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Current Concepts in Assisted Reproduction and Fertility Preservation (Part II)
Guest Editors: Sajal Gupta and Ashok Agarwal

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PREFACE

The Special Issue on "Recent Advances in Reproductive Endocrinology and Women's Health" published by the Current Women's Health Reviews is a series of two volumes containing articles on cutting edge technology and contemporary topics of importance to reproductive endocrinologists, general gynecologists and specialists alike. The second volume has excellent articles on many recent advances and state-of-the-art technologies related to the field of assisted production and fertility preservation in women.

Dr Kader et al. present recent updates on “Single Blastocyst Transfer: Contemporary Experience”. The authors state that the cryopreserved single blastocyst transfer has been developed as a practical method that can be used to optimize pregnancy outcomes following an unsuccessful initial transfer or in patients who do not meet initial transfer criteria.

In their article on “The role of oxidative stress in assisted reproduction”, Drs. Gupta et al. explain that sperm and oocytes used in assisted reproductive techniques are subjected to oxidative stress because gametes lack the natural antioxidant defences that are present during in vivo reproduction in the male and female reproductive tract. In the past, studies quantified OS using inconvenient and labour-intensive biochemical methods to measure reactive oxygen species (ROS) and assess the antioxidant status within follicular fluid and culture fluid. Metabolomic profiling is a faster, more accurate method of quantifying OS during ART and may be used to identify gametes and embryos that are more likely to contribute to successful implantation and pregnancy. Various studies on the benefits of oral antioxidant supplementation in male and female patients undergoing ART procedures have yielded inconsistent and conflicting reports, and further research is required.

Dr Ahmady et al. from the Macdonald IVF and Fertility program discuss pre-implantation and prenatal testing techniques that provide genetic information and detect birth defects or abnormalities in an embryo/fetus before implantation/birth. They emphasize that the limitations of FISH and PCR will lead to the use of whole-genome analysis/CGH in the setting of in vitro fertilization (IVF), replacing current PGD testing. Its preliminary application has been encouraging as more chromosomes can be analyzed with higher sensitivity and accuracy.

Dr Aboulghar et al. from the Egyptian IVF Center have contributed an excellent article on “coasting,” which is a popular method of preventing ovarian hyperstimulation syndrome (OHSS). The authors point out that while coasting does not totally avoid the risk of OHSS, it decreases its incidence in high-risk patients. They highlight the effectiveness of coasting in patients undergoing intracytoplasmic sperm injection (ICSI) and the fact that it does not jeopardize outcomes. Coasting for >3 days is associated with a moderate decrease in pregnancy rates. The authors state that coasting is a feasible option in patients undergoing IVF cycles with GnRH antagonists.

Dr Gardner et al. have contributed an excellent article that discusses state-of-the-art technologies such as metabolomics and their application in the ART field. The authors have stated that metabolomics technology is perceived as an important diagnostic tool in clinical IVF that has the potential to assess embryo viability prior to transfer or cryopreservation. The authors conclude that as an embryo selection technique, metabolomics screening will form an integral part of ART laboratories. This will lead to an enhanced ability to determine the functional metabolic phenotype of an embryo as a key indicator of viability. They also highlight that the affordability of the new techniques is likely to improve as they become more readily available and tailored to clinical IVF and as they integrate with other cost effective and efficient technologies such as chip-based analysis.

This special issue also contains 4 articles on fertility preservation by researchers from Cleveland Clinic, the University of Toledo and University Hospital-Case Western Reserve University. The article on the economics of female fertility preservation discusses the increasing demand of fertility preservation and obstacles that hinder the creation of a standard of care. The authors analyze the challenges to establishing higher quality care standards and provide suggestions for continued research and multidisciplinary collaboration for a larger patient population.

Dr Nasr and Dr Bedaiwy have provided an overview of new technologies for fertility preservation in an article titled “Emerging Technologies for Fertility Preservation in Female Patients.” The authors explain that most of the currently available strategies to preserve fertility in women are still experimental and do not guarantee subsequent fertility. The only established method in women is IVF with embryo cryopreservation prior to cancer therapy. Other proposed strategies to preserve fertility in women with cancer include storage of frozen embryos, frozen ovarian tissue or isolated follicles for in vitro growth and maturation. They emphasize that future research should focus on critically defining patient suitability, methods of tissue collection, optimal tissue size, choice of cryoprotectants and cryopreservation protocols, and the possible use of in vitro maturation (IVM) of oocytes for human ovarian tissue.

Dr Salle et al. from the Université Claude Bernard in Lyon France, write about the emerging technology of whole ovarian vitrification. They state that it is an experimental procedure and the advantages expected from this procedure have yet to be confirmed.

Dr Banerjee et al. delineated the importance of ovarian reserve testing in their article titled “Prognostic role of ovarian reserve testing.” The authors state that in clinical practice, each patient must be evaluated individually to utilize the best prognostic indicator of their reproductive outcome. They also express their view that with the development of predictive markers of ovarian reserve testing, ART will also require further improvement to improve their outcome.
We hope that the readers will enjoy reading the latest, informative and authoritative articles by some of the most recognized and prolific leaders in reproductive endocrinology from across the globe. We would like to extend our appreciation to all the authors for their hard work and valuable contributions. We are indebted to our colleagues and associates at Cleveland Clinic for their valuable contributions. We gratefully acknowledge the generous support of Ms. Amy Slugg Moore (Manager, Medical Writing Education Program) for her help. We are also grateful to Prof. Jose Belizan, Editor in Chief of Current Women’s Health Review, for his constant encouragement and support. We are most thankful to the editorial team of CWHR for their support and hard work. Finally, we extend our sincere thanks for the opportunity to serve as a Guest Editor on the special issue of CWHR. We are confident that readers will benefit from the latest knowledge in these valuable articles from the experts in their respective fields.

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Amr Kader, MD graduated from Alexandria University, Egypt in 1998 and joined the Cleveland Clinic’s Center of Reproductive Medicine and the Minimally Invasive Surgery as a Research Fellow in Gynecology from 2006 to 2009. During this period his research was focused on gametes and tissue vitrification and fertility preservation. He has many publications on vitrification and he holds a patent for the invention of the Ohio-Cryo, a closed vitrification device for tissue fragments. He earned many grants and awards such as the NIH trainee travel award (2007) and the ASRM best video presentation award (2009). He is currently an Ob-Gyn resident at the West Virginia University.

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Ali Ahmady is Assistant Professor of Reproductive Biology at Case Western Reserve Medical School, and Director of IVF & Andrology at University Hospital Case Medical Center, Cleveland, OH. He is a member of National Center for Regenerative Medicine. Dr. Ahmady produced the first live birth in the mouse using ICSI and investigated the fertilizing ability of dead sperm at the cell and molecular level. Dr. Ahmady is an author of a manual for IVF, co-author of a chapter in the atlas of embryology, and has published more than 20 peer-reviewed manuscripts.

Jashoman Banerjee, MD
Jashoman Banerjee, MD is a trained Ob-Gyn specialist from India. He is currently graduating as a chief resident in Ob-Gyn from the University of Toledo Medical Center in Toledo, Ohio. Dr. Banerjee will start his fellowship in reproductive endocrinology and infertility at Wayne state University. He has actively participated in extensive research involving endometriosis and infertility at the Cleveland Clinic. His other research interests are to explore effects of oxidative stress on oocyte quality and ovarian cryopreservation as means of fertility preservation. He has published his research work in peer reviewed journals.

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Dr. Bedaiwy received his medical degree from Assiut University School of Medicine, Egypt, where he graduated Valedictorian, summa cum laude. He is still on staff as an associate professor. He did a REI/MIS research fellowship at the Cleveland Clinic, during which he prepared a Doctorate thesis involving his research work in ovarian tissue cryopreservation and transplantation. He also completed a clinical fellowship in laparoscopic surgery in the same institution. Dr Bedaiwy has 67 peer-reviewed publications, 8 book chapters and 9 videos. He has received 15 national and international awards.

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Kathryn Coyne is a senior at the University of Notre Dame and is majoring in pre-professional studies. She is the President of the Irish Kids Fighting for St. Jude Kids club and volunteers at a clinic that serves uninsured residents of St. Joseph County. Kathryn interned at the Cleveland Clinic Center for Reproductive Medicine and past internships include working at the Spanish Red Cross in Toledo, Spain and the Ireland Cancer of University Hospitals of Cleveland. She has travelled on multiple medical brigades in Honduras and hails from Shaker Heights, Ohio. She plans on attending medical school in the future.
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Dr. Goldfarb is Director of Infertility Services and In Vitro Fertilization (IVF) for the Cleveland Clinic. He is also the current President of the Society for Assisted Reproductive Technologies. Dr. Goldfarb has been a pioneer in IVF. His program was responsible for the first IVF birth in Ohio (1983) and the world’s first IVF surrogate birth (1986). Dr. Goldfarb is also the co-founder of the Partnership for Families Program, a charitable organization that provides funding for second IVF cycles for qualifying couples and for fertility sparing procedures for qualifying cancer patients.

Sajal Gupta, MD

Dr. Gupta holds the position of Project Staff in the Glickman Urological Institute and serves as the Assistant Coordinator of Research at the Center for Reproductive Medicine. She has published over 40 review articles and scientific papers in peer-reviewed scientific journals. She is a member of several professional societies, including: American Society of Reproductive Medicine, American Society of Andrology, and the Society for the Study of Male Reproduction. Dr. Gupta is an ad hoc reviewer for Human Reproduction, Fertility & Sterility, and European Journal of Obstetrics and Gynecology. Dr. Gupta is a co-investigator or principal investigator on 8 research grants. Her current research interests include the role of oxidative stress in female infertility, endometriosis, assisted reproductive techniques and gamete cryobiology.

Kim D. Ly, BS, MBA

Kim D. Ly obtained her Bachelor of Science in Biology with a minor in Chemistry from Baylor University, Waco, TX, USA in 1998. She later obtained her Masters in Business Administration from the University of North Texas, Denton, TX, USA in 2000. She joins the Center for Reproductive Medicine Cleveland Clinic, Cleveland, OH, USA as a researcher with an interest in embryology, implantation failure, preimplantation genetic screening for aneuploidy, and blastocyst transfer.

Mohamed Aboulghar, MD

Dr. Mohammed Aboulghar is Professor of Obstetrics and Gynecology at Cairo University. He is the clinical director and founder of the Egyptian IVF-ET center at Maadi in Cairo, Egypt and the founder and first president of the Middle East Fertility Society (MEFS). He is also the editor in chief and the founder of the Middle East Fertility Society Journal (MEFS) since 1996. He has over 100 published papers in top international medical journals and over 100 papers in local and regional journals. He was awarded the Egyptian National Award for Excellency in Medical Sciences in 2000 and Honorary Membership of the European Society of Human Reproduction and Embryology at Berlin in 2004.

Bruno Salle, MD, PhD

Dr. Salle is a Professor of Reproductive Medicine at the Claude Bernard University, Lyon, France and the Head of the Fertility Clinic at the Hospices Civils de Lyon, University Hospital. He is in charge of a research program on fertility preservation at the INSERM. He has written many papers on ovary transplantation and ovarian cryopreservation or vitrification. He is a member of the European Society of Human Reproduction and Embryology, member of the task force of fertility preservation and a member of the International Society of Fertility Preservation.
George Thouas, PhD

George Thouas is a post-doctoral research fellow in reproductive biology, with specialization in oocyte and preimplantation embryo mitochondrial biology, and development of new ARTs. He has worked previously as a clinical embryologist, involved in specific research programs at Monash IVF, and embryo-toxicity testing for quality control. More recently, Dr Thouas has worked in biomedical engineering and biotechnology research, toward optimization of in vitro technologies for the life sciences. His latest appointment with Professor David Gardner at the University of Melbourne sees a return to his original specialization, to furthering the understanding of embryo metabolism and its application to embryo diagnostics.

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Ashok Agarwal is a Professor in the Lerner College of Medicine at Case Western Reserve University and the Director of Center for Reproductive Medicine, and the Clinical Andrology Laboratory at The Cleveland Clinic, Cleveland Ohio, United States. He has published over 500 scientific articles and reviews and is currently editing 10 text books in different areas of andrology/embryology, male and female infertility and fertility preservation. His research program is known internationally for its focus on disease oriented cutting edge research in the field of human reproduction. His team has presented over 700 papers at national and international meetings. More than 200 scientists, clinicians and biologists have received their training in Ashok’s Lab. His long term research interests include unraveling the role of oxidants-antioxidants, genomic integrity, and apoptosis in the pathophysiology of male and female reproduction.
Evidence-Based Management of Infertile Couples with Repeated Implantation Failure Following IVF

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Abstract: Embryo implantation depends on both embryo quality and the endometrial environment. Implantation failure has a complex, variable pathophysiology and is detrimental to the outcome of in vitro fertilization (IVF). Thus, patients with multiple implantation failure require an individualized approach to diagnosing and managing treatment options for future IVF cycles. These options should be based on concrete, unambiguous, consistent scientific evidence with randomized, controlled trials.

We review and discuss 14 treatment options: (i) blastocyst transfer, (ii) assisted hatching, (iii) co-culture, (iv) preimplantation genetic screening, (v) hysteroscopy, (vi) sildenafil, (vii) salpingectomy for tubal disease, (viii) oocyte donation, (ix) transfer of six or more embryos, (x) intratubal embryo transfer, (xi) natural cycle IVF, (xii) antiphospholipid antibodies (APA) testing and treatment, (xiii) allogenic lymphocyte therapy, and (xiv) IV immunoglobin therapy. The approaches were evaluated based on available information from studies, expert opinions, consensus, etc.

We conclude that blastocyst transfer, assisted hatching, salpingectomy for tubal disease, and hysteroscopy in IVF procedures are clinically effective. This review serves as a summary of current treatment options for clinicians to counsel patients and manage their expectations based on strong and reliable evidence.

Keywords: Repeat implantation failure, in vitro fertilization, endometrial receptivity, blastocyst transfer, assisted hatching, hysteroscopy, salpingectomy.

INTRODUCTION

The world’s first baby conceived by in vitro fertilization (IVF) was Louise Joy Brown in 1978. Today, more than 3 million children worldwide have been born as a result of IVF. The numbers continue to increase as science discovers ways to overcome barriers for various subgroups of infertile patients. However, despite the improvements in IVF technology and methods, many couples experience multiple IVF failures. After each failed IVF attempt, pregnancy rates in subsequent attempts decrease by as much as 57% with the most remarkable decrease after the third attempt [1, 2]. The main causes of multiple IVF failures include: (i) poor response to ovarian stimulation, (ii) repeated fertilization failure, (iii) repeated difficult transfers, and (iv) repeated implantation failures (RIF).

Implantation failure is a major limiting step for IVF [3]. The process of embryo implantation is described as having three phases: 1. Apposition: “unstable adhesion” of the transferred embryo to the surface of the uterine lining. 2. Attachment (adhesion): “stable adhesion,” believed to involve signaling back and forth between the embryo and the lining. 3. Penetration (invasion): invasion of the trophoderm cells from the embryo through the surface of the lining deeper into the stroma of the uterine lining, forming a vascular connection to the mother.

The etiological causes of implantation failure include embryo quality; endometrial receptivity; immunological factors; uterine, tubal and peritoneal factors; and culture media [3]. Poor response to superovulation and chromosomal aneuploidy due to advanced maternal age negatively affect embryo quality; suboptimal embryos are less likely to implant. The disruption in prostaglandin synthesis is one of many factors that decrease endometrial receptivity in some patients prone to RIF [4]. Other cellular and adhesion pathways affected by abnormal gene expression in the endometrium have been observed to be linked to RIF [5]. The cultured endometrial cells of RIF patients were found to have different gene expressions than those found in the cultured endometrial cells of women who miscarried or had an ongoing pregnancy [6]. From this observation, the differential gene expression of RIF patients is assumed to negatively affect critical signaling pathways important for the development of adhesion molecules in the embryo-endometrium bond and may be linked to implantation failures. Immunological factors such as antiphospholipid antibodies (APA), abnormal expression of endometrial natural killer cells, cytokines [3], local and systemic immune factors, anti-sperm antibodies, and anti-thyroid antibodies [7] have been found in significant amounts among RIF patients and have been reported to affect implantation. APA interferes with the normal function of blood vessels by either causing narrowing/irregularity of the blood vessels (vasculopathy) or by causing the develop-
ment of blood clots in the blood vessels (thrombosis). In the majority of cases, failed implantation appears to be related to the quality of embryos transferred rather than to the endometrial receptivity. Part of the evidence stems from the significantly higher implantation rates found in egg donation programs, even in couples that have failed IVF repeatedly using their own eggs.

In this paper, we focus on the options supported by clinical evidence to improve implantation and pregnancy rates for couples with multiple IVF failures. Evidence-based medicine has three levels of recommendation. Level A recommendations are based on good and consistent scientific evidence with randomized, controlled trials. At level B, recommendations are based on limited or inconsistent scientific evidence with clinical controlled trials, cohort, etc. Level C includes recommendations based primarily on consensus and expert opinion. Clinicians would benefit from knowing to which category a treatment option belongs to be able to counsel patients and manage their expectations for future IVF cycles. We will examine 14 approaches to repeated implantation failure in IVF: (i) blastocyst transfer, (ii) assisted hatching, (iii) co-culture, (iv) preimplantation genetic screening, (v) hysteroscopy, (vi) sildenafil, (vii) salpingectomy for tubal disease, (viii) oocyte donation, (ix) transfer of six or more embryos, (x) intratubal embryo transfer, (xi) natural cycle IVF, (xii) APA testing and treatment, (xiii) allogenic lymphocyte therapy, and (xiv) IV immunoglobulin therapy. (See also Table 1).

### Table 1. Fourteen Possible Approaches to the Management of Infertile Couples with Repeat Implantation Failure

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<td>Blastocyst transfer</td>
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<td>Assisted hatching (AH)</td>
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<td>Co-culture</td>
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<td>Preimplantation genetic screening (PGS)</td>
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<td>Hysteroscopy</td>
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### BLASTOCYST TRANSFER

Embryo transfer occurs either during the cleavage stage on Day 2/Day 3 or during the blastocyst stage (the embryo has inner cell mass, trophectoderm layer, and blastocoele) on Day 5/Day 6. Several studies have demonstrated higher rates of implantation and pregnancy with blastocyst culture and transfer compared with Day 3 cleavage stages [8-11].

Recent advances in culture technology have been made due to a better understanding of early embryo metabolism, and these advances have enabled researchers to culture zygotes to the blastocyst stage prior to implantation in the uterus. The original intent of blastocyst transfer was to select the healthiest embryos for transfer. Thus, a single or the fewest possible embryos may be transferred per cycle to reduce high order pregnancies, which are associated with very real, serious risks to mother and baby. Several advantages of culture and transfer of blastocysts make this option appealing for RIF patients. Self-selection of blastocysts increases endometrial receptivity. The trophectoderm cells of the blastocyst cross-talk with the endometrium and develop the ability to attach to the lining of the endometrial lining of the uterus, both of which are likely to improve implantation success. However, blastocyst culture and transfer usually is recommended for patients with a good prognosis—those who are younger in age with > 6 oocytes available from an uneventful ovarian stimulation. Sequential media, a two-part culture medium, commences with the embryo initially grown in a media rich with pyruvate to Day 3. The embryo is then transferred to the second media, rich with glucose, on Day 3 to support embryo growth from the eight-cell stage to the blastocyst stage.

A study by Weissman et al. observed a significant improvement in pregnancy rates from blastocyst culture and transfer to patients who were young, did not have multiple IVF failures, who produced multiple oocytes, and whose zygotes developed into good quality cleavage-stage embryos [1]. However, for patients in the general population with a single failed IVF attempt, pregnancy and implantation rates were observed to significantly decrease with subsequent IVF attempts employing blastocyst culture and transfer strategy [12]. In patients with multiple implantation failures, Cruz et al. reported a positive correlation between blastocysts and improved implantation and pregnancy rates for RIF patients in a non-randomized population [13]. However, a prospective, randomized control study by Levitas et al. that specifically examined the effects of blastocyst transfer for RIF patients compared with Day 2 embryo transfer found that blastocyst transfer was beneficial only in patients with an acceptable response to ovarian stimulation [14]. However, there was no difference in the incidence of multiple pregnancies between the groups. Blastocyst transfer on Day 5 for RIF patients permits the selectivity of a higher quality embryo after embryonic genomic activation has occurred [15]. It may also decrease the rate of ectopic pregnancy because of the larger diameter size of the Day 5 embryo; because the blastocyst normally resides in the uterus, an improvement to receptivity is also expected.

### ASSISTED HATCHING (AH)

Differences in embryo development during IVF treatment compared with in vivo are well-documented. One observed difference is the hardening of the zona pellucida (ZP), which prevents the blastocyst from escaping the ZP during hatching. The culture medium, which differs in its available nutritive source from the in vivo environment, and the cryopreservation method have been implicated as possible causes of implantation failure [16].

Assisted hatching was developed as a workaround to the hardened ZP of IVF embryos. It involves thinning or cre-
ating a hole in the ZP, either of which can be performed by a mechanical (glass pipette), chemical (acidic Tyrodes’s or enzymes), or laser method (contact and non-contact) with equal efficacy. The industry standard today includes the use of laser-assisted hatching for rapid and precise drilling. AH is not a benign procedure since the time between the zona drilling and embryo transfer is a vulnerable period for the blastocyst as it is exposed to foreign elements, undefined antibodies, or surveillance cells in the endometrial environment. However, blastocysts are believed to be more prepared for exposure to uterine lymphocytes and immune cells at this stage [17]. In addition, an increased incidence of monozygotic twinning may occur during the ablation procedure, which in some reports has caused herniation and division of the inner cell mass into two [18]. This may result from (i) a too-narrow opening of the ZP, trapping the blastomere in a figure-eight formation, thus causing a subdivision of the blastocyst to form twins or (ii) a premature hatching of the blastocyst from a too-large opening combined with loosening of the tight junctions between blastomeres to cause division of the inner cell mass to form twins [18].

A Cochrane review of AH that included 28 randomized control trials from 24 publications or abstracts shows an increase in clinical pregnancy rates for women with RIF but suggests additional research is needed [19]. There is no evidence supporting AH after one failed IVF attempt, but women with multiple implantation failures benefited most from AH [19]. The review’s validity, coupled with multiple good quality, randomized, controlled studies and strong recommendations, suggest that AH does improve implantation rates for women with multiple implantation failures (3+) due to hardening of the ZP, occurring mainly from cultured IVF treatment or methods of embryo cryopreservation.

CO-CULTURE

Co-culture refers to the placement of human and non-human live cell types (feeder cells) alongside the embryo during in vitro culture. It has been suggested that co-culture improves embryo growth and development by improving in vitro parameters—it removes toxic substances such as heavy metals and ammonium and free radicals [20].

Endometrial (Simon, Mercader et al. 1999, Weichselbaum, Paltieli et al. 2002) and granulose [21-23] cells are commonly used in co-culture. Studies have demonstrated conclusively the existence of cross-talk between endometrial cells and embryonic cells, resulting in a paracrine release of molecules believed to improve implantation [20]. The presence of uterine epithelial cells, the first cells in contact between maternal and fetal cells, may initiate a signaling cascade for cells from the blastocyst to become depolarized in preparation for endometrial attachment, thus improving embryo competence for implantation [24].

The heterogeneous co-culture cell lines that have been utilized include human and bovine oviductal cells [25-30], bovine uterine epithelial cells [31, 32], African Green Monkey kidney cells (Vero cells) [28, 33-35], ovarian cancer cells [36], buffalo rat liver cells [37], and human skin fibroblasts [38]. One randomized study using conventional media compared the various types of co-culture cell lines and found that granulosa cells and bovine oviductal uterine epithelial cells were associated with a higher percentage of early embryos developing to the eight-cell stage.

In some countries, including the United States, non-human cell types have been banned by the Food and Drug Administration due to concerns regarding disease transmission from the cell types to the developing embryo or mother. Consequently, the use of autologous endometrial cells has gained in popularity [39]. Several different human cell types are now available, including cumulus cells, luteinized granulosa cells, fallopian ampullary epithelial cells (FAEC), and endometrial epithelial cells [40]. No randomized controlled studies are available to compare and determine which of the human cell types provides maximum benefit.

In a study by Weichselbaum et al., very poor quality embryos, in which more than 50% of embryo volume had fragmentation and the blastomeres were of unequal size with grany to dark cytoplasm, were rescued from cleavage arrest and degeneration when co-cultured with fallopian ampullary epithelial cells. In addition, blastocyst formation increased by 56%, suggesting that co-culture may help embryos overcome developmental incompetence [40]. Ehyremendy et al. used the best embryos from women with multiple implantation failure to co-culture with monolayers of autologous endometrial cells [41]. Of the 68 patients who failed to become pregnant after multiple IVF treatments, 39 become pregnant, 19 attained a live birth, and 10 remained pregnant beyond 12 weeks. The study also suggested that an endometrial biopsy should be performed about 7 days after ovulation and that the co-culture medium contain both stromal and glandular cells in the monolayer to improve implantation rates [41]. In 2008, Desai et al. reported for the first time the success of a human endometrial culture system in a clinical environment, thus supporting the two previously mentioned studies [42].

Interestingly, although the benefits of co-culture media have been demonstrated over the years, its prevalence is not widespread, perhaps because it remains a labor-intensive and unproven technique [41]. Several limitations must be overcome to realize its benefit. The most serious limitation is that supplementation of human or non-human live cells with the culture media results in an uncontrolled and undefined alteration to the conditions of the media with unknown growth factors in unknown concentrations [43]. The introduction of sequential media in the past decade has slowly replaced not only non-human but also human cell types because of its safety and ease of use [44]. Additional research is needed in the area of co-culture, with special attention to the type of cells and type of culture media (conventional or sequential media), to support its use prior to its widespread adoption in clinical settings.

PREIMPLANTATION GENETIC SCREENING

Chromosomal abnormalities have been widely reported to be a major cause of early spontaneous abortions in as much as 60% of the general population [45]. In couples with multiple implantation failures undergoing fertility treatment, the frequency of chromosomal abnormalities appears to be higher regardless of maternal age [46-49]. Specifically, embryonic aneuploidy in patients with multiple implantation failures was 54-57% compared with 35% in a control group.
Thus, preimplantation genetic screening (PGS) could be utilized as an appropriate means for selecting normal embryos to improve implantation and pregnancy rates and, ultimately, live birth rates in couples with RIF. However, recent studies present conflicting data supporting the use of PGS for couples with RIF.

The European Society of Human Reproduction and Embryology (ESHRE) PGS Consortium Steering Committee reports the use of PGS as having little impact on improving the pregnancy rate for women with RIF [52]. In addition, Gianaroli et al. showed that PGS did not increase implantation or pregnancy rates per embryo transfer in RIF patients [53]. Other data from ESHRE’s review, ESHRE PGS Consortium IX, demonstrated that 57 IVF centers performed 748 IVF cycles with oocyte retrieval for RIF, which resulted in a 27% clinical pregnancy rate, 24% implantation rate, and an 11% delivery rate between January and December 2006 [54].

For comparison, the 27% clinical pregnancy rate for PGS in RIF patients is better than the 18% clinical pregnancy rate in the total patient population undergoing PGS for various reasons. This latest data collection from 2006 highlighted RIF as a main indication for PGS in fertility centers despite the ongoing debate regarding its efficacy [54]. In a retrospective study of 121 first PGS for RIF, multivariate logistic regression analysis was utilized to generate a predictive model [55]. The model demonstrated that to have a 90% probability of having an embryo transfer after PGS, the patient should have at least 10 mature oocytes, eight normally fertilized oocytes, and six embryos for biopsy on Day 3.

The cause of aneuploidy among RIF patients differs from other cases of random failed implantation. One main characteristic of chromosomal abnormality found in preimplantation embryos of couples with multiple implantation failures is the low probability of meiotic errors resulting in chromosomal abnormalities [56, 57]. Two studies by Mantzouratou et al. and Voullaire et al. have shown that meiotic errors are an unlikely cause in this group of patients and that chromosomal abnormalities are reflective of an inefficiency in mitotic division due to abnormal cell cycle regulation by the embryo. This may explain the lower rate of success in the management of fertility for couples with RIF in studies using polar body analysis.

In 2009, Fragouli et al. released a study comparing results of PGS between polar body I, polar body II and blastocyst stages in which the aneuploidy rates were 36.5%, 45.8%, and 45.2% after meiosis I, meiosis II, and mitosis, respectively [48]. Though the numbers appear to indicate that aneuploidy rates are higher after meiosis II, errors from meiosis I carried into meiosis II should be considered. The higher aneuploidy rate at the blastocyst stage may be explained by the fact that the embryo is more vulnerable to developmental arrest between the four-cell and eight-cell stage as it switches from maternal to embryo gene expression [58]. Maximum embryonic gene expression has been shown not to occur until the blastocyst stage. Therefore, the disturbed immature cell cycle regulation increases the likelihood of chromosomal abnormalities, which persists to the blastocyst stage, thus reducing the likelihood of successful implantation. These observations suggest that future studies should focus on understanding the embryonic role in RIF by sampling the blastocyst stage where maximum embryonic gene expression occurs [59].

The occurrence and pathology underlying complex chromosomal abnormality (three or more whole chromosome imbalance) was another characteristic abnormality found in patients with RIF in a recent study using array comparative genomic hybridization (CGH) analysis of chromosomal material in Day 3 cleavage-stage embryos. Chromosomal breakage and failure of mitotic cell cycle checkpoints to detect abnormalities has been suggested as the cause of mosaic complex chromosomal abnormalities in nearly 30% of RIF patients [57, 60]. This study was further supported by the use of CGH analysis of blastocysts [48]. Abnormal replication and segregation of chromosomes during early embryo development of RIF patients is likely caused by maternal cytoplasmic factors or mutation in the cell cycle control genes [61]. Thus, the use of PGS in identifying abnormal embryos in advance to improve implantation is restricted.

Fluorescent in situ hybridization (FISH) is the most commonly used method today to detect chromosomal aneuploidy. However, FISH techniques can only analyze a small subset of the chromosomes, usually the most commonly involved in aneuploidy. Embryonic mosaicism (multiple germ cell lines in one embryo) further complicates analysis of test results in terms of reliability since only one blastomere, representing the entire embryo, is removed for testing. According to one study, 38% of embryos were incorrectly graded as normal using a five-probe set on blastomeres [57]. In another study using a nine-probe set, 25% of embryos were misdiagnosed [62]. In the most recent study, which used a 12-probe set, 19% of embryos were incorrectly graded as normal [48]. The acceptable error rates from the studies suggest FISH is able to reliably detect aneuploidy in mosaic embryos and further implies that mosaic embryos have a sufficiently high ratio of abnormal-to-normal blastomeres for cleavage-stage biopsy to serve as clinically useful. Despite several recent advances in diagnostic methods, including whole genome amplification with comparative genomic hybridization and the use of microarrays to overcome the limitations in FISH, identifying complex chromosomal abnormalities has limited success, is labor-intensive, and costly [65].

Currently, convincing evidence for the wide use of PGS in RIF is insufficient. The technique did not improve implantation rates for RIF patients [53, 63, 64]. Moreover, some normal embryos might be lost due to the error rate. Furthermore, with the advent of less invasive methods for predicting better quality embryos such as metabolomics and proteomics, PGS may become a less popular option. It is not likely to happen anytime soon because no studies to date have identified the significant metabolites or proteins involved in early development that are unique to embryos with a low implantation success rate. The role of PGS in the management of RIF patients remains unresolved.

**HYSTEROSCOPY**

Hysteroscopy is an invasive diagnostic procedure that is used to visualize uterine pathologies, including submucous fibroids, polyps, intrauterine adhesions, and uterine malfor-
receptivity is believed to improve with increased blood flow. A dilator used to improve vascular supply [71]. Endometrial bleeding for the treatment of male erectile dysfunction, is a vaso-suppressant. Endometrial cellular matrix, regulate cell growth, and induce angiogenesis: (i) p53 codon 7 tumor suppressor factor, (ii) plasminogen activator inhibitor 1 (PAI-1), and (iii) vascular endothelial growth factor (VEGF) – 1154. The study found a link between one or more of the genes and patients with repeat implantation failure. A significantly higher correlation existed for RIF patients than the control group. More studies are needed to confirm the findings, which could facilitate the clinician’s ability to identify and counsel patients prone to implantation failure [72].

Several studies have focused on increasing endometrium thickness with sildenafil therapy for women with RIF. In 2000, Sher and Fisch applied sildenafil vaginally in women with RIF and a thin endometrium to increase blood flow for endometrial growth. They hypothesized that a thicker endometrium (> 8 mm) may improve implantation and pregnancy rates [73]. The preliminary study showed that three of the four women had a successful pregnancy outcome after sildenafil treatment during the proliferative phase. Two years later, Sher and Fisch released a follow-up study on 105 patients who were given sildenafil; 70% developed endometrial thickness ≥ 9 mm with the remaining patients (30%) being ≤ 9 mm. Of the patients who developed endometrial thickness ≥ 9 mm, 45% had a significant implantation rate [74]. The study does not explain why the majority (55%) of women treated with sildenafil experienced optimal endometrial growth but subsequent IVF failure. However, it highlighted the importance of endometrium quality [75].

In a case report on two patients with Asherman’s syndrome, sildenafil improved endometrial thickness but did so by a much smaller margin. The endometrium of one woman increased from 6.5 mm to 8.9 mm, and another woman’s increased from 5.0 mm to 6.6 mm. Despite suboptimal endometrial measurements of ≤ 9 mm, both women had healthy offspring [76]. In a study by Paulus et al., the data did not support Sher and Fisch’s findings or those of Zinger et al. In 10 women with at least one IVF failure, sildenafil increased endometrial thickness, but only three of 10 patients had a successful pregnancy. However, the heterogeneous and small patient population may have affected the results [77].

Natural killer cell activity levels, which have been reported as a predictor for recurrent miscarriages, may also be involved in RIF [78-80]. An increase in both peripheral blood natural killer cells and endometrial natural killer cells appears to be associated with lower pregnancy rates in patients with recurrent miscarriages. Upon activation with nitric oxide, the natural killer cells release cytokines such as tumor necrosis factor-α, which has been implicated as a cause of implantation failure [81]. One study extended the hypothesis to suggest that repeated implantation failures may be caused by high levels of peripheral natural killer cell activities. It showed that sildenafil lowered peripheral blood natural killer cell activity, thereby improving the local endometrial immunological environment in women with multiple IVF failure. However, more randomized control studies must be done to confirm these findings. It also will be important to determine the appropriate period to prescribe sildenafil therapy [75].

In a pilot study, the amount of NO released by the embryo in vitro correlated with implantation success. It was reported that higher quality embryos producing an elevated...
amount of NO in culture media had a higher success rate than the controls [82]. Thus, the use of sildenafil to block the breakdown of cyclic guanosine monophosphate (cGMP) causes an accumulation of NO. As a result of increased NO, radial uterine blood vessels dilate to increase the vasculature and perpetuate endometrial growth. However, as NO induces natural killer cells to produce cytokines that can cause implantation failure, it has been recommended that sildenafil not be used five or more days prior to embryo transfer [81]. On the other hand, one study using mouse embryos reported that higher concentrations of NO in vitro and in vivo resulted in lower implantation rates in a dose-dependent manner [81]. The same higher concentration of NO (1 mM) inhibited implantation in vitro as it did for mouse embryos. Cytostatic and cytotoxic effects resulting from an extended production period of NO in reproductive tissues to protect against infection, immunological reactions, and pathological conditions (e.g., endometriosis, reproductive tract infections) is a suggested cause for the lower implantation rates found in the mouse embryo study [81].

The effects of sildenafil therapy on the endometrium and nearby environments should be better understood before it is widely adopted in IVF clinics for RIF patients. Natural killer cells and NO play important roles in the female reproductive tract. Alterations in NO-synthase (an enzyme that converts the nitrogen in L-arginine to NO in the presence of NADPH and dioxygen) and/or NO production of these tissues could directly affect the development of human embryos, especially during the early stages of pregnancy.

**SALPINGECTOMY FOR TUBAL DISEASE**

Of all the different tubal pathologies, hydrosalpinx (disruption of the fingered portion of the fallopian tube due to an abnormal accumulation of fluid or water most likely resulting from an inflammatory response) has the most detrimental effect on implantation [83]. Generally, hydrosalpinx is caused by abortion, pelvic inflammatory disease, endometriosis, previous operations, a history of tuberculosis and peritonitis, or an unknown reason [84]. It usually occurs on both sides but can occur exclusively on one side. Interestingly, a one-sided hydrosalpinx will usually correspond with an abnormal fallopian tube on the opposite side. For women with infertility problems, removing the fallopian tubes is a major decision. Nonetheless, hydrosalpingectomy has been reported as a promising option for a subgroup of RIF women with severe tubal factor infertility [85, 86]. The main theoretical reasons for its use as a treatment option are (i) the embryotoxic effect of the fluid in the region by leakage of hydrosalpinx fluid, resulting in either endometrial alterations that are hostile to embryo implantation and development [87]; (ii) the mechanical wash out of the embryo [88] before it has a chance to implant; (iii) altered endometrial receptivity due to variations in the levels of certain biomarkers such as LIF [89] and αβ3 integrin [90, 91], (iv) release of intrauterine cytokines, prostaglandins, leukotrienes, and other inflammatory compounds directly to the endometrium or via the circulatory or lymphatic system [92, 93], or (v) chronic endometriosis caused mainly by asymptomatic Chlamydia trachomatis [94]. Thus, some studies support preventative salpingectomy for infertile patients meeting two criteria: (i) large hydrosalpinges visible by ultrasound and (ii) bilateral hydrosalpinges [85, 86]. The emphasis on the severity of the tubal disease (see Table 2 for hydrosalpinx scoring system) for salpingectomy as a treatment option is important since it limits or removes chances for a natural conception and is likely to result in disruptions to ovarian blood flow and nerve supply and reduce ovarian reserves [95] -- all of which are important for follicle production, hormone production, and the number and quality of the ova [84]. An additional criterion for irreversibility of the tubal condition should also be included so that patients have the option to maintain their fallopian tubes.

