The effect of LH on the fertilizability and developmental capacity of rat oocytes matured *in vitro*

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Summary. The effect of adding LH (10 µg NIH-LH-B8/ml) to the medium in which oocytes were undergoing maturation *in vitro* was studied. The fertilizability of the oocytes was evaluated in the sterile oviduct of a unilaterally ovariectomized, mated recipient. Freshly ovulated oocytes, used as a control of the method, were fertilized at a rate of 72%. Only 14% of oocytes matured in culture (without LH) were penetrated by spermatozoa, and 11% were fertilized normally. Addition of LH to the medium increased these proportions to 43 and 33% respectively. Oocytes matured in the presence of LH were able to develop into apparently normal rats. It is concluded that, although oocytes can mature *in vitro* spontaneously, and that these matured oocytes can be fertilized, addition of LH increases the numbers 3-fold. LH therefore has a direct maturation-promoting action on the rat oocyte–cumulus complex *in vitro*.

Introduction

Just before ovulation, the oocyte undergoes a transformation known as maturation, and becomes a fertilizable, secondary oocyte (Tsafiri & Kraicer, 1972). This change is recognized to be a response to the ovulatory secretion of LH (Lindner *et al.*, 1974). Another way of obtaining maturation of the oocyte is to isolate it from the follicle: such an oocyte undergoes an immediate spontaneous maturation in culture which does not depend on the presence of LH (Pincus & Enzmann, 1934; Chang, 1955; Edwards, 1965). The maturation in culture appears to be normal in that the oocytes have the structural and cytological characteristics of ovulated oocytes. These oocytes can be fertilized, but at a relatively low rate, compared to that of oocytes matured *in vivo* (mouse: Cross & Brinster, 1970; pig: Motlik & Fulka, 1974; rabbit: Thibault & Gérard, 1973; rat: Niwa & Chang, 1975).

In this study we have examined the effect of LH on the fertilizability of rat oocytes matured *in vitro* and fertilized in one ampulla of a host female by comparing it to that of oocytes maturing spontaneously, i.e. in the absence of LH, in the other oviduct.

Materials and Methods

The rats were cyclic albino females, originally of the Wistar strain, bred in the Experimental Animal Facility of the Zoology Department, Tel Aviv University. They were housed in air-conditioned quarters and supplied with laboratory animal pellets (Ambar, Israel) and tap water

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ad libitum. Artificial illumination was provided for 14 h daily, but the timing of the period of illumination of different groups of rats was altered to advance or retard times of ovulation. Animals were allowed at least 2 weeks to acclimatize to a change in their lighting schedule. All times cited in the text refer to the animals' diurnal cycle. Thus 00:00 h is the midpoint of the animals' dark period and 12:00 h is the animals' noon.

Time of ovulatory hormone secretion and of ovulation

The time of the ovulatory LH surge was established by the method of Everett & Sawyer (1950). Pro-oestrous rats were injected with 35 mg sodium pentobarbitone/kg body weight at various times between 12:00 and 17:00 h and were killed by cervical dislocation 1 day later. If the oviductal ampullae were visibly distended, they were excised and transferred to a drop of 0.15 M-NaCl solution in a well slide. The ovulated cumulus-oocyte complexes were liberated and the oocytes counted under a stereoscopic microscope. When no oocytes were found in the ampulla, the ovaries were isolated and the morphology of the cumulus-oocyte complexes in the largest Graafian follicles was examined by Nomarski interference-contrast microscopy; animals with follicles containing dictyate oocytes were considered to have responded to the sodium pentobarbitone. The time at which sodium pentobarbitone ceased to block ovulation was considered to be the time of secretion of an ovulatory quota of LH. The sigmoid curve of the waning of ovulation inhibition was fitted by probit analysis (Finney, 1971).

