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Mini Review—Developments in Reproductive Medicine

Investigation and treatment of repeated implantation failure following IVF-ET

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Pregnancy rate following one cycle of IVF and ET can be as high as 60%. But even in the very successful units, some couples fail repeatedly. The causes for repeated implantation failure (RIF) may be because of reduced endometrial receptivity, embryonic defects or multifactorial causes. Various uterine pathologies, such as thin endometrium, altered expression of adhesive molecules and immunological factors, may decrease endometrial receptivity, whereas genetic abnormalities of the male or female, sperm defects, embryonic aneuploidy or zona hardening are among the embryonic reasons for failure of implantation. Endometriosis and hydrosalpinges may adversely influence both. In this mini review, we discuss the suggested methods for evaluation and treatment of RIF: repeated hysteroscopy, myomectomy, endometrial stimulation, immunotherapy, preimplantation genetic screening (PGS), assisted hatching, zygote intra-Fallopian transfer (ZIFT), co-culture, blastocyst transfer, cytoplasmic transfer, tailoring stimulation protocols and salpingectomy for hydrosalpinges.

Key words: implantation/IVF failure/IVF treatment/repeated failure

Introduction

Treatment of infertile couples has progressed immensely during recent years. From close to 235,000 registered assisted reproductive technology (ART) cycles performed in Europe during the year 2001, nearly 29% resulted in a pregnancy (Andersen *et al*., 2005). Failure could be caused by many different factors such as inappropriate ovarian stimulation, suboptimal laboratory culture conditions and faults in embryo transfer techniques. These would usually result in a low pregnancy rate (PR) for the whole unit. But even in successful units with high pregnancy and delivery rates, some couples have repeated implantation failure (RIF). The aim of this article is to summarize the reported aetiologies for RIF, highlight the suggested investigations to be performed and review the recommended treatment strategies.

Definition of RIF

Failure to achieve a pregnancy following 2–6 IVF cycles, in which more than 10 high-grade embryos were transferred to the uterus was defined by various clinicians as RIF (Tan *et al*., 2005). Today with the tendency of transferring only one or two embryos, the definition of RIF is not apparent. Nevertheless, we suggest that after failure of three cycles in which reasonably good embryos were transferred, further investigation should be initiated.

Assumed aetiologies for RIF

Embryonic loss, which occurs repeatedly after assisted reproduction, may be attributed to many factors. These can be grouped into three categories: decreased endometrial receptivity, embryonic defects and factors with combined effect (Table I).

Decreased endometrial receptivity

RIF might be because of undiagnosed uterine pathology. In 18–27% of women with a normal initial hysteroscopy or hysterosalpingogram, repeated hysteroscopic visualization after RIF revealed uterine abnormalities, mainly hyperplasia, polyps, endometritis, synechiae and leiomyomata (Demirol and Gurgan, 2004). The effect of leiomyomata on implantation is uncertain (review Surrey, 2003). Specifically, the impacts of intramural lesions without cavity distortion (Eldar-Geva *et al*., 1998) or myomas of <4 cm (Oliveira *et al*., 2004) on RIF remain controversial.

The presence of a thin endometrium did not influence the cumulative PRs in a prospective large cohort studies (De Geyter *et al*., 2000), particularly when high-quality embryos were transferred (Zhang *et al*., 2005). Thin or hyperechogenic endometrium or persistent endometrial fluid impaired the outcome in tubal factor, but not in polycystic ovary syndrome (PCOS) (Akman *et al*., 2005) or ICSI (Rinaldi *et al*., 1996). However, the concept that a minimum thickness (4–8 mm) is

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Table I. Assumed aetiologies for repeated implantation failure (RIF)

Decreased endometrial receptivity
Uterine cavity abnormalities
Thin endometrium
Altered expression of adhesive molecules
Immunological factors
Thrombophilias
Defective embryonic development
Genetic abnormalities (male/female/gametes/embryos)
Zona hardening
Suboptimal culture conditions
Multifactorial effectors
Endometriosis
Hydrosalpinges
Suboptimal ovarian stimulation

required to establish a clinical pregnancy is still arguable and should be considered in RIF.

