
THE FUTURE OF ASSISTED REPRODUCTION

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The past three decades have witnessed revolution in reproductive medicine. Since the birth of Louise Brown in 1978 (1), there has been an expansion in the number of vitro fertilization treatment worldwide. In the first European Register publication (2) 203,893 IVF/ICSI were reported by eighteen European countries, and by 2002, this number rose to 324,238 cycles from twenty-five countries, accounting for almost 60 percent increase of registered cycles (3). In the last World IVF report from 2000, 460,157 cycles were carried out in forty-nine countries, and it was estimated that approximately 200,000 babies were born (4). Although neither European nor World coverage is complete regarding the register data, the expansion of IVF is evident, and the estimate is that more than three million children have been born as a result of assisted reproduction since the beginning.

The driving force of this medical field has always been better treatment outcome. Increasing the efficiency of the treatment is what the future holds for us.

DEFINITION OF SUCCESS

What is the definition of success in assisted reproduction? A debate was started in *Human Reproduction* in 2004, with the suggestion of Min et al., to define success as “BESST – birth emphasizing a successful singleton at term” (5). This sparked discussion of many renowned international groups, and a number of definitions were introduced – healthy lower order birth (6), number of elective single embryo transfers per center (7), and value of cryopreservation programs (8). Danish group suggested that reporting the number of oocytes, implantation rate, and number of deliveries per embryo transfer would cover all steps in ART: stimulation, laboratory, that is, in vitro and embryo transfer/outcome phase (9). Heijnen et al. went a step further and emphasized the need to focus on the whole treatment rather than on single cycle and to report success as singleton birth per started IVF treatment or per given period (10).

All these approaches have a common goal: to increase the efficacy and safety of the treatment on one hand while to decrease the risks on the other.

The future of assisted reproduction lies in this goal, and the accomplishment of it involves optimization of each treatment phase starting with ovarian stimulation through laboratory procedures, selecting the best embryo for transfer, embryo transfer, luteal phase support leading to pregnancy, and the birth of a healthy singleton baby.

OPTIMIZATION OF OVARIAN STIMULATION PROTOCOLS

GnRH Agonists

Ovarian stimulation has come a long way since its first attempts. In an elegant recollection of the past by Professor Howard Jones (11), he looks back at the beginning of IVF and the obstacles encountered on the path of improvement. In 1980, in their center in Norfolk, forty-one aspirations were carried out in a natural cycle, with thirteen transfers and no pregnancy. At the same time in Melbourne, there had been forty-eight transfers following stimulation with clomiphene citrate, resulting in three pregnancies, all of which ended in miscarriages with no term deliveries. By 1981, with ovarian stimulation, there had been no term deliveries anywhere in the world in normal menstruating women with stimulation combined with IVF (12). Jones’s group started using Pergonal in 1981 and in their thirteenth attempt were finally successful.

GnRH agonists have changed the course of ovarian stimulation for in vitro fertilization. Since 1984, they have been used to prevent premature surge of LH during controlled ovarian hyperstimulation (13). Profound stimulation regimens have been introduced with a large number of oocytes as the desirable outcome.

Amounting evidence grew with the clinicians experience, causing a shift in the attitudes, from an aggressive approach where a large number of oocytes is considered a criterion of success to a more moderate approach.

What is the optimal number of oocytes retrieved? Increasing number of oocytes gives rise in pregnancy rates, but it eventually levels off (14,15), while side effects and risks continue to increase. OHSS is a well-known short-term risk of COH, with the incidence of 2–5 percent (16). Evidence has arisen showing potential detrimental effect of COH on endometrial receptivity and embryo implantation (17,18). Currently, it is clinically accepted that appropriate ovarian response is achieved with retrieval of five to fourteen oocytes (19).

Figure 74.1 (20) illustrates this concept, from an ideal point of view that patients should be in the high-benefit, low-risk window.

Dutch group has proved this concept in the population of almost 7,500 women, showing that the mean number of oocytes associated with the highest chance of conceiving per embryo transfer (PR/ET) and per started cycle (PR/C) was 13.1 (Fig. 74.2). After this number of oocytes, the pregnancy rates level off

