REPRODUCTION

REVIEW

The impact of ovarian stimulation for IVF on the developing embryo

Margarida Avo Santos, Ewart W Kuijk and Nick S Macklon

University Medical Centre Utrecht, Reproductive Medicine and Gynaecology, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Correspondence should be addressed to M A Santos; Email: mavoribe@umcutrecht.nl

Abstract

The use of assisted reproductive technologies (ART) has been increasing over the past three decades, and, in developed countries, ART account for 1–3% of annual births. In an attempt to compensate for inefficiencies in IVF procedures, patients undergo ovarian stimulation using high doses of exogenous gonadotrophins to allow retrieval of multiple oocytes in a single cycle. Although ovarian stimulation has an important role in ART, it may also have detrimental effects on oogenesis, embryo quality, endometrial receptivity and perinatal outcomes. In this review, we consider the evidence for these effects and address possible underlying mechanisms. We conclude that such mechanisms are still poorly understood, and further knowledge is needed in order to increase the safety of ovarian stimulation and to reduce potential effects on embryo development and implantation, which will ultimately be translated into increased pregnancy rates and healthy offspring.

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Introduction

Some 30 years after the birth of the first 'test tube' baby, IVF has become a widely available treatment for most causes of subfertility. Despite ongoing advances in the associated assisted reproductive technologies (ART), pregnancy rates remain around 20–30% per started cycle. In order to compensate for inefficiencies in IVF procedures, high doses of exogenous gonadotrophins are administered to stimulate the development of multiple oocytes to mature in a single cycle. The use of such ovarian stimulation protocols enables the selection of one or more embryos for transfer, while supernumerary embryos can be cryopreserved for transfer in a later cycle (Macklon *et al*. 2006). In recent years, it has become evident that ovarian stimulation, although a central component of IVF, may itself have detrimental effects on oogenesis, embryo quality, endometrial receptivity and perhaps also perinatal outcomes. In this article, the impact of ovarian stimulation and underlying mechanisms will be reviewed. Strategies for reducing the impact of ovarian stimulation on IVF outcomes are also addressed.

Current regimens for ovarian stimulation

Ovarian stimulation with exogenous gonadotrophins promotes the growth of multiple follicles to the preovulatory stage by interfering with the physiological mechanisms, which normally ensure single dominant follicle selection. It is important to distinguish this from ovulation induction treatment, which aims to restore normal follicular growth in anovulatory women.

Current regimens are based on the administration of high doses of either urinary derived or recombinant FSH (recFSH). The aim is to raise serum FSH levels above the threshold required for follicle development for a prolonged period, in order to enable the growth and maturation of not just one, but the complete cohort of follicles that have reached the FSH-dependent stage of development (Fig. 1; Fauser *et al*. 2005). Starting doses of FSH usually vary between 150 and 450 IU/day (Verberg *et al*. 2009). In addition to FSH, LH may also be administered. However, LH has been shown not to be absolutely necessary for follicular development (Macklon *et al*. 2006).

Stimulation of the growth of multiple follicles leads to their production of supraphysiological serum oestradiol (OE_2) levels, which by means of positive feedback at the pituitary may cause a premature LH peak and hence premature luteinisation and ovulation. In order to prevent this, exogenous gonadotrophin treatment is usually supplemented by the administration of GNRH analogues. In the commonly employed 'long protocol', GNRH agonists are commenced in the midluteal phase of the preceding cycle leading to an initial 'flare' of gonadotrophin hypersecretion, followed by desensitisation of the pituitary, resulting in gonadotrophin

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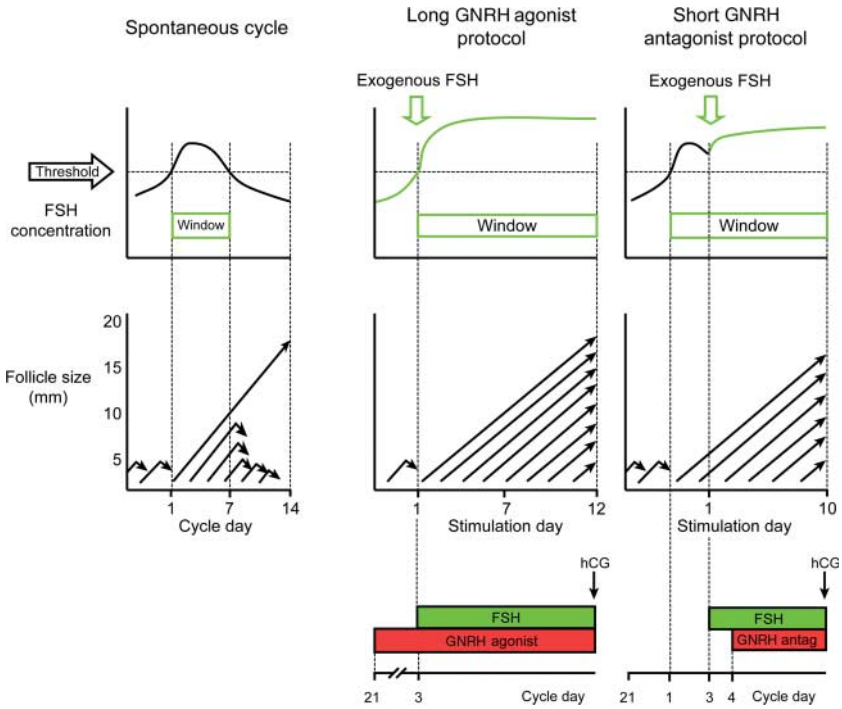