A study by Dechaud et al. reported significantly higher implantation rates per transfer among women <41 years of age who had experienced RIF after laparoscopic bilateral hydrosalpingectomy compared with those who did not [96]. The pregnancy rate was also higher for the group with salpingectomy than the group without (23.5% and 9.9%, respectively, P value = 0.01). Bilateral salpingectomy also was reported to not only increase implantation rates but also decrease the time to pregnancy. A group of women who underwent salpingectomy became pregnant within three IVF attempts as opposed to a group who did not undergo salpingectomy in which some patients required as many as 11 IVF attempts to become pregnant [96]. Furthermore, a recent Cochrane study for the surgical treatment of tubal diseases performed a meta-analysis on five randomized trials and found a significant increase in clinical pregnancy rates and ongoing pregnancy rates for patients with hydrosalpinges who underwent salpingectomy and IVF (for the first time) as compared with those who did not undergo surgical intervention [97]; thus, salpingectomy should be considered for all patients with ultrasound-visible hydrosalpinges.

In contrast, there is an argument in preference of tubal microsurgery, which is a less invasive procedure that pre-

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**Table 2. Hydrosalpinx Scoring System (Puttemans and Brosens 1996)**

| I. | Simple hydrosalpinx represented by a thin-walled translucent hydrosalpinx with flattened and separated mucosal folds in a single lumen but without mucosal adhesions. |
| II. | Hydrosalpinx foliculans represented by a thin-walled hydrosalpinx with mucosal adhesions which can be focal or extensive. As a result the tubal lumen may become divided into locules by agglutinated folds forming compartments or pseudoglandular spaces. |
| III. | Thick-walled hydrosalpinx represented by an ampullary wall >2 mm thick and absent mucosal folds or some fibrotic fold remnants at most; tubal distension is less marked and the amount of invisible intralumina fluid is less abundant. |

serves the fallopian tubes, for women with less severe tubal pathology [84]. The higher cost and greater time commitment make it a less popular option among clinicians. Also, no studies have been done to compare efficacy, recovery, psychological factors, risks, invasiveness of tubal microsurgery (a surgical procedure to repair and open the fallopian tubes that is sometimes referred to as tuboplasty) versus hydrosalpingectomy [98]. Perhaps a randomized controlled study to compare salpingectomy and tubal microsurgery for RIF patients with various severity of hydrosalpinx could determine the optimal procedure in the appropriate patient population. Additional randomized controlled studies are needed to clarify whether unilateral or bilateral salpingectomy is more appropriate and necessary than tubal surgery to minimize unnecessary removal of healthy fallopian tubes [97].

OOCYTE DONATION

In the past two decades, oocyte donation has become a valuable and effective therapeutic option for infertile patients who choose assisted reproductive technology (ART) and has become an acceptable indication for patients with multiple implantation failures [99]. Egg donors are prescreened for medical and physical history of infectious disease, genetic disorders, polycystic ovarian syndrome, serious malformations (resulting in severe functional or cosmetic handicap such as spina bifida or heart malformations), mental health, and uterine pathologies (for accessibility to oocyte retrieval) and then selected based on age and phenotypic match to the recipient [99, 100]. The screening process also extends to the oocyte recipients (medical, physical, psychological, and social) and their partners (for infectious disease and paternal factors) to identify other possible sources that may adversely affect the outcome.

Three groups of patients, those with (i) repeat implantation failure and no other indications, (ii) RIF in combination with advanced age, or (iii) RIF in patients with balanced translocations in homologous chromosomes, may benefit from oocyte donation [100-102] to overcome factors that have been implicated in implantation failure such as genotype and age of the oocyte. [100]. Two committees, the American Society for Reproductive Medicine (ASRM) and Society for Assisted Reproductive Technology (SART) published the 2008 Guideline for Gamete and Embryo Donation, which provides the most recent recommendations and information from the U.S. Center for Disease Control (CDC), U.S. Food and Drug Administration (FDA), and American Association of Tissue Banks (AATB) for optimal screening and testing of oocyte donors and recipients (see Table 3). The guideline specifically identifies women with “multiple previous failed attempts to conceive via ART” as an indication for oocyte donation [99].

More recently, the advancement of cryopreservation methods and technologies for freezing and storing of oocytes has increased the number of available unused oocytes [103]. Patients undergoing controlled ovarian stimulation to cryopreserve their oocytes prior to treatment for illnesses that pose a serious threat to their future fertility (e.g., cancer) have the option to donate their extra oocytes for research or a donor bank once they no longer need the oocytes. Accordingly, RIF patients have a choice between using fresh and cryopreserved oocytes. Although the latter option is available and supported by the ASRM as a fertility preservation strategy, there is not enough evidence to support its safety and efficacy at this time [104]. For the first time, a recent prospective, randomized study comparing fertilization and embryo development rates and ongoing pregnancy rates found no difference between fresh oocytes and vitrified oocytes fertilized via intracytoplasmic sperm injection and then developed in vitro [105]. Further clinical studies are needed to clarify the long-term safety concerns to support the use of cryopreserved oocytes.

TRANSFER OF SIX OR MORE EMBRYOS

A definitive answer to the question of what constitutes the proper number of embryos for transfer in IVF has eluded

Table 3.   ASRM and SART Guidelines for Evaluating the Oocyte Recipient

| I. | Provide psychological counseling by a mental health professional and further psychological consultation if necessary prior to consent. |
| II. | Obtain medical physical examination and reproductive history to detect reproductive abnormalities. Provide treatment as appropriate prior to use of donor oocytes. |
| III. | Complete a general physical exam and pelvic exam. |
| IV. | Assess the uterine cavity with hysterosalpingography or similar device to detect any significant uterine abnormality. |
| V. | Other recommended tests including: |
|   | a. Blood type, RH factor, and antibody screen |
|   | b. Rubella and varicella titers |
|   | c. HIV-1 and HIV-2 |
|   | d. Serologic test for syphilis |
|   | e. Hepatitis B surface antigen |
|   | f. Hepatitis B core antibody (IgG and IgM) |
|   | g. Hepatitis C antibody |
|   | h. Cervical cultures or similar tests for Neisseria gonorrhoeae and Chlamydia trachomatis |

In 1995, Azem et al. reported a significant increase in pregnancy rates with the transfer of six or more embryos in comparison with five in women with repeated implantation failure (at least four prior failed IVF-ET attempts) [106]. No other published evidence has demonstrated improved pregnancy or live birth rates after the transfer of more embryos in subsequent IVF-ET cycles than the number transferred in previous failed cycles. Despite this lack of evidence, the transfer of greater numbers of embryos than the recommended guidelines in women with multiple fresh IVF-ET failures is commonly performed in practice. This has also been extended to women predicted to have a poor conception prognosis based on indicators such as embryo quality, also with little supportive evidence regarding efficacy [107].

On the other hand, women undergoing IVF with multiple embryo transfer face an increased risk of higher-order multiple pregnancies (HOMP) with their known medical, social, and economic consequences.

The first guidelines on the number of embryos to transfer were issued by the ASRM in 1996 [108]. These guidelines were revised four times since then, with a subsequent reduction in HOMP. As recently as 2003, the triplet rate for IVF in women younger than 40 was approximately 6%. In 2007, it was less than 2%, and this decrease is directly related to the decrease in the number of embryos transferred per cycle [109].

In the latest ASRM guidelines, issued in November 2009 [110] (see Table 4) the maximum recommended number of embryos to transfer is five, and this number only applies to women aged 41-42. For all patients with one or two previous failed fresh IVF cycles, the guidelines also recommend transfer of one supplementary embryo (in comparison with the standard recommended number according to age group, see Table 4), after proper counseling regarding the risk of HOMP and justification in the patient’s medical records [110]. This only brings the number of embryos to transfer to six in the 41-42 age group if previous failed IVF attempts are documented. For all other age groups, the numbers are even more limited (as low as 1-2 transferred embryos for women younger than 35). The Society of Obstetricians and Gynaecologists of Canada (SOGC) goes even further in their restrictions by considering the transfer of more embryos than recommended only “in exceptional cases when women with poor prognoses have had multiple failed fresh IVF-ET cycles” with a level of evidence/recommendation III-C [111]. The most recent Cochrane systematic review on the number of embryos to transfer in IVF compares pregnancy rates and chances of multiple pregnancies following single versus double, three and four embryo transfer in fresh IVF treatments with various results, reflecting the experience of selected young women in a single fresh cycle of IVF/ICSI. As such, data concerning older women and women with previous multiple failed IVF attempts have yet to be assessed [112].

IVF practices will most likely be modified as scientific advances allow a more accurate assessment of the implantation potential of a given embryo, which will most likely include the determination of the embryonic genome and the metabolic and proteomic fingerprints of viable versus nonviable embryos using microarray technology.

**INTRATUBAL EMBRYO TRANSFER**

Embryo transfer is a vital step of IVF treatments. Therefore, in patients with repeated implantation failure, clinicians frequently focus on the transfer procedures to enhance embryonic implantation following IVF [113, 114]. Tubal transfer of embryos or zygotes has been widely utilized as part of treatments with various results, reflecting the experience of selected young women in a single fresh cycle of IVF/ICSI. As such, data concerning older women and women with previous multiple failed IVF attempts have yet to be assessed [112].

Zygote intratubal transfer (ZIFT) has been hypothesized to have many advantages over transcervical embryo transfer (ET), mainly that it provides a “natural” growth milieu for zygotes under physiological regulation with numerous growth factors and cytokines from the tubal fluid, which helps these zygotes attach to the uterus with greater synchronization, thus enhancing implantation potential [117]. After all, natural Day 1/Day 2 embryos belong in the fallopian tubes and not the uterus. Environment or *in vitro* culture systems play a crucial role in the early development stages of the embryo, and a suboptimal environment may have adverse effects. The ZIFT technique also prevents spillage of embryos after transcervical ET and solves the problem of

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*Favorable = first cycle of IVF, good embryo quality, excess embryos available for cryopreservation, or previous successful IVF cycle.
technically difficult ET in patients with cervical stenosis [61]. Nowadays, the environmental advantage of tubal transfer seems limited since laboratory conditions have improved along with the composition of culture media over the last two decades.

Although initial retrospective reports of ZIFT showed higher pregnancy rates than with intrauterine ET [118-120], other investigators have found that the main value of ZIFT is limited to RIF cases [115]. ZIFT was reported to be a valid alternative to standard ET in most subgroups of patients with either first or multiple failed attempts at IVF [114, 115]. It was demonstrated that in patients with tubal factor and a confirmed patency of one fallopian tube, ZIFT can be applied successfully as a treatment for RIF; the pregnancy rates and implantation rates for all ZIFT cycles in RIF patients were 35.1% and 11.1% - significantly higher PRs and IRs as compared to standard transcervical ET [121]. This study concluded that ZIFT should be recommended to IVF patients with a mild form of tubal factor and proved patency of one tube, whereas in severe forms, salpingectomy remains the recommended treatment.

The interest in intratubal transfer was greatly diminished after the results of a meta-analysis and a randomized, controlled trial that failed to demonstrate any advantage for ZIFT over standard IVF/ET [122]. Furthermore, a study by Aslan et al. including 229 patients with RIF showed comparable outcomes following ZIFT and transcervical ET [123]. However, these studies are not readily comparable due to different methods and selection criteria. It is likely that a patient’s age, number of previous IVF attempts and etiology of infertility may influence the results [123]. A number of drawbacks to the use of ZIFT exist, such as the need for general anesthesia, laparoscopy, and heavy medical equipment, which increases the medical risks to the patients, as well as the expenses related to the need for operative conditions [123]. An association between tubal embryo transfer and the increased risk of ectopic gestation has been inconsistently reported [122, 123].

ZIFT is normally performed in the pronuclear stage, one day after egg retrieval. For practical reasons, ZIFT is sometimes deferred by one day, and cleavage-stage embryos are transferred two days after egg retrieval—a procedure termed “embryo intra-Fallopian transfer,” or EIFT [114]. In a recent retrospective study by Weissman et al., ZIFT and EIFT transfers had comparable outcomes in regards to implantation and pregnancy rates as well as in all other study parameters such as miscarriage rates, ectopic pregnancy rates, and multiple pregnancy rates, which were found to be unacceptably high [124].

In conclusion, RIF patients are a heterogeneous group with unclear and various pathophysiological diagnoses. The potentially high efficiency of ZIFT in RIF patients is only partially understood, with a commonly accepted interpretation being that ZIFT embryos possibly are protected from expulsion from the uterine cavity by junctional zone contractions as well as from the introduction of cervical microorganisms into the uterine cavity by the transfer catheter [116, 125]. Meanwhile, and despite the scant available evidence, ZIFT continues to be proposed in clinical practice exclusively to RIF patients [124].

NATURAL CYCLE IVF

Despite the fact that the first IVF/ET baby was born in 1978 after a natural unstimulated cycle, this technique soon was practically abandoned, mainly because of the very high cancellation rates. Controlled pharmacological ovarian hyperstimulation became the standard treatment in IVF cycles of high- and normo-responder patients [126]. However, natural cycles have regained attention in poor-responder patients, where only very few follicles can be recruited and very few oocytes can be retrieved after controlled ovarian hyperstimulation. In these patients, natural IVF cycles may offer comparable outcomes (comparable number of follicles) [127-128], reduced side effects such as multiple pregnancy and ovarian hyperstimulation syndrome, and may represent a more cost-effective and patient-friendly alternative [129].

A recent retrospective study of 500 consecutive natural cycles demonstrated that IVF in natural cycles is an affordable and valid alternative in poor-responder patients [129], in accordance with the data reported in an earlier meta-analysis [130]. Controversial data exist as to the efficacy of cycles with minimal stimulation (i.e. GnRH antagonist plus mild gonadotropin stimulation), and there are currently no studies in the literature comparing natural versus minimal stimulation cycles [129].

Moreover, to address the specific issue of implantation failure, a better understanding of the key determinants of successful implantation is warranted, one of which is endometrial receptivity. The endometrium is receptive to blastocyst implantation during a limited spatio-temporal window, called “the implantation window” [131]. In humans, this period begins 6–10 days after the LH surge and lasts for 48 h [132, 133]. Successful implantation depends on synchronization between the developmental stages of the embryo itself and the complex endocrine environment [134, 135]. There is evidence in the literature suggesting that controlled ovarian hyperstimulation (COH) can alter endometrial receptivity [136, 137]. Supraphysiologic doses of hormones can cause asynchronism between the embryo and endometrium and altered concentrations of growth and adhesion factors, causing implantation failure [138, 139].

Ledee-Bataille et al. conducted a study in which endometrial CD56 bright cells (uterine natural killers or uNK) were immunostained. They found elevated numbers of endometrial NK cells in RIF patients after COH cycles and significantly lowered numbers after natural cycles [140]. Data suggest that uNK may be directly or indirectly involved in controlling the early steps of the implantation process, in part because of their role in vascular remodeling, specifically spiral uterine arteries [141]. Furthermore, on the molecular biology level, alterations in endometrial gene expression have been reported with the use of gonadotropins in stimulated cycles [142]. More recently, Haouzi et al., using DNA microarrays of endometrial biopsies, identified for the first time five genes that are up-regulated during the implantation window and proposed them as new biomarkers for exploration of endometrial receptiveness, including during a natural
cycle. This novel strategy could prove useful in patients with poor implantation after IVF or ICSI [143].

In conclusion, in light of the many potential advantages of natural cycle IVF, and with the many improvements in laboratory conditions and fertilization techniques such as ICSI, it seems worthwhile to re-evaluate the place of natural cycle IVF in the arsenal of fertility treatments, especially in RIF patients. A randomized, controlled trial, comparing natural cycle IVF with current standard practice, is justified.

ANTIPHOSPHOLIPID ANTIBODY (APA) TESTING AND TREATMENT

Antiphospholipid antibodies (APAs) [144] are acquired immunoglobulins or monoclonal antibodies (IgG, IgM, and/or IgA) directed against negatively charged membrane phospholipids, which were characterized as thrombophilic factors because of their association with slow progressive thrombosis and infarction in the placenta, leading to uteroplacental insufficiency [145]. With regard to implantation and pregnancy, it is more appropriate to classify APAs as autoimmune factors because of the complex nature of their interactions [7]. APAs have been shown experimentally to block in vitro trophoblast migration, invasion, and syncytialization, to reduce trophoblast production of the vital hormone human chorionic gonadotrophin [144] and activate complement on the trophoblast surface inducing an inflammatory response [146]. Increased concentration of APA in the follicular fluid, which was once thought to be an explanation for the direct effect of APA on the implanting embryo [147], is still debatable. A recent study demonstrated that this increased concentration had no adverse effect on the reproductive outcome of women undergoing IVF [148]; another study found a significant relationship between follicular fluid APA concentrations and fertilization rates in IVF failure patients [149].

APAs associated with reproductive failure are lupus anticoagulant (LCA), anti-cardiolipin (ACL), anti-phosphatidylserine (APS), anti-phosphatidyl inositol (API), antiphosphatidyl glycerol (APG), anti-phosphatidyl ethanolamine (APE), and phosphatidic acid (PA) [150]. Not only do APAs bind to their direct antigens, they also bind to plasma-bound proteins and co-factors such as β2 glycoprotein I (β2GPI), the most studied anti-phospholipid protein, and prothrombin, which is also important in this role [151]. There is evidence that the presence of β2GPI on trophoblast and decidual cell membranes might explain the clinical association between recurrent fetal loss and β2GPI-dependent APA and the pathogenic role for these antibodies at the same time [152]. Antinuclear antibodies (ANA) also may be associated with reproductive failure, but they were shown to be positive in 9% of normal fertile women and appear to lack specificity in low titre [153].

Even after 20 years of investigation, the role of APA remains elusive when it comes to assisted reproduction, mainly because most of the groups reported an increased prevalence of APA in infertile patients [154, 155], but the evidence is much less definite with respect to IVF outcome [156, 157]. An explanation for the conflicting evidence might be related to differences in antibodies tested. Some groups solely tested for ACL, LCA, or ANA [154], while others evaluated a more comprehensive range of APA [156]. Coulam et al. found a 22% prevalence of seven APAs in women experiencing implantation failure after IVF/ET. Only 4% of women with positive antibodies would have been detected if only ACL were tested and only 14% if APS were added to ACA [156]. Unfortunately, with the exception of ACL, no universally accepted standard for the determination of APA concentrations exists, which adds to the confusion. Actually, the most broadly used clinical assays for these antibodies test for ACL antibodies using enzyme linked immunosorbent assay (ELISA) and lupus anticoagulant (LA) [156]. It is noteworthy here to emphasize the fact that APA can be found in low concentrations in as many as 16% of “normal” controls, i.e. healthy fertile women [153].

The association of antiphospholipids with RIF has been shown in some early studies [145, 153, 154, 156, 158], but large prospective studies failed to reveal an association [155, 157, 159, 160]. A meta-analysis considered the effect of APAs on the likelihood of IVF success and concluded that testing for APAs was unjustified in patients undergoing IVF [161]. However, these results did not close the debate because of the heterogeneity of the cohort studies, the populations included, because the RIF group of patients was not addressed specifically [162]. A strong association was demonstrated between antibodies to the cofactor β2 glycoprotein I and IVF implantation failure [153]. Antibodies to annexin V, which acts as an inhibitor of phospholipid-dependent coagulation and may be necessary for trophoblast differentiation, were found to have a significantly greater incidence (8.3%) in women with RIF than in controls (1.1%) [163]. Other findings by Geva et al. suggest that although APAs may be important in recurrent fetal loss and spontaneous abortions, neither the serum concentration nor the number of positive APAs appear to have significance in recurrent implantation failure, cumulative pregnancy, or live birth rates [164]. According to the ASRM (2008), no association is present between APA abnormalities and IVF success, there is no indication for the assessment of APA in couples undergoing IVF, and therapy is not justified on the basis of existing data [165].

Despite the uncertainty concerning the pathophysiology of APAs in reproductive failure, their presumed thrombotic effects have led to the widespread use of heparin and aspirin for women with RIF [145]. Heparin is thought to protect the trophoblast from injury by inhibiting the binding of phospholipids with antibodies, thus promoting implantation and placentation [166]. Only a very few randomized, placebo-controlled studies evaluating the benefits of heparin and aspirin for APA-positive women with RIF have been undertaken. While some evidence exists that treatment with unfractionated heparin and low-dose aspirin can improve live birth rates [167], other studies have shown that neither implantation nor pregnancy rates are improved with heparin and aspirin [168, 169]. A recent randomized, placebo-controlled cross-over study in patients with strictly defined RIF did not show any benefit of heparin and low-dose aspirin in patients seropositive for at least one antiphospholipid (APA), antinuclear (ANA), or beta(2) glycoprotein I autoantibody, when the outcome measured was ongoing pregnancy or implantation rates [169].
The most recent Cochrane review by Empson et al. examined the outcomes of all treatments to maintain pregnancy in women with prior miscarriages and positive APA. The results found that only unfractionated heparin combined with aspirin appeared to reduce pregnancy losses (by 54%) when compared with aspirin alone. However, these results were only based on two small trials, one of which lacked satisfactory allocation concealment. Low molecular weight heparin (LMWH) combined with aspirin had no statistically significant effect when compared with aspirin or intravenous immunoglobulin (IVIg) [170]. Aspirin alone had no significant effect on any of the outcomes examined; corticosteroids did not show any benefit but demonstrated increased adverse outcomes [170]. IVIg did not significantly differ from prednisone or aspirin in outcomes [171] and was shown to have lower live birth rates than LMWH plus aspirin [172, 173]. The beneficial effect of IVIg has only been proved only in uncontrolled studies [174].

To date, the combined use of low-dose aspirin and heparin is considered standard therapy for women seropositive for APAs, despite the lack of adequate, prospective, randomized, placebo-controlled studies addressing the RIF group of patients specifically. Caution must be exercised in recommending any given treatment.

In conclusion, in women with RIF, no consensus exists regarding testing for APA, assays to be used, auto-antibodies to test, definitions of patient groups or therapy for seropositive patients [175]. Patients must, therefore, be counseled prior to starting any treatment that no clear evidence of benefit for anticoagulation exists [116].

ROLE OF IMMUNE MECHANISMS IN RECURRENT PREGNANCY LOSS AND RIF

Background

The immune system of a patient with a successful pregnancy has been considered a paradox since Medawar in 1953 described the embryo as a semi-allogen, but one that is protected from allogeneic recognition by antigenic immaturity, possibly explained by non-classical class I HLA molecules. Since then, numerous studies have supported the theory of alloimmune causes as an explanation for miscarriages and implantation failure. The hypothesis is that the absence of such alloimmunoprotective mechanisms would result in alloimmune-mediated miscarriage [176]. Moreover, Wegmann et al. suggested that successful pregnancy might result from the predominance of T helper 2 (Th2) cytokines over T helper (Th1) cytokines [177]. In spite of the central role attributed to immunology in reproductive failure and the intense debates on its scope, no appropriate diagnostic strategy has been established to date [176]. Genetic and immunological factors interact with each other in a complex network of antibodies, adhesion molecules, metalloproteinases, natural killer cells, and cytokines [150]. Other factors influencing reproduction and implantation include human leukocyte antigen expression, antisperm antibodies, integrins, leukemia inhibitory factor, cytokines, antiphospholipid antibodies, endometrial adhesion factors, mucin-1, and uterine natural killers [116, 150].

NATURAL KILLER CELLS

A difference is thought to exist between the uterine and peripheral natural killer (NK) cells of women with recurrent pregnancy loss (RPL) compared with controls. Higher numbers of uterine NK cells have been found in the preimplantation endometrium of women with RIF [178]. However, this abnormality was only part of a composite range of immune and vascular abnormalities found in the endometrium of RIF patients [178, 179]. The uNK from nonpregnant RPL patients who exhibit lower CD56 expression (classified as CD16+CD56dim) are more frequent than CD16+CD56bright, as opposed to fertile controls [180]. Non-pregnant RPL patients also show evidence of increased numbers of activated NK cells in peripheral blood mononuclear cells [181]. Kwak et al. also observed the up-regulated expression of CD56+, CD56+CD16+, and CD19+ cells in peripheral blood lymphocytes in pregnant women with RPL [182]. Moreover, Aoki et al. also reported that high preconceptional NK cell activity was associated with higher abortion rates in the next pregnancy [80]. Studies of immune factors investigated at the time of miscarriage showed that deficiency of CD56 bright natural killer cells in the decidua and high natural killer cell cytotoxicity in peripheral blood monocyte cells (PBMCs) increase the risk of euploid miscarriage [183-185].

CYTOKINES

Strong evidence exists that locally secreted cytokines control the implantation process and can cause implantation failure [186, 187]. The Th1 cytokines include IL-2, IFNγ and TNFα, and the Th2 cytokines include IL-4, IL-5 and IL-10. Evidence suggests that the mean of the Th1:Th2 ratio in patients with RPL and in patients with multiple implantation failure after IVF-embryo transfer [188] is significantly higher than in normal fertile women. This predominance of Th1 cytokines was demonstrated to exist in endometrial cells as well as peripheral blood mononuclear cells before pregnancy [187, 189, 190] and at the time of miscarriage in decidual cells [191]. However, there are significant discrepancies in the results of the different studies, as some suggest that Th1 cytokines production was higher in normal women than in RPL patients in early pregnancy [192], and others even found that the production of Th1 and Th2 cytokines was similar in RPL patients who subsequently had successful or failed pregnancies [193]. Th1 dominance may well be a result of the miscarriage rather than a cause, and much more basic knowledge is needed about the complex cytokine networks in pregnancy and the correlation between cytokine production in peripheral mononuclear cells and decidual lymphocytes [194] before tests measuring cytokines can be introduced in clinical practice. With further research and newly discovered cytokines, it is now clear that acceptance of the Th1:Th2 paradigm as a single explanation for implantation failure would be an overly simplistic approach to a very complex mechanism [195]. Other cytokines, particularly leukemia inhibitory factor (LIF), recently have been shown to play a role in women with RIF [196]. Mannose-binding lectin, a constituent of the innate immune system that modulates cytokine production by monocytes [197], was shown to have significantly lower levels in women with RPL [198-200].
TREATMENT WITH INTRAVENOUS IMMUNOGLOBULINS (IVIg)

The mechanism of action of IVIg, a fractionated blood product, is multifactorial [201]. It is involved in a number of processes, including modulation of T cells, B cells, NK cells, monocytes, and macrophages; down-regulation of antibody production; inhibition of antibody function; and modulation of complement activation [202]. Immunoglobulins develop their suppressive activity in vitro through the CD200 tolerance-signaling molecule, which is released from the surface of subsets of blood mononuclear leukocytes and may bind to IVIg [203]. CD200 is known to promote generation of regulatory T cells in mice [204]. A more recent report suggests IVIg suppresses NK activity, specifically the CD56 bright subset of NK cells found at the feto-maternal surface [205]. Additionally, one underlying mechanism may be the restoration of Th1/Th2 balance with dominant Th2 [201]. High-dose IVIgs nearly always have been combined with corticosteroids or anti-thrombotics, so that their precise efficacy cannot be readily estimated and is practically hard to assess [201].

A review of the literature concerning IVIg treatments yields conflicting results. A meta-analysis of six trials by Daya et al. demonstrated a lack of clinical efficacy of IVIg on live birth rates [206], and a prospective, randomized, double-blinded, and placebo-controlled study by Stephenson et al. observed no differences between the IVIg-treated and the placebo groups [207]. A prospective, randomized, double-blinded, and placebo-controlled study by Coulam et al. demonstrated the efficacy of IVIg treatment in increasing the percentage of live births among women experiencing unexplained RIF [208]. In a randomized study by Triolo et al., IVIg was less efficacious than low-dose aspirin and low molecular weight heparin in increasing live births rate [172]. On the other hand, a randomized controlled trial by Christiansen et al. showed no improvement in live birth rates with IVIg compared with placebo, but they suggested a possible beneficial role of IVIg in women with secondary recurrent miscarriage [209]. This effect was confirmed by the review of Hutton et al., although the data concerning primary recurrent miscarriage was inconclusive [210]. A meta-analysis of randomized and cohort controlled trials of IVIg in RIF patients showed a significant increase in the live birth rate per woman (p=0.012) and number needed to treat for one additional live birth = 6) [211]. Relevant variables appeared to be selection of patients with abnormal immune test results and properties of IVIg preparations, as different biological preparations vary significantly in their ability to suppress NK activity in vitro. Another variable is the scheduling of IVIg treatment, as it is argued that pre-conception treatment is better in both primary recurrent miscarriage patients and in IVF failure patients [211, 212]. An observational pilot study found that elevated numbers of NK cytotoxic CD16+ CD56+ cells are independent predictors of treatment success, and that IVIg ameliorated the numbers of these NK cells in RIF [213]. Another observational study by Winger et al. studied for the first time a subset of IVF patients with elevated Th1/Th2 cytokines and showed an improvement of implantation and live birth rates for the groups treated with IVIg and both IVIg and TNF-alpha inhibitors as compared with the no-treatment group [214]. However, the most current Cochrane review shows no significant increase in the overall pregnancy success rate over placebo or no treatment [215].

In conclusion, IVIg, an expensive treatment with possible side effects, is widely used off-label in the treatment of early reproductive failure. Systematic reviews have generated inconclusive results so evidence concerning its efficacy is controversial. Rigorous randomized, controlled trials studying the efficacy of the treatment on various subgroups of RIF patients and additional measurements of CD200-dependent IVIg effects should be undertaken to solve the current controversy [211].

ALLOGENIC LYMPHOCYTE THERAPY

During pregnancy, the paternal human leukocyte antigen (HLA) is recognized by the maternal immune system, which induces production of several alloantibodies. These alloantibodies include anti-paternal cytotoxic antibodies (APCA), anti-idiotypic antibodies (Ab2), and mixed lymphocyte reaction blocking antibodies (MLR-Bf). Once expressed, they may coat the trophoblast to render it undetectable by the maternal immune response system [216]. A reduction in or the absence of these alloantibodies during pregnancy may cause fetal loss [201, 217, 218]. As has been shown repeatedly, increased sharing of HLA may prohibit the mother from producing these alloantibodies, leading to an increased tendency toward repeated fetal loss [201]. Maternal immunomodulation via transfusion of paternal leukocytes (lymphocytes) prior to conception has been proposed as a solution to RIF [219-221], while the use of third party donor white cells or trophoblast membranes transfusions have been largely abandoned because of doubts about efficacy [201]. Allogenic lymphocyte isoinmunization (ALT) also has been proposed to solve the Th1/Th2 paradigm by shifting the balance towards Th2 cytokines, thus enhancing the implantation process [181, 188].

The first randomized, controlled study using ALT with paternal PBMCs showed a 24% increase in successful pregnancy rates [222]. However, subsequent trials provided conflicting results due to variations in cell numbers, number of injections, routes of administration, and types of placebo. These differences make comparisons and meta-analyses difficult to realize [209]. A subsequent intention-to-treat meta-analysis by the Recurrent Miscarriage Immunotherapy Trialists Group showed an increased live birth rate of approximately 9–10% [223]. The number needed to treat for an additional live birth was limited to three to four women with primary recurrent pregnancy losses who were seronegative for autoantibodies (ANA and ACL) [219]. Since then, the beneficial effects of ALT in RIF patients, previously demonstrated primarily by non-randomized studies. In a retrospective, non-randomized review of 686 couples referred for ALT, Kling et al. found a temporary beneficial effect lasting for six months after immunization; this effect seemed to be most pronounced in couples who failed three or more cycles of IVF-embryo transfer [224]. Despite the lack of randomized trials for RIF, a large randomized trial in RSA patients failed to show any beneficial effect of ALT [225]. Pooling the results of the randomized and non-randomized studies, Pandey et al. showed that 67% of RSA patients who re-
ceived paternal lymphocyte immunotherapy had successful pregnancy outcomes in comparison to 36% success in women with RSA in the control group who received either autologous lymphocytes or no therapy [220].

In a double-blind, placebo-controlled trial in women with unexplained RSA, immunization with paternal lymphocytes proved to be beneficial over autologous (maternal) lymphocyte therapy [221]. On the other hand, a Cochrane meta-analysis of relevant trials [215] concluded that paternal and third-party ALT provide no significant beneficial effect over placebo in preventing miscarriages. The results of this meta-analysis are extremely controversial because it included a large negative trial using immunization with paternal lymphocytes stored overnight [225]. This factor impairs the protective anti-abortive effect of the procedure and causes loss in surface CD200, at least in mice [226]. The results of the Ober study, despite the debate over its design, decisively influenced the issues of the most current Cochrane review cited above [215], as well as the US FDA regulation, [227] which states that administration of such cells as allogenic lymphocytes or cellular products in humans should only be performed as part of a clinical research project and then only if an Investigational New Drug application is in effect. Moreover, concerns over possible adverse effects of LIT have been raised. These include transfusion-related problems, autoimmune disorders, graft-versus-host reaction, and transmission of infection such as hepatitis B virus or HIV [176], or even cancer and gestational pathology [228]. Adverse neonatal outcomes are rare, but a case of neonatal alloimmune thrombocytopenia and intracranial hemorrhage in an infant whose mother received immunizations of paternal mononuclear cells has been reported [229]. However, a prospective study by Kling et al., with follow-up after 2-3 years, showed that the acute side effects of intradermal ALT were comparable to those reported after intradermal vaccination for infectious diseases and that specific risks for anaphylaxis, autoimmune, or graft- versus- host disease were not significant [228].

In conclusion, proposing ALT to RIF patients in the absence of standard and broadly applicable diagnostic tests of immune-mediated pregnancy losses, of reliable methods for judging the immunization effects, and of unified protocols of immunization should await further randomized, controlled trials based on adequate patient selection and more complete knowledge of the underlying pathophysiology of the assumed alloimmune causes of recurrent miscarriage.

CONCLUSIONS

Randomized, controlled trials have shown that blastocyst transfer, assisted hatching, salpingectomy for tubal disease, and hysteroscopy in IVF procedures are beneficial for improving treatment outcome in patients with repeated implantation failure. Studies also demonstrate that treatment with aspirin and heparin with IVIg does not have a clear impact on treatment outcome. Allogenic lymphocyte therapy, ZIFT/EIFT, co-cultures, sildenafil, use of donor oocytes, transfer of six embryos, natural IVF, and PGS await further clinical assessment. The management of RIF should be individualized because the pathophysiology is so variable and often complex.

EXPERT COMMENTARY

Having to endure not one but two or more failed rounds of IVF is painfully frustrating to the patient as well as to the clinicians and technicians involved. We discuss 14 current options that are supported by varying degrees of scientific evidence. Clinicians would benefit from knowing which treatment options are proven in order to counsel patients effectively and manage their expectations for future IVF cycles. Unfortunately, even the more promising ones are successful only to a certain subgroup of patients with specific conditions. Our understanding of the mechanism behind embryo implantation remains poorly understood, which hampers our ability to find a solution. To date, strong evidence supports the use of blastocyst transfer, assisted hatching, salpingectomy for tubal disease, and hysteroscopy in the management of couples with previous implantation failures and clearly dismisses the use of aspirin and heparin with IVIg. Other treatment options such as allogenic lymphocyte therapy, ZIFT/EIFT, co-cultures, sildenafil, use of donor oocytes, transfer of six embryos, natural IVF and PGS are controversial, and their efficacy remains to be elucidated.

FIVE-YEAR VIEW

Endometrial receptivity is a vital component for embryo implantation. A better understanding of the critical success factors required for successful implantation is recognized. What is the optimal condition of the embryo? What is the optimal condition of the endometrium? Recent genetic research focused on cultured endometrial cells from RIF women has demonstrated a difference in transcriptional activity during the implantation window. A possible disruption in genetic expression of specific genes that regulate the cell cycle has been implicated. Further research is needed to fully understand the genes involved in the defunct pathway of endometrial cells during the implantation window, thus resulting in implantation failure.

KEY POINTS

- Implantation failure and embryo quality are major limiting steps for IVF treatment.
- Etiological sources of implantation failure include embryo quality; endometrial receptivity; immunological factors; uterine, tubal, and peritoneal factors; and culture media. The treatment options discussed in the article are intended to address the particular causes and improve implantation rates.
- Differences in the local environment of the endometrium of RIF patients compared with other infertile patients have been found. These differences (i.e. gene expression) possibly affect cross-talks between the embryo and the endometrium and thus implantation.
- Blastocyst transfer, assisted hatching, salpingectomy for tubal disease, and hysteroscopy are highly recommended treatment options based on good, consistent scientific evidence with randomized, controlled studies.
Evidence-Based Management of Infertile Couples

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REFERENCES


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- options that have not been proven to benefit the couple. Reprod Biomed Online 2005; 11(3): 382-91.


- Urman B, Yakin K, Balabah B. Recurrent implantation failure in assisted reproduction: how to counsel and manage. B. Treatment


- Urman B, Yakin K, Balabah B. Recurrent implantation failure in assisted reproduction: how to counsel and manage. B. Treatment


- Urman B, Yakin K, Balabah B. Recurrent implantation failure in assisted reproduction: how to counsel and manage. B. Treatment


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Single Blastocyst Transfer: Contemporary Experience

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Abstract: Recent studies demonstrated an overwhelming success in single blastocyst transfer (SBT): implantation rates (IR) were 60.9%-70.5% and pregnancy rates (PR) were 60.9%-76% while the multiple pregnancy rates (MPR) were 0%-3.2%. Most of these studies involved good prognosis patients not more than 37 years of age. The results indicated that SBT decreased the number of multiple pregnancies while maintaining desirable pregnancy outcomes. However, SBT and cryopreserved single blastocyst transfer (cSBT) in the field of in vitro fertilization (IVF) are still in their infancy. Guidelines for the number of blastocysts being transferred and the techniques have not yet been standardized. The method to estimate the most viable blastocyst has not yet been proposed. The success of SBT also was found to be highly associated with the technique and patients’ and clinicians’ perceptions toward it.

