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In Vitro Fertilization

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INTRODUCTION

In vitro fertilization (IVF) was first developed to treat patients with tubal obstruction.[1] Since then, indications for IVF have expanded to include virtually all causes of infertility. In addition, conversion to IVF has become an option for patients who hyperrespond to superovulation and intrauterine insemination (SO-IUI), to reduce the risk of high-order multiple pregnancies and ovarian hyperstimulation syndrome (OHSS). Oocyte donation allows patients of advanced maternal age and/or diminished ovarian reserve who could not otherwise conceive to become parents. Preimplantation genetic

diagnosis (PGD) for a multitude of heritable diseases, such as sickle cell anemia and cystic fibrosis, is another expanding application of assisted reproductive technology (ART).

INDICATIONS FOR IN VITRO FERTILIZATION

Tubal disease

Prior to the advent of IVF, patients with irreparable bilateral tubal obstruction could not conceive. Women with less severe forms of tubal disease could occasionally conceive either spontaneously or with the aid of SO-IUI or controlled ovarian hyperstimulation and intrauterine insemination (COH-IUI), but the risk of ectopic pregnancy is high in such cases and pregnancy rates are disappointing.

Despite the unquestioned success of IVF for the treatment of tubal infertility, it has been shown that the presence of a hydrosalpinx has a negative impact on IVF success rates. Communicating hydrosalpinges may produce mechanical and/or embryotoxic factors that may interfere with implantation. In some studies, hydrosalpinx fluid has been shown to be toxic to both embryos and sperm *in vitro*.[2] Many clinicians advocate the removal of hydrosalpinges prior to IVF; however, most of the available data are retrospective with historical controls. One paper demonstrated that donor oocyte recipients with hydrosalpinges had a poorer outcome than donor recipients without hydrosalpinges, suggesting that the negative impact is indeed at the level of the intrauterine environment.[3]

Randomized controlled trials are available, and a Cochrane systematic review of these trials has been published.[4] In one study, Strandell and colleagues showed that pretreatment salpingectomy improved pregnancy outcome in patients with hydrosalpinges sufficiently large to be visualized on ultrasound.[5, 6] A subsequent systematic review combined three randomized controlled trials and demonstrated that patients with hydrosalpinges may benefit from salpingectomy pre-IVF.

Endometriosis

Endometriosis affects approximately 25% of infertile women compared with 5% of women in the general population. Therefore, options for women with minimal to mild endometriosis include expectant management, surgery, and COH-IUI. Unfortunately, as in many areas of reproductive medicine, there is again little information available in the form of randomized, controlled trials to suggest optimal management. In cases of minimal to mild endometriosis, Marcoux and co-workers demonstrated, in a well-conducted randomized controlled trial, that surgical ablation of laparoscopically documented minimal to mild endometriotic lesions results in higher cumulative pregnancy rates (30.7% versus 17.7%) over a period of 36 weeks, versus expectant management without ablation.[7]

Treatment of advanced-stage endometriosis remains challenging. No randomized, controlled data are available comparing surgery with IVF, and such studies would be difficult to undertake. Most of the available data are in the form of less robust retrospective case-control, cohort, and case series studies. Some authors have suggested conservative surgical therapy at the time of diagnosis to restore anatomy, followed either immediately, or after a period of observation, by IVF.

Some concerns have been raised regarding a possible adverse effect of endometriosis on IVF outcome, possibly through deleterious effects on egg quality and/or the number of oocytes retrieved. In an effort to assess for a possible detrimental impact on the endometrium, Diaz and associates undertook a study in which donor oocytes were split between recipient patients with and without endometriosis.[8] Here, pregnancy rates were similar; however, this study did not have sufficient power (57%) to exclude a difference. Other authors have suggested that oocyte quality may be affected in women with endometriosis, and embryotoxic factors from endometriosis may affect implantation.[9, 10] Available data regarding the effectiveness of IVF in the treatment of endometriosis-related infertility have demonstrated a poorer prognosis than for patients with tubal disease.[11] A large retrospective study from Cornell suggested that outcome was as good for patients with endometriosis as for patients with tubal infertility and did not vary appreciably by stage. Society for Assisted Reproductive Technology (SART) data support the latter concept.[12, 13]

The issue of whether pre-IVF resection of large endometriomas improves the response to controlled ovarian stimulation remains controversial. Nevertheless, these patients may benefit from gonadotropin-releasing hormone (GnRH) agonist suppression prior to stimulation.[14, 15, 16]

Male factor

Comparing semen analyses from fertile and nonfertile couples, Guzick and colleagues suggested that clinically significant semen analysis cut-offs are as follows: concentration less than 13.5 million sperm/mL, motility less than 32%, and less than 9% normal morphology according to strict World Health Organization (WHO) criteria.[17]

Male factor infertility is deemed etiologic in approximately 40–50% of infertile couples. Up to 57% of male infertility has an identifiable cause that might be amenable to direct treatment of the male.[18] Previously, successful treatment of most severe male factor infertility required therapeutic donor insemination.

Options for the treatment of severe forms of male factor infertility were therefore relatively limited before the advent of micromanipulation techniques such as intracytoplasmic sperm injection (ICSI) and, subsequently, sperm retrieval techniques such as testicular and epididymal sperm extraction combined with ICSI. Conventional IVF insemination rates are poor with motile sperm concentrations of less than 3 million per ejaculate.[19] ICSI is specifically addressed later in this chapter.

Other indications for *in vitro* fertilization

IVF has also been used for the preservation of reproductive capacity. Female cancer patients who wish to preserve their ability to procreate following antineoplastic therapy have the option of cyropreserving embryos. Current research efforts are focusing on the preservation of ovarian tissue and oocytes (e.g., pre-reproductive age patients and those without a male partner).

Patients who are unable to procreate with their own genetic DNA may alternatively be offered treatment with donor oocytes. The technique of oocyte donation was initially developed for patients with agonadism (e.g., premature ovarian failure). Currently, oocyte donation is most often used for the treatment of patients of advanced reproductive age or those who have repeatedly produced embryos of poor quality. The success of oocyte donation is dependent on the age of the donor rather than the recipient. The quality and number of oocytes diminish with age, and the rates of aneuploidy increase in resultant embryos. Ovarian reserve is the most important limiting factor for IVF. Oocyte donation may also be indicated in cases in which the mother

carries a genetic risk/disorder for which PGD is not available or as an alternative to PGD.

IVF for the treatment of irreparable uterine abnormalities is also possible. Patients with congenital absence of the uterus (Mayer-Rokitansky-Hauser syndrome) or patients who have undergone hysterectomy can undergo IVF with embryo transfer to a gestational surrogate resulting in the birth of genetic offspring. Women who have medical disorders that contraindicate pregnancy can also reproduce through surrogacy.

Women with ovulatory disorders (hypogonadotropic hypogonadism or polycystic ovarian syndrome) typically respond well to ovulation induction. For those women who have such an exaggerated response that the risk of higher-order multiples or ovarian hyperstimulation is significant, conversion from a stimulated IUI cycle to an IVF cycle is possible and can prevent cycle cancellation, given the ability to restrict the number of embryos transferred. Success rates from these converted cycles appear comparable with cycles initially started as IVF. In addition, patients who fail to conceive despite an ovulatory response to ovulation induction agents are reasonable candidates for IVF, if other treatable causes of infertility have been excluded.

Patients with unexplained infertility, some of whom failed COH-IUI, will frequently benefit from IVF. In addition, IVF may uncover an etiology such as fertilization failure or poor embryo quality in some of these cases. Overall, patients with a diagnosis of unexplained infertility are more likely to succeed with IVF than with more conservative measures.[20, 21, 22]

PGD allows the detection of significant genetic disease before embryo transfer and conception. PGD is most commonly used for autosomal recessive and sex-linked disorders. Other indications for PGD include the diagnosis of aneuploidy; the assessment of patients with a history of recurrent abortions, especially if a balanced translocation has been identified in one of the parents; and the evaluation of embryos of women with repeated, unexplained IVF failure despite good embryo morphology.

