Assessment and treatment of repeated implantation failure (RIF)

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Abstract Repeated implantation failure (RIF) is determined when embryos of good quality fail to implant following several in vitro fertilization (IVF) treatment cycles. Implantation failure is related to either maternal factors or embryonic causes. Maternal factors include uterine anatomic abnormalities, thrombophilia, non-receptive endometrium and immunological factors. Failure of implantation due to embryonic causes is associated with either genetic abnormalities or other factors intrinsic to the embryo that impair its ability to develop in utero, to hatch and to implant. New methods of time-lapse imaging of embryos and assessment of their metabolic functions may improve selection of embryos for transfer, and subsequent outcomes for IVF patients, as well as for those diagnosed with RIF. This review discusses the various causes associated with RIF and addresses appropriate treatments.

Keywords Implantation · Recurrent implantation failure · Repeated implantation failure · RIF · In vitro fertilization · IVF failure

Introduction

Repeated implantation failure (RIF) is determined when transferred embryos fail to implant following several in vitro

fertilization (IVF) treatment cycles. However, there are no formal criteria defining the number of failed cycles or the total number of embryos transferred in these IVF attempts. Accordingly, different fertility centers practicing IVF may use different definitions for RIF [93]. Considering the current success rate of IVF treatments and the mean number of embryos transferred in each cycle, we recommend defining RIF as failure of implantation in at least three consecutive IVF attempts, in which 1–2 embryos of high grade quality are transferred in each cycle.

The process of implantation involves two main components, a healthy embryo that should have the potential to implant and a receptive endometrium that should enable implantation. The “cross-talk” between the embryo and the endometrium that finally leads to apposition, attachment and invasion of embryos is mandatory for successful implantation and subsequent normal placentation. These processes are under thorough investigation and seem to involve many mediators originating in the embryo, as well as in the endometrium, and to also involve the maternal immunologic system [1, 37, 42]. Any abnormality attributed to the embryo, the endometrium or the immune system will result in implantation failure. Therefore, in assessing RIF, the embryo should be evaluated, with reference to the uterus and its functional endometrium. Accordingly, treatment of RIF should be targeted to the abnormality detected, and the correction of any potential malfunction that might contribute to the failure of implantation [61, 93].

Figure 1 represents a clinical approach for assessing and treating RIF. We set out to investigate maternal factors (including, but not limited to, factors relating to the endometrium) and embryonic factors that contribute to RIF. Treatment should be targeted according to the maternal or embryonic factors that are present, or that are considered to be potentially associated with RIF.

Capsule Successful implantation depends on well functioning endometrium and healthy embryo. An abnormality in one or both these variables can cause repeat implantation failure. The condition should be assessed for its different causes and be treated accordingly.

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Fig. 1 Evaluation and treatment of repeated implantation failure (RIF)

Maternal factors

Uterine anatomy

The first step in evaluation should include assessment of the uterus and the integrity of the uterine cavity. Anatomical malformations of the uterus, either congenital (septate uterus and bicornuate uterus) or acquired (uterine fibroids, especially submucous type, endometrial polyps, intrauterine adhesions and hydrosalpinx), may interfere with normal implantation [76, 84, 101]. When possible, the intrauterine abnormality should be corrected [30] and hydrosalpinx should be removed to enhance implantation [76].

Thrombophilia

Although the thrombophilic state, either inherited or acquired, has been mainly associated with repeated pregnancy loss, several investigators have reported an association between RIF and a tendency for hypercoagulable conditions [7, 43]. It is assumed that the mechanism of implantation failure is similar to that of pregnancy loss, namely, disturbed blood flow to the endometrium and placenta. Disturbed blood flow can, on one hand, hamper normal endometrial receptivity and, on the other, cause miscarriage. For patients with RIF, diagnosed with thrombophilia, treatment with heparin has been shown to significantly improve implantation, as well as the clinical pregnancy rate in subsequent IVF

attempts [16, 78]. Still, the association between the hypercoagulable state and RIF is still debatable, since other investigators did not find a higher prevalence of thrombophilia in patients with RIF compared to controls [62, 96]. The exact mechanism of such association, if it exists, is thus uncertain. Nevertheless, when summarizing all studies focusing on gene polymorphisms and the thrombophilia trait, it seems that prothrombotic disorders are more prevalent in RIF patients than in controls [106].

While patients with RIF who have prothrombotic disorder might benefit from heparin treatment, for those without this abnormality empiric treatment with heparin is not justifiable [13, 87, 108]. Altogether, it is recommended that patients diagnosed with RIF be investigated for acquired as well as hereditary thrombophilia disorders (Fig. 1), and be treated accordingly.

Immunological factors

A number of studies indicate a major role for the immunologic system in the process of implantation, and in the subsequent maintenance of pregnancy [21, 89, 97].