Keywords: Single blastocyst transfer (SBT), embryo transfer, blastocyst grading, cryopreservation, in vitro fertilization (IVF), assisted reproductive technology (ART).

DEVELOPMENT OF EMBRYO TRANSFER

Since the first success of human birth after the implantation of human embryo in 1978 [1], IVF has become the most effective treatment for infertile patients regardless of the cause of infertility [2]. To achieve higher IR, the practice of transferring multiple embryos per transfer cycle has been widely employed [3]. The results of multiple embryo transfer have to have an IR from 3% to 69% and a PR from 24% to 66% [4-10]. However, with the relatively satisfying success of pregnancy outcomes, multiple gestations have become a serious complication resulting from the transfer of multiple embryos at a time [3].

The MPR revealed by many studies ranged from 17% to 75% [6-10]. With this high MPR, studies have been advocating reducing the number of transferred embryos and defining the success of embryo transfer as the single live birth rate. Luke et al. analyzed 69,028 data entries regarding embryo transfer during 2004-2006 in the Society for Assisted Reproductive Technology (SART) Clinic Outcomes Reporting System (CORS) database. The single live birth rate was found to be 42.9%, 32.5%, 26.5%, and 23.2% for transferring of one, two, three, and more than four embryos at a time, respectively [11]. The results showed that the single live birth rate after single embryo transfer is significantly higher than those of multiple embryo transfer (p<0.0001). However, despite the support and advocating efforts in the literature, single embryo transfer still represented a minority of practice in the United States—12% overall in 2001 [12], and 4.4% during 2004-2006 [11].

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DEVELOPMENT OF BLASTOCYST TRANSFER

Blastocyst Culture: Higher Performance as the Culture Technique Improves

Recent improvements in culture technology have enabled more robust embryo development to the blastocyst stage. Furthermore, a better understanding of the dynamic uterine and endometrial environment as the embryo develops from a zygote to blastocyst has aided specifically in improvement of the required nutritive contents in culture systems to more closely mimic the reproductive tract. The addition of amino acids, sugars, and other compounds permits early embryos to develop into blastocysts [13], the embryonic stage known to be highly associated with a higher IR, without compromising PR [14]. With regard to nutrition requirements, the preimplantation period should be considered in two phases for correct medium formulation, pre and post-compaction [15]. Prior to Day 3 of embryo development the first couple of cell cycles utilize reserves containing maternal transcripts and stored mRNA; in other words, its metabolism is regulated at the post-transcriptional level [16]. During post-compaction, the embryonic genome has taken over and the embryo becomes more susceptible to transcriptional inhibition from nutrient deficiencies [13].

Two types of culture systems are involved in blastocyst culture, monoculture media and sequential media. In a monoculture system, a single medium is supplemented with all the required components to sustain the embryo for growth to the blastocyst stage. A sequential system requires growing the early embryo until Day 3 in culture with amino acids and low levels of glucose and phosphate to allow the embryo to use its reserve (maternal transcripts and stored mRNA) and then transfer the embryo to a more complex medium with numerous metabolites (citrate, malate, various lipids, etc.) to support higher demands and avoid metabolic blocks
[17]. As noted before, the embryo depends on its reserves prior to compaction, and so it is reasonable for embryos to grow in a medium with lower glucose levels during this period.

Sequential media systems were introduced in the late 1990s and became a controversial issue. How can a simple change in culture media have such a significant improvement in embryo development to ensure a quality embryo is cultured to the blastocyst stage? Opponents of blastocyst culture and transfer contend that the self-selection criteria, the main underpinning benefit for use of sequential media for blastocyst culture, is thwarted by the preimplantation embryo’s high incidence of chromosomal abnormality, an increased risk for monozygotic twinning, and an increased risk for epigenetic mutation. A prospectively randomized control study by Staessen et al. evaluated the incidence of chromosomal abnormality of cleavage-stage versus blastocyst-stage embryos in women of advanced maternal age and noted a difference: a reduction of aneuploidy incidence from 59% to 25% on Day 3 to 35% on Day 5 [18]. Proponents argue that considerable evidence suggests a significant improvement in IR without compromising PR in blastocyst transfer as compared with Day 2 or 3 transfer [19]. Nonetheless, sequential media is widely accepted today for blastocyst culture and transfer.

Early studies evaluating sequential media culture systems show evidence of improved PR and significant IR (32.3%) following blastocyst transfer in a heterogeneous group of infertile patients [20]. However, both Sepulveda et al. and Reed et al. found it was unnecessary to transfer the embryo to a subsequent media after Day 3 [21, 22]. Reed et al. demonstrated a significantly higher mean number of optimal blastocysts on day five with the single medium compared to the sequential medium (P<0.05) [21]. A total of 893 embryos from patients and donors were divided into three groups: (i) 232 patient embryos cultured for Day 3 transfer, (ii) 480 patient embryos for Day 5 transfer, and (iii) 181 donor embryos for Day 5 transfer. Each group of 232, 480, and 181 oocytes was split in half to be cultured in sequential and continuous media. Embryos in the sequential medium group were moved to the second sequential medium, while embryos in the single medium treatment remained in the original dish, without renewal of the microdrops. The mean number of embryos transferred for embryos cultured in sequential (1.3 embryos) versus continuous (1.6 embryos) for Day 3 transfer did not differ significantly (P>0.05). Also, the mean quality score of Day 3 transfer was 2.5 and 2.4 (P>0.05) for sequential and continuous medium, respectively; and did not show a significant difference. On the other hand, the mean number of Day 5 blastocysts was higher in the continuous media than in the sequential media. The same results were found for the mean number of Day 5 blastocysts transferred. Surprisingly, the mean number of Day 5 blastocysts to be transferred from continuous media was two times that of sequential media (P<0.01). Also, mean embryo quality scores did not differ between the media for Day 5 blastocysts [21].

Further support was demonstrated in a prospective, randomized, controlled study using donor oocytes cultured in single medium versus sequential media in which no significant difference was found in number or quality of blastocysts on Day 5 when comparing embryo culture in either system, single or sequential [22]. A total of 287 and 322 embryos were cultured in continuous and sequential media, respectively. By Day 5, 24.7% of the 287 embryos in the continuous medium and 13.7% of 322 embryos from the sequential medium (P=0.001) were in full to hatching status; thus, a greater portion of embryos developed to Day 5 blastocysts in continuous medium than in sequential [22]. In this study, embryos in both groups of single and sequential medium were moved to fresh droplets of single medium and the second sequential medium, respectively on Day 3. It is important to note that the single medium used in this study contained all 20 amino acids as opposed to the sequential media with significantly fewer amino acids (alanyl-glutamine only) [22]. To put it briefly, the single, continuous, uninterrupted culture system was just as good as or better than the sequential media system when it was properly formulated with all the required nutrients. If true, the monoculture system eliminates one step in handling the embryo - a more desirable option to reduce any unknown risks associated with moving the embryo to another medium. The extra step translates into more work, increased cost, and a theoretical chance for negative events to occur. A comparison of the efficacy of the variety of single and sequential medium for culture to the blastocyst stage also would be important.

Advance culture technology such as sequential media is not the only critical success factor for improving SBT and increasing IVF success. Prerequisites to successful blastocyst culture are dependent on (i) lab protocols, (ii) quality management system, and (iii) superior oocytes from a good ovarian response in good prognosis patients [13]. Other important features of the culture system also have an impact on IVF success per transfer cycle, including gas phase, embryo incubation volume and group size, and macromolecule supplementation [13]. The autocrine effect of volume and group size on embryo is only proven in mouse and remains controversial in humans. Quality control of procedures has long been declared as critical to protect embryos from potential toxins, especially during the move to sequential media [15]. For instance, lab conditions must be stable, and so close monitoring is required to protect against atmospheric fluctuations [21]. Unfortunately, comparisons of lab quality conditions and lab protocols among various IVF laboratories are not often studied and so only very limited reports are available; no standard laboratory requirements for performing single blastocyst culture and transfer are documented.

In conclusion, we noted that sequential media has had a positive effect on the outcome of single blastocyst culture and transfer for a select patient population. Yet, the majority of the studies regarding blastocyst culture and transfer have eliminated patients with advanced maternal age. This suggests that extending studies to determine whether SBT would be just as beneficial for older patients with good prognosis, good response to ovarian stimulation, an optimal number (10 or more) of high quality embryos, and no other known indications would be worthwhile. Comparing the efficacy of the variety of single and sequential medium for culture to the blastocyst stage in the future may also be beneficial, especially in media with undefined contents and/or concentrations to standardize the components in complex media.
Multiple Blastocyst Transfer: Widely Employed to Yield High IR

In the not too distant past, an IR above 10% was an acceptable milestone for an IVF clinic. Given this low IR, transferring two or more embryos was considered necessary to achieve acceptable IR or PR. Milki et al. found that the IR was 47% and the PR was 58% with 24 cycles of triple-blastocyst transfer [23]. This result demonstrated the success of multiple blastocyst transfer. Furthermore, Jain et al. reported the results of 75 cycles of blastocyst transfer with a mean number of 2.0±2 blastocysts being transferred per cycle. The IR was found to be 45.3%, and the clinical PR was found to be 50.7% [24]. In addition, Yamamoto et al. reported the results of 290 cycles of multiple blastocyst transfer with a mean number of 1.44±0.50 blastocysts being transferred per cycle. The IR was found to be 35.2%, and the clinical PR 42.4% [25]. These studies all indicated the success of utilizing multiple blastocysts in blastocyst transfer.

SBT: a Way to Curb High MPR in ART

Although great success was achieved with multiple blastocyst transfer, a high MPR also occurred. The high MPR has been a problem for the Assisted Reproductive Technology (ART) industry over the past decade. Ryan et al. reported that the MPR approached 40% after double blastocyst transfer (DBT) in their center [26]. Recently, IR above 50% for transfers of top-quality cleavage-stage embryos have been reported, while IR above 60% for transfer of selected blastocysts have been reported. Stillman et al. demonstrated that IR for double blastocyst transfers were almost 50% for all patients regardless of age or blastocyst quality [27]. IRs as high as these put patients at risk of multiple pregnancies if more than one embryo or blastocyst is transferred. Stillman et al. demonstrated that MPR were 44% among 4083 cycles of fresh double blastocyst transfers [27].

Monozygotic Twinning Caused by SBT

The only possible mechanism for SBT to result in twins is through monozygotic twinning (MZT). MZT is a rare phenomenon in humans, occurring in 0.42% of spontaneous pregnancies. Guerif et al. demonstrated that MZT in 218 cycles of SBT is higher, although not statistically significant, compared with 243 cycles of single cleavage-stage embryo transfer (3.8% vs. 1.6%) [36]. da Costa et al. suggested that one probable reason for the higher incidence of MZT in blastocyst transfer could be the hardening of the zona pellucida resulting from prolonged exposure to embryo culture [37]. As the blastocyst herniates through the abnormally hardened zona pellucida, it might facilitate the blastocyst’s division.

Moreover, Papanikolaou et al. demonstrated that MZT is not increased after SBT (n=271) compared with single cleavage-stage embryo transfer (n=308) (1.8% vs. 2.6%, p>0.50) [38]. It is important to note that assisted hatching and blastocyst coculture have not been performed in this study. Several studies reported monozygotic twins and triplets in association with SBT [39, 40]. Lee et al. reported three healthy boys each weighing 1.78 kg were born to a 28-year-old woman after a SBT [40].

To summarize, although current studies revealed that the incidence of MZT is increased following IVF compared with spontaneous conception, evidence is insufficient to support this observation. Further studies are needed to clarify the association between extended culture to the blastocyst stage and MZT.

CRYOPRESERVED SINGLE BLASTOCYST TRANSFER (cSBT)

With recent improvements in culture media and cryopreservation techniques, frozen embryo transfer has evolved. Blastocyst cryopreservation can be accomplished by slow-freezing or vitrification. Slow-freezing utilizes cryoprotectants to minimize intercellular ice crystal formation. Vitrification was first reported in 1985 utilizing high concentrations of cryoprotectants to achieve a ultra-rapid freeze to eliminate ice crystal formation [41, 42]. These two methods have been readily employed at the blastocyst stage [43, 44].

Although the results of transfer for more than one cryopreserved blastocysts are well-documented [25, 32, 36, 45], few studies have been published regarding the pregnancy outcomes of cryopreserved SBT. Desai et al. examined 56 slow-freezing cycles of cSBT [46]. A two-step glycerol freeze protocol was used in this study. The result demonstrated a 27% clinical PR and an 18% live birth rate.

Mukaida et al. showed that PR increased as the number of transferred blastocysts increased [47] when a Cryoloop technique was used to vitrify the blastocysts produced from 223 cycles. PR were 26% in cryopreserved single blastocyst transfer, 34% in cryopreserved double blastocyst transfer, and approaching 60% when four to five cryopreserved blastocysts were transferred. This study included blastocysts that were either re-expanded or not re-expanded before embryo transfer. In addition, Yanaihara et al. reported the pregnancy outcomes of 412 cycles of vitrified single blastocyst transfer.
in their center [48]. The PR and MPR were 40.7% and 2.3%, respectively. This study demonstrated that vitrification is a practical method for cSBT.

CRITERIA FOR SBT
Current Guidelines

Together, the American Society of Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) have published several versions of guidelines on the number of embryos to be transferred to maintain pace with changing technology. In the most current version, the number of blastocysts to transfer for ages <35, 35-37, 38-40, and 41-42 were 1, 2, 2, and 3 blastocysts, respectively, for patients with a favorable prognosis [49]. These include patients in their first cycle of IVF and/or those with high quality embryos, high number of embryos available, or have had previous successful IVF cycles. Alternatively, all other patients within the same age ranges should transfer the following (number of blastocysts): ages <35 (2), 35-37 (2), 38-40 (3), and 41-42 (3) [49].

A recent study was done to evaluate the prevalence of U.S. IVF clinics willing to act in accordance with the guidelines and led to additional interesting findings [51]. First, a majority of U.S. IVF clinics (94% of those responding to the survey) would follow the guidelines. Second, certain situations were likely to result in deviation from the guidelines; those identified from the study include patient request, IVF procedures involving the transfer of frozen embryos, and IVF cycles preceded by a failed previous IVF attempt. Third, a higher than expected number of patients (who had optimal conditions for single embryo transfer) with insurance coverage had deviated from the guidelines and had a double embryo transfer. Fourth, clinicians are not likely to communicate the single embryo transfer policy to their patients, only the risks involved with multiple pregnancies. These points indicate a potential for improvement of the guidelines to take patients’ and clinicians’ concerns into consideration in response to social, economic, and financial pressures. After the publication of the ASRM-SART guidelines, the number of higher order multiple deliveries in the United States declined during subsequent years indicating a voluntary adoption of single (or double) blastocyst transfers by IVF clinicians, embryologists, and patients [52]. The data reveals a decrease in the mean number of embryos transferred in women <35 years of age decreased from 3.12 in 1996 to 2.18 in 2003. During the same period, IR and delivery rate increased from 20.7% to 34.1% and from 30% to 40%, respectively [52]. This is good news for the future of IVF children who have no voice to deny a future of complications and the movement toward a single, healthy live birth for all patients.

Embryo Grading and Blastocyst Grading

IVF technique has long included embryo grading to aid in selection of the most excellent quality embryos for implantation for the best results. Good embryo quality is associated with a high IVF success rate [53]. Embryos can be graded at various stages (e.g. pronuclear, zygote, cleavage, and blastocyst) by taking into account various markers, including blastomere morphology, developmental rate, fragmentation, metabolic markers, genetic markers, and epigenetic markers [54]. Here we will focus on blastocyst grading.

The two most popular blastocyst embryo grading systems are the Dokras and Gardner grading system, both based on morphology [14, 55-57]. Dokras grading is based on the blastocoele’s rate of development and characteristics of the blastocoele cavity; whereas Gardner’s system focuses on blastocoele size and developmental characteristics of the inner cell mass and trophectoderm. In the Dokras system, blastocysts are graded as BG1, BG2, or BG3. BG1 and BG2 have been shown to have higher IRs and have been recommended for SBT [53, 58].

According to the Gardner grading system, the blastocoele size, degree of expansion, and hatching status is initially examined and graded from 1-6. Next, the blastocysts graded 3 through 6 are identified and their inner cell mass and trophectoderm are further graded.

Studies have shown that transfer one blastocyst with grades ≥ 3AA using the Gardner system has had a significantly higher IR (54.3%) and higher clinical PR (69.6%) than blastocysts with ≤3AA [56]. A more recent study by Urman et al. had similar results when two blastocysts graded as 3AA were transferred [54]. Finally, a randomized study comparing the two grading systems illustrates a superior Gardner system for predicting blastocysts with a higher chance of implantation (37.6% vs. 25%, P=0.01) and clinical pregnancy (66.7% vs. 53.0%, P=0.11) than the Dokras system. According to this study, both are valuable predictors for selecting blastocysts with high implantation potential and to implement elective SBT strategy to avoid multiple pregnancies [59].

In some countries where blastocyst selection is illegal (Germany), scoring is most effective when completed as close to latest stage permitted for selection. Zygote scoring for obtaining good quality blastocysts has not been promising [60-64]. The scoring system by Zollner et al. included an examination of the pronuclear zygote for the following: (i) number and size of pronuclei, (ii) juxtaposition of pronuclei, (iii) halo effect, (iv) alignment and number of pronuclei (evaluated separately) and nucleoli, (vi) appearance of vacuoles, and appearance of ooplasm. Each criteria was evaluated and assigned a score with 10 being the best and 31 the worst. A pronuclear score of >15 correlated with poor blastocyst development and a decrease in PR [62]. Even
though the study showed a correlation between pronuclear morphology and blastocyst development, it was not strong enough to identify the highest quality embryos with high IRs.

The pronuclear scoring system by Scott and Smith (1998) and Tesarik and Greco (1999) continue to be the most widely used methods. Scott and Smith’s is based on the combination of two scores, one for position of the pronuclei and the other score for cytoplasmic appearance when two pronuclei and two polar bodies are present [61]. Tesarik and Greco’s is based on the appearance and distribution of the nucleolar precursor bodies within the pronuclei and scored between 0 and 5 [63]. A score of 0 is associated with normal development, and all other patterns are considered irregular. The use of scoring systems with blastocyst culture and transfer is a valuable tool for the selection of an embryo with a higher probability to be normal and result in a live birth, but is not always reliable and should be pursued with caution. Patients should be informed of the circumstances prior to their commitment.

Patients’ Clinical Conditions

Most studies practiced SBT in patients not more than 37 years old [27, 30, 34]. However, a couple of studies practiced SBT in patients with ages up to 43 years [31, 35]. Kalu et al. compared PR after SBT in patients age 25-37 years and 38-43 years: 73.8% and 47.1%, respectively [31]. This study suggested that SBT could result in better pregnancy outcomes in young women. Furthermore, most studies are restricted to patients who had a good prognosis. In addition to young age, the good prognosis criteria often included no previous history of failed IVF cycles, no moderate or severe endometriosis, at least three (some studies at least five [34]), blastocysts available for transfer, and normal uterine cavity, etc [27, 30, 32]. However, Trout et al. also reported results in patients at risk of ovarian hyperstimulation syndrome (OHSS). Seven cases of SBT in women at risk of OHSS had an ongoing PR of 57% [65].

PATIENTS’ PERCEPTIONS TOWARDS SBT

Patient Perceptions Differ from Clinicians

The patient’s perception of blastocyst culture and transfer of one, two, or other multiples of embryos is important to consider. It aids the clinician’s communication with couples and improves patient satisfaction. In one study, men and women were interviewed separately to remove influence from their partners, and each was asked about their decision-making process in deciding on single or double blastocyst transfer [66]. Several interesting points resulted from the study. First, patients who chose a double transfer perceived a higher chance of pregnancy. Second, patients who chose a single transfer say it was a much more difficult decision than deciding on a double transfer. Those that tended to select the single transfer had similar characteristics, including: (i) having experienced a prior childbirth, (ii) being young, (iii) having no previous failed IVF treatment, and (iv) having spare embryos to freeze. Thirdly, patients who were more likely to accept a double transfer and perceive it as more desirable were older, had no spare embryos to freeze, or perceived a single embryo transfer might lower chances of pregnancy per transfer based on evidence either from a study or a previously failed IVF attempt [66].

In another study, de Lacey et al. identified three factors influencing the decision for a SBT, including repeated treatment, advanced age, and urgency to become pregnant [67]. This may be in relation to patients’ perception that the risk of maternal and neonatal complications is low to moderate in association with single or double transfers, despite being fully informed of the risks [67]. Several studies also support findings that patients are willing to accept a double transfer along with the maternal and neonatal risks involved to have children [66-68]. Thus, awareness of complications is not a deterring factor in the decision for SBT, and so identifying the points that influence patient decisions is important. Perhaps patients are more willing to accept SBT if there are no financial pressures in case of a failed cycle of SBT [27]. For this reason, determining what factors will motivate patients to accept SBT over DBT in particular circumstances will be important.

Business interests do not always coincide with patients’ best interests, and so clinicians may hesitate to decrease the number of blastocysts to transfer despite the risk for unhealthy babies being born [69]. However, a recent study by Stern et al. assessed the impact of embryo transfer guidelines designed to minimize multiple births by lowering the number of embryos transferred in IVF clinics. It reported a decrease in the number of embryo transfers and proved the effectiveness of voluntary reduction of blastocyst transfer [52]. Not surprisingly, this also has led to a decrease in the number of higher order multiple births.

Patient Education is Essential for SBT

Ryan et al. reported the results of an educational campaign with a two-fold process: First, a one-page description of the comparative risks of twins versus singletons to maternal, fetal, and neonatal health was given; second, this page was then discussed between the couples and physicians [26]. Pre- and post-educational campaign questionnaires (n=110) were collected and analyzed. Results showed that significantly more subjects ranked singletons as their most-desired treatment outcome post-educational campaign compared with pre-educational campaign (86% vs. 69%, p<0.001). This observation suggests that an improved understanding of twin risks appeared to affect patients’ declared desire for numbers of embryos to transfer and for twin pregnancies.

Stillman et al. demonstrated the effect of a policy recommending SBT to patients identified as high risks for twins [27]. In this study, an educational program for physician, staff, and patient also was implemented to increase the program-wide proportion of SBT. This education program for patients constitutes laminated sheets for each physician office outlining the recommendation criteria, statistics, and risks regarding SBT and DBT. In addition to online education and in-person consulting with patients, the sheets also were hung in each physician office. The education program for physicians and staff took place periodically at physician and staff seminars. Results have shown that the program-wide number of embryos transferred was decreased significantly post-policy (n=2923) compared to pre-policy (n=1556) (2.16±0.84 vs. 2.45±0.84, p<0.003). This observa-
tion suggested that patient education is an important factor that could pave the future for SBT among good-prognosis patients.

EXPERT COMMENTARY

The purpose of this article was to discuss various aspects regarding the current practice of single blastocyst transfer. Human blastocyst transfer has been evolving in the recent decade since the successful development of blastocyst culture and transfer techniques. Given the unwanted high multiple pregnancies resulting from multiple embryo transfer and multiple blastocyst transfer, single blastocyst transfer was proposed to be a prospective method to resolve this problem. Single blastocyst transfer was found to significantly decrease the number of multiple pregnancies while maintaining desirable pregnancy outcomes. A number of recent studies have demonstrated the potential role of single blastocyst transfer in young and good prognosis women.

Single blastocyst transfer and cryopreserved single blastocyst transfer in the IVF are still in their infancy. The guidelines for number of blastocysts being transferred and the techniques have not yet been standardized, and a method to estimate the most viable blastocyst has not yet been proposed. The success of single blastocyst transfer is highly dependent on the technique and patients’ and clinicians’ perceptions toward it. Further research is required to provide more data on single blastocyst transfer in different patient groups to standardize the practice based on different patient variables.

FIVE-YEAR VIEW

The practice of single embryo transfer is less common in the United States than in Europe. One reason for this has been the concern that transferring two or more embryos would lead to higher birth rates [12]. However, with more robust studies proving the effectiveness and the advantage of single blastocyst transfer [3], the unresolved problem of the optimal number of embryos to transfer is about to be debunked. Although the success of single blastocyst transfer has been overwhelming in controlled studies, it will require more mature blastocyst culture, grading, and cryopreservation techniques before gaining wide clinical acceptance. Clinics need to assess their own available techniques, laboratory conditions, and patients’ willingness to participate in single blastocyst transfer.

Traditionally, the success of blastocyst transfer was determined by comparing implantation rates and pregnancy rates. However, there has been a shift in focus to a single live birth rate as the variable to be compared. In addition to patient characteristics, including age, BMI, and clinical conditions that need to be reported, the single live birth rate also should be discussed in future studies to aid comparisons across studies. Given more and more studies discussing the effects of single blastocyst transfer in a variety of patient populations, generating a standardized guideline for practitioners may be possible in the near future. It also is hoped that many proposed blastocyst assessment methods can be improved to more accurately predict the most viable blastocyst to transfer.

KEY ISSUES

- Multiple embryo transfer was successful in terms of pregnancy rates; however, it resulted in unwanted high multiple pregnancy rates.
- Increased number of studies revealed that single embryo transfer would be beneficial for young patients with good prognosis.
- Two types of culture systems have been developed and are widely available for culturing blastocyst: single media and sequential media. The single culture system was just as good as or better than the sequential media system when it is properly formulated with all the required nutrients.
- Multiple blastocyst transfer was able to generate satisfying implantation rates and pregnancy rates; however, it resulted in the same problem encountered in single embryo transfer—high multiple pregnancy rates.
- The only truly effective means by which to avoid multiple pregnancies is believed to be transfer of a single embryo or blastocyst.
- Frozen single blastocyst transfer has been developed as a practical method to optimize pregnancy outcomes following the possible failed initial transfer or to be employed for patients with inappropriate initial transfer conditions.
- An improvement of current guidelines for blastocyst transfer is possible when taking patients’ and clinicians’ concerns into consideration in response to social, economic, and financial pressures.
- Determining for certain which blastocyst will implant is not possible; however, many grading methods, including morphology assessment could provide possible information on the blastocyst’s potential to implant.
- The patient’s perception of blastocyst culture and transfer of one, two, or other multiples of blastocyst is important to consider. Physicians should communicate with patients to educate them on the pros and cons of various blastocyst transfer choices.

REFERENCES

Single Blastocyst Transfer: Contemporary Experience


Milkii AA, Fisch JD, Belar B. Two-blastocyst transfer has similar pregnancy rates and a decreased multiple gestation rate compared with single cleavage-stage embryo transfer. Fertil Steril 2010; 93(2): 592-7.


The Role of Oxidative Stress and Antioxidants in Assisted Reproduction

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Abstract: Aim: Oxidative stress contributes to the high rate of failure seen in assisted reproductive techniques in achieving fertilization and pregnancy. Many studies have been done to elucidate the sources of oxidative stress in the setting of assisted reproductive technology (ART) and interventions to overcome its negative influence on the outcome of IVF and ICSI. This article explores the utility of metabolomics as a novel, non-invasive method of accurately and efficiently quantifying oxidative stress. The aim of this study was to review the current literature on the effects of various interventions, including the use of antioxidants supplementation of IVF culture media and patients to improve fertilization and pregnancy rates in subfertile patients undergoing ART.

Methods: Review of recent publications through Pubmed and the Cochrane data base.

Results: Oxidative stress is correlated with negatives ART outcomes. Both exogenous and endogenous sources of reactive oxygen species during IVF/ICSI are well established in the literature. Compared to IVF, ICSI is known to minimize the exposure of gametes to endogenous sources of oxidative stress. Strategies to control exogenous sources of oxidative stress within the ART setting include reducing visible/near UV light exposure, the addition of metal chelators to culture media, maintenance of low oxygen tension in the environment and the use of antioxidant therapy. Antioxidant supplementation of culture media with vitamin C, vitamin E, and melatonin has been investigated and yielded conflicting results. Whereas oral antioxidant supplementation of male patients has been accepted and is currently practiced, there is a lack of consensus regarding the effectiveness of supplementation of vitamin C, vitamin E and melatonin in females undergoing ART.

Conclusion: There is a need for further investigation with randomized controlled studies to confirm the efficacy and safety of antioxidant supplementation of culture media and patients as well as the need to determine the dosage required to improve fertilization rates and pregnancy outcome with IVF/ICSI.

Keywords: In vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), infertility, oxidative stress, antioxidants, metabolomics.

INTRODUCTION

Infertility is a universal issue, affecting millions of men and women in both developed and developing areas of the world. Recent advances in the field of reproductive medicine may allow for a select group of infertile couples to conceive and bear offspring by utilizing assisted reproductive techniques (ART). However, any hope offered by these procedures is limited by persistently poor fertility outcomes. In 2003, a report by the Society for Assisted Reproductive Medicine (SARMS), Cleveland, OH, 44195, USA; Tel: (216) 444-9485; Fax: (216) 444-4985; E-mail: agarwaa@ccf.org

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view to emulate the process of natural, in vivo reproduction. However, the in vitro environment exposes gametes and embryos to an excess of reactive oxygen species not normally experienced during in vivo fertilization and pregnancy. Although reactive oxygen species are physiologically required for various biochemical pathways necessary for reproduction, their in vivo levels are tightly controlled by enzymatic antioxidants that scavenge and neutralize free radicals to maintain an optimal, physiologic oxygen tension in the male and female reproductive system.

In contrast, in vitro ART procedures are carried out without the protection of enzymatic antioxidants normally found in vivo, leading to unopposed, elevated levels of ROS, which have been shown to adversely affect gametes, gamete interaction, fertilization and pregnancy rates. Given the impact of redox status on ART outcomes, it is crucial to elucidate the mechanism by which ROS are generated, methods to accurately quantify ROS levels, and strategies to regulate the amount of ROS experienced to enhance embryo quality, promote single embryo transfer and reduce multiple pregnancy rates with ART.

Poor fertility outcomes with ART are particularly problematic when the high risk of failure in achieving reproduction is placed in an economic context. The high cost of ART...
coupled with relatively low success rates is especially unacceptable in the developing world, where resources are scarce and a high prevalence of infertility endures. Thus, it is imperative to investigate new strategies to improve the rate of successful outcomes in ART. The aim of this discussion is to clarify what is currently known regarding the role oxidative stress plays in ART and potential ways to alleviate the effects of oxidative stress, with specific interest in the use of antioxidants to optimize ART outcomes.

WHAT IS ART?

Assisted reproductive techniques can serve as an effective method to overcome a variety of causative factors of infertility such as pathology of the fallopian tubes, endometriosis, and male factor and unexplained infertility [1]. The primary focus of this review is centered on the effect of oxidative stress on the outcome of in vitro ART procedures and the potential role of antioxidants to protect against oxidative stress to increase the likelihood of achieving successful pregnancy with ART.

Intrauterine Insemination

Intrauterine insemination (IUI) is performed by threading a very thin, flexible catheter through the cervix and injecting washed spermatozoa into the uterus. This technique requires large numbers of forward-progressing motile spermatozoa [2]. Moreover, higher sperm counts increase the success rates of this procedure. Therefore, male infertility patients with a low number of spermatozoa are not suitable candidates for IUI. In addition, this method is not suitable for semen samples high in free radicals due to the presence of large number of leukocytes, cellular debris, and/or immature germ cells [2].

In vitro Fertilization

(IVF) is another assisted reproductive technique in which sperm-oocyte interactions take place within culture media, leading to fertilization. Most IVF cycles utilize fertility drugs such as GnRH agonists and human menopausal gonadotropins to stimulate the ovaries to produce several mature eggs, in addition to the single egg normally produced each month. If ovarian stimulation is not done, the oocyte may simply be retrieved from the natural menstrual cycle, avoiding any risk of overstimulation and multiple pregnancy. The oocytes are collected into a specially prepared culture medium, which then is microscopically evaluated for maturity. Between 20,000-30,000 sperm are mixed with each oocyte in a drop of specially prepared culture medium and then incubated to ensure an optimal environment to facilitate fertilization. After fertilization is complete, the resulting embryos are qualitatively graded on the basis of morphology, and those chosen for transfer are loaded in a minute volume of medium into a transfer catheter. A catheter tip is advanced into the uterus and the embryos are expelled. The pregnancy rate increases with the number of embryos transferred.

This treatment is primarily for female-related infertility problems such as hydrosalpinges, damaged or inoperable fallopian tubes, endometriosis, and cervical mucus pathology. Idiopathic infertility, male infertility and immunologic infertility also respond well to IVF. A major disadvantage of IVF is the need for women to undergo ovarian stimulation with hCG or progesterone hormonal therapy to support the luteal phase, which can lead to ovarian hyperstimulation syndrome- the most common and potentially serious complication for conventional IVF treatment [3].

Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) is a laboratory procedure in which a single sperm is injected directly into an oocyte’s cytoplasm using a very fine needle. The process allows for oocyte fertilization regardless of the morphology and motility characteristics of the single spermatozoon injected [4]. This procedure is used in cases in which unsuccessful conventional IVF led to fertilization failure or in instances where a male-factor such as low sperm count is involved [4]. This procedure is also used for non-obstructive azoospermic patients, in which sperm may be retrieved using testicular sperm aspiration [5].

ICSI is particularly beneficial in infertile males with disordered sperm-zona pellucida interactions, in that it bypasses all of the preliminary penetration and fusion steps of fertilization. Despite these advantages, the injected sperm in ICSI are at an increased risk for having oxidatively damaged DNA which could adversely affect the outcome. ICSI circumvents the process of natural selection and may allow damaged spermatozoon to be directly injected into the oocyte, which can negatively impact embryo development. IVF is superior to ICSI with respect to avoiding this risk, as collateral peroxidative damage to the sperm plasma membrane will prevent fertilization by spermatozoa with DNA damage [2].

Similarities Between IVF and ICSI

Both IVF and ICSI are very costly procedures not covered by most health plans. They require special facilities and are subject to many regulatory restrictions. Although these procedures provide the possibility of a potential cure for infertile couples, there are many weaknesses and factors that often detract from successful reproductive outcomes. As in vitro procedures, the environment in which IVF and ICSI are conducted fails to mimic the intricate physiological conditions of an in vivo system. These methods are greatly affected by environmental factors without beneficial protective factors such as antioxidants provided by the in vivo environment. Measures aimed at minimizing differences between in vitro and physiologic in vivo conditions should be investigated and employed to maximize the efficiency of ART procedures. This article will review the role of oxidative stress in both conventional IVF and ICSI and approaches to optimize the chance of achieving successful pregnancy with these procedures.

WHAT IS OXIDATIVE STRESS?

Aerobic metabolism generates reactive oxygen species-hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide. They are small and highly reactive due to unpaired valence shell electrons that are capable of initiating an uncontrolled cascade of chain reactions. Normally, this is regulated by antioxidants, which have the ability to scavenge and neutralize free radicals [2]. The controlled production of
such compounds is known to play a role in physiological reproductive processes such as hormone signaling, oocyte maturation, folliculogenesis, tubal function, ovarian steroidogenesis, cyclical endometrial changes, and germ cell function. However, at higher levels ROS may overwhelm antioxidant capacity, leading to oxidative stress. The high energy electrons of ROS are capable of modulating gene expression and transcription factors, with the ability to modify and damage DNA [2].

Intracellular homeostasis is achieved through a balance between pro-oxidant compounds and antioxidants. Antioxidants have the ability to oppose the effects of pro-oxidants by hindering ROS production, scavenging ROS, and repairing cell damage caused by ROS. Non-enzymatic antioxidants consist of vitamin C, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase. Within the body, enzymatic antioxidants protect and support gametes and embryos during fertilization and pregnancy from OS-induced pathological changes. IVF and ICSI procedures lack these natural antioxidants and, therefore, expose gametes and embryos to a level of OS higher than that experienced in vivo during physiologic reproduction.

WHAT IS THE ROLE OF OXIDATIVE STRESS IN ART?

OS exerts toxic effects by altering cellular molecules such as lipids, proteins and nucleic acids. This can lead to an increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA, mitochondrial alterations, adenosine triphosphate depletion, and apoptosis. Free radicals are thought to act as a determinant in reproductive outcome due to their effects on oocytes, sperm, and embryos in their follicular fluid, tubal fluid, and peritoneal fluid microenvironments. Oocytes and embryos can be protected against OS by free radical-scavenging antioxidants that exist within the follicular and oviductal fluid [6]. However, this defense against OS is lost as sperm, oocytes, and embryos are removed from their natural microenvironments to be used for ART [6]. This loss of protection leads to increased exposure of gametes and embryos to OS, which can lead to impaired oocyte maturation and embryo development [7].

Oocyte quality is thought to act as the main determinant of IVF outcomes [8]. Generally, higher quality oocytes develop into healthy, high quality embryos, ultimately resulting in a higher birth rate [8]. The compound 8-OHdG acts as a sensitive marker of OS-induced DNA damage in granulosa cells during ovulation. A study by Seino et al. demonstrated an association between increased levels of 8-OHdG and decreased fertilization rates and embryo quality. Thus, increased OS in the granulosa cell environment that surrounds oocytes adversely affects the oocytes themselves, leading to lower success rates for IVF [8]. Wang et al. conducted a study exposing mouse embryos to PMA-activated leukocyte supernatant which generated ROS. The findings of the study relate increased OS levels with a decreased blastocyst development rate [6]. Thus, the literature implicates OS and its deleterious effects in low fertilization rates with IVF and ICSI. Therefore, a thorough analysis of the endogenous and exogenous factors that contribute to the oxidative stress faced by gametes and embryos during IVF and ICSI is necessary to appropriately manipulate the in vitro culture medium to minimize oxidative stress and improve ART outcomes.