PROGNOSTICATORS AND ASSESSMENT

Age

Reproductive success decreases with advancing maternal age. Age is the strongest predictor of ovarian reserve. Studies of populations who do not practice contraception have documented a definitive decrease in fecundity with age.[23] However, other measures of ovarian reserve are available that may predict a patient's prognosis and response to stimulation. Day 3 follicle-stimulating hormone (FSH) and estradiol levels have been correlated with IVF success in terms of pregnancy rates, number of oocytes retrieved, and peak estradiol levels.[24] For younger patients, FSH and estradiol levels may more accurately reflect a patient's ovarian reserve than her age alone. However, it must be emphasized that normal FSH and estradiol levels in the older patient do not override the impact of chronologic age on outcome.

Ovarian reserve

The total number of oogonia peaks at 6–7 million by midgestation. When puberty is attained, the number of oocytes has been depleted, through atresia, to approximately 300,000. At age 37–38 years, this loss accelerates, with a decrease in the total number of oogonia to approximately 25,000.

Competent follicles produce inhibin B. Inhibin B exerts a negative feedback on FSH secretion. FSH is required to recruit follicles for the next ovulatory cycle. As women age, the cohort of recruitable follicles diminishes and the levels of inhibin B decrease in parallel. FSH may increase in the late luteal phase of the previous cycle. This early increase in FSH in turn stimulates earlier recruitment of a dominant follicle. In a younger patient, the dominant follicle is usually recruited by approximately day 5. This concept forms the basis for the day 3 hormonal tests that have become an integral part of the infertility evaluation; elevated early follicular FSH levels at any age and/or estradiol levels, particularly in women older than 35 years, reflect diminished ovarian reserve.[24]

A study at the Cornell Center for Reproductive Medicine demonstrated that ongoing pregnancy rates per retrieval were higher for patients with basal estradiol levels less than 30 pg/mL versus 31–75 pg/mL. No pregnancies occurred when the unsuppressed day 3 estradiol exceeded 75 pg/mLl, and no pregnancies occurred when the day 3 estradiol was greater than 45 pg/mL and the FSH level was concurrently greater than 17 mIU/mL (Leeca assay).[25]

The clomiphene citrate challenge test (CCCT) can also be used to prognosticate ovarian reserve. Here, the FSH is measured on day 3 and then again on day 10 following administration of clomiphene 100 mg by mouth (PO) on days 5–9. Poor ovarian reserve is defined as a day 10 FSH level greater than 2 standard deviations above the mean. Navot and colleagues reported that of patients with an exaggerated response, only 5.5% subsequently conceived versus 42% of patients with a normal response.[26] Some authors have suggested that the CCCT might be more sensitive than basal FSH levels alone, but it is not clear whether basal estradiol levels were taken into account in these studies or whether basal FSH levels were assessed in more than one cycle.

As mentioned above, the total number of primordial follicles in the ovary decreases with age. Thus, the number of primordial follicles at any given time is an indicator of ovarian reserve. Unfortunately, primordial follicles cannot be measured directly by chemical tests or ultrasound. Antral follicle count (AFC), done by ultrasound, is thought to be a surrogate marker for primordial follicles as the total antral follicles observed derived from the pool of primordial follicles.[27] Thus, AFC has also been used as a marker for ovarian aging and also as a predictor for ovarian response after hyperstimulation.[28]

One of the best indicators of ovarian reserve is the response to ovarian stimulation.

Basic evaluation

The initial evaluation for IVF should include a thorough history including a review of previous pregnancies, pregnancy outcomes, and fertility treatments including SO-IUI and IVF. During the physical examination, particular attention is paid to abnormalities of the thyroid, galactorrhea, previous surgical scars, and the pelvic examination. The pelvic examination generally includes cultures for chlamydia, gonorrhea, and other organisms as indicated. Cervical cytology should be up-to-date. A sounding (trial transfer) of the endometrial cavity for depth, position, and accessibility should also be performed, once negative cultures have been obtained. An ultrasound assessment of the uterus, endometrial cavity, and adnexae may also be performed to rule out any abnormalities such as ovarian cysts. If previous hysterosalpingogram films are available, these should be reviewed for the presence of intracavitary defects and hydrosalpinges, with surgical correction if indicated.

The male partner should also be evaluated. A thorough history should be taken with emphasis on previously fathered pregnancies; history of mechanical or infectious testicular injury; prior surgery including varicoceles, vasectomy, and vasovasostomy; previous semen analyses and/or tests for anti-sperm antibodies; social habits including the use of tobacco, alcohol, prescription medications. and nonprescription substances; exposure to chemicals, toxins,

radiation, or extremes of temperature (e.g., saunas, hot tubs); and sexual function. A semen analysis should be obtained prior to IVF. Semen cultures may be performed as indicated.

CONTROLLED OVARIAN HYPERSTIMULATION FOR IN VITRO FERTILIZATION

History

Ovarian stimulation protocols have evolved considerably from the earliest days of IVF. The first IVF baby resulted from harvest of a single oocyte in a spontaneous, nonstimulated menstrual cycle.[1] The use of ovulatory agents subsequently permitted the harvest of larger numbers of oocytes with increased per cycle efficiency.

Natural-cycle IVF has been limited by relatively poor success rates.[29] It is limited by the retrieval of a single oocyte, which effectively eliminates the ability to select and/or cryopreserve embryos and limits the pregnancy rates to the implantation rate of a single conceptus. In addition, ancillary reproductive technology procedures such as PGD and ICSI optimally require the harvest of multiple mature oocytes. IVF pregnancy rates reflect implantation rates per embryo, and therefore, the transfer of more than one embryo generally improves overall success rates. Nevertheless, natural-cycle IVF remains an option for patients who respond poorly to ovarian stimulation (i.e., produce only one or two follicles) or who have medical reasons to avoid supraphysiologic estradiol levels. As described later, the introduction of GnRH antagonists may avoid cancellations owing to premature luteinizing hormone (LH) surges and improve success rates following natural-cycle IVF.

The use of ovulation-inducing medications such as clomiphene citrate (CC) and, more significantly, gonadotropins (human menopausal gonadotropin [hMG] and both urinary and recombinant FSH) has allowed the recruitment of multiple oocytes per IVF cycle, improving the chances for fertilization, increasing the number of embryos available for selection and transfer, and improving pregnancy rates. CC has been used alone or in combination with gonadotropins. However, CC cycles typically result in limited oocyte recovery (one to three oocytes) and guarded success.

Gonadotropin-releasing hormone agonists/antagonists

The development of GnRH agonists has enhanced the efficiency of IVF. Prior to the routine use of GnRH agonists, premature luteinization accounted for cancellation of up to 20% of IVF cycles.[30, 31] Currently, fewer than 2% of cycles are canceled owing to premature LH surges.[32] For over a decade, GnRH agonists have been used in the majority of ART stimulation protocols.

Native GnRH is a decapeptide produced in the arcuate nucleus of the hypothalamus and first characterized in 1967.[33] However, GnRH agonists (e.g., leuprolide acetate) and, more recently, GnRH antagonists did not become available until much later. The GnRH agonists are produced by substitution of the amino acids at positions 6 and 10. Native GnRH has a half-life of 2–4 minutes. The substituted agonists have half-lives of up to 3 hours. Agonists result in pituitary desensitization through prolonged receptor occupancy. However, the initial effect of GnRH agonists is a flare through the increased release of stored gonadotropins. This effect is especially noticeable if given during the early follicular phase. After approximately 1–3 weeks, the maximal suppressive effect of the GnRH agonist is observed. GnRH agonists do not block pituitary gonadotropin production completely, and small LH

pulses may still be observed. GnRH agonists can be used in long, short or ultrashort protocols, as described later, to maximize the benefit of their differential effects dependent on dose and duration of use.