A conception must be recognized as non-self in order to trigger an immunologic process that prevents the maternal immune system from rejecting it. The HLA compatibility system evidently plays a role in this recognition. Couples who share common HLA alleles may experience recurrent pregnancy loss [20], or as suggested by Elram et al. [34], may suffer from RIF. The exact molecular mechanism to explain why shared HLA alleles among couples are associated with recurrent pregnancy loss or RIF is still obscure. However, inadequate response of the maternal immune system to stimulation by paternal antigens, due to HLA sharing, has been implicated. Such inadequate response may involve the imbalance of T helper 1: T helper 2 (TH1:TH2) response, causing the maternal system to be more cytotoxic, or reducing expression of the less polymorphic HLA-E, HLA-F and HLA-G, which displays immunoregulatory function associated with maternal tolerance to the fetus [28].

Treatment with paternal leukocytes immunization, as previously suggested [20], is no longer recommended because of the possible side effects to the mother and the fetus due to an unpredicted immune response to either autologous or allogenic blood components [19, 73, 103]. On the other hand, high dose IVIg administration carries less risk and was found to benefit patients with RIF who share HLA alleles with their partner [34]. Though the number of shared alleles justifying administration of IVIg treatment has not been determined, we have successfully treated patients with as few as one shared allele. Treatment consists of 30 g of IVIg before embryo transfer (ET) and a second similar dose when a fetal heart rate is noticed [34].

IVIg treatment is mainly administered for recurrent pregnancy loss, defined as unexplained, or as associated with shared HLA alleles between the couple. The benefit of such treatment is still questionable, and the data are conflicting [4, 24]. Elram et al. [34] were the first to suggest that patients with RIF and HLA allele sharing can benefit from IVIg treatment. Nevertheless, due to the high costs of both HLA testing and IVIg treatment, we suggest that the assessment of the immune system contribution to RIF be carried out last, and only after all other causes are investigated, treated or ruled out.

The infusion of 20 % intralipid solution was recently reported to improve outcomes in women with RIF [68]. In a non-randomized trial, presented at a scientific meeting in the United Kingdom [68], a 50 % pregnancy rate and 46 % clinical pregnancy rate were achieved in patients with RIF who had an elevated TH1 cytokine response. Intralipid infusion was administered once between days 4 and 9 of the ovarian stimulation, and again within 7 days of a positive pregnancy test. In all cases ($n=50$), the TH1:TH2 activity ratio was decreased following treatment with intralipid. This cytokine activity alteration was considered responsible for the successful outcome that resulted. Routinely used for parenteral nutrition, intralipid is a fat emulsion that was reported to suppress abnormal NK cytotoxic activity in peripheral NK cells from women with recurrent reproductive failure both in vitro and in vivo [81]. Roussev et al. [81] have shown that infusion of 2–4 ml of 20 % intralipid solution dilutes in 250 ml of saline can effectively suppress natural killer (NK) cell activity in patients with abnormal NK cytolytic activity that is higher than 11 %. This modulation of the immune system achieved by a single dose of intralipid administration and lasting for several weeks is the basis for intralipid treatment in patients with RIF in which increased cytotoxic NK activity was found. The exact mechanism by which intralipid modulates the immune system is still unclear. Nevertheless, it has been hypothesized that fatty acids within the emulsion serve as ligands to activate peroxisome proliferator-activated receptors (PPARs) expressed by the NK cells. Activation of such nuclear receptors has been shown to decrease NK cytotoxic activity, which can consequently enhance implantation and maintain pregnancy [81]. The possibility that intralipid can modulate the immune system to positively affect implantation and the maintenance of pregnancy, is promising. However, after assessing the relevant available data, Shreeve and Sadek [92] concluded that these findings should be considered cautiously, and that large-scale confirmatory studies are necessary to prove the efficacy of intralipid before it should be recommended for routine use. Furthermore, many IVF programs, including ours, perform egg retrieval by general anaesthesia, applying propofol as a short acting hypnotic agent. Propofol solution contains the same components of intralipid emulsion (10 % soybean oil, and 1.2 % purified

egg phospholipid, with 2.25 % glycerol), supplemented with 1 % propofol. Although propofol is widely used, and in volume doses that are higher than those suggested for the treatment of RIF, the problem of unexplained RIF remains an issue to be addressed.

The endometrium

A functioning and receptive endometrium is crucial for embryo implantation. During the menstrual cycle the endometrium undergoes both morphologic and biologic changes that prepare it for interaction with the embryo, and ultimately for successful implantation. Once all biological changes transpire, the embryo can attach, invade the endometrium and finally implant. This crucial stage lasts for a few days and is referred to as the “window of implantation”. For occurrence of the “window of implantation”, the endometrium must proliferate, increase in thickness and then, after ovulation, adequately respond to progesterone and become receptive. Ultrasound examination of the thickness and appearance of the endometrium is an easily performed means of assessing morphological changes occurring in the endometrium during the follicular phase, and is thus used as a measure to predict successful implantation. Indeed, several studies have reported a strong association between endometrial thickness and successful implantation [12, 23, 32, 41, 69, 91]. However, others failed to confirm such an association [39, 53, 71, 115]. The minimal adequate endometrial thickness for successful implantation, as measured in the late proliferative phase, varies between studies, with a range of 6–8 mm. However, although rare, some investigators have reported successful implantation in an endometrium of no more than 5 mm thickness [33, 102].