WHAT IS THE ORIGIN OF ROS IN ART?

Endogenous Factors Affecting Both IVF and ICSI: Embryo Development

ROS play a significant physiological role in the modulation of gamete interaction and successful fertilization. Oocyte metabolism generates ROS. This, coupled with the lack of protective antioxidant mechanisms that normally would be found in vivo [9], results in an overall increase in OS in the in vitro environment. Preimplantation embryonic development is accompanied by a change in preference in energy metabolism pathways [10]. Oxidative phosphorylation is utilized throughout the preimplantation embryo development period. Increased energy demand is met by a significant shift from dependence on oxidative phosphorylation to glycolysis to generate ATP [7]. Oxygen is needed for oxidative phosphorylation to convert ADP to ATP, which is required for the processes of folliculogenesis and oocyte maturation. This use of oxygen results in ROS production, which in excess has a toxic influence on embryo development [7]. ROS are primarily produced at the inner mitochondrial membrane where electrons leak from the electron transport chain and are transferred to the oxygen molecule, resulting in oxygen with an extra unpaired electron [10, 11]. In addition, cytoplasmic or membrane-bound NADPH-oxidase, cytochrome p450 enzymes and the xanthine-xanthine oxidase systems [10] are also capable of generating an excess of ROS.

Endogenous Factors that Differ Between IVF and ICSI

There are differences between the potential sources of ROS derived from conventional IVF and ICSI [12] (Fig. 1). For instance, oocytes used in ICSI are initially denuded of their cumulus cells so that the only possible source of ROS is within the culture media environment, the oocyte, and the injected spermatozoa [9]. With IVF, however, ROS may arise from the multiple oocytes per dish, the large cumulus cell mass, and from the spermatozoon used for insemination. Unlike IVF, ICSI avoids contact time between the sperm and oocyte, thus minimizing the opportunity for ROS generation by defective spermatozooa [9, 13]. Lower sperm concentrations in the culture media are correlated with an improvement in fertilization, implantation, and pregnancy rates and with yielding higher quality embryos [14]. Thus the ICSI procedure, which utilizes single spermatozoa at a time, minimizes OS development from the gametes themselves.

Exogenous Factors Affecting Both IVF and ICSI

The external environment and culture media surrounding embryos during both IVF and ICSI are believed to significantly influence the outcomes of these procedures. IVF and ICSI do not significantly differ with respect to the level of exposure of sperm, oocytes, and embryos to ROS [8]- aside from the fact that the incubation time interval is shorter in the ICSI procedure, decreasing the amount of exposure of
the culture media to external environmental factors [9, 13]. An incubation time limited to 1-2 hours has been associated with better ART outcomes. With IVF and ICSI, ROS may arise from the male or female gamete, the embryo, or indirectly from the external environment, which includes cumulus cells, leukocytes, and the culture media.

As fertilization and embryo development in vivo are known to occur in an environment of low oxygen tension, it is hypothesized that OS experienced in the in vitro setting is a major contributor to the persistently high failure rate of ART. Therefore, ART outcomes should improve if in vivo conditions are emulated by avoiding the various factors that promote ROS production.

**OXYGEN CONCENTRATION**

ROS may be derived from exogenous factors that impact both IVF and ICSI. This effect may be more pronounced in IVF due to longer incubation time intervals leading to increased exposure to environmental oxygen concentration. A hyperoxic environment promotes enzyme activity, which generates an increased level of the superoxide radical. The growth of a first trimester embryo in vivo has been demonstrated to take place in a microenvironment with low oxygen tension [15]. This leads to the assertion that a low oxygen concentration is necessary during ART, which attempts to mimic the conditions of in vivo fertilization and pregnancy [15].

Studies have shown that embryos cultured in an environment with atmospheric oxygen concentration and exposed to increased levels of hydrogen peroxide radicals resulted in DNA fragmentation of approximately 20% of embryos, compared with DNA damage seen in 5% of embryos grown in an environment with low oxygen tension [16]. Reports of successful blastocyst development with embryos cultured in a low oxygen tension environment [16] imply that the oxygen concentration experienced in the in vitro setting plays an influential role in the outcome of ART procedures. A study by Noda et al. confirmed the advantage of lower oxygen concentrations in the in vitro environment by measuring OS levels encountered when using a new IVF culture system with low oxygen tension and a low level of illumination [17]. This manipulation of the culture media resulted in high blastulation rates and enhanced human embryo development [17]. Another study found that, in addition to improved blastocyst outcomes in low-oxygen environments (5%) versus high-oxygen concentrations (19%), human embryos cultured in low oxygen tension are associated with an increased live birth rate compared with embryos incubated under the influence of high oxygen concentrations [18].

**METALLIC CATIONS**

In the setting of ART, metallic cations may promote ROS generation and act as an exogenous source of OS. According to the Haber-Weiss reaction, traces of metallic ions such as iron or copper in the culture medium may lead to increased ROS generation [19]. The binding of metallic ions by metal chelators such as ethylenediamine tetra-acetic acid (EDTA) or transferrin can inhibit the ability of metals to react and produce ROS, suggesting their potential use within the embryo culture media to block the induction of ROS by metals present in the in vitro setting. Use of the iron chelator transferrin within the culture media has been shown to decrease lipid peroxidation and the formation of hydroxy radicals [20]. Minimizing the production of highly toxic hydroxy radicals is protective against free-radical-mediated damage to oocytes and preimplantation embryos and can lead to improved fertilization and pregnancy rates [20].

**ILLUMINATION**

Visible light acts as an exogenous source of OS in that it promotes ROS production, causing cellular damage through the oxidation of DNA bases and DNA strand breaks [21]. The findings of a study by Beehler et al. implicated UV
radiation in ROS-induced DNA base modifications. Furthermore, Takenaka et al. showed that smaller amounts of short-wavelength visible light are advantageous in ART as they expose oocytes and embryos to lower levels of OS than cool white light [22]. These results suggest that minimizing the exposure of oocytes, zygotes, and embryos to visible light and near-UV light will serve to better mimic in vivo conditions, thereby yielding more successful ART outcomes [22].

**SPERMATOZOA**

Sperm quality is a crucial determinant of the potential for successful fertilization and pregnancy achieved by ART [23]. At physiologic levels, ROS facilitates normal sperm function such as the acrosome reaction, oocyte fusion, and capacitation. Intracellularly, sperm generate ROS at the level of their plasma membrane and mitochondria. Leukocytes act as a source of OS by generating extracellular ROS in prostaglandin and seminal vesicle secretions [23, 24]. Leukocytes can cause ROS-induced damage when seminal plasma is removed during sperm preparation for assisted reproduction. Seminal leukocytes stimulate ROS production by spermatozoa.

Male germ cells are extremely vulnerable to OS as the sperm membrane is rich in unsaturated fatty acids [23] and lacks the capacity for DNA repair [25]. Human spermatozoa generate superoxide radicals that can cause lipid peroxidation, which occurs in a self-propagating manner. This is associated with a decrease in membrane fluidity and reduced activity of membrane and ion channels which impairs spermatozoan fertilization capacity. In addition to these effects, OS arising from spermatozoa induce peroxidative damage to the oocyte and its DNA, reducing the likelihood of successful fertilization [25]. A study conducted by Hammadeh et al. demonstrated that metaphase oocytes incubated with DNA-damaged spermatozoa during IVF were associated with high rates of failed fertilization, defective embryo development, implantation failure and early abortion [26]. Therefore, utilizing sperm preparation techniques that minimize ROS production within seminal fluid and by spermatozoa themselves is likely to improve the success of IVF and ICSI procedures.

Swim-up preparation is a sperm preparation technique in which a semen sample is centrifuged to form pellets that are coated with culture medium. The most motile spermatoza will swim up into the culture medium, and they are selected for use in the IVF procedure. This technique is not appropriate for semen samples with excessive leukocytes or immature or damaged spermatozoa [9] as close contact with defective spermatozoa and leukocytes [11] will lead to ROS-induced damage of functional spermatozoa. It is hypothesized that the risk of OS-induced damage may be reduced by incorporating the use of antioxidants in the swim-up technique [27]. ROS levels have been shown to be significantly lower in sperm suspensions washed with antioxidants than in the control group without antioxidant protection. Antioxidant supplementation was seen to significantly decrease DNA fragmentation rates in spermatozoa, whereas it had no effect on lipid peroxidation. These results suggest that antioxidant supplementation may serve a beneficial role in sperm preparation to improve ART outcomes by enhancing the overall functional parameters of spermatozoa through reducing levels of oxidative stress [27].

Density-gradient centrifugation is a sperm preparation technique used to isolate mature, leukocyte-free spermatozoa [9]. This method employs centrifugation to separate fractions of spermatozoa based on motility, size, and density [3]. The process of centrifugation can activate seminal leukocytes and disturb the redox balance within a semen sample, with the potential to adversely affect sperm function [28]. A study by Shekarziz et al. demonstrated that even a single-step centrifugation for a short period could significantly increase ROS formation in human semen [28]. Increased levels of ROS are also thought to arise during this type of sperm preparation technique due to mechanical perturbation of the sperm plasma membrane. Thus, minimizing centrifugation time minimizes the formation of excess ROS and may ensure the use of high quality sperm in ART [28].

In general, the literature confirms an inverse correlation between ROS levels and sperm functional characteristics, including density, motility, and morphology. Oxidative stress is related to DNA fragmentation of spermatozoa that is sub-optimal for use in IVF or ICSI [26]. Thus, strategies used to prepare sperm and decrease ROS levels in a semen sample may allow for an increased chance of successful fertilization and pregnancy with ART.

**HOW ARE ROS LEVELS MEASURED?**

**Follicular Fluid**

Oxidative stress is thought to occur within the follicular fluid of women undergoing IVF and ICSI [25]. The follicular fluid microenvironment plays an integral role in shaping the quality of the oocyte, which is a major determinant of successful ART outcomes [1, 25]. The results of a study by Van Blerkom et al. demonstrate that hypoxic conditions, as evidenced by low intrafollicular oxygenation, are related to an increased likelihood of oocyte cytoplasmic defects such as impaired cleavage and abnormal chromosomal segregation [29]. The degree of OS can be quantified by assessing biomarkers of lipid peroxidation and the total antioxidant capacity (TAC) within follicular fluid [25]. The compound malondialdehyde (MDA) is a by-product of lipid peroxide decomposition and can be used to measure ROS levels. Pasqualotto et al. discovered that the mean MDA levels of pregnant women were significantly lower than the mean MDA levels seen in non-pregnant control subjects [30]. In addition to its role as a biomarker for OS, MDA levels may be a predictive marker of ART outcome. Follicular fluid of oocytes that were later successfully fertilized exhibited an increase in TAC [1]. Thus, lower TAC, which would lead to a redox imbalance with overwhelming levels of unopposed ROS, can be related to decreased fertilization potential as an adverse effect of OS.

A study by Wiener-Megnazi et al. used a novel thermochemiluminescence assay to assess the degree of OS in follicular fluid [31]. Increased levels of OS within follicular fluid were significantly related to poor fertilization rates and post-fertilization outcomes, manifested as decreased blastocyst cleavage and development, and a lower likelihood for completion of a successful pregnancy [31].
Culture Fluid

Within the culture fluid, the TAC levels of Day 1 culture media are postulated to serve as a biochemical marker of protection against OS in the early stages of embryo development [25]. Day 1 TAC levels have been shown to significantly correlate with increased clinical pregnancy rates in ICSI cycles [25]. Bedaiwy et al. analyzed the effect of ROS levels on early human embryonic development in IVF and ICSI culture media on Day 1 post-insemination [12]. The findings implicate increased Day 1 ROS levels in decreased development rates, higher degrees of fragmentation, and reduced formation of morphologically normal blastocysts [12]. The results also showed that decreased fertilization outcomes in ICSI were significantly related to increased Day 1 ROS levels in the culture fluid. However, this apparent relationship was not exhibited by the conventional IVF cycles studied. Another study by Bedaiwy et al. used TAC levels in Day 1 culture media as a biochemical marker of OS to demonstrate an association between increased Day 1 ROS levels and slower development, higher fragmentation rates, and reduced formation of morphologically normal blastocysts [13]. Day 1 ROS and TAC levels within culture media serve as useful biomarkers of OS and can be utilized to quantify the degree of OS present in the setting of IVF and ICSI and relate OS to embryonic development parameters and thus ART outcomes.

Metabolomic Profiling to Assess ROS

In the past, evaluation of ROS and OS was based entirely on biochemical methods that are inconvenient and labor-intensive. Efforts have continued to determine a more efficient way of identifying and measuring biomarkers that accurately quantify and correlate OS to clinical reproductive outcomes. OS may be assessed on a molecular level using metabolomics - the systematic study of metabolites as small-molecule biomarkers that contribute to the functional phenotype of a cell, tissue, or organism [32]. The metabolome refers to the complete inventory of small molecules, metabolic intermediates such as amino acids, lipids and nucleotides, ATP, hormones, other signalling molecules, and secondary metabolites [33]. Low-molecular weight metabolites represent end products of cell regulatory processes, revealing the response of biological systems to a variety of genetic, nutrient, or environmental influences [34]. Therefore, the metabolome represents the end product of gene expression, illustrating the interaction of environmental conditions with physiology [35] (Fig. 2).

Metabolomic profiling is a rapid, non-invasive method of measuring OS markers such as -CH, -NH, -OH and ROH. OS is reflected by the CH:ROH ratio. Gas chromatography, high performance liquid chromatography, or capillary electrophoresis is used to separate biomarkers, while methods of spectrometry are used to identify and quantify them [36]. Metabolomic profiling has been investigated for its use in quantifying the degree of OS during ART and identifying those gametes and embryos most likely to contribute to successful implantation and pregnancy.

Currently, the morphology and cleavage rate of embryos are used as a predictive measure of implantation potential in IVF. Morphological assessment consists of observing the developmental pattern of embryos during culture, fragmentation, inclusion bodies, cell number, morphology of the inner cell mass and trophectoderm, and blastocoele expansion at the blastocyst stage [37]. However, these parameters may not accurately reflect functional status, as normal-looking gametes and embryos can still harbour genetic or epigenetic defects such as ROS-induced damage [38]. The inability to precisely determine the reproductive potential of gametes and embryos contributes to the high failure rate in IVF. To minimize the risk of implantation failure, multiple embryos often are transferred simultaneously, leading to an increased incidence of multiple pregnancies [35]. Metabolomic profiling used in conjunction with morphological assessment of gametes and embryos may serve to maintain or increase overall pregnancy rates while decreasing the unfavorable occurrence of multiple gestations [35].

Fig. (2). The use of metabolomic profiling to quantify oxidative stress.
A quantitative, objective assessment of gametes retrieved for use in IVF may provide a prognostic indication of IVF outcome and clarification of the reasons for success or failure of the procedure. Metabolomic profiling can be used to assess the impact of OS on sperm function and fertilization capacity. A study by Agarwal et al. demonstrates the existence of unique spectral 'signatures' of semen samples that indicate statistically significant differences in the levels of oxidative stress among men with various conditions known to be associated with altered redox status including varicocele, idiopathic male factor infertility, and vasectomy reversal. The various degrees of OS were evidenced by unique changes in the ratios of –CH to ROH. Significant differences in –CH, –NH, and –OH concentrations were observed among the groups of subjects [39]. These findings assert that metabolomic analysis is a rapid, non-invasive diagnostic method that can be used to assess semen for abnormalities related to reactive oxygen species.

The metabolome of an oocyte is thought to be ‘fingerprinted’ by its interaction with its IVF culture medium and exogenous factors such as environmental oxidative stress [40]. Metabolomic profiles of follicular fluid samples have demonstrated a significant relationship between high levels of OS in follicular fluid and decreased oocyte viability and poor embryo quality [41]. A study by Nagy et al. correlated the metabolome of oocytes with corresponding embryo fertilization, development, and viability and demonstrated the ability of NIR spectroscopy to predict fertility potential as early as the pre-fertilization stage of oocytes. Metabolomic analysis of the spent culture media of oocytes was shown to predict embryo development at Day 3 and Day 5, with the potential to indicate embryo viability. NIR analysis was shown to assess the metabolomic status of oocytes with high sensitivity and significant correlation with healthy embryo morphology and high implantation potential [40].

Embryo metabolomic profiles also have been shown to vary according to embryo implantation potential. A study by Scott et al. used metabolomic profiling to calculate a viability index for 41 spent media samples from 19 patients with known reproductive potential. Higher viability indices were observed in both Day 3 and Day 5 embryos with proven reproductive potential than in those that failed to implant. An overall diagnostic accuracy of 80.5% in predicting delivery or a failed implantation demonstrates the significant relationship between the reproductive potential of embryos and modifications of their culture media [42]. Seli et al. used a multivariate analysis approach to compare the spectral profiles between embryos that resulted in live births with those that failed to implant. Markers of OS discriminated between the two study populations and were most predictive of pregnancy outcome. CH:ROH content was significantly lower in the culture media of embryos that progressed to pregnancy than in the culture media of embryos that failed to implant [43]. Therefore, in vitro cultured embryos with high reproductive potential may alter their environment differently compared with embryos that do not result in pregnancy [44].

A similar investigation by Agarwal et al. used metabolomic profiling to identify the OS biomarkers R-OH, CH, OH, and NH in 228 embryo media, 72 follicular fluid, and 133 seminal plasma samples. The discarded culture media, follicular fluid, and seminal plasma were shown to consistently produce unique metabolomic OS profiles, which correlated well with pregnancy versus non-pregnancy outcomes [45].

In addition to indices of redox status, metabolomic profiling of amino acids may be helpful determining the functional status of embryos. During the IVF process, embryo culture media is often supplemented with mixtures of amino acids that influence blastocyst formation [46]. A study by Houghton et al. found that competent preimplantation embryos that develop into blastocysts demonstrate a lower rate of amino acid turnover than embryos that did not progress to the blastocyst stage. These results suggest that embryos with higher metabolic activity are more likely to arrest, contributing to failed outcomes in ART [47]. Brison et al. reported an association between decreased glycine and leucine and increased asparagine levels in the culture media with increased clinical pregnancy and live birth rates. Lower levels of amino acid metabolism in embryos were correlated with increased viability [48]. Cryopreserved embryos used in successful IVF procedures also have been shown to exhibit lower rates of amino acid metabolism compared with those that failed to implant [49]. Therefore, the use of metabolomic profiling to measure parameters such as CH:ROH and amino acid turnover may allow for better discernment when evaluating and selecting embryos for transfer in IVF. These methods of objectively quantifying embryo viability and implantation potential may minimize the number of embryos needed to be transferred, thereby decreasing the incidence of multiple infant births and improving overall pregnancy outcomes.

In addition to predicting the potential of gametes and embryos to give rise to favorable reproductive outcomes, metabolomic profiling of the uterine endometrial lining may indicate the degree of endometrial receptivity to blastocyst implantation, providing the opportunity to optimally time embryo transfer in ART [32]. Although the results of many studies suggest various ways in which metabolomic profiling may help to improve ART outcomes, this method of evaluating the functional status and potential of gametes and embryos has yet to be standardized and requires validation by further studies.

**WHAT IS THE ROLE OF ANTIOXIDANTS IN IVF AND ICSI?**

The culture media used in IVF and ICSI can be a source of ROS generation during ART procedures [50]. The in vitro environment exposes gametes to ROS in excess of what they would normally face in vivo [50]. An excess of ROS has the ability to damage lipids, proteins, nucleic acids, DNA, and RNA [25]. Thus, IVF protocols must be revised to incorporate strategies that decrease or prevent the generation of ROS [50].

Javiet et al. established that culture media riddled with a toxic level of OS compromises oocyte and embryo integrity. Because the success rates of IVF are influenced by the quality of the embryos transferred, antioxidant supplementation...
to neutralize the effects of ROS on oocyte quality may have a beneficial effect on ART outcome [25]. Repeated changes of the culture media and the use of sequential culture systems may help to minimize the ROS generated from within the culture media itself [11]. The use of supplemental antioxidants to modulate ROS levels in the culture media is hypothesized to promote an ideal environment for pre-implanted embryos produced by ART [25]. Ongoing trials continue to investigate the role for oral antioxidant supplementation in both infertile men and women with the aim of optimizing the success rates of IVF and ICSI procedures.

**Antioxidant Supplementation Within the Culture Media**

The success of ART depends on how closely the in vitro setting mimics in vivo conditions. Therefore, it is crucial to have physiological concentrations of individual amino acids, antioxidants, vitamins, and energy sources within the culture media of embryos to maximize blastulation hatching rates [51]. However, the literature presents conflicting reports regarding the validity of using any one specific antioxidant therapy to improve ART outcomes. However, given the strong evidence that OS plays a pathogenic role in decreasing the success rates of IVF and ICSI, the potential for the use of antioxidants to control OS in the in vitro setting is a promising concept that warrants further investigation in the field of reproductive medicine (Fig. 3).

Wang et al. elucidated the effects of adding the antioxidant vitamins C and E to culture media in ART. The results of this study demonstrated that these antioxidants led to increased rates of blastocyst development, by way of counteracting the embryotoxic effects of ROS [6]. Embryo culture supplementation with vitamin C was seen to exert a protective effect on embryo development in culture media containing an ROS-generating PMA-activated leukocyte supernatant [52]. Furthermore, the findings of Wang et al. suggest that the beneficial, ROS-neutralizing effect of vitamin C supplementation in culture media may be superior to the effects seen with vitamin E supplementation or simultaneous supplementation with both vitamins C and E [6]. The idea that there is a critical composition and dosage of antioxidant supplementation, below or beyond which the full potential protective benefit may not be realized, is confirmed by the findings of Choi et al. [53]. This study showed that vitamin C administered at a higher dosage than needed is capable of inducing damage. Moderate dosages, however, were seen to reduce oxidative damage in the culture media, with no detrimental effect on mouse oocyte quality [53].

The investigations of Olson et al. determined that vitamin E supplementation in culture media increased the number of bovine embryos that reached the fully expanded blastocyst stage [52]. This effect is thought to be mediated by the role vitamin E plays in protecting unsaturated membrane fatty acids. The peroxidation of these membrane lipids can lead to structural damage and affect its membrane function. Furthermore, the results showed that vitamin E supplementation alone improved conditions for embryonic development more than that seen when vitamin E and C were employed in combination.

Supplementing the culture media with melatonin, a powerful hormonal agent known to possess the ability to neutralize ROS, also has been shown to improve the efficiency of in vitro embryo production in experiments using buffalos [54]. This has led to studies investigating the merits of oral melatonin supplementation to improve fertility.

L-carnitine also has been evaluated for its potential use as a supplement in embryo culture medium and the effects of this supplementation on developing mouse embryos [55]. L-carnitine is known to possess antioxidant properties, consisting of free radical scavenging and metal-chelating properties [55]. Specifically, this compound has the ability to neutralize the embryotoxic effects of exogenous production of oxidative stress by hydrogen peroxide [55]. Embryo culture medium supplementation with L-carnitine has been shown to bring about a significant improvement in the blastocyst development rate compared with a control group without antioxidant supplementation. At moderate concentrations, L-carnitine is has been proven effective in blocking the effect of ROS, resulting in a decreased level of DNA damage [55].

![Fig. (3). Interventions to control OS during IVF/ICSI.](image)
Oral Antioxidant Supplementation

Studies investigating the benefits of oral antioxidant therapy in male and female patients undergoing IVF or ICSI procedures have yielded inconsistent data and conflicting reports.

FEMALE

Oral antioxidant supplementation in female patients undergoing conventional IVF or ICSI has been evaluated in terms of the effects on fertilization rates and pregnancy outcome.

Melatonin, a free radical scavenger that acts within the mitochondria to decrease protein damage, improve electron transport chain activity, and reduce mitochondrial DNA damage [56], has been shown to protect against the toxic effects of oxidative stress on oocyte maturation, thus improving oocyte quality and fertilization rates [56]. Tamura et al. used the biomarker 8-hydroxy-2-deoxyguanosine to evaluate the association between OS in follicular fluid and poor oocyte quality. Orally administered melatonin was seen to both the redox status within follicular fluid and oocyte quality [56]. In addition to confirming the toxic effects of OS on oocyte maturation, the study supported the idea that oral melatonin supplementation can be used to bring about a significant reduction in the number of degenerate oocytes and increase the number of fertilized embryos [56].

Despite the findings of Tamura et al., clinical trials have failed to demonstrate a specific regimen and dosage of antioxidant supplementation that will provide a definitive increase in ART success rates. Antioxidant supplementation with ascorbic acid has long been hypothesized to have a favorable influence on ART procedures for female factor infertility. A significant correlation is known to exist between the level of ascorbic acid in a woman’s blood serum and follicular fluid, with the follicles having a higher concentration of ascorbic acid [54]. Crha et al. conducted an investigation in which a group of women supplemented with vitamin C during the period of hormonal treatment in IVF had a statistically insignificant increase in the ability to achieve pregnancy compared with the control group that did not receive oral antioxidant supplementation [57].

Based on the premise that ascorbic acid confers an improvement in fertility and ART outcomes, Griesinger et al. evaluated the impact of ascorbic acid at different doses on women undergoing IVF procedures [58]. In contrast to the findings of Crha et al., the results of this study failed to reveal clinical evidence of any beneficial effect of ascorbic acid on IVF [58]. Furthermore, Tarin et al. found that the oral administration of pharmacological doses of vitamins C and E on mice had no effect on time to pregnancy, age of cessation of female reproductive life or pregnancy rate [59].

MALE

In contrast to the accepted management of female infertility, oral antioxidant supplementation in the management and treatment of infertile men is widely accepted and practiced. A study by Geva et al. demonstrated that oral antioxidant supplementation with vitamin E led to a significant increase in fertilization rates in IVF for male factor infertility [60]. The results of this study provide evidence for the potential of vitamin E therapy to improve fertilization rates of fertile normospermic patients with low fertilization rates after one month of treatment. Greco et al. demonstrated a significantly reduced percentage of DNA-fragmented spermatozoa in a study group treated with oral vitamins C and E compared with control group subjects who were not given oral antioxidant supplements [61]. However, this study failed to show any improvement in pregnancy rates with oral antioxidant therapy. Another study by Greco et al., which examined the beneficial effect of oral antioxidant treatment in cases of DNA-damaged sperm utilized for ICSI, revealed no differences in fertilization and cleavage rates or in embryo morphology with vitamin C and E antioxidant treatment. However, a marked improvement in the number of clinical pregnancies and implantation rates was observed in the antioxidant treatment group [62]. Vitamin E itself has been hypothesized to protect against the loss of sperm motility by lipid peroxidation and improve sperm motility and increase the possibility of successful fertilization with sperm from asthenospermic patients [63]. Furthermore, Suleiman et al. showed a significant decrease in ROS-induced sperm and improvements in spontaneous pregnancy rates during the next six months with oral vitamin E supplementation [63].

Tremellen et al. investigated Menevit (Bayer Health Care, Pymble, NSW, Australia), an oral antioxidant treatment used by male patients, and found no differences in quality of resultant embryos between the antioxidant-treated and control groups. However, supplementation was related to significant improvements in implantation rates and pregnancy outcomes. Therefore, treating infertile males with the oral antioxidant Menevit may serve to improve pregnancy rates by optimizing factors related to the amount of OS experienced during the course of IVF/ICSI treatment [64].

Meneco et al. [65] correlated oral vitamin intake with reduced levels of ROS-induced DNA fragmentation, leading to improved fertility. However, in addition to better semen quality, oral antioxidant supplementation also was associated with increased sperm decondensation, which may prevent IVF/ICSI success [25]. A study by Rolf et al. failed to demonstrate any improvement in semen parameters or survival rates of sperm from men with asthenozoospermia or moderate oligoasthenozoospermia who were supplemented with oral vitamin C and E [66]. Although considerable data suggest a possible benefit from vitamin C and E supplementation on ART outcomes, several investigations have yielded conflicting findings. This lack of consensus in the literature complicates the debate as to whether oral antioxidant supplementation has a definitive role in optimizing ART outcomes.

CONCLUSION

Despite the many advances in ART, the issue of using antioxidant therapy to alleviate the burden of infertility by improving IVF and ICSI procedures and their outcomes continues to be the subject of much debate. Although data from the existing literature fail to provide any definite conclusions regarding whether specific antioxidant supplementation of infertility patients and culture media used in ART will
increase successful ART outcomes, significant evidence suggests that it has the potential to combat oxidative stress, a known contributor to ART failure. There is no doubt that there is an underlying link between OS and difficulty in achieving fertilization and eventual pregnancy with IVF and ICSI. Further controlled evaluations using large sample size populations are needed to arrive at a consensus regarding the use of antioxidant supplementation in the culture media and oral administration of these compounds in both male and female patients undergoing ART.

**EXPERT COMMENTARY**

The purpose of this article was to discuss the role played by oxidative stress in assisted reproductive technologies such as IVF and ICSI. Controlled levels of OS are known to serve a physiological role in the various biochemical pathways that comprise human reproduction. However ART procedures lack the protection of the natural antioxidant defense of the female reproductive tract, the microenvironment where fertilization normally takes place. Therefore, gametes used in ART procedures are highly susceptible to damage from overwhelming and unopposed degrees of oxidative stress that may arise endogenously from the gametes themselves and from exogenous influences within the laboratory environment such as oxygen tension, light, and metallic cations. Increased OS has been shown to hinder sperm-oocyte interaction and adversely affect fertility and successful pregnancy rates. Given the evidence that implicates oxidative stress in producing poor ART outcomes, elucidating the precise mechanisms by which ROS arise in the ART environment, current methods to accurately quantify OS, and potential strategies to modulate the amount of OS experienced by gametes within both the human reproductive tract and the in vitro culture media must be a top priority.

The field of metabolomics has given rise to novel techniques to more accurately measure oxidative stress. Oxidative stress quantification may allow for the selection of the most viable gametes with the least OS-induced damage to be used in ART to optimize fertility outcomes and ensure greater efficiency. ART outcomes also may be improved by modulating the amount of OS experienced by gametes and embryos in vitro by supplementing the culture media with protective antioxidants. Furthermore, increased levels of oxidative stress that may be seen in vivo in both the male and female partner participating in ART may be treated by oral antioxidant supplementation. The use of oral antioxidants to treat male factor causes of infertility are well-established and in clinical use. However, the safety and efficacy of oral antioxidant supplementation to treat female infertility requires further investigation.

**FIVE YEAR VIEW**

Success rates in ART are influenced by maternal age, number of oocytes retrieved, and the quality of the embryos transferred. Embryo quality is influenced by extrinsic factors like culture media. A large number of extrinsic factors that modulate OS can influence successful outcomes of IVF and embryo transfer including oxygen concentration, ionizing radiation, and levels of the antioxidants EDTA, SOD, and catalase. Evidence suggests that media supplementation with antioxidants, disulphide reducing agents, or divalent chelators of cations may be beneficial to gametes/embryos. Repeated change of media and use of sequential culture systems may help reduce exposure to ROS. Oocytes also can be protected from oxidant-induced early apoptosis by supplementing media with vitamin E and C. In addition to strategies to modulate OS to reduce the chance of fertilization and pregnancy failure, more accurate methods to assess the degree of oxidative stress may allow for selection of healthy gametes and embryos for use in IVF or ICSI. Optimizing the procedure in this way may serve to reduce patient time and costs associated with the need for repeat use of assisted reproductive technologies. The significance of improved IVF/ICSI outcomes would be enormous to the many infertile couples who seek assisted reproduction due to infertility issues.

**KEY ISSUES**

- IVF and ICSI are assisted reproductive techniques that achieve fertilization by circumventing various anatomic, mechanical and functional obstacles to fertility in vivo.
- Oxidative stress arises when high levels of ROS overwhelm antioxidant capacity, resulting in modified gene expression and transcription factors and damaged DNA. ART procedures are susceptible to increased OS as gametes lack the natural antioxidant defence seen during in vivo reproduction in the male and female reproductive tract.
- Endogenous sources of oxidative stress in ART include the multiple oocytes per dish, cumulus cell mass, and spermatozoa used for insemination in IUI or incubation with oocytes in IVF. ICSI avoids excess ROS that may arise from defective spermatozoa in IVF.
- Exogenous sources of oxidative stress in ART include oxygen concentration, metallic cations, and illumination.
- In the past, studies quantified OS by inconvenient and labor-intensive biochemical methods to measure ROS and antioxidant status within follicular fluid and culture fluid. Metabolomic profiling is a faster, more accurate method of quantifying OS during ART and may be used to identify gametes and embryos more likely to contribute to successful implantation and pregnancy.
- The success of ART is increased when the in vitro culture setting mimics in vivo conditions. Therefore, the use of the following antioxidants in culture media may improve outcome: vitamin C, vitamin E, melatonin, and L-carnitine.
- Various studies on the benefits of oral antioxidant supplementation in male and female patients undergoing ART procedures have yielded inconsistent and conflicting reports, and further research is required.

**REFERENCES**

The Role of Oxidative Stress and Antioxidants in Assisted Reproduction

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Coasting: What is the Cost?

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Abstract: The objective of this article is to review the current literature on the value of coasting for the prevention of ovarian hyperstimulation syndrome (OHSS). Coasting is a common procedure that is performed in ovarian stimulation cycles at risk of OHSS. Coasting is done by stopping FSH injections and monitoring E2 daily until it drops below 3000 pg/mL then hCG is given. Depriving granulosa cells of the FSH stimulus results in their apoptosis, thus reducing levels of E2 and vascular endothelial growth factor (VEGF linked to the pathogenesis of OHSS). Meanwhile, the small follicles that are dependent on FSH will undergo atresia, while large follicles will not be affected. Coasting is effective in reducing the OHSS rate, but complete prevention is not possible. Prolonged coasting is associated with a significantly lower pregnancy rate.

Keywords: Coasting, E2, Stoppage of FSH, Antagonist.

INTRODUCTION

Ovarian hyperstimulation syndrome is the most serious complication of ovulation induction for in vitro fertilization (IVF) [1] as well as non-IVF cycles [2]. Rarely OHSS occur after stimulation with clomiphene citrate or in a spontaneous pregnancy [3]. Several methods have been used for prevention of OHSS. Identification of patients at risk of OHSS, namely, women with polycystic ovarian syndrome or a history of previous OHSS, is the first step in prevention.

One of the effective measures for prevention is using a small starting dose of FSH for stimulation together with close monitoring. Other measures used for prevention of OHSS include intravenous albumin [4], the use of GnRH antagonist as a protocol of stimulation [5] and in patients treated by a long GnRHa protocol with very high E2 levels toward the end of ovarian stimulation [6]. Cancelling of the cycle is the only choice if the risk of OHSS is very high. Coasting was introduced in the 90s as a novel method of OHSS prevention, and it gained wide acceptance worldwide. The objective of this chapter is to review the literature on coasting and discuss its place in OHSS prevention.

DEFINING COASTING

Coasting is a technique that involves withdrawing exogenous gonadotrophins and withholding hCG until the patient’s serum estradiol concentration decreases to a safer concentration. Many studies have been published on the role of coasting for OHSS prevention. The technique appeals to physicians and patients, and it also allows for the timely transfer of fresh embryos [7]. Coasting is performed when a large number of follicles and/or rising serum estradiol concentrations are observed, and it is the most widely favored and used preventive measure for OHSS, as well as the most cost-effective [8-10].

COASTING IN NON-IVF CYCLES

Coasting was introduced for gonadotrophin-induced cycles that were liable to develop hyperstimulation [2]. Treatment with HMG was stopped for 2–10 days, and pregnancies occurred in three patients whose serum oestradiol concentrations continued to rise until the day of hCG. Urman et al. (1992) [11] in an attempt to avoid cancellation of 40 cycles that had been overstimulated with exogenous gonadotrophins in patients with PCOS. HCG was administered after a mean 2.8 days. The clinical pregnancy rate per cycle was 25%, and only one patient (2.5%) developed severe OHSS.

It has been noted that prematurely reducing or withholding gonadotrophins prior to follicular maturation may lead to arrest of follicular growth [12-16].

COASTING AND IVF

It has been suggested that coasting in GnRH agonist/HMG/FSH cycles might prevent life-threatening complications of OHSS [17]. These authors withheld menotrophins in 17 patients whose serum E2 levels exceeded 6000 pg/ml, and continued daily GnRH agonist treatment until serum E2 levels had fallen below 3000 pg/ml hCG was then administered to trigger ovulation. The coasting period lasted between 4 and 9 days, after which six of the 17 cycles (35%) produced viable pregnancies. All 17 patients developed signs of grade 2 or 3 OHSS, but none developed severe OHSS. In another trial, the same group [17] treated 51 women at a high risk of developing OHSS who underwent coasting until plasma E2 level fell to <3000 pg/ml. The mean number of embryos transferred per procedure was 5.4, and there were 21 clinical pregnancies (41% per oocyte retrieval), but none of the women developed severe OHSS.

In a pilot study, 66 at-risk patients were coasted, receiving hCG when the serum E2 level reached 2500 pg/ml, but four patients developed OHSS [12]. In a retrospective study,
120 women considered to be at risk of OHSS [15] were coated when serum E2 levels exceeded 2500 pg/ml, and hCG was delayed until E2 levels fell below 2500 pg/ml. Outcomes were compared with those from 120 matched OHSS high-risk patients, but without coating. Coasting significantly decreased the incidence of both moderate and severe OHSS.

In a multicenter trial [16], 65 IVF cycles were severely hyperstimulated, and coating was carried out until the serum E2 level fell below 10,000 pmol/l (mean 4.3 days). Four cycles were cancelled, and a pregnancy rate of 42% per started cycle with an implantation rate of 31% was achieved. Only one patient developed severe OHSS.