The development of well-tolerated, effective GnRH antagonists has now occurred. The second-generation compounds exhibit side effects of histamine release and potentially anaphylaxis. Third-generation compounds are now available for clinical use in the United States. Side effects appear to be mainly limited to those of estrogen withdrawal and localized, short-duration erythema at the injection site.[33] GnRH antagonists have altered amino acids at positions 1, 2, 3, 6, 8, and 10. They inhibit pituitary gonadotropin output through competitive inhibition of the pituitary GnRH receptors but, unlike the agonists, are not accompanied by the “flare” effect, that is, the suppression is immediate. The half-life is 6–30 hours for the nondepot form. Within 6 hours of administration, LH levels drop by 70%, whereas the FSH drops by only 30%.

There are several potential advantages to the use of an antagonist in IVF. The duration of administration is reduced in comparison with agonists because the antagonist can be initiated later during the process of stimulation (days 6–8). When an antagonist is used as an adjunct to ovarian stimulation, estrogen withdrawal symptoms are eliminated because the patients already have elevated estrogen levels by the time the antagonist is started. Lower total doses of gonadotropins may be needed; treatment duration is decreased because the ovaries are not suppressed prior to stimulation. Fewer ovarian cysts form as a result of therapy because there is no pretreatment luteal flare as seen with luteal initiation of the agonists. The literature suggests that the incidence of OHSS may also be decreased with the use of antagonist relative to the agonist.[34] Recently, antagonist has been used to suppress endogenous gonadtropin release, so as to allow the use of GnRH agonist as an alternative to human chorionic gonadotropin (hCG) to induce oocyte maturation in patients at high risk for OHSS.[35] The antagonist provides an alternative approach to the stimulation of poor responders and may be considered as an alternative approach to the flare and microdose agonist protocols in these difficult cases.[36, 37]

Bovine embryos have been shown to contain GnRH receptors. Some studies have suggested that, although fertilization rates are not affected in GnRH antagonist cycles, implantation rates might be reduced as demonstrated by somewhat lower clinical pregnancy rates and deliveries. A meta-analysis suggested that antagonist cycles had a 5% lower pregnancy rate when compared with agonist cycles.[38] Whether this phenomenon is due to decreased endometrial receptivity, interference with embryonic cleavage, or other effects has yet undetermined. In at least one study, cryopreserved embryos from GnRH antagonist cycles did not exhibit lower success rates compared with agonist cycles. The half-lives of the available antagonists suggest that minimal GnRH antagonist activity should be available in the peri-implantation window.[39]

Other potential disadvantages accompany the use of GnRH antagonists. Strict patient compliance is required because a missed injection may permit an LH surge. Close surveillance is required, so as not to miss a potential surge and premature luteinization. The debate regarding the importance of LH for ovarian stimulation is ongoing. The GnRH agonist does not completely abolish endogenous LH production, whereas the antagonist does. At many centers, antagonist cycles are supplemented with exogenous LH (e.g., hMG) at the time of initiation of the antagonist, if recombinant FSH is being used as the sole initial agent. Without adjuvant LH, estradiol levels may drop, although the clinical significance of this phenomenon is not clear.[40, 41]

Protocols are optimally selected according to a patient’s age, history of response to stimulation, and assessment of ovarian reserve. For patients who are likely to have a good response, one of the objectives is to avoid overstimulation, including full-blown OHSS, which is potentially life-threatening. One commonly utilized protocol is the long GnRH agonist protocol. Here, the agonist is initiated 1 week following ovulation (e.g., documentation of the LH surge).

Once adequate suppression has been documented by withdrawal bleeding and appropriately suppressed estradiol, exogenous gonadotropins are initiated at a dose and in a combination tailored to the individual patient. A typical starting dose is 3–4 ampules (225–300 IU) of FSH and/or hMG per day. Either a fixed, a step-up, or a step-down protocol may be used, that is, the gonadotropins can be started at a low dose and increased according to estradiol levels, or a protocol with a higher starting dose can be employed with the dosage decreased according to the response. We typically employ the step-down protocol approach. Once the lead follicles attain a mean diameter of approximately 17 mm, hCG is administered, usually at a dose of 5000–10,000 IU. This allows maturation of the oocytes (resumption of meiosis) for retrieval 34–36 hours later.

Patients are advised not to try to conceive in the periovulatory window of an agonist-start cycle. The agonist may rescue the corpus luteum and therefore rescue a very early pregnancy. Whereas it is prudent to avoid administering GnRH agonists during a known pregnancy, patients have nonetheless taken GnRH agonists inadvertently during very early pregnancy and adverse effects have not been reported.

High responders

Several stimulation approaches have been utilized in an effort to improve outcome in the high responder who is at increased risk for OHSS. Dual suppression with both an oral contraceptive pill overlapping with a GnRH agonist has been shown to attenuate the response to stimulation and reduce the risk of excess numbers of oocytes and excessive estradiol concentrations.[42] Lower doses of gonadotropins are administered in polycystic ovary-like patients (e.g., 150 IU/day). Patients who achieve estrogen levels exceeding 3000 pg/mL before follicular maturation is attained may be “coasted.” The GnRH agonist is continued but the gonadotropins are withheld for 1–3 days. The larger follicles are allowed to grow, while attenuating the growth of the smaller and intermediate follicles. hCG is administered once the estradiol drops to a relatively safe level (e.g., <3000 pg/mL). This strategy results in a cancellation rate of approximately 20–30%, but usually avoids the potentially life-threatening complications of OHSS, and satisfactory pregnancy rates are maintained. Other strategies to avoid OHSS include delay of the embryo transfer until day 5, so that the patient may be clinically reassessed for significant signs and symptoms prior to transfer. Alternatively, the embryos may be cryopreserved for future replacement since the risk for OHSS is significantly elevated in cycles of conception. The brisk responder, however, ultimately has a very good prognosis. Even if the initial cycle is canceled, a high response indicates a good probability of success in the future with a more conservative stimulation.

Poor responders

The poor responder poses a far greater challenge. One approach is to reduce the degree of ovarian suppression by adjusting the dosage of GnRH agonist. A low-dose luteal lupron protocol, for example, reducing the initial dose from 1 to 0.5 mg, can augment the response. A short follicular phase protocol, which exploits the agonist’s flare effect, can also be employed. In this protocol, leuprolide acetate is administered at 1 mg subcutaneously on days 2–4 and then decreased to 0.5 mg subcutaneously on day 5, and gonadotropins are started on day 3. The oral contraceptive pill (OCP) microdose protocol involves 14–21 days of ovarian suppression with an OCP, followed by microdose leuprolide (40 μg subcutaneously twice daily) beginning on 3rd day off the OCP, and then high-dose gonadotropins (e.g., 450 IU) on day 3 of leuprolide administration. More recently, antagonists have been applied to the treatment of the poor responder in order to avoid intial ovarian suppression. Gonadotropins are initiated in the early follicular phase of a spontaneous cycle, and a GnRH antagonist is started on cycle days 6–8, usually once the lead follicles have attained a mean diameter of 12–13 mm. Success rates with these approaches have been nonetheless limited, largely owing to the significantly poorer prognosis for patients with decreased ovarian reserve.

In some low-response patients, a combined clomiphene-gonadotropin protocol may enhance oocyte recruitment.[43] There is a consensus in the literature that simply increasing the dose of gonadotropins, for example, beyond 300–450 IU, is not helpful.

One meta-analysis suggested that the long agonist protocol is more successful than the short protocol in IVF patients overall.[44] All unstratified infertility patients were included in the randomized, controlled studies reviewed in this analysis, without reference to prognosticators. Therefore, these conclusions are not translatable specifically to low responders.