Thin and unresponsive endometrium, especially following surgical interventions in the uterine cavity [94], is difficult to treat and obviously contributes to implantation failure. Several approaches have been implemented to increase endometrial thickness, and presumably prepare it for the “window of implantation”. Treatment with high dose oral estrogen or vaginal estradiol application, intended to increase the estradiol level in the serum, as well as in the vicinity of the endometrium, has demonstrated only marginal success [22, 107]. Similarly, treatment with low dose aspirin [114] or vaginal sildenafil [90], which presumably increases blood flow to the uterus, consequently improving the response to estradiol, were rather disappointing [86]. Our approach is to freeze embryos when a thin endometrium is noticed, and to transfer them in a natural cycle, if possible; or alternatively, in an artificial cycle, while applying increased dosages of estradiol, for as long as three weeks before progesterone is added. Once progesterone is added, estradiol treatment dose can be lowered, or switched again from the vaginal to the oral route.

Nevertheless, once all modes of treatment fail, surrogacy should be offered as an option.

As previously mentioned, the “window of implantation” is characterized by morphological and histological changes of the endometrium that are associated with intense biological activity originating in the maternal immune system, the endometrium and the embryo itself. The cross-talk between the endometrium and the developing embryo is mediated by many substances, including cytokines (IL1, IL6 and its product LIF), integrins, adhesion molecules, metallo proteins, growth factors, prostaglandins and hormones such as hCG, all of which support the process of apposition, adhesion and invasion [1].

Exploration of stages of the complex process of implantation has inspired researchers to use some of the mediators to facilitate implantation. When Simon et al. [95] used hyaluronic acid in the transfer media to replace albumin as a macromolecule, they found no increase in pregnancy rate. Similarly, the same group [11] reported comparable pregnancy rates between patients injected with hCG during the secretory phase in a hormonally modulated cycle of frozen thawed embryo transfer, and untreated patients. Aware that endometrial secretion of LIF might be low in patients with RIF [46], other investigators tested the systemic administration of LIF to patients with RIF. A multicenter, randomized, double-blinded, placebo-controlled study failed to demonstrate that r-hLIF administration after embryo transfer improved implantation and pregnancy rates compared with a placebo [17]. However, since in the latter studies, both hCG and LIF were administered systemically, their direct local effect on the endometrium is not clear. On the other hand, when the uterine cavity was flushed with medium supplemented with hCG just before embryo transfer, the pregnancy rate as well as implantation rate were increased as compared to controls [60]. Although promising, it is yet to be determined whether local delivery before embryo transfer, of substances involved in the implantation process, increases implantation efficiency. Gleicher et al. [38] suggest that local delivery of various mediators by flushing the uterine cavity may be of real benefit to RIF patients. They used endometrial perfusion with granulocyte colony-stimulating factor (G-CSF) to induce thickening of the endometrium. Successful expansion of endometrial thickness to at least 7 mm after uterine perfusion with G-CSF was observed in four patients previously resistant to treatment with estrogen and vasodilators. The fact that all four patients conceived, though one pregnancy required termination, due to intramural corneal ectopic location, suggests that endometrial perfusion with G-CSF may improve implantation.

In a separate study, G-CSF administered systemically to patients with RIF benefitted only patients with deficiencies in three critical activating killer-cell immunoglobulin-like receptor (KIR) genes [119]. In those patients, interaction

between the embryonic trophoblast, through its HLA-C expression and the natural uterine killer (uNK) cells, was hampered, resulting in implantation failure or miscarriage. The impact of G-CSF on NK cells has not been totally elucidated. Recent data, however, suggest that G-CSF may be a strong inhibitor of NK cell activity [85]. Whether the effect of G-CSF on uNK cells depends on the presence or absence of specific NK cell receptors in RIF patients, remains to be explored [106].

Several investigators have suggested that patients with RIF may benefit from endometrial stimulation induced by local injury resulting from a uterine endometrial biopsy catheter in the cycle that precedes the actual treatment cycle [9, 58, 66, 80]. In a randomized controlled trial that included 100 patients with a diagnosis of RIF, Narvekar et al. [66] performed two endometrial samplings, one in the follicular phase and the other in the luteal phase of the cycle, antecedent to the embryo transfer cycle. The authors reported significant improvement in the clinical pregnancy rate (32.7 % vs 13.7 %), the live birth rate (22.4 % vs 9.8 %) and the implantation rate (13.07 % vs 7.1 %) in patients who underwent the intervention as compared to those who did not. The exact mechanism for such appreciative effect is not fully understood. However, it is postulated that performance of a biopsy induces an inflammatory response that may facilitate the preparation of the endometrium for implantation. Elevated proinflammatory cytokines, as well as increased MIP-1B expression in the regenerative endometrium, could play a role in implantation competence [40]. Interestingly, this beneficial inflammatory effect lasts at least one additional month, thus improving implantation in the subsequent cycle. The exact mechanism of such positive effect on implantation following endometrial sampling, as well as the number of such endometrial stimulations and their timing along the cycle preceding that of embryo transfer, still needs to be determined [67].

