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Experimental extension of the time interval between oocyte maturation and ovulation: effect on fertilization and first cleavage

Nava Dekel, Ph.D.*†
Daniel Ayalon, M.D.‡
Orly Lewysohn, B.Sc.‡
Nava Nevo, D.V.M.*
Ruth Kaplan-Kraicer, M.Sc.§
Ruth Shalgi, Ph.D.§

The Weizmann Institute of Science, Rehovot; Timsit Institute of Reproductive Endocrinology, Ichilov Hospital; and Tel Aviv University, Tel Aviv, Israel

Objective: To test the hypothesis that impaired fertility in human patients with high LH concentrations throughout the follicular phase of the menstrual cycle reflects premature maturation of their oocytes.

Design: Previous information that resumption of meiosis is induced by lower hCG concentrations than that required for stimulation of follicular rupture was confirmed and used for establishment of a rat animal model in which oocyte maturation and ovulation can be separated experimentally. In further experiments hypophysectomized, pregnant mare serum gonadotropin (PMSG)-primed, immature female rats injected with 1.1 IU of hCG, a dose found to induce maturation in 72.9% ± 6% of the rats with no effect on ovulation, were administered with a second injection of an ovulatory dose (4 IU) of hCG, 24 hours later. The ovulated eggs were subjected to IVF.

Results: Fertilization and first cleavage in oocytes recovered from our experimental animal model were similar to that observed in control PMSG-primed, either hypophysectomized or intact rats, treated by a single injection of 4 IU of hCG.

Conclusions: The extension of the time interval between oocyte maturation and ovulation in the rat does not result in a lower rate of fertilization or a reduced incidence of cleavage. However, an inferior developmental capacity of these embryos cannot be ruled out.

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Key Words: Oocyte maturation, delayed ovulation, fertilization, LH surge

Both oocyte maturation and ovulation are induced by the preovulatory surge of the pituitary gonadotropin, LH (reviewed by Dekel [1]). However, these two biological responses of the mammalian ovary take place at different time intervals after the hormonal stimulus. In the rat, the first meiotic division of the oocyte is initiated by 4 hours and completed by 9 hours after the LH surge, whereas ovulation occurs only 3 hours later (2, 3). The above-mentioned sequence between oocyte maturation and ovulation ensures that a premature oocyte is not released from the ovarian follicle into the site of fertilization. Furthermore, as the life span of the unfertilized ovum is limited, a strict control of the time interval that elapses between these two events is probably essential for successful reproduction. Our study aimed at examination of this hypothesis. Specifically, our initial experiments were conducted to confirm previous reports that oocyte maturation and ovulation are stimulated by different doses of LH and hCG (4, 5)

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* Department of Hormone Research, The Weizmann Institute of Science.
† Reprint requests: Nava Dekel, Ph.D., Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel (FAX: 972-8-344116).
‡ Timsit Institute of Reproductive Endocrinology, Ichilov Hospital.
§ Department of Embryology, Sackler School of Medicine, Tel Aviv University.

and to characterize the sensitivity range of these two responses to hCG in our rat colony. Further experiments were designed for the establishment of an animal model, in which the time interval between oocyte maturation and ovulation can be extended. The ability of the egg to be fertilized and the capacity of the zygote to cleave in these experimentally manipulated rats have been tested.

MATERIALS AND METHODS

Human Chorionic Gonadotropin-Induced Oocyte Maturation and Ovulation: Dose-Response Analysis

Immature (25-day-old) Wistar-derived female rats were hypophysectomized by the transauricular approach. Hypophysectomy was followed immediately by an injection of 15 IU SC of pregnant mare serum gonadotropin (PMSG; Chromo-gest, Intervet, Holland) in 0.1 mL 0.9% (wt/vol) NaCl. An intraperitoneal injection of 0.1 mL 0.9% (wt/vol) NaCl containing various doses (ranging between 0 and 4 IU) of hCG (Pregnyl, Organon, Holland) was given 50 hours later. The rats were killed 20 hours after administration of hCG. Each animal was inspected for remnants of pituitary tissue, and those suspected of incomplete hypophysectomy were excluded.

The oviductal ampullae were removed and the ovulated eggs released into a drop of 0.9% of NaCl. The number of eggs present in each oviduct was counted using a stereoscopic microscope. The results are presented both as the mean number of eggs (±SEM) found in both oviducts of the individual rats (ovulation size) and as the fraction of ovulating rats at each time point (ovulation rate). Rats were considered as ovulating in the presence of ≥2 eggs in the oviducts. Ovulation rate and size also were analyzed in a group of similarly treated intact rats.

