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Embryonic development of fertilized rat oocytes induced to mature by an analogue of gonadotrophin-releasing hormone

R. Shalgi* and N. Dekel†

**Department of Embryology and Teratology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel-Aviv 69978, Israel; and †Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel*

Summary. A gonadotrophin-releasing hormone analogue (GnRHa) was administered to hypophysectomized immature rats. Postovulatory mature oocytes obtained under these conditions were exposed *in vitro* to a sperm suspension for fertilization. Developmental ability of the fertilized ova was studied by transfer of the 2-cell stage embryos to oviducts of foster mothers. The potential of oocytes, undergoing maturation in response to GnRHa, to develop into 2-cell embryos was similar to that of oocytes stimulated by hCG (76·4% and 83·1% respectivey). The 2-cell stage embryos obtained from such oocytes were equally able to implant in the uteri of foster mothers (25·7% and 21·2% respectively) and subsequenty develop into live embryos (15·3% and 15·2%, respectively, at Day 20 of pregnancy).

Keywords: GnRH; oocyte maturation; rat; embryos

Introduction

The physiological stimulus for resumption of meiosis is provided by luteinizing hormone (LH) (Lindner *et al.*, 1974), although it has been reported that gonadotrophin-releasing hormone (GnRH) can also promote oocyte maturation. Hillensjo & LeMaire (1980) found that exposure of isolated ovarian follicles to GnRH or its agonist analogues *in vitro* resulted in maturation of the oocytes within these follicles. The direct stimulatory action of GnRH on the ovary has also been demonstrated *in vivo*. Oocyte maturation and ovulation were induced in hypophysectomized rats after administration of GnRH agonists (Corbin & Bex, 1981; Ekholm *et al.*, 1981; Erickson *et al.*, 1983). Like the earlier reports (Hillensjo & LeMaire, 1980; Corbin & Bex, 1981; Ekholm *et al.*, 1981) we have also demonstrated that GnRH agonist analogues are potent inducers of resumption of meiosis in follicle-enclosed oocytes *in vitro*, as well as stimulators of oocyte maturation and ovulation *in vivo*, in hypophysectomized rats (Dekel *et al.*, 1983, 1985).

The studies discussed above demonstrated that, like LH, GnRH and its agonist analogues can stimulate the oocyte to mature and can trigger the follicle to release the mature oocyte. LH action results in the release of a functional fertilizable oocyte; after sperm penetration this ovum will develop into a normal embryo. The diagnosis of GnRH-induced oocyte maturation in all the studies mentioned above was based only on morphological markers and the functional performance of the matured oocytes was not tested (Hillensjo & LeMaire, 1980; Ekholm *et al.*, 1981; Dekel *et al.*, 1983). In a previous study we assessed the ability of oocytes undergoing maturation in response to GnRH to be fertilized (Dekel & Shalgi, 1987). Our present study is aimed at analysis of the potential of such fertilized oocytes to cleave into 2-cell embryos, to implant in uteri of foster mothers and to develop further to term.

Materials and Methods

Hormones. The GnRH agonist analogue [D-Ser(t-Bu)^6]des-Gly^{10}-GnRH-N-ethylamide (GnRHa), used in this study was purchased from Hoechst AG (Frankfurt, West Germany). Pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were purchased from Organon (Oss, The Netherlands).

Animals. Wistar-derived female rats were housed in air-conditioned rooms and supplied with laboratory animal pellets (Ambar-Israel) and tap water *ad libitum*. The 25-day-old rats were anaesthetized with ketamine HCl (100 mg Vetalar/kg body wt: Parke-Davis, NJ, USA) in 2% Rompun (Bayer, Germany), and hypophysectomized by the transauricular approach. The rats were injected with PMSG (15 i.u./rat) immediately after hypophysectomy and with (A) GnRHa (2 μg/rat), (B) hCG (4 i.u./rat) or (C) saline, 48 h later as described previously (Dekel *et al.*, 1985). The rats were killed 20 h after GnRHa/hCG administration. Each animal was inspected for remnants of pituitary tissue and those suspected of incomplete hypophysectomy were excluded. Completeness of hypophysectomy was also indicated by the fact that ovulation was not obtained in any of the rats of Group C. The ovulated oocytes from Groups A and B were recovered from the oviducts and transferred for in-vitro fertilization.