Embryonic factors

Genetics

The second player in the equation of successful implantation is the embryo. Implantation is in fact not an efficient process, as the natural conception rate, within timed intercourse in otherwise healthy young couples, is only about 20–25 % per cycle. Similarly, the average implantation rate in IVF is about 25 % [29]. Inadequate uterine receptivity, as discussed earlier, is responsible for approximately two-thirds of implantation failures, whereas the embryo itself is responsible for only one-third of these failures [1]. Abnormal karyotype of the embryo is one of the major reasons for failure of implantation and miscarriage. This random abnormality can

occur during early fertilization or meiosis, at the stage that the oocyte completes its first meiotic division. Nevertheless, both male and female partners may produce genetically aneuploid gametes if they are carriers of balanced translocation. Indeed, in that case, according to the chromatid segregation during the first meiotic division, about two thirds of the produced gametes will be genetically imbalanced and only one third will be balanced (either normal or balanced translocation). Thus, two thirds of the generated embryos following fertilization will be genetically abnormal and either fail to implant or be aborted. Women with RIF and their spouses should undergo karyotype testing to rule out balanced chromosomal translocations. When such an abnormality is detected the couple should be offered preimplantation genetic diagnosis (PGD).

Increased incidence of chromosomal translocations, mosaics, inversions and deletions of genetic material were reported in patients with RIF [79]. Moreover, almost twice as many chromosomal abnormalities were detected in embryos from RIF patients as in embryos of controls (67.4 % versus 36.3 %) [74]; and significantly more complex chromosomal abnormalities (which involve three or more chromosomes) were reported in cleaved embryos of RIF patients [112]. Thus, preimplantation genetic screening (PGS), in the presence of normal karyotype, has been suggested for these patients. However, though primary reports were encouraging, subsequent large controlled studies not only did not confirm the advantage of PGS for RIF patients, but also reported lower clinical pregnancy rates, compared to controls [113, 120]. Thus, a recommendation of PGS for cleaved embryos is currently not justified for patients with RIF with normal karyotype.

Zona pellucida

Hatching of the blastocyst through the zona pellucida (ZP) is an essential step preceding implantation. Hatching involves both mechanical and chemical reactions, which eventually lead to ZP thinning followed by its rupture and blastocyst hatching. Abnormalities of the hatching process may contribute to failed implantation in IVF attempts. This is supported by the early reports that observed a significant improvement in human embryo implantation rates when assisted hatching (artificial thinning or breaching of the ZP) was attempted [25, 26, 70, 100]. Many assisted reproductive technology (ART) programs have since incorporated the use of assisted hatching in efforts to improve clinical outcomes [47]. However, differences in patient populations, operator experience, hatching technique and study design make it difficult to compare findings from different centers. Based on a review of the literature, the American Society for Reproductive Medicine (ASRM) practice committee concluded that the published evidence currently available does not support the routine or universal application of assisted

hatching in all IVF cycles. Nonetheless, assisted hatching may be clinically useful in patients with a poor prognosis, including those with >2 failed IVF cycles and poor embryo quality, and in woman of an advanced age (≥ 38 years of age) [75]. Thus, at our medical centre, we routinely offer laser assisted hatching to women with RIF in whom we plan to transfer early cleaved embryos.

Embryo culture and transfer

The definition of RIF refers to the transfer of good quality embryos that do not result in implantation. Embryo quality is assessed by morphologic criteria, under the assumption that the embryos, although transferred on either day 2 or 3 after fertilization, would continue to develop in utero, reach the blastocyst stage and then implant. However, even embryos that are morphologically defined as good quality may cease to develop in utero and fail to progress into a blastocyst stage. This may be due to either suboptimal local conditions or intrinsic factors within the embryos. Several approaches have been suggested for overcoming these obstacles, among them zygote intra fallopian transfer (ZIFT), blastocyst transfer, sequential ET and embryo co-culture system.

Zygote intra fallopian transfer (ZIFT)

Transfer of embryos or zygotes directly into the fallopian tubes (ZIFT) seems more physiological than placing embryos of day 2–3 cleavage stage into the uterine cavity. When the ZIFT procedure is applied, the fertilized egg benefits from endosalpingeal secretions and reaches the endometrium as a blastocyst at a time that coincides more closely with the window of implantation. While in the general population, tubal versus uterine embryo transfer failed to show any advantage [45]; in patients with RIF, ZIFT has been reported superior to trans-cervical embryo transfer [57]. Levran et al. [57] reported significantly higher pregnancy and implantation rates when ZIFT was applied (34.2 % and 8.7 % respectively) compared to transcervical cleavage stage embryo transfer (17.1 % and 4.4 %, respectively). In a later publication, the same group of researchers reported pregnancy and implantation rates for ZIFT cycles in patients with RIF as 35.1 % and 11.1 %, respectively [36]. They concluded that ZIFT can be considered an effective a mode of treatment for patients with repeated failure of implantation in IVF-ET. However, the risks and complications of laparoscopy, due to the necessity of general anaesthesia one day after egg retrieval preclude its unrestricted use.