In the nonovulating rats, the ovaries were removed and the oocytes from the large antral follicles (40 follicles per rat) were recovered. Maturation, as indicated by the absence of the germinal vesicle in the individual oocytes, was analyzed by Nomarski interference contrast microscopy (6). The results are expressed as the fraction of rats containing oocytes (follicular, oviductal, or both) with germinal vesicle breakdown (GVBD) observed in response to the various concentrations of hCG. The mean number of GVBD oocytes per rat (±SEM) also was calculated. Oocyte maturation and ovulation also were examined in intact rats, injected SC with Nembutal (35 mg/1 kg body weight) at 48 hours after PMSG administration.

Experimentally Induced Delayed Ovulation

Hypophysectomized, PMSG-primed female rats, prepared as described previously, were subjected to a sequential administration of hCG as follows: The first injection of hCG, at a dose that was found to induce oocyte maturation in a maximal fraction of the rats with no effect on ovulation (1.1 IU/rat; see Results), was administered at 50 hours after PMSG. A second injection of hCG, at a dose that was fully effective in stimulating ovulation (4 IU/rat; see Results) was administered 24 hours later. Ovulation in these rats was examined at 20 hours after the second injection of hCG. Using this protocol, we established an animal model in which mature oocytes were "trapped" in the ovarian follicles for an extended period of about 24 hours.

In Vitro Fertilization

The poor mating performance exhibited by the hypophysectomized rats did not allow us to test in vivo the fertilizability of the mature oocytes subjected to delayed ovulation. These experiments were, therefore, performed using an IVF system.

Spermatozoa for these experiments were collected from the uteri of mature-cycling rats soon after mating and diluted in modified rat fertilization medium (7), to a final concentration of 7 to 12 $\times$ 10^5 spermatozoa/mL. Aliquots of sperm suspension (100 μL) were incubated in Petri dishes under heavy paraffin oil (BDH, Poole, United Kingdom) at 37°C for 5 hours in a humidified atmosphere of 5% CO_2 in air to allow capacitation (8).

Ovulated eggs were recovered from the excised oviductal ampullae of the above mentioned two groups of hypophysectomized rats (normal or delayed ovulators) and introduced to the sperm suspensions (10 to 15 eggs per 100-μL drop). Forty hours later, the eggs were examined by Nomarski interference-contrast microscopy for fertilization and first cleavage. Eggs containing a sperm tail in the vitellus were classified as fertilized. The number of fragmented eggs also was recorded. Fragmented eggs contained more than two fragments with a sperm tail present.

Oocytes that underwent maturation and ovulation in intact rats served as a bridge control. These oocytes were recovered from 28-day-old female rats injected with 4 IU of hCG 50 hours after PMSG (15 IU/rat) administration, and sacrificed 20 hours later. This group was used for normalization of the results according to daily variations in fertilization rate, which may reflect the use of different males as donors of spermatozoa.

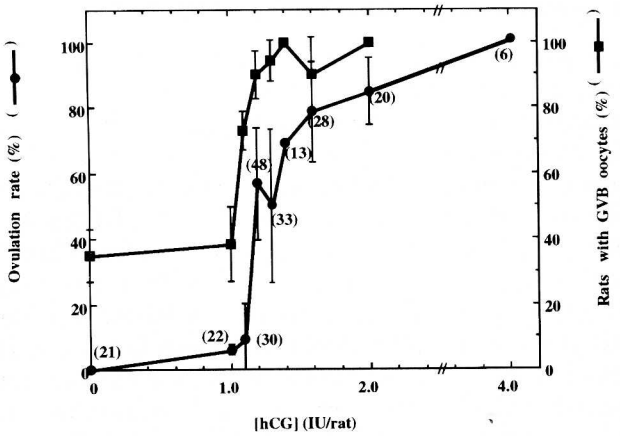

Figure 1 Dose-response of hCG-induced oocyte maturation and ovulation. Hypophysectomized, PMSG-primed rats were administered with the indicated hCG concentrations. Ovulated eggs were recovered from the oviductal ampullae 20 hours later. Nonovulating rats were examined for the presence of mature oocytes in their ovarian follicles. The results are expressed as the means ± SEM of the fraction of rats containing ovulated or GVBD oocytes (follicular, oviductal, or both) observed in response to the indicated concentrations of hCG, in at least three individual experiments. The total number of rats examined for each hCG concentration is indicated in parentheses.

Statistical Analysis

Statistical differences in ovulation size, in response to different doses of hCG, were analyzed by Student's *t*-test. Analysis of variance was employed for comparison of the results obtained after IVF of eggs acquired from the experimental and the two control groups of rats.

RESULTS

Dose-response analysis of hCG-induced oocyte maturation and ovulation was performed in a group of 208 hypophysectomized, PMSG-primed female rats administered with increasing concentrations of hCG. We found that induction of ovulation was observed initially in response to a dose of 1.1 IU/rat. A full ovulatory response was stimulated by 4 IU of hCG/rat, with ED_{50} at 1.3 IU of hCG (Fig. 1). A similar ovulation rate was observed in intact PMSG-primed rats (83% n = 6, 83% n = 6, and 100% n = 8 administered with 1.6, 2, and 4 IU of hCG, respectively).