The time of ovulation in rats to be used as hosts for transplanted oocytes was determined by killing 67 rats during the night between pro-oestrus and oestrus. Their oviducts and/or ovaries were examined as described above. Animals which had not ovulated and whose follicles contained only dictyate oocytes were not included. The results were analysed by probit analysis. Since the animals were maintained on a reversed light cycle, the curve describing their cumulative frequency of ovulation as a function of time was compared to that of the standard for the colony by the *t* test.

Maturation of oocytes in culture

Oocytes were obtained from the follicles of rats killed by cervical dislocation between 12:00 and 13:30 h on the day of pro-oestrus. Their ovaries were placed in a well slide containing approximately 0.5 ml sterile culture medium. The large preovulatory follicles were incised under a stereomicroscope and the cumulus-oocyte complexes were liberated. Extraneous follicle cells, debris and follicular fluid were removed by three transfers to fresh medium. They were handled gently with wide-bore polyethylene micropipettes (0.8–1.1 mm i.d.) to avoid loss of cumulus cells. The cumulus-oocyte complexes were then incubated in plastic culture chambers (Sterilin, U.K.) at 37°C in water-saturated air.

The culture medium consisted of Eagle's Basal Medium with Hank's salts (Gibco, U.S.A.), buffered with 5% (v/v) 154 mM-sodium Tes (*N*-Tris-hydroxymethylmethyl-2-aminoethanesulphonate) to pH 7.2 and supplemented with 15% (v/v) fetal calf serum (Wellcome, U.K.), 100 i.u. penicillin-K/ml and 50 µg streptomycin/ml. For cultures with LH, the hormone (LH-NIH-B8, 10 µg/ml) was added to the medium immediately before use. Media were sterilized by positive-pressure membrane filtration (pore size 0.22 µm).

Transplantation

Between 06:00 and 23:00 h on the day of pro-oestrus host females were caged with males of proven fertility; copulatory plugs or spermatozoa in the vagina were accepted as evidence of insemination. Ova from donor rats or from cultures were transplanted into the right oviducts, as follows. The hosts were anaesthetized with tribromoethanol solution 1–3 h before the time of

ovulation and the right ovarian adnexa were exposed through a lumbar incision. The ovarian bursa was incised over the ostium of the oviduct and the oocyte–cumulus complexes were transferred, in a minimal volume of medium, into the infundibulum of the oviduct by a finely drawn, heat-polished glass pipette. Immediately after transplantation, the right ovary was removed, care being taken to avoid bleeding. Since the ovary was removed before ovulation, the ampulla on this side could contain only transplanted oocytes.

Two types of oocyte–cumulus complex were transferred—those which had matured *in vivo* (in the follicles of pro-oestrous rats) and those which had undergone maturation in culture. Oocytes which had matured *in vivo* were obtained from donor rats kept in a light cycle 6.5 h earlier than that of the host rats. Oocytes were removed from the oviducts of the donor rats between 2.5 and 4.3 h after the median time of ovulation and were rinsed by transfer to fresh medium at least once before transfer to recipient females. The oocytes which matured in culture were incubated with or without LH for 15.5–17 h. They were then picked up in a polyethylene micropipette, placed in a fresh drop of medium and transferred to a recipient rat. Dead oocytes, naked oocytes and oocytes whose cumuli had not matured in the presence of LH (Dekel & Kraicer, 1977, 1978) were not transplanted.

Fertilizability of transplanted oocytes

One group of recipients was killed 24 h after transplantation. Their oviducts were isolated intact and flushed with 0.2 ml culture medium. The oocytes were mounted for microscopic examination. They were classified as fertilized only if a sperm tail was seen in the vitellus. Animals were considered only if normal fertilization of the recipient's own oocytes (in the left oviduct) was confirmed. The capacity of transplanted oocytes to develop further was tested. At laparotomy, implantation sites were counted on Days 9 or 12 and embryos were counted on Day 16. On Day 21, fetuses were delivered by a Caesarean section, and the progeny from transplanted ova were distinguished from the hosts' own fetuses. The neonates were placed with post-parturient foster mothers for nursing.