Some cases of RIF were related to local dysregulation of the normal expression or action of various cytokines. Elevated endometrial NK cells, dysregulation of interleukin (IL) 12, 15 and 18 (Ledee-Bataille *et al*., 2005), high IL-1β and low interferon-γ and IL-10 (Inagaki *et al*., 2003) were found in RIF. Failure of appearance of a specific integrin –αVβ3 in the endometrium at the time of implantation was suggested as a cause of implantation failure (Tei *et al*., 2003; Thomas *et al*., 2003). High levels of aromatase p450 mRNA (Brosens *et al*., 2004), changes in pinopode expression (Pantos *et al*., 2004) and high matrix metalloproteinases (Inagaki *et al*., 2003) have been suggested to be associated with RIF.

The role of immunological causes and thrombophilia in implantation failure through mechanisms similar to recurrent miscarriages has been the focus of many recent research efforts. The association of antiphospholipid or other autoantibodies with RIF has been shown in some early studies, but large prospective studies failed to reveal an association (Denis *et al*., 1997; Eldar-Geva *et al*., 1999). Part of this confusion relates to differences in antibodies tested. Eldar-Geva *et al.* (1999) could not find any association between any of 18 specific antiphospholipid antibodies and RIF, whereas Stern *et al*. (1998) found that β2-glycoprotein-I antibodies were related to IVF failure. Antibodies to annexin-V, which acts as an inhibitor of phospholipid-dependent coagulation and may be necessary for trophoblast differentiation, were found in greater incidence (8.3%) in women with RIF than in controls (1.1%) (Matsubayashi *et al*., 2001). T-helper 1 and 2 (Th1, Th2) intracellular cytokine expression was increased in peripheral lymphocytes (Kwak-Kim *et al*., 2003). An association between peripheral natural killer (NK) cells and RIF was suggested in several small observational studies; however, in a recent review, Rai *et al*. (2005) contended that there is no scientific evidence for such an association (Rai *et al*., 2005).

Carp *et al*. (1994) suggested that couples sharing HLA alleles are at high risk of RIF and recurrent biochemical pregnancies. However, their preliminary report has never been confirmed. Increased rates of hereditary thrombophilia in women with RIF were found in recent two case-control studies (Grandone *et al*., 2001; Azem *et al*., 2004) but not in another large one (Martinelli *et al*., 2003). The prevalence of PAI-1 mutation and multiple thrombophilic gene mutations among patients with RIF was significantly higher than among fertile controls (Coulam *et al*., 2006). Hence, screening of thrombophilia in RIF is still controversial.

The significantly decreased expression of specific endometrial molecules suggested that functional, not only morphological, endometrial defects may be associated with unexplained infertility or RIF. Further work is required to confirm a causal relationship.

Defective embryonic development

Chromosomal abnormalities of the male or female partner, the gametes or the developing embryo may burden embryogenesis. Increased frequency of female chromosomal abnormalities such as translocations, mosaics, inversion, deletion and chromosomal breakages, particularly at the centromere region were observed in young women with high-order RIF (Tarlatzis *et al*., 2000; Raziel *et al*., 2002). Increased incidence of sperm chromosomal abnormalities in patients with normal karyotype and RIF was also observed (Rubio *et al*., 2001).

Using fluorescence in-situ hybridization (FISH) for chromosomes 13, 16, 18, 21, 22, X and Y on blastomeres from biopsied embryos, Gianaroli and later Pehlivan found that the percentage of embryonic aneuploidy was higher in RIF (54–57%) compared with controls (36%) (Gianaroli *et al*., 1997; Pehlivan *et al*., 2003). Using comparative genomic hybridization (CGH), chromosome abnormalities have been detected in 76/126 (60%) of single blastomeres biopsied from embryos before implantation in 20 women with RIF (Voullaire *et al*., 2002). The disruption of the normal sequence of chromosome replication and segregation in early human embryos, caused by maternal cytoplasmic factors or mutations in cell cycle control genes, might be a common cause for RIF. Thus, it can be assumed that many patients with RIF develop a high percentage of chromosomally abnormal embryos that fail to implant despite good morphology and developmental rate.

The zona pellucida, which surrounds the mammalian oocyte, hardens naturally after fertilization to prevent polyspermic fertilization and to protect the integrity of the preimplantation embryo. Increased zona thickness was associated with lower implantation rates (Cohen *et al*., 1989). Zona hardening, which may be induced by *in vitro* culture or by *in vivo* ageing, can also affect hatching (De Vos and Van Steirteghem, 2000). Thus, failure of the zona to rupture has been suggested as a possible cause of RIF.