No adverse effects on children born from GnRH antagonist cycles have been reported.[45]

THE IN VITRO FERTILIZATION PROCEDURE

Oocyte retrieval

Oocyte retrieval is generally performed 34–36 hours after hCG administration. Either urinary hCG or recombinant hCG may be used. The interval between hCG administration and retrieval allows resumption of meiosis I. One study suggested that 36 hours might be suboptimal for retrieval.[46] However, a randomized controlled trial suggested that retrieval at 36 or 37 hours was both safe and equally efficacious in terms of pregnancy outcome, but that retrieval at 35 hours had a worse pregnancy prognosis owing to immaturity of oocytes. Also, no cases of spontaneous ovulation were observed between 36 and 37 hours.[47]

Originally, retrievals were performed by laparoscopy. Transvaginal ultrasound has since become the standard route of oocyte harvest and requires minimal anesthesia and recovery time. The patient is prepped and draped in the dorsal lithotomy position under intravenous sedation. Although antiseptics are toxic to oocytes, we have not found the use of povidine iodine to be detrimental as long as the vagina is copiously irrigated with sterile saline solution following its application. A transvaginal ultrasound probe with a high-frequency transducer (5–7 MHz) and needle guide is used to identify the follicles and align them in their largest diameter. The follicles are then aspirated under negative pressure (100–120 mmHg) with flushing of the tubing following each withdrawal of the needle to maximize oocyte recovery. Empty follicle syndrome is a clinical scenario in which, despite the presence of ovarian follicles, no oocytes are retrieved at harvest; although this could rarely be due to technical difficulties, most often it occurs when the patient fails to appropriately administer the hCG. We therefore measure serum LH and/or hCG levels the day before retrieval to ensure adequate hCG exposure.

The debate regarding the need for prophylactic antibiotics is ongoing. One study did demonstrate colonization of the transfer catheter tip. It suggested that hydrogen peroxide-producing *Lactobacillus* was associated with better pregnancy results, whereas *Streptococcus viridans* was inversely associated with ongoing pregnancy rates. In either case, doxycycline (commonly used for prophylaxis at retrieval) was not specifically associated with better outcomes and did not target the bacteria previously discussed.[48] Another uncontrolled cohort study prospectively evaluated routine antibiotic prophylaxis at retrieval with 2 g of intravenous ceftriaxone and 1 g of intravenous metronidazole by studying the cultures of the catheter tip from the mock transfer at the time of retrieval and also those of the actual embryo catheter tip (48 hours later). They showed that prophylactic antibiotics reduced embryo transfer catheter–positive cultures and that negative cultures at the time of mock transfer or actual embryo transfer improved

implantation rates.[49]

We typically advocate a 4-day course of oral tetracycline started immediately following retrieval. We routinely use preretrieval intravenous antibiotic prophylaxis (e.g., cefoxitin) for high-risk patients, that is, those with a history of pelvic inflammatory disease and/or endometriomas. It has been shown that endometriomas are a risk for abscess formation postretrieval.[50, 51] Postoperative complications are reported to occur in 0.3–3% of cases.[52] The most common complication postretrieval is pelvic infection.[53] Bleeding is also a risk and may result from injury to uterine, vaginal, infundibulopelvic, or iliac vessels as well as from the ovary itself, the vascularity of which is increased during stimulation. Injury to abdominal viscera is extremely rare but possible. In addition, anesthesia carries some inherent risks.

Oocyte maturity

Oocyte maturity has previously been classified on the basis of the oocyte-corona-cumulus complex. However, correlation between nuclear maturity and expansion of the cumulus cells is imperfect. Oocyte maturity grading is important for the timing of insemination and particularly critical for the timing of ICSI. Failure to incubate the sperm with the oocyte at the appropriate time may lead to poor fertilization. Approximately 20–30% of oocytes are meiotically immature at the time of harvest, reflecting disparities in the synchronization of follicle development at the time of hCG administration.[54] Generally, a small cohort of immature follicles is acceptable because prolongation of stimulation may lead to postmaturity of the leading follicles, in addition to an increased risk of OHSS.

The primary oocyte is arrested at prophase I of the first meiotic division until ovulation. The administration of hCG allows resumption of meiosis I. A maturation score can be developed from the size of the follicle, expansion of the cumulus mass, radiance of the corona cells, size/cohesiveness of associated granulosa cells, and shape/color of the oocyte. Alternatively, if the cumulus mass has been mechanically and/or enzymatically removed, as in the case of ICSI, the oocyte can be graded according to the presence or absence of the first polar body and germinal vesicle.

A metaphase II oocyte is a preovulatory oocyte that has extruded the first polar body and is in the resting phase of meiosis II. The first polar body may remain attached to the ooplasm through a cytoplasmic bridge. The cumulus cells are typically expanded and luteinized, and the corona radiata is in a sunburst configuration. A metaphase I oocyte is of intermediate maturity, with more dense cumulus cells and no evidence of the first polar body, but the germinal vesicle (nuclear membrane) and nucleolus have faded. A late metaphase I oocyte may require between 1 and 15 hours of incubation prior to insemination, whereas an early metaphase I may require 15–42 hours. Periodic examination of the oocyte is important to document extrusion of the first polar body. A prophase I oocyte is grossly immature, with a compact corona and only a few layers of cumulus cells. Here the germinal vesicle and nucleolus can be easily visualized. Dissolution of the germinal vesicle indicates the resumption of meiosis I. On the extreme end of immaturity, prophase I oocytes yield significantly reduced pregnancy rates, despite the fact that with additional incubation, more than 80% will mature to metaphase II.[55, 56, 57] If inseminated at the prophase I stage, the sperm will fail to decondense. Polyspermia may also result from fertilization of either postmature or immature oocytes.

In vitro insemination and fertilization

As described previously, harvested oocytes exhibit various stages of maturity and therefore require varying intervals of preincubation, up to 36 hours, before insemination. For mature oocytes (metaphase II), the oocytes are incubated briefly and insemination is performed at approximately 4 hours

(range 2–8 hours) following retrieval.

A semen sample should be obtained by masturbation just before or after retrieval. It is usually collected in a sterile plastic jar or a Silastic condom. The sample is allowed to liquefy at room temperature before preparation. Two methods are commonly used for sperm preparation: the swim-up method or the gradient centrifugation method. Several studies suggest that the gradient method may be superior for semen with abnormal parameters.[58, 59] The objective is to isolate a highly motile fraction of sperm for insemination. The highly motile fraction is then incubated in a high-protein supplemented media for 30 minutes to 4 hours to initiate capacitation.

Generally, each oocyte is incubated with between 50,000 and 200,000 motile sperm for a period of 12–18 hours at 37°C, 5% CO_2 in air, and 98% relative humidity. The acrosome reaction, which is necessary for the spermatozoa to penetrate the zona pellucida, is initiated by contact between the zona pellucida and the sperm. Exocytosis of cortical granules from the ooplasm (cortical reaction) causes the zona pellucida to become relatively refractory to polyspermy. Occasionally, incubation with greater than 200,000 sperm per oocyte is undertaken in male factor cases to improve fertilization rates. This practice can result in a higher incidence of polyspermy.

Sperm penetration of the oocyte induces oocyte activation and initiates the second meiotic division, which then separates the chromatids between the oocyte and the second polar body. Oocytes are evaluated for fertilization at 18 hours postinsemination. The presence of two pronuclei, one each from the oocyte and the spermatozoa, and two polar bodies in the perivitelline space indicates normal fertilization.

Pre-embryos must be carefully inspected for the presence of extra pronuclei (i.e., three pronuclei is indicative of triploidy) because polyploid embryos may otherwise cleave normally and be undiagnosed at later stages. Polyploidy occurs in 5–10% of IVF embryos, with 1–2% in mature oocytes and up to 30% in immature oocytes.[60, 61] In addition to polyspermy, polyploidy may result from digyny, with origin of the extra chromosomal complement from the oocyte, which may occur owing to meiotic spindle errors or failure to extrude a polar body.[62] These events are more common in aging oocytes or immature or postmature oocytes.[63] ICSI may result in a polyploid embryo owing to digyny (retention of the second polar body).

The process of fertilization takes approximately 24 hours and is completed with the initiation of the first mitotic cleavage.

Embryo transfer

Embryo transfer is most commonly performed after 72 hours (day 3 postretrieval). Blastocyst transfer is generally performed at 120 hours (day 5 postretrieval). Blastocyst transfer is detailed later; the principal advantage of blastocyst transfer is the replacement of fewer embryos (generally one or two), given their apparent higher implantation potential. Transfer of fewer day 3 embryos reduces the incidence of higher-order and twin multiple gestations. In our experience, transfer of two day 5 blastocysts results in a comparable rate of twins but fewer triplet pregnancies.

