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Gonadotrophin-releasing hormone antagonists inhibit sperm binding to the human zona pellucida

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Previous work from our laboratory indicated that gonadotrophin-releasing hormone (GnRH) increases human sperm–zona pellucida binding. Here we present evidence that GnRH antagonists inhibit sperm–zona pellucida binding in humans. Motile spermatozoa (10^7 cells/ml) were incubated in modified Tyrode's medium at 37°C, in 5% CO_2 in air. After 4.5 h, aliquots of spermatozoa were treated with saline (control) or with different concentrations of GnRH antagonists (test). Each sperm aliquot was then tested in the hemizona binding assay. In this assay, the control aliquot was incubated with half a human zona pellucida (hemizona) and the test aliquot was incubated with the matching half. After 20 min, the hemizonae were withdrawn and the number of zona-bound spermatozoa counted using phase-contrast microscopy. In addition, the effect of GnRH antagonists upon the pattern of sperm movement, frequency of sperm–zona pellucida collisions, and percentage of living and acrosome-reacted spermatozoa was determined. The results indicated that treatment with GnRH antagonists decreased the number of zona-bound spermatozoa and did not change the pattern of sperm movement, frequency of sperm–zona collisions, and percentage of acrosome-reacted spermatozoa. We suggest that this action of GnRH antagonists may be due to an effect on zona receptors on the sperm plasma membrane.

Key words: GnRH antagonists/human zona pellucida/sperm–zona collisions/sperm–zona binding

Introduction

Sperm–oocyte binding is a crucial step in the process of mammalian fertilization (Yanagimachi, 1994). This interaction between gametes is thought to involve specific receptors on the surface of the sperm plasma membrane and glycoproteins of the zona pellucida (ZP) (for references, see Morales and Llanos, 1996). In the majority of the mammalian species studied to date, sperm binding to the ZP triggers exocytosis of the acrosome. The acrosome reaction, a crucial event for the success of fertilization, takes place after the fertilizing spermatozoon binds to the surface of the ZP, induced by the protein component of ZP3, one of the zona glycoproteins (Yanagimachi, 1994). Thus, molecules that modify the ability of spermatozoa to bind to the ZP are of physiological significance, as they could interfere with the outcome of the fertilization process.

Gonadotrophin hormone-releasing hormone (GnRH) is a decapeptide of hypothalamic origin, whose structure is: pyro-Glu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-amide. The amino acids 1 and 10 are recognized by the receptor, and amino acids 2 and 3 are responsible for the biological activity (Schaison, 1990). GnRH binds to a specific receptor located on the pituitary gland, causing release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The effect of GnRH is mediated by intracellular Ca^{2+} influx (Conn *et al*., 1979; Nikolics and Conn, 1990).

The presence of GnRH or 'GnRH-like' molecules has been detected in human follicular fluid (Ying *et al*., 1981) and seminal plasma (Izumi *et al*., 1985; Sokol *et al*., 1985). Although its origin is still unknown, it has been suggested that the GnRH-like material in seminal plasma may originate in the prostate, an organ in which the pro-hormone and its mRNA have been identified (Azad *et al*., 1993). Moreover, local production of GnRH-like material has been reported in the ovary (Hsueh and Schaeffer, 1985; Saint *et al*., 1988; Bahk *et al*., 1995) and testis (Aten *et al*., 1986, 1987; Li *et al*., 1993) where it is produced by granulosa and Sertoli cells respectively. These observations suggest that GnRH-like molecules may play an endocrine and/or paracrine function in these extrapituitary tissues (Siler-Khodr *et al*., 1990).

Recently, GnRH and GnRH agonists have been shown to enhance bovine in-vitro fertilization through an effect on the cumulus–oocyte complex (Funston and Seidel, 1995). In addition, a tripeptide that is of prostatic origin and structurally related to thyrotrophin-releasing hormone, pyroglutamylglutamylproline-amide, has been implicated in the process of mouse and human sperm capacitation (Green *et al*., 1996; Fraser *et al*., 1997). Our own work indicates that GnRH enhances sperm–ZP binding ability in humans (Morales, 1998). This effect of GnRH only takes place when there is calcium in the culture medium, and is mediated by a calcium influx (Morales *et al*., 1998).