***Fertilization* in vitro.** Spermatozoa were collected from the uteri of mated females (Shalgi *et al.*, 1981) The sperm cell concentration was adjusted to 4–7 × 10^5/ml by dilution with rat fertilization medium (RFM) (Kaplan & Kraicer, 1978). Samples of sperm suspension (200 μl) were incubated under heavy paraffin oil at 37°C in an atmosphere of 5% CO_2 in air for 3 h. Ova with associated cumuli isolated from the oviducal ampullae of the immature treated rats as mentioned above were introduced to the sperm suspension.

A control group of oocytes (isolated from oviducts of 28-day-old, intact PMSG/hCG-treated rats) was included for each experiment. This group was used to standardize the results according to the daily variations in fertilization rates which may result from the use of different donors of spermatozoa (Shalgi *et al.*, 1981).

The eggs were examined by interference contrast microscopy for fertilization and first cleavage at 27–28 h after exposure to spermatozoa. Ova were considered fertilized when a sperm flagellum was seen in the vitellus (Shalgi & Phillips, 1982).

Transfer of zygotes and examination of embryonic development. Two-cell embryos were transferred to the oviducts of pseudopregnant recipients, 26–28 h after exposure of the cumulus-enclosed oocytes to the sperm suspension. Pseudopregnancy was induced by stimulation of the cervix by using a glass rod. The stimulation was performed twice, once at mid-morning on the day of pro-oestrus and then at mid-morning on the day of oestrus. Pseudopregnancy was verified from daily vaginal smears.

The hosts were anaesthetized with chloralhydrate (Merck) and the adnexa was exposed through a pair of lumbar incisions. The embryos were transferred in a small volume of RFM (50–100 nl) to the infundibulum of the oviduct using a finely drawn flame-polished glass pipette. Transfers were performed under a Zeiss dissecting microscope as previously described (Shalgi *et al.*, 1979). Embryos derived from oocytes isolated from GnRHa-treated rats were transferred to one oviduct and embryos derived from another group (hypophysectomized, hCG-treated or intact hCG-treated) to the contralateral tube of the same animal. To prevent transfer of unfertilized ova, only 2-cell zygotes were selected. To minimize damage to the transferred embryos, we limited manipulation and exposure to light and the 2-cell zygotes were selected by a brief observation using a dissecting binocular microscope (× 40).

One group of the recipient rats was killed at Days 12–13 of gestation. The number of implanted embryos was monitored and their size examined. Embryos with a size smaller than the expected average were classified as resorbing. The embryos were processed for histological examination. Following fixation in 4% formalin, and embedding in paraffin wax, 6 μm sections were prepared, stained with haematoxylin and eosin and examined according to Muntener & Hsu (1977) and Pijnenborg *et al.* (1981). Other recipient rats were examined on Days 12–14 by laparotomy. The number of embryonic implantation sites was recorded and their size examined. On Day 20, these hosts were killed. The embryos were counted, released from their embryonic investments, and examined. The embryos were processed for histological examination as described above.

Results

As previously reported (Dekel & Shalgi, 1987), the rate of fertilization of oocytes induced to mature by GnRHa was not significantly different from that of oocytes induced to mature by hCG. A similar fertilization rate was also obtained in the control group of oocytes isolated from intact PMSG–hCG-treated rats (Table 1). Examination of the zygotes 27 h after exposure to spermatozoa *in vitro* revealed that the ability of the zygotes of all 3 experimental groups (hypophysectomized, GnRHa/hCG-treated and intact hCG-treated) to develop into 2-cell embryos was >75% with no significant difference between them (Table 1).

In 12 individual experiments a total of 423 two-cell zygotes were transferred to 67 oviducts of 37 recipients: 10 recipients were examined for development of their embryos twice (Day 12–14 and

Table 1. Number of zygotes and 2-cell rat embryos after incubation of ovulated oocytes in sperm suspensions for 27 h

	Hypophysectomized		Intact control
	GnRHa	hCG	
No. of cultures	24	19	22
No. of experiments	6	6	6
Fertilized oocytes (%)†	182/216 (84·3)	118/129 (91·5)	132/141 (93·6)
2-cell embryos (%)‡	139/182* (76·4)	98/118* (83·1)	105/132* (79·5)

†Of total oocytes incubated with spermatozoa.
‡Of fertilized oocytes.
*$\chi^2 = 0{\cdot}37$, N.S.

