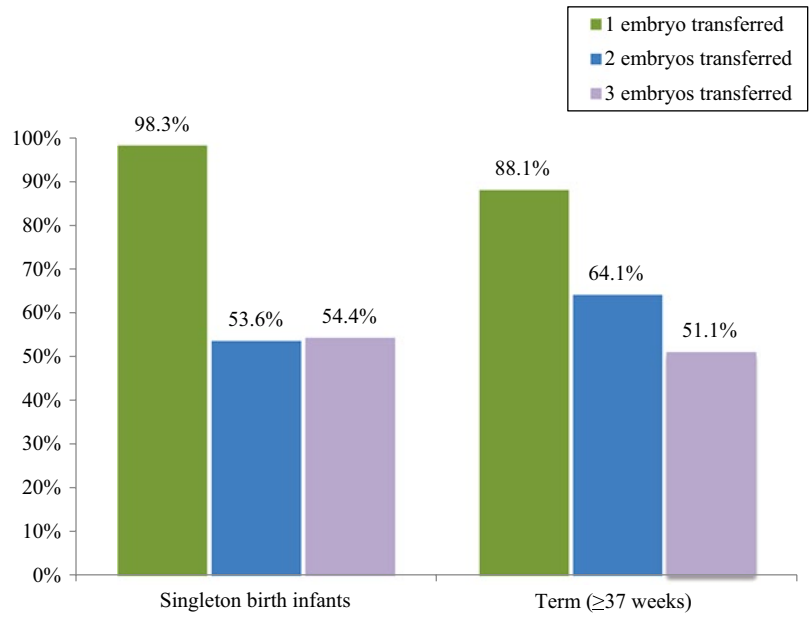

Fig. 12.3 Characteristics of ART-conceived live births, favorable-prognosis (favorable-prognosis patients include those who underwent first assisted reproductive technology (ART) cycle and had extra embryo(s) cryopreserved) patients younger than 35 years who used fresh blastocyst-stage (day 5) embryos from autologous oocytes, by number of embryos transferred. (*Source*: United States, National ART Surveillance System, 2011 [61])

National Vital Statistics System, the risk of neonatal death is 6 times higher among twins (19.3 per 1,000) and 14 times higher among triplets (48.9 per 1,000) than among singletons (3.5 per 1,000) [14]. Compared to singleton births, multiple births, including twin births, are associated with increased risk of cerebral palsy, birth defects, autism spectrum disorders, and other adverse short- and long-term outcomes [15–25].

The maternal risks of multiple gestation pregnancies and births include an increased risk of gestational hypertension, preeclampsia, gestational diabetes, hemorrhage, cesarean delivery, and maternal hospital admission, among other factors [15, 20, 26–29]. In addition to serious somatic consequences of multiple births, caring for twins, triplets, and higher-order multiples can negatively affect mental health of parents [24].

Although eSET will not be able to completely eliminate the risk of adverse outcomes of ART due to the inherent contribution of infertility and/or ART procedure itself, minimizing the risk of multiple births can substantially improve ART outcomes for both ART patients and their children [30–33].

12.2.2 Elective Single-Embryo Transfer Improves ART Success

One of the main arguments of opponents of eSET is that transferring one embryo instead of two reduces the overall success of ART [34]. This argument is based on the assumption that ART success is measured in terms of achieving a pregnancy followed by a live birth. Indeed, data from randomized controlled trials indicate that pregnancy and live birth rates are significantly lower for cycles in which a single embryo is transferred compared with those in which two embryos are transferred [35, 36]. However, these traditional measures of ART success, pregnancy and live birth rates, do not reflect substantial risks related to multiple gestations and multiple births and triggered significant debate in the ART community as to more appropriate measures of ART success [37–46].

There is general consensus among experts and professional societies that the optimal outcome of ART, as well as any other fertility treatment, is healthy singleton birth [12]. One of the

proposed measures of ART success that reflect this goal is BESST (birth emphasizing a successful singleton at term), which is defined as a singleton term (≥37 weeks of gestation) live birth rate per cycle [45]. Another proposed measure of ART success that balances effectiveness (live birth rates) and risks (multiple births, prematurity, and low birth weight) of ART is good perinatal outcome, defined as live birth of a term (≥37 weeks of gestation) normal birth weight (≥2,500 g) singleton per transfer [47]. Using one of these measures of ART success in the decision-making process about the optimal number of embryos to transfer may result in fewer preterm births.

If we define ART success as a healthy singleton infant, it will be clear that among women younger than age 35 with favorable prognosis, eSET leads to higher success rates than double-embryo transfer (Fig. 12.4). This statement is true for both cleavage- and blastocyst-stage transfers. Having eSET as the standard of care for appropriate ART patients will reduce multiple births and associated complications and adverse outcomes.

It is also important to note that *elective* single-embryo transfer implies that at least one additional embryo is available for transfer in a subsequent ART cycle. When an unsuccessful single-embryo transfer is followed by an additional frozen/thawed single-embryo transfer cycle, the pregnancy and live birth rates are comparable with that following double-embryo transfer [35, 36]. In addition, findings from a recent randomized controlled trial suggest that there is no difference in pregnancy rates when a single euploid blastocyst is transferred compared with two untested embryos [48].

12.2.3 Elective Single-Embryo Transfer Reduces Healthcare Costs

One of the important economic benefits of eSET in appropriate groups of patients is the ability to reduce healthcare costs by minimizing multiple births and the associated adverse outcome of prematurity. Preterm birth is one of the most seri-

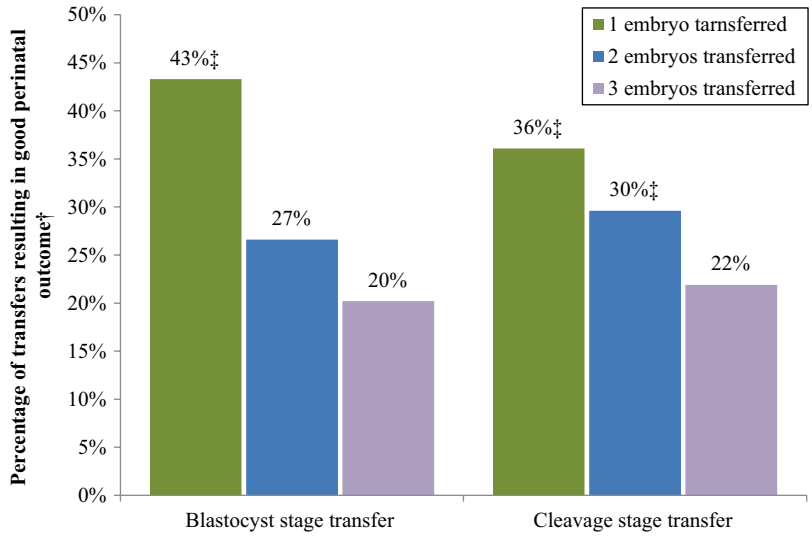

Fig. 12.4 Patients with a favorable prognosis (patients with a favorable prognosis include those who underwent a first assisted reproductive technology cycle and had extra embryo(s) cryopreserved): percentage of transfers resulting in good perinatal outcome (good perinatal outcome is defined as live birth of a term (at least 37 weeks of gestation) and normal birth weight (at least 2,500 g) singleton) among patients younger than 35 years who used fresh embryos from autologous oocytes for blastocyst-stage (day 5) and cleavage-stage (day 3) embryos by the number of embryos transferred, United States, 2011 (from Kissin DM, et al. [47]). ‡$P < .05$, comparison with the proportion associated with transfer of additional embryo; if not marked, $P \geq .05$

ous and costly complications of ART. The societal economic burden of ART-conceived preterm infants in the United States amounts to over $1.3 billion per annum [49, 50]. Estimates of the societal economic burden of preterm birth were established by the Institute of Medicine's Committee on *Understanding Premature Birth and Assuring Healthy Outcomes* and are "above and beyond what would have been expended had these infants been born at term" [51]. This measure included the costs of medical care, maternal delivery, early intervention services, special education services, and labor market productivity associated with preterm birth.

Several studies compared repeated single-embryo transfer with double-embryo transfer and found no statistical differences in live birth rate, significant reductions in multiple birth rates, and substantial cost savings with repeated eSET in good-prognosis patients [52–57]. The estimated savings depend on the characteristics of patients, the rates of live birth and multiple births, cost of the procedures, and the method of calculation (inclusion or exclusion of certain costs and the assessed time period). However, the studies consistently showed cost-effectiveness of repeated single-embryo transfer due to the very high cost of multiple births.

Based on the economic and health benefits of eSET, several countries implemented IVF coverage policy in exchange for strict restrictions on the number of embryos allowed for transfer. This resulted in a dramatic increase of single-embryo transfer and a similarly dramatic decrease of ART-related multiple births and related healthcare costs [58–61].

12.2.4 Appropriate Candidates for Elective Single-Embryo Transfer

Elective single-embryo transfer may not be beneficial for all ART patients. The decision regarding whether eSET is appropriate for a patient should take into account multiple factors, including the patient's age, the patient's prior experience with ART, the source of the oocytes (autologous or donor), the availability of supernumerary embryos for cryopreservation, the cycle type (fresh or frozen/thawed), the stage of embryonic development (cleavage or blastocyst), and the embryo quality, among other factors [12].

Patients who have a greater likelihood of achieving a healthy singleton live birth with one embryo rather than with two are suitable candidates for eSET. According to the recent study using 2011 data from the US National ART Surveillance System [47], among fresh autologous ART cycles, the following groups of patients had a higher chance of delivering a term normal birth weight singleton after transferring one embryo than transferring two embryos: (a) patients younger than 35 years of age with favorable prognosis (those undergoing their first ART cycle and having extra embryo(s) cryopreserved) who had either blastocyst- or cleavage-stage embryos, (b) patients 35–37 years of age with favorable prognosis who have blastocyst-stage embryos, and (c) patients younger than 35 years of age with average prognosis [those undergoing their first ART cycle and having no extra embryo(s) cryopreserved, those who had previous ART cycle(s) and/or no previous live birth(s) and have extra embryo(s) cryopreserved, and those who had previous ART cycle(s) and previous live birth(s)] who have blastocyst-stage embryos.

Since the likelihood of implantation, pregnancy, and live birth is largely determined by the age of the woman producing the oocyte, recipients of donor oocytes have a good chance of successful ART cycle regardless of their own age [62]. Patients undergoing ART cycles with donor oocytes were shown to have a higher chance of a healthy singleton live birth when a single embryo was transferred [63].

Significant advances in cryopreservation and the absence of the potentially negative effect of controlled ovarian hyperstimulation make it possible to achieve comparable or higher success rates with frozen/thawed embryo transfer cycles than with fresh embryo transfer cycles. Frozen/thawed embryo transfer cycles were shown to have higher clinical and ongoing pregnancy rates

[64] and no adverse effect on perinatal outcomes, including preterm birth, low birth weight, and small for gestational age compared with fresh embryo transfers [65]. In one retrospective analysis of frozen/thawed cycles, the authors found no difference in live birth rates but significantly lower multiple-birth risk following eSET compared to double-embryo transfer and concluded that eSET can be a viable option in frozen/thawed cycles [66]. Although there are few studies on the effectiveness of eSET in frozen/thawed ART cycles, the existing evidence suggests that the same criteria that are used to define the best candidates for fresh embryo transfer cycles can be used to define the best candidates for frozen/thawed cycles [10].

12.3 Overcoming the Barriers Toward the Widespread Use of Elective Single-Embryo Transfer

Despite a growing body of evidence on the efficacy and safety of eSET, there are many barriers to the widespread adoption of eSET, including financial pressure that may compel patients to transfer more embryos in order to maximize ART success per cycle, as well as the desire of some patients to have twins instead of one singleton infant at a time.

12.3.1 Removing Financial Pressure from Patients Undergoing ART

ART treatments are expensive, and, in the absence of public funding or insurance coverage, rates of utilization are inversely associated with costs [67–69]. For example, in the United States, it has been estimated that one fresh IVF cycle accounts for over half of an individual's disposable income for those living in states without an infertility insurance mandate [70]. In contrast, for those living in a state with a mandate requiring full or partial coverage of IVF services or for those living in a country with public funding for IVF cycles, a fresh cycle represents 13 % or less of an individual's disposable income. Therefore, for patients who are paying out of pocket for some or all of the costs associated with IVF, there is a considerable financial pressure to achieve success in a single cycle. Because pregnancy and live birth rates are lower for a single eSET cycle compared with a double-embryo transfer cycle, patients may prefer to transfer two embryos as a means of increasing the likelihood of a live birth for that particular cycle. Indeed, countries with supportive public funding or insurance coverage for IVF have higher rates of eSET than those without such funding, even without corresponding limitations on the number of embryos transferred [70, 71]. Furthermore, it has been shown that expanding the amount of insurance coverage increases a patient's desire for eSET [72].

In light of persistently high twin rates and corresponding risks for maternal and infant morbidity and mortality, a number of developed countries have sought to increase eSET rates via reimbursement strategies coupled with limits on the number of embryos to transfer [58–61, 71]. The success of these countries in curbing ART-related multiple births provides evidence in support of such policies, which are advocated by many experts in the field [12, 73–76].

12.3.2 Educating Patients About the Benefits of Elective Single-Embryo Transfer and Risks of Twin Births

Another barrier to the implementation of eSET is the lack of patient awareness about the health risks associated with twin births which to some patients are preferred over the singleton option [77]. While the risks of triplet and higher-order births are generally understood, many patients are not aware of the potentially adverse outcomes associated with twin births [78] or elect to ignore these risks [79, 80]. Findings from one study indicate that women would rather give birth to twin infants affected by physical, cognitive, or visual impairments than remain childless [81]. Furthermore,

patient preferences for twins or singletons have been found to change over the course of the ART cycle and may be adjusted based on the information they receive about the chances of pregnancy or, if they become pregnant, based on the expected outcome of their pregnancy [82].

Patient perception of the acceptability of eSET is also highly variable although it has been shown that many patients would choose eSET if pregnancy and live birth rates were consistent with those for double-embryo transfer [83, 84]. Among patients that express a preference for eSET, the primary motivation is often a desire to avoid a twin pregnancy and the potential risks for the mother and infant [77, 85]. Notably, economic considerations were not found to be important predictors of patient preference for twins in some studies, thereby suggesting that, for some patients, certain factors play a larger role in decision making than treatment costs [86–88].

Clearly, the need for patient education on the risks of multiple births and the benefits of eSET cannot be overstated. Studies show that patient education can be successful in promoting utilization of eSET [86, 89–91].