Pre-embryos transferred on day 3 have generally cleaved to six to eight cells. Techniques for grading of the quality of embryos vary from center to center. Morphologic characteristics utilized in most grading schemes include cell number, symmetry and shape of the blastomeres, the presence of cytoplasmic fragmentation in the perivitelline space, and rate of cleavage.[54] The grading system employed at Cornell is based on the morphology of the cleaving pre-embryos as follows: grade 1, pre-embryos with blastomeres of equal size and no cytoplasmic fragmentation; grade 2, pre-embryos with blastomeres of equal size and minor cytoplasmic fragmentation covering 10% or less of the pre-embryo surface; grade 3, pre-embryos with blastomeres

of distinctly unequal size and variable fragmentation; grade 4, pre-embryos with blastomeres of equal or unequal size and moderate to significant cytoplasmic fragmentation covering greater than 10% of the pre-embryo surface; and grade 5, pre-embryos with few blastomeres of any size and severe fragmentation covering 50% or more of the pre-embryo surface.

The objective of embryo transfer is to maximize the chance for pregnancy while limiting the number of multiple gestations. Both of these outcomes are directly correlated with the number of pre-embryos transferred.[64] The optimal number of embryos to transfer is individualized, based on the patient's expected implantation rate per embryo. Maternal age and embryo quality are important factors determining the implantation potential for each embryo.[65] Some centers calculate the number of embryos for transfer on the basis of a cumulative embryo score. The cumulative embryo score is derived from morphologic analysis of the embryo as well as the number of blastomeres. Maternal age is also an important predictor for implantation potential. Fewer embryos are generally transferred to younger patients and some authors advocate the replacement of only a single embryo in these cases; this cannot be universally applied to all patients, given different anticipated implantation and pregnancy rates.[66]

Schoolcraft and associates published a summary of the literature regarding variables that can affect the success of embryo transfer.[67] Whereas much of the literature is based on retrospective observational data, this paper attempts to address such issues as bed rest posttransfer, physician factor, catheter type, loading of the catheter, placement of the catheter tip, trial transfer, uterine contractions, effect of blood or mucus on or in the catheter, and perceived difficulty of transfer. Frank blood on the catheter may be an indicator of endometrial trauma.

Uterine contractions are known to decrease in frequency later in the luteal phase. Fanchin and coworkers sonographically quantified uterine contractions during embryo transfer and noted a negative correlation with pregnancy rate.[68] Blastocyst transfer is potentially advantageous because day 5 is normally when the pre-embryo enters the uterine cavity, and therefore, uterine contractions are already reduced. Catheters that touch the fundus during transfer increase the frequency of uterine contractions. Ultrasonography at the time of transfer has been suggested to improve implantation rates, but randomized controlled trials are lacking.[69] It is likely that ultrasound guidance is most useful for difficult transfers, as occur with a tortuous cervical canal.

The transfer is generally performed with the patient in the dorsal lithotomy position. The cervix is cleansed with transfer medium, and a mock transfer may be performed to ensure accessibility of the cavity and the correct bend, if necessary, of the catheter. We perform most transfers with a soft catheter. Soft catheters have been shown to be associated with less local trauma and higher pregnancy rate.[70, 71] Embryos are loaded into a sterile catheter in a small volume (20–50 µL) of transfer medium (75% serum concentration). The catheter is gently advanced through the cervix toward the fundus but stopped before the fundus is reached. A trial transfer guide aids this process. The pre-embryos are then slowly injected using a syringe attached to the catheter. The catheter is then microscopically examined and flushed to ensure that no pre-embryos are retained. The patient is transferred to a holding area, where she remains supine for 30 minutes or more. The interval of rest following transfer does not appear to be clinically important.

Zygote intrafallopian tube transfer (ZIFT) and gamete intrafallopian tube transfer (GIFT) entail the laparoscopic transfer of the zygotes and oocytes/sperm, respectively. At one time, these techniques were advocated as being more successful than IVF for patients with normal fallopian tubes, but advances in the IVF laboratory have all but relegated these procedures to the archives.

Blastocyst transfer

The first human IVF pregnancy was achieved after blastocyst transfer.[1] Since then, most transfers have been performed with day 2 or 3 pre-embryos owing to difficulties in successfully maintaining pre-embryos in culture to the blastocyst stage. Newer sequential media have led to renewed interest in blastocyst transfer.[72]

Compaction of the pre-embryo usually occurs at the 8–16-cell stage. Before the 8-cell stage, assessment of the pre-embryo is preliminary because the embryonic genotype has not yet been activated. Therefore, it is difficult to specifically predict pregnancy success rates from a day 3 pre-embryo assessment.

The development of effective sequential media has enhanced the culture of pre-embryos beyond the day 3 stage. The cavitating morula forms a blastocele cavity with an inner and outer cell mass composing the blastocyst. There are several reasons why blastocyst transfer may be more successful than day 3 pre-embryo transfer. As mentioned previously, the developmental potential of a given embryo can be more fully documented on day 5. Transfer on day 3 introduces the pre-embryo into the endometrial cavity earlier than would occur in a natural cycle, which could lead to dysynchonicity between the pre-embryo and the endometrium.[73] In addition, uterine contractions may decrease by day 5.

Other advantages to blastocyst transfer include excellent implantation rates in good prognosis patients, an extended window of opportunity for ancillary procedures such as PGD, and a decrease in the number of embryos transferred owing to better implantation rates. A recent meta-analysis demonstrates a significantly higher rate of live births per couple with blastocyst transfer (36.0% vs. 29.4%, OR 1.35, CI 1.05–1.74), while the multiple-pregnancy rate, high-order multiple pregnancy rate, and miscarriage rate are similar.[74] Disadvantages of blastocyst transfer include a decreased rate of embryo freezing, failure to transfer any embryos per couple, increased rate of monozygotic twins, and altered sex ratio of infants.[73, 75, 76]

Patients who have a good response to stimulation and at least four good-quality pre-embryos on day 3 may be good candidates for blastocyst culture; it should be noted that generally fewer than 50% of *in vitro* fertilized oocytes will attain the blastocyst stage even in sequential media. The debate is ongoing as to whether patients with poor prognosis might benefit from blastocyst culture. For pre-embryos with a poorer prognosis (i.e., slower cleavage rates, more fragmentation, irregular-shaped blastomeres), earlier placement in the uterus may be advised.

Luteal phase support

Centers vary in their approach to management of the luteal phase. The prevalent practice is to provide luteal phase support in the form of either supplemental hCG or progesterone until sonographic documentation of pregnancy and placental progesterone production (approximately 7–8 weeks' gestation).[77]

There are several theories behind luteal phase support for IVF cycles. One theory is that the supraphysiologic estradiol levels, due to controlled ovarian hyperstimulation, require counterbalance with supplemented progesterone. Aspiration of follicles may debulk some of the granulosa-theca cells destined to produce progesterone, but the presence of multiple potential corpora lutea may counteract this effect.

Luteal phase support may be accomplished with additional hCG injections or daily progesterone supplementation. With the former approach, the risk of OHSS is increased because exogenous hCG restimulates the ovaries. Progesterone supplementation may be administered once daily as an intramuscular dose of 25–50 mg, or it can also be given as oral or, more commonly, vaginal, micronized progesterone, for example 100–200 mg three times daily.

Studies to date have suggested superior efficacy of intramuscular progesterone.[78, 79] However, it is possible that vaginal progesterone could be as effective as intramuscular progesterone if the timing of the first dose is adjusted. Vaginal progesterone produces higher local uterine concentrations of progesterone compared with intramuscular progesterone.[80] Thus, the initial dose given the evening postretrieval may induce levels that shift vis-à-vis endometrial receptivity for the embryo transfer. Patients who experience local reactions to intramuscular progesterone in oil may be switched to vaginal progesterone after the initial doses.