Blastocyst culture

Under the assumption that embryos that cease to develop in utero, result in implantation failure, culturing them

to the blastocyst stage will serve two goals. First, it will enable better selection of embryos for transfer, and second, it will promote more physiologic synchronization with the endometrium and capability of achieving the “window of implantation”. Currently, as stated above, morphologic criteria helps in the selection of embryos for transfer. However, in RIF patients, intrinsic factors may prevent some embryos, even ones with high morphologic scores, from cleaving normally. To overcome the problem of random selection of impaired embryos, more embryos can be transferred in patients with RIF, though the risk of multiple pregnancies must be acknowledged.

The transfer of embryos that are cultured to the blastocyst stage improves selection and decreases the risk of multiple pregnancies. Indeed, several studies have investigated the benefit of blastocyst transfer to women with RIF [27, 44, 56]. The study by Cruz et al. [27], although not randomized, showed significantly increased pregnancy and implantation rates for women who underwent blastocyst transfer ($N=15$), compared to those in which embryo transfer occurred on day 3 ($N=22$)(40 % vs 9.1 % and 11.3 % vs 3.4 %, respectively). In a larger, prospective, but again non-randomized study, that included 276 patients defined as having RIF, Guerif et al. [44] reported a higher live birth rate, as well as a higher implantation rate for patients who opted for day 5–6 blastocyst transfer compared to those in whom day 2 embryos were transferred. In a prospective randomized study, Levitas et al. [56] recruited 54 patients who failed to conceive in 2–3 cycles of cleavage stage embryo transfers and allocated them to undergo embryo transfer either on day 2–3 or day 5. A significantly higher implantation rate (21.2 % vs 6 %) was recorded for blastocyst transfers compared with day 2–3 transfers. Pregnancy rates per oocyte retrieval and per embryo transfer, although higher in the blastocyst group, did not reach statistical significance. The embryo transfer cancellation rate was also higher (26 % vs 6.4 %) in the blastocyst transfer group. The latter finding highlights the disadvantages of culturing embryos to the blastocyst stage; this approach is only suitable for patients who produce many embryos, and patients may end up without any embryos suitable for transfer.

To overcome these problems, several authors have suggested the use of sequential embryo transfer for patients with RIF [2, 59]. Almog et al. [2], as well as Loutradis et al. [59] reported increased pregnancy rates for sequential embryo transfer, compared to a control group in which a similar mean number of embryos were transferred. The mechanism for the greater success is not totally understood. The assumption, however, is that double transfer increases the chance of hitting the “window of implantation”, since timing may vary among patients according to the endometrial response to the steroidal hormones.

Co-culture conditions

Improved culture media and conditions have increased the efficiency of in vitro fertilization treatment. However, for women with RIF, the culture media routinely used might not be of sufficient quality for the production of healthy embryos with the potential to successfully implant. Apparently, for these women, intrinsic factors hinder implantation, even of high quality embryos. Co-culture of embryos on different types of feeder cell layers has been suggested as a means of improving culture conditions and subsequent implantation rates [109]. Possible benefits of co-cultures to the embryo include secretion of trophic factors such as nutrient substrates, growth factors and cytokines, and the removal of potentially toxic substances by feeder cell layers. Opinions as to the beneficial effect of co-culture for early embryonic development are conflicting [52]. While some authors reported improved outcome using co-culture [64, 116, 122], randomized controlled studies failed to demonstrate a significant benefit for such conditions in unselected patient populations undergoing IVF–ET [82, 110]. This incongruity emanates from several confounding variables, including differences among the studies in the cell lines and culture media used. In addition, most co-culture studies have been poorly controlled and retrospective in nature, which raises questions about their clinical validity. Nevertheless, in contrast to an unselected group, IVF fertilization cultivated by co-culture was found to be highly beneficial for patients with RIF [10, 35, 99]. Recently, in a non-randomized, prospective study in which women served as their own controls, Eyheremendy et al. [35] reported significantly higher pregnancy (51 % vs 5 %) and implantation rates per embryo transfer (23.7 % vs 2.1 %) for cycles applying co-culture with autologous endometrial cells, than cycles under routine culture conditions for the same patients. Although co-culture may benefit patients with RIF, unfortunately, most IVF programs do not have the equipment, facilities, and personnel required for routine use of the co-culture system.

