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 trophictoderm 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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<th>Option</th>
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<td>Blastocyst transfer</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 trophoderm 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 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 in vitro 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 Tyrosides’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 (FAEAC), 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 grainy 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]. Eyheremendy 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) PGD 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 PGD 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-
motions (i.e. septate uterus) that may be associated with infertility. Studies have shown the procedure to be more sensitive, specific and accurate than pelvic ultrasound in evaluating uterine pathology for patients with RIF [65]. Currently, hysteroscopy has not been adopted as part of the routine workup for infertility but rather as a secondary investigative measure to evaluate patients after three or more implantation failures [66, 67].

Women with RIF have been shown to have a higher incidence of uterine pathologies. In the most recent observational study of 1475 women with RIF, an abnormality was found in the uterus of 36.6% of these women (16.7% endometrial polyps, 12.5% endometrial adhesions, 1.5% endocervical adhesions, 4.3% endometritis, 0.9% uterine septa, and 0.8% submucous fibroids) [68]. The study reported that 22.2% of the population had a prior ultrasound screening with a false negative result and subsequent hysteroscopy intruterine pathology (endometrial adhesions in 12.5%, endometritis in 4.3%, endometrial polyps in 3.3%, endocervical adhesions in 1.5%, uterine septa in 0.5%, and submucous myomas in 0.1%). The same study also compared the use and non-use of hysteroscopy for women with RIF in a new IVF attempt and showed a significant increase in the implantation rate and pregnancy rate in the former group [68]. It strongly suggests the use of hysteroscopy for women after two failed IVF attempts for two reasons: (i) a significant number of uterine pathologies are undetected by ultrasound and (ii) the significant success rate of ongoing pregnancy after hysteroscopy [68]. For women with endometrial polyps, a hysteroscopic polypectomy (surgical removal of polyps) may be more efficacious than non-intervening hysteroscopy [69].

A novel approach to the use hysteroscopy for evaluating endometrial cell integrity has been described [70]. The technique involves the application of chemical agents to highlight cellular-level or mucosal anomalies of the endometrium (chromohysteroscopy). Preliminary studies on a small patient population have shown promising results for women with RIF [70].

SILDENAFIL (VIAGRA)

Sildenafil (Viagra®, Pfizer, New York, N.Y.), most notable for the treatment of male erectile dysfunction, is a vasodilator used to improve vascular supply [71]. Endometrial receptivity is believed to improve with increased blood flow in the uterine arteries of women with thin endometrium to increase the thickness of the endometrium, the portion of the uterus associated with successful implantation. Both the quality and quantity of endometrial tissue are important to consider in a strategy aimed at improving the endometrial environment for a successful pregnancy.

A recent case control study by Goodman et al. investigated three gene polymorphisms that have been linked to patients with repeat implantation failures in prior individual studies [72]. Successful implantation depends on the blastocyst’s ability to infiltrate the endometrium and develop a sustaining blood supply, which requires the following genes to produce the necessary proteins for digesting the 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 [74]. 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 (dilatation 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 salpingectomy 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 distention is less marked and the amount of invisible intralumenal 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

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**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.

### Table 4. Recommended Limits on the Numbers of Embryos to Transfer

<table>
<thead>
<tr>
<th>Prognosis</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 37 yrs</td>
</tr>
<tr>
<td>Cleavage-stage embryos</td>
<td></td>
</tr>
<tr>
<td>Favorable*</td>
<td>1-2</td>
</tr>
<tr>
<td>All others</td>
<td>2</td>
</tr>
<tr>
<td>Blastocysts</td>
<td></td>
</tr>
<tr>
<td>Favorable*</td>
<td>1</td>
</tr>
<tr>
<td>All others</td>
<td>2</td>
</tr>
</tbody>
</table>

*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 phospholipidosis, 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 antiphospholipid 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 births 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 allogenic 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 fetomaternal 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 birth rates [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 has been 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-
received 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/ETFT, 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/ETFT, 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.
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