The size of ovulation in response to the various hCG concentrations is shown in Table 1. The mean ± SEM number of ovulated eggs, recovered from the oviducts of rats administered with hCG doses ranging between 1.1 and 1.6 IU/rat, was 16.3 ± 1.5 with no significant differences between the various groups of treatment (Student's *t*-test, $P > 0.05$). However, a sharp increase in ovulation size was obtained in response to a dose of 2 IU of hCG (43.4 ± 5.4). No further elevation in ovulation size was observed in response to increasing concentrations of hCG. The size of ovulation observed in intact PMSG-primed rats administered with 1.6, 2, and 4 IU of hCG was 26 ± 5.8 (n = 5), 46.2 ± 7.2 (n = 5), and 62 ± 7.8 (n = 8), respectively.

The large antral ovarian follicles of those rats that did not ovulate were analyzed for the presence of mature, GVBD oocytes. We found that the ovaries of 100% of the rats injected with 1.4 IU of hCG contained mature oocytes. The ED_{50} for the hCG-induced oocyte maturation was at 1.02 IU (Fig. 1). Some GVBD oocytes (4.4 ± 0.5/rat) were found in 33% of the rats injected with the vehicle only (0.9% NaCl). The ovaries of a similar faction (40%, n = 10) of intact PMSG-primed rats, treated with a dose of Nembutal that totally blocked ovulation, also contained GVBD oocytes (2.0 ± 0/rat).

Determination of the concentration dependency of oocyte maturation and ovulation enabled the establishment of an experimental animal model in which oocytes, induced to mature by hCG (1.1 IU/rat), were kept in the ovarian follicles. These oocytes were further stimulated to ovulate by an additional injection of 4 IU of hCG administered 24 hours after the first injection of this hormone. A total of 674 eggs, recovered from 31 such rats, were subjected to IVF in 6 individual experiments. As seen in Table 2, fertilization diagnosed as described in Materials and Methods, was observed in 51.1 ± 5.8% of these eggs. A high rate of first cleavage (93.5% ± 4.7%) was obtained in those eggs that have been fertilized. Unexpectedly, this rate of successful fertilization, is not significantly lower ($P > 0.5$) than that demonstrated in the two control groups: hypophysectomized and intact rats (55.9% ± 6.5% and 55.1% ± 5.6%, respectively) in which ovulation has not been delayed (see

Table 1 The Size of Ovulation in Rats Administered with Different Doses of hCG

hCG	No. of ovulating rats	No. of eggs/rat*
IU/rat		
1.1	3	14.3 ± 9.1
1.2	22	13.8 ± 2.2
1.3	11	23.0 ± 3.7
1.4	9	14.2 ± 2.9
1.6	15	16.5 ± 3.2
2.0	18	43.4 ± 5.4
4.0	6	35.5 ± 6.2

* Values are means ± SEM.

Table 2 Rate of Fertilization, First Cleavage, and Fragmentation After IVF

Rats	No. of eggs*	Fertilization†	First Cleavage‡	Fragmentation†
Intact	947 (23)	55.1 ± 5.6	83.6 ± 4.7	15.6 ± 3.1
Hypophysectomized, normal ovulation	406 (22)	55.9 ± 6.3	83.1 ± 9.3	21.5 ± 5.2
Hypophysectomized, delayed ovulation	674 (31)	51.1 ± 5.8	93.5 ± 4.7	16.8 ± 4.7

* Number of rats in parentheses.
† Values are mean % of total ± SEM.
‡ Values are mean % of fertilized ± SEM.

Materials and Methods). Similar to the experimental group, the rate of first cleavage in these two control groups was also high (83.1% ± 9.3% and 83.6% ± 4.7%, respectively). The rate of fragmentation ranged between 15.6% ± 3.1% and 21.5% ± 5.2%, with no significant differences between the control and experimental groups ($P < 0.5$).

DISCUSSION

Our study presents a rat animal model in which resumption of meiosis and ovulation that normally occur in succession in response to LH and hCG can be separated experimentally. Establishment of such animal model is based on previously reported information that these two biological responses exhibit different sensitivities to the hormone. Induction of oocyte maturation that is stimulated by lower levels of hCG than those required for follicular rupture has been shown initially in vivo in rabbits (4) and later demonstrated in rat perfused ovaries exposed to LH in vitro (5). Confirming these findings we demonstrate herein that the ovulatory response exhibits a lower sensitivity to hCG than that of oocyte maturation. The maximal effective dose required for reinitiation of meiosis in our rat colony was found as 1.4 IU of hCG. A full ovulatory response, on the other hand, was not obtained by doses that were lower than 4 IU of the gonadotropin. The LH requirement for induction of ovulation in the rat has been studied previously (9–11). However, in all these studies oocyte maturation has not been analyzed. Titration of LH requirements for oocyte maturation, but not ovulation, has been performed in primates (12).