12.4 Conclusions

Elective single-embryo transfer allows favorable-prognosis infertility patients to achieve the goal of a healthy singleton infant in the most safe and cost-effective way. Reliance on eSET versus double-embryo transfer in good-prognosis patients can substantially reduce the serious adverse consequences of multiple births and improve the likelihood of an optimal outcome. In addition, eSET can substantially reduce healthcare costs. Combining expanded insurance coverage for ART along with limits placed on the number of embryos to be transferred has been shown to constitute a feasible and effective strategy to improve ART outcomes. Since the public health goal is for all children to be healthy, it is important to educate infertility patients about the potential benefits of transferring one embryo at a time.

Disclaimer The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Elective Single-Embryo Transfer Should Not Be the Standard of Care For All Patients

13

G. David Adamson

13.1 Introduction

Since the birth of Louise Brown in 1978, assisted reproductive technology (ART) scientists, embryologists, and clinicians have made incredible progress improving pregnancy rates. As a result, ART is now established as a mainstream medical specialty that has contributed over five million babies to the world and been recognized by a Nobel prize to its physiologist pioneer, Sir Robert Edwards [1, 2]. However, ART's successes have not been accomplished without controversies and problems. The use of donor gametes, gestational surrogacy, cryopreservation, intracytoplasmic sperm injection, preimplantation genetic diagnosis and screening (PGD and PGS), and gender selection have fundamentally challenged many traditional, historical perspectives, values, morals, and ethics regarding families, parenting, children, reproductive rights, the role of science, and religion.

However, perhaps no controversy has been greater than the multiple pregnancy rate associated with ART treatment. Scientific and laboratory progress brought dramatic increases in implantation rates in the 1990s, resulting in twin rates approaching 50 % and triplet rates 10 % in some populations [3]. Numerous studies documented the increased neonatal prematurity and other problems resulting in significant maternal and perinatal morbidity and mortality [3]. Professional and media attention on ART shifted from extolling its accomplishments to criticism of its complications. Some jurisdictions, notably those in northern Europe and Australia, responded by replacing fewer embryos at embryo transfer. This had the predictable effect of reducing multiple pregnancy rates but also created concern over potential reduction in live birthrates [4].

In the United States (USA), the emphasis on obtaining the highest possible pregnancy rate almost always took precedence over the risk of twins, which were an outcome desired by many patients.

However, there are many factors influencing multiple birthrates: egg source, especially age [5, 6]; embryo quality (probably most important) [7]; number of embryos transferred [5, 6]; patient demand, 69 % want twins [8]; insurance/government financial coverage [3, 9]; country demographics, culture, economy, and regulations [9]; practice and selection biases; marketplace pressures; different perspectives of multiple risk [10]; infertility specialists' lack of involvement in follow-up care [3]; patients' and physicians' underestimation of negative consequences of twin pregnancies [8, 11]; focus on live birthrate per cycle rather than cumulative live birthrate [12]; patient race and comorbidities (age, obesity, hypertension, diabetes) [13, 14]; patient dropout

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_13

rates [15, 16]; and lifestyle factors (alcohol, smoking, caffeine, exercise) [17].

Consequently, while the number of embryos transferred has gradually been reduced over the past 20 years, resulting in a falling triplet rate, the twin pregnancy rate and the accompanying sequelae have remained fairly constant or even increased in ART [3]. Only in some jurisdictions in which ART services are widely covered through third-party funding has eSET been widely adopted, for example, in northern Europe and Australia/New Zealand, or when private payors have required eSET in order to receive payment [18].

Multiple scientific studies have confirmed that the quickest way to reduce the twin rate is to perform elective single-embryo transfer (eSET), but this strategy also results in significantly lower pregnancy rates which are not acceptable to many patients or IVF programs [18–21]. While additional pregnancies occur from frozen embryo transfer (FET) of the next best untransferred embryo in eSET patients, historically the cumulative pregnancy rate only approximates, and is likely less, than that from DET, except possibly in the best prognosis patients [22].

Recent improvements in ART, including higher pregnancy rates with blastocyst transfer, egg and embryo vitrification, PGS, and other methods to assess embryo implantation potential such as time-lapse photography, have all increased the rationale for performing eSET. Some European countries, Australia/New Zealand, and Japan have successfully implemented widespread eSET, resulting in twin pregnancy rates of approximately 5 %. Even though the USA twin rate is over 30 %, resulting in approximately 45 % of all American ART babies being twins, implementation of eSET in the USA and some European and many other countries remains far below the level needed to reduce significantly the twin pregnancy rate. This ongoing problem has caused some to argue that the benefits of eSET are so great that eSET should be standard for all patients. While being a strong proponent for many years of the need to increase eSET utilization, this chapter will make the case that, while eSET is an excellent clinical strategy that should be considered for all patients and should definitely be performed on many more patients, especially in the USA, it is not the best treatment for all patients undergoing ART [23, 24].

13.2 Are Twin Pregnancies Desirable?

The first issue to address is that some have argued that "twin pregnancy, contrary to consensus, is a desirable outcome in infertility" and should be encouraged [25, 26]. Cogent arguments are made that patients suffering from long-term infertility often want two children, so having twins is cost effective; most risk assessments after fertility treatment use spontaneous conceptions rather than those in the infertility population; IVF twins have 40 % lower outcome risks; the correct outcome denominator to assess is born children, not pregnancy, and; two children born with twins effectively halves the risk for babies and mothers. While not agreeing that twins should be encouraged, but believing patients should have as many babies as they want, one at a time, eSET should be considered for every patient every time, but is not the best treatment for every patient every time. Why is this so?

13.3 What Do Women Want?

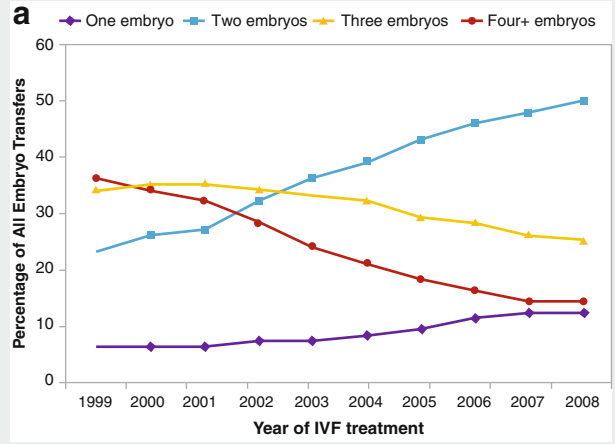
First, patients have different perspectives and preferences. What do women want? Many studies in the USA and elsewhere show that the majority of infertile women want more than one child [8, 27, 28]. In Denmark, 58.7 % preferred twins so their children would have siblings and less IVF was needed [29]. In Holland, 61 % opted for double-embryo transfer (DET) over eSET on the first cycle [30]. In the UK, only neonatal death was considered a worse outcome than treatment failure [31]. Even in Sweden, the quality of life for parents who had undergone DET and SET was similar and not different than that in the general population [32].

US live birthrates are higher than in countries with eSET as the standard; for the USA in 2001 and 2002, live birthrate, the number of embryos transferred, and multiple pregnancy rate are higher than in Europe ($P<0.001$) [33, 34]. The higher pregnancy rates are not explained by a larger number of embryos transferred alone. The live birthrate in the USA in 2006 at 35.4 % was higher than Sweden at 27.2 %, although the singleton rate was the same at 24.6 % and 25.6 %, respectively, and

the twin rate was much higher in the USA at 30.6 % compared with 5.8 % [3].

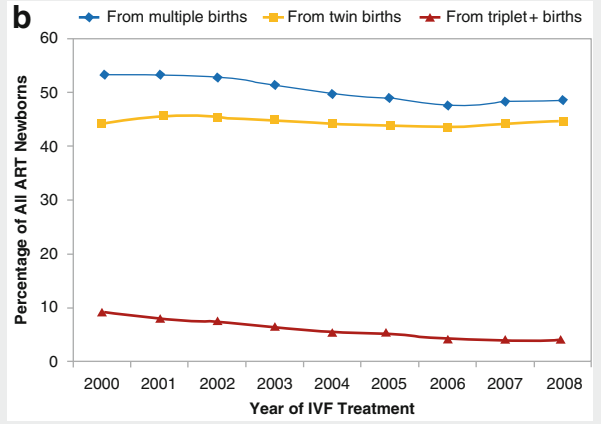
13.4 Guidelines Are Working to Reduce the High-Order Multiple Pregnancy Rate Without Requiring eSET as the Standard

In the USA, the Society for Assisted Reproductive Technology (SART) guidelines have consistently recommended fewer embryos be transferred. This has resulted in a continuous reduction in the number of embryos transferred and associated reduction in high-order multiple pregnancy (Fig. 13.1a, b) [18]. Furthermore, guidelines are constantly being revised based on new information gained from basic scientific research, clinical research, and national and global registries so that guidelines reflect treatment based on the best possible evidence. Recent technology changes that have influenced practice guidelines include blastocyst culture and vitrification improvements. Current technologies being assessed that might change guidelines toward more eSET include PGS and time-lapse photography. The most recent recommendation from SART is that "eSET should be considered seriously for good prognosis patients, assuming the availability of



Proportions of transfers of one, two, three, or four or more embryos among all IVF cycles performed in the United States, 1999–2008.

Practice Committee. eSET. Fertil Steril 2012.



Proportions of all liveborn children resulting from assisted reproductive technologies in the United States that were members of multiple births.

Practice Committee. eSET. Fertil Steril 2012.

Fig. 13.1 (**a**) Trends in the USA of the number of embryos transferred. (**b**) Proportion of US live born children from ART as member of multiple birth. (Reprinted from Fertility and Sterility, Vol. 97, Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine, Elective single-embryo transfer, pp. 835–42, Copyright 2012, with permission from Elsevier)

effective cryopreservation protocols that will help to maximize cumulative pregnancy rates" [3]. More specifically, SART stated: elective SET should be offered to patients with a good prognosis and to recipients of embryos from donated eggs; IVF centers should promote eSET when appropriate through provider and patient education, and improvements in embryo selection should further increase the application of eSET [18].

13.5 Every Patient Is Different

Patients need individualized care and so eSET is not appropriate clinically for all patients. Many biological, psychological, health, socioeconomic, and personal financial factors affect patient care. Most importantly in ART, patient age has a profound impact on egg quality [5, 7, 9, 13–17, 35]. Excellent US data confirmed by many demonstrate that there is no single correct number of embryos to transfer in all patients at all ages [36] (Fig. 13.2). Setting a standard that mandated eSET for all patients would clearly result in much lower pregnancy rates and fewer infertile patients realizing their dream of having a child.

13.6 Multiple Birth Is Only One of the Many Pregnancy Risks

While multiple pregnancy has approximately doubled the risk of singleton pregnancy for women, it is only one of the many risks faced. Indeed, all infertile women have an increased risk in pregnancy regardless of how they got pregnant or whether or not they have a multiple pregnancy [3]. Other health factors that can significantly increase risk include advanced maternal age, obesity, smoking, diabetes mellitus, hypertension, and other medical disorders. These conditions are routinely and generally without much question managed in non-infertile, pregnant women. Are we discriminating against infertile women when rules, regulations, and standards are used to reduce their chances of reproduction

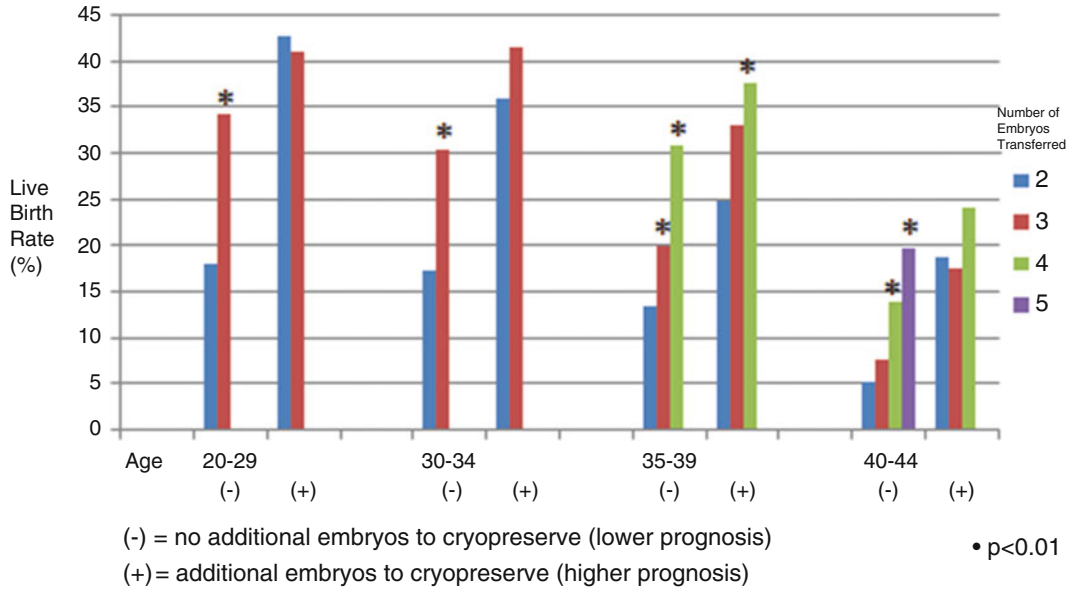


Fig. 13.2 Live birthrate by number of embryos transferred, age, and presence of embryos to cryopreserve (data from [36])

but there are no such rules for other non-infertile pregnant women?

13.7 Other Technologies Can Help Determine the Optimal Number of Embryos to Transfer

While eSET is clearly the most effective way, it is not the only approach to solving the problem of multiple births. Currently, assessment of embryo quality through PGS with comprehensive chromosome screening is helping to identify embryos with the likelihood of a higher implantation rate, but also those with lower rates [37–41]. In clinical situations in which no top quality or good prognosis embryo is identified, it would be appropriate to replace additional embryos. Additional new technologies such as time-lapse photography that improve our ability to identify embryos with high, medium, or low probability of implantation and pregnancy without having to grow to blastocyst or perform an invasive procedure are also now available [42–45]. Other technologies such as metabolomics and proteomics may also bring advantages in the future. All of these technologies require further high-quality, clinically based studies to determine their optimal utilization in the general infertility population undergoing ART [46]. It is almost certain that the treatment models developed subsequent to embryo assessment will not optimize outcomes or care if they require eSET for every patient.