Day 20) throughout their pregnancy. Laparotomy on Day 12–14 revealed a total of 23 fully developed embryos (Table 2) with 10 smaller intrauterine swellings that were classified as resorbing embryos. The number of fully grown embryos in these 10 recipient rats did not differ on Day 20, but only 8 of the resorbing embryos could still be identified at this time. Histological examination of the resorbing embryos on Day 20 showed hardly any embryonic remnants. Since the numbers of viable embryos on Day 12–14 were representative of those at term, the remaining recipients were killed at Day 12–13 instead of Day 20 to permit histological examination of the resorbing embryos.

Table 2. Survival and development to term of transferred embryos

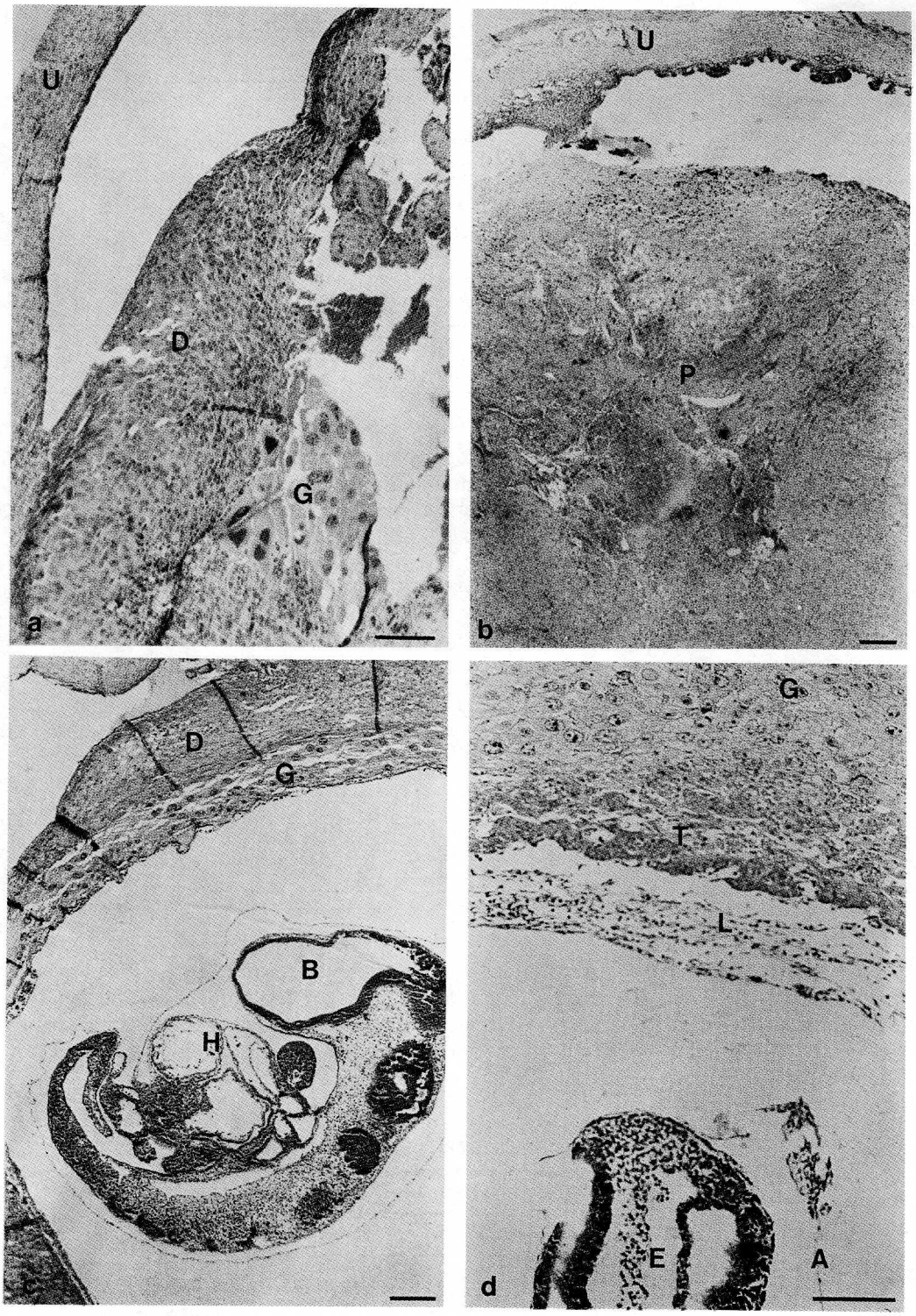
	Day 12–13		Day 19–20	
	Normal	Resorbing	Normal	Resorbing
Control (intact)	7	2	7	1
hCG (hypophysectomy)	9	4	9	3
GnRHa (hypophysectomy)	7	4	7	4
Total	23	10	23	8

