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The effect of pre-treatment with a gonadotrophin-releasing hormone agonist on reproductive functions in mature cycling rats

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In order to investigate the performance of follicles in a rat model in which gonadotrophin-releasing hormone agonist (GnRHa) was used for hypothalamic–pituitary–ovarian axis suppression, three groups of mature cycling rats were studied. One group was treated with buserelin followed by pregnant mare's serum gonadotrophin (PMSG), and the second group was treated with PMSG alone. Both these hormonally treated groups received human chorionic gonadotrophin for induction of ovulation. The third group received no hormonal treatment. The average number of ovulated oocytes recovered from rat oviducts pre-treated with GnRHa was significantly higher than that in rats treated with the gonadotrophin alone, in spite of the larger number of pre-ovulatory follicles present in the gonadotrophin-treated group. The morphology of both the pre-ovulatory and the post-ovulatory cumulus–oocyte complexes in the three groups appeared similar. No difference in the capacity of follicles of the three groups to synthesize progesterone *in vitro* in response to luteinizing hormone could be observed. We conclude that ovarian morphology and function are not impaired by pre-treatment with buserelin.

Key words: GnRH agonist/rat follicle biosynthesis/rat oocyte cytology

Introduction

The chronic administration of gonadotrophin-releasing hormone agonists (GnRHa) for suppression of the hypothalamic–pituitary–ovarian axis is widely used in humans (Lunenfeld and Vickery, 1990). Since rat ovarian tissue has been shown to contain high-affinity receptors for GnRH, a direct effect of these agents at the ovarian level also exists (Koves *et al.*, 1989; Dekel *et al.*, 1989).

Our study was designed on the basis of observations made in our in-vitro fertilization (IVF) unit, that the cumulus–oocyte complexes retrieved from follicles exposed to GnRHa are morphologically different from those not exposed to this agent. Since the availability of human follicles and ovulated oocytes for research is quite limited, we used rats pre-treated with buserelin (a GnRHa) to test whether their follicles and oocytes were different to those from spontaneously ovulating rats, or from rats in which ovulation was induced by gonadotrophin treatment.

Materials and methods

Animals

The effect of pre-treatment with a GnRHa was studied on mature (90 day old) cycling female Wistar rats from the colony of the Department of Hormone Research, at the Weizmann Institute of Science, Israel. The animals were kept under a controlled photoperiod (14 h of light/day). The average weight of each rat was ~200 g and the length of their oestrous cycle was 4 days. Starting on the day of oestrus, for the next 3 days, the rats were each given one daily s.c. injection of 2 μg of buserelin [D-Ser (T-Bu)6] des-Gly-10 *N*-ethylamide (Hoechst, Ag, Frankfurt, Germany). According to our previous experience, this protocol of treatment results in suppression of the oestrous cycle. The next pro-oestrus in this animal model is observed on the fourth day after the last buserelin injection (unpublished data). On the third day, in addition to buserelin, the rats received 30 IU of pregnant mare's serum gonadotrophin (PMSG, Gestyl; Organon, Oss, The Netherlands). These rats are referred to as the GnRHa/PMSG group. Two additional groups, meta-oestrous rats injected with 30 IU of PMSG (PMSG group) and untreated spontaneously ovulating rats (spontaneous group), served as controls. In order to obtain mature oocytes, an i.p. injection of 4 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon) was given to the hormonally treated rats 50 h after PMSG administration.

Morphological examination

For recovery of ovarian follicles, the rats from the hormonally treated groups were killed 48 h after PMSG administration. The untreated rats were killed on pro-oestrus. The ovaries were removed and transferred to Leibovitz-L-15 tissue culture medium. These ovaries consisted mainly of small pre-antral follicles with a small population of large, transparent antral follicles that were easily distinguished. These large antral follicles were dissected under a stereoscopic microscope and counted. Some of the follicles were punctured and their content was morphologically analysed. Another group of antral follicles was further incubated as described in detail in the following section.

For examination of mature oocytes, rats were killed 17 h after HCG administration. The spontaneously ovulating rats were killed in oestrus. In those rats the ovulated cumulus–oocyte complexes were recovered from the oviductal ampulla and counted.