Taken together, these observations have led us to postulate that human spermatozoa might interact with GnRH (or GnRH-like molecules) during their journey through the male and female genital tracts. This interaction with GnRH might confer on the spermatozoa increased zona-binding capabilities.

Conversely, prevention of this interaction might render the spermatozoa unable to initiate binding to the ZP and therefore decrease or totally inhibit the possibility of conception. In this work, we report observations which indicate that GnRH antagonists inhibit sperm–ZP binding *in vitro*. Part of this work has been presented previously (Morales *et al.*, 1999).

Materials and methods

The following GnRH antagonists were purchased from Sigma Chemical Co. (St Louis, MO, USA): D-Phe^{2,6}, Pro^{3}-GnRH (Phe-Pro); Ac-D-Nal^{1}-Cl-D-Phe^{2}-3-Pyr-D-Ala^{3}-N-e-Nicotinoyl-Lys^{5}-N-e-Nicotinoyl-D-Lys^{6}-N-e-Isopropyl-Lys^{8}-D-Ala^{10}-GnRH (Antide); and Ac-D-Nal^{1}-Cl-D-Phe^{2}-3-Pyr-D-Ala^{3}-Arg^{5}-D-Glu(AA)^{6}-GnRH (Nal-Glu). The GnRH antagonist Ac-^{3,4}-dehydro-Pro^{1},-*p*-fluoro-D-Phe^{2}, D-Trp^{3,6}-GnRH (4pF), was obtained from Bachem Bioscience Inc. (Torrance, CA, USA).

Source and preparation of biological material

Human zonae pellucidae

The zonae pellucidae used in this study were recovered from human cadaveric ovarian tissue as described previously (Morales *et al.*, 1989, 1991). Briefly, ovarian tissue was placed on ice and dissected immediately following a published protocol (Overstreet *et al.*, 1980). Zona-intact, immature oocytes were denuded of granulosa cells, placed in capillary tubes containing 2 mol/l dimethylsulphoxide in phosphate-buffered saline, and stored at –80°C. Before use, the oocytes were cut to obtain two equal halves or hemizonae (see below).

Human spermatozoa

Samples were obtained from ten donors with normal semen according to WHO guidelines (WHO, 1992). Motile spermatozoa were selected using a two-step Percoll gradient as described previously (Suarez *et al.*, 1986; Morales *et al.*, 1991). The spermatozoa were then resuspended at 10^7 cells/ml and incubated in modified Tyrode's medium (Suarez *et al.*, 1986), supplemented with 2.6% bovine serum albumin (BSA), at 37°C, in 5% CO_2 in air.

Sperm–zona pellucida binding assay

To test the effect of GnRH antagonists on sperm–ZP binding ability, the hemizona assay was used (Burkman *et al.*, 1988; Franken *et al.*, 1989). Briefly, 100 µl droplets of control and test sperm suspensions were held under oil in a plastic Petri dish. One hemizona was then added to the control sperm droplet, and the matching hemizona was added to the test sperm droplet. Control and test sperm droplets containing hemizonae were incubated for 20 min at 37°C, in 5% CO_2 in air. After incubation, each hemizona was removed and gently washed with a wide-bore pipette. The tightly bound spermatozoa on the outer surface of each hemizona were counted under a phase-contrast microscope.

Effect of GnRH antagonists on the gametes

General procedures

95 µl droplets of spermatozoa suspended in modified Tyrode's medium containing 2.6% BSA were treated by adding 5 µl of GnRH antagonists (test) or saline (control).

Sperm–ZP binding

Aliquots of sperm suspensions capacitated for 4.5 h were incubated with GnRH antagonists or saline for 20 min and then tested in the hemizona assay. To test whether GnRH antagonists have an effect on the ZP, one hemizona was incubated with a given GnRH antagonist and the control hemizona with saline for 20 min. The hemizonae were then washed in culture medium before adding untreated spermatozoa.