Sophisticated culture media have been shown to be superior to others, especially in ICSI (Aoki *et al*., 2005). Several quality control methods have been suggested for identifying suboptimal components of a culture system (Gardner *et al*., 2005). We assume that, in some cases, tailored specific culture conditions are needed for optimal embryonic development and lack of these conditions may be the cause of RIF.

Multifactorial causes

Endometriosis as a cause for RIF has not been investigated directly; however, all markers of reproductive process, including

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ovarian response, embryo quality, implantation and PRs, are decreased in endometriosis, especially in severe disease (Barnhart *et al*., 2002; Kuivasaari *et al*., 2005).

Patients with hydrosalpinges have lower implantation and PRs (Zeyneloglu *et al*., 1998). Hydrosalpinx fluid is commonly slightly alkaline and may contain cytokines, prostaglandins or other inflammatory compounds. These compounds may have either direct embryo-toxicity or adversely affect the endometrium (Meyer *et al*., 1997). Reflux of hydrosalpinx fluid into the uterine cavity may result in diminishing embryonic endometrial apposition.

Endometrial and embryo qualities may be harmed by certain drugs given for ovarian stimulation. Recent randomized controlled trials (RCTs) found no evidence of clinical superiority for rFSH over urinary-FSH/hMG (Al-Inany *et al*., 2003), for recombinant-hCG over urinary-hCG (Al-Inany *et al*., 2005) or for FSH/GnRH-antagonist over FSH/GnRH-agonist protocols (Barmat *et al*., 2005). However, the importance of different drugs in RIF is unknown.

Suggested methods for investigation and treatment of RIF (Table II)

Improving endometrial receptivity

Hysteroscopic correction of cavity pathology

Four hundred and twenty-one patients with RIF who had a normal hysterosalpingogram were prospectively randomized into office hysteroscopic evaluation (n = 210) or nothing (n = 211) (Demirol and Gurgan, 2004). Patients who had abnormal hysteroscopic findings (n = 56) were operated on during the procedure. Clinical PR was significantly higher in the treatment group (30.4% following normal hysteroscopy and 32.5% following hysteroscopic operation) compared to that in the controls (21.6%). Hence, treatment of intrauterine pathologies found by hysteroscopic evaluation improved the pregnancy outcome.

Table II. Suggested methods for treatment of repeated implantation failure (RIF)

Improving endometrial receptivity
Hysteroscopic correction of cavity pathology
Myomectomy
Treatment of thin endometrium
Endometrial stimulation (biopsy)
Immunotherapy (intravenous immunoglobulin, steroids, aspirin and heparin)
Treatment of the embryos
Preimplantation genetic screening
Assisted hatching
Zygote intra-Fallopian transfer
Co-culture
Blastocyst transfer
Cytoplasmic transfer
Improving ET technique
Multifactorial treatment options
Treating endometriosis
Danazol
Salpingectomy in case of hydrosalpinges
Tailoring the stimulation protocols
Psychological assistance

Myomectomy

The favourable PRs obtained after myomectomy lead many clinicians to believe that removal of myomas increases pregnancy and live-birth rates (review Donnez and Jadoul, 2002). However, no appropriate prospective studies have been performed. Furthermore, no information on the value and complications of myomectomy in RIF is available, although most clinicians recommend hysteroscopic removal of submucous fibroids distorting the uterine cavity.

Treatment of thin endometrium

To improve uterine blood flow which may boost endometrial development, low-dose aspirin (Weckstein *et al*., 1997) and vaginal sildenafil (Sher and Fisch, 2002) were suggested in cases of RIF with thin endometrium. Many freeze all embryos when the endometrium is less than 7 mm and transfer them after stimulation with high-dose estrogens. Vaginal administration of micronized estradiol to maximize estrogenic effect (Tourgeman *et al*., 2001) or antifibrotic treatment with pentoxifylline and high-dose vitamin E (Ledee-Bataille *et al*., 2002) has been shown to increase PR in cases with a thin endometrium.