ANCILLARY TECHNIQUES AND MICROMANIPULATION

Embryo coculture systems

Standard culture media for human pre-embryos are typically formulated to mimic human tubal fluid. In a natural cycle, the oocyte is usually fertilized in the distal third of the fallopian tube. Ham's F-10 and HTF were media commonly used in US IVF programs. Maternal serum or protein substitutes are often used to supplement the media. A sequential media system using Gardner1/Gardner2 media has replaced the traditional monoculture system using Ham's F-10 and HTF as the media of choice. The sequential media system has been shown to better support the growth of blastocyst as well as improve implantation rate.[81, 82, 83] Embryo coculture is an attempt to decrease fragmentation and to improve cleavage and implantation rates by coincubation of pre-embryos with another *in vitro* cell system.

The initial concept for coculture was to utilize tubal epithelial cells in the dishes. However, harvest and culture of tubal cells are impractical. Bovine uterine cells were used, as well as several alternative cell systems from other species (e.g., rats, monkeys). We have used biopsied autologous endometrial cells. A good mix of glands and stroma is preferred. Clinical trials suggest that coculture may be of particular benefit to patients with poor IVF prognosis, particularly those who have failed multiple previous cycles.[84, 85, 86] Nevertheless, the overall benefit of coculture is still controversial and awaits larger randomized, controlled trials.[87, 88] Current US Food and Drug Administration (FDA) regulations have significantly limited the application of nonautologous coculture systems, for example, utilizing cells from other species, given the concern for transmission of potential infectious agents.

Assisted hatching

Observations of improved implantation rates in pre-embryos that had undergone partial zona drilling led to the concept of assisted hatching (AHA).[89] AHA involves the thinning or focal disruption of the zona pellucida just prior to embryo transfer. The objective of this procedure may be twofold. It may improve implantation rates for patients with a thick zona pellucida and allows removal of fragments from the perivitelline space. Such fragments have been correlated with embryo implantation failure (although they may be increased with chromosomal abnormalities, this correlation is not perfect). AHA may be performed early or late (two-cell embryo to a blastocyst), but it is generally performed on day 3 embryos. The size of the zona gap should not be too small because it would interfere with escape of the embryo from the zona pellucida. Complications of AHA include embryo loss at the time of transfer if the hole is too large. Also, if the hole is too small, the blastocyst may be trapped. The incidence of monozygotic twinning appears to be increased by AHA.[90] Studies have been conducted regarding the size of the aperture, and smaller disruptions are associated with better pregnancy rates

than larger disruptions.[91] In the early to mid-1990s, AHA gained popularity, especially for the following indications: advanced maternal age, poor reproductive history with IVF, poor ovarian reserve, poor embryo morphology, increased cytoplasmic fragmentation, and thick/abnormal zona pellucida. However, some more recent, small, randomized controlled trials have challenged this concept, and AHA at our institution is now performed less frequently and selectively, particularly for cases with significant fragmentation.[92, 93, 94, 95]

Assisted fertilization

During the last two decades, rapid advances have been seen in micromanipulation technology.

Prior to the advent of assisted fertilization, couples who suffered from severe male factor infertility experienced very limited success with IVF. The initial experiences with assisted fertilization involved zona drilling (ZD), partial zona dissection (PZD), and subzonal sperm injection (SUZI). ZD and PZD involve disruption of the zona pellucida followed by the usual IVF insemination techniques. SUZI is the insertion of several motile sperm under the zona of the oocyte. The objective of these techniques was to improve fertilization in men with oligospermia. These procedures are of historic interest owing to polyploidy, relatively low fertilization rates, and the subsequent introduction of ICSI.

The first human births from ICSI were reported in 1992.[96] ICSI involves the injection of a single, viable sperm into a single oocyte. The development of ICSI has revolutionized the treatment of male factor infertility. Previously, semen concentrations of less than 5×10^6 sperm/mL were associated with poor IVF outcome.[97] With ICSI and, more recently, advances in sperm retrieval directly from the testes or epididymis (testicular sperm extraction [TESE], microsurgical sperm aspiration [MESA]), even men with obstructive or nonobstructive azoospermia have the potential to fertilize oocytes and father their own genetic offspring.

The indications for ICSI are varied and differ slightly from center to center.[98] In general, they include (1) previous failed IVF fertilization, (2) sperm concentrations less than 2–5 x× 10^6 sperm/mL, (3) motility less than 5%, (4) normal morphology less than 4% by Kruger's strict criteria, (5) use of surgically retrieved and, therefore, a relatively limited number of immature spermatozoa, and (6) PGD because conventional insemination techniques may result in extra spermatozoa attached to the zona and therefore contaminate the sample for polymerase chain reaction (PCR) diagnosis. There is a general concern that rescue ICSI, to fertilize oocytes that have not been fertilized by conventional IVF insemination methods, is relatively ineffective.[99] At Cornell, ICSI is used only for research purposes because it introduces the risk of fertilizing aged oocytes.[100]

The factors that affect the success of ICSI have been shown to be relatively independent of the semen parameters and to be mostly dependent on factors of egg quality such as maternal age. Nevertheless sperm viability is critical and can be difficult to document in the absence of motility.

The procedure involves two stages: (1) oocyte processing to remove the corona radiata and the cumulus mass with nuclear grading and (2) microinjection of a single spermatozoa. Nagy and associates reported that success rates are improved if the sperm is injected close to the mitotic spindle.[101] Generally, the oocyte is stabilized with the polar body at the 6 or 12 o'clock position, and the spermatozoa injected at the 3 o'clock position, following immobilization of the sperm by compression of the sperm tail. Because the process of gamete fusion is bypassed with this procedure, a method to initiate oocyte activation is necessary; stimulation of the ooplasm by pipette suction and reinjection has been shown to accomplish this.

Complications from ICSI include those associated with IVF in general (i.e., perioperative complications, OHSS, and multiple births) and those specific

to the ICSI procedure. The latter include (1) oocyte infection from disruption of the corona radiata, cumulus mass, and zona pellucida; (2) oocyte damage – in experienced hands, this is rare, but oocyte disruption may occur in 7–14%; (3) concerns regarding the possible induction of congenital anomalies by insult of the ooplasm and by sperm selection. We recommend prenatal diagnosis (amniocentesis, chorionic villous sampling) for those patients who undergo ICSI. The incidence of sex chromosomal abnormalities appears to be increased after ICSI, especially in cases of male infertility.[102] Fifteen per cent of azoospermic males have chromosomal abnormalities such as 47,XXY (Klinefelter's). Also, there is a higher incidence of aneuploidy in oligospermic men (3–6%). Y microdeletion studies of azoospermic or severely oligospermic men have also identified a higher incidence of nonkaryotypic genetic abnormalities (up to 13%). The partners of male patients with congenital unilateral or bilateral absence of the vas deferens should be screened for cystic fibrosis, as should the partners of patients with idiopathic epididymal obstruction because, a significant proportion of the time, these men are carriers of the cystic fibrosis mutation.

Much attention has been focused on the outcome of ICSI pregnancies. Several initial reports did not observe untoward neonatal effects from ICSI; an update of a study done at Cornell again confirmed these findings.[103, 104] This study also suggested a lower incidence (0.17%) of sex chromosomal abnormalities than the previous *Lancet* article.[102] Palermo and colleagues[104] did not observe a higher incidence of other chromosomal abnormalities, spontaneous abortions, or congenital anomalies when compared with IVF or general population statistics for women of similar ages. Bowen and coworkers evaluated, at 2 years of age, medical and developmental outcome of children born from ICSI.[105] They compared children born from ICSI, IVF, and natural conception. Although there were no significant health problems and the mean Bayley MDI for development was within the normal range for most children, the ICSI subset did have a significantly lower score than the IVF and natural conception group. Fathers of ICSI children were found to be significantly different from the other groups in that the former were more likely to hold "unskilled occupations." Nevertheless, when the subgroup analysis was done, a significant difference remained. Another study suggested that the incidence of major birth defects is increased after ICSI.[106] Nevertheless, this study did not differentiate major from minor birth defects and was subject to multiple biases characteristic of observational data, such as surveillance bias and confounding, as well as lack of an appropriate control group. Musculoskeletal and urogenital defects, the latter of which might be expected in the offspring of subfertile males, were the two areas in which the malformation rate appeared to be higher.