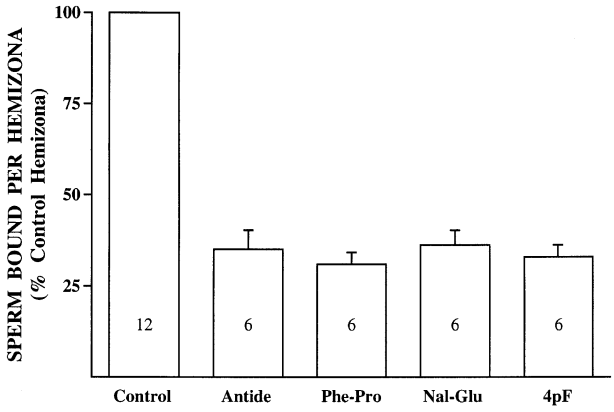

Figure 1. Number of human zona pellucida-bound spermatozoa in response to different GnRH antagonists. Data are expressed as a percentage (mean ± SEM) of the control hemizonae. Spermatozoa were capacitated for 4.5 h and then treated either with saline (control), 200 nmol/l Antide, 200 nmol/l Phe-Pro, 200 nmol/l Nal-Glu or 200 nmol/l 4pF for 20 min before the hemizona assay. The figure within each bar indicates the number of experiments in each group. All test groups were significantly different from the control group ($P < 0.0001$).

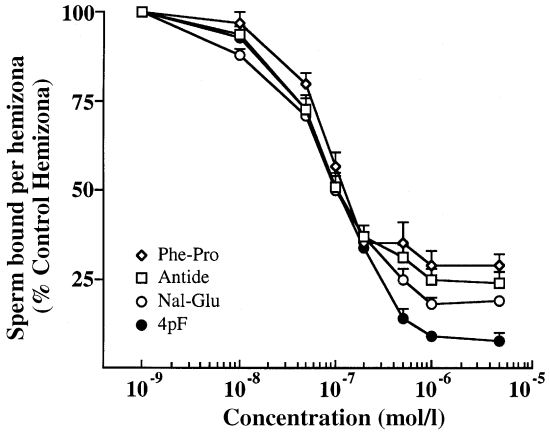

Figure 2. Effect of various doses of different GnRH antagonists on sperm binding to the human zona pellucida. Data are expressed as a percentage (mean ± SEM) of the control hemizonae. Sperm suspensions were incubated for 20 min with saline (control) or with different concentrations of Antide (□), Phe-Pro (◇), Nal-Glu (○), and 4pF (●) before the hemizona assay. In one experiment all doses of a single antagonist were tested; each experiment was repeated six times.

Acrosome reactions

Aliquots of spermatozoa incubated for 4.5 or 20 h were incubated with GnRH antagonists or saline for 20 min. As a positive control, 20 h-capacitated spermatozoa were treated with 20% (v/v) human follicular fluid (Morales *et al.*, 1992). The acrosomal status of the sperm suspensions was determined as described before (Cross *et al.*, 1986; Morales *et al.*, 1992). Briefly, aliquots of sperm suspensions were incubated with the supravital stain Hoechst 33258 (H258; Sigma Chemical Co.) and with the fluoresceinated *Pisum sativum* agglutinin (PSA; Vector Laboratories, Inc., Burlingame, CA, USA) to label dead cells and the acrosomal content, respectively. H258 and FITC-PSA fluorescences were examined using an Olympus fluorescence microscope.