In another study [18], four out of 20 patients developed severe OHSS despite coating. The mean duration of coating was 3 days and hCG was administered on the day that serum E2 levels began to fall. The authors concluded that this was too early to administer hCG.

Three groups of IVF–ET (embryo transfer) patients, including a group of highly responsive coated patients, a group of equally responsive non-coated patients, and an age-matched normally responsive control group, were also studied [19]. Two groups of coated patients also were compared to assess the effect of estradiol concentrations at the time that they met the follicular criteria for hCG administration. Lastly, the effect of varying coating duration was examined by regression analysis. The authors concluded that coating had no detrimental effect on cycle outcome in the subset studied. Regression analysis, however, suggested an inverse relationship between coating duration and the number of mature oocytes retrieved, as well as the clinical pregnancy rate.

In another study, 112 severely over-stimulated IVF/ intracytoplasmic sperm injection (ICSI) patients were treated with coating when the serum E2 level was >3000 pg/ml and the leading follicles had attained a diameter of >18 mm [20]. Fertilization failure was noted in six couples, and in another 10 cases it was decided to freeze all of the embryos. A pregnancy rate per patient of 30.4%, with an implantation rate of 18.1%, was reported, while six patients developed moderate and two severe OHSS.

Coasting was effective in preventing OHSS in five patients with polycystic ovary syndrome (PCOS) who had developed severe OHSS in a previous stimulation cycle [21]. Delvigne and Rozenberg, [22] assessed whether physicians modify their preventive attitude in relation to clinical factors and to the E2 response chart. Three case scenarios with three levels of risk factors for OHSS were constructed. At random, three out of the 12 artificially constructed case scenarios were sent to 573 physicians who are members of the European Society of Human Reproduction and Embryology (ESHRE). Coasting was by far the most popular choice (60%) among the selected preventive measures. Most of those physicians surveyed who would use coating selected an E2 level of 3000 pg/ml as a safe value for the hCG administration.

A prospective, randomized study was conducted to evaluate the incidence of OHSS and the cycle cancellation rate in 49 high-risk patients using a reduced HMG dose in one arm and continuation of the same dose in the other arm before coating [23]. The duration of coating was significantly reduced when the HMG dose was reduced.

A systematic review was conducted to analyze whether evidence is sufficient to justify the general acceptance of coating. The studies, which involved 493 patients in 12 studies, are very heterogeneous in the characteristics and number of patients in the ovulation stimulation schemes. Study designs, control groups, selection criteria for coating, and the OHSS classifications were variable. In most studies a threshold value of E2 was used (often 3000 pg/ml and/or the number of follicles were considered. The fertilization rates (36.7-71%) and the pregnancy rates (20-57%) were acceptable in terms of IVF results in comparison with those of other large IVF databanks. In 16% of the cycles, ascites was described and 2.5% of the patients required hospitalization. In conclusion, while coating does not avoid totally the risk of OHSS, it decreases its incidence in high-risk patients. Many questions remain unanswered about how coating should be managed, and we suggest that a randomized prospective multicenter study is required [24].

**DURATION OF COASTING AND IVF OUTCOME**

The length of the coating interval has been a matter for research in recent years. If this period is too short, it may not be effective in preventing OHSS, while a prolonged duration may have deleterious effects on oocyte quality and/or endometrial receptivity [10]. Previous studies have suggested that a duration of more than 4 days has a less favorable outcome [19, 25, 26].

To investigate the effect of coating on IVF outcome in GnRH agonist cycles, in a retrospective analysis [27] the average length of coating was 2.2 days. Age and baseline FSH were comparable to control cycles. The coating group had e were more follicles and oocytes, but the numbers of fertilized oocytes and embryos transferred were similar. Implantation rate (22.4% vs. 13.9%) was higher in the control group, but the pregnancy rates were comparable (45.1% vs. 38.5%). Within the coating group, baseline, stimulation, and embryology parameters were comparable between successful and unsuccessful cycles. Pregnancy rates were comparable after 1, 2, and 3 or more days of coating (36.3% vs. 38.4% vs. 40%). Pregnancy rates also were comparable (28.5% vs. 35.7% vs. 44.4%) when groups were compared based on change in E2 (<25%, 25%-50%, >50%). The authors concluded that coating for 3 days can be used successfully in the management of hyper-responding patients during IVF [27].

Another retrospective study compared 94 patients in whom coating was applied (group 1), a control group of 22 patients in whom coating was not applied despite E2 > 3000 pg/mL (group 2), and a second control group of 111 normally responsive patients with peak E2 < 3000 pg/mL level (group 3). When E2 levels were > than 3000 pg/mL in the presence of at least 20 follicles, each measuring ≥10 mm in diameter with ≥20% of them of diameter ≥15 mm, recombinant FSH administration was discontinued while GnRH agonist was maintained. There was no statistically significant difference between number of total oocytes retrieved, metaphase II oocytes, and fertilization rates among group 1 vs.
group 2. However, implantation rates and pregnancy rates were significantly higher in group 1 compared with group 2. Group 1 had more total oocytes retrieved and metaphase II oocytes compared with group 3. However, there was no significant difference in implantation and pregnancy rates between groups 1 and 3. The authors concluded that coasting does not adversely affect assisted reproductive technology outcome and can be applied safely to high-responder patients in ICSI [28].

A retrospective study evaluated the impact of the duration of coasting on IVF cycle outcome in 132 patients who showed a high response (estradiol >4500 pg/ml and/or more than 20 follicles >17 mm) to ovarian stimulation and were coasted due to their high risk of developing OHSS. Additionally, serum LH and progesterone concentrations were studied to investigate whether premature luteinization was present in these cycles and whether it might be related to coasting duration. A significant decrease in implantation rate was found when coasting was required for more than 4 days, together with a trend towards a higher cancellation rate. Premature luteinization was significantly elevated in women undergoing coasting compared with control women (34% vs. 15.6%, \( P < 0.05 \)). In the majority of patients who showed premature luteinization, coasting lasted ≥3 days. The conclusion was that prolonged coasting may affect the endometrium, affecting the implantation window [29].

Mansour et al. (2005) [30] reported a large series of patients \((n = 1223)\) at risk of developing OHSS who underwent coasting. Coasting started when the leading follicle reached 16 mm and continued until estradiol concentration fell to 3000 pg/ml. It fell to 2755 ± 650 pg/ml on the day of hCG injection, after 2.89 ± 0.94 days. Results were analyzed according to the duration of coasting (Group I: \(n = 983\), 3 days or less; Group II: \(n = 240\) more than 3 days). Number of oocytes retrieved was 16.45 ± 6.25 and 14.93 ± 6.01 in groups I and II, respectively (\( P < 0.05 \)). Fertilization rates were 63% and 65% in groups I and II, respectively. Implantation and clinical pregnancy rates were 26% and 52% in Group I compared with 18% and 36% in Group II, respectively (\( P < 0.05 \)). Severe OHSS occurred in 16 patients, representing 0.13% of all stimulated cycles and 1.3% of patients who were at risk of developing OHSS. Coasting was an effective measure in OHSS prevention, without jeopardizing the ICSI outcome. Coasting for ≥3 days is associated with a moderate decrease in pregnancy rate.

Furthermore, whether there is a threshold for the percentage of estrogen decrement that would reduce oocyte quality or that would have a negative impact on the implantation capacity of the embryos transferred remains to be clarified. Some investigators claim that there is no harm with extended coasting, whereas others have demonstrated decreased fertilization, implantation, and pregnancy rates with coasting lasting >4 days [19, 25, 27, 9, 29]. Similarly, the percentage of E2 decrement during coasting did not jeopardize IVF cycle and did not alter pregnancy rates [24, 31].

On the other hand Atabekoglu et al. [32] reported two patients with ongoing pregnancies despite a dramatically sharp decrease in E2 levels after coasting. They were a 30-year-old and a 25-year-old woman, both with unexplained infertility, in whom E2 levels increased up to 6345 and 14,275 pg/mL during ovarian hyperstimulation and decreased by 79.5% and 75.5%, respectively, after coasting. The result was two pregnancies ongoing at gestational weeks 20 and 14, respectively. The authors believe that a sharp decline in E2 levels after coasting does not have deleterious effects on implantation [32].

Using the oocyte donation model, prolonged coasting (>4 days) was found to affect both implantation and pregnancy rates in oocyte recipients whose endometrial receptivity was not altered by the coasting procedure. Thus, extended coasting duration with prolonged follicular deprivation of gonadotrophins had an impact on oocyte quality [25].

Endometrial receptivity also may be affected by the coasting procedure. Despite numerous efforts to study endometrial receptivity by immunohistochemistry and electron microscopy, and more recently by oligonucleotide microarrays and electrophoresis of secretory proteins, an unequivocal marker has not yet been found. Many authors have demonstrated that endometrium on the day of oocyte retrieval shows a ≥2 day advancement as compared with natural cycles [33]. This advancement was more pronounced in those cycles with premature serum progesterone rise on the day of hCG administration [34].

From the literature, it seems clear that coasting does not compromise IVF cycle outcome as long as its duration is not extended [9, 16, 25, 26, 35, 36].

WITHDRAWING BOTH FSH AND GnRH-agonist FOR COASTING

A retrospective study evaluated the effect of short coasting by withdrawing both gonadotropins and GnRH-agonist. When > or = 20 follicles >15 mm with E2 level of 4000 pg/mL were detected, both gonadotropins and GnRH agonist were withheld for 1 to 2 days. The mean serum E2 level fell from 7915 pg/mL at the onset of coasting to 3908 pg/mL on the day of hCG administration. Eighteen patients became pregnant (43.9%), and the implantation rate was 12.7%. Twenty-eight patients were coasted for 1 day, and 13 were coasted for 2 days. After coasting, three mild and two severe cases of OHSS occurred [37].

Coasting was tried with continuation versus stopping of GnRHa during coasting. Fifty-nine IVF (ICSI) patients were coasted for 3 or more days. In the GnRH-a withdrawal group, E2 levels decreased by 63% (18,043–6,656 pmol/L) without cycle cancellations or cases of severe OHSS. In the agonist continuation group, the E2 decrease was 29% (14,205–10,132 pmol/L) with cycle cancellation and severe OHSS rates of 9.5% (4/42) and 4.8% (2/42), respectively. Oocyte retrieval, fertilization, embryo transfer, and clinical pregnancy rates were not compromised by stopping the agonists. Withdrawal of GnRH-a during coasting interrupted increasing E2 levels, prevented cycle cancellation, and mitigated the risk of OHSS in this high-risk group without compromising oocyte retrieval, fertilization, embryo transfer, or pregnancy rates. The authors postulated that during prolonged coasting [2] GnRH-a stimulated cycles [38] and withdrawal of the agonists could interrupt the E2 increase and prevent cycle cancellation in these patients.
HOW COASTING WORKS

The rationale behind coasting is similar to the step-down protocols: mature follicles will survive for a few days without exogenous gonadotrophins while smaller follicles will enter apoptosis/necrosis, reducing the granulosa cell population that will release VEGF among other vascular mediators after hCG administration [36]. A total of 160 women (patients and oocyte donors) undergoing coasting and 116 controls were included in the study. Serum, follicular fluid and granulosa cells were collected on the day of oocyte retrieval. VEGF concentrations were determined using an enzyme-linked immunosorbent assay (ELISA). Real-time PCR was performed to evaluate VEGF gene expression in granulosa cells. Cell death was studied by flow cytometry using annexin V-fluorescein isothiocyanate (FITC) and counterstaining by propidium iodide, and double staining with CD45 monoclonal antibody was performed to distinguish the contamination of apoptotic leukocytes. Follicular cells aspirated for granulosa and luteal cell cycle status. The authors observed that follicular cells from coasted patients showed a trend in favor of apoptosis, especially in smaller follicles (48% vs. 26%, p < 0.05). Follicular fluid determinations confirmed that coasting reduces VEGF protein secretion (1,413 vs. 3,538 pg/mL, P < 0.001) and gene expression (twofold decrease by real-time PCR) in granulosa cells.

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GNRH ANTAGONIST FOR OHSS PREVENTION

In one case report, a GnRH antagonist was used to stop the LH surge in a patient who had been stimulated using the FSH/GnRH-antagonist protocol and who was at risk of OHSS [39]. HCG was withheld and antagonist continued, whereupon the serum estradiol concentrations fell and OHSS was prevented. Coasting was also effective in preventing OHSS in two high-risk patients receiving a GnRH-antagonist/FSH protocol [40].

In another study Garcia-Velasco et al. [41] evaluated 151 coasted IVF cycles from January 2001 to January 2004, of which 17 were patients who had been treated with GnRH antagonist (cetrorelix 0.25 mg since stimulation day 6) and 144 were long standard protocols. The criteria for initiating coasting were the same in both groups of patients (serum E2 levels >4,000 pg/mL and/or >20 follicles >17 mm). Patients treated with GnRH antagonists received a significantly lower dose of gonadotropins (1,780 IU vs. 2,682 IU, P = 0.012), were coasted for a shorter period (1.7 vs. 3.9 days, P = 0.001), had a lower serum E2 level at the onset of coasting (4,099 vs. 6,241 pg/mL, P = 0.001) but a similar E2 level on the day of hCG administration (2,382 vs. 2,754 pg/mL, P = ns), similar number of oocytes retrieved (19.7 vs. 20.6), comparable numbers of mature oocytes (15.4 vs. 17.1), and analogous implantation rates (25.2% vs. 26.1%). Thus, although serum E2 levels are usually lower in IVF cycles with GnRH antagonists, coasting is a feasible option in these patients to prevent OHSS.

It is believed that there is a need for a protocol that avoids prolonged coasting with its drawbacks in women at high risk of OHSS. The hypothesis of such a suggested protocol is that by using GnRH antagonist, estradiol concentration could be reduced rapidly to a safe level [42]. Meanwhile, the granulosa cells will not be totally deprived of FSH by the administration of 75 IU of HMG daily, thus maintaining oocyte quality and providing better quality embryos. The shorter intervention period in the antagonist arm and the administration of a small dose of HMG continuously may maintain the granulosa cells and support the production of good quality oocytes and embryos [6]. The mechanism of action of the antagonist that resulted in the fall in estradiol concentration is not clear. The mean drop in estradiol concentration in 94 women after 1 day of antagonist administration was 36% in the present study [6]. The reason for the immediate effect of GnRH antagonist on estradiol concentration is not completely clear.

Aboulghar et al. [6] evaluated possible advantages of GnRH antagonist administration as an alternative to coasting in prevention of severe OHSS in women undergoing IVF/ICSI. In a prospective, randomized study comparing a coasting (group A, n = 96) and a GnRH antagonist administration (group B, n = 94) in patients at risk of OHSS, the primary outcome measure was high quality embryos. The secondary outcome measures were days of intervention, number of oocytes, pregnancy rate, number of cryopreserved embryos, and incidence of severe OHSS. The numbers of
high quality embryos (2.87 ± 1.2 vs. 2.21 ± 1.1; \( P < 0.0001 \)) and oocytes (16.5 ± 7.6 vs. 14.06 ± 5.2; \( P = 0.02 \)) were significantly higher in group B as compared with group A. Days of coasting were higher than days of antagonist administration (2.82 ± 0.97 versus 1.74 ± 0.91; \( P < 0.0001 \)). In conclusion, GnRH antagonist was superior to coasting in producing significantly more high quality embryos and more oocytes as well as reducing the time until hCG administration. There was no significant difference in pregnancy rate between the two groups. No OHSS developed in either group [6].

**WHEN TO START AND WHEN TO STOP COASTING**

Coasting is generally initiated when follicles are 15 to 16 mm in diameter and serum E2 levels are extremely high (>3000 pg/mL). The large follicles have a low dependency on FSH, and they can tolerate a few days without gonadotrophins administration. The immature follicles enter atresia as they are very dependent on FSH hormone, and the mature follicles will progress and be ready for oocyte retrieval [41]. The majority of publications suggest that E2 of 3000 pg/mL is the limit below which coasting could come to an end and hCG is administered.

**EXPERT COMMENTARY**

The purpose of this paper is to discuss the role of coasting in the prevention of OHSS. Unfortunately, there are no available randomized studies that compare coasting versus no coasting or any other procedure that could be an alternative to coasting in patients at risk of OHSS. However, multiple controlled studies and comparative studies show that coasting is effective in OHSS prevention. The proposed action for coasting is apoptosis of granulosa cells which are producing E2 and a possible significant decrease in VEGF, which is thought to be the main etiological factor underlying the development of OHSS. Coasting does not completely prevent OHSS but markedly reduces its incidence. It could be used effectively with GnRH agonist or GnRH antagonist protocols.

**FIVE-YEAR VIEW**

The procedure of coasting has been used extensively with several modifications to improve its effectiveness. Major changes to the procedure are unlikely in the near-term future. On the other hand, it is likely that new drugs will be developed to prevent VEGF expression and secretion, and this will be a real breakthrough in the prevention of OHSS. However, this substance or drug to be developed should not have an effect on the quality of the embryo or its implantation in the endometrium.

**KEY ISSUES**

1. Coasting is a technique that involves withdrawing exogenous gonadotrophins and withholding hCG until the serum estradiol concentration decreases to a safer concentration in patients at risk of OHSS.

2. Coasting was by far the most popular choice (60%) among the selected preventive measures for preventing OHSS.

3. While coasting does not avoid totally the risk of OHSS, it decreases its incidence in high-risk patients.

4. Coasting is an effective measure in OHSS prevention, without jeopardizing the ICSI outcome. Coasting for >3 days is associated with a moderate decrease in pregnancy rate.

5. During coasting mature follicles will survive for a few days without exogenous gonadotrophins while smaller follicles will enter apoptosis/necrosis, reducing the granulosa cell population that will release VEGF.

6. Coasting is a feasible option in patients undergoing IVF cycles with GnRH antagonists to prevent OHSS.

**REFERENCES**


PGD and Prenatal Diagnosis: Comparison and Review in Different Genetic Disorders

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Abstract: Pre-implantation and prenatal testing provide genetic information and detect birth defects or abnormalities in an embryo/fetus before implantation/born. In this review, the process and details of the two testing are discussed.

Keywords: PGD, Prenatal Diagnosis, Blastomere Biopsy, FISH, CGH, Chorionic villus sampling, Amniocentesis.

INTRODUCTION

Prenatal diagnosis is a technique for couples to avoid having a child affected by severe disease; it has been applied routinely for more than 30 years in many countries [1]. Ideally, prenatal diagnosis aims to provide an accurate and rapid result as early in pregnancy as possible. However, therapeutic abortion cannot be avoided if genetic disease is diagnosed, which could hurt the woman physically as well as emotionally. In the early 90s, following widespread use of in vitro fertilization (IVF), a new diagnostic technique was developed in England termed pre-implantation genetic diagnosis (PGD), allowing genetic diagnosis to be made prior to embryos being transferred into the uterus [2]. Because only unaffected embryos are selected for transfer to the uterus for implantation, it eliminates the dilemma of pregnancy termination following unfavorable prenatal diagnosis. The techniques used in PGD are much different than those in prenatal diagnosis. This review discusses the details of these two techniques.

PRE-IMPLANTATION GENETIC TESTING

Pre-implantation genetic diagnosis was first introduced by Handyside in 1990 [3] to sex embryos for X-linked recessive disorders by PCR for Y-sequence amplification after blastomere biopsy. The first diagnosis for Mendelian traits involved α-1-antitrypsin deficiency by Verlinsky et al. [4] and ΔF508 homozygosity cystic fibrosis by Handyside et al. [5]. Fluorescence in situ hybridization (FISH) with chromosome specific probes was used [6, 7] to detect aneuploidy.

Two different terms are used in pre-implantation genetic testing. The term “pre-implantation genetic diagnosis” (PGD) applies when one or both genetic parents carry a gene mutation or an unbalanced chromosome. The embryos are tested to determine whether the mutation or an unbalanced chromosome is passed to the offspring. PGD usually is offered for three major disease groups: sex-linked disorders, single-gene defects, and chromosomal disorders. The term “pre-implantation genetic screening” (PGS) applies to normal genetic parents whose embryos are screened for aneuploidy.

Two main procedures are used in pre-implantation genetic testing: Biopsy is the removal of a cell or material from an embryo or blastocyst. This may include removing a polar body, blastomeres, or cells from the trophectoderm. After biopsy, diagnosis can be made by PCR for single-gene disorders or FISH for chromosomal abnormalities.

Polar Body Biopsy

The oocyte extrudes polar bodies during meiosis. For female inherited mutations or chromosomal disorders, genetic analysis can be performed on the first or, sometimes, the second polar body. However it has limitation of providing maternal information only [9].

Cleavage Stage Biopsy

Cleavage stage biopsy is the most widely used technique, usually involving biopsy of a single blastomere from day 3 embryos [2]. The advantage of cleavage stage biopsy is that the genetic constitution of the embryo is complete, and the timeframe for diagnosis is up to 72 hours (biopsy usually is performed on day 3 and transfer on day 4, 5, or 6). The deci-
sion to remove one or two blastomeres depends on the quality of the embryo and the disease of interest.

**Blastocyst Stage Biopsy**

On day 5 after oocyte retrieval, if the embryos have developed to the blastocyst stage with presence of inner cell mass and trophoderm, some of the trophectoderm cells can be removed from the embryo for analysis. The inner cell mass that develops into the fetus is left undisturbed [10]. This method avoids a possible harmful effect from losing cells in early day3 embryos. The trophectoderm biopsy potentially can provide up to five cells for genetic analysis.

**Fluorescence In Situ Hybridization (FISH)**

FISH is employed for analysis of chromosomal disorders. Cells are lysed after biopsy and their nuclei are fixed on a glass slide individually with the cytoplasm dispersed. Chromosomal-specific DNA probes labeled with different fluorochromes can bind to the homologous denatured DNA sequences unique to each chromosome. The number of fluorescent signals of a particular color reflects the number of copies of each of the chromosomes [11]. Sex determination in cases of sex-linked disorders, chromosomal disorders, and PGS for aneuploidy can be diagnosed by this method. Chromosomal disorders involve a variety of chromosomal rearrangements, including translocations, inversions, and deletions. Usually telomeric probes and breakpoint-related probes specific to the loci site are employed [12]. A mother who carries the abnormal X chromosome for an X-linked disease has a 50% of chance of passing it to her son. If the father is the one affected, the sons should be healthy, but the daughters have a 100% risk of being carriers. Specific centromere probes are usually used for sexing the embryos.

Aneuploidy increases with maternal age and is one of the most common causes for early pregnancy failure, recurrent early pregnancy loss, and repeated failed IVF cycles despite of transfer of high-quality embryos (based on morphology) [13]. There are, however, no specific indications for PGS. It has been proposed for patients at risk for increased prevalence of aneuploid embryos, including women of advanced maternal age and those with a history of recurrent early pregnancy loss, repeated IVF failure, or severe male factor infertility. However, no prevailing evidence supports the use of PGS to increase live birth rates as compared with control groups where no PGS is performed. Therefore, PGS currently is not recommended by ASRM [14].

**Polymerase Chain Reaction (PCR)**

PCR is used to identify specific gene mutations, such as cystic fibrosis, Tay-Sachs disease, sickle cell anemia, and Huntington’s disease. A small portion of the genome that contains the target gene of interest is amplified or replicated to provide a suitable sample for analysis. The blastomeres(s) are lysed and the gene amplified, along with a gene from normal samples that is amplified simultaneously as a control. Subsequently double stranded DNA gel electrophoresis is applied. A mismatch due to a genetic deletion or substitution will result in a differential migration on the gel, thus permitting diagnosis [15, 16]. Because only one to two cells are amplified in PGD, the accuracy of PCR diagnosis can be hampered by amplification failure, DNA contamination, and allele dropout [17].

**Limitation of PGD/PGS Technique**

Mosaicism is very frequent in in vitro generated embryos, meaning that normal blastomeres can co-exist with abnormal ones [18]. The true proportions of normal and abnormal cells cannot be determined unless all of the cells are analyzed. In some cases, embryos identified as aneuploid at the cleavage stage were “self-corrected” at blastocyst stage re-analysis [19]. This is one of the reasons that day 3 single-cell diagnosis has an inevitable false positive rate up to 10% [20].

**Future Prospects of PGD**

Only up to five chromosomes can be enumerated simultaneously on a single biopsied cell by FISH. Although multi-

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**Table 1. Comparison of Pre-Implantation Genetic Diagnosis and Prenatal Diagnosis**

<table>
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<tr>
<th>Pre-Implantation Genetic Diagnosis</th>
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<td><strong>Samples</strong></td>
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<td><strong>Testing Time</strong></td>
<td>D0 or D1 after oocyte retrieval</td>
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<td><strong>Invasive</strong></td>
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<tr>
<td><strong>Testing Method</strong></td>
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<td><strong>Cell Number</strong></td>
<td>1 or 2</td>
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<td><strong>Advantages</strong></td>
<td>Diagnosis before implantation, no need to abort</td>
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<tr>
<td><strong>Limitations</strong></td>
<td>Amplification failure, ADO, mosaicism and self-correction. Need to confirm by prenatal diagnosis</td>
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ple (3 to 4) rounds of FISH testing can be used, it cannot screen all 24 chromosome pairs. This is achieved by comparative genomic hybridization (CGH) to get a complete 24 chromosome karyotype [21]. The limitation of CGH is that it is time consuming, as the whole procedure takes 48 to 72 hours to complete. Whole-genome analysis using multiple displacement amplification (MDA) sequencing with gene chips is another new aspect of PGD testing. It can offer greater accuracy, more information, and rapid throughput testing in the near future [22].

**Prenatal Diagnosis**

Compared with the PGD procedure, prenatal diagnosis is carried out after the pregnancy is established. Prenatal diagnostic tests can be categorized into non-invasive and invasive tests. Non-invasive diagnosis is achieved by using maternal blood plasma and medical imaging, including routine obstetric ultrasound scan, echocardiography, and MRI, which do not harm the fetus or mother. Alternatively, invasive diagnosis includes amniocentesis or chorionic villus sampling for genetic analysis. These tests are conducted only in pregnancies that are considered to be at high risk for chromosomal abnormalities or after a PGD to confirm the diagnosis.

**Ultrasound**

Ultrasound or ultrasonography is the most popular and widely used technique in prenatal diagnosis. It is a non-invasive and cost-effective procedure that is considered harmless to both the fetus and the mother. Fetal structural malformations generated by an abnormal gene or chromosome may present, especially in the second trimester of gestation [23, 24]. Alternatively, fetal structural malformations may arise during embryonic differentiation.

A limitation to ultrasound is that often subtle abnormalities may not be detected until later in pregnancy or not at all. A good example of this is Down’s syndrome (trisomy 21) where the morphologic abnormalities are usually not so remarkable, thereby evading detection by ultrasound. Often only subtle appearance, such as nuchal thickening, is noted [25]. Other sonographic markers include nuchal translucency and nasal bone abnormality [26]. The finding of structural malformations increases the likelihood of aneuploidy and warrants genetic counseling and consideration of amniocentesis [26]. In recent years three-dimensional (3D) and four-dimensional (4D) ultrasound have been employed in prenatal diagnosis. These methods can be used to evaluate visual facial features, central nervous system abnormalities, and skeletal defects [27]. Fetal echocardiography can be performed at 18 to 23 weeks gestation to detect the presence of heart defects in high-risk pregnancies. When this technique is used with duplex or color flow Doppler, it can identify a number of major structural cardiac defects and rhythm disturbances [28].

**Biochemical Markers of Maternal Blood Plasma**

Some alternative approaches, such as endocrine or biochemical markers for detection of Down’s syndrome or maternal serum beta hCG, should be considered, especially for at-risk pregnancies.

**Maternal Serum Alpha-Fetoprotein (MSAFP)**

The fetus can produce alpha-fetoprotein (AFP). Ordinarily, only a small amount of AFP can cross the placenta to the mother’s blood and amniotic fluid. In cases of a fetal neural tube defect, however, more AFP escapes into the amniotic fluid as well as into maternal serum. The MSAFP test has the greatest sensitivity between 16 and 18 weeks gestation, but can still be useful between 15 and 22 weeks gestation [29].

**Maternal Serum Beta-HCG**

The trophoblast should produce beta-HCG when embryo implantation occurs. It reaches a peak at 10 weeks of gestation, and then declines. Therefore, maternal serum beta-HCG can be used to screen for fetal abnormalities, especially for Down’s syndrome when coupled with MSAFP testing. An elevated beta-HCG with a decreased MSAFP suggests Down’s syndrome [30, 31].

**Unconjugated Estriol**

Combined evaluation of unconjugated estriol (uE3), beta-HCG, and MSAFP in maternal plasma is called triple testing. uE3 tends to be lower when Down’s syndrome is present and in the presence of adrenal hypoplasia with anencephaly [31].

**Inhibin-A**

Inhibin-A is secreted by the placenta and the corpus luteum. An increased level of inhibin-A is associated with an increased risk for trisomy 21. It is increasingly utilized with the previous three markers, named quadruple testing when performed together [31].

**Pregnancy-Associated Plasma Protein A (PAPP-A)**

Low levels of PAPP-A as measured in maternal serum during the first trimester may be associated with fetal chromosomal anomalies [32].

**Amniocentesis**

Amniocentesis is an invasive procedure, usually performed between 14 and 20 weeks of gestation. A sample of about 15 ml of amniotic fluid is obtained transabdominally under ultrasound guidance. The amniotic fluid is sent for biochemical and molecular biologic analysis, and the fetal cells from amniotic fluid are cultured for chromosome analysis [32]. Risks of amniocentesis, although low, include fetal loss and maternal Rh sensitization.

**Chorionic Villus Sampling (CVS)**

This procedure usually is performed between 8 and 11 weeks of gestation under ultrasound guidance. The sample of placenta usually is obtained by a catheter through the cervix or by alternative approaches, such as transvaginally and or transabdominally. Similar to amniocentesis, cells from CVS can be analyzed by a variety of techniques [32]. The major advantage of CVS over amniocentesis is that the diagnosis is performed at an earlier stage of gestation. The disadvantage of CVS is that it is an invasive procedure which could induce pregnancy loss. The risk of pregnancy loss is about 0.5 to 1%, which is higher than that of amniocentesis [32]. Rarely, CVS also can be associated with limb defects of the fetus [33]. The possibility of maternal Rh sensitization is present.
Misdiagnosis may occur because of placental mosaicism and sample contamination by maternal tissue [32].

Percutaneous Umbilical Blood Sampling (PUBS)

PUBS, also named cordocentesis [34] is performed after 18 weeks of gestation. A needle is inserted into the umbilical cord under ultrasound guidance, and a sample of 0.5-1 ml fetal blood is collected from the umbilical vein for further diagnosis. It is useful for evaluating fetal metabolism and hematologic abnormalities.

The optimal approach to prenatal diagnosis is to first refer the woman for genetic counseling and then to employ non-invasive methods. In cases of abnormal screening results, invasive methods should be used to confirm the diagnosis. The use of PGD and prenatal genetic diagnosis have been dramatically prevented genetic diseases and improved the quality of life for many families.

EXPERT COMMENTARY

Although PGD is often regarded as an early form of prenatal diagnosis, the nature of the PGD request often differs from prenatal diagnosis, which is accomplished when the mother is already pregnant. Some widely accepted indications for PGD would not be acceptable for prenatal diagnosis, such as screening for diagnosis of late-onset disease or predisposition syndromes (like the BRCA mutation for breast cancer).

FISH is the most widely used method to determine an embryo’s chromosomal constitution. The main problem with cleavage embryo PGD is the high rate of mosaicism, up to 70% of analyzed embryos. It has been shown that 40% of the embryos diagnosed as aneuploidy on day 3 turned out to have euploid inner cell mass at day 6. The possibility of discarding a viable embryo due to the limitation of the technique following PGD on a day 3 embryo is a major concern. One or two blastomeres are not representative of the complete embryo. Biopsy at the blastocyst stage would provide more accurate diagnosis as more cells can be biopsied.

Currently, a large number of probes are available for different segments of chromosomes; however, the limited number of fluorochromes constrains the number of signals that can be analyzed simultaneously. To overcome this problem, up to three consecutive rounds of FISH can be carried out on the same sample. Overall, 13 is the maximum number of chromosomes that can be analyzed by FISH. Whole-genome analysis using multiple displacement amplification (MDA) sequencing with gene chips would allow for analysis of all 24 chromosomes.

FIVE-YEAR REVIEW

Due to the limitation of FISH and PCR, we expect to witness the entry of whole-genome analysis/CGH into the mainstream of IVF to replace current PGD testing. Its preliminary application has been encouraging as more chromosomes can be can be analyzed with higher sensitivity and accuracy. However, it takes longer to have results, which may constrain fresh embryo transfer. Additionally, whole-genome analysis/CGH is not cost-effective for the patient. More research hopefully will help us overcome these obstacles in the near future.

KEY POINTS

Prenatal diagnosis consists of a range of non-invasive and invasive testing that is used for diagnosing diseases or conditions in a fetus prior to birth.

Pre-implantation genetic diagnosis (PGD) refers to diagnostic procedures performed on oocytes, embryos, or blastocysts before implantation to enhance the transfer of a normal embryo into the uterus and avoid selective pregnancy termination.

In FISH, the most widely used method for PGD, cells are fixated on glass microscope slides and hybridized with fluorescence DNA probes for analysis under the fluorescence microscope.

Comparative genomic hybridization (CGH) is a molecular cytogenetic method for genetic screening that analyzes all 24 chromosomes to produce a map of DNA sequence copy number as a function of chromosomal location throughout the entire genome.

REFERENCES


Options to Prevent Multiple Pregnancies with ART

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Abstract: Multiple pregnancies have been and remain the most common and serious complication of assisted reproductive technologies (ART). Prematurity is the major complication of multiple pregnancies although there are other problems affecting the children and parents also increase significantly. The scope of the problem of ART and multiple pregnancies is discussed as well as the specific issues involving children and parents. Progress in decreasing ART multiple pregnancies and suggested steps to further decrease ART multiple pregnancies are also discussed.

Keywords: Multiple pregnancy, assisted reproductive technologies, in vitro fertilization, prematurity.

INTRODUCTION

Multiple pregnancies are the most common and serious complication of assisted reproductive technologies (ART). The significant stress of not being able to get pregnant can, not infrequently, make both infertile couples and health care providers forget the obvious --- the goal of ART is not merely getting pregnant but having healthy children. Either due to desperation for getting pregnant or lack of knowledge regarding the morbidity of multiple pregnancies, couples seem to lack appropriate concern about multiple pregnancies. A survey by Goldfarb et al. of 77 women embarking on gonadotropin therapy or in vitro fertilization (IVF) showed that 95% of women preferred getting pregnant with triplets to not getting pregnant, and 68% of women preferred getting pregnant with quadruplets to not getting pregnant [1]. Looking at this issue in a different way, Ryan et al. [2] asked new infertility patients as to their preference for treatment outcomes. More than 20% of women embarking on infertility treatment preferred multiple pregnancies to a singleton pregnancy. Very significantly, 4% of those favoring multiple pregnancies ranked quadruplets as their most desired outcome. Based on these observations, the fact that couples undergoing IVF are often very aggressive regarding the number of embryos to transfer is not surprising.

Besides the above described attitudes regarding multiple pregnancies, many couples are of the philosophy that, because they have never been able to get pregnant at all, they do not need to be worried about multiple pregnancies. Similarly, patients who failed to get pregnant with the transfer of two embryos in a first IVF cycle often do not feel comfortable unless they transfer more embryos in a subsequent cycle. These understandable emotional issues, in combination with the overwhelming financial investment for IVF, result in higher than necessary multiple pregnancy rates in the United States.

Further exacerbating this trend is the pressure on IVF programs to report high success rates to stay competitive. While there is an increasing emphasis on the undesirability of multiple pregnancies with IVF, success of IVF programs still is undoubtedly judged predominantly by the “take-home-baby rate” which does not take into consideration the significant financial costs and morbidity of multiple pregnancies.

SCOPE OF THE PROBLEM OF MULTIPLE PREGNANCIES WITH ART

Multiple pregnancies have increased greatly in the United States since 1980. From 1980 to 1999 the overall multiple pregnancy rate increased from 1.9% to 3.1% of all births, an increase of approximately 60%. Numerically, most of the increase was in twins. However, percentage-wise the increase in higher-order pregnancies (triplets or more) was more than 400%, increasing from 37 to 193 per 100,000 births [3]. While a small part of the increase in multiple pregnancy rates is due to other factors, such as increased maternal age, the vast majority of the increase in multiple pregnancies is due to infertility treatments. Infertility treatments involve more than ART and, indeed, ovulation induction, according to a review by Norwitz [4] may be responsible for twice as many high-order pregnancies than is ART. The impact of ART, however, is extremely significant. In 2006, infants conceived with ART accounted for approximately 1% of the total births in the United States. However, the proportion of twins and triplets or higher order multiples resulting from ART were 17% and 38%, respectively [5].

While an increased number of dizygotic multiples are expected with ovulation induction and ART, the number of monozygotic twins is also increased with ART. An increased number of monozygotic twins also has been reported with ovulation induction. Derom et al. [6] reported a 1.2% incidence of monozygotic twinning after ovulation induction versus the generally accepted 0.4% incidence in the general population [7]. All these incidences may be understated to some degree since diamniotic-dichorionic monozygotic gestations can easily be overlooked [8].

Ovulation induction itself may be one factor causing the increased incidence of monozygotic twins in ART, but it is not the only one. It seems the incidence of monozygotic
twins is higher in ART than in ovulation induction, especially when ART involves blastocyst transfer. Milki et al. [7] found monozygotic twinning occurred in 5.6% of pregnancies conceived after blastocyst transfer compared with 2% after cleavage-stage transfer. Further exacerbating this problem is the finding of Milki and others [7-9] that the incidence of monozygotic twinning seems to be highest when more than one gestational sac is present, thus leading to a relatively higher rate of triplets than twins caused by monozygotic twinning. Another negative factor regarding monozygotic twinning associated with ART is the possibility that the incidence of monoamniotic twinning, and all it associated problems, may be higher with ART than with natural monozygotic twinning [10].