Figure 1 Concentration of FSH, number and size of follicles during the follicular phase of the menstrual cycle in three different situations: natural cycle; long GNRH agonist regimen; GNRH antagonist regimen. The threshold represents the concentration of FSH in serum above which ongoing gonadotrophin-dependent follicle development is stimulated. The window represents the duration of time at which FSH concentrations are above the threshold. Each arrow represents a developing follicle. Adapted from Macklon *et al.* (2006).

suppression and prevention of a premature LH surge. Although associated with hypo-oestrogenic side effects and a considerable patient burden, this protocol still remains the most widely used stimulation regimen in contemporary practice (Macklon *et al.* 2006).

In recent years, GNRH antagonists have become established in clinical practice. In contrast to GNRH agonists, antagonists are immediately effective in reducing endogenous gonadotrophin production, and their administration can hence be limited to the mid-to-late follicular phase of the menstrual cycle. They do not therefore suppress the endogenous intercycle rise in FSH, and, as a result, less exogenous FSH may be required in association with GNRH antagonist versus agonist co-treatment (Fig. 1; Macklon *et al.* 2006).

An alternative approach advocated by some is 'natural cycle IVF'. In contrast to the aims of ovarian stimulation, this treatment is aimed at aspirating the single oocyte, which has developed during a spontaneous cycle. Although appealing in terms of cost and burden of treatment, frequently, no oocyte will be obtained. To reduce the risk of losing the oocyte to premature ovulation, 'modified' natural cycle IVF employs GNRH antagonists together with a low dose of exogenous gonadotrophins aimed at maintaining development of the follicle despite GNRH antagonist suppression of endogenous gonadotrophins. Pregnancy rates using this approach are just 7% per cycle (Pelinck *et al.* 2002). However, it has been suggested that women who respond poorly to exogenous gonadotrophins may be good candidates for natural cycle IVF (Schimberni *et al.* 2008). Approximately, 10% of women undergoing ovarian stimulation for IVF will demonstrate a poor response defined as the production of fewer than four follicles (Pellicer *et al.* 1987), and a low level of serum OE_2 (Hanoch *et al.* 1998). Although more frequent in older women (40 years old or more), poor ovarian response can also occur unexpectedly in younger women. Natural cycle IVF may be therefore an alternative to ovarian stimulation or egg donation, as it has been shown to be as effective as ovarian stimulation in terms of pregnancy rates in this group of patients (Schimberni *et al.* 2008). Furthermore, in older poor responders, natural cycle allows the retrieval of the dominant follicle only, allowing fertilisation of the putatively most competent oocyte available for retrieval.

How does ovarian stimulation affect early oocyte and embryo development?

In recent years, the previously prevailing paradigm of stimulating hard to obtain large numbers of oocytes for IVF has been increasingly questioned. A number of studies have demonstrated the high burden, risk and costs of this approach and a detrimental effect of ovarian stimulation on oocyte development. Pellicer *et al.* (1989) showed that the retrieval of >10 oocytes in women was correlated with oocytes of lower quality, as decreased fertility rates were reported in this group, when compared with two other groups of women in whom one to five or six to ten oocytes were retrieved. Similarly, our group has recently shown that the optimum chance of conceiving after the long protocol occurs associated with a harvest of 13 oocytes, and that a fall in pregnancy

rates was observed when more than this number was obtained (Fig. 2; van der Gaast *et al.* 2006). This could be indicative of a detrimental effect of supraphysiological OE_2 levels on oocyte quality or indeed endometrial receptivity, as discussed later. A potentially lethal complication of ovarian stimulation, which is encountered in 1–2% of women undergoing IVF treatment, is the so-called ovarian hyperstimulation syndrome (OHSS). OHSS is associated with excessively high OE_2 serum concentrations, which could explain the significantly lower percentages of good-quality oocytes and fertilisation rates observed in cycles complicated by OHSS compared to control groups (Aboulghar *et al.* 1997). In contrast, Ng *et al.* (2003) have reported normal nuclear maturity of oocytes and fertilisation in patients with high OE_2 serum concentrations.

The detrimental effects of exogenous gonadotrophins on embryo development have been best characterised in rodent models. *In vitro* studies showed that ovarian stimulation disrupts (Ertzeid & Storeng 1992) and delays (Van der Auwera & D'Hooghe 2001) the development of one- or two-cell mouse embryos into blastocysts. Likewise, embryos from superovulated hamsters had significantly reduced mean cell numbers than the controls (McKiernan & Bavister 1998). *In vivo* studies are concordant, indicating that ovarian stimulation delays embryo development (Van der Auwera & D'Hooghe 2001, Ertzeid & Storeng 2001). Furthermore, analysis of the surface architecture of mouse embryos showed a reduction in the number of cells and of microvilli on blastocysts from gonadotrophin-treated females, compared to those from spontaneously ovulating females (Champlin *et al.* 1987). However, the results of human studies assessing possible effects of ovarian stimulation protocols on embryo development are inconsistent with mouse studies. A retrospective study comparing human embryo quality in the natural versus long GNRH agonist-stimulated IVF cycle revealed no differences in cleavage rates, developmental capacity (number of blastomeres) or degree of fragmentation of the embryos (Ziebe *et al.* 2004). Additionally, an excessive response to ovarian stimulation was shown to have no negative impact on embryo quality as assessed by morphology (Ng *et al.* 2000, 2003).