Pattern of sperm movement

Motile sperm suspensions, obtained as described, were incubated with GnRH antagonists or saline for 20 min. Sperm movement was

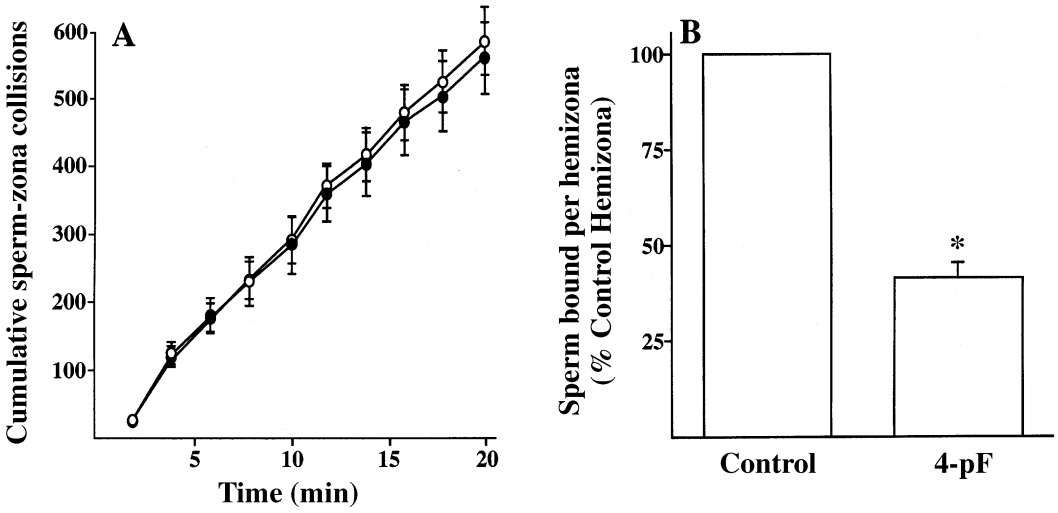

Figure 3. Frequency of sperm–zona pellucida collisions versus number of zona-bound spermatozoa. (**A**) Mean number of cumulative sperm–zona collisions per minute. Sperm suspensions were incubated for 20 min with saline (○) or 200 nmol/l 4pF (●) before recording sperm–zona interaction. Each symbol represents the mean ± SEM of six experiments. (**B**) Number of zona-bound spermatozoa (mean ± SEM). Data are expressed as a percentage (mean ± SEM of six experiments) of the control hemizonae. Sperm suspensions were incubated for 20 min with saline (control) or 200 nmol/l 4pF before the hemizona assay. *, significantly different from its respective control ($P < 0.01$).

then recorded using positive phase-contrast optics at 37°C. Videotape recording was carried out as described previously (Katz *et al*., 1982; Morales *et al*., 1988) and the videotapes were analysed manually, frame by frame. Twenty-five spermatozoa were analysed for each condition. Each spermatozoon was analysed for straight-line velocity, linearity of the trajectory, rolling frequency of the sperm head, flagellar beat frequency, and amplitude and flagellar curvature ratio. A detailed description of these procedures has been published previously (Katz *et al*., 1982; Morales *et al*., 1988).

Sperm–zona collisions versus binding

These experiments were designed to determine whether the effect of GnRH antagonists was related to a decrease in the number, or in the effectiveness of sperm–ZP collisions (defined as number of zona-bound spermatozoa divided by number of spermatozoa colliding with the zona×100; see Morales, 1998). Aliquots of test and control sperm suspensions, obtained as described before, were added to a hemizona attached to a plastic dish and incubated for 20 min at 37°C in 5% CO_2 in air, under oil. The sperm–hemizona interaction was recorded at a rate of 60 fields/s using a high-speed video camera (Tritronics, Burbank, CA, USA). A record of elapsed time in 0.01 s intervals, generated by a video time generator (For A, Los Angeles, CA, USA), was recorded simultaneously on a video cassette recorder (Victor Co., Tokyo, Japan). At the end of the recording period, the videotapes were analysed manually frame-by-frame to determine: (i) the number of sperm–ZP collisions; and (ii) the total number of zona-bound spermatozoa. A detailed description of these procedures was published previously (Morales, 1998)

Statistical analysis

Percentages were subjected to arc-sin transformation before analysis (Snedecor and Cochran, 1980). Bartlett's test for homogeneity, followed by the *F*-test and then the paired *t*-test and/or Dunnett's multiple comparison tests were used to compare the number of bound spermatozoa in the control and treated groups. Differences were considered significant at the 0.05 level of confidence.

