Embryo implantation and GnRH antagonists

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When gonadotrophin-releasing hormone (GnRH) was discovered, the agonist and antagonist of GnRH were developed to control the release of FSH and LH by the gonadotrophs. More than 10 years of research were needed to develop a GnRH antagonist free of histamine release. Recent studies have shown that these GnRH antagonists are effective in preventing a rise in LH during ovarian stimulation in IVF. However, a decrease in ongoing pregnancies seems to suggest that implantation rates per transferred embryo are reduced in GnRH antagonist-stimulated cycles. In my opinion, these data highlight an area less well known to clinicians: the role of the GnRH antagonist at the cellular level in extrapituitary tissues. There are sufficient data in the literature suggesting that GnRH antagonist is an inhibitor of the cell cycle by decreasing the synthesis of growth factors. Given that, for folliculogenesis, blastomere formation and endometrium development, mitosis is everything; the interaction between the GnRH antagonist and the GnRH receptor (present in all these cells and tissues) may compromise the mitotic programme of these cells. This is the Rubicon for the GnRH antagonist: to demonstrate irrevocably that, at the minimal doses necessary to suppress LH release, it does not affect processes such as implantation, embryo development and folliculogenesis.

Key words: embryo implantation/GnRH agonists/GnRH antagonists

Introduction

When Julius Caesar was marching against Pompey, he was stopped by a natural barrier: the Rubicon river. At that moment, he knew that an irrevocable step had to be taken. A decision with only two possibilities: to conquer or perish. Since then, the term ‘Rubicon’ is applied to a situation that is resolved by a decisive step. Implantation is the Rubicon for the gonadotrophin-releasing hormone (GnRH) antagonist.

Shortly after GnRH was discovered, the agonist and antagonist of the GnRH were developed to control the synthesis and release of FSH and LH by the gonadotrophs (Karten and Rivier, 1986; Arimura, 1991; Reissmann et al., 1995). As everyone is aware, the results did not turn out to be quite as the research team planned. A GnRH agonist, after a burst of stimulation of FSH/LH (flare-up), behaves as an antagonist, and the GnRH antagonist develops an unpleasant ‘gusto’ for histamine release (Morgan et al., 1986; Rivier et al., 1996). Nevertheless, the suppression of FSH and LH secretion by the GnRH agonist generated a lot of success in clinical practice, and was soon used as the standard treatment to control the growth of hormonally-dependent tumours (Filicori et al., 1983; Broekmans, 1996). Later, GnRH agonists were introduced in the protocols for ovulation induction as a means of avoiding premature luteinization (Meldrum, 1994; Fauser et al., 1999). They have been indispensable ever since.