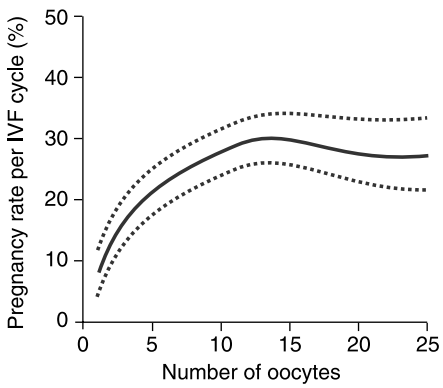

Figure 2 Number of retrieved oocytes in relation to pregnancy rate per started IVF cycle. Adapted from van der Gaast *et al.* (2006).

The discovery of extra-pituitary GNRH receptors in tissues such as the uterus, endometrium, oocytes–cumulus complex, pre-implantation embryos and placenta (Casan *et al.* 1999, Raga *et al.* 1999, Grundker *et al.* 2002) has led to growing concern about possible detrimental effects that GNRH antagonist may have on embryo development and implantation. *In vitro* studies have shown that GNRH antagonist is responsible for an inhibitory effect on pre-implantation development of mouse embryos (Raga *et al.* 1999). In an attempt to explain such results, Yang *et al.* (2009) have recently hypothesised that GNRH antagonists could interfere with cell growth by decreasing the synthesis of insulin-like growth factor (IGF) and epidermal growth factor receptors, which are involved in the MAP kinase-mediated mitogenic cascade. However, the developmental potential of human pre-implantation embryos does not seem to be limited by putative detrimental effects of GNRH antagonist (Yang *et al.* 2009). Additionally, high doses of GNRH antagonist were shown not to harm the implantation potential of embryos in frozen–thawed cycles (Kol *et al.* 1999), and a recent meta-analysis showed no significant differences in live birth rates following co-treatment with GNRH agonists versus GNRH antagonists (Kolibianakis *et al.* 2006).

The concern that suppressed LH concentrations in the late follicular phase may be detrimental to clinical IVF outcomes lead to the development of stimulation protocols including exogenous LH (Macklon *et al.* 2006). Supplementation of LH activity may be advantageous to some patients by accelerating large follicle development and decreasing the duration of treatment (Filicori *et al.* 1999). Moreover, LH alone has been shown to be effective in monofollicular stimulation as part of a sequential ovarian stimulation protocol following initiation with recFSH (Sullivan *et al.* 1999). Recent studies have indicated that stimulation protocols that include LH may increase the percentage of diploid (Weghofer *et al.* 2008) and top-quality (Andersen *et al.* 2006) pre-implantation embryos. It has been proposed that such protocols may be beneficial to some women who respond poorly to standard 'FSH-only' regimens (Mochtar *et al.* 2007). On the other hand, elevated follicular phase LH levels have been associated with reduced fertility and an increased risk of miscarriage (Regan *et al.* 1990), which has been confirmed by recent data showing treatment with recLH alone in the late follicular phase to be detrimental to preovulatory follicle development (Hugues *et al.* 2005, Rao & Tan 2005). The contradictory findings regarding LH supplementation to ovarian stimulation protocols support the concept of a 'window' for LH, since there seems to be a threshold LH level below which OE_2 production is inadequate, and a

'ceiling' level above which LH may be detrimental to follicular development (Shoham 2002).

In order to reduce the exposure of the patient to the risks and side effects of exogenous gonadotrophin treatment, the maturation of oocytes can be performed *in vitro*. This approach normally involves the administration of a short period of low-dose gonadotrophins sufficient to stimulate multiple follicles to grow to a diameter of 12 mm stage at which the oocytes are aspirated. Women with anovulatory infertility due to polycystic ovary syndrome (PCOS) are known to be at increased risk of developing OHSS and may therefore benefit from *in vitro* maturation (IVM) as an alternative to conventional IVF (Reinblatt & Buckett 2008). An early case–control study comparing IVF and IVM in PCOS patients showed lower implantation rates with IVM (Child *et al*. 2002). However, a recent meta-analysis comparing live birth rates after IVM to conventional IVF or ICSI in women with PCOS has emphasised the need for controlled trials in this field (Siristatidis *et al*. 2009). It therefore remains unclear whether IVM is beneficial for women with PCOS as an alternative to conventional IVF. One concern with the approach is the relatively high rate of developmental incompetence observed in oocytes subject to IVM. Li *et al*. (2006) raised concerns regarding possible deleterious effects that IVM might have on the organisation of the meiotic spindle and chromosomal alignment. Although, at present, there are no indications of increased risk of congenital malformations in children conceived by IVM, the processes involved and the long-term outcomes are still poorly understood (Reinblatt & Buckett 2008), and data from ongoing follow-up studies are awaited. Although there seems to be some evidence that IVM could provide a promising alternative to conventional IVF, particularly in women with PCOS, or others at increased risk of developing OHSS, prospective randomised controlled trials are needed before it can be recommended for clinical practice.