Therefore, the use of technology to assess each embryo's ability to result in a live birth will not promote eSET as the standard, but will help identify the optimal number of embryos to transfer. This will, almost certainly, result in more eSET and a fewer average number of embryos transferred, but not eSET for all patients. Individual physicians can and should transfer fewer embryos, and professional associations can continue to modify voluntary guidelines as new technology and clinical understanding is developed and validated, but it is important to avoid mandatory standards set by insurance companies or regulatory bodies that cannot possibly optimize the care each individual patient has a right to expect.

13.8 The Role of Multifetal Reduction

A very difficult and sensitive topic is that of induced multifetal reduction, also known as elective fetal reduction or pregnancy reduction. This procedure is performed around the end of the first trimester and can be used to reduce the number of fetal sacs in a patient with ongoing multiple pregnancy [47]. It is controversial because it can be considered a "partial abortion," and while there is much support for this aspect of reproductive choice, there are also many who are opposed. The procedure is usually done only for high-order multiple pregnancy (three or more sacs) but can be used to reduce to a singleton pregnancy. While the procedure markedly reduces the multiple pregnancy risk, it does not reduce the risk back to what it would have been if the multiple pregnancy had not occurred in the first place. Also, there is an approximately 5 % chance of losing the entire pregnancy as a result of the procedure itself. Furthermore, even though patients who undergo this procedure are generally satisfied that they did, there are clearly major psychological issues associated with it. Therefore, for effectiveness, safety, psychological, moral, ethical, and religious reasons, this procedure should be performed very infrequently and as a last resort. It should not be used as "insurance" or an escape mechanism for inappropriately replacing too many embryos.

13.9 Blastocyst Transfer: Progress and Problems

Improvements in blastocyst culture have led to a dramatic increase in day 5 versus the historic day 3 transfers. The driver for this is the higher implantation and live birthrate of day 5 blastocyst transfer, odds ratio (OR) 1.35 (95 % CI 1.05–1.74 [48]). This allows for fewer embryos to be replaced, including eSET, while maintaining live

birthrates. However, blastocyst transfer still has some challenges. Only about half of day 3 embryos grow to day 5, and, while it is generally felt and data tend to support that mostly only poor-quality embryos don't grow to day 5, it is likely that there is a small but real loss of reproductive potential when some embryos that might have implanted with a day 3 transfer do not grow to a blastocyst. There is also a higher chance of having no blastocysts to transfer, OR 0.35 (95 % CI 0.24–0.51) [49]. Additionally, rates of cryopreservation on day 3 are understandably higher than on day 5, meaning there are more often cryopreserved embryos for transfer with cleavage stage cryopreservation, OR 2.88 (95 % CI 2.35–3.51) [49]. The cumulative pregnancy rate from fresh plus frozen transfers favors cycle day 3 transfer, OR 1.58 (95 % CI 1.11–2.25) [49]. There are also still nagging issues over the possibility of epigenetic imprinting disorders occurring more often in blastocysts than in day 3 embryos and of the overall health of blastocyst babies, with some data suggesting an increased rate of adverse neonatal outcomes compared with naturally conceived babies: cycle day 3 transfer, OR 1.11 (95 % CI, 1.02–1.21), and cycle day 5 transfer, OR 1.53 (95 % CI 1.23–1.90) [3, 18, 50, 51]. Overall, the ASRM Practice Committee has concluded that there is emerging evidence that in selected patients, blastocyst culture may be applicable for SET. Despite its increasing popularity, it is reasonable to conclude from careful assessment of the current evidence that the optimal role for blastocyst transfer is not yet clearly defined.

13.10 eSET Dropout Rates Are Higher

Patient dropout rates have been reported to be 37–68 % in ART treatment. Patients drop out of treatment because of cost, because of physician recommendation often related to prognosis, and also for physical reasons and psychological reasons related to the stress of ART treatment [52]. Dropout rates are a major confounding variable on the cumulative live birthrate with eSET because all of these causes of dropout are potentially deleterious to cumulative live birthrates. Concerns have been expressed that if pregnancy rates are reduced by the replacement of fewer embryos in order to reduce multiple rates, more ART cycles, both fresh and frozen, are required. So the need for additional cycles almost certainly increases dropout rates, resulting in fewer babies born and families created [16, 52, 53].

13.11 Not All Twins Are Unhealthy and Not All Singletons Are Healthy

The risk of twin pregnancy is higher than singleton pregnancy. But the reality is that the vast majority of twins are healthy (Table 13.1) [3]. Therefore, any assessment of the benefits and risks of twins versus singletons must make the comparison based on the difference between the two in terms of the number of healthy babies and the number of babies with morbidity and mortality. Such comparisons have not been commonly done, but unless one can make the argument that eSET should be done for 100 % of patients on every

Table 13.1 Major perinatal morbidity and mortality outcomes in multiple pregnancies

	Singleton	Twin	Triplet
Prospective risk of fetal death (%)[a]	0.03	0.09	0.14
Gestational diabetes (%)	0.06	0.31	1.38
Neonates <2,500 g (%)	6.2	53.2	93.2
Neonates <1,500 g (%)	1.2	10.5	37.5
Average gestational age (weeks)	39.1	35.3	32.2
Average birth weight (g)	3,358	2,347	1,687

Source: Adapted from Fertility and Sterility, Vol. 97, Practice Committee of the American Society for Reproductive Medicine, Multiple gestation associated with infertility therapy: an American Society for Reproductive Medicine Practice Committee opinion, pp. 825–34, Copyright 2012, with permission from Elsevier

[a]Prospective risk of fetal death between 24 and 43 weeks' gestation in a singleton pregnancy, at 41 weeks in a twin pregnancy, and at 38 weeks in a triplet pregnancy; prospective risk calculated as a proportion of all fetuses still present at a given gestational age because gestational age varies by the number of fetuses

cycle, such comparisons become necessary in order to make good clinical decisions.

13.12 ART Babies Are Not an Economic Burden to Society and Twin ART Babies Are Not an Economic Burden to Society

The economic burden of twin pregnancy and twins is real. Both maternal and neonatal complications cost money, and usually those in society other than the patient pay through either public or private funding. So it is legitimate to ascertain the additional financial cost of twins. However, such financial cost must be compared with the costs of singletons, and the difference balanced against the benefits of twins versus singletons. In essence, not only are the costs double for twins, but so are the benefits.

The cost to obtain each child through ART does not differ for eSET vs. DET in selected populations [54, 55]. However, eSET has been shown to only be cost effective for young women who also have extra embryos to cryopreserve [56, 57]. When direct and indirect costs (e.g., lost productivity) are considered, eSET costs more than DET for each baby born [56]. Therefore, overall, eSET costs more than DET to create each baby [18]. Of course, twins increase indirect and long-term costs to approximately 2–3 times per baby because of neonatal intensive care unit and longer hospitalization costs, special education, and other costs for disability pay, day care, and incremental health costs. However, even when these costs are calculated to age 20, the incremental cost per live birth for DET compared with eSET is approximately $45,000 for a 32-year-old, $30,000 for a 36-year-old, and $25,000 for a 39-year-old [58]. There are also psychological and family consequences that create costs that are difficult to quantify but certainly occur.

Using Thurin's data, the difference between eSET and DET on the fresh transfer is that DET would result in 15 more live births, and therefore families, in the infertile couples and 61 more babies born, both of which could be considered benefits. However, these gains must, of course, be balanced against the costs which are a multiple birthrate that is 25.2 % higher with its known associated burdens, including two more neonatal deaths, 13.7 % more preterm births, and 3.7 % more very preterm births [22].

Despite the increased costs of twins, a balanced assessment requires calculation of the societal benefit of twins. While seemingly impossible and somewhat arbitrary, the reality is that all societies place financial value on human life through regulations, legal cases, insurance, and other means. While a detailed analysis of the value of a person is beyond the scope of this article, it can be stated that the economic value of a human life is between $400,000 and $10 million with an average range of approximately $2–3 million up to $6 million [59–65]. Furthermore, the cost of ART treatment is insignificant compared to the lifetime tax contribution of ART children. A recent study calculated the lifetime net taxes paid from a child relative to the child's initial ART investment which represented a 700 % net return to the government in discounted US dollars from fully employed individuals [66]. In the UK, the discounted net tax revenue was $208,400 which was eight times the return on investment for those otherwise not conceiving [67]. It is clear that by even the most conservative measurement, the economic value of each ART baby greatly exceeds $1 million and is at least one or two orders of magnitude the cost of obtaining that child through ART. Furthermore, even the increased costs of twins with or without morbidity or mortality, including immediate, short term, and lifelong, are dwarfed by the economic benefit of the individual to society. And, of course, with twins, that is two individuals rather than one [60, 68–76]. It is beyond the scope of this chapter to detail the case, but many Western societies today have low birthrates and can benefit from additional citizens. Furthermore, even in societies with overpopulation, social justice does not require the infertile to have no children, while others have too many. Finally, the noneconomic benefits to families of children born to ART are substantial, and this is generally still true even if the children have health issues [29–33, 52, 53, 77–79].

13.13 No Countries Use eSET Exclusively

Sweden, Australia/New Zealand, and Japan have led the way internationally in the promotion and implementation of eSET. Despite their widespread support of eSET and three very different socioeconomic environments, the overall rate of eSET is approximately 75 % in these three countries [80, 81]. Furthermore, live birthrates are lower in these countries than in the USA. Therefore, even in countries with almost a complete commitment to eSET, there is clearly selection taking place based on multiple factors that affect clinical decisions. So it is not possible even in these countries to establish eSET as the standard for all patients, and clinical decisions must be made regarding which patients will or will not have eSET.

13.14 SET as a Universal Standard Will Encourage Cross-Border Reproductive Care

Cross-border reproductive care is an increasing phenomenon in the world. Factors driving this are the unavailability of the desired service as a result of regulation or mandatory standards that prevent or affect the provision of the service, for example, illegality of gamete donation, absence of payment for gamete donors, and non-anonymity in third-party reproduction; unaffordability of the service through lack of insurance/public coverage and/or high cost; or perceived higher pregnancy rates in other jurisdictions [82, 83]. Patients required to have eSET even with a lower prognosis or after failed cycles will have increased motivation to consider cross-border care. Cross-border care is generally considered to be less than optimal because it forces the patient to have care away from her support network in unfamiliar circumstances, may involve difficulties because of language and other cultural problems, limits follow-up in case of posttreatment problems, complicates monitoring of quality of care and outcomes, and may result in complications of care being managed by those with limited knowledge of the clinical situation [82–84].

13.15 An eSET Standard Will Not Solve the Twin Problem

While multiple births from ART contribute to the number of twins born, it is far from the most significant way that twins occur. Most twins occur spontaneously, but after that controlled ovarian stimulation with or without IUI contributes the next largest number of twins, followed by ART (Fig. 13.3) [85]. Of course, this does not mean that the twin rate should not be reduced, because it needs to be, and part of the solution is eSET. But eSET will only help with a minor part of the overall multiple birth problem.

13.16 Why PGS and Single Blastocyst Transfer Might Become the Standard, but Is Not Yet

Studies have shown that embryos with certain types of chromosomal abnormalities are negatively selected during preimplantation embryo development but that still a remarkable percentage of chromosomally abnormal embryos can develop normally to blastocyst stage with high probability of implantation and pregnancy [86]. Furthermore, it appears that selective transfer of euploid embryos results in implantation rates that are not significantly different between reproductively younger and older patients up to age 42 years [87]. Accumulating evidence suggests that comprehensive chromosome screening (CCS) is accurate, is safe, and has good predictive value and clinical efficacy [88]. As a result, a new paradigm involving blastocyst biopsy, CCS, cryopreservation, and delayed frozen/thaw blastocyst transfer has been proposed for ART cycle management that would potentially help increase healthy pregnancy rates [89]. Investigators claim additional advantages of higher implantation and pregnancy rates and lower miscarriage rates [90]. Proponents of this approach not only make the case but address concerns raised by critics of this new approach [41, 91]. Others have noted the relatively poor correlation of blastocyst morphology, euploidy, and implantation which complicates blastocyst selection for transfer [92].

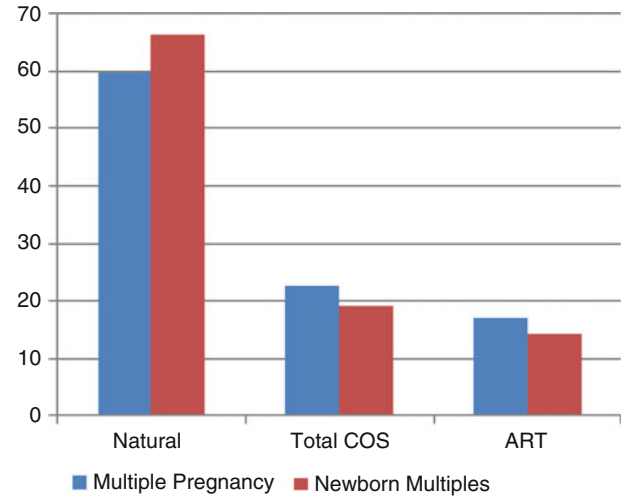


Fig. 13.3 Natural vs. treatment sources of multiple pregnancies and neonates (data from [85])

Despite great interest in this new approach and its increasing utilization, it is not clear that it should yet be widely adopted. Current studies have largely been performed in favorable populations and have often involved the transfer of more than one screened blastocyst with accompanying high pregnancy rates but also exceedingly high multiple rates. Most importantly, there are not yet any studies beginning with an unselected population and randomized blindly from intention to treat to outcome including transfer of all embryos obtained from a single oocyte retrieval cycle. Declining pregnancy rates with age occur not just because of aneuploidy but also because women with poor ovarian reserve and/or small embryo numbers may have increased their failure rates by further culture and biopsy [93, 94]. Additionally, there are concerns about loss of reproductive potential from the need for intracytoplasmic sperm injection (ICSI) in CCS patients to minimize the risk of DNA contamination from sperm at the time of fertilization, loss of embryos in culture from day 3 to day 5, imprinting injury, damage from trophectoderm biopsy required for CCS, damage from cryopreservation of blastocysts, the frequency of having no normal blastocysts to transfer, inaccurate test results because of mosaicism or test platform error, and potentially more prematurity and abnormal babies born subsequent to blastocyst transfer [95–100]. These patient selection issues, cost-effectiveness, informed consent concerns, ethical and legal questions, and the potential role of other, noninvasive technologies such as time-lapse photography/analysis and other biomarkers cloud the optimal role of these new technologies. Therefore, it is not yet time to establish them as a standard because further research is needed, but they are very promising and almost certainly will have a future role in patient management [83, 100, 101].