Endometrial stimulation

Endometrial injury or stimulation may cause a pseudo-decidual reaction that enhances implantation. We have described a protocol for RIF (Friedler *et al*., 1993) that included hysteroscopy, curettage, triple antibiotic and estrogen treatment. Of 14 patients who had RIF in 98 transfer cycles following this procedure, six patients conceived (PR 43%). Barash *et al.* (2003) performed repeated endometrial biopsies in 45 cases. Pregnancy and live birth rates in the IVF cycle following the biopsy were doubled. They concluded that local injury to the endometrium increased the incidence of implantation. There is a need for a prospective controlled study to prove the value of this procedure.

Immunotherapy

Because there is evidence to suggest that immunological factors may be involved in RIF, immunotherapy with intravenous immunoglobulin (IVIG) has been introduced empirically into IVF programmes. Preliminary studies found variable success with IVIG (Coulam *et al*., 1994). Elram *et al*. (2005) describe 10 couples with seven or more IVF failures and HLA similarity. Treatment with IVIG resulted in seven ongoing pregnancies. Yet, Stephenson and Fluker (2000) in a double-blind, placebo-RCT including 51 couples with RIF found that IVIG did not improve the live birth rate. Thus, the effectiveness of IVIG treatment in RIF is still unresolved.

Combined treatment of glucocorticosteroids and aspirin has been reported to improve PR in autoantibody seropositive patients who have RIF (Geva *et al*., 2000). Two large RCTs indicated that heparin and aspirin did not improve pregnancy or implantation rates in RIF (Urman *et al*., 2000), even for autoantibody-positive patients (Stern *et al*., 2003). Similarly, immunotherapy using partner's leukocytes was not shown to affect RIF (Carp *et al*., 1994). Yet, the effects of heparin are not restricted to anticoagulation. It is involved in the adhesion of the blastocyst to the endometrial epithelium and the subsequent

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invasion. Fiedler and Wurfel (2004) showed that prolonged heparin treatment increased the PR.

Wurfel *et al*. (2001) found that the administration of leukocyte ultrafiltrate significantly improved treatment results. They suggested that growth factors and cytokines secreted by leukocytes have an important influence on embryonic implantation and growth.

Treatment of the embryos

Preimplantation genetic screening

Patients with RIF develop a high percentage of chromosomally abnormal embryos that fail to implant despite regular morphology and developmental rate. Using preimplantation genetic screening (PGS) and selecting chromosomally normal embryos for replacement significantly increased the implantation rates in RIF when 3–8 chromosomes were analysed (Munne, 2003; Pehlivan *et al*., 2003; Wilding *et al*., 2004). Taranissi *et al*. (2005) have shown that PGS for chromosomes 13, 16, 18, 21 and 22 was associated with improved outcome (PR of 43% and delivery rate of 32%) in young women with RIF. However, Caglar *et al*. (2005) reviewing the literature on PGS in RIF concluded that the data in the literature did not provide firm evidence that patients with RIF will benefit from PGS. They state that PGS can be useful to clarify the reason for recurrent failures.

Wilton *et al*. (2003) showed that CGH was able to identify many chromosomal abnormalities that would have been missed if those cells had been analysed by FISH. The clinical pregnancy and implantation rates were 11 and 7% for embryos analysed by FISH and 21 and 15% for embryos analysed by CGH. However, CGH remains technically challenging and, in its current form, is likely to be performed in only few laboratories.

Assisted hatching

Failure of the embryonic zona pellucida to rupture following blastocyst expansion has been suggested as a possible cause for RIF (De Vos and Van Steirteghem, 2000). To help the release of the embryos from their zona, different types of assisted hatching (AH) have been developed. These involve the creations of an opening in the zona pellucida either mechanically (partial zona dissection) or chemically (zona drilling with acid Tyrode) or by laser 'damage' of the zona pellucida, before ET. Three RCTs have shown that, in cases of RIF, AH significantly increases the pregnancy and implantation rate (Chao *et al*., 1997; Magli *et al*., 1998; Nakayama *et al*., 1999). However, in a systematic review of 23 RCTs (2572 women), although clinical PR was significantly higher following AH, there was no effect on the 'take-home-baby rate' (Edi-Osagie *et al*., 2003). No benefit of laser-AH in RIF could be found in a large European multicentre randomized trial (Primi *et al*., 2004).