Results

Treatment with 200 nmol/l of several GnRH antagonists significantly decreased the number of zona-bound spermatozoa (Figure 1). This effect depended upon the concentration of antagonist used (Figure 2). Doses of antagonists equal to or lower than 10 nmol/l did not affect the number of zona-bound spermatozoa. Doses between 50 nmol/l and 1000 nmol/l, however, had a significant inhibitory effect. Higher concentrations of the antagonists did not further inhibit sperm–zona binding (Figure 2). The inhibitory effect of the antagonists never reached 100%, even at the highest concentration tested (5 μmol/l). The data indicated that 4pF was the most potent inhibitor of sperm–zona binding, reaching a maximum inhibition of about 90% (Figure 2). This effect of the GnRH antagonists was specific on the spermatozoa; treatment of the ZP with any of the GnRH antagonists did not affect sperm–zona binding capacity (data not shown).

Analysis of the video tapes indicated that treatment with GnRH antagonists did not modify the pattern of sperm movement (Table I). There were no significant differences between the control and GnRH antagonist-treated groups in any of the sperm movement parameters studied (Table I). In addition, spermatozoa treated with 200 nmol/l 4pF did not differ from control spermatozoa in terms of frequency of ZP collisions (Figure 3A). This was true whether the number of sperm–zona collisions per minute (data not shown) or the cumulative frequency of collisions were compared (Figure 3A). The mean (± SEM) number of sperm–zona collisions per minute was 31 ± 3 in the control group, and 29 ± 4 in the experimental group. The total number of spermatozoa colliding with the zona during the 20 min recording period was 582 ± 44 and 553 ± 51 in the control and experimental groups respectively (Figure 3A). However, spermatozoa treated with 200 nmol/l 4pF bound to the ZP in significantly lower numbers (7.1 ± 1.6) than did control spermatozoa (18.6 ± 3.7) ($P < 0.001$) (Figure 3B). Based on these data, we calculated that the effectiveness of the sperm–ZP binding process was 3.2% in the control group and 1.3% in the experimental group. Thus, in the control group, approximately 1 of 31 spermatozoa that

Table I. Effect of GnRH antagonists upon the pattern of sperm movement

Parameters	Control[a]	Antide	Phe-Pro	Nal-Glu	4pF
Motile spermatozoa (%)	83 ± 5	84 ± 3	86 ± 4	82 ± 3	85 ± 4
Straight-line velocity (μm/s)	76.9 ± 3.9	75.0 ± 3.5	74.1 ± 4.2	78.4 ± 3.1	69.6 ± 3.4
Linearity of the trajectory	0.85 ± 0.03	0.88 ± 0.03	0.86 ± 0.03	0.82 ± 0.02	0.79 ± 0.01
Rolling frequency (rolls/s)	7.3 ± 0.3	6.8 ± 0.6	7.0 ± 0.6	7.1 ± 0.7	6.8 ± 0.5
Flagellar beat frequency (beats/s)	13.4 ± 0.7	14.2 ± 1.1	12.6 ± 1.1	12.9 ± 1.0	10.8 ± 0.4
Flagellar amplitude (μm)	9.0 ± 0.3	8.7 ± 0.6	8.6 ± 0.5	9.1 ± 0.4	7.4 ± 0.9
Flagellar curvature ratio	0.80 ± 0.02	0.85 ± 0.01	0.84 ± 0.02	0.81 ± 0.02	0.80 ± 0.02

[a]Spermatozoa were capacitated for 4.5 h and then treated either with saline (control), 200 nmol/l Antide, 200 nmol/l Phe-Pro, 200 nmol/l Nal-Glu, or 200 nmol/l 4-pF for 20 min. The results represent the mean ± SEM of five experiments. In each experiment, 25 control and 25 test spermatozoa were analysed.