13.17 Conclusions

All progress begins with the truth. The truth is the burden of infertility is very high on those affected and significantly impacts the quality of life. Social justice calls for treatment, but infertility is generally globally not given the attention and support that would be justified by its impact on individuals and society [102]. Therefore, patients struggle financially and otherwise to obtain treatment that will result in a baby. Healthcare providers need to do everything possible to overcome the challenges to a successful live birth, and so treatment needs to result in a baby as often as possible.

At the same time, the truth is that twins have higher complication rates and costs for babies, mothers, families, and societies. The ART

community needs to take action to reduce the twin rate. The single most effective way to reduce the twin rate is to perform eSET.

eSET with additional FET can result in pregnancy rates that are almost equivalent to DET and with dramatically lower twin and triplet rates. Therefore, eSET must be central to the strategy to reduce twins. However, eSET is not appropriate for all patients for the many reasons outlined in this chapter. Therefore, our challenge is to identify which patients will have more benefit than cost when treated by eSET. While we have many new technologies to assist us with eSET, we do not yet have sufficient good evidence to know exactly when to apply eSET and other ART technologies to which patients.

Currently, it seems reasonable to recommend eSET to good prognosis patients less than age 38 with at least one top quality embryo, however determined, and at least one additional good embryo for cryopreservation. eSET would seem appropriate for at least the first two cycles, be they fresh or following vitrification. For young patients, those with a prior ART baby and for egg donors, eSET is likely appropriate for up to three cycles. The above would be especially applicable if blastocysts, and in particular blastocysts screened genetically or with time-lapse photography, are being transferred. Recommendations such as those above must be modified for individual program results, embryo quality, and specific patient situations.

To achieve widespread eSET implementation, we must understand and respect each patient's individual values, challenges, and dilemmas. The majority of patients will tell us they desire twins. This is understandable but this is before they have to manage the reality of twins, especially if there is a bad outcome. Both patients and physicians underestimate the risks and family burdens of twins, healthy or not. Some of the burdens last a lifetime. Therefore, informed choice is essential for patients. This takes time by clinic staff but most importantly by physicians. Through education, more patients will choose eSET [103]. Patient autonomy in reproductive choice is a human right, but patient responsibility to themselves, their potential babies, their families, and their society must also be taken into account.

Additionally, better insurance and/or public coverage will make ART more accessible and increase eSET [104–106]. All stakeholders in ART need to advocate for better financial coverage for ART and eSET. Where coverage is not available, clinics should try to address the financial challenges patients face, provide solutions for them as possible, and ensure that their patient pricing is reasonable and fair.

If the above steps are taken, eSET will become much more common, such that the majority of patients will likely undergo eSET. Others will have the transfer of two embryos with a small increase in twin risk if they are appropriately selected. Few patients, almost all much older and with poorer prognosis for multiple reasons, will have the transfer of three embryos. Transfer of four or more embryos will become historical. By constantly improving our scientific and clinical understanding, informing our patients with the best evidence available, respecting their autonomy, and working for social justice, we will continually get closer to achieving our goal of having a healthy singleton baby for as many of our infertility patients as possible.

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Klinefelter Syndrome: Early Treatment of the Adolescent Is Warranted

14

Darius A. Paduch and Christopher T. Ryan

14.1 Introduction

Klinefelter syndrome (KS) is an important cause of primary hypogonadism and is the most common sex chromosomal abnormality, affecting approximately 1 in 660 males [1]. The prevalence of KS among men presenting with infertility is estimated at 3 % and up to 11 % in men with nonobstructive azoospermia [2, 3]. Many patients with KS remained undiagnosed, and it is estimated that only 25 % of men with KS will be diagnosed during their lifetime and fewer than 10 % will be diagnosed before puberty [1]. KS is caused by an increased gene dosage of X chromosomal material; 80–85 % of patients possess an additional X chromosome (47,XXY), and the remainder exhibit higher-order aneuploidies or 47,XXY/46,XY mosaic forms [4–6]. The pedagogical description of Klinefelter syndrome is a triad of hard, small testes with hypergonadotropic hypogonadism, learning disabilities, and a tall, eunuchoid stature [4, 5, 7]. Most patients exhibit normal timing of pubertal initiation and normal hormonal levels at the beginning of puberty, but the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) begin to increase and serum testosterone (T) to decline compared to normal boys due to pathological hyalinization of seminiferous tubules and loss of germ cells [7]. Patients can also have an increase in estradiol (E2) levels due both to increased adiposity and overexpression of aromatase CYP19 leading to an altered T/E2 ratio [4]. This pituitary-testicular axis dysfunction and hormonal imbalance result in poor pubertal progression leading to compromised virilization and delayed psychosexual development. Seminiferous tubule hyalinization leads to characteristic shrinking and hardening of the testes in KS during puberty as well as impaired spermatogenesis and infertility [7]. KS is also associated with a wide range of other complications including decreased muscle mass, decreased bone density, propensity to insulin resistance and abdominal adiposity, and expressive language and learning issues [4, 8]. Considering historically high prevalence of comorbidities associated with KS in adults, it is intriguing if early diagnosis and aggressive multidisciplinary and individualized management can improve health and overall quality of life in adolescents and men with KS.

14.2 Diagnosis and Screening

Early diagnosis is essential for instituting early treatment of KS and minimizing the physical and psychosocial implications of the disease. Currently, however, KS remains an underdiagnosed condition, and the majority of diagnoses

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_14

occur in adulthood. Based on prevalence estimates established by national registry studies, 10 % of expected KS diagnoses occur prenatally, 10 % in children and adolescents, and 20 % in adult populations, with the remaining 60 % going undiagnosed in their lifetime [9]. This is partially due to a wide variability in the clinical presentation of KS, particularly milder forms, as well as poor awareness of KS signs and symptoms among general practitioners [2]. In order to capture the benefits of early treatment of children and adolescents with Klinefelter syndrome, the pediatric diagnosis rate must be improved by increasing awareness among general practitioners of the various manifestations of KS in all stages of life.

14.3 Early Treatment Principles

Multidisciplinary management of Klinefelter syndrome patients including physical, speech, occupational therapy and hormonal therapy beginning at pubertal initiation is essential to ensure proper pubertal progression, normal physical development, psychosocial development, and academic progress [4, 7, 10]. An example of clinical treatment algorithm recently proposed by Mehta et al. for the initiation of testosterone replacement therapy (TRT) is presented in Fig. 14.1; however, the treatment plan has to be optimized to individual needs and goals [11].

Prior to the start of puberty, adolescents with KS should undergo a complete hormonal profile including LH, FSH, T, estradiol, prolactin, inhibin-B, insulin-like growth factor-1 (IGF-1), and cortisol levels, and a basic set of hormonal evolution and markers of response should be repeated every 6 months thereafter till reaching adulthood [4]. Based on our experience with over 200 children treated by us, we believe that testosterone supplementation should be initiated at the beginning of puberty (approximately 11 years of age) and the dosage titrated to maintain normal physiological serum testosterone, gonadotropin, and estradiol levels throughout puberty, unless adolescent advances through puberty at a normal pace. Signs and symptoms (like body hair, muscle strength and tone, acne, nocturnal emissions, penile growth, Hct) of peripheral action of T rather than absolute serum testosterone level should guide the therapy. The target for serum testosterone should be age-specific high-normal values, as men with KS seem to exhibit partial androgen resistance to T. Puberty is a progressive process requiring stepwise adjustment of T dose to mimic normal pubertal timing and avoid acceleration of delay in puberty. Topical testosterone formulations are preferred as they allow flexible dosing starting at lower doses and progressively increasing daily dose. T levels with topical forms of supplementation rarely lead to supraphysiological levels of T; thus, necessary

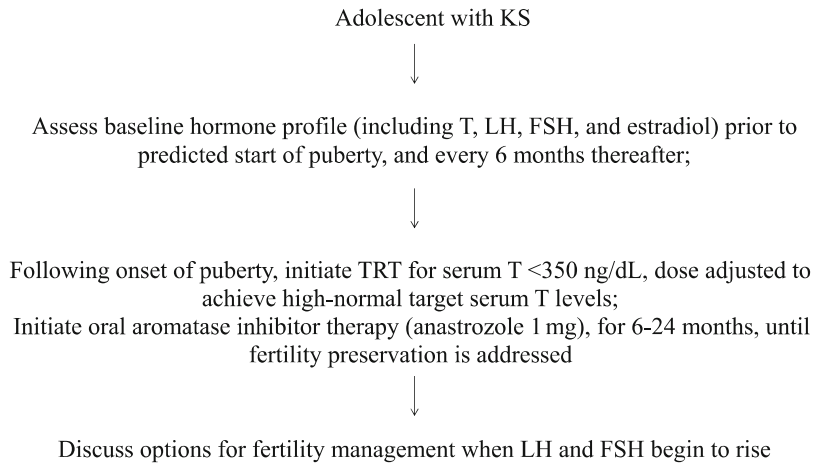

Fig. 14.1 Clinical treatment algorithm for TRT in adolescents with KS [11]

levels of serum T can be achieved without full suppression of LH and FSH, unlike with injectable forms of testosterone [4, 11, 12]. For those patients unable to achieve proper serum T levels with maximum dosing of exogenous testosterone and for those patients experiencing gynecomastia or central obesity issues, an aromatase inhibitor can be added to the therapeutic regimen [4].

Testosterone supplementation treats only the signs and symptoms of Klinefelter syndrome, and underlying infertility must be addressed separately in patients wishing to conceive. Psychosocial issues like emotional immaturity, delayed development of boundaries, executive skills deficits, and attention deficit hyperactivity have to be addressed as necessary working with experienced child and adolescent psychiatrists. In early puberty, sperm is found in the ejaculate in some patients, but less than 5 % of subjects have adequate sperm density for successful cryopreservation of ejaculate, but semen analysis and cryopreservation of ejaculate should be always considered as a first step in these individuals. In the remainder, testicular sperm extraction (TESE) or microscopic testicular sperm extraction (mTESE) can be used to harvest sperm for cryopreservation or immediate use in intracytoplasmic sperm injection in men with KS who have a female partner.

14.4 Benefits of Early Speech Therapy and Educational Planning

Availability of speech therapy is critical to the development of children with Klinefelter syndrome because the majority of patients (up to 70–80 %) suffer from a language disorder of some form, which manifests as delays in early language and speech milestones [8, 13]. Deficits in older boys manifest as difficulties with higher-order aspects of expressive language and verbal intelligence quotient [8]. This results in delays in academic progression and socialization. Patients with KS are more likely to have repeated at least one school grade than the general population [9]. Speech therapy can help children with KS with vocabulary accrual, verbal fluency, and complex language skills in order to minimize academic lag behind their peers. Estimated 33–52 % KS patients receive speech therapy at some point in their lives [5, 9]. Correspondingly, it is important that students with KS are provided with proper educational support to target their specific learning challenging and allow for modifications that allow for their individual learning styles [8]. These strategies can help to reverse the trends of lower educational attainment and negative socioeconomic trajectory observed in KS populations [14]. With early diagnosis and management, over 70 % of subjects in our cohort finish high school and enter college; however, this may be a selection bias of higher socioeconomic status of parents of children attending our clinic.

14.5 Benefits of Early Psychological Evaluation and Treatment

An increase in psychiatric morbidity has been reported and observed in adult KS populations due to an increased prevalence of depression, anxiety disorders, ADHD, autism spectrum disorders, and schizophrenia [10]. In our experience, 90 % of adolescents with KS had ADHD of varied severity, and most benefit from therapeutic interventions including stimulants. Out of 200 boys and adolescents seen over the decade, only 2 had psychotic disorder specifically auditory hallucinations, and two other adolescents had severe depression and anxiety requiring hospitalization. No adolescent or young adult was diagnosed with schizophrenia in our cohort. These problems are present beginning in childhood and adolescence in these patients and require evaluation and intervention. A retrospective chart review of 28 patients with KS aged 12–18 found that 63 % exhibited psychiatric and/or behavioral problems, most commonly aggression/impulse control issues and attention deficit disorder [9]. A prospective evaluation of 51 children and adolescents aged 6–19 years with KS diagnosis confirmed by karyotype analysis found that rates of psychiatric disorders in pediatric populations

as assessed by structured psychiatric interviews mirror the elevated rates found in adult populations [13]. Specifically, 63 % of patients met standard criteria for attention deficit hyperactivity disorder, 24 % for depressive disorder, 18 % for generalized anxiety disorder, 12 % for schizophrenic spectrum disorder or nonspecific psychotic disorder, and 27 % for autism spectrum disorder [13]. These rates are markedly elevated compared to the normal population and can lead to significant morbidity in KS patients. For all KS patients, regardless of whether they met standard criteria for psychiatric illness, the diagnosis of KS is difficult and can cause significant psychological distress because of the connotation of possessing "female" characteristics [6]. This has led some to contend that psychological and education support should be a standard part of the management of KS from the time of diagnosis [6]. Discourse with a psychiatrist or other mental health professional knowledgeable about the natural history of KS can help to establish a positive and proactive framework for the patient from which to view his disease, as well as providing appropriate pharmacotherapy when needed in cases of psychiatric morbidity.

There is a significant debate as to the impact of hormone therapy on the neurocognitive and behavioral manifestations of KS both in children and adults. Benefits such as increased energy and endurance as well as improved mood have been anecdotally observed in adolescents during testosterone treatment [6, 10, 15]. There is a lack of randomized controlled trials, however, and current studies exploring investigating the effect of testosterone therapy on neuropsychological phenotype have been cross-sectional and utilized nonstandard testosterone dosages and formulations [10]. Further research is needed to corroborate anecdotal reports of mood benefits of testosterone therapy before any conclusions can be reached. Testosterone therapy does, however, allow for the proper progression of puberty and *development of a masculine phenotype, which removes a psychosocial stressor from KS patients and helps facilitate socialization*.