Zygote intra-Fallopian transfer

In contrast to standard IVF-ET, zygote intra-Fallopian transfer (ZIFT) allows early embryonic growth in the natural tubal environment and transport of the embryos into the uterine cavity under natural physiologic regulation. This technique also prevents spillage of embryos after transcervical ET and solves the problem of technically difficult ET because of cervical stenosis. Initial retrospective studies reported superior results of implantation and PRs after ZIFT as compared to standard IVF-ET (Asch, 1991). Others have found that the main value of ZIFT is in RIF cases (Friedler *et al*., 1991; Levran *et al*., 1998). This enthusiasm later was curtailed by the results of a series of RCTs that failed to demonstrate any advantage for ZIFT (Habana and Palter, 2001; Dale *et al*., 2002). These studies, together with the complexity and cost of ZIFT, led to the discontinuation of this method in most IVF units. Furthermore, a recent study including 229 patients with RIF showed comparable outcome following ZIFT and IVF-ET (Aslan *et al*., 2005).

Co-culture

One of the methods suggested to improve culture conditions has been the development of co-culture systems in which a variety of different cells have been used. The suggested beneficial effects of the co-culture include the secretion of embryotrophic factors such as nutrients, growth factors and cytokines and detoxifying of free radicals and potentially harmful substances (Simon *et al*., 1999). The most promising co-culture method seems to be homologous endometrial cells (Jayot *et al*., 1995). Using this method, Spandorfer *et al*. (2004) reported 49% PR in 1030 patients with RIF. However, most IVF units do not have the needed facilities and experience for co-culture. Controversies exist regarding the benefit of various sequential or sophisticated culture media.

Blastocyst transfer

Since the introduction of IVF, embryos have been routinely transferred into the uterus around the 2–8-cell stage (day 2–3), at the time when they would naturally be in the Fallopian tube. Transfer of embryos at the blastocyst stage is a more physiological approach because the human embryos enter the endometrial cavity only 5 days after fertilization, at the morula-blastocyst stage. Activation of the embryonic genome occurs at the 8–10-cell stage. Up to this stage, embryonic development depends only on the oocyte genome. Culturing the embryos to the blastocyst stage examines the propriety of the whole embryonic genome.

Two large RCTs have shown that blastocyst transfer after RIF following day 2–3 transfer carried significantly higher implantation and live birth rates (Guerif *et al*., 2004; Levitas *et al*., 2004). Improved embryo selection and uterine receptivity may explain the benefit of embryo transfer at the blastocyst stage for couples with RIF.

Cytoplasmic transfer

RIF might be because of compromised ooplasmic components in some patients. The introduction of a small amount of ooplasm from a donor oocyte or zygote may alter the function of probable deficient oocytes (Cohen *et al*., 1998). This technique has led to the birth of at least 30 healthy babies worldwide (Barrit *et al*., 2001). However, the transferred cytoplasm may contain mRNAs, proteins and mitochondria, as well as other factors and organelles. Thus, cytoplasmic transfer is still considered an experimental procedure because it is not known whether the physiology of the early embryo is affected.

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Improving ET technique

Obviously, the best ET technique is essential in each cycle and must be reconsidered in RIF. Meta-analysis of randomized trials has shown that significantly higher PRs were obtained when an atraumatic ultrasound guidance technique was used and the embryos were deposited in the middle part of the uterine cavity (Sallam, 2005). In an RCT, Bar-Hava *et al*. (1999) showed that fibrin glue doubled PR in RIF. Many clinicians transfer large number of embryos after RIF; however, no comparative study has been published yet.

Multifactorial treatment options

Treating endometriosis

The administration of GnRH agonists for 3–6 months before ART in women with endometriosis significantly increases the ongoing PR (Surrey *et al*., 2002). No deleterious effect on ovarian response was observed. A recent meta-analysis of three RCTs indicated that this treatment increased the odds of clinical pregnancy by 4-fold (Sallam *et al*., 2006). Most investigators agree that there is no benefit in the removal of endometriomas before IVF (Garcia-Velasco *et al*., 2004; Wong *et al*., 2004), whereas the role of laparoscopic treatment of non-ovarian endometriosis in patients with failed IVF is controversial (Adamson, 2005; Littman *et al*., 2005). Furthermore, surgery might be deleterious for ovarian reserve.