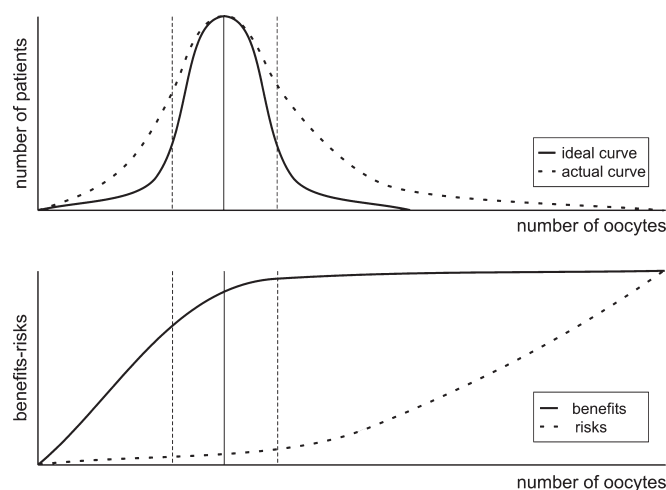


Figure 74.1. Distribution of oocytes, benefits and risks – the present and the ideal situation. Popovic-Todorovic et al. (20).

and this is not due to the embryo transfer rate since it remains stable at 93–95 percent when four or more oocytes were obtained (21) (Fig. 74.2).

In practice, COH is not always “controlled,” and a range of inappropriate ovarian responses is often present. At one end of the spectrum, we have inadequate response with retrieval of few oocytes, and increased treatment cancellations, and on the other end, a proportion of exaggerate responses is observed increasing the risk of OHSS. The variability of responses may be due to inherent biological mechanisms in relation to differences in the number of recruitable follicles, follicle sensitivity to FSH, and pharmacodynamics, but it may also be due to factors that may be predicted and at least partially controlled.

During recent years, data have accumulated showing that to some extent we are able to predict ovarian response, investigated factors being age (22), ovarian volume (19,23–27), antral follicle count (19,27–29), evaluation of stromal blood flow (19,28,30), cigarette smoking (31), hormonal markers assessment FSH (32,33), LH (34), inhibin B (35), and AMH (36–38).

How and to what extent is it possible to reduce the incidence of inappropriate responses by administering an appropriate starting dose of FSH?

Starting FSH Doses

Although COH has been in practice for many years, the optimal starting FSH dose has not been established since there have been no randomized controlled trials in the early IVF years (39). After introduction of the rFSH preparations, a number of studies have attempted to define an optimal starting dose (40–44). The doses range from 100 to 250 IU/day, reflecting the range of policies from “friendly IVF” with a minimal dose, to an approach where a large number of oocytes is considered criterion of success. Regardless of the dose used, a variability of responses is present, ranging from one oocyte at retrieval to more than thirty.

Most clinics have opted to use a “standard” dose for a “standard” patient who is below forty years of age, with two ovaries, a normal serum basal FSH, and a regular menstrual cycle. Dose adjustments are common clinical practice, higher doses being given to older patients. The cutoff value for

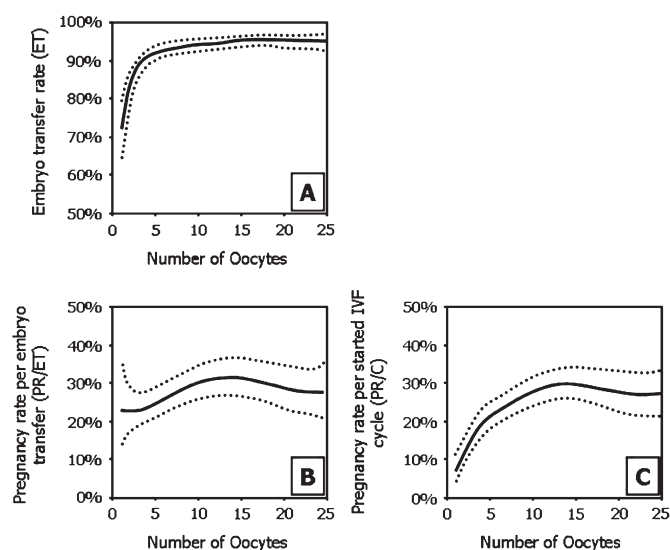


Figure 74.2. Optimum number of oocytes for a successful first IVF treatment cycle. The number of retrieved oocytes (mean with 95 percent CI) in relation to embryo transfer rate (A); pregnancy rate per embryo transfer (B); pregnancy rate per started IVF cycle (C). The optimal number of obtained oocytes to conceive is 13.1. Van Der et al. (21). Reprinted from an article in *Reproductive BioMedicine Online* by Van der Gaast et al., with permission from Reproductive Healthcare Ltd.

age is usually thirty-five years, that is, patients aged less than thirty-five years are given 150 IU/day, while those aged less than thirty-five years are started on a higher rFSH dose (usually 225–300 IU/day) (45).