14.6 Benefits of Early Hormonal Manipulation

Testosterone therapy beginning at pubertal onset allows for the proper progression of puberty and development of secondary sexual characteristics [3, 4]. The natural history of hypogonadism in KS results in sparse facial, body, and genital hair as well as eunuchoid body proportions and gynecomastia due to altered T/E2 ratio. Testosterone therapy is titrated throughout puberty to maintain patients' serum T within the physiological range and prevent these direct manifestations of hypogonadism. This results in an acceleration of pubic/axillary hair and penile Tanner staging leading to a more age-appropriate masculine appearance in patients [16]. Testicular size does not advance in Tanner staging due to the arrested growth in testicular size resulting from seminiferous hyalinization and testicular atrophy. It is important to remember that Tanner staging in KS is not prototypically synchronized when evaluating the effects of and titrating testosterone therapy. Additionally, serum T levels may not sufficiently define hypogonadism in KS patients as a certain degree of androgen resistance is common [17]. Androgen sensitivity and response to testosterone replacement are inversely associated with the length of the CAG repeat polymorphism located in the X-linked androgen receptor gene [18]. Due to androgen resistance, patients with "normal" serum testosterone levels may still exhibit signs and symptoms of hypogonadism and benefit from testosterone therapy. Because of this interpatient variability in androgen sensitivity, clinical signs and symptoms should primarily drive implementation and dosage titration of testosterone therapy with serum T measurements serving as a secondary indicator. Better understanding of inactivation patterns of additional X chromosome may lead to individualized treatment protocols and is an active area of research. Preliminary results of X ch methylation analysis performed in our laboratory showed significant differences in methylation patterns of genes

involved in androgen signaling like Filamin A, thus explaining spectrum of phenotypic response to androgens observed clinically (own data presented at ASRM, 2014).

Testosterone supplementation has also been shown to support proper muscle development and muscle mass attainment [7]. This is important because KS patients demonstrate various motor difficulties, most notably in running speed and agility and overall strength, which can prevent participation in sports, lower self-esteem, and contribute to stigmatization and social isolation [8]. Sports participation and the ability to exercise regularly are particularly important due to the increased rates of truncal obesity and unfavorable muscle/fat ratios observed in KS patient populations beginning in childhood and extending throughout their lifetimes [19, 20].

Gynecomastia is more common in KS patients than the general population and is reported at a prevalence of 38–75 %, depending on the population examined [5, 7, 9]. It is hypothesized that gynecomastia is the result of elevated estrogen levels and an abnormal E2/T ratio and/or an imbalance between estrogen receptors and androgen receptors [2]. Anecdotal evidence suggests improvement in some cases of gynecomastia with testosterone replacement therapy, but this benefit is unlikely in patients without pronounced hypoandrogenism [6, 16]. In patients with severe or psychologically distressing gynecomastia, antiestrogen therapy such as tamoxifen or aromatase inhibitors like anastrozole can be added to the treatment regimen [4, 6]. Prompt treatment of gynecomastia is important to the psychological development of KS patients as this feminine-appearing characteristic can have a detrimental impact on masculine self-image for the patient and acceptance from peers. In fact, gynecomastia is one of the leading signs leading undiagnosed children and adolescents to seek an initial consultation [21].

The impact of testosterone therapy on bone density is controversial, but gradual and consistent evidence points toward a positive, protective effect when given during puberty. KS patients have a high risk of developing osteoporosis and osteopenia leading to an increased rate of fractures [17]. This results in increased mortality from osteoporotic hip fractures in men with KS and reveals a need for preventive bone health interventions [6, 10, 22]. Research supports the idea of a "critical period" in bone development during puberty within which testosterone is very important for periosteal bone formation and attainment of peak bone mass by early adulthood [10, 17, 23]. Androgens are necessary for radial bone growth through direct interaction with osteoblasts and osteocytes as well as more complicated effects within the hormonal framework of osteogenesis. After puberty, testosterone is necessary for the maintenance of bone mass [23]. Observational data in hypogonadal non-KS men has shown improvements in cortical and trabecular bone mass during testosterone supplementation [17]. In KS populations, there has been some disagreement as to the effects of testosterone therapy on BMD. Wong et al. examined 14 patients with clinically diagnosed KS and described no beneficial effect of testosterone therapy on BMD in KS men with low testosterone levels and low BMD [24]. Van der Bergh et al. examined 52 karyotype-confirmed KS patients and found that a large percentage exhibited low BMD despite adequate long-term testosterone supplementation, suggesting that therapy was not sufficient to reverse osteoporosis or osteopenia [25]. Both of these studies, however, examined the effect of testosterone supplementation initiated in adulthood, and thus, the patients would not have been receiving during the "critical period" of bone mass attainment during puberty. No patients in the Wong et al. study received testosterone therapy before the age of 20 years. Fourteen patients in the van der Bergh study had started testosterone therapy before the age of 20, but the mean age of initiation was 17.1 years, likely too late for adequate BMD accrual [25]. Treatment of other causes of hypogonadism with testosterone therapy has shown that the beneficial effects on BMD, increases in both cortical and trabecular bone density, are fully realized only

when therapy is initiated at a younger age, before closure of epiphyseal plates [26]. Kubler et al. showed that the BMD of KS patients receiving testosterone therapy instituted prior to the age of 20 does not differ from a reference population [27]. In contrast, those subjects receiving testosterone therapy after the age of 20 had a lower average BMD equal to 81.1 % that of the reference population, statistically significantly lower than both the reference population and the patients receiving early testosterone [27]. Given the known molecular impact of testosterone on bone growth and the successful use of androgen therapy in other forms of hypogonadism to support BMD gains, it is reasonable to conclude that early testosterone therapy will have a beneficial effect by allowing KS patients to attain a greater peak bone mass. Because of the importance of the pubertal period in establishing lifelong bone density, it seems this is an area of KS management in which early testosterone treatment is particularly warranted and important, though randomized clinical trials are necessary to confirm the currently observed positive trends toward meaningful therapeutic effect.

Many patients with KS exhibit an altered body composition with increased total body and truncal fat and reduced lean muscle mass [6, 10]. They also experience a greater degree of insulin resistance, and it is estimated that almost half of adults with KS meet standard criteria for the metabolic syndrome, compared to 10 % of the general population [6]. To date, no randomized controlled trials have explored the impact of hormonal therapy on this altered metabolism, and benefit has not been firmly established. In the general population, a negative correlation has been demonstrated between serum testosterone levels and abdominal adiposity, and prospective studies show that low levels of testosterone serve as a predictive factor for the development of metabolic syndrome and type II diabetes [10]. In studies of patients with hypogonadism of causes other than KS, treatment with testosterone has been shown to increase lean body mass and decrease abdominal adiposity [28]. This effect is seen primarily in obese patients and not replicated in lean populations [10]. An experimentally induced model of hypogonadism in healthy young men demonstrated a dose-dependent increase in lean body mass with testosterone supplementation and a negative correlation of fat mass with testosterone dosage [28]. Though these studies have yet to be repeated in KS populations, extrapolation of the results is reasonable, and some have advocated testosterone therapy for adolescents with KS with low serum testosterone or increased LH and concurrent changes in body composition and weight gain [28]. However, KS patients exhibit increased rates of obesity and other signs of altered body composition prior to the onset of puberty and the development of hypogonadism, indicating that independent genetic effects may play a significant role in their altered metabolic state [10]. Additionally, testosterone treatment during adolescence can only partially correct the unfavorable muscle to fat ratio in patients with KS [6]. In obese adolescents with KS exhibiting weight gain and increases in fasting insulin, initiation of metformin treatment may be warranted, though there have been no studies to examine the efficacy of this strategy [28]. For all patients, lifestyle modifications should be stressed from the time of diagnosis including addition of weight-bearing exercise. Testosterone therapy may have an indirect effect here, as increased muscle mass and strength can facilitate more regular exercise and sports participation, thus easing lifestyle changes.

14.7 Lack of Adverse Effects of Hormonal Therapy

Potential adverse effects of testosterone include aggressive behavior, hypercoagulability, and the suppression of native testicular function. A recent review of the safety of testosterone replacement found no adverse events associated with testosterone therapy in 110 patients aged 10–21 years with average treatment duration of 23 months [11]. Cessation of testosterone supplementation due to adverse effects was not required in any case. This is not unexpected, as testosterone dosage is titrated to the individual patient and serum T kept within the normal physiological range with biannual serum hormone panels and clinic visits.

There are currently no studies evaluating the safety of aromatase inhibitor therapy specifically in KS patients. Aromatase inhibitors have, however, been used without significant side effects to treat teenagers presenting with short stature [4, 29].

14.8 Hormonal Therapy and the Impact on Fertility Potential

Approximately 97 % of men with Klinefelter syndrome are affected by infertility due to testicular failure during puberty [4]. Cases of spontaneous pregnancy have been reported in KS patients, primarily in those with mosaic forms, but remain exceedingly rare [30]. Recent developments in testicular sperm extraction (TESE) and microsurgical testicular sperm extraction (mTESE) followed by intracytoplasmic sperm injection (ICSI) have opened the possibility of biological fatherhood to KS patients, and over 100 such births have occurred worldwide [30]. In experienced hands, mTESE men with KS have approximately 66 % sperm retrieval rate (SRR) and a greater than 50 % chance of eventual conception and live birth, which is considered equivalent to the rate in men with nonobstructive azoospermia of any other cause [4, 31].

There is a considerable debate as to the effect of adolescent hormonal manipulation therapy on fertility potential and sperm retrieval rate (SRR), and there is currently a lack of randomized controlled trials addressing the matter [3]. Concerns are rooted in the hypothesis that exogenous testosterone administration will suppress any remaining spermatogenesis and thus squander the already limited fertility potential of the patient. Suppression would be mediated by a suppression of the gonadotropins LH and FSH, such as seen in depot administration of testosterone [4]. With topical formulations of testosterone, however, suppression of LH and FSH is not observed [4, 11]. Without suppression of endogenous gonadotropins, there is unlikely to be a suppression of spermatogenesis, particularly with serum T levels being maintained within the physiological range during treatment. There have been two studies with a total of 13 testosterone-treated patients demonstrating a negative impact on SRR, but these involved mixed populations and unknown dosages, and duration of testosterone treatment and testosterone therapy was halted 6 months before mTESE in both. Ramasamy et al. examined SRR from mTESE in 68 adult men with KS, 8 of which had previously received TRT [31]. Only two of these patients had successful sperm retrieval leading to a calculated SRR of 25 %, well below the 66 % overall rate. However, the rationale for initiation of testosterone therapy was not recorded in these patients, and it is possible they represent a patient population with a more severe phenotype necessitating testosterone therapy. Thus, the requirement of testosterone therapy could reflect an overall defect in hormonal and spermatogenesis function rather than suppression of sperm production by exogenous testosterone [31]. Schiff et al. reviewed the SRR of 42 patients with KS, 5 of which had previous testosterone therapy [32]. Only one of these patients had successful sperm retrieval, leading to an SRR of 20 % for testosterone-treated patients. All five patients had received testosterone ethanoate injections, however, which have been shown to suppress gonadotropins, and thus, a suppression of spermatogenesis would be expected in these patients and not translatable to KS patients receiving topical testosterone therapy. A recent case series directly exploring the effect of testosterone therapy investigated the fertility outcomes of 10 patients with an average age of 15.1 years having received at least 1 year of topical testosterone therapy prior to mTESE and concomitant aromatase inhibitor therapy. Sperm was successfully retrieved for cryopreservation in seven patients, for an overall SRR of 70 % [33]. Exogenous testosterone supplementation with aromatase inhibitor usage did not appear to suppress spermatogenesis in this group as this rate is comparable to that reported for KS patients overall. Additionally, these patients were receiving testosterone therapy at the time of mTESE, unlike in the other studies where testosterone therapy was halted at least 6 months prior to the procedure.

Younger age is a positive predictor of successful sperm retrieval in men with KS, and early to mid-puberty may be the best time to consider sperm retrieval due to a brief increase in testicular size and temporarily normal hormone levels before seminiferous tubule atrophy begins in earnest [4, 34]. The strategy of retrieval and banking via cryopreservation at early puberty is analogous to that employed in other diseases causing primary gonadal failure, such as Turner's syndrome [35]. When possible, retrieval can be performed before initiation of testosterone replacement therapy until the effects on fertility are definitively established with controlled trials.

14.9 Conclusions

Klinefelter syndrome is a disorder with a wide array of manifestations that, when left untreated, can cause significant impairment in quality of life and increased morbidity and mortality. Early treatment and multidisciplinary management beginning in adolescence are necessary to support normal academic advancement and social development, allow for normal pubertal and physical development, and avoid serious complications from the pleiotropic effects of the syndrome. Speech therapy and proper educational planning as well as psychiatric evaluation must be instituted from diagnosis to avoid lagging in knowledge acquisition and socialization. Hormonal therapy ensures proper pubertal progression, helping to establish a masculine phenotype and avoid stigmatization and social isolation. The beneficial impact of early treatment on bone density, in particular, depends on administration during the "critical period" of bone mass acquisition during puberty. Deficits in bone mass due to delays in treatment initiation cannot be compensated for by later treatments. Thus far, adverse effects from early testosterone therapy have not been observed, and recent studies refute any adverse impact on fertility. A treatment algorithm involving early multidisciplinary management and initiation of hormonal therapy at pubertal onset represents an effective clinical strategy for Klinefelter syndrome patients.

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Klinefelter Syndrome: Early Treatment of the Adolescent Is Not Warranted

15

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15.1 Introduction

Klinefelter syndrome (KS) was first described in 1942 by Harry F. Klinefelter as the clinical presentation of gynecomastia, small testes, elevated gonadotropins, and azoospermia [1]. Studies show that approximately 80 % of KS patients have a numerical chromosome abnormality with a karyotype of 47,XXY, and the remaining 20 % have higher grade chromosome aneuploidies or mosaicisms [2, 3]. KS is the most common sex chromosomal disorder in men, diagnosed in 1 of 600 male newborns [4]. KS is seen in 3 % of infertile men, and in 11 % of men with nonobstructive azoospermia [4, 5]. This syndrome, however, remains underdiagnosed because of the lack of screening programs and wide variety of phenotypic presentation. It is estimated that only 25 % of males with KS receive a diagnosis, and less than 10 % are diagnosed before puberty [6, 7].

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Although there is wide variability in phenotypic expression in KS males, almost all men with non-mosaic KS are azoospermic if they undergo fertility evaluation. Boys with KS present with an apparently progressive decline in spermatogenic capacity that is associated with increasing FSH and decreasing inhibin B and antimullerian hormone levels [8, 9]. Bastida et al. reported on the changes in hormone levels of adolescents with KS during puberty (Fig. 15.1) [8]. Histology of testis can change in KS males from early puberty to mid-puberty. Testis biopsies may initially contain some normal seminiferous tubules, reduced germ cells, and normal Leydig/Sertoli cells, but later on in puberty, histology of testis can have fibrosis with extensive hyalinization of the seminiferous tubules [14]. It appears that early germ cell differentiation is arrested at either the spermatogonium or early spermatocyte stage often by 14–15 years of age [15, 16]. Developmental studies suggest that spermatogenic degeneration occurs before Tanner stage III for young males with KS, raising significant concern as whether a window of early intervention to preserve mature sperm is even possible.