The morphology of either pre-ovulatory or post-ovulatory cumulus–oocyte complexes was analysed by Nomarski interference contrast microscopy. Each post-ovulatory cumulus–oocyte complex was inspected before and after the addition of hyaluronidase to the incubation medium for removal of the cumulus cells. The parameters analysed were ooplasm homogeneity, presence of the germinal vesicle in the oocytes, appearance of the zona pellucida and the density of the cumulus mass (Veeck *et al.*, 1983).

Follicle culture/progesterone biosynthesis

Large antral follicles, dissected as previously described, were placed in Leibovitz-L-15 tissue culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Seralab, London, UK), penicillin (100 IU/ml) and streptomycin (100 µg/ml; Gibco). Ten replicates of three follicles each were placed in 1 ml of the above medium with or without 10 µg/ml ovine luteinizing hormone (LH S-24; NIH) in 25 ml flasks, gassed with 50% O_2 and 50% N_2. Incubations were carried out at 37°C in an oscillating waterbath. At 4 h of incubation, 200 µl of the medium of each flask were removed and kept at −20°C for progesterone assay. Oocytes recovered following exposure to LH *in vitro* were analysed for maturation. Maturation was indicated by the absence of the germinal vesicle in the individual oocytes, as observed by Nomarski interference contrast microscopy. Progesterone concentrations were determined by radioimmunoassay (Lindner and Baumginger, 1974). The sensitivity of the assay was 15 pg/ml; intra- and interassay coefficients of variation were 2 and 20% respectively.

The progesterone values presented for each day of the experiment represented an average of five samples of three follicles, each incubated with and without LH. Progesterone concentrations induced by LH on each day were normalized according to the concentrations of progesterone in the follicles incubated in parallel in LH-free medium.

Results

Number of the pre-ovulatory follicles as compared to the size of ovulation

The average number of large antral follicles dissected from the GnRHa/PMSG group of rats was 10.7 ± 2.7 per rat (range 8–13), compared with 13.35 ± 3.8 per rat in the PMSG group (range 11–17), as shown in Table I. In the spontaneously ovulating rats we found 11.75 ± 3.4 large antral follicles per rat.

The average number of mature oocytes isolated from the oviducts of the GnRHa/PMSG group was 13.3 ± 4.8 (range 10–18), compared with 9.9 ± 4.7 (range 6–15) in the PMSG group and 10.00 ± 4.6 (range 9–10) in the spontaneously ovulating rats (Table II).

Using the Scheffe procedure (to detect pairs of groups different at the $P < 0.05$ level), no significant difference was found between the number of follicles in each of the hormonally treated groups (GnRHa/PMSG or PMSG) and the spontaneously ovulating group. However, the number of antral follicles isolated from the PMSG group was significantly higher than that of the GnRHa/PMSG group ($P < 0.01$). In contrast, the number of mature oocytes in the GnRHa/PMSG group ($P < 0.001$) was significantly higher than that in either the PMSG group or the spontaneously ovulating group.

Table I. The average number of follicles isolated from the ovaries of untreated rats and GnRHa/pregnant mare's serum gonadotrophin (PMSG) or PMSG-treated rats

GnRHa/PMSG	PMSG alone	Spontaneous ovulation
12.0 ± 0.28 (n = 6)	17.2 ± 1.56 (n = 6)	10.2 ± 1.75 (n = 5)
8.8 ± 0.89 (n = 5)	11.4 ± 1.43 (n = 9)	14.8 ± 1.82 (n = 5)
11.4 ± 0.45 (n = 5)	12.5 ± 1.26 (n = 6)	10.4 ± 1.70 (n = 5)
9.7 ± 0.88 (n = 6)	13.2 ± 0.82 (n = 5)	11.7 ± 1.70 (n = 5)
9.3 ± 1.76 (n = 6)		
13.0 ± 1.58 (n = 5)		
Average ± SD		
10.7 ± 2.7*	13.35 ± 3.8*	11.75 ± 3.4

*Significantly different at $P < 0.01$ (Scheffe procedure); Cochrans C and Bartlett–Box F used to show homogeneity of variances.
n = number of rats in different cohorts