Cryopreservation

The first pregnancy from a cryopreserved human conceptus was reported in 1983. Advances in the success of cryopreservation have significantly improved the efficiency and safety of IVF. Approximately 60–70% of pre-embryos survive the thaw process. In most centers, once the conceptus has survived the thaw process, the success rate of a frozen-cycle transfer is approximately half that of a fresh-cycle transfer. However, this is not reflected in the SART statistics. Success of frozen-cycle transfers increases the cumulative pregnancy rate per retrieval.[107, 108] In countries in which the governments limit the number of pre-embryos to be transferred, authorities recommend limiting the number of pre-embryos transferred to one or two, with the remainder of the conceptuses being frozen for later cycles. Success of cryopreserved pre-embryos achieved through assisted fertilization (ICSI) is not compromised compared with standard IVF insemination techniques.[109] Patients who are identified as having a significant risk for OHSS may benefit from cryopreservation of all pre-embryos with transfer in a later frozen cycle since OHSS rates and severity are greater in stimulated conception cycles.[110] Conceptuses may be frozen safely for at least 7 years and perhaps indefinitely.[111] Frozen cycles for which the fresh transfer was successful are also more likely to be successful. Recently, oocyte cryopreservation has been reported. [112, 113] Unforunately, the clinical pregnancy rate using cryopreserved oocyte is still low and thus, should only be used in special circumstances.

Conceptuses may be frozen at any stage from prezygote (prior to cleavage) to blastocyst. Intuitively, those embryos that survive culture longer and develop well may be more likely to result in pregnancy. Nevertheless, at least two studies have suggested better success rates with cryopreservation of zygotes.[114, 115] Recently, a successful experience with refreezing of thawed pre-embryos has been reported.[116]

The process of cryopreservation involves two stages: freezing and thawing. Objectives of freezing are to avoid ice crystallization of intracellular water, which can lead to cellular damage. The methods of freezing and the cryoprotectants used depend on the stage of development, which affects cellular permeability. A cryoprotectant (e.g., dimethyl sulfoxide [DMSO], 1,2-propanediol glycerol) replaces the intracellular water by osmosis to prevent intracellular seeding of ice crystals. The cryoprotectant is added by pipetting the embryos through gradually increasing concentrations of the agent. Conceptuses are then sealed in glass ampules and cooled to temperatures between −30°C and −110°C with storage in liquid nitrogen. The higher temperature should be achieved quickly because ice formation is more likely, whereas the lower temperature is attained more slowly. Thawing is achieved by reversing the process. The cryoprotectant is removed sequentially by decreasing the concentration, and the embryos are cultured for an interval pretransfer.

Frozen pre-embryos may be transferred into the endometrial cavity in a monitored natural cycle for patients with normal ovulatory function. For patients without regular cycles, ovulation may be suppressed and the cycle programmed with the use of exogenous estrogen and progesterone. The transfer is timed so that the endometrium is synchronous with the embryonic age of development as per the number of days postinsemination. In appropriately selected patients, success rates appear comparable.[115]

Preimplantation genetic diagnosis

PGD has become an indication for IVF. PGD allows diagnosis at three levels: sex chromosome abnormalities/aneuploidy, structural chromosomal abnormalities, and single-gene diagnosis. The first reported cases of PGD were undertaken for sex determination of embryos to prevent transmission of X-linked genetic disorders. These initial cases were reported in 1989. Subsequently, PGD was used to prevent single-gene disorders such as cystic fibrosis. The two most common single-gene disorders diagnosed by PGD are cystic fibrosis and sickle cell disease.[117] Whole-genome amplification with comparative genomic hybridization has been used to derive the entire karyotype, but the results are still preliminary and the procedure is lengthy.[118] Thus, the indications for PGD have been expanded to include the diagnosis of embryo aneuploidy in women of advanced maternal age, previous IVF failures, and history of previously affected embryos or offspring. Diagnosis of structural chromosomal abnormalities in couples with balanced translocations is also possible with PGD, particularly in the treatment of recurrent miscarriage. Interestingly, a recent, randomized, double-blinded, controlled trial conducted by Mastenbroek *et al.* demonstrates that ongoing pregnancy rate and live birth rate in advanced materal age women undergoing IVF with preimplantation genetic screening for aneuploidy are significantly reduced in comparison to those without screening.[119] With 408 women undergoing a total of 836 IVF cyles, the ongoing pregnancy rate and live birth rate in the screening group vs. control group were 25% vs. 37% and 24% vs. 35%, respectively.[119]

Although current evidence does not support the use of PGD for aneuploidy screenning, PGD has many potential advantages. It can limit the occurrence of known lethal or severely disabling inherited genetic diseases and, potentially, even completely remove these diseases from a familial lineage. PGD can be used to identify some specific causes of recurrent implantation or pregnancy failure. This ability may serve two purposes: (1) it may help resolve issues for such couples who have been unable to succeed with reproductive technology and allow them closure so that they may decide to proceed with

alternatives such as oocyte donation or adoption; (2) once we are able to identify a particular disorder, we may be able to improve the implantation and ongoing pregnancy rates by transferring only those embryos that are genetically normal. This reduces the emotional burden of recurrent miscarriages for affected couples. Another advantage of PGD is the ability to avoid later pregnancy termination in patients who have a high risk of genetically abnormal conceptuses by preventing transfer of an identified genetically abnormal embryo. Many couples may consider PGD a preferable alternative to prenatal diagnosis and pregnancy termination. PGD to avoid a particular disease, for example, Fanconi's anemia, while at the same time producing an offspring who is human leukocyte antigen (HLA)-compatible with an affected sibling is also possible and has been reported.[120]

PGD has several potential limitations. PGD opens many ethical controversies with regards to selection for traits that do not specifically affect the health of offspring. Also, with the availability of HLA typing, the possibility exists of couples reproducing for the express purpose of providing a tissue donor or rescue sibling for treatment of an affected sibling. PGD has several technical limitations. The technology has been mostly limited to centers that are expert in both molecular genetic and reproductive technology, although collaboration between centers has been reported. The relatively small number of PGD cycles completed and the pregnancy success rate (which rarely exceeds 30%) have limited the availability of outcomes studies. This has several implications. Conventional prenatal diagnosis is still recommended to confirm the accuracy of PGD. PGD may be likened to chorionic villous sampling in that error is possible through several mechanisms. For single-gene defects, PCR is used to amplify the genetic signal. Contamination from several sources is possible, including the maternal cumulus cells, copies of paternal DNA available from extra sperm attached to the zona pellucida (ICSI is therefore utilized); and the operator or any contacts with the PCR procedure may contribute exogenous DNA. Also, the DNA may be obtained at different stages: from the polar body, from the cleavage stage (six- to eight-cell) embryo or from the blastocyst trophoectoderm. The sources may not accurately reflect the DNA constitution of the remainder of the embryonic cells, for example, mosaicism may exist, similar to that seen with chorionic villous sampling. For single-gene diagnosis, allelic dropout poses a threat of misdiagnosis. A limited number of live-born infants have been available to ensure that there are no long-term consequences of these procedures and not all outcomes have been reported.[121, 122, 123]

The diagnoses are also limited by incomplete identification of all possible mutations. For example, for a single-gene defect such as cystic fibrosis, the most common allelic mutation is the deltaF 508; however, several other mutations may lead to compound heterozygotes who also may be affected. The ability to identify these compound heterozygotes depends on the availability of the appropriate probes and the fact that certain mutations have not yet been identified. Also, when one is searching for structural abnormalities, the error rate for aneuploidy may be as high as 15%. Generally, one or two blastomeres are removed from a cleavage-stage embryo and multiple probes for different chromosomes are used in the same recycled blastomere, for example, 13, 16, 18, 21, X, and Y. Generally, the number of probes that can be used on a single cell is limited to approximately seven. Therefore, only those numeric chromosomal abnormalities related to the tested chromosomes can be identified. Errors due to background signals, weak signals, and the like, can interfere with the diagnosis. Often, polar body biopsy is used for preimplantation genetic screening (PGD of aneuploid embryos in couples with no specific family history but who may have, for example, advanced maternal age) because the abnormal chromosomal complement usually comes from the mother. Nevertheless, errors in diagnosis can occur because of recombination events or because the paternal complement has not been examined. The number of embryos obtained also limits PGD. For example, a couple with advanced maternal age who have few embryos (e.g., <5) risks reduced viability of even a normal embryo from the procedure.