Does ovarian stimulation disrupt chromosomal competence of the oocyte and embryo?

Bidirectional signalling between oocytes and granulosa cells is essential for follicular development and the acquisition of oocyte competence (Eppig 2001). The nuclear and cytoplasmic maturity of the oocyte that accompanies follicular development plays a crucial role in facilitating fertilisation and the early stages of embryonic development (Albertini *et al*. 2003). Exposure of the developing oocyte to supraphysiological concentrations of gonadotrophins may disturb oocyte maturation and the completion of meiosis leading to chromosomal aneuploid oocytes and/or embryos (Hodges *et al*. 2002).

Several studies in the mouse have investigated whether ovarian stimulation could induce chromosomal malsegregation during meiotic maturation. Early studies showed no increase in the incidence of non-disjunction in mouse oocytes obtained after ovarian stimulation versus spontaneous ovulation (Hansmann & El-Nahass 1979, Golbus 1981). However, more recent studies indicate that exogenous gonadotrophin treatment contributes to increased frequency of chromosomal abnormalities. Mouse embryos originating from stimulated females showed a fourfold increase in sister chromatid exchange frequency than embryos from spontaneous ovulations, which is suggestive of induced-DNA lesion by ovarian stimulation (Elbling & Colot 1985). Moreover, when compared to zygotes derived from spontaneous ovulation, mouse zygotes obtained after ovarian stimulation showed an increased rate of chromosomal aberrations in the female pronucleus and compromised embryo development (Vogel & Spielmann 1992). Likewise, *in vitro*-matured mouse oocytes exposed to high concentrations of FSH showed accelerated nuclear maturation and increased aneuploidy (Roberts *et al*. 2005).

Although advanced maternal age is the only clearly identified risk factor for chromosomal aneuploidy in the human embryo (Hassold & Hunt 2001, Champion & Hawley 2002), a number of studies have reported particularly high rates of chromosomal aneuploidy and mosaicism in early human IVF embryos (Munne *et al*. 1997, Katz-Jaffe *et al*. 2005, Baart *et al*. 2007). Recently, post-zygotic chromosome instability has been observed to be a common feature of early human embryogenesis, leading to chromosomal disorders such as mosaicism and uniparental disomies in the majority of cleavage-stage embryos (Vanneste *et al*. 2009). Although the mechanisms underlying aneuploidy are still poorly understood, it has been hypothesised that increased rates of embryo aneuploidy could also result from the interference of ovarian stimulation with the natural selection of good-quality oocytes or from exposure of growing follicles to detrimental effects of hyperstimulation on oocyte maturation (Verberg *et al*. 2009). In order to investigate the role of ovarian stimulation as a possible cause of chromosomal malsegregation in human IVF cleavage-stage embryos, Baart *et al*. carried out pre-implantation genetic screening (PGS) for aneuploidy using fluorescent *in situ* hybridisation (FISH) for ten chromosomes in two blastomeres biopsied from viable embryos derived from two different stimulation protocols. A significantly higher proportion of aneuploid embryos following conventional high FSH dose long protocol was observed compared with that found after exposure to a mild, lower FSH dose ovarian stimulation protocol (Baart *et al*. 2007). The increased number of abnormal embryos was mainly due to a higher incidence of mitotic segregation errors, leading to mosaicism. These findings supported both previous reports of an association between ovarian stimulation regimens and chromosomal mosaicism in human embryos (Munne

et al. 1997), as well as reports indicating an association between meiotic and mitotic chromosome 21 cell division errors with significantly higher FSH doses daily (Katz-Jaffe *et al*. 2005). Thus, milder ovarian stimulation regimens seem to be less detrimental to the vulnerable process of nuclear maturation and chromosomal segregation.

As mentioned previously, the value of LH supplementation to FSH stimulation protocols remains unclear. In an attempt to address this question from a cytogenetic viewpoint, Weghofer *et al*. (2008) evaluated the effect of ovarian stimulation on the ploidy of cleavage-stage embryos after long agonist downregulation combined with either recFSH or human menopausal gonadotrophin (hMG). In this small study, a higher rate of diploidy and ongoing pregnancies per cycle was seen in women treated with hMG, suggesting that LH-containing ovarian stimulation protocols may be beneficial for achieving higher diploidy rates in pre-implantation embryos.

A significant increase in the proportion of morphologically abnormal oocytes after repeated rounds of ovarian stimulation has been reported both in the cow and the mouse (Lubbadeh *et al*. 1980, Kanayama & Osada 2000). In an attempt to determine whether repeated ovarian stimulation affected oocyte competence also at the nuclear and cytoplasmic levels, Van Blerkom & Davis used a mouse model to study the effects of four rounds of ovarian stimulation on cytoplasmic and spindle organisation. *In vivo*-matured oocytes were reported to suffer a progressive and significant increase in the frequency of spindle defects with each additional round of ovarian stimulation (Van Blerkom & Davis 2001). In humans, a number of studies confirmed the results from animal studies, with pregnancy and implantation rates reported to significantly decline in cycle 2 compared with cycle 1 (Shapiro *et al*. 2001, Silberstein *et al*. 2005, Wang *et al*. 2008), reaching a plateau for cycles 3–5 at a rate lower than in cycle 2 (Silberstein *et al*. 2005). However, other studies do not show significant declines on ovarian response to gonadotrophin stimulation with repeated cycles, either in terms of the number of oocytes retrieved or in the quality of the embryos based on morphological criteria (Hoveyda *et al*. 2002, Kolibianakis *et al*. 2002, Kolibianakis & Devroey 2004, Doldi *et al*. 2005). None of these studies looked into the cytogenetic outcomes of the embryos generated, and it therefore remains unclear whether repeated cycles of ovarian stimulation may interfere with oocyte and/or embryo chromosomal competence.