More that 10 years of research by a group of selected scientists were needed to untie the labyrinth of chemistry to develop a GnRH antagonist free of histamine release. More than 10 years of research were needed to develop a GnRH antagonist free of histamine release (Bajusz et al., 1988; Schally et al., 1989; Rivier et al., 1996). Finally, they have arrived, i.e. Cetrorelix® (ASTA Medica AG; Frankfurt, Germany) and Ganirelix® (Organon, Oss, The Netherlands). Recent studies have shown that these GnRH antagonists are effective in preventing the LH rise during ovarian stimulation for IVF (Frydman et al., 1991; Diedrich et al., 1994; Leroy et al., 1994; Olivennes et al., 1994; Felberbaum et al., 1995; Diedrich and Felberbaum, 1998; Felberbaum and Diedrich, 1999) and their clinical efficacy has been confirmed by large multicentre phase III clinical trials (Ganirelix® Dose-Finding Study Group, 1998). So far, in all the clinical studies with the GnRH antagonists (independent of the dose used; 0.25 mg/day or 3.0 mg as depot), LH has been successfully suppressed, a lower amount of FSH was required, patient satisfaction was highly rated (no histamine release) and ovarian hyperstimulation syndrome (OHSS) was cut by half, when compared with patients treated with the GnRH agonist. Furthermore, no significant differences in the number of oocytes retrieved, fertilization rates and embryo quality between patients treated with GnRH agonist or GnRH antagonist, were found. However, a decrease in oestradiol concentrations, pregnancy rates and ongoing pregnancies seem to suggest that implantation rates per transferred embryo are reduced in GnRH antagonist-stimulated cycles (Ganirelix® Dose-Finding Study Group, 1998; Felberbaum and Diedrich, 1999; Fauser et al., 1999). Although not statistically significant (with 0.25 mg or 3 mg of GnRH antagonist), it is worth mentioning that these parameters were aggravated in a dose-dependent manner. For example, implantation rates varied from 1 to 20% when 2 or 0.25 mg/day of the GnRH antagonist was administered respectively (Ganirelix® Dose Finding Study Group, 1998; Felberbaum and Diedrich, 1999). In my opinion, these data bring to light a little known area to clinicians: the
role of GnRH antagonists at the cellular level in extrapituitary tissues. The notion that the mechanism of action by which the GnRH antagonist controls the growth and differentiation of tissues and organs by suppressing gonadotrophin and steroid production is incomplete; the GnRH antagonist is more powerful than that. There are sufficient data in the literature to support the notion that GnRH antagonists are a potent inhibitor of the cell cycle by decreasing the synthesis of locally-produced growth factors, in a dose dependent manner (Emons et al., 1992; Hershkovitz et al., 1993; Kleinman et al., 1993; Emons and Schally, 1994; Moretti et al., 1996). Given that folliculogenesis, blastomere formation and endometrium development, mitosis is everything; and that GnRH receptors are present in all these cells and tissues (Dekel et al., 1988; Emons et al., 1992; Emons et al., 1993; Minaretzis et al., 1995a; Murdoch, 1995; Emons et al., 1997; Ortmann and Diedrich, 1999; Casañ et al., 1999), the possibility of an interaction between the GnRH antagonist and the GnRH receptor is possible and manifested as lower implantation rates. This is the Rubicon 1993; Horvath, 1999), the possibility of an interaction between the have been used (Fekete et al., 1995; Emons et al., 1997; Reismann et al., 1992; Emons et al., 1993; Horvath et al., 1995). In these in-vitro studies, GnRH antagonists restrain cell growth by decreasing the synthesis and the growth stimulatory effect of insulin-like growth factors (IGF); probably through effects on a post-receptor mechanism (Hershkovitz et al., 1993; Kleinman et al., 1993). Furthermore, inositol 1,4,5-trisphosphate (a second messenger of GnRH) was inhibited by GnRH antagonists (Beckers et al., 1995). Moreover, receptors for epidermal growth factor (EGF) were significantly down-regulated, and a decrease in EGF receptor mRNA and EGF peptide to non-detectable values was seen in culture in the presence of the GnRH antagonist (Pinski et al., 1994; Shirahige et al., 1994; Moretti et al., 1996). Taken together, these studies demonstrate that GnRH antagonist is able to control the growth of the cell by governing the production of growth factors. Interestingly, IGF and EGF are peptides of a family of growth factors that exert their action through receptors with intrinsic tyrosine kinase activity (LeRoith et al., 1995). These receptors that phosphorylated tyrosine residues form the basis for the generation of an important mitogenic cascade that involved p21-ras, MAP kinases (MAPK) and activation of c-fos and c-jun. MAPK are some of the central enzymes in the growth factor-induced signalling pathway, because they control the concentrations of cyclin D/Cdk4 and cyclin E/Cdk2. In the G1 phase of the cell cycle, these cyclins are important because they control the overall proliferation process, since entry into the S-phase requires both cyclins activated (Van Zoelen, 1999). In fact, embryonic fibroblast from the mutants lacking IGF-I receptors showed that the cell cycle is 2.5-fold longer than normal, indicating that this signalling system influences the most important determinant: the rate of cellular division that increases total cell number (Sell et al., 1994).

Theoretically, if IGF and EGF (which control the cell cycle by activation of MAPK and cyclins) are inhibited by the GnRH antagonist (Schally and Vargas, 1999), a direct effect of the GnRH antagonist in the cascade that regulates the transition through the checkpoints of the cell cycle in folliculogenesis, implantation and embryo development cannot be excluded. In my opinion, these are the reproductive targets have been demonstrated in ovary, testis, uterus, in human endometrium of fertile patients, mammary gland and a number of different malignant cells (Hsueh and Jones, 1981; Dekel et al., 1988; Fekete et al., 1989a,b; Srkalovic et al., 1990; Emons et al., 1992, 1993; Minaretzis et al., 1995a; Murdoch, 1995; Ortmann and Diedrich, 1999).

Although the concentration of hypothalamic GnRH in the systemic circulation is considered to be too low to interact with the extrapituitary GnRH receptors, it is important to keep in mind that the amount of GnRH antagonist used in reproductive medicine may lead to values that can activate the extrapituitary GnRH receptor (Rivier et al., 1996; Ortmann and Diedrich, 1999).