The significance of differences between experimental groups was analysed by the χ^2 test.

Results

Time of ovulation

The time of spontaneous cyclic ovulation in rats of our colony is checked periodically. During the period in which this study was performed the time limits of ovulation (2.5–97.5% of the rats, as estimated from the probit curve) were from 23:50 h of pro-oestrus to 03:55 h of oestrus. The median time of ovulation (50% of the rats) was 01:53 h $\pm$ 10 min (standard error of estimation). Since the host rats used in this study were on a different lighting schedule from that of the main rat colony, the time of their ovulation was determined. In relation to their dark/light cycle, there was no difference in either the median ($P = 0.3$) or the slope ($P = 0.35$) of their time curve of cumulative frequency of ovulation.

The effect of blocking of ovulation with sodium pentobarbitone treatment (Table 1) showed that the median time for secretion of an ovulatory quota of LH was 15:05 h $\pm$ 10 min (s.e. of estimation) on the day of pro-oestrus. Oocytes were therefore always isolated for culture $3\frac{1}{2}$ –5 h before the end of the dark period on the day of pro-oestrus so that they were not affected by the endogenous surge of LH secretion.

Transplantation success

The success rate of transplantation could be estimated as (1) the proportion of host rats still bearing transplanted oocytes 24 h after transfer or (2) the proportion of transferred oocytes later

Table 1. The critical period for LH secretion as determined by sodium pentobarbitone (35 mg/kg) injection at various times on the day of pro-oestrus

Time of injection (h)	No. of rats	No. of rats ovulating	Inhibition of ovulation (%)*
11:30	7	7	0
12:00	9	1	90
12:30	7	0	100
13:00	9	1	90
13:30	7	0	100
14:30	12	7	40
15:00	11	7	35
15:30	66	5	15

* Rounded-off to nearest 5%.

recovered. Significantly more ($P < 0.005$) of the hosts given ovulated oocyte-cumulus complexes lost all of the transplants ($10/27 = 37\%$) than those given complexes which had been cultured for 24 h ($14/86 = 16\%$). On the other hand, partial loss from a group of transplanted oocytes was much more frequent ($P < 0.001$) among hosts given oocytes matured in culture (80% versus 37%). The reasons for this are believed to be as follows. Postovulatory cumuli had a much larger diameter than cultured cumuli, necessitating both a coarser pipette (o.d. 1.1 versus 0.8 mm) and a larger volume (1.8 versus 0.9 μ l) of medium. Both these factors would encourage loss of oocytes by regurgitation. Furthermore, the cohesion of the ovulated cumuli would make all-or-none losses preponderate. Cultured cumuli were isolated individually into the transfer pipette. As a result, partial losses of oocytes were more probable.

Fertilization

We distinguished sperm penetration from fertilization. An ovum was classified as penetrated if at least one spermatozoon traversed the zona pellucida, whether or not the oocyte was fertilized. The criteria for normal fertilization were two: (1) normal ovular morphology for the time of isolation and (2) presence of the sperm flagellum in the ooplasm. Cleavage or other signs of apparently normal development but without the presence of the flagellum were considered to be signs of parthenogenetic activation.

Spermatozoa penetrated the zona pellucida of 72% of the transplanted ovulated oocytes and this was therefore taken as the measure of the maximum expected rate of sperm penetration under the technical conditions employed in this study. Only 14% of the 162 oocytes cultured without LH were penetrated compared to 43% of the 139 oocytes cultured with LH. As ratios of the control rate (72%) these rates are 19 and 60% respectively. There were distinct differences between eggs which had not been penetrated; gross abnormalities, such as fragmentation and degeneration of the vitellus, were seen in much higher frequency among the oocytes cultured in the absence than in the presence of LH. The difference in percentage fertilization between oocytes incubated with LH and those isolated after spontaneous ovulation was also significant (Table 2; $P < 0.001$). Although addition of LH greatly enhanced the fertilizability of oocytes matured in culture, from 11 to 33% ($P < 0.001$), this proportion was still less than the control value of 61%. The percentage of normal fertilization among the penetrated oocytes of all three treatment groups was the same (85% of post-ovulatory ova; 75% matured in culture with LH; 75% matured in culture without LH; $P = 0.7$). The developmental stages of the fertilized oocytes varied from two intact pronuclei, through the stage of cleavage to two blastomeres. There was no difference between native and transplanted oocytes.