The two most common methods of PGD are first polar body biopsy and, more commonly, blastomere biopsy.[124] First polar body biopsy is often used for structural chromosomal analysis for anomalies of maternal origin because fertilization has not yet occurred. First polar body biopsy is often therefore

referred to as *preconception genetic diagnosis*. Blastomere biopsy may be performed at the six- to eight-cell stage and is necessary for assessment of disorders of paternal origin but is also more accurate for numeric, structural, and single-gene diagnosis. Theoretically, biopsy of one blastomere should represent all other embryonic cells at this stage, although embryonic chromosomal mosaicism can interfere with the diagnosis. Structural chromosomal analysis is generally achieved with florescent *in situ* hybridization (FISH) because the results are available in 12 hours. PCR is required for single-gene diagnosis.

OOCYTE DONATION

Oocyte donation has proved to be a successful option for women who cannot conceive with their oocytes owing to advanced age, diminished ovarian reserve, or genetic disease. Oocyte donation allows the female partner to carry and deliver a pregnancy with her husband's genetic contribution.

The success of oocyte donation is mainly limited by the age of the donor, who should optimally be younger than 35 years. Although endometrial receptivity may diminish somewhat with age, the contribution of this uterine factor appears minimal in comparison with oocyte quality.[125] For this reason, the optimal number of embryos to be transferred to the recipient is also principally determined by the donor's age rather than that of the recipient.[126]

Oocyte donors may be either known or unknown to the recipient. Known donors are often biologically related (e.g., sister or cousin). Because most donors, and particularly anonymous donors, are young, their oocytes and resultant embryos have a good prognosis. The risks of the ART procedure for the donor are confined to the risks associated with stimulation and retrieval because the donor does not carry the pregnancy. The primary risk of donation for the recipient is the transmission of infection. Despite the fact that the donors are screened at multiple intervals for infectious disease, the fact that fresh embryos are used, owing to lower implantation rates with frozen embryos, give rise to a small, theoretical risk of transmission of diseases such as human immunodeficiency virus (HIV), although such transmission has not been documented with oocyte donation. The surrounding cells are removed completely from the oocyte prior to insemination, and the oocytes are washed of any blood cells. In contrast to semen, isolated oocytes do not represent a leukocyte-rich source. Although this risk appears to be mainly theoretical, the alternative of cryopreserving and quarantining embryos resulting from donor oocytes should be discussed with recipients, including a discussion of relative implantation and survival rates with freezing and thawing conceptuses.

A key component of successful oocyte donation is synchronization of the recipient's menstrual cycle with the donor's cycle. In a recipient with intact ovarian function, this can be achieved through GnRH agonist down-regulation followed by hormonal support with exogenous estrogen and progesterone. The start date of exogenous progesterone administration is designated as day 15. The greatest success rates for cleavage stage embryos appear to occur with transfer on day 17, 18, or 19. A mock (prep) cycle may be undertaken to ensure that the recipient responds appropriately to hormonal support and that her endometrial lining develops adequately.

Estrogen and progesterone support is continued throughout the first trimester, until approximately 10 weeks, and is discontinued once adequate placental steroidogenesis is documented.

IN VITRO FERTILIZATION OUTCOME

IVF outcomes have improved throughout the years. In 2005, a total of 97,442 fresh, nondonor IVF cycles were performed in the USresulting in 33,101 pregnancy and 27,047 live births.[127] The overall clinical pregnancy rate was 34% (20.5% single-fetus prenancy, 11.2% multiple-fetus pregnancy, 2.3% pregnancy ended in early miscarriage). The ectopic pregnancy rate was 0.6%. The live birth rate per cycle, retrieval, and transfer was 28%, 32%, and 34% respectively. Although advancing maternal age has a negative effect on prognosis, analysis of outcomes over the past 3 years shows a steady improvement in outcomes for each of these maternal age groups: younger than 35 years, 35–37 years, 38–40 years, and older than 40 years. Factors that have been shown to affect outcome include maternal age, clinic size, the use of fresh versus cryopreserved embryos, and oocyte donation.[128, 129]

A major concern regarding reproductive technology has been an increased incidence of multiple births. As mentioned earlier, the success rate per cycle depends on the implantation rate per embryo. Both pregnancy rates and multiple birth rates are directly correlated to the number of embryos transferred. In 2005, of the 27,047 live births, the rate of singletons, twins, and triplets or more was 68.0%, 29.6%, and 2.4%, respectively. Morbidity and mortality are significantly increased in pregnancies complicated by multiple gestations. Approaches to decrease the number of multiple births may also decrease the number of successes per fresh IVF cycle, for example, by decreasing the number of embryos transferred. However, as success rates of cryopreserved embryos increase (19% overall but higher in some clinics), frozen cycles may become a more viable option to increase pregnancy yield per retrieval while still limiting the number of multiple births. Employment of blastocyst transfer also allows replacement of fewer embryos (e.g., two) but has been associated with a higher rate of monozygotic twinning. Serious debate and concern have been engendered over the issues of multiple gestations.[130, 131, 132]

Other complications that relate to IVF include ectopic pregnancies. The incidence of ectopic pregnancies appears to be lower than previously thought. The most recent report from SART cited an incidence of 0.6%. Little practical information is available regarding the overall incidence of heterotopic pregnancies from IVF. Most of the available data are in the form of case reports. We have recently reviewed our statistics and found an incidence of 0.18% heterotopic pregnancies. Interestingly, tubal disease was a risk factor in all of the heterotopic pregnancies, and the intrauterine pregnancies were all delivered successfully at term following surgical intervention for the ectopic pregnancy. Early monitoring with ultrasound surveillance is important to diagnose heterotopic and ectopic pregnancies.

Data evaluating the outcomes of children born from IVF are available. One study specifically assessed the growth and physical and developmental health of children born from cryopreserved embryos compared with fresh IVF cycles and also compared these with spontaneous conceptions. There were no significant differences with respect to chronic illness, congenital anomalies, chromosomal anomalies, and neurologic or developmental health among the three groups. However, statistically significant differences were found for growth in the cryopreserved embryo group. The authors concluded that these small differences, however, were not clinically significant.[133] Three other cohort studies have suggested that outcomes of IVF children, in some cases, specifically ICSI children, may be different from spontaneous conception with regards to neurologic development, congenital anomalies, and low birth weight.[106, 134, 135] As with most observational data, however, these studies suffer from multiple biases including surveillance bias, confounding, reporter bias, and misclassification, and lack of an adequate control group. One consistent finding that is concerning, however, is that singleton births from IVF do appear to have lower gestational ages and birth weights compared with those from spontaneous conceptions.[135]

Available data concerning the link between ovarian cancer and ovulation induction have proved to be reassuring. Several meta-analyses have shown that the risk appears to be related more to infertility than to the use of fertility drugs.[136] In fact, a meta-analysis by one of the authors suggests that women who suffer from infertility may be protected from ovarian cancer if they conceive with therapy.[137] Preliminary data from a large, prospective, cohort study conducted with the National Institutes of Health are also reassuring.

CONCLUSIONS

ART has evolved immensely during the past two decades. The development of GnRH antagonists, sequential media for blastocyst culture, assisted fertilization, and advances in cryopreservation have contributed to the increased success rates of IVF. In addition, ancillary techniques such as PGD allow us not only to effectively treat infertility but also to prevent potentially devastating and tragic diseases. Oocyte donation allows couples who cannot conceive with their own oocytes to gestate and deliver a pregnancy.

ART combines both art and science. Meticulous clinical and laboratory management are both required to maximize the potential of the existing technology.

Future directions such as cytoplasmic transfer, nuclear transfer oocyte reconstitution, and oocyte/ovarian tissue freezing are on the horizon. No doubt, the next decade will also prove exceedingly fertile in our advances to alleviate the suffering of couples with infertility.

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