Despite the fact that a lot of research has been carried out in order to establish the predictive factors of ovarian response, they have not been really used in developing dosage nomograms. Firstly, it was done in ovulation induction for PCO patients. The Dutch group has developed a model based on pretreatment clinical and endocrine and sonographic screening characteristics in order to predict FSH threshold dose in normogonadotropic anovulatory infertile women (46). The FSH threshold (75 to >187 IU/day) was determined on the basis of body mass index, presence or absence of resistance to clomiphene citrate, initial free insulin-like growth factor-I, and basal FSH.

There is only one prospective randomized trial that tested the use of a dosage nomogram in ‘standard’ patients comparing the individual dose, from 100 to 250 IU/day, based on the predictive factors versus a standard dose of 150 IU/day (20). Firstly, a prospective trial was conducted in order to establish the predictive factors of ovarian response in first IVF/ICSI treatment cycle of standard treated with 150 IU/day of rFSH (19). The constructed FSH dosage nomogram consisted of the following parameters: age, antral follicle count, ovarian volume, power Doppler score, and smoking status (19). The nomogram was subsequently tested, and the results showed that an individual dosage regimen in a well-defined first IVF/ICSI cycle standard patient population increased the proportion of appropriate ovarian responses and decreased the incidence of dose alterations during the course of COH. Although the trial was not designed to study a difference in pregnancy rates, higher ongoing pregnancy rate was observed in the individual dose group (20).

A retrospective analysis of eleven randomized phase II–IV trials has been performed in order to define the predictive factors of ovarian response by Howles et al. (47). Predictors were established by scoring their potential influence on a number of dependent variables – fourteen in total, although the primary outcome variable was the number of retrieved oocytes. A dosage calculator based on basal FSH, BMI, age, and antral follicle count is currently being tested.

It is necessary to have this issue further explored; a number of different nomograms have to be tested in prospective randomized trials in order to have tailor-made starting FSH doses already from the first treatment cycle.

GnRH Antagonist Protocols

GnRH antagonists have been introduced recently in ovarian stimulation for pituitary suppression. They compete directly with endogenous GnRH for receptor binding (48), their pharmacological effect being characterized by rapid and reversible blockade of pituitary GnRH receptors, and as such are used to prevent premature LH surges. The clinical acceptance of GnRH antagonists has been slow and mostly due to the initial meta-analysis (49), which has observed 5 percent difference in clinical pregnancy rates. This meta-analysis included five RCTs, and the difference in live birth rate was 3.8 percent higher in agonist cycles, but it is not statistically significant.

Initially, GnRH antagonists were often implemented in poor-prognosis patients, who have already had a number of unsuccessful trials, as was shown by the data from German registry (50). The last meta-analysis showed that among the patients treated for IVF with gonadotropins and GnRH analogues, the probability of live birth does not depend on the type of analogue used (51).

Antagonist protocols are novel compared to more than twenty years of agonist protocols, and optimization progresses with knowledge accumulation. Steroid levels in the antagonist cycles differ from the downregulated levels in the agonist cycles since the antagonist cycle is preceded by a luteal phase of the natural cycle. It was shown by Kolibianakis et al. (52) that a proportion of patients (5 percent) who exhibit elevated progesterone level at the onset of stimulation have a decreased probability of pregnancy in relation to patients with normal progesterone levels (5 vs. 31.8 percent). This issue needs to be further explored.

There have been only two prospective randomized trials, which compared the use of 150 versus 200 IU/day and 150 versus 225 IU/day in standard patients (53,54). Higher doses yielded more oocytes, but pregnancy rates remained the same. These studies were underpowered to assess the impact of starting dose alterations on the pregnancy rates. Prospective studies establishing predictive factors of ovarian response in antagonist cycles are needed. Furthermore, individual dosing approach has not been explored in GnRH antagonist protocols, and further optimization should evolve in tailor-made dose approach from the first treatment cycle.

Fixed antagonist protocol was introduced empirically on day 6 of stimulation since it was assumed that by that time there would be sufficient production of estradiol, which would induce premature LH rise. In a flexible protocol, onset of administration of antagonist is determined by the follicular size, usually when the leading follicle is 14–15 mm. Meta-analysis of four randomized controlled trials of fixed versus flexible

protocol shows a trend for increased pregnancy rates in the fixed protocol, although the power of the analysis is too low for definite conclusions (55). Another methodological issue is that by day 6, approximately 50 percent of the patients have a follicle of 15 mm (56), and that lowers the chance of detecting an existing difference between the two protocols.

Timing of the antagonist as well as timing of hCG administration require further exploration. Only one trial assessed the impact of delaying hCG and showed that prolongation of follicular phase has a negative impact on pregnancy rates (57). The use of GnRH agonists to trigger final oocyte maturation has a potential benefit in patients at risk for OHSS, but the current evidence suggests that it leads to lower pregnancy rates (58).