The ability of oocytes to form functionally normal embryos was assessed for oocytes

Table 2. Effect of LH on maturation of rat oocytes in culture, assessed 24 h after their transplantation into the oviducts of mated host rats

	With LH	Without LH
No. of oocytes cultured	139	162
No. of oocytes penetrated	60*	23
Fertilized and normal	45*	18
Fertilized and abnormal	8	2
Unfertilized†	7	3
No. of oocytes not penetrated	79	139
Apparently normal	28	53
Parthenogenetic	23	26
Grossly abnormal	28*	60

* Significantly different from value without LH, $P < 0.001$.

† Free spermatozoon in perivitelline space.

matured *in vivo* (7 rats) and *in vitro* with LH (19 rats). From Table 3 it can be seen that some of the fertilized oocytes which implanted developed into fetuses. The proportion of zygotes developing into normal embryos was not different ($P > 0.5$ by the binomial test) for oocytes matured *in vivo* or *in vitro* with LH. The embryos appeared entirely normal with respect to size, external appearance and developmental stage compared to the autologous embryos in the contralateral uterine horn.

Table 3. Implantation and development of oocytes matured *in vitro* with LH or *in vivo* after transplantation into the oviducts of mated host rats

	No. transplanted	Day 9 or 12	Day 16	Day 21
Matured <i>in vivo</i>	84			
Embryos observed		38	10	10
Embryos normal		10	3	3
Matured <i>in vitro</i> with LH	255			
Embryos observed		139	59	59
Embryos normal		13	5	5

Discussion

It is well established that explantation of oocytes into culture medium results in immediate resumption of meiosis, the product of which is a mature secondary oocyte (Pincus & Enzmann, 1935). Although morphologically normal (Zamboni, Thompson & Moore-Smith, 1972), these oocytes are fertilized at a lower rate than oocytes normally matured *in vitro* (Niwa & Chang, 1975), and the developmental capacity of these ova, even when fertilized, is very low. Since the lack of gonadotrophic stimulation *in vitro* is an obvious difference between maturation *in vivo* and *in vitro*, it is curious that so few attempts have been made to study the effect of addition of gonadotrophin to the culture medium (Pincus & Enzmann 1934; Thibault & Gérard, 1973).

Assessment of the fertilizability of oocytes would be most simply done *in vitro*. A system for fertilization of rat oocytes *in vitro* has been described (Toyoda & Chang, 1974), but we have not been able to use this method with consistent results. We, therefore, designed our own procedure for fertilization of oocytes *in vivo*. Furthermore, the developmental capacity of the oocytes could be assessed without additional manipulation. Because oocytes were transferred into only one oviduct, the contralateral oviduct, containing autologous ova, served as a quality control for the spermatozoa and for the maternal environment. The timing for the transfer of oocytes

matured in culture into ampullae of mated hosts must be precise. Earlier results indicated that maximal frequency of maturation, as judged by the presence of the polar body, was after culture for 8 h (with or without LH) (Kaplan, Dekel & Kraicer, 1977). *In vivo* the polar bodies are extruded by 4 h before follicular rupture (Tsafiriri & Kraicer, 1972). Cultured oocytes therefore achieve a developmental stage parallel to that of newly ovulated eggs after culture for 12 h. *In vivo*, a further 4 h elapse before 50% of the ovulated oocytes are fertilized (Shalgi & Kraicer, 1978). In the present study, therefore, oocytes were cultured for 16 h before transplantation.