Ovarian stimulation and epigenetics

Epigenetic mechanisms regulate gene activity in a hereditary fashion without affecting the genetic constitution (Lucifero *et al*. 2004). Gene imprinting is an epigenetic process, which allows a subset of genes to be expressed in a monoallelic parent-of-origin manner (Lawrence & Moley 2008). Imprinting occurs in genes that have been shown to be essential for embryonic growth and development, placental function and postnatal behaviour (Isles & Holland 2005, Fowden *et al*. 2006, Smith *et al*. 2006). The main epigenetic mechanisms controlling imprinting are DNA methylation and histone modification. DNA methylation is the best characterised epigenetic modification and in many cases occurs in a differentially methylated region (DMR; Lucifero *et al*. 2004).

In the mouse, methylation patterns of imprinted genes are erased in the germ line. In the male, remethylation starts early during embryonic development in the gonocytes and continues up to the spermatogonia stage; whereas, in the female, it begins after birth, early in the oocyte growth phase, continuing throughout oocyte growth (Zamudio *et al*. 2008). In humans, little information is available on imprinting dynamics, but existing data suggest some conservation of the epigenetic mechanisms described in the mouse (Lucifero *et al*. 2004).

Genes that acquire their imprints late in oocyte development are believed to be the most susceptible to perturbations on their imprints (Gosden *et al*. 2003, Fortier *et al*. 2008). Ovarian stimulation regimens promote the development of many oocytes in a non-physiological endocrine milieu. Therefore, it is possible that the acquisition of methylation imprints in oocytes may be disturbed by ovarian stimulation. Methylation defects at the DMRs of *SNRPN* (Angelman syndrome), *KCNQ1OT1* (Beckwith–Wiedemann syndrome) and *PEG1/MEST* (Silver–Russel syndrome) have been identified in affected children conceived with ART (Lawrence & Moley 2008). Two-cell mouse embryos from superovulated female mice showed a correlation between the number of abnormally methylated embryos and embryo loss during pre-implantation, indicating that ovarian stimulation may lead to epigenetic abnormalities (Shi & Haaf 2002). Determination of DNA methylation profiles of the DMRs of maternally (*PEG1*) and paternally (*H19*) imprinted genes in both mouse and human oocytes demonstrated imprinting reversal upon ovarian stimulation (Sato *et al*. 2007). Monoallelic expression of *Snrpn* and *H19* imprinted genes in the mouse placenta seems particularly susceptible to perturbation following ovarian stimulation (Fortier *et al*. 2008). These and previous results from Mann *et al*. (2004) suggest that trophectoderm-derived tissues are more susceptible to imprinting disruption (Fortier *et al*. 2008).

Ovarian stimulation has also been suggested to have an epigenetic effect on folliculogenesis and gametogenesis by possibly interfering with the homocysteine pathway. A number of intermediates of this pathway are directly involved in processes such as protein and DNA synthesis, and oxidative stress balance, which have important roles in gametogenesis (Ebisch *et al*. 2007).

Ovarian stimulation has been shown to alter folate metabolism in the follicle, which may be a further mechanism by which normal folliculogenesis is disrupted (Boxmeer *et al*. 2008).

Endometrial receptivity and embryonic implantation

Increasing evidence points to pre-clinical pregnancy loss rather than failure of conception as the principal cause for the relatively low fecundity observed in humans. In natural cycles, up to 55% of conceptions are estimated to be lost due to implantation failure or pre-clinical miscarriage (Fig. 3a; Macklon *et al*. 2002). In a recent study by Boomsma *et al*. (2009) it was shown that in stimulated cycles, the contribution of implantation failure for the numbers of conception losses is higher (50%) than described for natural cycles (30%; Fig. 3b). This suggests that in patients undergoing ART, not only the quality of the embryo is crucial for achieving successful implantation and clinical pregnancy, but the endometrium also plays an important role.