To determine the mechanism of action of GnRH antagonist at the cellular level, human ovarian cancer, breast cancer, prostate carcinoma, and JAR human choriocarcinoma cell lines have been used (Fekete et al., 1989a; Sharoni et al., 1989; Emons et al., 1992; Reismann et al., 1992; Emons et al., 1993; Horvath et al., 1995). In these in-vitro studies, GnRH antagonists restrain cell growth by decreasing the synthesis and the growth stimulatory effect of insulin-like growth factors (IGF); probably through effects on a post-receptor mechanism (Hershkovitz et al., 1993; Kleinman et al., 1993). Furthermore, inositol 1,4,5-trisphosphate (a second messenger of GnRH) was inhibited by GnRH antagonists (Beckers et al., 1995). Moreover, receptors for epidermal growth factor (EGF) were significantly down-regulated, and a decrease in EGF receptor mRNA and EGF peptide to non-detectable values was seen in culture in the presence of the GnRH antagonist (Pinski et al., 1994; Shirahige et al., 1994; Moretti et al., 1996). Taken together, these studies demonstrate that GnRH antagonist is able to control the growth of the cell by governing the production of growth factors. Interestingly, IGF and EGF are peptides of a family of growth factors that exert their action through receptors with intrinsic tyrosine kinase activity (LeRoith et al., 1995). These receptors that phosphorylated tyrosine residues form the basis for the generation of an important mitogenic cascade that involved p21-ras, MAP kinases (MAPK) and activation of c-fos and c-jun. MAPK are some of the central enzymes in the growth factor-induced signalling pathway, because they control the concentrations of cyclin D/Cdk4 and cyclin E/Cdk2. In the G1 phase of the cell cycle, these cyclins are important because they control the overall proliferation process, since entry into the S-phase requires both cyclins activated (Van Zoelen, 1999). In fact, embryonic fibroblast from the mutants lacking IGF-I receptors showed that the cell cycle is 2.5-fold longer than normal, indicating that this signalling system influences the most important determinant: the rate of cellular division that increases total cell number (Sell et al., 1994).

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where the GnRH antagonist may impact when used in assisted reproductive techniques.

**At the level of the ovarian cells**

Growth factors are important for ovarian cells physiology (Adashi et al., 1991; Giudice 1992; Hernandez et al., 1992; Ojeda and Dissen, 1994; Hernandez, 1995; Baker et al., 1996). For example, EGF and IGF induce the differentiation of granulosa and theca–interstitial cells in conjunction with FSH and LH (Hernandez et al., 1988; Bendell and Dorrington, 1990; Mason et al., 1990; Adashi et al., 1991). Probably, IGF are one of the signals that may induce the granulosa cells of the primordial follicles to exit the G0 phase of the cell cycle and enter the G1/S phase, by activation of cyclins. Interestingly, *cyclin D2* mRNA was specifically localized to granulosa cells of growing follicles and in mice null for cyclin D2, granulosa cell proliferation is impaired and ovulation does not occur (Rebecca and Richards, 1998). Like FSH, IGF-I is an anti-apoptotic agent which preserves the granulosa cells to enter in apoptosis (Tilly and Robles, 1999). Given that the granulosa cells harbour GnRH receptors (Minaretzis et al., 1995a; Ortmann and Diedrich, 1999), a direct effect of the GnRH antagonist in these cells can not be excluded. If GnRH antagonists inhibit the synthesis of growth factors involved in the cell cycle of the granulosa cells, the result will be seen as follows: follicles arrested in the primordial stage, increase in apoptosis (aggravated by the fact that the GnRH antagonist also induce apoptosis; Redding et al., 1992), decrease in the total number of granulosa cell per follicle and finally less oestradiol circulating. Curiously, some clinical data published reflect this hypothetical situation. For example, when 2 mg/ day of the GnRH antagonist was used, no increase in oestradiol secretion and no follicle growth was seen, an effect that was alleviated when the doses of the GnRH antagonist were decreased (Ganirelix® Dose Finding Study Group, 1998; Felberbaum and Diedrich, 1999). Although no explanation was given at that time, it seems to me that a direct effect of the GnRH antagonist (receptor-mediated) in the granulosa cells could be the cause of oestradiol decrease and follicle quiescence. So far, studies done with cultured luteinized human granulosa cells are controversial, because it has not been demonstrated that GnRH antagonist inhibits oestadiol production by human granulosa cells. Minaretzis found that GnRH antagonist was able to inhibit aromatase activity, but Ortmann did not find any significant effects of the GnRH antagonist on steroids production (Minaretzis et al., 1995b; Ortmann and Diedrich, 1999). Nevertheless, these experiments were more to determine the role of the GnRH antagonist in the differentiation programme of the granulosa cells and not in the cell cycle. Hence, a direct impact of the GnRH antagonist in cell division (where it probably is working) still cannot be excluded. A further clarification about the effect of the GnRH antagonist in the luteinized granulosa cells is needed, because IGF-I plays an important role in the differentiation programme of corpus luteum formation and function (Talavera and Menon, 1991; Sugino et al., 1999). Since the first stages of corpus luteum activity will occur in the presence of significant circulating concentrations of the GnRH antagonist, inhibition of IGF action may lead to a decrease in the production of progesterone by the luteinized granulosa cells, disrupting the transformation of the endometrial cells and making implantation more difficult. Nevertheless, more in-vivo and in-vitro data must be performed and collected to test and confirm this hypothesis.