Historically, KS males were considered infertile. There are, however, now well-documented series with successful sperm retrieval and subsequent pregnancies resulting from men with KS [17, 18]. Multiple studies have reported the finding of isolated foci of spermatogenesis in the testis of KS patients [19, 20]. The fertility outlook for KS patients has changed dramatically

D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_15

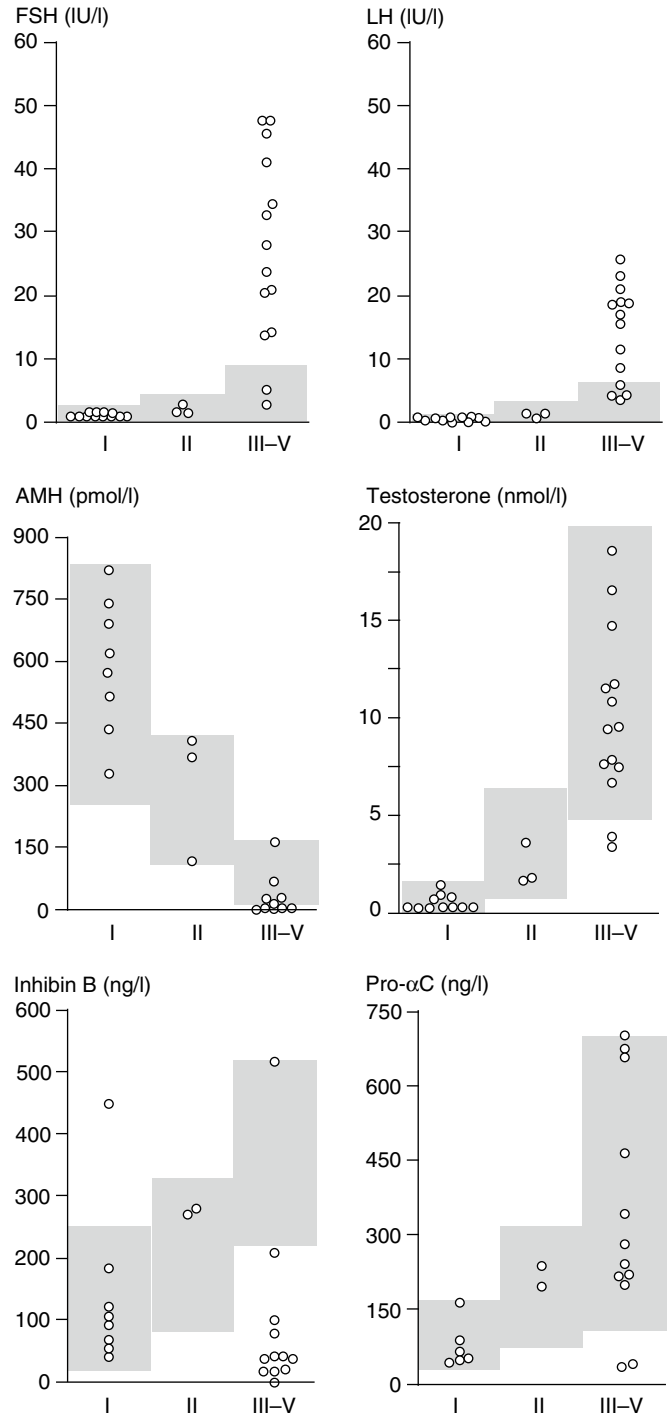

Fig. 15.1 Bastida et al. show serum levels of FSH, LH, anti-Müllerian hormone (AMH), testosterone, inhibin B, and Pro-αC in patients with Klinefelter syndrome at different stages of pubertal development based on tanner staging [8]. *Gray* areas represent reference values for pubertal stage [10–13]. (From Batista et al. [8] and reprinted with permission from John Wiley & Sons, Inc.)

as extracted sperm can be used with assisted reproductive technology, specifically intracytoplasmic sperm injection (ICSI).

There is debate in the literature as to the optimal time to begin fertility treatment in patients with KS. One school of thought is to begin treatment early in adolescence soon after initiation of puberty. Supporters of early treatment recommend early semen analysis for cryopreservation and/or sperm retrieval with testicular tissue cryopreser-

vation [21–23]. Alternatively, treatment can be commenced in adulthood when the KS patient becomes interested in paternity. This chapter will argue for treatment of KS in adulthood. Treatment in adulthood should focus on hormonal optimization and helping men achieve fertility goals.

15.2 Medical Therapy for Adolescents with Klinefelter Syndrome

Children with KS appear to have normal hormone levels in the prepubertal period, as well as a normal serum testosterone response to hCG stimulation [24]. While there are sufficient levels of circulating testosterone to initiate puberty, KS boys typically fail to progress through all stages of puberty. In addition to the poor development of facial hair and masculinization, there is a progressive increase in LH and FSH and a decrease in inhibin B levels [15]. Testosterone replacement therapy has been utilized in boys with KS in order to promote age-appropriate development of secondary sexual characteristics, although Bastida's data suggest maintenance of endogenous testosterone production despite spermatogenic failure during mid-puberty. Many published studies recommend the initiation of hormone replacement therapy in early to mid-puberty [25, 26]. In addition to promoting the development of secondary sexual characteristics, the goal of hormone replacement therapy is to stimulate linear growth, proper bone development, and increase muscle bulk.

However, the evidence to support use of early androgen therapy is limited. Controlled studies of hormone therapy to promote the progression of puberty are lacking. There is little evidence to support hormone therapy for fertility treatment in adolescents with KS. On the contrary, there is a possibility that exogenous testosterone may have an unfavorable effect on fertility by further inhibiting testicular function [23]. Azoospermia is seen to develop in up to 40 % of patients on testosterone replacement therapy [27]. Exogenous testosterone may irreversibly suppress sperm function by decreasing gonadotropin release and impairing germ cell maturation [28, 29].

The effect of testosterone therapy on future testicular sperm extraction rates is still unclear. We have published two studies that have shown that prior testosterone treatment was associated with a decreased sperm retrieval rate even when using a microdissection testicular sperm extraction (TESE) procedure [30, 31]. In a study done by Schiff et al., it was found that of the five patients who received prior testosterone therapy, only one had extraction of sperm after micro TESE. This is in contrast to an overall sperm retrieval rate of 72 % per TESE attempt for the study cohort [31]. In the study by Ramasamy et al., there was a similar decrease in retrieval rate of sperm for men with prior testosterone treatment. Of the eight men with prior testosterone therapy, only two had sperm retrieved with micro TESE, while the overall sperm retrieval rate was 68 % per TESE attempt in men with KS [30]. It is important to note that the reasons for initiation of testosterone therapy and duration of therapy were unknown in these reports. It was observed that men who had normal testosterone at baseline had a sperm retrieval rate of 86 %.

Hormonal therapy to enhance sperm production in adolescent patients has been attempted in uncontrolled reports. A small case series by Mehta et al. suggested the use of topical testosterone replacement therapy and aromatase inhibitors (anastrozole 1 mg PO QD) for a period of 1–5 years before micro TESE. Their results suggest that treatment did not appear to decrease sperm retrieval rates. In this study, seven out of ten patients had successful sperm retrieval [32]. The study was small, evaluated only ten patients, and was a retrospective case series which may have led to selection bias. Additionally, no control group was used to compare the sperm retrieval rates in young KS patients who did not receive hormonal therapy. While the high retrieval rate is encouraging, critical aspects of this proposed treatment need to be better elucidated. A few key clinical questions that need to be answered include: is testosterone replacement along with aromatase inhibition helpful? Can sperm be effectively frozen? Are these patients just as likely to have sperm found with later micro TESE? Are there long-term hormonal treatments

that can maintain sperm production seen in early adolescents with KS? Future larger studies should be performed to explore these key issues.

Some studies have explored treatment regimens to minimize the deleterious effects of testosterone therapy on spermatogenesis; however, there is no standard accepted protocol for adolescents. One possible proposed solution is to give patients concomitant intramuscular injections of human chorionic gonadotropin. In a retrospective analysis of 26 men treated with testosterone replacement therapy along with human chorionic gonadotropin, there were no differences in semen parameters seen after 1 year of follow-up [33]. None of the patients in the study became azoospermic, and 9 of the 26 patients contributed to a successful pregnancy. In a randomized controlled trial, it was found that low-dose human chorionic gonadotropin helped to maintain intratesticular testosterone levels in men treated with testosterone enanthate [34]. The downside to treatment with human chorionic gonadotropin is that it may decrease FSH stimulation of the testis and requires frequent injections. Other possible treatment regimens involving clomiphene citrate and anastrozole may be explored as they preserve the hypothalamic–pituitary–gonadal axis [35, 36]. The hormonal therapy given to children with KS must be further analyzed in controlled studies to explore the effects it may have on spermatogenesis and future fertility.

15.3 Cryopreservation of Spermatogenic Stem Cells

Cryopreservation of sperm is currently offered to patients interested in fertility preservation and is considered a valuable measure for early adolescents with KS [37]. Current recommendations are to bank sperm for all males at or above Tanner stage III [38]. Cryopreservation of mature sperm has been reported for over 30 years, and there has been a case report of successful fertilization and birth after sperm were cryopreserved for 28 years [39]. While cryopreserved sperm is generally acknowledged to have lower viability and impaired fertility potential in comparison to fresh sperm, it is still a valuable option [40]. When no sperm is detected, testicular tissue cryopreservation can be offered in the hope of preserving spermatogonial stem cells (SSCs). It has been proposed that SSCs could be used to restore spermatogenesis, or they could be matured in vitro to produce viable sperm [41]. The use of SSCs remains experimental and has no current use in clinical medicine.

For adolescent patients with KS, cryopreservation of mature sperm should be offered if viable sperm are found on semen analysis. Invasive therapies to retrieve mature sperm from adolescents should not be offered because of the negative effects of the procedure and high retrieval rates seen in adulthood. Some pundits argue for cryopreservation of SSCs from KS patients; however, there is no fertility treatment available to use these SSCs now or in the foreseeable future. Firstly, it is unlikely that transplanting SSCs into the testis of the adult KS patient will be feasible. The testes of adult patients with KS are characterized by extensive fibrosis and hyalinization of the seminiferous tubules, making transplantation unlikely to be successful. Therefore, in order to use cryopreserved testicular samples containing no mature germ cells for infertility treatment, in vitro maturation of spermatozoa would be required.

While advancements towards in vitro maturation of SSCs have been made, the future of this therapeutic option remains unknown. In a study by Sadri-Ardekani et al., it was found that testicular cells taken from adult human testes could be cultured and propagated [42]. This was the first study to show the propagation of human SSCs in vitro. In a follow-up study, the same research group found similar results in samples taken from two prepubertal boys [43]. In another study, a soft agar culture system was developed for mouse SSCs that enabled maturation of spermatozoa from stem cells [44]. While these advancements are promising, as of now, in vitro maturation of humans SSCs is not possible. In addition to developing a new technique to mature human SSCs in vitro, future studies would need to determine whether the sperm generated maintain DNA integrity and would be suitable for in vitro fertilization. Overall, there is no tangible use of cryopreserved SSCs from KS children at present, and the future of this treatment option remains questionable.

Testicular biopsy in children with KS has resulted in limited extraction of SSCs. In one study of 14 boys with KS aged 10–14 years old, only 50 % were found to have germ cells following testicular biopsy [45]. Another study of 11 boys with KS found that none of the boys had germ cells after the age of 2 years old [46]. In a population of seven pubertal KS boys aged 13–16 years old, only one was found to have spermatogonia in non-degenerating seminiferous tubules [47]. The data available show that there is no assurance that SSCs will be retrieved from the testes of children/adolescents with KS.

In addition to the low reported retrieval rate of SSCs, testicular biopsy and dissection for sperm extraction have known negative effects on testicular function. There is a known temporary decrease in serum testosterone that will typically recover within 12–18 months [48]. In a study of 24 KS patients undergoing TESE, serum testosterone levels decreased in all patients postoperatively and had not returned to baseline levels after 12 months [49]. In another study of 69 patients undergoing TESE, testosterone levels were found to have recovered to 50 % of baseline 12 months postoperatively [50]. This drop in testosterone levels could be detrimental to an adolescent KS patient who is already dealing with poor progression of puberty and development of secondary sex characteristics. In addition, there are known histological changes seen in the testes after TESE. One study found that TESE results in decreased seminiferous tubular volume within the testicular parenchyma adjacent to the biopsy site and a trend toward decreased germ cells per a tubule after the procedure [51]. All of these negative effects should provide caution against testicular biopsy and sperm extraction in adolescent patients.

15.4 Ethics of Cryopreservation

It is important to evaluate the ethical question of whether collecting, preserving, and possibly later using testicular tissue from the adolescent KS patient is in the best interest of the child. To ethically support cryopreservation of either mature sperm or SSCs, the benefit to the child should outweigh the costs. This means that semen/testicular tissue collection, storage of tissue, and financial costs should be offset by the possibility of future paternity [52]. The utilization rate of the preserved tissue will have a great effect on the balance of these costs and benefits. The scale shifts toward greater cost as the utilization rate of the cryopreserved tissue decreases. The utilization rate will be affected by KS patients' available alternatives and desire to father a child. One possible alternative is to have a TESE procedure in adulthood when interested in fatherhood. The high retrieval rate seen in KS adults may decrease the utilization rate of cryopreserved tissue [30]. Additionally, one study found that of men with KS at an average age of 40 years old, only 52 % had a partner, therefore making the need for fertility options less likely [53]. These two findings combined likely predict a low utilization rate of cryopreserved tissue.

Overall, it seems hard to justify cryopreservation of SSCs in KS adolescents given the unknown future use of stored tissue. There is currently no practical use of cryopreserved SSCs from KS patients for fertility treatment. Cryopreservation of SSCs is completely experimental with no expected human clinical application in the near future. In addition, the collection of tissue requires a surgical procedure in a minor. Even if the patient does undergo the procedure, some studies have found that SSCs are extracted in only 50 % of cases [45]. An unsuccessful procedure can result in a negative emotional response in the patient and the patient's family.