There are some indications that high OE_2 levels resulting from ovarian stimulation may impair endometrial receptivity (Pellicer *et al*. 1989, 1996, Paulson *et al*. 1990, Simon *et al*. 1995, 1998). Once the threshold level of OE_2 is exceeded, progesterone receptors may be prematurely induced leading to an increased sensitivity to progesterone and thus early endometrial secretory advancement. This has been described to occur not only during GNRH agonist/gonadotrophin protocols in the preovulatory phase, but also during GNRH antagonist/recFSH stimulation (Macklon *et al*. 2006, Hayden 2008). In mice, levels of OE_2 have been shown to have a critical role in regulating the window of uterine receptivity (Ma *et al*. 2003). Using a delayed implantation model, low levels of OE_2 were shown to maintain uterine receptivity for a longer period of time; whereas high OE_2 levels lead to a refractory state, leading to implantation failure (Ma *et al*. 2003). Moreover, OE_2 was shown to have a detrimental effect on embryonic adhesion in mice, with both embryo and endometrium being affected (Gidley-Baird *et al*. 1986, Ng *et al*. 2000), although the latter was affected at higher OE_2 concentrations only (Valbuena *et al*. 2001). Ertzeid & Storeng also observed reduced implantation and increased embryo mortality in superovulated recipient mice compared to controls. These authors proposed that decreased uterine receptivity after exogenous administration of gonadotrophins could be caused by altered expression of cytokines in the endometrium of superovulated mice. Additionally, they have also shown that embryos from superovulated donors transferred to control recipients had a lower implantation rate when compared to that of embryos from control donors (Ertzeid & Storeng 2001). Therefore, it seems that gonadotrophin stimulation compromised not only uterine receptivity but also oocyte/embryo developmental competence.

According to Simon *et al*. (1998) low implantation rates in high responders can be improved by the use of a step-down regimen in a subsequent cycle, which has been shown to result in lower OE_2 levels. An *in vitro* mouse model mimicking early and late embryonic transfers supports these findings, showing that reduction of embryonic exposure to OE_2 in late embryo transfers seems to attenuate the toxic effect of OE_2 on embryo implantation (Valbuena *et al*. 2001). Thus, implantation rates in high responders may be improved either by reducing OE_2 levels (Simon *et al*. 1998) or by reducing the time of exposure of the embryo to OE_2 (Valbuena *et al*. 2001).

Perinatal outcomes

The evaluation of the use of gonadotrophins for ovarian stimulation as a risk factor for perinatal outcomes is complex due to the difficulty of eliminating other confounding risk factors such as maternal age, parity and *in vitro* procedures. Furthermore, ART patients with

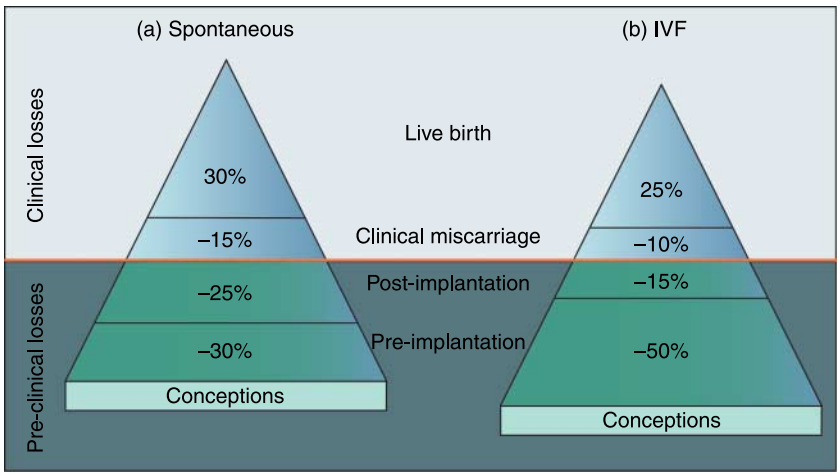

Figure 3 The pregnancy loss iceberg: an overview of the outcomes of spontaneous versus IVF pregnancies. (a) A total of 70% of conceptions are lost prior to live birth. The majority of these losses occur prior to the time of the missed menstrual period, and are not revealed. (b) In stimulated cycles, the iceberg 'sinks' mainly due to increased pre-implantation losses, which results in 75% of conceptions being lost prior to live birth. Adapted from Macklon *et al*. (2002).

a history of subfertility have been associated with several foetal and neonatal abnormalities (Lambert 2003, Shiota & Yamada 2005). Subfertility might therefore partially contribute for the association between assisted conception and poor perinatal outcome of singletons. However, several studies seem to indicate that ART itself, including ovarian stimulation, also has an important effect (Kapiteijn *et al*. 2006).

The use of gonadotrophins for ovarian stimulation is the most important cause of multiple pregnancies in ART patients in the United States, with one-third of multiple pregnancies being caused by non-IVF ovarian stimulation (Ombelet *et al*. 2006). Multiple pregnancies are associated with increased risk of miscarriage, growth retardation and preterm delivery (Fauser *et al*. 2005). However, even singletons are at higher risk of low birthweight, premature birth and perinatal mortality and morbidity in the subfertile population using ART (Schieve *et al*. 2002, Helmerhorst *et al*. 2004, Jackson *et al*. 2004, Kapiteijn *et al*. 2006, Ombelet *et al*. 2006). Mouse studies are concordant, as the mean weight of live foetuses was shown to be significantly lower for foetuses obtained from superovulated recipients, compared to that of those obtained from control recipients (Ertzeid & Storeng 2001). Several studies suggest that low birthweight in IVF singletons is associated with ovarian stimulation (Wennerholm *et al*. 1997, Kallen *et al*. 2005*a*, Wang *et al*. 2005, Kapiteijn *et al*. 2006). Nonetheless, a recent study has shown no correlation between ovarian stimulation parameters and birthweight (Griesinger *et al*. 2008). These authors suggest that the results from previous studies indicating an association between ovarian stimulation and low birthweight could be possibly explained due to confounding by the infertility background of the study population. Further studies are therefore needed to confirm the effect of ovarian stimulation on birthweight of IVF babies.