Table II. The average number of mature oocytes recovered from the oviducts of untreated rats and GnRHa/pregnant mare's serum gonadotrophin (PMSG) or PMSG pre-treated rats

GnRHa/PMSG	PMSG alone	Spontaneous ovulation
11.6 ± 6.5 (n = 10)	6.4 ± 4.7 (n = 9)	9.4 ± 4.5 (n = 8)
12.0 ± 3.7 (n = 10)	9.25 ± 3.7 (n = 8)	9.6 ± 4.5 (n = 8)
12.4 ± 3.6 (n = 7)	10.80 ± 3.8 (n = 9)	10.2 ± 4.2 (n = 9)
16.9 ± 4.2 (n = 10)	15.00 ± 8.2 (n = 4)	10.6 ± 4.8 (n = 6)
18.4 ± 3.2 (n = 5)	9.70 ± 0.8 (n = 6)	10.4 ± 5.0 (n = 6)
10.3 ± 2.4 (n = 6)	11.70 ± 3.5 (n = 6)	12.0 ± 1.4 (n = 5)
Average ± SD		
13.3 ± 4.8*	9.9 ± 4.7	10.0 ± 4.6

*Significantly different from both other groups at $P < 0.001$ (two-tailed Student's t-test).
n = number of rats in different cohorts

Morphology of the pre-ovulatory cumulus—oocyte complexes

The cumulus–oocyte complexes isolated from pre-ovulatory follicles pre-treated with GnRHa/PMSG were found to be morphologically identical to those isolated from rats exposed to PMSG alone or from spontaneously ovulating rats. In all cases the cumulus–oocyte complexes contained a round, immature oocyte with homogeneous cytoplasm and a nucleus with one or sometimes two nucleoli. The oocyte was surrounded by a well-defined zona pellucida and a compact structure of cumulus cells. In ovulated oocytes retrieved from the oviducts of rats pre-treated with GnRHa/PMSG, we found a subgroup of cumulus–oocyte complexes containing 'pear-shaped' oocytes

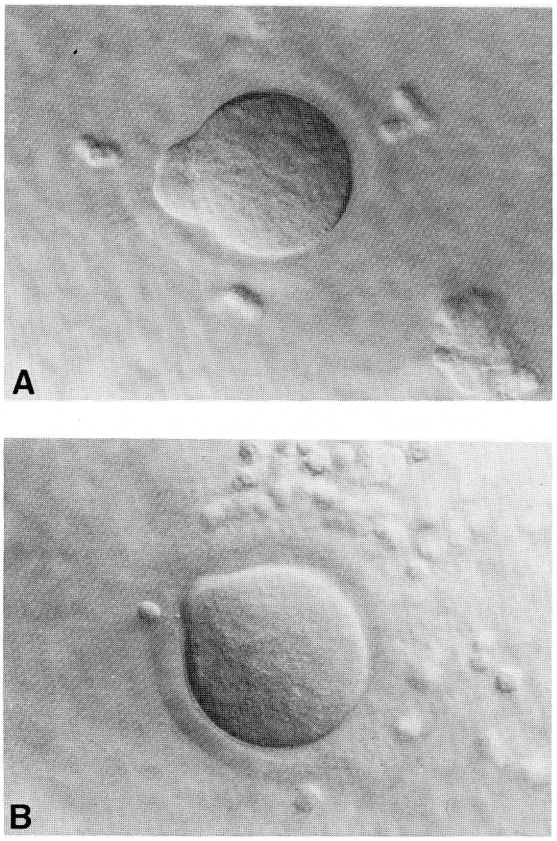

Figure 1. (**A** and **B**) Post-ovulatory 'pear-shaped' rat oocytes isolated from rats pre-treated with GnRHa/pregnant mare's serum gonadotrophin.