Table II. Effect of GnRH antagonists on the percentage of living and acrosome-reacted spermatozoa

Condition[a]	Control[b]	Antide	Phe-Pro	Nal-Glu	4pF	Follicular fluid
Living spermatozoa						
4.5 h (%)	89 ± 3	89 ± 4	92 ± 3	88 ± 4	90 ± 4	80 ± 3
20 h (%)	83 ± 3	77 ± 5	71 ± 4	81 ± 3	78 ± 2	78 ± 4
Acrosome-reacted spermatozoa						
4.5 h (%)	7 ± 0.2	5 ± 1	6 ± 1	7 ± 3	8 ± 0.3	8 ± 0.3
20 h (%)	12 ± 0.4	15 ± 1	12 ± 2	13 ± 2	8 ± 0.4	26 ± 1[c]

[a]Spermatozoa were capacitated for 4.5 or 20 h and then treated either with saline (control), 200 nmol/l Antide, 200 nmol/l Phe-Pro, 200 nmol/l Nal-Glu, 200 nmol/l 4-pF or 20% (v/v) human follicular fluid for 20 min. The percentage of living and acrosome-reacted spermatozoa was determined using the supravital dye Hoechst 33258 and the lectin PSA-FITC.
[b]The results represent the mean ± SEM of six experiments.
[c]Significantly different ($P < 0.005$) from all the other groups.

collided with the ZP remained bound to it. In the 4pF-treated group, however, only about 1 of 78 spermatozoa that collided with the ZP remained bound to it. Similar results were found for the other GnRH antagonists (data not shown).

The percentage of living spermatozoa did not change after treatment with GnRH antagonists (Table II). In addition, treatment with GnRH antagonists did not significantly change the percentage of acrosome-reacted spermatozoa in comparison with controls (Table II). This was observed in spermatozoa capacitated for 4.5 h and 20 h. In contrast, 20 h-capacitated spermatozoa significantly increased the percentage of acrosome reactions after treatment with follicular fluid (Table II).

Discussion

In the present study we have found that GnRH antagonists have a significant inhibitory effect upon the process of sperm–ZP binding in humans. This effect depended on the concentration of antagonists used, and was specific to the sperm cells, since zonae incubation with the antagonists did not cause binding inhibition. The mechanism by which these compounds exerts their effect is not known. However, the results indicate that the following are unaffected by GnRH antagonists: (i) pattern of sperm movement; (ii) percentage of acrosome reactions; (iii) frequency of sperm–zona collisions; and (iv) inhibition or fatigue of the flagellar motor apparatus of the spermatozoa. These observations agree with previous results which indicated that GnRH treatment increased sperm binding to the human ZP, and that this effect was inhibited by GnRH antagonists (Morales, 1998).

One of the effects of the GnRH antagonists was a diminution in the efficiency of the sperm–ZP binding process; this is in agreement with previous results showing that the efficiency of the process in humans is very low (Morales, 1998). In controls, only about 1 in 30 spermatozoa that collided with the ZP remained bound to it, compared with only 1 in 78 spermatozoa after treatment with GnRH antagonists. The reason why most spermatozoa that collide with, but do not bind to, the ZP in a given period remains unknown. An analogous situation occurs regarding the ability of human spermatozoa to undergo the acrosome reaction. Only a fraction of a sperm population is able to acrosome-react in response to any physiological inducer of the acrosome reaction. For example, treatment with follicular fluid usually increases the percentage of acrosome reactions to 20–40% (see references in Meizel *et al*., 1990; Morales *et al*., 1992). Considering that the basal percentage of acrosome reactions is about 5–15%, then only 15–25% of spermatozoa are able to undergo the acrosome reaction at a given time. There is no direct evidence in the literature as to whether the efficiency of sperm–zona binding changes over capacitation time. Data are available, however, which seem to indicate that for the first 5–6 h of sperm capacitation, the number of zona-bound spermatozoa remains constant (Singer *et al*., 1985; Morales *et al*., 1994). With longer capacitation times, the efficiency of sperm–zona binding may decrease, as evidenced by a reduction in the zona-binding capacity of the spermatozoa (Singer *et al*., 1985).