PROBLEMS ASSOCIATED WITH MULTIPLE PREGNANCIES

Prematurity

Clearly the major cause of morbidity with multiple pregnancies is prematurity. The relative risk of birth at gestational age less than 32 weeks is increased 7-, 23-, and 40-fold for twins, triplets, and quadruplets, respectively. Fully one-third of triplets and two-thirds of quadruplets will be born at less (and often considerably less) than 32 weeks [11]. Approximately 25% of twins, 75% of triplets, and essentially 100% of quadruplets or more will be admitted to a neonatal intensive care unit. According to Newman and Luke, the average days in the NICU average 18, 30, and 58 for twins, triplets, and quadruplets, respectively [12].

Small for Gestational Age

In addition to prematurity, multiple births increase the number of small for gestational age (SGA) babies, and the incidence seems to increase with gestational age. Alexander et al. in reviewing United States live births from 1991 to 1995 found 28% of twins were SGA by 34 weeks and 40% were SGA by 37 weeks. They found that 50% of triplets were SGA by 35 weeks gestation [13].

Cerebral Palsy and other Handicaps

Of all the sequelae of prematurity, cerebral palsy seems to be the most studied. Pharoah and Cooke reported in 1996 the rate of cerebral palsy was increased 5.5-fold for twins and almost 20-fold for triplets [14] Similar numbers were reported by Peterson et al. in 1993 [15].

Other handicaps also clearly are increased by multiple births. Luke and Keith [16] using data from the US Congress, Office of Technology Assessment, found that overall, handicaps were increased by 39% in twins and by 97% in triplets. Severe handicaps were increased by 30% and 71%, respectively.

Birth defects are also increased by multiple gestations. Monozygotic twins are twice as likely as dizygotic twins to be born with congenital malformations. In one study, the risk of significant congenital anomalies among triplets was 5% [17].

Infant Mortality

Infant deaths (deaths at less than 1 year of age) are not as commonly discussed as are the other sequelae of multiple pregnancies. Not surprisingly, however, infant death statistics are just as impressive. Keith and Luke [16], using Vital Statistics of the United States, 1998, calculated the number of infant deaths to be 6-fold higher in twins and 17-fold higher in triplets compared to singletons. The perinatal mortality for triplets, including all intrauterine demises and deliveries occurring after 20 weeks, is greater than 10% in many studies [17].

Maternal Complications

Maternal morbidity, while of significantly less magnitude than that of the babies, is nevertheless a consideration. Essentially all maternal complications of pregnancy are increased by the occurrence of multiple pregnancies. In addition to premature labor, complications include preeclampsia, gestational diabetes, anemia, postpartum hemorrhage, and even the relatively rare acute fatty liver of pregnancy [17].

Parenting Issues

The psychological impact of multiple pregnancies has been well described. Cook et al. [18] evaluated parents of IVF twins and reported that these couples found parenting to be not as satisfying as they expected. Furthermore, they found that parents of IVF twins seem to be more stressed than were parents of naturally conceived twins.

FINANCIAL COSTS

While not as important as the morbidity caused by multiple pregnancies, the financial costs of multiple pregnancies also are overwhelming. Callahan et al. [10] evaluated the births at a Boston hospital from 1986 to 1991 and found that added costs of multiple pregnancies were approximately $38,000 and $110,000, respectively, for twins and higher-order pregnancies. Goldfarb et al. [19] estimated the added cost (above normal costs for a singleton delivery) per woman delivered of pregnancies from an IVF program during the years 1991 and 1992. Estimated added costs included the cost of the IVF procedure, admissions for premature labor, time off of work, and, very importantly, costs for ongoing care of premature infants. The authors found the added cost per delivery of IVF patients was approximately $39,000 for single and twin deliveries versus approximately $343,000 for triplet and quadruplet pregnancies.

STEPS TO MINIMIZE THE PROBLEM OF ART-ASSOCIATED MULTIPLE PREGNANCIES

There is little debate as to the significance of the problems resulting from multiple pregnancies and ART. There is, conversely, a great deal of debate as to the best way to minimize the problem. Issues such as patients’ rights to decide on the number of embryos and trade off between higher pregnancy rates and higher multiple pregnancy rates result in controversy over embryo transfer policy. Legislation in some European countries strictly limits the embryo transfers to two embryos for most patients.

The American Society of Reproductive Medicine has established guidelines for number of embryos to transfer in an IVF cycle, the most recent of which was published in 2008 [20]. These guidelines recommend, for example, the
transfer of one embryo for younger patients with a “favorable prognosis” (Table 1). Many are strongly advocating even stricter guidelines because of the feeling that the goal of ART should be a healthy singleton birth, and the fact that increasingly good success rates can be achieved by transferring a single embryo, particularly at the blastocyst stage [21].

Of course, as stated earlier, even a single embryo transfer, particularly at the blastocyst stage, does not completely eliminate multiple pregnancies.

Several questions arise regarding how insurance coverage influences the number of embryos transferred. Reynolds’s et al. [22, 23] showed a lower incidence of transfer of three or more embryos in insured versus non-insured states. However, the effects of insurance coverage on multiple pregnancies were modest. Many feel a substantial effect on multiple pregnancies would occur only if insurance coverage was combined with mandated limits regarding number of embryos to transfer.

Another way to decrease the number of multiple pregnancies is to revise the way program success is assessed. In the United States the “take-home-baby rate” is the standard by which a program is judged; the multiple pregnancy rate of a program is much more rarely examined. Embryo implantation rate, a very good indicator of the quality of an IVF program, has been incorporated into the Society of Assisted Reproductive Technologies (SART) reporting system but is not prominently displayed. The CDC does not include implantation rates in its report to the public.

Despite the still significant barriers to decreasing multiple pregnancies, considerable progress has been made in decreasing triplet births. The SART 2007 report shows a triplet birth rate of less than 2% compared with almost 8% 8 years earlier. Twin pregnancy rates, however, remain unchanged at more than 30% for patients under 38 years old.

Clearly there is no simple answer to the problem of further decreasing ART multiple pregnancies. Efforts on many fronts are necessary. Insurance coverage with a reasonable limitation to the number of embryos transferred combined with a SART and CDC reporting system that emphasize implantation rates and/ or singleton pregnancy rates would be very helpful. Also, very importantly, educating our patients as to the risks of multiple pregnancies is imperative. We must not forget, our goal is not to get patients pregnant but for them to have a healthy family.

EXPERT COMMENTARY

The purpose of this article was to bring attention to the increase in multiple pregnancies caused by assisted reproductive technologies and to increase awareness of the significant health and financial costs of multiple pregnancies. Couples and health care workers involved with ART often tend to focus on pregnancy rates and often do not give as much consideration to trying to decrease the incidence of multiple pregnancies. Efforts that include guidelines by the American Society of Reproductive Medicine on maximum number of embryos to transfer have been successful in decreasing high-order multiple pregnancies from ART. However, as yet, these efforts have not been successful in decreasing the incidence of ART twins.

FIVE-YEAR VIEW

In the next 5 years it is anticipated there will be increasing emphasis on a singleton birth as the goal of ART. Acceptance of the importance of this goal will be accomplished through several avenues:

• Education of physicians and patients regarding the health risks of twins.

• Changes in the reporting of ART success rates that will incentivize ART programs to strive for singleton pregnancies.

• Further improvements in embryo culture methods resulting in better implantation rates per embryo.

• Better methods of evaluating embryos to increase ability to choose the best embryos for transfer.

Table 1. Recommended Limits on the Numbers of Embryos to Transfer

<table>
<thead>
<tr>
<th>Cleavage-Stage Embryos</th>
<th>Age</th>
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<tbody>
<tr>
<td>Prognosis</td>
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<tr>
<td>Favorable</td>
<td>&lt;35</td>
<td>35-37</td>
<td>38-40</td>
<td>&gt;40</td>
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<tr>
<td>1-2</td>
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<tr>
<td>All others</td>
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<td>3</td>
<td>4</td>
<td>5</td>
</tr>
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<table>
<thead>
<tr>
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<th>Age</th>
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<tr>
<td>Prognosis</td>
<td>&lt;35</td>
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<tr>
<td>All others</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

1See text for more complete explanations. Justification for transferring more than the recommended number of embryos should be clearly documented in the patient’s medical record.

2Favorable = First cycle of IVF, good embryo quality, excess embryos available for cryopreservation, or previous successful IVF cycle. ASRM Practice Committee Guidelines on number of embryos transferred. Fertil Steril 2008.
• More stringent guidelines from professional groups, insurance companies, and possibly government regarding maximum number of embryos to transfer.

KEY ISSUES
• Multiple pregnancies are the most common and serious complication of assisted reproductive technologies (ART).
• The major cause of morbidity with multiple pregnancies is prematurity, but other complications, including small for gestational age, also are increased with multiple pregnancies.
• There has been good success in reducing the number of high-order multiple pregnancies (triplets or more) resulting from ART, but little progress has been made in reducing the number of twins.
• A combination of actions is needed to decrease the number of twins resulting from ART. These actions include acceptance by patients and doctors that the goal of ART should be a singleton pregnancy.

ACKNOWLEDGEMENT

REFERENCES
[23] Reynolds MA, Schieve LA, Peterson HB. Insurance is not a magic bullet for the multiple birth problem associated with assisted reproductive technology. Fertil Steril 2003; 80: 32.
Metabolomic Profiling for Selection of the Most Viable Embryos in Clinical IVF

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Abstract: Along with standard morphological indicators of preimplantation embryo development, information gained from novel “Omics” platforms is providing more detailed functional characterizations of embryo phenotype. Since embryo metabolism is a critical driver of development and implantation, it is proposed that analysis of the embryo metabolome may reveal several physiologically relevant markers. Metabolomics analysis is currently showing significant promise in this context, providing a systematic method of screening for low molecular weight metabolic by-products, in isolated cell extracts and biological fluids. Correlations between in vitro development and pregnancy outcomes have already been described after retrospective data comparison with metabolomic profiles. This article summarizes progress and current findings of metabolomic analysis as a new and complimentary technology for screening embryo cohorts in clinical IVF, to facilitate prognostic selection of a single embryo for transfer.

Keywords: Implantation rate, metabolomics, pregnancy, preimplantation embryo, viability.

INTRODUCTION

Analysis of blastomere morphology and in vitro growth rate of the preimplantation embryo have been the key indicators of embryo quality in clinical IVF laboratories for over 30 years [1-5]. However, morphological evaluation remains a subjective metric and consequently there is an ongoing need for more systematic quantitative assays of the biochemical and molecular processes within embryos that underlie these phenotypes. Over the five day period between fertilization and blastocyst formation, the preimplantation embryo relies on energy generation from oxidative metabolism of carbohydrates and amino acids [6], with the notable shift from three-carbon to six-carbon sugars at Day 3 and an increase in aerobic glycolysis at the blastocyst stage [7, 8]. This latter change in metabolism is attributed to both an increased demand for biosynthesis, consistent with an increased growth trajectory and in preparation for implantation [9]. Drawing from this data, human embryo culture media formulations have been improved significantly, which in turn has led to increased success rates of in vitro embryo production, cryopreservation survival and post-transfer pregnancy outcomes. Improvements in laboratory conditions have also led to the ability to culture human embryos to the blastocyst stage [10]. One advantage of such an approach is the ability to apply alpha-numeric grading systems, capable of grading both the trophectoderm and inner cell mass, which have been shown to be of value in blastocyst selection for transfer [5].

Although blastocyst transfer has been adopted for several groups of patients, a significant number of transfers are performed on either day 2 or 3 of development [11]. At these early stages of development there is, perhaps, a greater need for systematic assays of embryo quality and prospective viability, considering that the cleavage stages tend to be morphologically similar. To this end, application of emerging “Omics” technologies, benchmarked by functional genomics (whole genome analysis, transcriptomics) and proteomics [12] are starting to provide innovative and clinically amenable technologies for obtaining functional biomolecular profiles that correlate with embryo development. In combination with detailed bioinformatics sorting and statistical analysis of datasets, fine differences between profiles of individual embryos can be detected using an array of methods [13].

METABOLOMICS AS A CLINICAL BIOASSAY SYSTEM

Overview of Analytical Principles

Metabolomics profiling is emerging as a highly useful adjunct to standard clinical diagnostics, yielding a wealth of phenotypic information. Like other “Omics" techniques, metabolomics relies on the chemical purification of cell extracts or biological fluids, followed by spectroscopic analysis [14]. In some cases, spectroscopy can be performed directly without the need for sample purification, such as the case of buffers with low levels of secreted metabolites. Also, the purification method itself can yield sufficient information on fractions based on molecular weights and charge distributions on molecules. For example, chromatographic methods rely on separation of different fractions based on their binding affinity with a porous matrix through which the sample flows, very similar to the way different molecular weight DNAs are separated using electric fields in gel electrophoresis. While specific extraction protocols are required for isolating unique cellular fractions (e.g., DNA, protein, lipids and polysaccharides) the method of spectroscopic analysis of those fractions varies dramatically, depending on the chemical properties of the molecular constituents.

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Briefly, spectroscopy relies on probing a solid or fluid sample with a specific range of optical or non-visible wavelengths, followed by analysis of the resultant energy output to obtain an absorbance profile. Different absorbance patterns correlate with the abundance and chemical structure of the complete set of molecules in the sample. For example, non-visible infrared wavelengths (greater than visible region, >800 nm) interfere with the vibrational frequency of chemical bonds, providing information about the abundance and type of bonding between individual atoms and side groups in the sample. Shorter wavelengths tend to interfere with higher energy bonds, such as atomic-scale associations. There are some exceptions, which could be regarded as both purification and spectroscopic methods in one. For example, mass spectrometry (MS) uses high energy electrons and other chemical methods to ionize samples into their constituent atoms, providing a distribution map of all atoms in the sample. On the other hand, nuclear magnetic resonance (NMR) spectroscopy uses very low energy waves to detect single elements such as hydrogen protons, using proton movement to reveal precise information on organisation and structure. In general, the complex chemical structure of molecules and abundance of elements can be quantitated using combinations of chemical purification and spectroscopy.

Biospectroscopy as a Clinical Diagnostic Tool

Overall, there are several terms that describe the approach adopted in the adaptation of these spectroscopic techniques to biological systems (sometimes termed “biospectroscopy”) – the term itself refers to the nature of the energy profiles obtained from samples as “spectra”, since they are typically over a specific wavelength range, and have a characteristic pattern. Chemical extraction of whole cells or biological fluids can yield a spectral representation (profile) of the entire metabolome, often termed a metabolic “fingerprint” [15, 16], as distinct from a metabolic “footprint”, which can refer to subsets of extracellular metabolites produced by cells or tissues [16]. For simplicity, we will use the term “metabolomic profile”, in the same context as the term for the applications described in this article. Generation of either type of profile, usually over an arbitrary range (spectrum) of wavelengths, is generally classified as a “bottom-up” (discovery driven) or “top-down” (hypothesis driven) exercise [17]. The same spectroscopic approaches can be used in both contexts, such as in generation of distributions of broad compositional data for basic comparisons (e.g., control versus treatment), or analysis of specific elemental abundance (eg specific metabolic responses to a drug).

For biomedical applications, profiles of spectral data obtained from diseased versus healthy biological samples forms the basis of a diagnostic assay, as has been demonstrated successfully for cancer diagnosis using Fourier-transformed infrared spectroscopy (FTIR) and Raman spectroscopy directly on preserved histological specimens [18]. In conjunction with these comparative assessments is the need for further computer-based statistical processing (commonly termed “chemometrics”) which can standardize and increase the power of the comparisons to detect minute differences in the profiles. A similar approach has been used extensively in functional genomics, where whole genome maps of changes in gene expression can be related to single genes based on differential optical fluorescence of base pairs, and later fed into gene databases. In this way, a bottom-up approach can rapidly yield candidates for further top down studies [19], such as in the systematic search for biomarkers of cancer cells [20].

Metabolomics: Analysis of Metabolite Presence and Abundance

Specific types of spectroscopy are amenable for screening of the largely non-protein pool of approximately 2500 or more small molecule metabolites (<800 kDa) that constitute the metabolome, synthesized endogenously within the cytoplasm, and often secreted from cells [15]. Metabolomics has been used to great success in plant biology, such as for assessments of metabolism relative to environmental stress or chemical toxicity, as recently demonstrated in an intricate biochemical pathway analysis [21]. In relation to clinical bioassays, metabolomics screening has proven useful diagnostically, in the search for metabolic biomarkers of tumour phenotype [22], as well as in treatment, for assessing pharmacotherapy outcomes in vivo [23]. In combination with metabolome and biochemical pathway databases [24, 25], it is now possible to use metabolomics data to piece together entire intracellular pathways, a powerful approach in any clinical application.

Common spectroscopic techniques used in metabolomics include gas chromatography/mass spectrometry (GCMS) and liquid chromatography/mass spectrometry (LCMS). GCMS and LCMS have become particularly useful for metabolite screening due to their ability to separate low molecular weight polar molecules and organic compounds, a criterion that satisfies most metabolites [15]. Atomic scale techniques such as NMR, tandem mass spectrometry (MS/MS), time-of-flight mass spectrometry (TOF/MS) and other combinations are also amenable to metabolites, based on their ability to further discriminate organic compounds by charge versus molecular weight.

Aside from cost and time expenditure, the main limitations on the choice of platform to use clinically include the molecular weight range of interest, the yield of data versus the amount or availability of sample, and the extent of sample degradation following sample processing such as solvent and heat treatments [26]. Conversely, while combinatorial methods like GCMS and LCMS may reduce the yield and require more starting sample, there is an improvement in purity.

APPLICATION OF METABOLOMICS TO ASSISTED REPRODUCTION

In a similar manner to the systematic application of genomics [27], transcriptomics [28] and proteomics [12] analysis, traditional metabolomics approaches are becoming widely used as a diagnostic tool in assisted reproduction for two main purposes: (i) the determination of aetiological biomarkers of infertility, and (ii) the analysis of gamete and embryo quality, for prognostic screening to assist in the treatment of infertility using IVF and other clinical labora-
tory interventions. In the first instance, information gained from metabolomic analysis of sperm samples has revealed potential links to biochemical factors associated with poor sperm quality in infertile men, including reactive oxygen species [17]. Similarly, oocyte specific markers may be predictive of subsequent post-fertilization survival of oocytes based on factors found in follicular fluid [29]. In the second instance, several techniques have been adopted for the selection of embryos based on metabolomic profiles [30] which will be discussed further in the following section.

EMBRYO SELECTION USING METABOLOMICS

General Considerations

From a research perspective, metabolomics would seem a logical progression in the development of viability assays given the wealth of information in the field of preimplantation embryo metabolism. Much of this data has been obtained through the use of microfluorimetry (MF) to quantify metabolic rates and exchange of specific metabolites from individual embryos in vitro [31-33]. This approach has revealed key findings including a strong correlation between glucose metabolism and morphological grade of human blastocysts [34]. A dramatic improvement in pregnancy outcomes following the transfer of blastocysts selected according to their metabolic efficiency, using rodent models has also been shown [35, 36].

Methods to screen embryo physiology and genetics are generally classified as “invasive” or “non-invasive” [6], and “direct” or “indirect” [37] depending on the degree of manual interference with the embryo and its culture microenvironment. Invasive assay ranges from whole or partial embryo analysis (e.g., extracts from whole embryos, biopsied blastomeres or polar bodies). Non-invasive assay involves sampling of spent culture medium used to incubate live embryos, and remains the most ideal approach for clinical IVF embryo assessment. Advantages of non-invasive sampling include its close correlation with morphological parameters of individual embryos, its high sensitivity and the potential for performance of multiple assays on the same embryo as it develops in vitro, to obtain a kinetic profile of metabolism. The main disadvantage lies in the fact that it remains an indirect measure of embryo performance in an in vitro system, typically based on substrate consumption and metabolite release rather than metabolic rate, and that until recently such analyses were only feasible in a handful of laboratories world-wide.

Similar considerations apply to metabolite screening using metabolomics. In terms of purification yield, regardless of the degree of sample invasiveness, there are major limitations due to the amount of embryo (picogram) or medium (microlitre) starting material, dictating that the analytical technique be sufficiently sensitive and miniaturized, making MS and small scale optical spectroscopies (Raman, NIR and FTIR spectroscopy) good candidate technologies. By the same token, purity is less of an issue with medium samples, making them highly amenable to single-step methods, compared to combinatorial ones [5]. While indirect screening may as yet not provide a complete diagnostic profile of metabolism, it may provide sufficient information to make discriminations in spectral differences that are non-specific or broad. Furthermore, direct methods such as optical biospectroscopy are particularly rapid, providing metabolomic profiles in minutes, which can later be quantified statistically as necessary.

Beyond the technical hurdles are considerations such as how metabolomic information integrates with data on human embryos obtained from existing Omics platforms which have led to the postulation of a “secretome” [12, 38] and embryo-specific “implantome” [39], which could make the basis of an embryo “selectome” (see Fig. 1). How the human embryo metabolome ultimately overlaps with these subsets of information and forms a universal selectome is unclear at this stage, although the schema proposed represents a simplified view of possible classifications. These classifications are likely to become more complicated and integrated as more data is obtained and related to pregnancy outcomes. In relation to a selectome, the basis of choosing a profile feature based on non-specific changes remains to be fully justified, since metabolism itself is an associative physiological indicator of the embryo and does not consider maternal factors that play a role in implantation and ongoing pregnancy. Also, during early cleavage stages, it may not be feasible to use metabolomics data as a prognostic marker of how a blastocyst is expected to implant [10, 13]. Therefore the metabolomics profiles should be considered in tandem with proven endpoints at specific stages to improve clinical efficacy.

Specific Metabolomic Methods used in Embryo Selection

Several analytical methods have been used successfully to obtain metabolomic profiles of preimplantation human embryos (summarized in Table 1) [13, 17, 40]. High performance liquid chromatography (HPLC) has been used non-invasively to analyse global amino acid exchange by single human embryos [41-43]. Of these, the study by Brison and colleagues demonstrates that embryos with profiles showing selective changes in three specific amino acids were the most viable after transfer [42]. Most recently, Stokes and colleagues show that metabolic rates extrapolated from the HPLC amino acid profiles of single embryos were highly predictive of embryo survival to Day 5 following Day 3 cryopreservation [43], in confirmation of the original report by Houghton and colleagues [41]. In terms of the attainment of embryo viability with progressive development of embryos toward Day 5, these findings provide close correlation to the stage-specific changes in embryo metabolism already understood from MF studies. Using LCMS, as a combinatorial MS approach with higher resolution than HPLC alone for amino acid analysis, it has recently been shown that significant differences in amino acid uptake occur between viable and non-viable human blastocysts [44], in parallel with increased glucose uptake as measured using MF (data not shown).

Like MS, NMR spectroscopy has become one of the most widely used metabolic tools at the elemental and atomic level [15], particularly for hydrogen detection, making it extremely useful for metabolite screening. Proton NMR spectroscopy has been used to demonstrate significant correlations between uptake of a subset of six metabolites in single embryos with pregnancy outcomes after transfer [48].
although further studies are required to validate these preliminary findings. Other combinatorial techniques such as MS/MS and GCMS may become more feasible based on the increased sensitivity of MS signatures from fractionated samples further sorted into known metabolite subsets.

Another innovation in non-invasive metabolomics analysis has been the implementation of variants of optical spectroscopy including FTIR, Raman and Near Infrared (NIR) methods, which deal with emission profiles beyond the visible end of the electromagnetic spectrum, as discussed earlier [13]. These methods have previously dominated biospectroscopy, because of their efficiency, sensitivity and amenability to milli- and micro-scale volumes. Seli and colleagues were the first to report comparisons of metabolomic embryo implantome/selectome, such metabolomic markers may prove useful as embryo viability indicators for non-invasive diagnostic assay.

Table 1. Summary of Metabolomics Methods Analysis of Viable Embryos in Clinical IVF

<table>
<thead>
<tr>
<th>Method</th>
<th>Purification</th>
<th>Optical Emission</th>
<th>Direct or Indirect</th>
<th>Clinical Analysis</th>
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MF: microfluorimetry; MS: mass spectrometry; HPLC: High Performance Liquid Chromatography; LCMS: Liquid Chromatography followed by Mass Spectrometry; IR: infra-red spectroscopy (includes near-infrared and Fourier-transformed infrared; NMR: nuclear magnetic resonance.

\(^1\)All references are cited in the text.
between pregnancy outcomes [45]. More recently, Vergouw and colleagues [46] showed similar correlations between NIR spectra and pregnancy outcomes, with a somewhat lower accuracy than the previous study, despite having a tenfold larger patient cohort. It remains to be determined whether such inter-laboratories variations are clinically significant, and whether the choice of chemometrics based on the outcomes of one study are suitable for universal applications.

Raman spectroscopy is slightly different from FTIR and NIR methods, in relation to its use of visible light excitation and scattering, and its lack of interference from water, making it ideal for biological systems for invasive and non-invasive analysis [49]. While Scott and colleagues showed similar results to the described infrared spectroscopy studies in terms of significant correlation between metabolomic profiles and pregnancy outcomes, the method is somewhat limited due to its notoriously low detection sensitivity [47]. Undoubtedly, further optimization in terms of choice of specific metabolomic screening methods using these popular optical methods will require validation across several laboratories, to generate a larger pool of data towards a global selectivity index for embryos.

General Considerations for Selecting the Optimal Embryo

In terms of the biological relationship between embryo metabolism and implantation potential, metabolomics profiles represent a potential wealth of information that can be drawn from, in the search for both selective and diagnostic biomarkers of embryo health. As a selection tool, Raman and infrared spectroscopy may provide a practical clinical solution as a non-invasive, bench-top approach. Prospective studies will determine the accuracy and reproducibility of such methods. Important considerations include the need to relate this metabolomic data to implantation markers that have arisen from other Omics platforms, such as TOF/MS proteomics and single embryo metabolite analysis using MF and LCMS [34, 44], as well as to global datasets appearing in new metabolome and pathway databases [24, 25]. While selecting an appropriate tool may be as difficult as using one to select the most viable embryo, it is heartening that such an array of innovative analytical biotechnology is currently available, and being tailored to this single objective.

EXPERT COMMENTARY

Metabolomics is perceived as an important diagnostic tool in clinical IVF, with the potential to calculate embryo viability for the purposes of screening prior to transfer or cryopreservation. Regardless of how adequately the metabolomic data is resolved with morphological and developmental scores, sufficient information can allow embryos of like morphology to be discriminated and selected. Affordability of the new techniques is likely to improve, as they become more readily available and tailored to clinical IVF, and as they integrate with other cost effective and efficient technologies such as chip-based analysis [50]. This latter approach has the further advantage of increased analytical sensitivity. Regardless of which technique is adopted, some specific requirements include that:

- The technology is reproducible, rapid and provides quantitative data that is complementary to other patient specific laboratory data.
- The technology interfaces easily within the context of working ART laboratories.
- The methods can discriminate between samples from different patient subpopulations in the context of their individual clinical diagnosis.
- The methods can distinguish variation within a patient’s cohort of embryos with similar developmental rates and morphologies, but different implantation outcomes.

FIVE-YEAR VIEW

As an embryo selection technique, metabolomics screening will form an integral part of ART laboratories. This will improve the ability to determine the functional metabolic phenotype of an embryo as a key indicator of viability. A 5 to 15% increase in the efficiency of clinical IVF laboratories to generate pregnancies based on this information would not be an unrealistic outcome.

Beyond embryo selection, there is also the potential to analyse a range of patient specific material, including sperm extracts and purified sperm samples, testicular biopsies, follicle fluid, endometrial tissue and fluids, ovarian biopsies and cumulus cell extracts. The same methods may also be useful retrospectively in the determination of unexplained recurrence of pregnancy loss by analysis of maternal serum or urine. Overall, a holistic approach in correlating metabolomics profiles within and between patients will be a continued requirement, both prospectively in the context of laboratory indications, as well as retrospectively in relation to clinical and pathological indications.

KEY ISSUES

- Metabolomics is one of an emerging set of technologies that are becoming important diagnostic tools in clinical ART, for the detection of abundance and type of metabolites in biological samples as correlates of a functional or physiological state.
- As an “Omics” technique, metabolomics integrates with existing bioinformatics platforms, providing global information on functional phenotypes of cells and tissues of interest.
- Metabolic profiling, combined with statistical data analysis, can be performed non-invasively on spent embryo culture medium samples, making it suitable for single embryo diagnosis of implantation potential.
- An increase in the sensitivity of embryo viability detection can be expected, which will complement existing morphological criteria, especially among embryos of like morphology; this will improve the ability to choose a single viable embryo for transfer.
- Potential improvements to patient outcomes include incremental increases in pregnancy rate and foetal normality following single embryo transfer.
REFERENCES


[47] Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using...


Creating A Standard of Care for Fertility Preservation

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Abstract: Fertility preservation options for women are currently only routinely offered to patients who face iatrogenic fertility loss, and most options are considered experimental. The most common modalities for female fertility preservation are embryo cryopreservation, oocyte cryopreservation, and ovarian tissue cryopreservation, the later two of which remain in the experimental arena. Natural fertility loss is affecting women as significantly as premature fertility loss. Increasing cancer survival and the modern reproductive trend of delaying childbearing are indications for the need and demand for fertility preservation. Advances in the field are necessary to respond to this demand and include superior cryopreservation techniques and fertility preservation technologies, customized guidelines, comprehensive care plans, and availability of more cost-efficient procedures. The obstacles to creating a standard of care for fertility preservation are as broad as the field itself. Lack of patient awareness, limited physician experience and knowledge, inadequate counseling, costs, and ethical issues are some examples of the many challenges to establishing a standard of care. With continued research and multidisciplinary collaboration, a higher quality of care may be provided to a larger patient population who wishes to maximize their fertility potential in the future.

Keywords: Fertility preservation, standard of care, treatment options, challenges, assisted reproductive technology (ART), patient expectations.

CURRENT STATUS OF FERTILITY PRESERVATION

The technologies of fertility preservation include all methods and efforts to maintain the ability to reproduce even after natural or iatrogenic loss of fertility. The many methods to preserve fertility for women include gonadal protective measures during potentially harmful therapeutic treatments and cryopreservation of the ovary, oocyte, or embryo. Of the cryopreservation options for females, embryo cryopreservation is the only form currently accepted as an established practice [1]. Oocyte and ovarian tissue cryopreservation are still considered experimental forms of preservation due to the less than satisfactory outcomes of cryopreserved oocytes in assisted reproductive technologies (ARTs) and the short lifespan of ovarian tissue transplantation grafts [2]. Additionally, this option is offered mainly for patients who face iatrogenic loss from treatments related to cancer and other diseases. Fertility preservation is less available for patients who wish to delay their childbearing years at the risk of becoming sterile.

Currently there is an increased demand for a broader range of options and availability of treatment for both cancer survivors and healthy individuals [3, 4]. Translational research and improvements in methods and technologies in the last decade have provided a diversity of potential fertility preservation options; however, at present these options remain limited and suboptimal [5]. Continued research and success present the need to establish standards of care for the many available fertility preservation techniques.

NATURAL AND PREMATURE FERTILITY LOSS

Natural fertility loss occurs for all women with advancing age. For most women, menopause occurs during their mid-50s; however, fecundity occurs some 10 years before the onset of menopause [6]. A natural atresia of oocytes over the years accounts for the decrease in fertility potential. As the quantity and quality of oocytes declines as age advances, the possibility of conceiving a healthy child decreases.

Premature fertility loss occurs before the naturally occurring time and may be induced by gonadotoxic treatments necessary for cancer and other diseases, from genetic causes such as Turner’s syndrome [7], or from environmental hazards. With premature fertility loss women not only lose their reproductive potential, but, in addition, the cessation of ovarian function induces menopause symptoms, including osteoporosis, which may have greater consequences when endured at a younger and more active age [6].

The most common forms of cancer that a female may suffer during her reproductive years that require gonadotoxic treatment are breast and cervical cancer [3, 8]. Most diagnoses of these cancers occur before the age of 35, and many others occur during the prepubertal and adolescent years, including hematologic malignant conditions, sarcomas, central nervous system lesions, renal cancer, and bone cancer [4]. Autoimmune diseases that require similar chemotherapeutic treatments include rheumatoid arthritis, systemic lupus erythematosus, steroid-resistant glomerulonephritis, inflammatory bowel disease, and pemphigus vulgaris [3, 8].

Treatment for these diseases may wreak havoc on the female reproductive system. Chemotherapy, radiation therapy of the pelvic region, gonadotoxic medication, and surgical incisions of or near the gonads or reproductive system
produce a high risk for induced fertility loss. Among women who undergo combination chemotherapies, 20-50% under the age of 20 and 80-90% over the age of 25 will present with amenorrhea after treatment [9]. Radiation may cause apoptosis to occur, decreasing the amount of primordial follicles available to mature and resulting in premature ovarian failure (POF) [3, 10-12]. Gonadotoxic drugs such as alkylating agents arrest cell proliferation and may also induce POF [3, 9].

INDICATIONS FOR DEMAND FOR FERTILITY PRESERVATION

In the last decade cancer treatment and ART have made significant advances and achieved higher success rates than ever [13]. The goal of cancer treatment is first and foremost survival, yet the inevitable gonadotoxicity of the treatment harms the potential for survivors to genetically parent their own children. Until an effective treatment for cancer can be developed to target tumor cells specifically, sterility will remain a possible consequence of successful cancer treatment [14]. Many patients undergoing treatment are nulliparous but desire to have children in the future.

With the increase in cancer survival more attention is being given to the patient’s quality of life post-treatment [15]. Current survival rates for patients with Hodgkin’s disease, lymphoma, and leukemia are as high as 90% [5], and of 10,700 children diagnosed with cancer in 2008, approximately 80% of them were expected to survive [4]. Additionally, the incidence of cancer in the adolescent and young adult population has increased steadily over the last 25 years [13]. Thus, as more patients are undergoing treatment and more of those patients are living longer, fertility preservation must become an integral part of holistic cancer treatment.

Concurrently with increased cancer survival, the modern reproductive trend of delaying childbearing necessitates a greater need for fertility preservation. Women all over the world, especially in developed countries, are choosing to defer pregnancy in pursuit of academic or occupational achievements or for lack of a suitable partner [7, 9, 16, 17]. With advancing reproductive age the ability to conceive greatly decreases due to the naturally accelerating atresia of primordial follicles that occurs in the late 30s and early 40s of a woman’s lifetime [9, 12]. During this time the possibility of spontaneous abortion and aneuploidy increases [9, 12]. Cryopreservation of oocytes at a maternal age of 35 years or younger for fertility preservation may provide the potential for women to reproduce even after the biological clock has run down [17].

The positive risk-benefit ratio for the cryopreservation of oocytes to be electively self-donated to healthy individuals at a later date calls for an increased availability of these services for all women who desire its potential benefits [18]. As oocyte cryopreservation has been considered experimental, the Practice Committee of the American Society for Reproductive Medicine did not recommend it to postpone reproductive aging and asserts that it should be offered only in an experimental setting and with the approval of an institutional review board [2]. The Practice Committee recently purported that oocyte cryopreservation appears promising and may assist in reducing the rising number of cryopreserved embryos [19]. The Practice Committee is prepared to reconsider its position on oocyte cryopreservation with continued supportive evidence.

NECESSARY ADVANCES IN THE FIELD

Fertility preservation strategies emerged from techniques used for infertility treatment, yet the development of specific protocols and technologies is necessary for this advancing and distinct field of medicine [20]. The complexity and broadness of the field of fertility preservation requires that its technology and research be particular and organized. Novel techniques have been developed that are promising, yet challenges remain to applying these techniques routinely and successfully [21]. Research findings must be applied in protocol to improve the efficacy of the current strategies, as well as to develop new methods and tools [15].

For women undergoing cancer treatment, bridging the treatment gap between the oncologists and the fertility specialist is imperative [13]. By establishing a multidisciplinary team that includes oncologists, fertility and oncology nurses, social workers, reproductive endocrinology and infertility specialists, embryologists, and researchers, a specific treatment plan may be established that provides the highest possibility for success [13]. The members of the team that provide fertility preservation care for the female cancer patient must work together and keep the patient and the center of their focus; refer to Fig. (1). The goal is to provide a multidisciplinary treatment plan for the female cancer patient that maintains the greatest survival potential, preserves fertility, and offers the best quality of life with the least long-term suffering post-treatment [4, 22].

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**Fig. (1).** The members of the multidisciplinary team that provide fertility preservation care for the female cancer patient must work together and keep the patient and the center of their focus.

With a cohesive and knowledgeable team, an individualized care plan based on case-specific motives, situations, age, disease, and other necessary medical treatments can be established for each patient [11, 23]. Counseling is a fundamental aspect of a standard of care because it ensures that the woman may make the most informed decisions based on an
understanding of realistic expectations and after weighing the risk-benefit ratio [2, 9, 10, 24].

An established standard of care may allow more women who might desire the benefits of fertility preservation to utilize the services [5]. The standard must also be flexible to adjust to new technologies and discoveries, as well as to adapt to each patient in her specific socio-economic case. With increased awareness, success, and use of fertility preservation with a standard of care, the different options may become more clinically available and affordable for those who desire to maximize their fertility potential later in life.