The results reported here make it clear that rat oocytes matured *in vitro* without LH were only poorly penetrable. Addition of LH to the medium increased this proportion and the resulting zygotes developed into normal offspring in high proportions. Noyes (1952) showed that follicular oocytes of pro-oestrous rats which had responded to endogenous LH and whose cumuli were mucified became progressively more fertilizable with time. Niwa, Miyake, Iritani & Nishikawa (1976) showed similar acquisition of fertilizability *in vivo*. In our study, LH had at least two beneficial actions on cultured rat oocytes: there was marked inhibition of degeneration coupled with greatly improved fertilizability and developmental potential.

In speculating on the mechanism whereby penetration of sperm into oocytes is enhanced by LH *in vitro*, the relative roles of the cumulus and of the oocyte should be considered.

Oocytes will mature *in vitro* even after removal of the cumulus cells (Donahue, 1968) although maturation *in vivo* always occurs within the cumulus oophorus and it has been suggested that the oocyte-cumulus complex should be viewed as a functional unit (Dekel, Hultborn, Hillensjö, Hamberger & Kraicer, 1976; Phillips, Shalgi, Kraicer & Segal, 1978). Although little is known concerning the direct response of the oocyte to LH, recent studies suggest that oocyte maturation is the result of withdrawal of an inhibitor normally present in the follicle (Tsafiriri & Channing, 1975). The role of LH in eliminating this inhibitor, thereby fostering oocyte maturation, is not understood.

On the other hand, the response of the cumulus to LH, *in vivo* and *in vitro*, is clear. This response can be summarized as a series of structural changes which facilitate the passage of spermatozoa between the cumulus cells to the zona pellucida. In this study, only ova surrounded by cumulus cells were tested for fertilizability. The most significant effect of LH detected was to permit spermatozoa to penetrate the cumulus and zona pellucida of a larger proportion of oocytes. The proportion of oocytes fertilized among those to which spermatozoa had penetrated was not different in oocytes matured *in vivo* or *in vitro*, in the presence or absence of LH. In addition to the likelihood that LH acts via the cumulus cells, there is at least suggestive evidence that the oocyte itself responds to LH. In this connection it is significant that LH appeared to enhance the survival of oocytes in culture, causing a highly significant decrease in the number of grossly abnormal oocytes. Obviously, LH, by reducing the number of these abnormal oocytes, increased the proportion of potentially fertilizable ova. The observations of Thibault & Gérard (1973) also indicate that the ooplasm of the maturing oocyte of the rabbit responds to gonadotrophin. The action of LH on the oocyte need not be direct. The innermost cumulus cells, the corona radiata, and their oocyte are closely associated through gap junctions between the oolemma and extensions of the cumulus cells (Phillips *et al.*, 1978). By this means, metabolic responses of the cumulus cells to LH could be relayed to the vitellus. LH could also act on the oocytes by causing withdrawal of a meiosis inhibitor (Tsafiriri, 1978; Dekel & Beers, 1978; Gilula, Epstein & Beers, 1978).

In conclusion, maturation of oocytes in the presence of LH permits sperm penetration through the cumulus oophorus to the oocyte and enhances fertilizability of the oocyte. The close correlation between sperm penetration, diagnosed morphologically and normal embryogenesis indicates that fertilizability, as measured here, is a reliable index of the normality of LH-associated maturation *in vitro*.

Mr Y. Lelyveld, the Head of our Animal Facility and Mr Y. Navon were responsible for selection of the animals and the manipulation of the photoperiods. This work was supported, in

part, by a grant from the Chief Scientist's Bureau, Ministry of Health, of which P.F.K. is an established investigator. The LH used in this study was a gift of the Hormone Distribution Program, NIAMDD, NIH, Bethesda, U.S.A.

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Received 2 August 1978