Male contribution to embryo competence for implantation

A number of investigators have suggested that low sperm quality can decrease the success of assisted reproductive techniques (ART) due to abnormalities in chromatin arrangements and compactions in the sperm, as well as increased DNA fragmentation [77, 105]. DNA fragmentation was found to be associated with reduced natural conception, IUI outcome, and fertilization and pregnancy rates in ART cycles [18, 51, 54, 77, 111]. The performance of sperm cells of poor morphology is low and is correlated with a high rate of DNA fragmentation. It is reported that 28 % of the sperm cells of infertile men exhibit DNA

fragmentation, compared to only 13 % of those of fertile males [77]. The observation that poor morphologic sperm that exhibit a high rate of DNA fragmentation are associated with low rates of fertilization and implantation, and poor embryo quality, is not surprising. However, less expected is the observation of Avendaño et al. [6] who reported a high rate of DNA fragmentation in male partners of infertile couples although they exhibited normal sperm morphology. In a later study conducted by the same group [5] to include patients with sperm morphology within the normal range, the DNA fragmentation rate was higher in male partners of women who failed to conceive than in couples who achieved pregnancy (33.8 % vs 18.9 %). Though DNA fragmentation can affect embryo morphology, it is possible that the negative effect is only expressed when the paternal genome is activated (day 3 after fertilization). Thus, the embryo can be morphologically normal when transferred, but cease to progress beyond day 3 in utero. This scenario can occur in couples diagnosed with RIF. Male partners of women with RIF were indeed reported to have increased incidence of sperm chromosomal abnormalities [83], which can contribute to failure of implantation.

The group of Berkovitz et al. [14, 15] were the first to suggest that intracytoplasmic morphologically selected sperm injection (IMSI), using high magnification of $\times 6000$, can improve fertilization and pregnancy rates in ART cycles. Others have substantiated these findings and shown a significant and clear trend for better results in the IMSI group, particularly for ongoing pregnancy and live birth rates (both nearly doubled) and miscarriage (about 50 % reduced) [65, 98, 117]. Patients with poor sperm quality have been reported to benefit the most from the IMSI procedure [8, 88], and indeed, this procedure is mainly offered to couples with male factor infertility and embryos of poor morphological quality.

Male partners of patients with RIF may exhibit either normal sperm analysis or abnormal sperm parameters. Thus, the IMSI procedure should be assessed for its contribution to improve outcomes for this particular group of couples diagnosed with RIF. Routine sperm cell assessment defines normal spermatozoa using low magnification. However, some hidden anomalies can only be detected at higher magnification. By excluding sperm with higher probabilities of DNA fragmentation and abnormal chromatin condensation, sperm sorting for IMSI can yield embryos with a higher developmental capacity.

Two studies [104, 121] reported superiority of the IMSI procedure compared to regular ICSI in patients with previously repeated failure of ICSI cycles. However, both studies included patients with and without male factor infertility, thus making it difficult to adequately assess the real contribution of the IMSI procedure in cases where the male factor was primarily defined to be normal. In a recent study comparing

outcomes of IMSI and ICSI for RIF patients, Oliveira et al. [72] assessed the contribution of IMSI in a subgroup of patients with no male factor. Their results suggest that, compared to ICSI, IMSI does not significantly improve either laboratory or clinical outcomes. Nonetheless, they reported clear trends for lower rates of miscarriage (14.3 % vs 25 %) and higher ongoing pregnancy (19 % vs 10.9 %) and live birth rates (17.5 % vs 9.1 %) in the IMSI compared to the ICSI group. They concluded that the use of morphologically-selected spermatozoa with IMSI would benefit those with poor reproductive prognosis, though further confirmation in randomized large-scale trials is still needed.

Until the real benefit of IMSI for RIF is confirmed, we recommend for patients with RIF and normal sperm parameter assessment of sperm cells at higher magnification and consideration of their suitability for IMSI, rather than unrestricted implementation of the procedure.

It seems that patients who are diagnosed with RIF that is associated with male factor will benefit from the procedure, while the value of IMSI to RIF without male factor infertility still awaits confirmation.

Future considerations

Time-lapse imaging

Currently, in most IVF units, selection of embryos for transfer is based mainly on morphologic criteria of embryos on the day of transfer, and the timing of cell divisions of early cleaved embryos [63]. However, embryo morphology and timing of cell division are not sufficient criteria, since they cannot truly determine the molecular signature of a human embryo, or accurately predict its implantation potential. The current scoring system analyzes the fertilized egg and the embryo at a few predefined time points, thus, missing all the events that occurred between these points. Continuous image monitoring of the cultured embryo may provide a complete picture of the developmental kinetics that the embryo undergoes. Processing the data retrieved on the day of embryo transfer might help improve selection of embryos possessing the highest potential for implantation [55, 118]. Applying a time-lapse imaging system, Meseguer et al. [63] identified the morphokinetic parameters specific to embryos that were capable of implanting, and proposed a multivariable model to classify embryos according to their probability of implantation. Although the authors considered the model promising, the efficacy of time-lapse analysis has still to be evaluated in prospective randomized studies, to determine whether it can improve implantation rates. Patients with RIF might benefit from such an assessment, since routine embryo selection as currently applied may be deceiving, especially for this group of patients.