Danazol

Immunosuppressive effects of danazol *in vitro* were shown long ago (Hill *et al*., 1987), when it was used for endometriosis suppression. In an RCT of 81 patients with RIF, danazol treatment significantly increased PR (40 versus 19.5%) (Tei *et al*., 2003). Danazol was found to increase receptivity of the endometrium and upgrade the endometrial $\alpha V\beta 3$ integrin.

Salpingectomy of hydrosalpinges

Strandell and co-investigators were the first to show in an RCT that salpingectomy of hydrosalpinges increased PR (Strandell *et al*., 1999). In a recent meta-analysis (Johnson *et al*., 2004) of three RCTs involving prophylactic salpingectomy in 295 patients with hydrosalpinges, the pregnancy and live birth rates doubled following prophylactic salpingectomy. Laparoscopic salpingectomy is now recommended in all women with hydrosalpinx before IVF treatment, certainly following RIF.

Tailoring the stimulation protocols

Takahashi *et al*. (2004) showed that using GnRH-antagonist protocols improved blastocyst quality and pregnancy outcome after RIF with GnRH-agonist protocols. Natural cycle was also suggested (Kadoch, 2003), particularly to patients with high uterine NK cell count (Ledee-Bataille *et al*., 2004). There are no controlled studies to prove that changing any specific medication or stimulation protocol can improve treatment outcome. Even so, we assume that certain patients are more vulnerable than others to certain medications, and thus, there might be a place for 'personal' protocol in RIF.

Psychological assistance

It seems obvious that stress can interfere with infertility treatments. Boivin (2003) analysed 380 studies dealing with the effect of psychological treatments for infertile individuals. She indicated that psychological interventions had little influence on PRs. de Liz and Strauss (2005) performed a meta-analysis to evaluate the efficacy of group and individual therapy on the possible promotion of pregnancy. The main result suggested that psychotherapy (group and individual) reduces anxiety and depression and possibly enhances conception success. Many recommend psychological interventions and various relaxation techniques, but proof of their efficacy is lacking.

Personal experience

There are many reasons for RIF. We believe that we do not have the tools to diagnose in each case the exact cause for the repeated failure. At the IVF unit in Shaare-Zedek Medical Center, about 10% of the cycles are of patients with RIF. Because the regulations in Israel force the medical insurance companies to finance IVF treatment, if needed, until a couple has two children, patients have no financial restrictions on the number of IVF cycles that they undergo.

In the older age group, we assume that the major cause for RIF is chromosomal abnormalities of the embryos. Because we do not have the ability to do PGS at our unit, we transfer in these cases as many embryos as possible.

In cases with any hint of autoimmune disease, we treat with steroids (0.5 mg dexamethasone/day or 5–10 mg prednisone/day) and aspirin (100 mg/day) during the whole cycle. During the past years, we have occasionally performed ZIFT to patients who failed five or more ET especially (but not only) if the embryo transfer was difficult. Of 86 ZIFT cycles, 20 pregnancies were achieved (23% PR). AH by mechanical PZD was performed in 71 cases of young (<35 years) women with more than three failures. Twenty-three (32%) pregnancies have been achieved. During the last year, we performed endometrial stimulation (biopsies) on days 12 and 21 of the cycle preceding the IVF treatment. Of 30 women who underwent the biopsies, 10 conceived (33%).

Conclusions

There are many known and unknown reasons for RIF, and we do not have the tools to diagnose in each case the exact cause for the repeated failure. However, we think that after failure of three transfers of good-quality embryos in a unit with a PR of at least 30%, one should take some special measures. There are no hard data from RCTs that any of the treatments has a significant value, but on the contrary, everyone agrees that taking a different approach achieves a pregnancy in many cases that failed repeatedly.

After three failures, repeated hysteroscopy and a try of blastocyst transfer are highly recommended. A change in the stimulation protocol has a place. AH, PGS and co-culture are probably beneficial in experienced hands. Long-term use of danazol or GnRH agonists probably has a place in repeated failures with endometriosis. The use of IVIG is very controversial but may be justified after many failures in specific cases.

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Steroids might have a place in patients with any sign of autoimmunity, and ZIFT has a place in cases of difficult embryo transfers.

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