**At the oocyte level**

By the studies performed in the *Xenopus laevis* oocyte, it is known that growth factors with intrinsic tyrosine kinase activity and p21ras (IGF-I induced) are important signals for oocyte maturation (Sadler, 1991). In the mouse and human oocytes this pathway is not so well defined, but there are reports suggesting that the IGF and EGF may be part of an auto-paracrine signal for oocyte maturation (Dekel and Sherizly, 1985; Das et al., 1991; Goud et al., 1998; de la Fuente et al., 1999). Given that receptors for GnRH have been found in oocytes (Dekel et al., 1988), and that GnRH antagonist decreased EGF peptide and message (Pinski et al., 1994; Shirahige et al., 1994; Moretti et al., 1996), the possibility exits that the GnRH antagonist interferes with the mechanisms involved in germinal vesicle breakdown as well as the cell signalling pathway driving the oocyte into metaphase II. Nevertheless, clinical results have not shown any significant differences in number of MII oocytes collected in women treated with the GnRH antagonist versus the agonist (Ganirelix® Dose Finding Study Group, 1998). Undoubtedly, more research needs to be done to determine the role of GnRH antagonist in the maturation process of the human oocyte.

**At the embryo development level**

If we have learned anything with the inactivation of a given gene by homologous recombination technology, it is the importance of growth factors and their receptors in embryo development (Kane et al., 1997). For example, knock-out mice for the IGF-I and IGF-II do not grow properly and are infertile. invalidation of the IGF-1 receptor gene is incompatible with life, due to respiratory failure (De Chiara et al., 1990; Baker et al., 1993; Liu et al., 1993; Wolf et al., 1994; Baker et al., 1996). IGF-II is very important in embryo development, because it has been demonstrated that it stimulates the growth and metabolism of pre-implantation mouse embryo (Funk et al., 1992; Harvey and Kaye, 1992). As in the murine model (Heyner et al., 1989; Bondy et al., 1990), the presence of the IGF system in the human embryo has been shown by polymerase chain reaction (Liu et al., 1997). Given that the GnRH antagonist is able to inhibit the synthesis of these growth factors, a deleterious effect of the GnRH antagonist in the mitotic programme of the developing embryo cannot be excluded. To the best of my knowledge, no publications dealing with the impact of the GnRH antagonist in the human embryo exist. However, an important study was recently published exploring the role of GnRH in murine preimplantation embryonic development (Raga et al., 1999). In this study, Raga has reached two interesting conclusions in the understanding of the role of GnRH antagonist in embryo development: (i) the
presence of GnRH receptors in all the developmental embryonic stages studied and (ii) a complete block of embryo development produced by increasing concentrations of the GnRH antagonist in vitro. Nevertheless, the drastic effect of the GnRH antagonist in embryo development, as described (Raga et al., 1999), may be due to the in-vitro conditions (close contact between embryo and GnRH antagonist), and might not mirror the clinical situation. In fact it is important to bear this in mind, because in IVF cycles the GnRH antagonist is suspended when human chorionic gonadotrophin (HCG) is applied, the half-life of the GnRH antagonist is ~30 h (Hermann et al., 1996) and the embryo is transferred between 5–7 days after the last doses of GnRH antagonist. Therefore, a potential harmful effect of the GnRH antagonist in the developmental programme of the human embryo is unlikely (Kol et al., 1999). In fact, the same number of good embryos were transferred to patients under GnRH agonist or GnRH antagonist regimens (Ganirelix® Dose Finding Study Group, 1998). Although this line of reasoning may be good for IVF cycles, things are not so clear for intrauterine insemination (IUI) cycles (although no data is available on the use of GnRH antagonist for IUI). Firstly, because it has been shown that GnRH antagonists inhibit sperm binding to the human zona pellucida (Morales et al., 1999) and, secondly, because it has been proposed that EGF (expressed and synthesized by the human oviduct epithelium in an oestradiol-dependent manner) may be involved in early embryonic development (Morishige et al., 1993). Hence, in IUI cycles a direct impact of GnRH antagonist in the fertilization process and early blastomere formation cannot be excluded completely (Casaná et al., 1999). If this hypothesis is correct, to induce the ovulation with GnRH to displace the GnRH antagonist for the GnRH receptor may be necessary. Although this has already been done (Olivennes et al., 1995, 1996), more clinical trials comparing the pregnancy rates in cycles where ovulation was provoked with HCG or GnRH need to be performed.