Table III. Progesterone concentrations (ng/ml) produced by rat follicles *in vitro*[a]

Group	With LH	Without LH	Relative increase
GnRHa/PMSG	170	3.8	45
	150	2.8	53
	80	1.0	80
	270	2.6	103
	190	2.5	76
	165	1.68	98
Average relative incease ± SD			75.83 ± 21.65
PMSG alone	80	1.10	73
	42	0.62	68
	96	1.10	87
	51	0.44	116
	47	0.60	87
	10.4	0.15	69
Average relative increase ± SD			83.33 ± 16.82
Spontaneous	180	1.82	99
ovulation	24	0.3	80
	50	0.6	83
Average relative increase ± SD			87.33 ± 8.63

[a]Data from different cohorts of rats mentioned in Table I.
LH = luteinizing hormone; PMSG = pregnant mare's serum gonadotrophin; SD = standard deviation.

(Figure 1A and B). These oocytes were also found in the PMSG group, but in a smaller proportion. No such oocytes were detected in spontaneously ovulating rats. The proportion of 'pear-shaped' oocytes did not change in oocytes retrieved later at 21 h after administration of HCG.

Progesterone synthesis of follicles exposed to LH in culture

Progesterone concentrations and the average relative rate of increase in progesterone production under LH is depicted in Table III. Progesterone concentrations in the follicles isolated from GnRHa/PMSG rats and exposed to LH *in vitro* ranged between 80 and 270 ng/ml as compared to 1–3.8 ng/ml without LH. The average relative increase in progesterone was calculated as 75.83 ± 21.65 ng/ml (range 45–103). Progesterone concentrations in the follicles isolated from PMSG rats and exposed to LH *in vitro* ranged between 10.4 and 96 ng/ml as compared to 0.15–1.1 ng/ml without LH. The average relative increase in progesterone in this group was found to be 83.33 ± 16.82 ng/ml (range 73–116). In the untreated rats, progesterone concentrations secreted by the follicles *in vitro* ranged between 50 and 180 ng/ml in response to LH as compared to 0.3–1.82 without LH. The average relative increase in progesterone was 87.33 ± 8.63 ng/ml (range 83–99). There was no significant difference between the three groups in terms of average relative increase in progesterone production in response to LH.

Discussion

The average number of mature oocytes found in rat oviducts pre-treated with GnRHa was significantly higher than in rats stimulated by gonadotrophins alone. This finding is in agreement with the observations in humans as demonstrated both by large treatment series of different individuals (Salat-Baroux *et al.*, 1987; Caspi *et al.*, 1989) and repeated stimulation protocols practised in the same woman (Ron-El *et al.*, 1990). However, the average number of large antral follicles in the GnRHa/PSMG pre-treated rats was found to be lower than that observed in rats given PMSG alone. The discrepancy between the relatively low number of pre-ovulatory follicles in the presence of the relatively high number of ovulations in the GnRHa/PMSG rats could result from wrong selection of the follicles that was based on their inspected volume. Follicles of a relatively smaller volume that were not chosen may also have ovulated later in response to HCG. On the other hand, some of the follicles diagnosed as antral in the PMSG group could possibly represent ovarian cysts, thus explaining our finding that the average number of mature oocytes in this group was lower than the average number of follicles diagnosed as pre-ovulatory.

The cumulus–oocyte complexes isolated from follicles of rats pre-treated with GnRHa/PMSG were not morphologically different from cumulus–oocyte complexes aspirated from follicles of rats exposed to PMSG alone or those from spontaneously ovulating rats. We noticed a prominent subgroup of 'pear-shaped' oocytes recovered from the oviducts of the rats pre-treated with hormones (either GnRHa/PMSG or PMSG groups). This appearance resembles the 'fertilization cone' which appears 1 h after sperm attachment and represents the site of the polar body extrusion (Stefini *et al.*, 1969; Shalgi,

1977). In the absence of spermatozoa in our oocyte preparations, we assumed that hyaluronidase, used *in vitro* to remove the cumulus cells, could possibly have induced the formation of the observed bulge. However, the presence of 'pear-shaped' oocytes in preparations that were not exposed to this enzyme did not support our hypothesis.

With regard to steroid production, there was no statistically significant difference in the average rate of increase in response to LH between the various groups analysed. However, the absolute concentrations of progesterone in the GnRHa/PMSG rats appeared higher than those of the PMSG group, possibly representing a higher state of sensitivity to LH.

Thus our conclusion from the present study is that in the rat, oocyte morphology and ovarian progesterone production are not impaired by treatment with GnRHa.

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