There is a need for a number of RCTs to explore further the issues of hormonal assessment at the beginning of the antagonist cycle, fixed or flexible antagonist administration, timing and doses, final oocyte maturation triggering as well as the use of GnRH agonists.

With increased knowledge and experience accumulation of the clinicians, GnRH antagonists will be increasingly used in clinical practice.

OVARIAN STIMULATION, ENDOMETRIAL RECEPTIVITY, AND LUTEAL PHASE

Although ovarian stimulation protocols have evolved, the influence of ovarian stimulation on the endometrial receptivity is not fully understood. Implantation involves a specific interaction of the human blastocyst and maternal endometrium. The window of implantation is defined as the period when the uterus is receptive, and it occurs eight to ten days after ovulation. The importance of the embryo quality has been demonstrated (59), but even when high-quality embryos are transferred (60), the increase in implantation rates levels off. Implantation failure remains the main limiting factor of the success of assisted reproduction.

Priming of endometrium toward the window of implantation is of maternal origin. High implantation and pregnancy rates in oocyte donation cycles irrespective of acceptors' age (61) imply that ovarian stimulation impairs endometrial receptivity in stimulated cycles.

Normal hormonal milieu and a normal endometrium give rise to a functional luteal phase. This is altered by ovarian stimulation at a number of levels. GnRH agonist theoretically could directly interact with GnRH-like peptide receptors in granulosa and theca cells and human endometrium (62). COH induces supraphysiological levels of steroids during follicular phase, resulting in advanced endometrial development regardless of the type of GnRH analogue used. In GnRH agonist cycles, endometrial biopsies taken in the preovulatory phase prior to hCG injection show accentuated proliferative aspects and early secretory changes even before rise in progesterone occurs (63).

Biopsies taken on the day of oocyte retrieval show endometrial advancement in more than 90 percent of the cases, with no pregnancy occurring if the advancement is exceeded by three days (64). These findings were confirmed in GnRH antagonist cycles (65).

Increased sensitivity to progesterone resulting in secretory advancement could be induced by elevated estrogen concentrations (66). Clinical studies in oocyte donation programmes show that increased estrogen levels have a negative impact on

implantation rates without affecting the embryo quality (67). Additionally, a step-down regimen improved pregnancy rates (68). Moderate responders exhibit less pronounced endometrial changes compared to high responders (69).

It has been established that corpus luteum support is required following ovarian stimulation and GnRH agonist cotreatment (70,71) due to the prolonged pituitary recovery from downregulation and the lack of support of corpus luteum. Due to the fact that after discontinuation of GnRH antagonists, pituitary recovery occurs within hours (72) it has been speculated that luteal phase support is not needed in GnRH antagonist cotreated cycles. Evidence has shown that this is not the case, and in nonsupplemented GnRH antagonist cycles, luteolysis is induced prematurely and pregnancy rates are severely affected (73).

The series of events leading to a deficient luteal phase include ovarian stimulation per se and removal of granulosa cells during follicular aspiration (74), and a high number of corpora lutea (75) during the early luteal phase could directly inhibit LH release via negative feedback actions at the hypothalamic-pituitary axis (76).

Although there is a lot of heterogeneity in the studies on endometrial morphology in stimulated cycles, a general trend involves endometrial advancement in the peri- and postovulatory period followed by a “normal” aspect of endometrium in the early luteal phase and frequent glandular-stromal dyssynchrony in the mid- and late luteal phase (77). Luteal support is necessary for a regular endometrial development as is shown by normal in-phase endometrial histology, irrespective of the luteal support used (78).

The deleterious effect of ovarian stimulation lies in the elevated steroid levels of the follicular phase, which subsequently cause a chain reaction, leading to a defective endometrium receptivity and an insufficient luteal phase to support embryonic development.

The future of ovarian stimulation must focus on developing milder ovarian stimulation protocols. Introduction of GnRH antagonists allows implementation of this approach with onset of FSH administration later in the follicular phase (79). In a randomized noninferiority effectiveness trial, Heijnen et al. (80) have shown that there is no difference in the cumulative live births between a mild stimulation protocol and a standard stimulation. The question that remains unanswered is what is the optimal ovarian response? In the light of discussed evidence, the definition of success must move to a milder ovarian stimulation, with fewer oocytes retrieved and a more physiological hormonal milieu.

If we alter the hormonal milieu and endometrial receptivity in such a severe way with various ovarian stimulation protocols, cryopreservation programs will play an increasing role in the future. Elective cryopreservation of cleavage embryos in cycles with the risk of OHSS is a well-established clinical entity (81).