At the endometrial cell level

In my opinion, the endometrial epithelium (with the granulosa cell) is the target tissue where the GnRH antagonist may be more disrupting. Firstly, because if the GnRH antagonist decreases the production of oestradiol by the granulosa compartment, the circulating concentrations of this steroid may be insufficient to develop an ideal endometrium to maintain the life of the incipient human embryo. Secondly, because the GnRH antagonist is administered at a moment when the endometrial cells are obliged to perform synchronous waves of mitosis to form a ripe endometrium, and the GnRH antagonist may inhibit this commitment. In fact, GnRH antagonist inhibited human endometrial cancer cell lines, as previously mentioned, and GnRH receptors have been described in the human endometrium of fertile patients and murine endometrium (Murdoch, 1995; Casaná et al., 1998; Raga et al., 1998; Dong et al., 1998). Moreover, EGF and IGF genes and peptides are produced by the endometrial cells in an oestradiol-dependent manner (Mukku and Stancel, 1985; Murphy et al., 1987; Kapur et al., 1992; Kleinman et al., 1996; Rajkumar et al., 1996) and IGF-II gene (expressed in human placenta) is very active in the cytotrophoblast (Shen et al., 1986; Ohlsson et al., 1989). As a result, the possibility exists that the GnRH antagonist may be disrupting an auto/paracrine loop (oestrogen growth factors) that is essential for the mitotic programme of the endometrial cells and it is manifested by a decrease in the pregnancy rates and an increase in the abortion rates. In-vitro studies need to be designed as soon as possible to determine the effect of the GnRH antagonist at the doses of 0.25 mg/day in the mitotic programme of the endometrial cell. This is very important to decipher, as years of research to improve culture conditions, blastocyst transfer and embryo selection by genetic analysis is meaningless if the end result is compromised by an unfavourable endometrium.

In conclusion, given that GnRH receptors are ubiquitously localized in the reproductive tract, oocyte and embryo, activation of the GnRH receptor by the GnRH antagonist may be possible. As a result, this binding will decrease the synthesis of growth factors involved in the control of the cell cycle, compromising the mitotic programme of granulosa and endometrial cells (in IVF cycles) and fertilization mechanism and zygote development (in IUI cycles) as well.

Future perspectives

The good news is that, with the minimal effective doses of GnRH antagonist (2.5 mg/day and 3 mg/depot) to inhibit LH secretion, embryo losses seem to be low (Ganirelix® Dose Finding Study Group, 1998; Felberbaum and Diedrich, 1999). The biological clues behind these phenomena are difficult to explain, but are probably due to mechanisms of redundancy of function (a loss of growth factor is substituted by another), type of ovulation induction protocols and endometrial epithelium more advanced than others (frequently seen in IVF cycles). In any event, more basic research needs to be done to determine: (i) the impact of this minimal dose in the production of growth factors and in the mitotic programme of the granulosa and endometrial cells, embryo development and oocyte maturation; (ii) whether ovulation stimulated with GnRH (to displace the GnRH antagonist for the GnRH receptors) rather than HCG is reflected in better pregnancy rates; and (iii) whether decreasing the number of days of GnRH treatment (to <1 week) may improve implantation. In my opinion, all these considerations are important because the pregnancy rates in IVF–embryo transfer in humans are rigidly limited by the barrier of implantation. Furthermore, it is well known that not all IVF clinics procure the same pregnancy rates; hence, a small decrease in ongoing pregnancies may be important in the final results of some groups. This is the Rubicon, the natural barrier the GnRH antagonist is confronting: pregnancy rates. A decrease in clinical pregnancies (significant or not) in the assisted reproduction field, where the final goal is to increase the number of pregnancies, is difficult to accept. GnRH antagonist will have to prove itself as useful as GnRH agonist to cross the Rubicon and, as Caesar did on a different occasion, proclaim: vidi, vini, vici.
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