**MOST COMMON MODALITIES FOR FERTILITY PRESERVATION**

1. **Embryo Cryopreservation**

As the only established form of cryopreservation, embryo cryopreservation is the most commonly utilized option based on its predictable and reproducible success [5, 9, 25, 26]. The human embryo is highly resistant to cryoinjury and thus has a high cryosurvival rate [12]. Cryopreserved embryos produce pregnancy rates similar to those of fresh embryos [3]. With embryo cryopreservation, stimulation is required to retrieve mature oocytes from the woman to be fertilized and then frozen until the embryo is ready to be transferred to the uterus in attempt for pregnancy. The stimulation cycle requires time that may delay cancer treatment and also may induce ovarian hyperstimulation syndrome (OHSS).

Depending on the time allowed for stimulation, multiple cycles may be utilized to preserve many embryos for multiple attempts. The number of oocytes necessary can be estimated based on fertilization, cryosurvival, and implantation rates for the woman’s age, along with consideration of the woman’s desired family size.

From an economic perspective, the most expensive aspect of treatment is the stimulation protocol for retrieval of mature oocytes. The cost of medication may constitute half the total cost and is dependent on age as higher doses of stimulation are required and more attempts are necessary with increasing age [27]. If multiple stimulation cycles are desired to maximize the potential for achieving multiple pregnancies, the costs increase exponentially.

In long-term studies of children born from cryopreserved embryos, no negative effects have been reported on cognitive, psychomotor, or neurodevelopmental outcomes [22].

2. **Oocyte Cryopreservation**

Oocyte cryopreservation remains in the experimental arena; however, recent promising and repeated successes have moved this method closer to becoming established [17]. Oocytes may be retrieved in the mature or immature stage of development. Hormonal stimulation is required to retrieve mature oocytes. This is the traditional method; however, as one of the largest and most complex human cells, the mature oocyte is particularly susceptible to cryoinjury [7]. Cryopreservation techniques cause a disorganization of the meiotic spindle, depolymerization of chromosomes, and a hardening of the zona pellucida [12, 25, 26]. With novel techniques such as vitrification to evade ice crystallization and the use of intracytoplasmic sperm injection (ICSI) to bypass a hardened zona pellucida, these complications may be resolved [26].

Immature oocyte retrieval requires little or no stimulation, and the smaller, less developed oocyte is less susceptible to cryoinjury [7, 28]. For fertilization to occur, the immature oocyte must be matured through in vitro maturation (IVM), which unfortunately has low success rates [7, 11, 29]. If optimized, this option would allow immediate retrieval with no delay in treatment and elimination of the risk of OHSS that could be induced through stimulation of mature oocytes [3].

Vitrification of oocytes has been proven to increase success in oocyte cryopreservation, producing reliable results and excellent obstetric and perinatal outcomes over the last decade [30]. Additionally, recent data suggests the repolimerization of the meiotic spindle upon thawing of the mature oocyte [7]. No known congenital or developmental deficits were reported from any child born from fertilized cryopreserved oocytes [28].

Utilizing immature oocytes decreases costs by eliminating the need for stimulation [17]. Cryopreservation of oocytes also reduces ethical dilemmas with children and women with no partner at the time of preservation. With improved techniques, this option is likely to become the most routine and reliable fertility preservation method within the next three to five years [25], and a universal vitrification protocol is expected to be established [7].

3. **Ovarian Tissue Cryopreservation**

Ovarian tissue is the definitive source of female fertility, and the ability to preserve and restore the endocrine and reproductive function of the ovary after removal and cryopreservation would be an ideal option if optimized [3, 31]. Ovarian tissue may be harvested immediately, as no stimulation is required, so cancer treatment would not be delayed [3]. Gonadal tissue may be retrieved and cryopreserved as a whole ovary with its vascular pedicle, as cortical strips, fragments, or as isolated primordial follicles [11, 23]. Primordial follicles in the ovarian cortex are more resistant to cryoinjury than the mature oocyte [12]. The tissue may be autotransplanted to the woman later in either an orthotopic or heterotopic location [1, 9]. Autotransplantation of the whole ovary orthotopically has the advantage of immediately resuming blood flow, thus avoiding ischemic damage; pregnancy also may occur spontaneously with this method [1, 9]. Transplantation to a heterotopic location presents a higher risk of ischemic damage, and this method relies completely on ART to achieve pregnancy [1, 31].

The risks involved with ovarian tissue cryopreservation include the risks of a surgical procedure, as well as the possibility of reintroducing malignant cells upon autotransplantation [11, 25]. This option should not be offered to patients with ovarian cancer [3], and it should be utilized with caution for other cancer patients, especially if the cancer has metastasized [4]. Cancer recurrence as a result of ovarian tissue grafts after autotransplantation has not been reported since the technique’s introduction [32].
Despite current reports of success, evidence of reproducible results is still limited, and a standard protocol has yet to be established [12, 23]. Most cases evidence only a short longevity of the ovarian grafts and produce low success rates to be established [11, 12, 25]. Only seven live births have been reported to date [34], yet this still may provide some hope for young girls as this is the only option suitable for pre-pubertal girls undergoing treatment [11, 12, 25].

The most significant advances in cryopreservation over the next several years most likely will occur in ovarian tissue cryopreservation [25]. Advances may result from innovations in surgical technologies and research in tissue engineering to promote better uptake of ovarian grafts and reduced loss of primordial follicles from ischemic damage [11, 23].

**OBSTACLES TO FERTILITY PRESERVATION**

Obstacles to fertility preservation include, but are not limited to, lack of patient awareness, limited physician experience and knowledge, inadequate counseling about fertility preservation options, the costs of the procedures involved, and ethical dilemmas.

The patient’s own lack of awareness of the option for fertility preservation is the first challenge for ensuring fertility preservation for all those who may desire it. For patients who have been told about fertility preservation at the time of diagnosis, looking beyond their own survival can be difficult. These patients may not adequately assess the importance of fertility preservation before treatment, especially if it causes a delay in treatment.

Secondly, the physician’s own lack of knowledge relating to fertility preservation options constitutes another hurdle in the effort to avoid sterility. An oncologist may not refer a patient to a fertility specialist because of the risk of delaying treatment, the patient’s limited resources, or feeling that a discussion of fertility preservation is inappropriate in the midst of fighting a disease [35]. The vast majority of studies examining the discussion and referral of fertility preservation among oncologists and patients indicate that less than half of eligible patients are presented with this information [36].

Discussion is more common than an actual referral to a reproductive endocrinologist, and, according to a survey of oncologists, patients may be willing to sacrifice more in survival than would the physician [37]. A recent study determined that with supportive resources available, the time required for stimulation of mature oocytes for cryopreservation does not significantly extend the period of time between diagnosis and the start of chemotherapy [38].

It is the physician’s role to be knowledgeable about the options and adequately present fair information to the patient or couple, or at least to refer the patient to a more expert specialist [39]. This is often the best solution as many techniques involved in fertility preservation are only performed at specialized centers and the oncologist may have limited access to the most current developments in the field.

Discussion and management of preserving reproductive potential are challenging aspects of care, and inadequate counseling results from the lack of a multidisciplinary approach to providing fertility preservation [40]. Information about actual clinical success rates, outcomes, and risks must be imparted to the patient so that she may make an informed decision founded in practical expectations and an understanding of the risks and benefits [41]. In a study on the experience of receiving fertility preservation options around the time of diagnosis, women were likely to report low levels of comprehension regarding the physiological impact of treatment as well as express distress from lack of services available [40]. The findings of this study suggest that young women may cope with information about fertility preservation alongside cancer diagnosis if there is sufficient professional and familial support [40].

Professional counseling is vital as much of the information that patients may find in the media may be biased or scientifically unfounded [41]. Specifically with healthy women who elect to cryopreserve oocytes, misunderstanding the actual potential can cause emotional devastation if they are unable to conceive later in life [41].

Another significant obstacle to fertility preservation is the high cost of services and the lack of insurance coverage. In the United States, there are currently no states that offer insurance for fertility preservation for cancer patients, although 15 states have laws for insurance coverage of IVF and/or other ART services [35]. Loss of fertility is an inevitable sequela of cancer treatment and as such may justify the coverage of fertility preservation by insurance. Especially with cancer treatment versus normal infertility services, the patient may not have the time to attain the necessary monetary resources required to undergo fertility preservation. The costs may deter both cancer patients and healthy patients alike until these services can be made more affordable.

Many ethical dilemmas currently present obstacles to achieving fertility preservation. For young girls and females without a partner at the time of preservation, embryo cryopreservation may not be an acceptable modality if the female is unwilling to use a donor sperm. In the case of children, it may be difficult to accurately gain informed consent or even for a parent to make an informed decision [22]. The use of oocytes for cryopreservation avoids this ethical issue, yet oocyte cryopreservation remains experimental and is not offered routinely [10]. Ovarian tissue cryopreservation also avoids the dilemma concerning donor sperm, yet it is also experimental, and the faint risk of reintroducing malignant cells should be considered.

In the case of cryopreserved embryos, the future fate of unused embryos presents a dilemma. The options of disposition of the embryo include using all the embryos in an attempt to conceive, donating embryos to another couple or to research, discarding the embryos, and continuing cryopreserved storage indefinitely [26]. Couples often have difficulty resolving this issue before undergoing IVF [42] as the outcome of the treatment is indeterminate.

With optimized outcomes and safe procedures involved with oocyte preservation, arguments against elective self-donation of oocytes are unjustified on clinical or ethical grounds [18, 29]. A person is free to choose when and whether to reproduce if medical and technological means...
exist to execute that choice. With this principal of reproductive autonomy, oocyte cryopreservation for the healthy individual represents a legitimate use of physician and medical resources [41]. Recognizing the medical insufficiencies and the limitations of current fertility preservation technologies, balancing realistic expectations with the desire to conceive and the right to maximize the potential to do so is a wise approach.

EXPERT COMMENTARY

The aim of this article is to elucidate the necessity for and the challenges to establishing a standard of care for fertility preservation. Currently embryo cryopreservation is the only routinely performed procedure for fertility preservation, yet oocyte and ovarian tissue cryopreservation are promising modalities that would increase the ease and availability of fertility preservation for a more diverse patient population. These experimental options have the potential to avoid the risks associated with hormonal stimulation and/or a delay in cancer treatment and to eliminate the ethical dilemmas of embryo cryopreservation, including embryo disposition and use of donor sperm in the case of children or single women. These options also allow healthy patients to maximize their potential to achieve pregnancy later in life. The techniques and methods of fertility preservation evolved from the use of ART for infertile patients, yet the circumstances involved with preservation solicit the need for specific standards, research, and multidisciplinary care. Fertility preservation is emerging as a distinct and specialized field of reproductive medicine, necessitating further research and standards of practice that may lead to better patient care.

FIVE-YEAR REVIEW

Attention to and demand for fertility preservation have increased significantly in the past few years. This was compelled by the increase in cancer survival and the modern reproductive trend of delaying childbirth, and indicated by the boom in research and collaborative efforts. Of specific note, the founding of the International Fertility Preservation Society points to the worldwide importance of preserving the potential to genetically parent one’s own child [20]. This collaborative effort allows clinical and research experts to work together to develop standards of care that provide minimal risk and greater efficiency. In addition, public education should be improved to confer objective and analytical information about current advances in technologies, the lack of evidence of long-term outcomes, and the ethical dilemmas a patient faces [5]. With the advancement of technology and establishment of a standard of care, the cost of procedures and treatment may fall, allowing more patients to take advantage of these benefits with greater ease and accessibility.

KEY POINTS

• Fertility preservation is the preservation of the potential to genetically parent a child beyond natural fertility and despite iatrogenic loss through the means of ART.
• Fertility loss occurs naturally with age or prematurely from gonadotoxic treatment, genetic disorders, or environmental hazards.

• Increased cancer survival and cancer diagnoses in the adolescent and young adult population, as well as the modern reproductive trend of delaying childbirth, indicate a growing need for fertility preservation.
• For women, embryo cryopreservation is the only established technique, and options for oocyte and ovarian tissue cryopreservation are still considered experimental.
• The standardization of oocyte and ovarian tissue modalities of cryopreservation would increase the availability of fertility preservation for a greater patient population while reducing risks and eliminating ethical issues.
• Necessary advances in the field include improved cryopreservation techniques and fertility preservation technologies, comprehensive care plans and customized guidelines, and the availability of more cost-efficient procedures.
• Obstacles to creating a standard of care consist of lack of patient awareness, limited physician experience with new modalities, inadequate counseling, the costs of procedures involved, and ethical dilemmas.
• Continued collaborative efforts among disciplines will assist in greater utilization of research discoveries in clinical applications and support multidisciplinary standards of care for fertility preservation patients.

REFERENCES


Prognostic Role of Ovarian Reserve Testing

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Abstract: The existing quantity of follicles and their response to stimulation at a particular age predicts the ovarian reserve. In vitro fertilization is emerging as a common method of treatment of infertility. This technique is not 100% accurate and puts the patient and the couple in both physical and financial burden. It is important to predict the outcome of a cycle of IVF to counsel a patient prior to proceeding. The ovarian reserve is the main functional component which might guide the outcome. There may be many factors affecting ovarian reserve but the age of the patient is the most important one. Various markers have been utilized to determine ovarian reserve in women which are endocrine, radiological or dynamic tests. No perfect marker has been identified yet but some have proven to be better than the other. This review will discuss the different methods of testing ovarian reserve and the current research that might help the clinician to predict outcome prior to initiating IVF.

Keywords: Ovarian reserve, anti mullerian hormone, antral follicle count, basal follicle stimulating hormone, ovarian volume.

INTRODUCTION

The ovarian reserve describes the functional potential of the ovary at a particular age reflecting the quantity and quality of existing oocytes. The human ovary undergoes a steady decline of follicles with advancing age. This decline is marked after the age of 30. Women are currently deferring their reproductive functions for pursuing career goals. More women are adopting in vitro fertilization (IVF) as an option to conceive at an older age. Extensive utilization of IVF has also encouraged women with cancer at younger ages to preserve their ovaries for future reproductive functions. Hence existing ovarian reserve during diagnosis may help provide information regarding outcomes. In case of oocyte donors ovarian reserve testing may help individualize laboratory protocols and help improve cycle cancellation rates. Since a considerable percentage of women are adopting IVF, which is an expensive modality of treatment with lower outcomes at ages above 40, it is also expected from physicians to counsel patients regarding outcomes and success rates. Ovarian reserve testing may serve as a useful guide for physicians to give their patients an estimate of outcome and expectation.

Various biochemical and radiological parameters have been used to measure ovarian reserve. But the most important factor is the patient’s age at initiation of treatment. Commonly used serum markers are basal follicle stimulating hormone (FSH) on day 3 of the cycle, inhibin-B, and serum anti-mullerian hormone (AMH). Radiological parameters include estimating the ovarian volume and antral follicle count (AFC). Some dynamic tests have been used like the clomiphen citrate challenge test (CCCT), GnRH agonist stimulation test (GAST) and exogenous FSH ovarian reserve test (EFORT), but these have fallen out of favor. This article will focus on current methods of ovarian reserve testing, their application as a guide in fertility preservation. We will discuss the major parameters namely age, day 3 FSH, inhibin-B, AMH, AFC, ovarian volume and dynamic testing and their advantages and disadvantages in predicting poor outcomes after in vitro fertilization.

Age

An increasing incidence of sub fertility in the past 3 - 4 decades has been due to the delay in child-bearing as result of changing societal shift. This postponing has been attributed to the changing career priorities, work force equality and widely available birth control. Many studies conducted on populations in whom birth control measures have not been used, show that optimal fertility occurs from 18 to 30 years, followed by declining fertility after 31 years and sterility at a mean age of 41 years [1, 2].

The normal reserve of ovarian follicles in an embryo at the age of 8 weeks and 5 months has been reported to be 600,000 and 7 × 10⁶ oocytes respectively [3, 4]. At birth, this reduces to 1 × 10⁶ and at the beginning of menarche are around 300,000 follicles [5]. Although, one egg is released in a month by the process of ovulation, a thousand additional eggs are lost during each cycle, due to follicular atresia. During midlife, there is an acceleration of follicle degeneration, when the numbers fall less than 25,000. The onset of menopause is heralded when the follicles reach approximately 1000 in number [6].

It is known that some women will not be able to conceive in their thirties, while others conceive in their forties. This may be partly explained by the above mathematical model, which gives more importance to the critical number of follicles, rather than the age, in determining the onset of menopause. However, others consider that ovarian reserve is not
merely a function of follicular quantity but also the quality of the oocytes, and is dependent upon a complex, dynamic process [7, 8].

Epidemiological studies have reported on the relationship between increased maternal age and increased incidence of chromosomal abnormalities [9, 10]. Several mechanisms have been proposed to explain the increased risk of these abnormalities with increased maternal age: impaired perifollicular microcirculation, abnormalities in follicular development resulting from the aging of the somatic cells surrounding the oocytes and hormonal imbalances [11]. Although the major contributing factor to the age related decline in female fecundity is the gradual but constant degradation of cohesins and other factors holding the four chromatids together during the metaphase, recently it has been proposed that shortening of telomeres may have important role [12].

Bukman et al. have used prognostic models in patients with subfertility for predicting response to the chances of pregnancy [13]. To determine the prognostic values of different tests, they have described a ratio, termed, Likelihood ratio (LR). This ratio, for a positive test, describes the chances of having a positive test while having the disease, compared to the chances of having a positive test and being healthy. A test with high LR could be considered appropriate for screening for patients with diminished ovarian reserve.

In a subgroup of population receiving ART, age was found to be poor predictor for pregnancy than basal serum FSH levels in some studies, although LR could not be calculated [14, 15]. In others, though calculated LR was low, age was considered to be a predictor for pregnancy rate [16-18]. Age was considered to be an important prognostic factor for pregnancy rate, among the general infertile population, for women with normal ovarian reserve. However, in the same group, women with poor ovarian reserve, were considered to have a poor prognosis, independent of age [19].

In summary, age is held to be a better predictor of ART outcome than basal FSH levels in younger women, and seems to affect egg quality more than quality [20]. Women younger than 35 years can still have a favorable IVF outcome, even with elevated basal FSH levels [21].

Day 3 FSH

This is the most commonly used test to assess ovarian reserve. It is an indirect estimate of the ovarian reserve. Normally, in women with reproductive potential, FSH values on the 3rd day of cycle are expected to be below 10 mIU/ml. If however, there is a reduction in the oocytes pool, due to the reduced feedback from estrogens and inhibins, there is corresponding increase in FSH [22, 23]. Thus, it is important, before analyzing FSH levels, that, corresponding estradiol levels (normally, low on day 3) are also assessed. In a patient with infrequent menstruation, an FSH level and estrogen level can be measured at random and is valid if the estrogens levels are low.

A majority of the data in the literature with regards to day 3 serum FSH concentration with pregnancy rates relates to subgroup of population on ART cycles [15, 17, 20, 24, 25]. No similar studies have been performed in women of the general infertile population. There was a better response to ovulation induction, demonstrated by the number of mature oocytes, in patients with low basal FSH levels while others [26] reported basal FSH to be the main predictor for pregnancies in patients with high 3 day FSH concentration (> 20 mIU/ml) as none of these patients had pregnancies. A combination of early follicular FSH and age has been said to be better than age alone in predicting outcome in women undergoing IVF [20].

A significant limiting factor for interpreting the values of day 3 FSH serum values relates to the intercycle fluctuations, which are greater in the presence of high basal concentrations [27] although, a low inter-cycle variability was found in patients with normal basal FSH levels [28]. In addition, interpretations of multiple cycles are also controversial, although it is recommended that fertility estimates may be more reliable if based on the highest FSH concentration and not the lowest. The specific FSH assay used in any laboratory can also affect the boundary between normal and abnormal results. [29, 30] FSH concentrations predict low fertility when its values are high, but do not accurately predict fertility when normal. Finally, the finding of normal FSH concentrations in older women with poor ovarian reserve emphasizes the importance of looking beyond age and FSH.

Inhibin B

Inhibin A and B are both released by the granulose cells of 2 – 5 mm ovarian follicle. They are dimeric peptides composed of an alpha (A) subunit (Inhibin A) and beta (B) subunit (Inhibin B) [17]. Inhibin A seems to be secreted by the dominant follicle, since it increases just after the rise in estradiol concentration during the late follicular phase and thus may mark follicular maturity [17, 31]. Inhibin B is possibly secreted by the developing cohort of follicles in a cycle, since its concentration rises across the luteal-follicular transition and peaks in the mid-follicular phase [32]. So, Inhibin B may indicate the number or quality of developing follicles [17, 31].

Early follicular inhibin B correlates inversely with early follicular FSH levels in perimenopausal women and has been promoted as an early indicator of poor ovarian reserve [33, 34]. It has been demonstrated that women with a low cycle day 3 inhibin B concentrations show a poorer response to ovulation induction than do women with a high day 3 inhibin B concentration [34].

Hofmann et al. investigated inhibin B concentration during clomiphen citrate challenge test in women with normal and poor ovarian reserve and reported that women with poor ovarian reserve had lower inhibin B concentrations than the former [35]. Decreased inhibin B concentrations were also found in women with poor ovarian reserve, despite having non-elevated FSH concentrations, suggesting that a fall in inhibin B concentration might be an earlier marker for limited ovarian reserve than was elevated FSH concentration [34]. Inhibin B concentration is also found to be a better predictor for cancellation than age [15], although its value is still questioned [36]. Although, inhibin B might provide an additional tool for analyzing ovarian reserve, more data is necessary in order to establish normal ranges in clinical practice.
**Anti-Mullerian Hormone**

AMH is a dimeric glycoprotein of the transforming growth factor –alfa family which plays a major role in male sex differentiation. In the female ovary it is expressed as early as 32 weeks of gestation from the granulosa cells [37]. Its role is primarily related to inhibition of progression of primordial follicles to primary follicles as evidenced by animal studies [38]. It can also influence follicle dominance and recruitment [39]. It reduces responsiveness of follicles to FSH in dominance selection [40].

Role of AMH to determine ovarian response have been extensively studied. It has been noted that AMH levels decline throughout the reproductive age and is almost non detectable after menopause [41]. This same group of researchers also noted that the AMH levels correlate well with antral follicle count (AFC) and that day 3 serum AMH also declines as reproductive age advances [41]. The level of AMH shows a steady decline over time compared to other markers both in women <35 yrs and >40 [42]. Due to this characteristic of lower levels corresponding to age, many researchers have suggested that AMH levels may predict onset of menopause [43, 44].

Role of AMH in ovarian reserve testing has been studied in IVF setting in predicting number of oocytes available for retrieval. The first study was done by Seifer et al. who proposed that a higher day 3 AMH corresponded to more oocytes retrieved [45]. Other groups established this above fact including predicting number of embryos for IVF but failed to provide information on oocyte quality and fertility outcomes [46]. Silberstein et al. demonstrated that higher AMH levels during HCG administration could predict better embryo scores [47]. In patients susceptible to develop ovarian hyperstimulation syndrome (OHSS), serum AMH may be used as a predictive marker as does estradiol levels and follicle numbers [48-50].

AMH levels may also be used as markers of treatment and progression in patients with polycystic ovarian syndrome (PCOS). Since AMH has significant role in primordial follicle transition and recruitment, it is hypothesized that abnormal AMH may hamper maturation of follicle and ultimately ovulation in PCOS patients. The serum level of AMH is 2 to 3 fold greater in PCOS compared to normal patients [51, 52]. Studies have suggested that in absence of ultrasonographic aid, in patients with oligoamenorrhoea and hyperandrogenism, serum AMH levels can help diagnose PCOS [53]. This can also serve as a prognostic indicator of recovery of menstrual function after weight loss treatment [54].

AMH estimation is technically advantageous secondary to its very low intra and inter assay variability [55]. Other advantages are its comparability to antral follicle count (AFC) and capacity to predict menopause as mentioned above. But there are certain disadvantages. Serum AMH fails to determine the exact cohort of follicles 2-5 mm in size where smaller follicles may also secrete AMH [51]. Besides the cost of estimation, the variability in levels in a single cycle has not been studied yet and its clinical application may be limited [46].

**Ovarian Volume**

It is a well known fact that ovaries get smaller with aging. This may also correspond to the number of follicles in them [56, 57]. Various ultrasound modalities are used to measure ovarian volume. Three dimensional ultrasound with power Doppler angiography is the most recent type. This method has advantages over color Doppler of being more sensitive and the three dimension feature adds to the details and accurate measurement of volume and blood flow [58]. There has been no definite cutoff established by measurement of the ovarian volume as it is an age dependant measurement. Kupesic et al. in a prospective study in women undergoing IVF found statistically significant differences in total ovarian volume (TOV) in women of different age groups utilizing three dimensional power Doppler ultrasound [59]. Another group of researchers did find declining ovarian volumes in women in perimenopausal age groups when compared to women of younger age and calculated a 0.2 cm³ decline with increasing age [60].

Though the measurement of ovarian volume is quite accurate, the main question is whether it has any prognostic role in determining outcomes of assisted reproduction techniques. Some studies documented that women with ovarian volumes <3 cm³ had higher cancellation rates [61] and there may be a correlation with success of assisted reproduction cycles but not significant for pregnancy outcomes [62, 63]. A prospective cohort study in women undergoing IVF correlated IVF parameters with mean ovarian volumes and failed to establish a cutoff volume which could predict pregnancy outcome [64]. It has also been documented that ovarian volume estimation has similar sensitivity and specificity to basal FSH in predicting menopause [65].

It is currently evident that though ovarian volume measurement may indicate available oocytes at a particular age group but may not be good in providing pregnancy outcomes after IVF.

**Total Antral Follicle Count**

Total antral follicle count during the follicular phase is a sonographic parameter of estimating ovarian reserve and an excellent marker of chronological age [66]. Scheffer et al. found that the antral follicle pool declines at 4.8% per year before 37 years and at a rate of 11.7% after the age of 37 [66].

Ultrasound guidance to measure the follicles have also been recommended. Antral follicles measuring 2-10 mm in diameter measured by transvaginal ultrasound are the normal standard [67, 68]. Some studies have also predicted that diameters less than 5 mm may result in no pregnancy [69, 70] and some group has suggested that practically the range 2-10 mm actually represent the smaller follicle pool of 2-6 mm that has a better representation of the ovarian reserve compared to those that are larger [71]. Inter observer and biological variations in measurement might be the only disadvantage of AFC [72]. Technically most commonly used methods utilize 2D or 3D measurements but recent research has postulated that Sono-AVC (Sono – Automated Volume Calculation) technique may reduce inter and intra observer variations [67].
Various studies have postulated that AFC is still the best test to predict fertility outcomes after IVF. A standardized AFC measurement is superior to basal FSH, inhibin-B in measuring ovarian response after stimulation [72]. AFC has also been studied to be a superior predictor of poor outcome when compared to basal FSH [73] and ovarian volume [74] in two meta-analysis. It has also been documented to be superior when compared with clomiphen citrate challenge test, inhibin-B, and ovarian volume in predicting poor outcome [75].

A retrospective study of 278 patients undergoing IVF established that an AFC of 11 or more is a better predictor of live birth after in vitro fertilization and embryo transfer (IVF-ET) and is a good number to counsel patients about success rates [76]. Combination of other parameters with AFC has also been studied. A day 7 follicle count with a basal AFC together had a high positive predictive value and was a better predictor of cycle cancellation in a retrospective study of 91 IVF cycles [77].

AFC and serum AMH have been documented to be strong predictor of poor ovarian response after IVF having similar capacity individually though combining them did not augment the predictive value in a prospective study of 135 women undergoing first IVF. They found that AFC has a sensitivity of 93% and a specificity of 88% while AMH had that of 100% and 73% respectively. This group also documented a cutoff of 10 for AFC and 0.99 ng/ml for AMH [78].

As discussed above AFC is a great marker of chronological age [66]. Relationship of AFC with menopause has also been studied [56, 79]. Maternal age of menopause and declining AFC may indicate genetic trait in patients. Women with a lower maternal age of menopause had low AFC and a slower decline in the number as documented in a recent cross sectional study [79].

Dynamic Testing

These are a set of tests which utilizes exogenous stimuli to stimulate folliculogenesis and measure hormone levels and or antral follicle counts to get an indirect indication of the nature of ovarian response. These tests namely the exogenous FSH ovarian reserve test (EFORT), clomiphen citrate challenge test (CCCT) and GnRH agonist stimulation test (GAST) are rarely used today since individual parameters like AFC and or serum AMH levels are better indicators.

Exogenous FSH Ovarian Reserve Test (EFORT)

This test can be used to predict ovarian reserve in patients prior to undergoing IVF treatment. It was documented that if estradiol levels increased above 30 pg/ml after administration of 300IU of exogenous FSH on day 3 of the cycle, the pregnancy and implantation rates were better [80]. A baseline FSH was also measured. Some studies also recommended measuring inhibin-B levels after EFORT as a guide for ovarian reserve testing and possibly a better predictor compared to clomiphen citrate challenge test (CCCT) [81]. Recent studies utilizing EFORT to predict pregnancy outcomes are currently lacking both in general and subfertile population.

GnRH Agonist Stimulation Test (GAST)

This test is performed by administering supraphysiologic doses of GnRH agonist and measuring change in levels of estradiol from day 2 to day 5 of the cycle. An increase in these levels is considered a good response. Controversies exist based on earlier and newer studies in patients undergoing assisted reproduction regarding the benefit of this test. Some groups did not find this to be useful [23, 82] whereas others thought the opposite [83, 84]. Hendriks et al. compared day 3 AFC and inhibin –B with the GAST and did not find it to be superior and in fact it could not predict ongoing pregnancy outcomes well. Thus GAST has fallen out of favor compared to serum AMH, AFC or inhibin-B estimation.

Clomiphen Citrate Challenge Test (CCCT)

100 mg of clomiphen citrate is administered orally from days 5 to 9 after estimating basal FSH on day 2 or 3 of a cycle. If an increase of FSH is found on day 10 by more than two standard deviations from normal the test is declared as abnormal. Jain et al. published a meta-analysis including 12 studies for basal FSH (6296 patients) and 7 studies for CCCT (1352 patients). Their outcome was to predict pregnancy. They concluded that both basal FSH and CCCT had equal capacity to predict achievable pregnancy though a normal test was not useful but an abnormal test was more confirmatory for failure to achieve pregnancy [85].

CONCLUSION

Infertility is a growing problem and age has been a main factor. Ovarian reserve declines with age and so does the success of pregnancy after IVF. Various parameters have been studied to predict outcomes after IVF which may reduce cancellation rates and develop protocols in infertility clinics to achieve better success rates. Though routine testing for ovarian reserve is not recommended, individualization of cases may indicate the need to do so. Serum basal FSH, AMH and AFC are still the most common parameters utilized due to evidenced based studies advocating their superiority. Dynamic testing has fallen out of favor due to advancements in estimation of endocrine markers and ultrasonography. Though studies have attempted to define cut off values for each parameter larger population based studies are required to get a standard definition for each. Various factors may influence the estimation of these markers which need to be considered. In clinical practice each patient need to be evaluated individually to utilize the best prognostic indicator of their reproductive outcome. Along with development of predictive markers of ovarian reserve testing, assisted reproduction techniques also require further development to improve their outcome.

REFERENCES

Prognostic Role of Ovarian Reserve Testing


Whole Ovarian Vitrification: A Viable Option for Fertility Preservation?

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Abstract: Ovarian transplantation may be the future of fertility preservation. Although ovarian fragments graft is known to restore fertility, but a large number of primordial follicles are lost during the neo vascularisation period and the life span of the transplant is usually short. Whole ovary transplantation may restore ovarian function immediately and during long time. Nevertheless results of whole transplantation are still disappointing. Only one gestation in animal had been reported.

Keywords: Whole ovary, vitrification, transplantation, gestation, delivery.

INTRODUCTION

Many girls and young women have been or will be treated for cancer. Depending on the type of treatment, early menopause can occur. Thus preserving female fertility is a major issue that concerns most reproductive medicine teams worldwide. One fertility preserving option that is being studied is ovarian tissue cryopreservation followed by implantation.

In one type of cryopreservation procedure, ovarian tissue fragments are taken from the patient and frozen. Indeed, many pregnancies and deliveries have been obtained in a number of species after freezing and implantation of ovarian tissue fragments [1]. Freezing cortical fragments enables a large number of primary follicles to be stored with relative ease due to the thinness of the tissue [2]. Cryoprotectants can easily penetrate the thinned cortex to protect the primary follicles [3]. The first human live birth using this procedure was reported by Jacques Donnez in 2007 [4]. Since then, many grafts have been performed in humans, and the number of births published or under publication is estimated at around 13 [5].

Ovarian tissue grafting, however, poses many problems. The functional lifespan of the grafts is sometimes short, requiring repeated grafting. For many women who choose to undergo in vitro fertilization after the ovarian freeze/thaw procedure, the results are poor. The follicles may be empty, and oocytes can be abnormal or immature, thereby greatly reducing the rates of pregnancy by transfer [6].

Whole Ovary Cryo-Preservation (WOCP) with vascular anastomosis, on the other hand, offers what may be a better alternative. With WOCP, the entire follicular reserve is preserved and transferred, and revascularization after implantation is almost immediate. WOCP could thus restore ovarian function entirely and over the long term.

WOCP with autograft has been performed in small mammals such as the rat and mouse with relative success for several years. With such small ovaries, the freezing technique is straightforward and the cryoprotectants penetrate easily. In larger species such as sheep and humans, however, the process is more technically challenging. The human ovary is composed of various types of tissues, such as vessels (arteries and veins), the ovarian medulla and follicles. Cryoprotectants do not always completely penetrate these tissues. We have shown using VS4 as cryoprotectant solution that VS4 perfused ovarian cortex fragments did not vitrify but formed ice crystal around in 18% [14]. The surgical demands are also considerable. When transplanting ovarian fragments, surgery is quick and simple and can be performed using laparoscopy. WOCP, on the other hand, requires termino-terminal arteriovenous anastomosis, which is technically challenging. The difficulty lies in the microscopic size of the vessels and the length of the operation. Additionally, laparotomy is required.

Arav [8] was the first to report results on WOCP, which was performed in 8 ewes. Cyclic progesterone secretion was documented in only 3 of the ewes and then only between 24 and 36 months after surgery. Oocytes were harvested from of the 2 ewes, and MRI found intact ovaries with functioning vessels in the 3 same ewes. In 2003, Bedaiwy demonstrated the feasibility of performing WOCP. But, only 27% of the whole ovary transplants had normal vascularization at 10 days [9]. In 2008, the same team recorded follicular survival rates in whole ovaries and in tissue fragments that had been frozen and transplanted in female patients—the rates were identical. Imhoff reported gestation and birth following WOCP in one ewe: the gestation occurred 2 years after transplantation. The results of Imhoff study suggest that recovery of ovarian function can be slow [10].

We have performed 12 ovarian transplantations in ewes (Fig. 1): 6 of the transplantations used vitrified-thawed ovarian fragments (Fig. 2), and 6 used fresh whole ovary (the right ovary was immediately attached to the left pedicle after the left ovary was removed). Follicle survival at 1 year was near zero in the vitrification group, with, obviously, no pregnancies whereas in the slow cooling group, ovarian function recurred in 5 of 10 ewes, and 2 gestations were documented, leading to healthy live birth [11, 12]. At the time of this writing (unpublished data), we have also
transplanted 12 ewes using a slow-freezing protocol in 6 and a vitrification protocol in the other 6. In both groups, cyclic steroid secretion has occurred, and gestations are expected for 2010.

WHY VITRIFICATION?

Deciding whether to use slow freezing or vitrification is tricky [13]. In the literature, most teams have performed WOCP using a slow-freezing protocol, including the ewe studies that resulted in gestations. We consider vitrification better suited to WOCP because of the variety of tissues that must be preserved. Using a vitrification solution called VS4, we achieved a 78% follicle survival rate--a rate that is no different than a fresh ovary follicle count. We demonstrated, however, that the cooling speed did not result in complete vitrification, which may account for the failure of transplantation using VS4 [14]. VM3 is a new vitrification solution containing so-called “super-iced molecules”, and it seems to yield much better results. Most ewes transplanted using VM3 have shown restored ovarian function (unpublished data). This is in line with Onions’ theory according to which follicle loss may be due not only to large-vessel thrombosis but also to cryodamage to subcortical ovarian microvascularization [15].

IS WHOLE OVARY FREEZING WORTH CONTINUING?

When comparing the results of slow tissue freezing with those of WOCP, the evidence suggests that the former is more advantageous, as witnessed by the live births obtained in animals and humans. Only one animal gestation and birth has been obtained with WOCP; an additional 2 were reported after fresh ovary grafting. The advantages expected from WOCP have yet to be confirmed. Functional recovery, including hormonal secretion, is no faster, which may be explained by the time needed for primary follicles to enter their growth phase and reach maturity. Primary follicle destruction rates are the same in both cases, but the mechanisms differ. In WOCP the problem lies in the difficulty of freezing primary follicles under the cortex or with damage to subcortical microvascularization. The choice between slow cooling and vitrification favors slow cooling. New vitrification solutions such as VM3, however, could improve outcomes. It is still too early to say that the future for ovary grafting lies with WOCP. Many further studies will be needed to improve both vitrification / freezing and surgical protocols. In our opinion, the future of ovarian vitrification lies not in transplantation so much as in ex corporis whole ovary culture, which could provide oocytes ad libitum for egg donation or cloning.

REFERENCES


Emerging Technologies for Fertility Preservation in Female Patients

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Abstract: A wide variety of fertility preservation options in women is available; however, most of the currently available strategies are still experimental and do not guarantee subsequent fertility. The only established method is in vitro fertilization with embryo cryopreservation prior to cancer therapy. Other proposed strategies to preserve fertility in women with cancer include: storage of frozen ovarian tissue or the whole ovary for future transplantation, storage isolated follicles for in vitro growth and maturation and ovarian transposition before radiotherapy. The effectiveness of ovarian protection during chemotheraphy with GnRH analogs is yet to be shown.

Keywords: Embryo cryopreservation, ovarian transplantation, chemotherapy, radiotherapy, premature ovarian failure.