Rapidly evolving technologies in the cryopreservation field have allowed development of different treatment approaches in assisted reproduction. In 1985, ice-free cryopreservation of mouse embryos at -196°C by vitrification was reported in an attempted alternative approach to cryostorage (82). Since then, vitrification is steadily becoming the mainstream of assisted reproduction techniques as an alternative cryopreservation method to traditional slow-cooling/rapid-thaw protocols (83).

It can be postulated that implantation rates can be improved by electively freezing the embryos and transferring in

a natural cycle where the endometrial receptivity has not been hampered by ovarian stimulation. Although this approach may seem far fetched, evidence is emerging that it can overcome our current treatment limitations. A German group has vitrified all two PN oocytes in patients at risk of developing OHSS, treated with GnRH antagonist protocol where final oocyte maturation was induced with GnRH agonist. All frozen-thawed embryo transfers were performed following spontaneous menses in an artificial cycle where endometrium was primed with transdermal estradiol patches, followed by addition of vaginal progesterone from day 15 onward. In total, nineteen patients underwent twenty-four FT-Ets and the cumulative ongoing pregnancy rate was 36.8% (84).

Endometrial Receptivity

Currently, there is no easily applicable clinical marker of endometrial receptivity. Endometrial biopsy remains the most used method despite its limitations. It is a method established by more than fifty years ago (85), with only infertile patients included in Noyes' criteria, and is a subject of intra- and inter-observer variations (86). Furthermore, it shows questionable relationship to endometrial receptivity (87). Most importantly, it is an invasive method and as such cannot be routinely used.

There is an urgent need to establish a clinically useful, applicable in daily routine, marker of endometrial receptivity since all the known markers can be used only for research purposes [pinopodes (88,89), integrins (90,91), leukemia-inhibiting factor (92,93)]. Transvaginal ultrasonography is a noninvasive technique, but the parameters that have been studied so far such as endometrial thickness, endometrial pattern and, endometrial and subendometrial blood flow (94–96) have a low positive predictive value (97,98).

Introduction of three-dimensional ultrasound (99) has opened new possibilities in studying the endometrium. In order to evaluate endometrial receptivity, endometrial volume and subendometrial and endometrial vascularization have been assessed. Regarding the endometrial volume, most studies to date conclude that it does not predict endometrial receptivity (100,101). The reports on the role of endometrial and subendometrial vascularity assessment in predicting pregnancy are conflicting, with some studies finding that endometrial/subendometrial vascularity is increased in conception cycles (102,103), while others found no differences (104). The controversy arises from methodological heterogeneity of the studies, especially due to the timing of ultrasound examination. Since 3D ultrasound is still a novel technique, with growing experience consistency of data will increase.

In the near future, correlation of 3D ultrasonographic data and histological dating of endometrium needs to be established, and if the results are encouraging, a novel clinical marker of endometrial receptivity may be founded.

EMBRYO TRANSFER – HOW, WHEN, AND HOW MANY?

Embryo transfer procedure plays a pivotal role in the success of assisted reproduction. Accumulated evidence shows that a number of factors influence the embryo transfer technique. A recent meta-analysis comparing the use of soft and stiff embryo transfer catheters was in favor of the soft ones regarding the pregnancy rates [odds ratio (OR) 1.34, 95 percent confidence intervals (CI) 1.18–1.54] (105).

Empirically, embryo transfer is performed blindly with the aim to deposit the embryos 1 cm away from the fundus. Recent research has shown that improvement is observed if the distance from the fundus is increased (106,107). In contrast, placement of the embryos in lower segment of uterine cavity may increase the risk of placenta previa (108).

Ultrasound-guided embryo transfer is routine in many infertility centers. A large number of studies has dealt with this issue, and a meta-analysis of four randomized controlled trials has shown increased pregnancy and implantation rates with the use of ultrasound during ET (109). Our own experience is that there is no outcome difference in ultrasound versus “clinical touch” embryo transfer technique (110).

It has been recognized and clinically accepted that embryo transfer should be performed in atraumatic way, minimizing uterine contractility. A large proportion of IVF patients have persistently high uterine contraction frequency at the time of day 3 transfer. Furthermore, the higher the frequency of uterine contractions, the lower the pregnancy rate (111). The contractility of the uterus decreases toward day 7 following the hCG injection, with the uterus reaching a nearly quiescent status for the day 5 transfer (112).

The future of the procedure will focus on further minimizing this contractility pharmacologically and further defining the correct position for deposition of the embryos.