'Omics' technologies

Throughout its developmental stages, the embryo is constantly dynamic, changing its morphology upon cell divisions and activating transcription of its genome to produce proteins that are then exploited by the embryo. In culture, the developing embryo is metabolically active, utilizing both the substances it produces and those supplemented in the culture media, and secreting its metabolites to the adjacent vicinity.

In recent years, transcriptomic and proteomic, as well as metabolomic, approaches were developed to promote investigation of the expression of thousands of genes, proteins and other metabolites. Study of the 'omics' group has contributed to our understanding of the gene regulatory structure involved in the embryo implantation process. It is currently possible to assess nutrient uptake as well as the metabolic production of the embryo. Exploring appropriate gene activation and expression, either in the embryo [3, 49] or in the endometrium [31, 48], may help improve selection of embryos for transfer at the appropriate phase of the secretory endometrium. The implementation of such an approach may improve success rates, especially where single embryo transfer is advocated to reduce multiple pregnancy rate complications. In patients with RIF, the 'omics' approach may provide a tool for better selection of the embryo to be transferred. This will improve the outcome and avoid the transfer of a high number of embryos as a means of overcoming recurrent implantation failure. Nevertheless, the 'omics' approach requires sophisticated, complex and expensive systems, making it difficult to apply routinely in every IVF program set-up.

Genetic considerations

PGS has been advocated for couples with RIF for whom the karyotype of both partners is normal. It is assumed that these patients might produce more genetically abnormal embryos than does the general population, either originating from abnormally functioning oocytes or from genetically abnormal sperm [74, 79, 112]. However, while early non-controlled studies supported PGS for several indications, including RIF, a more recent randomized control study showed that PGS in a cleaved embryo stage did not increase delivery rates [50]. An explanation for the latter finding is that the chromosomal pattern of embryos in the early cleavage stage does not exactly represent the final pattern in a later stage of blastulation. It seems that genetically aneuploid embryos diagnosed in the early cleavage state might correct themselves within cell divisions and exhibit a normal chromosomal pattern in the blastocyst stage. Another explanation for such a discrepancy is that the genetic constitution of the biopsied blastomere was not representative of the

blastomeres that were left intact. Thus, currently, PGS of early cleaved embryos should not be offered to patients with RIF, due to its ineffectiveness in increasing live birth rates.

Comparative genomic hybridization (CGH) can promote detection of genetic abnormalities (deletions or excess of DNA content) across the genome. A more sophisticated method, array CGH (a-CGH), can detect changes (excesses or deletions) of smaller fractions on the chromosomes. Thus, even if the karyotype is normal, a-CGH might reveal small divergences in the genome content that are missed by the karyotype, but that may still reduce the implantation potential of the embryo, or lead to miscarriage. Although it is currently possible to perform a-CGH on a single cell, it is prudent, as mentioned before, to perform it at the blastocyst stage, to obtain accurate information on the pre-implanted embryo. While a-CGH is a promising technique, it still has some drawbacks. For one, the test is very expensive and usually takes more than 24 h. Therefore, if performed at the blastocyst stage, the embryos should be frozen and transferred at a later stage. This is especially true if the assay is performed in a central laboratory and the DNA material has to be shipped. Better culture conditions that may increase the proportion of embryos reaching the blastocyst stage, as well as the vitrification technique that increases survival rates of frozen-thawed embryos, may ease the decision making process. In addition, CGH tests cannot detect balanced translocations or inversions, as the total amount of DNA tested is the same as in the control sample. Furthermore, these systems cannot detect gains or losses in regions of the genome not covered by the array [50]. Lastly, patients applying CGH tests should be counseled that the tests might detect abnormalities in the genome other than those sought, and that the upshot of these changes is not yet known. Acknowledging its limitations, in the near future, a-CGH performed at the blastocyst stage might help resolve RIF in some patients.

Summary and clinical approach

Successful implantation is a complex process involving two main players, the mother as a host and the embryo. Problems originated from the host environment, such as abnormal uterine anatomy, non-receptive endometrium and the medical condition of the mother (such as thrombophilia and abnormal immunologic response) can adversely affect the cross-talk between the embryo and the endometrium that is crucial for successful implantation. Similarly, this endometrium—embryo interaction may be hampered if the embryo is disordered. Embryo abnormality can originate from either paternal sperm factors, or from the oocyte and its capability of being fertilized normally and cleave. Accordingly, the investigation and treatment of patient with RIF

should focus on both male and female risk factors that once identified should be managed and treated appropriately (Fig. 1). According to our definition, and following three consecutive IVF failures, patients should undergo hysteroscopy to assess the uterine cavity. Three dimensional ultrasonographies, as well as hysterosalpingography, are complementary tools to be performed as needed. Once an abnormality associated with implantation failure is recognized, treatment options should be considered to include uterine septectomy, removal of intra-uterine adhesions, endometrial polypectomy or myomectomy (particularly the submucous type), and excision of hydrosalpinx.