Ovarian stimulation has been shown to lead to imprinting defects in the mouse placenta (Fortier *et al*. 2008). Elevated expression levels of paternally imprinted gene *IGF2* in the placenta have been correlated with foetal growth restriction in humans (Street *et al*. 2006) and sheep (de Vrijer *et al*. 2006) and with early embryonic lethality of somatic cell nuclear transfer-derived cows (Oishi *et al*. 2006). Since low birthweight in humans may be an important risk factor for the development of neurological disorders and adult-onset diseases such as coronary heart disease, stroke, hypertension, type II diabetes and osteoporosis, ovarian stimulation could even have adverse effects in adult life (Fleming *et al*. 2004).

Confined placental mosaicism (CPM) has also been associated with intrauterine growth retardation (Lestou & Kalousek 1998). Although our group hypothesised that increased rates of CPM may occur after ovarian stimulation due to the persistence of chromosomal mosaicism present in pre-implantation embryos into later gestation, and that this mechanism may underlie the reported increase in intrauterine growth retardation in IVF singletons, a large review of national databases were unable to confirm this (Jacod *et al*. 2008).

A large Swedish cohort study comparing the risk of congenital malformations in infants born after IVF with that of controls showed an association between birth defects and ART (Kallen *et al*. 2005*b*). More recently, a multicenter American case–control study has corroborated these observations (Reefhuis *et al*. 2009). Nevertheless, none of the studies looked into a possible association between the administration of drugs used for ovarian stimulation and the incidence of congenital diseases. Data from a meta-analysis by Elizur & Tulandi (2008) suggest that the risk of congenital diseases caused by drugs commonly used in infertility treatments such as aromatase inhibitors, GNRH agonists and antagonists, oestrogen and progesterone may be null or minimal. Clomiphene treatment was the only exception, as it might be associated with a slightly higher risk of neural tube defect and hypospadia.

In order to determine the details of adverse birth events in children conceived by ART, the majority of studies mentioned above consulted national or regional registries (Wennerholm *et al*. 1997, Kallen *et al*. 2005*a*, 2005*b*, Wang *et al*. 2005, Griesinger *et al*. 2008, Jacod *et al*. 2008). The studies by Kapiteijn *et al*. (2006) and Reefhuis *et al*. (2009) were predominantly based on interviews of mothers, who were asked to recall information regarding the preconceptional and pregnancy periods (method of conception, ethnicity, parity, duration of gestation, birth weight, etc). This method of data collection can lead to significant biases, and therefore extrapolations based in this kind of analysis have to be moderate.

Overall, however, from the studies done so far, it seems that the risk of birth defects in children conceived by ART is very small, just 1–2% greater than reported in naturally conceived children (Elizur & Tulandi 2008). However, follow-up studies in adulthood are crucial for a real evaluation of possible long-term effects of ART.

Conclusions and future perspectives

Since the birth of Louise Brown in 1978, significant advances have been made in both clinical and laboratory aspects of IVF treatment. However, pregnancy rates remain relatively low, showing there is still much to be learned about the endocrinology of follicle development, oocyte maturation and ovulation, as well as embryo development and implantation. New advances in molecular biology (genomics, epigenetics, proteomics and pharmacogenomics) will contribute to increase our knowledge on ovarian and endometrial physiology and the impact of stimulation regimens at the molecular level, which is still poorly understood. With this

knowledge, milder ovarian stimulation regimens can be designed, which reduce the potentially adverse effects (Table 1) on embryo development. Furthermore, as different patients show distinct responses to the same stimulation protocol, a better understanding of the mechanisms that are affected by ovarian stimulation will help in the development of patient-specific treatments.

A major determinant of IVF success is the accurate selection of the most competent embryos for embryo transfer. Morphology and development rate remain the cornerstones of embryo selection, but are a limited measure of embryo competence. Other techniques such as FISH have been employed to assess the chromosomal constitution of embryos prior to selection for transfer. This is termed as PGS. Although a number of observational and uncontrolled studies have suggested higher pregnancy rates and reduced miscarriage rates could be achieved after PGS (Devroey & Fauser 2007), large randomised trials have shown no benefit for

Table 1 Summary of the possible impact of ovarian stimulation used for IVF on embryo development.