Some may argue that cryopreservation of mature sperm should be offered since there is a potential application with the use of intracytoplasmic sperm injection. Since KS adolescents are azoospermic, collection of sperm will likely require TESE. There are known negative effects to the procedure as discussed above. There may also be a negative emotional impact of failure to extract sperm, both in the patient and the family. Additionally, there may be a loss in viability of the cryopreserved sperm, creating a need for another TESE procedure in adulthood [40]. If a child is able to produce viable sperm through ejaculation as seen on semen analysis, then it is

likely ethically sound to cryopreserve the sperm. However, if the patient requires an invasive procedure to retrieve sperm, the costs may outweigh the benefits.

15.5 TESE in Adulthood

Intracytoplasmic sperm injection (ICSI) has long been described as a successful option to treat male factor infertility [54]. In cases of azoospermia, TESE can be utilized to find viable sperm for ICSI. TESE combined with ICSI has been reported to be a successful fertility option for men with non-mosaic KS [55]. When a KS patient decides that he is interested in paternity, TESE can now be offered as a viable treatment option. The recovery rate of sperm in men with KS is comparable or better than that observed for other men with idiopathic nonobstructive azoospermia [52].

Men with low serum testosterone levels who are scheduled for a TESE procedure can be treated medically preoperatively. Patients are treated with aromatase inhibitors, human chorionic gonadotropin, or clomiphene for a 2-month period before surgery. Aromatase inhibitors cannot be used for longer than 2 months because of tachyphylaxis and their ability to block estrogen production. Aromatase inhibitors have been found to increase testosterone-to-estradiol ratio, sperm concentration, and motility in controlled studies [56]. Numerous studies have reported that treatment with clomiphene and human chorionic gonadotropin resulted in improvement in semen quality and testosterone levels [57–59].

Sperm retrieval rates in men with KS have been reported to be at least comparable to rates seen in other men with nonobstructive azoospermia. In a recent study, sperm was retrieved via micro TESE in 66 % of men with KS and in 68 % of TESE attempts [30]. This same study found that for patients who had successful sperm extraction, 57 % had a subsequent pregnancy via IVF and 45 % resulted in live births. It was also found that men with normal baseline testosterone levels and men who responded to hormonal therapy had up to 85 % retrieval rates [30]. Although other studies have reported lower sperm retrieval rates with different surgical approaches, the chance of finding sperm in KS males is typically better than in other males with idiopathic nonobstructive azoospermia [60]. Overall, the data supports the notion that TESE along with ICSI is an important treatment option for men with KS who are interested in paternity. Men who respond to medical therapy have retrieval rates similar to those with baseline normal testosterone levels. Men with low serum testosterone can receive medical therapy to enhance endogenous testosterone production and have an increase in testosterone levels. Taken together, baseline testosterone and response to medical therapy are good predictors of sperm retrieval in men with KS. While most studies have yet to uncover statistically significant factors that predict successful sperm retrieval, future studies should continue to assess factors such as age, previous medical therapy, hormone levels, and testicular volume.

15.6 Conclusions

Early treatment of adolescents with KS should be judiciously recommended based on data currently available. Sperm retrieval in adults with KS is the proven gold standard for fertility management. Early intervention in adolescents including testicular biopsy can result in negative effects and further reduce future fertility options. Cryopreservation of SSC retrieved via TESE is of unknown benefit and subjects the adolescent to an experimental and invasive procedure. If an adolescent is interested in fertility preservation, semen analysis of ejaculated sperm and cryopreservation is appropriate. However, recommending adolescent boys with KS to get a testicular biopsy is both impractical and costly, as older patients still have greater than 50 % retrieval rates, and the procedure itself has known negative effects. Sperm retrieval with cryopreservation is only warranted when testosterone replacement is necessary for symptomatic hypogonadism. Medical and surgical treatment of adolescents with KS can only be considered useful if it is evaluated in controlled studies.

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Are Dietary Supplements Beneficial for IVF Patients?

16

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16.1 Introduction

Approximately 15 % of couples of reproductive age in the United States are infertile, defined as trying unsuccessfully to conceive for over 12 months, while having unprotected regular sexual intercourse with the same partner [1]. Since the first child was born via assisted reproductive technology (ART) in 1981, the number of fertility clinics and the number of couples using ART have increased significantly [2]. In 2012 alone, a total of 176,247 ART procedures were performed in 456 fertility clinics, resulting in 51,267 live birth deliveries and 61,160 infants [3]. However, while the proportion of successful ART procedures rose steadily throughout the 1990s, it has remained stagnant at around 30 % for the past decade. In addition, as more women delay childbearing into their late 30s and early 40s [4], ART success rates will likely stay the same or even decrease, given that age is the one of the strongest predictors of pregnancy through ART [5].

The most common type of ART is in vitro fertilization (IVF). In IVF, oocytes are harvested from the ovaries and fertilized by spermatozoa in a laboratory. Once the egg is fertilized, the embryo is cultured for 2–5 days in the laboratory and then transferred into the uterine cavity. Undergoing IVF procedures can result in temporary increases in anxiety and depression among women who do not achieve conception and live birth. Interestingly, successful conception and live birth can result in long-term improvements in mental health among women who do give birth, or those who stop trying, compared to women who continue through additional unsuccessful cycles [6]. Perceived stress may be a sequelae of unsuccessful IVF and may lead to women seeking alternative/complementary treatments as well as dietary supplements to improve their outcomes and/or stress [7]. According to the American Society for Reproductive Medicine, an average IVF cycle costs $12,400. Therefore, given the substantial economic, psychological, and health-related consequences of IVF treatment, a more thorough understanding of modifiable factors that affect its success is of major public health importance. In addition, the same factors that affect fertility in the general population may not apply to couples

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3_16

undergoing IVF cycles; therefore, it is important to consider this population separately.

Recent investigations have considered whether intake of dietary supplements affects outcomes of IVF cycles. Dietary supplements are products that are intended to supplement the diet with a wide variety of substances, including vitamins, minerals, herbs, other botanicals, and amino acids. According to the Dietary Supplement Health and Education Act of 1994, dietary supplements are classified as food instead of drugs and are therefore largely unregulated [8]. They require no efficacy or safety testing by the Food and Drug Administration before being marketed and sold to the public. Therefore, the supplement industry has flooded the marketplace with a plethora of dietary supplements for which no scientific evidence is available.

A variety of micronutrients that are found in the diet, and can also be taken via supplements, are known to be beneficial to the health of mothers and fetuses during pregnancy. For example, folic acid supplementation during early pregnancy prevents neural tube defects, and it is recommended that all women of reproductive age take folic acid supplements for this reason [9, 10]. In addition, sufficient iodine intake during pregnancy is essential for the synthesis of maternal thyroid hormones and proper brain development of the fetus, and the World Health Organization recommends that women who are pregnant or breastfeeding take 250 μg/day [11]. Lastly, many prenatal vitamins contain omega-3 long-chain fatty acids including docosahexaenoic acid due to their role in proper brain and retinal maturation [12]. However, it is unclear whether these dietary supplements could also be helpful for fertility, and specifically, for women undergoing IVF.

Hundreds of dietary supplements specifically marketed towards women who are trying to conceive are available for purchase. These supplements claim to improve fertility, optimize a woman's chances of becoming pregnant, and improve overall reproductive health. A search for "fertility supplements for women" on Amazon returns almost 300 results, primarily supplements in the form of capsules and teas. These products contain mostly herbs, such as red clover blossom, ginseng, ginkgo biloba, and chasteberry, and vitamins and minerals, such as folic acid, myo-inositol, melatonin, vitamin E, zinc, and selenium. With few exceptions, these products have not been tested for efficacy or safety.

The purpose of this review is to summarize the scientific literature on dietary supplementation and IVF outcomes. It is important that couples attempting to conceive be given high-quality scientific information regarding how different types of supplements can affect their chance of success and if taking supplements puts them at risk for adverse events. To assess the available evidence, we performed a search using PubMed. We searched for MeSH terms "Fertilization in vitro" and "Dietary supplements" or "Folic acid" or "Antioxidants" or "Micronutrients" or "Vitamins" or "Minerals" or "Phytoestrogens." We reviewed the results to identify epidemiologic studies assessing the effect of dietary supplement use on IVF outcomes. We identified additional articles from the reference lists of articles identified using the PubMed search. Studies of dietary patterns are beyond the scope of this review.

16.2 Folic Acid

Folic acid, an essential B vitamin, plays a crucial role in DNA and RNA synthesis, amino acid metabolism, and cell proliferation. Therefore, processes that require rapid cell division, such as gametogenesis and early embryo development, are especially susceptible to folic acid deficiency [13]. Folic acid plays a crucial role in the breakdown of the amino acid homocysteine. Hyperhomocysteinemia is associated with several adverse pregnancy and perinatal outcomes, including recurrent miscarriages, pregnancy-induced hypertension, and congenital malformations including neural tube defects [14–16]. The United States Centers for Disease Control and Prevention and the United States Preventative Services Task Force recommend that all women of childbearing age take 400 μg of folic acid daily [17, 18]. All enriched cereal grain product flour in the United States has been fortified with folic acid since 1998, which has been effective in increasing folic acid levels in blood and reducing the prevalence of neural tube defects [19].

There is some evidence that folic acid supplementation may also improve fertility. In animal studies, folic acid supplementation has been found to beneficially affect ovulation [20], litter size [21], and embryo survival and normal development [22]. Among women in the Nurse's Health Study who attempted pregnancy during the 8-year follow-up, those who took a multivitamin supplement regularly had a reduced risk of anovulatory infertility compared to women who did not take a multivitamin (adjusted OR = 0.65, 95 % CI: 0.53, 0.80) [23]. An analysis of the total intake of individual B-vitamins suggested that folic acid may be responsible for part of this association. Similarly, in a prospective cohort of healthy women, dietary folate intake was associated with reduced odds of anovulation [24].

A few studies have been conducted specifically assessing the association between folic acid intake, or biomarkers thereof, and IVF outcomes, with conflicting results. Thaler and colleagues were the first to report that folic acid or its metabolism may have an impact on IVF outcomes [25]. In a group of 105 women who underwent 269 IVF cycles, those who were homozygous for the wild type C allele in methylenetetrahydrofolate reductase (MTHFR) C677T required lower gonadotropin (rFSH) doses for controlled ovarian hyperstimulation, produced significantly more oocytes, and had higher peak estradiol levels than homozygous women for the variant T allele; this association was stronger among women of 35 years or older. Subsequent work by this group demonstrated that this common mutation is also associated with lower basal and stimulated estradiol production by granulosa cells [26] and lower anti-Müllerian hormone levels [27].

Work from other groups is also supportive of the role of folic acid and its metabolism on IVF outcomes. In a prospective cohort study of 181 women undergoing IVF or intracytoplasmic sperm injection (ICSI) in the Netherlands, blood folate levels were significantly associated with better embryo quality, and a twofold increase in monofollicular folate levels was associated with 3.3 times the odds of achieving pregnancy [28]. A separate report from this group also found that follicular fluid homocysteine levels (a consequence of low folic acid status, dietary, or otherwise) were also related to lower day 3 embryo quality [29]. Likewise, in a cohort of 439 infertile Estonian women undergoing IVF or ICSI, the MTHFR 677 heterozygous CT genotype was associated with good quality embryos compared to the CC genotype and with increased chance of clinical pregnancy compared to either CC or TT homozygous genotypes [30]. Women with the cystathionase (CTH) 1208 heterozygous GT genotype had an increased chance of clinical pregnancy compared to women with the GG genotype.

All of the studies discussed above that suggest an effect of this pathway on IVF outcomes have been conducted in countries without a supplemented food supply, raising the possibility that the effect of folate metabolism genes and biological markers of the status of this pathway (such as folic acid or homocysteine levels) may be limited to "low" folate environments. However, a recent study from the United States, where food supply is supplemented with folic acid and intake levels are substantially higher than in Europe, also suggests that folic acid may be important in assisted reproduction. Gaskins and colleagues conducted a prospective cohort study in the US among 232 women undergoing a total of 353 IVF or ICSI. In this study, pretreatment intake of folic acid was positively associated with implantation, clinical pregnancy, and live birth rates [31]. A few additional points are worth noting. First, the association was driven by supplemental folic acid intake and was essentially null for folic acid from food sources. Second, the association was not linear; the largest benefits were observed at intakes above 800 μg/day but appeared to plateau at about 1,200 μg/day of supplemental folate. Lastly, analyses of intermediate outcomes revealed that most of the association was explained by a higher fertilization rate in conventional insemination cycles coupled with a drastically lower failure rate prior to embryo transfer among women in the highest category of folic acid intake (2 %) than among women in the lowest category of intake (15 %). No association with twin birth rate was found in this study.

However, not all studies of folic acid or folic acid metabolism have showed a potential beneficial

effect. In a Swedish cohort of 167 women with unexplained infertility who underwent ART, no association was found between use of folic acid-containing supplements and odds of clinical pregnancy or live birth [32]. Similarly, in a prospective cohort of 602 women undergoing IVF in the UK, folate intake, plasma folate levels, and red blood cell folate levels were not associated with chance of a live birth. However, plasma folate levels were associated with an increased risk of twin vs. singleton birth [33]. In addition, women homozygous for the variant CC genotype in MTHFR C1298A had reduced odds of viable pregnancy and live birth compared to women with the wildtype genotype.

It is important to consider the potential reasons for inconsistency in results across studies. The most salient difference between studies finding an association between folate and IVF and those finding no relation is their exclusion criteria. Specifically, all studies reporting no association employed exclusion criteria that could reasonably be expected to bias results towards the null. The Swedish study was restricted to women in couples with unexplained infertility who, relative to couples with other common diagnoses such as male factor and tubal factor infertility, have worse prognosis resulting in fewer live births overall. The clearest example of potential bias introduced by exclusions is the UK study, where investigators excluded cycles with a gestational sac but no fetal heart, cycles ending in chemical pregnancy, ectopic pregnancy, termination, stillbirth, or neonatal death, oocyte donor cycles, and cycles without embryo transfer which, in total accounted for 15 % of all eligible women. The exclusions in the UK study would bias the results towards the null if folate's main impact is on outcomes that take place before embryo transfer or the clinical recognition of a pregnancy (as suggested by the German, Dutch, and American group) or on pregnancy survival upon its clinical recognition (as suggested by a protective effect of preconception folic acid on clinical pregnancy loss seen in some studies [34]).

These methodological considerations notwithstanding, the biggest limitation of the current literature is the lack of randomized clinical trials evaluating this relation. Given the potential clinical and public health implications, randomized trials evaluating the questions raised by the existing observational research are warranted. Specifically, randomized trials evaluating whether or not folic acid may improve live birth rates in assisted reproduction and evaluating issues of dose and whether or not this purported relation depends on background food supply supplementation or genetic background (e.g., functional MTHFR polymorphisms) are needed.