The day of embryo transfer differs between the centers, and it includes day 2, day 3, and day 5. In the early years, most centers replaced the embryos on day 2 following fertilization, and with the improvement of culture media and laboratory techniques, this has moved to day 3 and in the last couple of years to blastocyst transfer on day 5.

The main disadvantage of transferring cleavage-stage embryos is that current morphological criteria are highly subjective with high inter- and moderate intraobserver variability (113) and in high percentages of the cases do not reflect the euploidy status of the embryo (114). In contrast, the risk of transferring on day 5 is that a number of embryo will not reach the blastocyst stage and there will be increased risk of cycle cancellation. Our group has carried out a prospective randomized controlled trial comparing the day 3 versus day-5 single-embryo transfer in patients younger than thirty-six years. The results showed significantly higher pregnancy and delivery rates among women undergoing transfer of a single blastocyst-stage embryo (60).

Although it is fair to say that presently blastocyst transfer may not be applicable to all patient populations, further research needs to be conducted in different patient populations. High blastocyst pregnancy rates reflect high standards of laboratories, and with further improvement, blastocyst transfer should have wider application in the future.

The leading complication of assisted reproductive techniques is high multiple pregnancy rates, which amounts to 24.5 percent (3,115). Increased perinatal morbidity, maternal complications, and medical costs are too high a price to pay for individuals, society, and medical profession (116).

The milestone in assisted reproduction is the introduction of single-embryo transfer as a strategy to reduce multiple pregnancy rates. Since the only variables predictive of multiple birth are age and the number of good-quality embryos transferred (117), the largest RCT on single-versus double-embryo transfer in women younger than thirty-six years (118), including the frozen-embryo transfer cycle in the SET group, showed that

$1 + 1 = 2$. This means that the cumulative ongoing pregnancy rate in one fresh and one frozen SET was 38.2 percent, which was not significantly different from the 42.9 percent ongoing pregnancy rate in the dual-embryo transfer group (DET). Only one twin birth was registered in the eSET group compared with 33.1 percent in the DET group.

Introducing SET reduces the twinning rate without affecting the pregnancy rates. This trial recognized the paramount importance of a well-functioning freezing program.

Implementation of SET is gaining momentum in some countries, although a large proportion of IVF society is still oblivious to this approach. It has to be recognized that there is a need to have a degree of flexibility over the number of embryo transferred, depending on pregnancy prognosis. The Belgian model recognizes the need for this since it is stratified by age and also by the cycle number, so that lack of success enables an increase in the number of embryos transferred. This legislation was introduced to improve financial access to assisted reproduction treatment and reduce multiple IVF and ICSI pregnancies. It has been estimated that the reduction in costs associated with multiple pregnancies will provide the means for treatment reimbursement. Since July 1, 2003, laboratory costs for IVF and ICSI have been refunded for six cycles in a lifetime for patients younger than forty-three years. For those aged less than thirty-six years, the first two cycles must be SET, but if these fail, cycles three to six can be dual-embryo transfer. For patients aged thirty-six to thirty-nine, the first two cycles can be up to two embryo transfers and cycles three to six can be up to three ET. Patients older than thirty-nine years can have up to three embryos transferred already from first treatment cycle.

In a publication by our group (119), a fifteen-month period was analyzed before and after the legislation was implemented. Overall, the multiple pregnancy rates were reduced from 29.1 to 9.5 percent (all patients) and from 28.9 to 6.2 percent in women younger than thirty-six years. Most twins were observed in the third cycle of patients younger than thirty-six years and in the first three cycles of patients of thirty-six to thirty-nine years. Overall, a significant decline in multiple gestations was mainly observed in the less than thirty-six population. Pregnancy rates were not compromised by the new law. This study also raised the issue for introducing SET for a certain proportion of the thirty-six to thirty-nine year population.

With these results, it is increasingly difficult to accept the rates of multiple pregnancies seen around the world. The financial aspect of the Belgian model clearly shows that the lowering of treatment cost by reducing the incidence of multiple pregnancies provides means for increasing availability of treatment.

EMBRYO SELECTION

The past twenty years have been marked by the immense development in the assisted reproductive techniques. Preimplantation genetic diagnosis (PGD) was introduced to prevent the inheritance of sex-linked diseases, the first successful pregnancy being achieved in 1990 (120).

PGD for aneuploidy screening (PGD-AS, PGS) aims to evaluate numerical chromosomal constitution of the cleavage-stage embryo through removal of a blastomere/s and subsequent analysis by the use of fluorescence in situ hybridization (FISH).