Parameters correlated with ovarian stimulation	Main outcomes	References
Oocyte development	Retrieval of high numbers of oocytes correlated to lower quality and lower chances of conceiving	Pellicer *et al.* (1989) and Van der Gaast *et al.* (2006)
Embryo development	No affect of ovarian stimulation administration on normal cleavage rates, morphology, developmental capacity, or degree of fragmentation	Ng *et al.* (2000, 2003) and Ziebe *et al.* (2004)
	No effect of high doses of GNRH antagonist on implantation potential of embryos in frozen–thawed cycles	Kol *et al.* (1999)
	Early human IVF embryos with high rates of chromosomal aneuploidy and mosaicism	Munne *et al.* (1997), Katz-Jaffe *et al.* (2005) and Baart *et al.* (2007)
	Possible association between ovarian stimulation protocols and chromosomal mosaicism	Munne *et al.* (1997) and Baart *et al.* (2007)
	Higher doses of FSH used in the long GNRH agonist stimulation protocol lead to increased proportion of aneuploid embryos	Baart *et al.* (2007)
	Higher doses of FSH associated with meiotic and mitotic chromosome 21 cell division errors	Katz-Jaffe *et al.* (2005)
	Repeated rounds of ovarian stimulation may compromise oocyte competence. Implantation and pregnancy rates decline from cycle 1 to 2 and reach a plateau after cycle 2	Shapiro *et al.* (2001), Silberstein *et al.* (2005) and Wang *et al.* (2008)
Epigenetics	Methylation defects at the DMRs of *Snrpn* (Angelman syndrome), *Kcnq1ot1* (Beckwith–Wiedemann syndrome) and *Peg1/Mest* (Silver–Russel syndrome) identified in affected children conceived with ART	Lawrence & Moley (2008)
	Correlation between the number of abnormally methylated embryos and embryo loss during implantation	Shi & Haaf (2002)
	Loss of *Peg1* and gain of *H19* methylation in oocytes obtained after ovarian stimulation	Sato *et al.* (2007)
	Trophectoderm-derived tissues more susceptible to imprinting disruption following ovarian stimulation	Mann *et al.* (2004) and Fortier *et al.* (2008)
	Putative folliculogenesis disruption due to alteration of folate metabolism after ovarian stimulation	Boxmeer *et al.* (2008)
Endometrial receptivity	Oestrogen levels above the threshold lead to endometrial secretory advancement	Macklon *et al.* (2006) and Hayden (2008)
	The reduction of embryo exposure to high levels of oestrogen using a step-down regimen in a subsequent cycle improves implantation rates	Simon *et al.* (1998)
Perinatal outcomes	ART enhances the risk of multiple pregnancies, which are associated with increased risk of miscarriage, growth retardation and preterm delivery	Fauser *et al.* (2005)
	Singleton IVF babies are at higher risk of low birthweight, premature birth and perinatal mortality and morbidity in the infertile population	Schieve *et al.* (2002), Helmerhorst *et al.* (2004), Jackson *et al.* (2004), Kapiteijn *et al.* (2006) and Ombelet *et al.* (2006)
	Low birthweight in IVF singletons associated with ovarian stimulation	Wennerholm *et al.* (1997), Kallen *et al.* (2005*a*), Wang *et al.* (2005) and Kapiteijn *et al.* (2006)
	Infants born after IVF have a higher risk of developing congenital malformations	Kallen *et al.* (2005*b*) and Reefhuis *et al.* (2009)
	The risk of congenital diseases caused by aromatase inhibitors, GNRH agonists, GNRH antagonists, oestrogen and progesterone may be null or minimal. Clomiphene was the only drug associated with a slightly higher risk of neural tube defect and hypospadia	Elizur & Tulandi (2008)
	The overall increased risk of congenital diseases in children conceived by ART is 1–2%	Elizur & Tulandi (2008)

pregnancy and delivery rates (Staessen *et al*. 2004, Mastenbroek *et al*. 2007). This could be explained by possible damage to the embryo during blastomere biopsy; limitations of FISH technology (only a few chromosomes can be analysed); and the phenomenon of chromosomal mosaicism (Devroey & Fauser 2007). Future studies focusing on a better understanding of the mechanisms and clinical significance of chromosomal mosaicism in early stage embryos may aid interpretation of PGS data, while alternative techniques to FISH such as comparative genomic hybridisation (CGH) offer the ability to analyse the complete set of chromosomes. An alternative to the invasive PGS approach is provided by the analysis of cumulus cell gene expression, which has been proposed as a non-invasive way of accessing embryo quality. Cumulus cells are closely associated with oocytes, and oocyte–cumulus cell communication has been shown to be essential to oocyte development (Hutt & Albertini 2007). Therefore, the study of differential expression of genes involved in key cumulus cell regulatory pathways using the real-time quantitative PCR offers a promising approach to increase our understanding of the factors controlling follicular development. This would allow not only identification of the most competent oocytes but also monitoring the consequences of different stimulation protocols on the cohort of oocytes retrieved, ultimately contributing to a better understanding of the impact of ovarian stimulation on embryo development.

Although most of the research has been mainly focusing in investigating oocyte and embryo development, in an attempt to explain relatively low pregnancy rates, the success of ART does not depend solely on the quality of the embryo, as pre-clinical losses rather than failure of conception are suggested as the main limiting factor (Macklon *et al*. 2002). The crosstalk between the embryo and the endometrium seems to be of major importance for achieving implantation and successful pregnancy. However, there is still poor knowledge of the mechanisms involved in such communication. Therefore, new studies exploring the molecular interactions occurring at the embryo–endometrial interface will be crucial to explain low implantation rates and hopefully improve pregnancy rates in patients undergoing ART (Teklenburg & Macklon 2009).

According to the studies published so far, most medications used in ART appear to be safe. However, it is necessary to carefully reassess the safety of ovarian stimulation on the first generation of ART-generated children that is now reaching adulthood. The true impact of ovarian stimulation on the development of the offspring will only become clear when the offspring of this generation have reached maturity. Until then, while major detrimental effects appear to be limited, caution continues to be required when developing and administering novel ovarian stimulation regimens for IVF.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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