INTRODUCTION

Female fertility can be adversely influenced by cancer therapies that affect the ovary and uterus. At the ovarian level, pelvic irradiation and chemotherapy can decrease ovarian reserve and result in ovarian failure. Moreover, pelvic irradiation can adversely affect the uterus, increasing the subsequent risk of obstetrical complications such as intraperineal fetal growth restriction and preterm birth [1]. A number of potential options are currently available, however, to help preserve a woman’s future fertility, thanks to a better understanding of the intricate mechanisms governing human reproduction. They are: [1] storage of frozen embryos, [2] storage of frozen ovarian tissue or the whole ovary for future transplantation, [3] storage of frozen ovarian tissue or isolated follicles for in vitro growth and maturation, [4] ovarian transposition before radiotherapy, [5] ovarian protection during chemotherapy with either GnRH analogs or antiapoptotic agents (e.g., sphingosine-1-phosphate), [6] uterine transplant after successful cancer treatment where a functional uterus no longer exists, and [7] in vitro fertilization (IVF) with embryo cryopreservation prior to cancer therapy.

Of those, only the last one—IVF followed by embryo cryopreservation—is considered a clinically proven option for women requiring cancer treatment [2]. The remaining strategies are considered experimental largely due to unproven efficacy or lack of adequate long-term follow-up.

In this review, the consequences of irradiation and chemotherapy on ovarian function and fertility as well as current and future fertility preservation strategies in female cancer patients will be discussed.

EVOLUTION OF HUMAN OOGENESIS

By the end of the first trimester of pregnancy, the human ovaries are almost fully formed. By the seventh week of intrauterine life, primordial germ cells reach their final destination in the gonadal ridge after migrating from the yolk sac endoderm. They differentiate into oogonia and eventually into primary oocytes by mitosis. Before the twelfth week, some primary oocytes enter their first meiotic division, which becomes arrested in the dictyotene stage of prophase I. This process resumes with the first ovulation. Contrary to spermatogenesis, oogenesis in humans does not continue after birth. The maximum number of primary oocytes (6 to 7 million) is attained at 20 weeks of intrauterine life. That number falls dramatically to 400,000 at birth and approximately 200,000 at the time of puberty [3,4]. Oocyte reserve continues to diminish both in quantity and quality throughout the remaining life cycle.

A multitude of endocrine, paracrine, autocrine and intracellular factors govern the fate of each ovarian follicle [5]. During the menstrual cycle, follicles mature in a series of stages (primordial, primary, secondary and tertiary) in a process called folliculogenesis which is independent of gonadotropin stimulation. The vast majority of follicles undergo atresia after differentiation into tertiary follicles with an antral cavity. During any ovarian cycle, one or two follicles mature to the preovulatory stage as a result of gonadotropin-induced folliculogenesis [5]. Throughout reproductive life, the main source of ovarian estrogens remains the mature Graafian follicle [6]. Many genes, hormones, proteins and factors are responsible for governing the intracellular process of folliculogenesis in humans [7-14]. Intrafollicular communication between the cells controls follicular dynamics; exposure to cancer therapy frequently results in the death of many neighboring cells, too [6, 7, 12, 13]. Traditionally, it has been well established that the germ cells (primordial follicles) are non renewable. This concept was challenged by 2 recent reports [15, 16] stating that germ cells may be renewed locally [15] or from bone marrow stem cells [16]. However, this new and exciting findings were not reproduced by other groups and it was challenged itself by the opinion of some other experts in the field [17-19].
ANTICANCER THERAPY-INDUCED PREMATURE OVARIAN FAILURE (POF)

POF Induced by Chemotherapy

Premature ovarian failure (POF) occurs after exposure to many anticancer drugs. Unfortunately, chemotherapy-induced ovariotoxicity is almost always an irreversible insult. After treatment with ovariotoxic drugs, the ovary exhibits a spectrum of histological changes ranging from decreased numbers of follicles to absent follicles to fibrosis [20]. The variable effects of different anticancer regimens on ovarian functions have been reported by many other investigators [21, 22].

The incidence of chemotherapy-induced POF cannot be precisely estimated because of the multifactorial nature of the insult. However, a number of factors can increase the risk:

- The cumulative dose.
- The patient’s age at the start of treatment. POF increases with advancing age owing to progressive age-dependent follicular depletion.
- The degree of gonadotoxicity. This varies from one chemotherapeutic drug to another. Cell cycle specificity of the chemotherapeutic agent majorly determines the magnitude of ovarian injury; cell cycle–nonspecific chemotherapeutic agents are more ovariotoxic than cell cycle–specific ones. Alkylating agents are the most gonadotoxic of all cell cycle–nonspecific anticancer drugs. Cyclophosphamide, a commonly treatment for breast cancer, is the most ovariotoxic of its group. Given the fact that breast cancer is the most common malignancy in women, most cases of chemotherapy-induced ovariotoxicity are induced by cyclophosphamide. Patient age and cumulative dose most strongly influence the POF rate in those taking this drug [20]. Less than 50% of women treated with cyclophosphamide who are younger than 30 years develop POF. On the other hand, approximately 60% of women between the ages of 30 and 40 years will develop POF and hypergonadotropic amenorrhea [20].

Consequently, the risk of POF is not the same in all patients receiving multiagent gonadotoxic chemotherapy. Women with the highest risk for developing POF are those who receive high-doses of alkylating agents with pelvic irradiation. On the other hand, young women with Hodgkin’s disease treated with multiagent chemotherapy and radiation to a field that does not include the ovaries will mostly remain fertile, although usually for a shorter period of time than that of age-matched controls [23]. In one case report, a young woman treated with repeated courses of ifosfamide combined with pelvic irradiation for Ewing’s sarcoma of the pelvis who developed POF was able to achieve a spontaneous pregnancy [24].

POF INDUCED BY RADIOTHERAPY

Among young women of reproductive age, radiotherapy is standard treatment for many genital as well as extragenital cancers including cervical, vaginal, and anorectal cancers, some germ cell tumors, Hodgkin’s disease, and central nervous system (CNS) tumors. Pelvic radiotherapy can not only damage the ovaries but also the uterus. The patient’s age, total dose of radiation reaching the ovaries and the number of sessions needed to deliver the total dose are the prominent factors that determine both the extent and duration of ovarian damage. This is quite similar to the impact of chemotherapy.

Fractionating the total dose of radiotherapy significantly reduces the extent of ovarian damage [25]. Single-dose radiation is more toxic than fractionated therapy. The approximate threshold for radiation-induced ovarian failure is 300cGy to the ovaries; only 11% to 13% of women developed POF at doses below 300cGy versus 60% to 63% at doses above that threshold [26]. Standard pelvic irradiation to the ovaries will consistently result in POF; this is compounded by co-administered chemotherapy [27, 28].

Ionizing radiation is particularly deleterious to ovarian follicles, resulting in radiation-induced DNA damage, ovarian atrophy and a significant depletion of ovarian follicles [29]. Oocytes exhibit a rapid onset of pyknosis, disruption of the nuclear envelope, chromosome condensation, and cytoplasmic vacuolization. Within four to eight weeks after exposure to radiation, serum levels of FSH and LH progressively rise while serum E2 levels fall [29, 30]. Radiotherapy results in a dose-dependent reduction in the follicular pool [30]. Fifty percent of the oocyte population is destroyed by a radiation dose < 2 Gy (LDL50 < 2 Gy) [31].

CHEMOTHERAPY/RADIOThERAPY-INDUCED POF: DIAGNOSIS AND PREDICTION

The diagnostic and prognostic implications of predicting POF in cancer patients can not be overemphasized. Researchers are currently looking for a biomarker that can reliably predict chemotherapy- or radiotherapy-induced POF with satisfactory sensitivity and specificity. Among those potential markers are anti-Mullerian hormone (AMH), serum FSH, inhibin B and basal antral follicle counts (AFC).

AMH is produced by the granulosa cells of virtually all types of follicles, from the primary to early antral stages. Growing follicles progressively lose their ability to produce AMH, which is why peripheral AMH concentrations decrease during ovarian stimulation. Unlike all other hormonal biomarkers, which are dependent on the stage of follicular development, AMH is independent of FSH, LH, and inhibin levels.

Serum FSH levels were elevated in cancer survivors with regular menstrual cycles whereas AMH levels were lower than those of age-matched controls [32]. Despite the smaller ovarian volume in cancer patients, AFC were similar between cancer survivors and controls [32]. In prepubertal girls receiving anticancer chemotherapy, serum levels of inhibin B transiently decreased. Consequently, serum FSH, coupled with serum inhibin B, was proposed as a potential biomarker of the ovariotoxic effects of cancer chemotherapy in prepubertal girls [33].

Basal AFC has been used extensively alone or with other markers to predict POF. Measurement of serum AMH levels could be a quantitative and possibly a qualitative biomarker of granulosa cell health and activity [34]. For prepubertal
girls undergoing sterilizing cancer therapy, AMH may have promising prospects.  

**WHEN IS FERTILITY PRESERVATION INDICATED IN WOMEN?**

Breast cancer is the most common malignancy seen during the reproductive years that requires an immediate fertility-preserving intervention. One of every seven breast cancer patients is younger than forty years [35, 36]. Another common malignancy seen in younger women that requires fertility-preserving intervention is cervical cancer. Of the 13,000 new cervical cancer cases diagnosed in the United States each year, 50% are estimated to occur in women younger than 35 years of age [37]. Moreover, owing to the peculiar anatomic location of the cervix, fertility preservation is even more challenging in those cases.

The indications of fertility preservation in women currently include ovariotoxic radiotherapy/chemotherapy for cancer as well as for the treatment of a multitude of non-malignant conditions including systemic lupus erythematosus, acute glomerulonephritis, and Behcet’s disease. On the other hand, many other patients with extra-genital cancers are candidates for fertility-preserving procedures including those with hematopoietic cancers such as leukemias and lymphomas, musculoskeletal cancers such as Ewing’s sarcoma and osteosarcoma, neuroblastomas, and Wilms’ tumor. Women receiving chemotherapy while undergoing bone marrow transplantation or umbilical cord stem-cell transplantation are potential candidates as well as patients with non-gynecologic cancers including lymphomas, sarcomas and colorectal carcinoma. Some of the salient indications of fertility preservation in women are summarized in Table 1.

### Table 1. Situations Necessitating Fertility Preservation in Women

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<td><strong>A-MULTI-SYSTEM DISORDERS:</strong></td>
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<tr>
<td>Acute glomerulonephritis</td>
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<td>Behcet’s disease</td>
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<td>Systemic lupus erythematosus (SLE)</td>
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<td><strong>B-TRANSPLANTATION PROCEDURES:</strong></td>
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<td>Bone marrow transplantation (BMT)</td>
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<td>2. MALIGNANCIES TREATED WITH OVARIOTOXIC RADIOTHERAPY OR CHEMOTHERAPY:</td>
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<td><strong>A-ADULT CANCERS:</strong></td>
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<tr>
<td>1. GYNECOLOGIC CANCERS:</td>
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<td>Breast cancer</td>
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<td>2. NON-GYNECOLOGIC CANCERS:</td>
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<td>Sarcomas</td>
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<td>Lymphomas</td>
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<td>Colorectal cancer</td>
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<td><strong>B-CHILDHOOD MALIGNANCIES:</strong></td>
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<tr>
<td>1. HEMATOPOIETIC CANCERS:</td>
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<tr>
<td>Lymphomas</td>
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<td>Leukemias</td>
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<td>2. MUSCULOSKELETAL CANCERS:</td>
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<td>Osteosarcoma</td>
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<td>3. OTHER CANCERS:</td>
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<td>Wilms’ tumor</td>
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<td>Neuroblastomas</td>
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<td>3. BILATERAL OOPHORECTOMY FOR BENIGN OVARIAN TUMORS, ENDOMETRIOSIS, OR PROPHYLAXIS.</td>
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STRATEGIES FOR FERTILITY PRESERVATION

A spectrum of potentially promising strategies has been assessed for fertility preservation. Although the currently available armamentarium encompasses an ever expanding list, most of those are more experimental than practical. Moreover, none has been tested in a prospective randomized controlled trial (RCT).

PHARMACOLOGICALLY-BASED PROTECTION

The theoretical rationale behind pharmacologically-based protection came from the intriguing observation that the premenarchal ovary is less sensitive to the effects of ovariotoxic agents. Consequently, researchers have studied pre-treatment with a gonadotropin-releasing hormone (GnRH) agonist, as well as many other medications that suppress the hypothalamic pituitary ovarian axis, in an endeavor to maintain the prepubertal ovarian quiescence during chemotherapy [38, 39]. Meanwhile, a direct ovarian effect has also been postulated [38, 39]. However, theory and practice do not always meet. The few observational studies addressing this issue have come to conflicting conclusions. Blumenfeld and colleagues reported a diminished frequency of POF in patients who received a GnRH agonist prior to chemotherapy in their excellent analysis of all studies on GnRH agonist use. Most of those studies, however, were limited by short follow-up period in the GnRH group and the retrospective nature of the control groups [40]. Other investigators [41, 42] have come to similar conclusions. On the other hand, a prospective controlled study found that GnR analogue were not effective in the prevention of POF [43]. That particular study however was limited by a small sample size.

Patients and oncologists strongly advocate GnRH use; oncologists are satisfied with starting cancer therapy without avoidable delays caused by standard ART protocols, and patients find it simple. On the other hand, counter-arguments downplaying the use of GnRH agonists are centered on the fact that FSH suppression will not protect the primordial follicles constituting the ovarian reserve. The debate on the effectiveness of GnRH agonists will continue until it is settled by the results of prospective randomized controlled trials that are sufficiently powered. Empirical use of other suppressive drugs such as combined oral contraceptives (COCs) or progestins has not been successful in preventing chemotherapy/radiotherapy-induced ovarian damage.

TRANPOSITION OF THE OVARIES (OOPHOREXY)

Another possible fertility preservation option for women scheduled to undergo sterilizing ovariotoxic radiotherapy is surgery in which the ovaries are moved away from the intended irradiation field. This technique helps curtail ovarian radiation exposure in patients with low intestinal and gynecological malignancies and in those with Hodgkin’s disease receiving pelvic irradiation. The radiation dose to the ovaries is reduced to approximately 5% to 10% following transposition [44]. Given an initial dose of 4,500 cGy, the dose to each transposed ovary is 126 cGy for intracavitary radiation, 135–190 cGy for external radiation therapy and 230–310 cGy with para-aortic lymph node inclusion in irradiation [45]. Lateral transposition of the ovaries was shown to be more effective than medial transposition—with the latter, the ovaries are sutured posteriorly to the uterus and shielded during treatment [46, 47].

Lateral ovarian transposition can be performed during staging laparotomy for Hodgkin’s disease (which is not customary nowadays) and during radical hysterectomy for cervical cancer [48]. Laparoscopic ovarian transposition could be considered a valuable option before starting radiotherapy. Moreover, immediate postoperative radiotherapy could be initiated, which could furthermore help prevent POF if the ovaries were to migrate back to the irradiation field [28, 49, 50]. Moreover, the same anesthetic can be used for laparoscopic ovarian transposition as that used to insert a brachytherapy device in cases of cervical or vaginal cancers treated by brachytherapy [51]. It can also be performed on an outpatient basis in patients with Hodgkin’s disease, leading to a better cosmetic effect and earlier recovery with minimal cost and discomfort.

Following laparoscopic ovarian transposition, almost all women with Hodgkin’s disease (stage I and stage II) treated with radiation alone or with minimal chemotherapy retain their ovarian function and fertility [28]. Laparoscopic ovarian transposition is not a complication-free procedure, however. POF is still a concern, and symptomatic ovarian cysts may develop as well. There are many potential causes of ovarian failure following ovarian transposition [52], and they are summarized in Table 2. Although COCs can help suppress cyst formation, the underlying etiology remains largely obscure [53].

Table 2. Potential Causes of Ovarian Failure Following Ovarian Transposition Before Radiotherapy

<table>
<thead>
<tr>
<th>A-VASCULAR INSULT:</th>
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<tr>
<td>1. Jeopardizing ovarian vessels</td>
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<td>2. Radiation-mediated injury of the vascular pedicle</td>
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<tr>
<td>B-ANATOMICALLY-MEDIATED MECHANISMS:</td>
</tr>
<tr>
<td>1. Transposed ovaries not moved far enough</td>
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<tr>
<td>2. Ovarian migration back to original position following use of absorbable sutures</td>
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</table>

ASSISTED REPRODUCTIVE TECHNOLOGIES (ART)

In patients who wish to proceed to fertility preservation, ART is probably the most commonly adopted policy.

Oocyte Cryopreservation

In unmarried women and those without a male partner, freezing mature or immature oocytes may be the only practically option. A multitude of factors come into play to determine the efficacy of oocyte cryopreservation, given the complex structural nature of the ovary. In contrast to preimplantation embryos, subcellular organelles in the oocytes are far more complex and perhaps more sensitive to thermal injury [54, 55]. Oocytes can be cryopreserved for years and still...
retain their reproductive potential. Moreover, the duration of oocyte cryopreservation does not seem to interfere with oocyte survival; a number of pregnancies have been reported several years after oocyte cryopreservation using liquid nitrogen [56].

In its recent evaluation of the currently available evidence, the ASRM practice committee concluded that oocyte cryopreservation is associated with a limited number of established pregnancies and deliveries resulting from cryopreserved oocytes. However, no increases in chromosomal abnormalities, birth defects, or developmental deficits have been noted so far among offspring. It also states that oocyte cryopreservation should still be considered an experimental technique that should only be performed under an investigational protocol approved by an internal review board (IRB) [57].

Despite an unacceptably low initial pregnancy rate per vitrified–thawed oocyte, recent technical modifications have resulted in improved oocyte survival, fertilization, and pregnancy rates. Intriguingly, pregnancy rates are steadily increasing, whether slow-freeze or vitrification methods are used [58]. Therefore, cryopreservation of oocytes has its place not only as an additional protocol in conventional IVF but also as a viable option for fertile women at risk of losing their fertility. The results of larger prospective controlled clinical trials focusing on efficacy and long-term safety of oocyte cryopreservation are needed. Oocyte cryopreservation can only be considered to have practical value when an adequate number of births is achieved and followed-up. Until then, it remains basically an experimental procedure that can only be carried out under strict surveillance [59].

Embryo Cryopreservation

In 2005, embryo cryopreservation was described by the ASRM as the only established evidence-based and clinically valuable fertility preservation option for women with a male partner. It is presumably the most effective means of fertility preservation in women undergoing ovarian stimulation regimens for IVF, and it offers them a satisfactory chance of success. Delivery rates per embryo transfer have been reported by the society for assisted reproduction technology (SART), using cryopreserved embryos, to be 31.8% for women under 35 years of age [60]. Post-thaw embryo survival rates range between 35% and 90%, and implantation rates range between 8% and 30%; cumulative pregnancy rates have been reported to be more than 60% [61, 62].

Consequently, embryo cryopreservation is considered the fertility preservation option with the best outcome. Moreover, long-term follow up data on the outcome of children born from these procedures are reassuring and confirm that the procedure is safe. A number of limitations do, however, exist. Firstly, it is unsuitable for prepubertal, adolescent and unmarried females and women without a partner. Secondly, ovarian stimulation protocols in IVF inevitably lead to extremely high estradiol levels, which may be dangerous for women with estrogen-sensitive tumors such as breast cancer. The typical time interval between breast cancer surgery and initiation of chemotherapy is six weeks. In lieu of the standard protocol, a short flare protocol is adopted, which usually requires less time to achieve follicle recruitment [63]. Conventional controlled ovarian hyperstimulation (COH) produces exceptionally high levels of estrogen, potentially affecting the overall prognosis [64]. Therefore, some centers offer unstimulated natural-cycle IVF, where a single oocyte is aspirated. Cancellation rates are, however, high, and pregnancy rates very low (7.2% per cycle and 15.8% per embryo transfer) [65, 66].

The nonsteroidal antiestrogen tamoxifen [66] and the aromatase P450 inhibitor letrozole [67] were used as potential alternatives for ovulation induction in the breast cancer patient. Oktay and colleagues reported, in their prospective controlled study comparing tamoxifen to letrozole as ovarian stimulation agents in breast cancer patients, that when combined with low-dose FSH, both drugs had a better outcome than when tamoxifen was used alone. Letrozole was preferred to tamoxifen because its use was accompanied with significantly lower peak serum estradiol levels, which is a potential asset for breast cancer patients [67]. They further compared the safety and success of ovarian stimulation with letrozole and tamoxifen in breast cancer patients undergoing IVF to cryopreserve their embryos for fertility preservation and reported similar cancer recurrence rates between those who underwent COH and those who did not [68].

CRYOPRESERVATION AND OVARIAN TISSUE Transplantation

Ovarian tissue cryopreservation and transplantation have emerged as promising experimental fertility preservation procedures in women at risk of losing their reproductive capacity. Given the fact that ovarian tissue transplantation is an autotransplantation rather than transplantation between two genetically distinct humans, immunosuppression is unnecessary. In contrast, long-term immunosuppression is required after transplantation of reproductive organs between genetically distinct humans. Cryopreservation of ovarian tissue is, therefore, an indispensable prerequisite to the potential autotransplantation process, allowing preserved germ cells to be replaced after completion of the ovarotoxic cancer therapy. Owing to their smaller size and lack of follicular fluid, better survival is expected to occur with primordial follicles occupying the ovarian cortical strips [69].

Animal models have provided invaluable information about transfer methods. Sheep ovaries, which resemble human ovaries, have been used as a model for ovarian tissue cryopreservation and transplantation by Gosden and colleagues [70]. Survival and endocrine activity of the follicular apparatus as well as pregnancy and delivery after transplantation of cryopreserved-thawed ovarian cortical strips have been successfully reported [70, 71].

MINIMIZING ISCHEMIC DAMAGE IN OVARIAN TISSUE TRANSPLANTATION

In some human ovarian transplants where nonvascularized grafts were used, an initial ischemic insult was noted, which was postulated to be, at least in part, responsible for the limited lifespan of ovarian function. Therefore, it is vital to reduce the duration of ischemia and provide immediate revascularization of the grafts to prolong their longevity and
help preserve their function for as long a duration as possible. To minimize ischemic damage of the grafts, Almodin and associates described a novel technique in which frozen-thawed fragments of one ovary were injected into the cortex of the remaining sterile ovary. In that experimental trial, radiotherapy was administered to ewes to induce ovarian failure in one ovary while fragments of the other ovary were kept frozen. Consequently, injection of the thawed fragments of the frozen ovary inside the cortex of the remaining irradiated ovary was carried out adopting a ‘‘sowing’’ procedure, which did require suturing. Rams were used to impregnate the ewes six months after grafting. By this novel procedure, they documented that prevention of ischemic damage was successful via intracortical grafting of the germinative ovarian tissue [72]. Transplantation of ovarian tissue into angiogenic granulation tissue during the process of wound healing was improvised to help prevent the initial ischemic insult to oocytes. The duration of ischemia was reduced by 24 hours thereby significantly increasing the healthy primordial follicular pool and the reperfused area of the transplanted grafts. Two days after transplantation, the graft became revascularized (functional blood vessels were seen) and functional integrity returned [73].

**INTRODUCTION OF VASCULAR GRAFTS IN ANIMAL MODELS**

As a potential way of avoiding ischemic damage to the graft, vascularized grafts have been successfully developed. With these grafts, there is immediate revascularization of the grafted ovarian tissue. The principle of vascularized grafting was challenging in two distinctive perspectives, despite its theoretical plausibility. First, the whole ovary along with its vascular pedicle must be cryopreserved, which is a technically demanding procedure. Reproductive surgeons are, therefore, required to be skillful at microvascular anastomosis; experience can readily be obtained from vascular and plastic surgeons with their vast experience in microsurgery on small vessels.

The ‘‘ischemia time’’ is defined as the duration of time in which the grafted ovarian tissue can withstand ischemia without resultant ischemic tissue damage. Large ovarian cortical strips were reported to withstand an ischemic insult for variable durations [74] without inducing significant histological or molecular changes [75]. Transplanting an intact fresh ovary together with its vascular pedicle using microvascular reanastomosis has been successfully performed. As with other tissue grafting procedures, if the vascular graft was technically feasible and successful, survival of the ovarian graft was assured [76].

Preservation of ovarian function after grafting is all important. Restoration of ovarian function after transplantation of a cryopreserved intact sheep ovary with its vascular pedicle has been documented [77]. Consequently, transplantation of fresh as well as cryopreserved-thawed (C-T) intact ovaries to heterotopic sites was established as a relatively simple, easily accessible, and technically feasible procedure with a short operative time [77]. Moreover, the intricate procedure of orthotopic autotransplantation of an intact frozen-thawed ovary to the upper genital tract using conventional microvascular principles has also been documented in rats [78].

A number of different techniques of autotransplantation of an intact fresh or frozen-thawed ovary together with its vascular pedicle using microvascular anastomosis in animal models were recently published. Special attention was directed towards identifying potential heterotopic locations containing adequate blood vessels that can be used to host and vascularize human ovarian grafts [79]. Moving one step further, contralateral orthotopic autotransplantation of cryopreserved whole ovaries with microanastomosis of the ovarian vascular pedicle has been successfully performed with very promising results, emphasizing the feasibility, practicality and reproducibility of that principle. Intriguingly, luteal function was demonstrated in four sheep, and delivery of a healthy lamb less than two years after transplantation following spontaneous intercourse has recently been reported. Unfortunately, histological verification of ovarian tissue 18 to 19 months after transplantation has shown that the life span of the graft was short, and the follicular survival rate in the grafted ovaries was only 1.7% to 7.6% [80]. Despite a functioning vascular anastomosis and fertility, the grafts still had a limited survival that could only be maintained for a finite length of time. Apart from decreased graft longevity, the concept of whole organ cryopreservation became established as a promising new popular fertility preserving option. In the same context, recent models for cryopreservation of an intact uterus [81] as well as a whole rabbit ovary have been documented [82].

**THE USE OF VASCULARIZED GRAFTS IN HUMAN TRIALS**

A number of human trials using cryopreserved autotransplanted ovarian cortical strips have been carried out, based on previous successes with animal models using C-T ovarian cortical strips with reported follicular survival, preservation of endocrine function as well as restoration of fertility [70, 71].

Many techniques can be adopted to make use of the cryopreserved ovarian tissue: transplantation back into the host; *in vitro* maturation (IVM) of primordial follicles, and xenografting into a host animal. Considering the first principle, cryopreserved ovarian tissue may be transplanted back into patient, with the obvious limitation of reintroducing cancer in malignancies known to preferentially involve the ovaries such as leukemias and possibly breast cancer. Ovarian tissue strips are removed from the women and kept frozen in small strips prior to chemotherapy using novel techniques. When pregnancy is desired, the ovarian tissue strips are retransplanted into the patient in an orthotopic or heterotopic site. Given the fact that such ovarian tissue grafts are avascular, any ischemic insult to the transplanted tissue would invariably result in irreversible loss of the whole growing follicle population together with a significant number of primordial follicles.

Three different surgical techniques of transplanting ovarian cortical strips have been improvised by Oktay and associates [83]. Those are orthotopic transplantation into the pelvis and heterotopic transplantation either into the arm or abdominal wall. In one study, the orthotopic transplant stopped
functioning nine months after transplantation [83]. In the heterotopic transplant, however, function was preserved and resulted in the generation of a four-cell stage embryo that was transferred without pregnancy occurring. Multiple ovarian stimulation cycles were undertaken before this one embryo was obtained [84].

A 32-year-old woman from Belgium treated for Hodgkin’s lymphoma was reportedly the first lady to give birth to a baby after successful orthotopic autotransplantation of cryopreserved ovarian tissue obtained prior to the start of ovarioxic chemotherapy. Despite developing chemotherapy-induced POF, the reimplantation of her ovarian tissue was successful in resuming ovulatory activity five months later. She became pregnant 11 months after retransplantation by natural fertilization and gave birth to a healthy baby 7 years after ovarian tissue banking [85].

Another pregnancy was reported in a woman with non-Hodgkin’s lymphoma in which a refined IVF stimulation protocol was used following orthotopic autotransplantation of cryopreserved-thawed ovarian cortical strips [86]. Some critics were doubtful as to the exact site of origin of the oocytes that resulted in the two pregnancies in the abovementioned reports. The reason for such skepticism was based on reports of documented spontaneous pregnancies in women with POF after repeated courses of ovarioxic radiotherapy and/or chemotherapy and the fact that the tissue transplanted to the native ovary does not rule out the chance of resumption of native ovarian activity [24].

A recognized complication of ovarian tissue cryopreservation and transplantation is loss of a considerable part of the follicular apparatus during the initial post-transplantation ischemic insult, which can seriously limit its use. After transplantation, almost 70% of follicles are lost. Freezing is not the major cause of such follicular loss, though [70, 87, 88]. Therefore, ovarian tissue freezing has been recommended only for women under the age of 35 [83]. Proposed sites of ovarian tissue transplantation with and without vascular anastomosis were reported previously by us and others.

HETEROLOGOUS OVARIAN TRANSPLANTATION

Silber and colleagues reported on the success of ovarian transplantation from a healthy fertile 24-year-old woman to her monozygotic twin sister, who had suffered from POF at the young age of 14. Monozygotic twins with discordant ovarian function have provided the basis of this work. Via minilaparotomy, ovarian cortical tissue was transplanted from the fertile sister to her sterile twin sister. Resumption of normal menstrual cyclicity was witnessed three months after transplantation, with a consequent drop of serum gonadotropin levels back to normal levels. During the second cycle, she conceived, and the pregnancy progressed uneventfully until delivery of a healthy female baby at 38 weeks’ gestation [89]. Such an outstanding success only helps to emphasize the concept of transplanting large segments of ovarian tissue between monozygotic twins without the need for immunosuppression.

The same group recently reported on ovarian cortex transplantation in a cohort of seven sets of twins discordant for POF in which ovarian cortical tissue was transplanted from the twin with normal ovarian function to the sister with POF. Similar to their previous reports, folliculogenesis, hormonal functions, and menses were restored in all recipients. Five spontaneous pregnancies were documented in this cohort [90, 91]. Donnez and associates [92] have recently reported on a successful ovarian allograft between two non-identical twins. Ovarian cortical tissue from the donor sister who had already been the donor of bone marrow for a transplant was used. The recipient had received chemotherapy, total body radiation, and bone marrow transplantation. The recipient developed spontaneous cycles after receiving the transplant. Moreover, two oocytes and two embryos were obtained [90, 92]. However, in most patients with an intact immune system, the potential for acute graft rejection and risks of long-term immunosuppressive complications in the mother such as infection and obstetrical complications may seriously limit its use [90]. Following documented ovarian function in a small number of cases following both orthotopic and heterotopic transplantation of thawed ovarian cortical strips, the ASRM practice committee recommended that the procedures of ovarian tissue cryopreservation or transplantation are to be regarded as experimental and should be performed only under IRB surveillance [57].

Del Priore et al. performed uterine extirpation during a multiorgan retrieval from a cadaver and demonstrated the technical feasibility in eight donors. A number of vascular pedicles were utilized including the ovarian, uterine, or internal iliac vessels. Serial histology sections throughout the period of cold ischemia, taken every 15 to 30 minutes, showed no significant change over 12 hours of cold ischemia. They concluded that the human uterus can be obtained from local organ donor networks using existing protocols [93]. Since a multitude of methodological and technical difficulties are associated with this procedure, the technique of human uterus transplantation is more experimental than practical at this point.

IN VITRO MATURATION OF OOCYTES (IVM)

The rationale behind in vitro maturation (IVM) is the fact the primordial follicles obtained from the frozen-thawed ovarian cortical strips can be allowed to mature in vitro. However, this procedure is still under investigation and will only become available in the future. In severe combined immune deficiency mice (SCID), transplantation studies demonstrated follicular maturation and completion of meiosis I in preparation for ovulation and potential fertilization [94]. Concerns have been raised pertaining to technical problems and potential viral infection transmission. To avoid spreading malignant cells, ovarian tissue culture with in vitro follicle maturation can be performed. Isolated follicle culture from the primordial stage has been tried, given the fact that the primordial cells represent >90% of the total follicular pool and by virtue of their ability to withstand cryoinjury [95]. However, isolated primordial follicles do not mature properly in culture [96]. Originally described for patients with polycystic ovary syndrome (PCOS), IVM of antral follicles may have applications for cancer patients. Unfortunately, its implementation is still being studied. IVM of oocytes could be considered a fertility preservation strategy as well; it is a safe and effective treatment offered in some
fertility centers for assisted reproduction. Potential advantages include avoidance of ovarian stimulation with expensive, and at times dangerous, gonadotropins, side effects of the medications, and risks such as ovarian hyperstimulation syndrome (OHSS).

Although primary candidates for IVM of oocytes have classically been PCOS patients with multiple antral follicles, the spectrum of IVM indications is expanding to include women with primarily poor-quality embryos in repeated cycles and poor responders to stimulation. Two new applications for IVM, especially in women with cancer who are undergoing ovariotoxic therapy, are oocyte donation and fertility preservation. It is combined with oocyte vitrification in younger women without partners needing this treatment for fertility preservation. Clinical pregnancy rates per cycle in women choosing IVM are related to their age: approximately 38% for infertility treatment up to the age of 30 and around 50% in recipients of IVM egg donation.

The issue of clinical applicability and practical value of IVM as a fertility preservation strategy is still unsettled [97,98]. Xu et al. improvised a method using tissue engineering principles for the culture of immature ovarian follicles followed by fertilization of oocytes in vitro [99]. This methodology is a great step forward in the search for new fertility preservation options in female cancer patients [99-101]. An additional strategy of fertility preservation, which combines ovarian tissue cryobanking with retrieval of immature oocytes from excised ovarian tissue, followed by IVM and vitrification has recently been described [102]. Recently, the first healthy baby was delivered following retrieval of immature oocytes in a natural menstrual cycle, followed by IVM and cryopreservation of the oocytes by vitrification [103]. This provides proof-of-principle evidence that the novel fertility preservation strategy of immature oocyte retrieval, IVM, and vitrification of oocytes can lead to a successful pregnancy and healthy live birth [103].

EXPERT COMMENTARY

Most of the currently available strategies to preserve fertility in women are still experimental and do not guarantee subsequent fertility. The only established method in women is IVF with embryo cryopreservation prior to cancer therapy. Other proposed strategies to preserve fertility in women with cancer include: [1] storage of frozen embryos, [2] storage of frozen ovarian tissue or the whole ovary for future transplantation, [3] storage of isolated follicles for in vitro growth and maturation, [4] and ovarian transposition before radiotherapy. The effectiveness of ovarian protection during chemotheraphy with GnRH analogs is yet to be shown.

FIVE-YEAR VIEW

Development of more successful strategies for fertility preservation among cancer survivors remains a challenging problem. Currently, ovarian tissue cryopreservation and transplantation are considered experimental. Future research recruiting larger numbers of patients may help determine whether acceptable longevity can be achieved with both pelvic and forearm ovarian cortical transplant procedures and whether fertility can be reliably restored. Research should focus on better defining patient suitability, methods of tissue collection, optimal tissue size, choice of cryoprotectants and cryopreservation protocols, and possible IVM of oocytes for human ovarian tissue.

In addition, research is needed to enhance the revascularization process with the goal of minimizing the follicular loss that occurs after tissue grafting. Appropriate current experimental indications primarily focus on providing an alternative for women who will immediately face near term medical therapies that clearly threaten their future fertility [57]. In humans, it is essential to generate active research to fill the currently available gaps and to optimize the results of cryopreservation and ovarian tissue transplantation. Of particular importance is the ideal transplantation site, which should be easily accessible via a simple and minimally invasive surgical procedure. Graft establishment, survival, and long-term function depend on adequate perfusion ensured by a generous blood supply to the recipient site. Development of new cryopreservation strategies, novel cryoprotectants, and new transplantation techniques tailored to decrease ischemia, particularly the use of vascularized grafts, are needed [76-78].

In IVM, future research is necessary to define the prerequisites of adequate follicular growth and maturation and to decipher the exact role of supporting theca and granulosa cells in this process.

KEY ISSUES

1. There is increasing need for fertility preservation in women for a number of benign conditions and malignant diseases.
2. Exposure to ovariotoxic chemotherapy and/or radiotherapy can seriously jeopardize future female fertility.
3. Despite the multiplicity of potential fertility preservation strategies, only few are of practical significance and the majority remains experimental.
4. Only in vitro fertilization followed by embryo cryopreservation is considered a clinically proven option for women requiring cancer treatment.
5. There is generally insufficient time available to allow for ovarian stimulation, oocyte retrieval, and embryo freezing for women who are scheduled to receive ovariotoxic chemotherapy and/or radiotherapy.
6. Despite being largely experimental, ovarian tissue cryopreservation and oocyte cryopreservation are promising avenues for future fertility preservation in women.
7. In a small number of cases, ovarian function has been reported following both orthotopic and heterotopic transplantation of thawed ovarian cortical strips.
8. Ovarian tissue cryopreservation and transplantation and oocyte cryopreservation are to be envisaged as experimental procedures that can only be performed under institutional review board (IRB) guidance.
9. In IVF, improved oocyte survival, oocyte fertilization, and pregnancy rates from frozen-thawed oocytes...
are the result of dramatic achievements in various laboratory techniques.

10. To date, no increase in chromosomal abnormalities, birth defects, or developmental defects was witnessed in pregnancies resulting from cryopreserved oocytes.

11. Fertility preservation is a new application for IVM, especially in women with cancer who are undergoing ovarioctomy therapy. Tissue engineering principles for the culture of immature ovarian follicles followed by fertilization of oocytes in vitro is an emerging new technology for fertility preservation in female cancer patients.

REFERENCES


Emerging Technologies for Fertility Preservation in Female Patients