Apparently, approximately a third of all IVF produced embryos is chromosomally abnormal (121,122). In the

poor-prognosis IVF population, which includes patients of advanced maternal age (AMA), recurrent implantation failure (RIF), recurrent miscarriage (RM), and testicular sperm extraction, the incidence of chromosomal abnormalities rises to 70 percent (123).

The current morphological criteria for choosing the best embryo for transfer are often unable to allow selection of euploid embryos, that is, morphologically best embryos are aneuploid in 25 percent of the cases (124). The rationale for introducing PGS has been that selection and transfer of euploid embryos would improve implantation and pregnancy rate and decrease miscarriage rate as well as multiple pregnancy rates. PGS is currently being done by an increasing number of infertility centers in the world, and there is a need to assess its effectiveness (124,125).

A large number of comparative studies has investigated the use of PGS in patients of AMA (126–130), RIF (126,127,131,132), RM (122,133,135), and testicular sperm extraction (136,137).

The results have shown initial optimism, but a number of issues have to be addressed in order to interpret the findings and conclusions of different trials.

Certain methodological concerns such as sampling variability (wide array of sample sizes present) and clinical heterogeneity (different study populations, number of blastomeres assessed, number of probes used, methodology used, randomization procedures, etc.) are present in the studies assessing the use of PGS.

In accordance with the methodological concerns, the Cochrane review (138) could only include two randomized prospective clinical trials of Staessen et al. (123) and Stevens et al. (139). There is another small prospective randomized controlled trial by Werlin et al. (140), but it does not provide sufficient data on the methodological quality.

The two randomized controlled trials included in the meta-analysis (138) represent 428 patients, with the majority of patients coming from the study of Staessen et al. ($n = 389$). PGS in the two studies was performed for AMA of thirty-seven years or more in Staessen et al. and more than thirty-five years in Stevens et al. There was no difference in the live birth rate in PGS versus non-PGS group, 11 versus 15 percent, respectively (OR 0.65; 95 percent CI: 0.36–1.19), ongoing pregnancy rate per woman 15 percent in the PGS group versus 22 percent in control group (OR 0.42, 95 percent CI 0.12–1.51).

The trial by Werlin et al. randomized the three categories of patients, AMA, RM, and RIF, to PGS versus non-PGS group. Although this is the only study that randomized recurrent miscarriage patients, 11 versus 8 controls, number of pregnancies 63.6 percent in the study versus 37.5 percent in controls ($P = 0.07$), it is difficult to interpret these results. The sample size was too small, the randomization procedure was not given, and all patients received corticosteroids and low doses of aspirin. Regarding the patients with RIF ($n = 19$, 11 study patients, 9 were controls), number of pregnancies was 20 percent in the study versus 0 percent in the control group.

Furthermore, results of the prospective cohort studies and retrospective studies showed that PGS improves the pregnancy rates, but study design does not allow these results to be used for recommending PGS as a routine procedure.

A recent publication by Munne et al. (141) analyzed 2,279 PGD cycles, which were carried out in patients older than

thirty-five years from hundred infertility centers in United States. Majority of the centers had fewer than Thirty cycles per center, 89 of them, while number of cycles per center ranged from 30 to 531 for the remaining eleven centers. In total, 1,886 cycles ended in embryo transfer (82.2%), of which 608 resulted in pregnancy, but only 562 cycles with known pregnancy outcome were included. Results were compared with general IVF population, nondonor fresh cycles, 7,682 from thirty-five to forty years and 1,024 cycles from older than forty years were used as a control group. The mean pregnancy loss for the PGS group (16.7 percent) was significantly lower than for general IVF group (21.5 percent, $P < 0.001$). When stratified for age, in the thirty-five to forty group, rate of pregnancy loss was 14.1 versus 19.4 in the control group ($P = 0.03$), and for patients older than forty, it was reduced from 40.6 percent in the control group to 22.2 percent ($P < 0.001$). Although this trial has a large sample size as such, there was a large heterogeneity in the results of individual clinics, the pregnancy rate varying from 11 to 57 percent.

There have been no RCTs evaluating PGS in couples with NOA and OA, although there is substantial evidence of an increase in aneuploidy and mosaicism of embryos derived from azospermic men compared to fertile men (137). Surprisingly, Platteau et al. showed that the aneuploidy frequency in embryos from NOA was 53 percent and in OA 60 percent, despite young age of their female partners.

The results of all these studies confirm high rate of aneuploidies in these patient populations. A recent study of Baart et al. (114) has shown that among young patients, the rate of aneuploidies is 64 percent in embryos that were not selected for transfer.

Although extensive research has been conducted in this field, there is a need for more well-designed randomized prospective trials, which will assess the value of PGS in well-defined patient populations, with delivery of a healthy child as the primary outcome.