Thin unresponsive endometrium is hard to manage, and if all available treatments (i.e. high dose estrogen, the application of vaginal estrogen pills, aspirin and other medications that may increase blood flow to the endometrium, and mechanical endometrial stimulation by mean biopsy sampling) fail, then surrogacy is a reasonable option.

In recurrent miscarriages, patients are advised to undergo blood tests for thrombophilia as well as for connective tissue diseases that involve antiphospholipid antibodies. However, as mentioned earlier, thrombophilia and antiphospholipid antibodies may also be associated with risk of RIF. Once detected, a consultation with a hematologist and connective tissue disease specialist is advocated and treatment with low molecular weight heparin (LMWH) is recommended. When thrombophilic trait is detected, treatment with prophylactic dose of LMWH is sufficient and seems to improve IVF outcome. However, when anti phospholipid antibodies (APLA) syndrome is diagnosed, a concomitant treatment with mini dose aspirin and/or corticosteroids should be considered. In addition, once hypercoagulability state is diagnosed, the appropriate treatment protocol for ovarian stimulation should be implemented to minimize the risk of ovarian hyperstimulation syndrome (OHSS). Initiation of LMWH should be considered from the early stimulation period or from the day of embryo transfer. The approach should be determined following appropriate consultation. A patient's family and personal medical history, particularly her previous IVF experience, are important for reaching a decision. Patients with no history of thrombotic events, personally or among close relatives, and who already experienced several uneventful IVF treatments, may be considered suitable to start LMWH on the day of ET. Patient with APLA syndrome, or with a history of a disease that can be attributed to a hypercoagulability trait, should start anticoagulation concomitant with gonadotropin administration. Treatment with LMWH should be stopped 24 h before egg retrieval and reinitiated the day following ovum pick-up. Empirical treatment with LMWH, aspirin or corticosteroids was not found to be effective, and is not advocated for women with RIF who were negative for thrombophilic tests.

A couple diagnosed with RIF should have karyotype testing to rule out structural anomalies of the chromosomes. Although structural disorders generally lead to habitual abortions, they may also prohibit implantation. If a structural anomaly is detected, then PGD should be offered. CGH testing should not be advocated for the couple, as it cannot detect balanced translocations. Similarly, the efficacy of PGS of early cleaved embryos in patients with normal karyotypes has not been established and therefore, at present, this technique should not be applied for RIF patients. In the future, when higher rates of blastocysts will be obtainable in culture media, and the new advent of freezing method by vitrification will be routinely practiced, array CGH applied on a blastocyst stage biopsy to detect minor differences in DNA content should be considered for RIF in order to better choose the embryo for transfer.

Investigation of causes of RIF may include some forms of advanced morphologic analysis of the sperm, since the contribution of the sperm cell to the production of normal and healthy embryo is crucial. Testing the sperm cells for DNA fragmentation and abnormal chromatin packaging is reasonable if RIF seem to be associated with male factor. This test, however, should also be carried out in patients with apparently normal sperm parameters. If advanced morphologic evaluation of the sperm or other methods of assessing DNA integrity reveal a high percentage of abnormal sperm cells, IMSI should be considered as a means of improving implantation. We routinely employ the IMSI procedure in severe cases of RIF defined as ≥ 5 IVF failures.

If the results of all tests mentioned above are normal, consideration of a possible contribution of the couple's immunologic system to implantation failure is recommended. This can first be performed by checking for the presence of an immunologic reaction following a mutual cross match between the serum and lymphocytes of the couple. If no reaction results, then the maternal immune system is apparently nonresponsive to paternal antigen components. This may be due to the couple's similarity in human leukocyte antigen (HLA) components. In such case, a similarity of alleles in Class I and Class II HLA compatibility should be tested. HLA similarity is reported to be associated mainly with recurrent abortions. However, in the extreme situation, it may interfere with implantation, since dissimilarity of HLA is crucial also at the very beginning of the implantation process, during which the immune system plays an important role. If such a similarity is found, high dose IV immunoglobulin (IVIg) should be offered before embryo transfer as suggested by Elram et al. [34], followed by an additional dose as soon as a heart beat is visualized, at about 6 weeks of gestation. Preliminary results using intralipid infusion to support implantation are rather encouraging. However, the real benefit of such treatment in patients with increased NK cytotoxic activity experiencing RIF has not been proved yet in large-scale randomized controlled studies.

Better selection of the embryo for transfer is expected once the new methods of time-lapse imaging and 'omics' technology are applied. These methods can accurately assess embryo morphology and its the metabolic activity. In the future, within the implementation of these modalities, implantation rates for IVF patients including those with RIF are expected to increase. 'Omic' technologies may also improve our understanding of the processes involved in the endometrium during implantation. This will promote better recognition of the "window of implantation" and may suggest options as to how manipulate this crucial period in order to facilitate the cross-talk between the embryo and its platform, resulting in improved implantation rates.

Better culture conditions, increased success rates of embryo freezing by vitrification, as well as innovative methods in molecular genetic biology, such as array CGH, will help achieve the goal of obtaining normal, healthy embryos, capable of implanting following transfer.

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