16.3 Phytoestrogens

Phytoestrogens are nonsteroidal compounds present in a variety of dietary products that possess estrogenic activity in animals. In IVF cycles in which gonadotropin-releasing hormone agonist is used for pituitary downregulation to obtain controlled ovarian stimulation, progesterone supplementation during the luteal phase is commonly prescribed [35]. However, the importance of estradiol supplementation remains controversial. Estradiol levels in the luteal phase have been positively correlated with conception cycles compared to non-conception cycles in fertile women [36–38], negatively correlated with implantation in women undergoing oocyte donation [39], and positively correlated with higher pregnancy rates in women undergoing IVF [40–42]. Therefore, phytoestrogen supplementation during IVF cycles has been hypothesized to improve the success of implantation through its estrogenic properties.

To date, only one randomized placebo-controlled trial has been conducted assessing the relationship between phytoestrogen supplementation and IVF outcomes [43]. Study participants were Italian women of <40 years of age who were using a gonadotropin-releasing hormone analog for pituitary downregulation. Patients were randomized using a randomization table. Those in the control group received 50 mg/day of progesterone and a placebo pill ($n=98$) and those in the treatment group received 50 mg/day of progesterone and a phytoestrogens tablet containing 1,500 mg of soy isoflavones ($n=115$). Treatment started on the evening of oocyte retrieval and was terminated upon either a negative serum pregnancy test or

confirmation of an embryonic heartbeat. Individuals randomized to receive phytoestrogens had a statistically significantly higher implantation rates (25.4 % vs. 20.2 %), clinical pregnancies (39.3 % vs. 20.9 %), and ongoing pregnancies/deliveries (30.3 % vs. 16.2 %). Given the small sample size, larger trials are needed before drawing any conclusions about the effect of phytoestrogens on IVF outcomes.

It is worth noting that another study investigated the role of phytoestrogens on infertility treatment, although not IVF [44]. Patients with unexplained infertility managed by clomiphene ovulation induction and timed sexual intercourse were randomized to receive either 150 mg/day of clomiphene alone ($n=60$) or 150 mg/day of clomiphene plus 120 mg/day of phytoestrogen ($n=59$). In an analysis excluding women who failed ovarian stimulation with clomiphene citrate, those who were randomized to the treatment group were more likely to have a clinical pregnancy compared to women who were randomized to the control group (36.7 % vs. 13.6 %, $p<0.001$). Pregnancies were not followed through delivery, so live birth rates could not be reported. It is known that clomiphene acts as an estrogen antagonist at the level of the endometrium [45–47]. It is plausible that phytoestrogen supplementation may mitigate the endometrium-level antiestrogenic effect of clomiphene. In fact, Unfer and collaborators also documented an increase in endometrial thickness following isoflavone supplementation among women undergoing IUI [43].

Overall, there is limited evidence of an association between phytoestrogens and IVF outcomes. Although both RCTs suggest that a relationship could exist, both trials are relatively small. A larger, well-controlled clinical trial is needed before phytoestrogen intake can be recommended for women undergoing IVF in relation to both early reproductive outcomes and live birth. In addition, since similar effects were observed in the trial among women undergoing IVF and the trial among women undergoing timed intercourse with clomiphene citrate, with a tenfold difference in phytoestrogen dose between trials, it is also important that future trials address the question of minimal effective doses, if an effect does exist.

16.4 Antioxidants

Antioxidants are compounds that inhibit the oxidation of other molecules and thereby reduce oxidative damage. Low levels of antioxidants can lead to oxidative stress, and recent evidence suggests that oxidative stress may be involved in the pathogenesis of infertility [48]. Antioxidants are thought to play a crucial role in regulating many processes related to reproduction [49]. Compared with healthy women, lower antioxidant levels have been found in women with polycystic ovary syndrome (a known cause of oligo- or anovulatory infertility) and in women with idiopathic infertility [50, 51]. Other risk factors for infertility, including obesity and age, are thought to exert pathological effects through increased oxidative stress [52, 53]. The predominant antioxidants evaluated for female subfertility are vitamins A, C, and E; *N*-acetyl-cysteine; melatonin; myo-inositol; zinc; and selenium.

A recent systematic review summarized randomized controlled trials that have been conducted comparing the effect of antioxidant supplementation on fertility in subfertile women attending fertility clinics but who may or may not be undergoing ART procedures [54]. A total of 28 trials were included in the review, comprising 3,548 participants. The trials were quite heterogeneous in study design, intervention type, comparison group, and indications for subfertility. Pooled data suggest that there is no association between taking antioxidant supplements and live birth (OR = 1.60, 95 % CI: 0.70, 3.69) or clinical pregnancy (OR = 1.12, 95 % CI: 0.92, 1.36) compared to placebo, no treatment, or standard treatment. Sub-analyses showed no effects for any individual antioxidants on either pregnancy or live birth outcomes. Adverse events were not well reported across studies, but pooled data demonstrated no overall association with antioxidant treatments.

Among the subset of studies in this review that included women undergoing IVF/ICSI, there was no evidence of an association between antioxidant intake and clinical pregnancy rate (OR = 0.97, 95 % CI: 0.74, 1.27). Only one study including women undergoing IVF assessed the relationship of antioxidant intake with live birth outcome; this

study found no relationship between the chance of live birth and L-arginine supplementation compared to placebo [55].

Overall, the quality of evidence was graded as "low" to "very low." This was primarily due to the inclusion of a large number of small studies, inadequate reporting of outcomes, a high risk of bias within studies (primarily due to issues with allocation concealment, blinding, and selective reporting), and high heterogeneity in the pooled analyses. Very few trials examined the same intervention making it difficult to interpret the pooled results and even calls into question the decision to pool the results at all. Additionally, the investigators used an extremely generous definition of "antioxidant" in this meta-analysis, which had the effect of including studies evaluating almost any substance that can be packaged as a supplement and sold over the counter, including substances that were clearly not antioxidants but could rather be categorized as nutrients or anti-inflammatory substances. Hence, it is not possible to know from this meta-analysis whether the lack of association with IVF outcomes reflects a true null association or is due instead to heterogeneity in the exposures under study.

Since the publication of this review, to our knowledge, no new randomized controlled trials on this topic have been published. However, secondary data analysis from women who were part of the FASTT trial (an RCT assessing an accelerated treatment strategy compared to standard treatment in couples with unexplained infertility) investigated the relationship between antioxidant intake and time to conception [56]. Antioxidant intake was measured from a dietary questionnaire, and total, dietary, and supplemental intake of β-carotene, vitamin C, and vitamin E were calculated. In the analysis of all 437 women who participated in the study, there was no relationship between any of the antioxidants under study and time to conception. However, a significant association did exist in certain age and body mass index (BMI) strata. In normal-weight women (BMI of less than 25 kg/m^2), supplementary intake of vitamin C was associated with reduced time to conception; dietary and total intake had no effect. Among overweight women (BMI of at least 25 kg/m^2), supplementary intake of β-carotene, but not dietary or total intake, was associated with shorter time to conception. Supplemental and total intake of β-carotene and vitamin C were also associated with shorter time to conception among women who were less than 35 years of age; however, in older women (at least 35 years of age), a positive relationship existed with supplemental and total vitamin E. This suggests that dietary supplements may be effective at increasing the chances of success with IVF among certain groups of women, but not others. More research is needed to evaluate this issue, as no other studies have investigated effect modification by age or overweight status.

The literature on the relationship between antioxidant intake and IVF outcomes is inconsistent. Overall, there is no evidence at this time to recommend that women undergoing IVF take antioxidant supplements. However, it is important to note that no adverse effects of these supplements have been reported. Large, well-designed randomized controlled trials that have clearly defined treatment and control groups, proper blinding and randomization protocols, and sufficient sample size to assess effect modification by other infertility risk factors are needed.

16.5 Vitamin D

Vitamin D is a fat-soluble secosteroid responsible for intestinal absorption of many compounds including calcium, iron, and zinc. It is essential for a variety of biological processes, including reproduction [57]. Animal studies provide substantial evidence that vitamin D could be involved in the pathogenesis of infertility. Vitamin D receptor knockout mice experience uterine hypoplasia, impaired folliculogenesis, and infertility [58–60]. In humans, the vitamin D receptor is present in several locations along the reproductive tract, suggesting that vitamin D could play a crucial role in human reproduction [61, 62]. Vitamin D may also play a role in the transcription of genes involved in regulation of implantation and placentation [63]. Supporting this hypothesis is evidence that pregnancy rates and quality of embryo ratings after IVF vary with total number

of light hours, with higher pregnancy rates in the spring and lower rates in the autumn [64, 65].

To our knowledge, no studies have investigated vitamin D intake or supplementation on IVF outcomes, but several studies have investigated the relationship between serum and follicular fluid levels of 25-hydroxyvitamin D (25(OH)D), the main circulating form of vitamin D, and chances of pregnancy and live birth after IVF. In two cohort studies in the US, women undergoing IVF in Los Angeles ($n=188$) and New York ($n=84$) had 25(OH)D measured in their serum and serum/follicular fluid, respectively. In the Los Angeles study, after adjusting for maternal age, number of embryos transferred, embryo quality, and diagnosis of diminished ovarian reserve, vitamin D deficiency (defined as serum 25(OH)D levels <20 ng/ml; compared to vitamin D repletion, defined as serum 25(OH)D levels >30 ng/ml), were significantly associated with lower rates of pregnancy (21 % vs. 55 %) and live birth (14 % vs. 47 %) among non-Hispanic white women, but not women of other races/ethnicities [66]. In the New York Study, each ng/ml increase in 25(OH)D levels was significantly associated with a 7 % increase in odds of clinical pregnancy following IVF, after adjusting for age, body mass index, race, and number of embryos transferred [67]. In a Canadian cohort of 173 women undergoing IVF, women with sufficient levels of vitamin D (serum 25(OH)D ≥75 nmol/l) had higher clinical pregnancy rates compared to women with insufficient levels (52.5 % vs. 34.7 %) [68]. Multivariate analyses adjusting for age, BMI, and day of embryo transfer showed a small but significant association [adj-OR=1.01 (1.00, 1.03)]. In a cohort of 99 southern California women who were undergoing IVF and were recipients of egg donation, recipient vitamin D deficiency was associated with significantly lower rates of clinical pregnancy and live birth, compared with recipients who were vitamin D replete [69]. This suggests that vitamin D could exert positive effects of IVF outcomes through the endometrium, rather than through oocyte quality.

Null associations between vitamin D levels and IVF outcomes have also been reported in two Iranian cohorts [70, 71]. Contrary to the findings described above, a prospective cohort study of 101 women in Greece, women with follicular fluid levels of 25(OH)D >30 ng/ml had lower pregnancy rates compared to women with 20.1–30 ng/ml and <20 ng/dl (14.5 % vs. 32.3 % vs. 32.7 %) [72]. However, there was no control for confounding in this analysis; therefore, the chance for bias in study results is high.

Since no studies have looked specifically at vitamin D intake and IVF outcomes, we do not recommend vitamin D supplementation at this time, despite some positive findings in the studies described above. However, given the recent increase in prevalence of vitamin D deficiency in the U.S., mainly due to changes in BMI, milk intake, and sun protection [73], the role of vitamin D supplementation in IVF outcomes is an important topic to investigate. Large, well-controlled prospective studies are needed to investigate this issue.

16.6 Conclusions

Simple behavioral changes that may improve chances of becoming pregnant are very appealing to couples experiencing infertility and undergoing ART. The public health implications for finding an effect of a modifiable risk factor on IVF outcomes are enormous.

However, the literature on the relation between dietary supplements and IVF outcomes is inconclusive and leaves many questions unanswered. Most of the studies reviewed above do not adequately control for confounding by lifestyle characteristics, hormone levels, BMI, and age. Many are limited by small sample size. A few assess effect modification by other infertility risk factors or by infertility subtype. Even for dietary factors for which evidence is stronger, questions regarding residual confounding, detailed characterization of dose–response relationships, and effect modification by background food supply fortification or genetic background remain. As a result, at this time, there is no sufficient evidence to recommend changes in clinical practice in order to recommend widespread use of specific dietary supplements for the purpose of improving IVF outcomes (Table 16.1). Nevertheless, further observational research for some nutritional factors may clarify the need for randomized trials.

Table 16.1 Summary of studies on dietary supplements and IVF outcomes

Supplement	Biologic plausibility/ location of effect	Studies to support	Clinical recommendation	Recommendation for research
Folate	• MTHFR genotype related to mother's ability to produce high-quality embryos • Hyperhomocysteinemia • Improved embryo quality, embryo survival • Improved survival of clinically recognized pregnancies (?)	Thaler et al. [25] Hecht et al. [26] Pavlik et al. [27] Boxmeer et al. [28] Ebisch et al. [29] Murto et al. [32] Gaskins et al. [31] Haggarty et al. [33]	Women undergoing IVF *should* take folic acid to prevent NTDs. Current evidence *does not* support the use of folic acid supplements to improve IVF outcomes	Randomized trials are needed to address causality, detailed dose–response relation, and potential for effect modification by background food supply fortification and genetic background (e.g., MTHFR polymorphisms)
Phytoestrogens	• Mechanism unknown • May improve success of implantation through estrogenic properties	Unfer et al. [43] Shahin et al. [44] Unfer et al. [43]	There is *no evidence* to support phytoestrogen supplementation by women undergoing IVF	Randomized trials and observational studies are needed to assess reproducibility of findings, detailed dose–response relations, and timing of supplementation
Antioxidants	• Ameliorate oxidative stress	Showell et al. (review) [54] Ruder et al. [56]	There is *no evidence* to support antioxidant supplementation by women undergoing IVF	Further observational research can help narrow down the list of antioxidants that may have an effect on IVF and provide information on dose–response relationship
Vitamin D	• Embryo quality • Activating innate immune response in endometrium • Regulation of transcription of genes responsible for implantation and placentation	Rudick et al. [66] Ozkan et al. [67] Garbedian et al. [68] Rudick et al. [69] Firouzabadi et al. [70] Aleyasin et al. [71] Anifandis et al. [72]	There is *no evidence* to support vitamin D supplementation by women undergoing IVF	Further observational research could address residual confounding, particularly, residual confounding by BMI

In addition, large, well-designed randomized controlled trials are needed to address outstanding questions of causality for the most promising dietary factors.

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D.T. Carrell et al. (eds.), *Biennial Review of Infertility: Volume 4*,
DOI 10.1007/978-3-319-17849-3

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