Major limitation of PGS is mosaicism, estimate running as high as 50 percent of all the cleavage embryos (142). Mosaicism is the result of presence of euploid and aneuploid cells or distinct aneuploidies on different blastomeres, so that cells analyzed by PGS do not represent genomic content of the rest of the embryo. The mechanisms underlying this phenomenon are mitotic non disjunction and anaphase lagging. Coonen et al. demonstrated that anaphase lagging accounts for 56 percent of the mosaicism in blastocysts (143).

Mosaicism often leads to misdiagnosis, up to 60 percent (144), giving rise to false-positive and false-negative results. It has been argued that removal of only one blastomere is not representative of the embryo and two blastomeres need to be removed. Due to the fact that this removal is not carried in random order, when two blastomeres are analyzed, there is a 25 percent probability of removing both reciprocal daughter cells, resulting in the euploid status of previously mosaic embryo (114). There is also a chance of aggravating existing mosaicism by removal of normal blastomeres (144) and reducing number of healthy embryos for transfer.

It has to be reiterated that the current high incidence of mosaicism after PGS can be an overestimation since the embryos that have been analyzed in majority of the studies are discarded for transfer or cryopreservation. Staessen et al. have shown that in patients of advanced maternal age, the rate of mosaicism is 10.7 percent (123), which is in agreement with the

control group for recurrent miscarriage patients, by Pehlivan et al. (131), of 10.8 percent. Although the populations studied are different, mosaicism rate was established in good-quality embryos and as such may be more representative.

There are technical limitations of the procedure itself, which have been acknowledged, namely signal overlapping and signal splitting (145).

Number of probes used varies among different groups; currently, FISH is able to analyze up to ten chromosomes, 1, 7, 13, 15, 16, 18, 21, 22, X, and Y. Irrespective of the number of probes used, not all chromosomes can be assessed by PGS at the moment. Comparative genomic hybridization (CGH) may overcome this since it allows complete chromosomal status assessment, but there are still issues that prevent CGH from being routinely used such as long period of hybridization, necessity of embryo freezing prior to transfer, and inability to distinguish diploid cells from haploid or tetraploid (146). The number of euploid embryos is lower than after FISH analysis, approximately 25 percent (147,148). The first birth following CGH and a modified freezing protocol has been documented by Wilton et al. in 2001 (149).

In conclusion, PGS technique cannot be recommended as a routine clinical procedure. Current clinical evidence shows no benefit in the pregnancy rates in poor-prognosis patients, but lack of well-designed randomized controlled trials hinders definitive conclusions from being made.

Mosaicism of the cleavage embryos remains a great source of misdiagnosis and cannot be overcome by removing two instead of one blastomere.

The most important misinterpretation of the results is linked to the fact that the euploidy status of the blastomere does not correspond to the euploidy status of the embryo due to mosaicism and probably due to the fact that the embryo is self-correcting.

Finally, with the current technology available, including comparative genomic hybridization, screening of blastomeres will not lead to the evaluation of the entire embryo. It is foreseeable that if more randomized controlled trials will be available, the final answer will confirm our interpretation. The future of the embryo selection has to focus on development of new genetic tools for embryo selection.

FINAL CONCLUSIONS

The future developments in assisted reproduction should encompass individualized approach to ovarian stimulation, vitrification, single blastocyst transfer, and development of new tools for genetic testing.

Since ovarian stimulation has a detrimental effect on the endometrial receptivity, development of milder stimulation protocols with tailor-made approach will mark the near future of ovarian stimulation. A fine-tuning should allow synchronization of ovarian folliculogenesis and endometrium. An optimal hormonal milieu needs to be achieved in order to allow harmonious endometrial development with optimal conditions during the implantation window. A step further will be vitrification of all embryos and transfer in a natural or a substituted cycle where the effect of ovarian stimulation on the endometrial receptivity will be completely circumvented.

Overall, the major disadvantage of classical IVF/ICSI is the occurrence of multiple pregnancies, which should be overcome by transfer of one blastocyst.

Embryo selection is in particular a crucial intervention. The main drawback of single or dual blastomere testing is the presence of mosaicism, and although comparative genome hybridization is tempting, it will not solve this problem. Since PGS did not show improved pregnancy rates, future research in this area has to be focused on developing new tools for genetic testing.

KEY POINTS FOR CLINICAL PRACTICE

The future of assisted reproduction should move in the direction of:

- Development of milder stimulation protocols with tailor-made dosing approach.
- Vitrification of embryos and embryo transfer in a natural cycle.
- Transfer of single blastocyst.
- Development of new genetic tools for embryo selection.

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