GnRH exerts its action on pituitary cells by binding to specific receptors located on the plasma membrane. The net effect of GnRH action is the release of LH and FSH (Schaison,

1990). This effect of GnRH is mediated by an intracellular Ca^{2+} influx (Conn *et al*., 1979; Nikolics and Conn, 1990). Through the binding to the same specific receptors, both agonists and antagonists of GnRH inhibit the release of gonadotrophins and, therefore, gonadal function. However, the inhibitory mechanism of gonadotrophin release is different for agonists and antagonists. The agonists initially stimulate the release of LH and FSH, followed by downregulation of the GnRH receptors in the pituitary gonadotrophs (Duello *et al*., 1983; Conn *et al*., 1987). This loss of receptors causes the suppression of gonadotrophin release (Loumaye and Catt, 1983; Conn *et al*., 1987). On the other hand, GnRH antagonists inhibit gonadotrophin secretion directly by competing with endogenous GnRH for binding to receptors on pituitary gonadotrophs. They are not internalized, and do not seem to induce any form of downregulation (Heber *et al*., 1982; Fraser, 1988). Several studies with GnRH antagonists have revealed a rapid inhibition (within minutes) of LH/FSH secretion (Danforth *et al*., 1991; Gordon *et al*., 1991a,b, 1992). The antagonists remain bound to the plasma membrane of the gonadotrophs for a long time (Loumaye *et al*., 1984). Then, the antagonists can be dissociated from the receptor without concomitant loss of binding sites for GnRH (Clayton *et al*., 1982).

We have reported previously that GnRH increases the ability of spermatozoa to bind to the human ZP (Morales, 1998), and that this effect was inhibited by the GnRH antagonist 4pF. In this work, we have extended those observations to show that several GnRH antagonists significantly decreased the capacity of the spermatozoa to initiate binding to the human ZP. These findings raise a challenging question as to how GnRH and its analogues act on the spermatozoa. The answer to this question is still unknown. However, we have suggested that GnRH could bind to specific sites on the sperm plasma membrane. Although there is no evidence of the existence of such receptors on human spermatozoa, preliminary observations from our laboratory indicated the presence of mRNA for the GnRH receptor on rat and mouse testicular germ cells (Huyser *et al*., 1998). The binding of GnRH to its receptor could increase the affinity of zona ligands on the spermatozoa, or could expose zona ligands previously masked. These changes in zona ligands could be due to direct configuration changes in the ligand itself, or to the production of second messengers in the sperm cells. In the gonads, the action of GnRH is mediated by an increase in the intracellular calcium concentration (Clapper and Conn, 1985; Hawes *et al*., 1992; Hawes and Conn, 1993). Preliminary data from our laboratory indicate that GnRH provokes an influx of calcium into the spermatozoa, and that this influx is blocked by the GnRH antagonist 4pF (Morales *et al*., 1998). Thus, GnRH antagonists may bind to a putative GnRH receptor on the sperm plasma membrane, thus inhibiting the release of second messengers, such as calcium. Additional experiments are necessary to elucidate the mechanism of action of GnRH and analogues upon human spermatozoa.

Finally, the information obtained in this study may help to develop new alternatives to inhibit conception in humans. It might be possible to administer GnRH antagonists to men, for example as a component of a vas occlusion plug. Thus, even when sperm numbers in the ejaculate do not fall to zero because of the vas occlusion, the remaining spermatozoa might have impaired zona-binding ability. It may also be possible to administer GnRH antagonists to women, either permanently as part of an intrauterine device, or just before intercourse, perhaps as part of a vaginal preparation. It has been suggested that the vaginal or uterine absorption of certain substances is poor (Bourin *et al*., 1983; Hsu *et al*., 1983; Corbo *et al*., 1989; Lau *et al*., 1992); therefore, the risk of the antagonists being absorbed through the mucosa into the systemic circulation and having a subsequent effect on pituitary secretion may be small.

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