The earliest morphological marker for reinitiation of meiosis in oocytes is GVBD. This parameter, which is used commonly for the diagnosis of mature oocytes, also has been employed in our study. However, disappearance of the germinal vesicle also characterizes oocytes that reside within atretic follicles. Because the vast majority of ovarian follicles in mammalian species undergo atresia (13), it seems likely that atretic follicles were probably the source for the GVBD oocytes recovered in our study from rats injected with vehicle only (0.9% NaCl). The disappearance of the germinal vesicle in these animals apparently does not represent a response to a discharge of LH possibly induced by hypophysectomy because the ovaries of the same fraction of intact rats treated with Nembutal also contained a similar number of GVBD oocytes.

Both oocyte maturation and ovulation represent cyclic adenosine 5′-monophosphate (cAMP)-mediated responses. However, distal to activation of the cAMP second-messenger system different intracellular biochemical pathways are used for the transduction of these LH actions. Rupture of the ovarian follicle is subsequent to a prostaglandin-mediated cascade of proteolytic reactions whereas the postreceptor events involved in resumption of meiosis are independent of prostaglandin synthesis and do not involve protease activation (reviewed by Tsafriri and Dekel [14]). Stimulation of each of the above mentioned biochemical pathways requires apparently a specific LH concentration explaining the different sensitivities to the hormone exhibited by these two biological responses.

The fact that induction of oocyte maturation requires a relatively lower threshold level of LH than ovulation determines, apparently, the sequence of these two biological events. Being sensitive to lower doses of LH, characteristic to the initial phase of the midcycle surge of this gonadotropin, resumption of meiosis takes place before the release of the mature egg from the ovarian follicle. Furthermore, the presence of the two specific thresholds for induction of oocyte maturation and for ovulation, not only determines their sequence but also controls the timing of these two responses. Since the rate of increase of LH concentration is species specific, in any given species (15) the profile of the LH surge apparently determines the time interval between completion of the first meiotic division and ovulation. Since, once the oocyte has resumed meiosis, it remains fertilizable for only a limited time, strict control of the temporal relationships between these two events is essential to ensure successful reproduction.

These hypotheses have been used in an attempt to explain the poor reproductive performance in human patients demonstrating a disturbed profile of the LH surge. Specifically, an impaired fertilization ability followed by implantation failure has been described in patients with raised basal LH levels during the follicular phase (16). Consistent with this observation, pregnancy failures associated with a significantly higher LH output before hCG administration also has been reported (17). Along with this line, a significant reduction in the rate of embryo cleavage has been correlated with the occurrence of an LH surge at over, but not less than, 12 hours before hCG administration (18). Similarly, a high incidence of an atypical LH rise before hCG administration in patients whose oocytes failed to fertilize in vitro also has been observed (19). One possible explanation provided in these reports is that the poor reproductive performance in all these cases may reflect premature oocyte maturation. Relatively high concentrations of LH throughout the follicular phase that allow "premature" resumption of meiosis resulting in ovulation of an "aged" egg, also has been proposed to explain the high rate of miscarriages in patients with polycystic ovarian disease (PCOD) that are characterized by elevated LH concentrations throughout the menstrual cycle (20–22). The presently available information that oocyte maturation can be induced by relatively lower (submaximal) concentration of LH, while higher (peak) concentrations are required for stimulation of ovulation clearly supports this theory (4, 5 and our present study). It was therefore surprising to find in our present study that neither the ability of the egg to be fertilized nor the capacity of the zygote to develop further into a two-cell embryo was impaired by the extension of the time period between oocyte maturation and ovulation by 24 hours.

Aging of the ovum can take place either intrafollicularly, when the mature oocyte is retained in the follicle beyond the normal time, or intratubally, when the egg is shed on time but fertilization is delayed. Delayed fertilization has been found to be associated with an increased proportion of chromosomal anomalies (23, 24) and postimplantation losses (25). Our results may suggest that unlike the postovulatory egg, the mature oocyte "trapped" experimentally within the ovarian follicle is somehow protected against aging. On the other hand, it is still possible that such damage occurring in intrafollicular-aged eggs does not interfere with their fertilizability or cleavage capacity but is rather manifested at later stages of pregnancy. Unfortunately, further embryonic development, implantation capacity and postimplantation events could not be tested in our animal model. Therefore, the possibility that the high incidence of miscarriages observed in PCOD (20–22), as well as other patients, exhibiting raised concentrations of LH during the follicular phase of the menstrual cycle (16, 17), represents ovulation of premature matured oocytes of low quality, is left open.

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