The survival rate of the control group of embryos was 20·5%, while 12·8% of the transferred zygotes of this group were recovered as resorbing embryos. The results obtained for oocytes from hypophysectomized GnRHa/hCG-treated females were lower but there was no difference between the two hormonal treatments (Table 3) and they do not differ from the results obtained in the earlier experiment (Table 2).

Table 3. Development of transferred zygotes on Day 12–13

	Hypophysectomized		Intact control
	GnRHa	hCG	
No. transferred	221	151	105
Resorbing embryos (%)	23 (10·4)	9 (6·0)	15 (12·8)
Normal embryos (%)	34 (15·3)	23 (15·2)*	24 (20·5)*

*$\chi^2 = 0{\cdot}5$, N.S.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Histological examination of the resorbing embryos on Day 12–13 allowed in all cases identification of embryonic remnants such as secondary giant cells of trophoblastic origin (Fig. 1a) or derivatives of the trophospongium that exhibited changes similar to those observed after removal of the fetus (Davies & Glasser, 1968) (Fig. 1b), thus providing evidence for embryo implantation and the start of placental formation. In some cases remnants of embryonic tissue could be observed (Fig. 1d). No gross malformations and no evident organic abnormalities were observed in the embryos of normal size (Fig. 1c).

Discussion

GnRH, released from the hypothalamus into the portal system, stimulates the pituitary to synthesize and secrete LH. This gonadotrophin, in turn, triggers the ovarian oocyte to resume meiois. The systemic concentrations of GnRH are too low to elicit any peripheral responses. The reports that, in addition to its LH-mediated action, GnRH can induce oocyte maturation (Hillensjo & LeMaire, 1980; Ekholm *et al.*, 1981; Dekel *et al.*, 1983; Erickson *et al.*, 1983) via a direct interaction with ovarian receptors (Clayton *et al.*, 1979; Jones *et al.*, 1980; Clayton & Catt, 1981) were, therefore, very surprising.

In all the studies mentioned above, GnRH-induced oocyte maturation was diagnosed using morphological parameters such as germinal vesicle breakdown and/or polar body formation. We thought it necessary to assess the nature of the response of the oocyte to GnRH in functional terms. In a previous study (Dekel & Shalgi, 1987) we showed that the potential of oocytes undergoing maturation in response to GnRH to be fertilized is similar to that of oocytes stimulated by hCG. Our present report further demonstrates that these oocytes can cleave into 2-cell embryos, implant in the uterus and develop to term, at a similar rate of success as hCG-stimulated oocytes.

Apart from being a contribution to basic knowledge, functional evaluation of GnRH-induced oocyte maturation may have a significant clinical importance. Different protocols of treatment with GnRH and its agonist analogues are commonly practised today in fertility clinics. Based on its role in activation of the pituitary–gonadal axis, pulsatile administration of GnRH is used to treat infertile women with gonadotrophin deficiency (Ekstein *et al.*, 1985). More recently, based on its desensitization effect, a chronic pretreatment with potent agonist analogues of GnRH has been included in many in-vitro fertilization programmes to increase the number of oocytes available for aspiration (Lewinthal *et al.*, 1988). The use of GnRH agonists is not limited to infertile human patients. Massive doses of this hormone are administered daily to young girls with idiopathic precocious puberty (Yen, 1983). Receptors for GnRH in human ovaries have not been demonstrated (Clayton & Huhtaniemi, 1982) but a biological response of human granulosa cells to this hormone has been reported (Tureck *et al.*, 1982). Considering that GnRH can possibly act also on extrapituitary targets, such as the ovary, it was necessary to analyse carefully the nature of its action on the oocyte and eliminate the possibility that there may be any clinical hazard in using this hormone for therapy. Our studies clearly demonstrate that the ability of oocytes to be fertilized and develop into normal embryos is not impaired by their exposure to GnRHa. In fact, they seem to suggest that GnRHa can actually mimic the action of LH to induce development of functional mature oocytes.

Do these two hormones, LH and GnRH use similar mechanisms to induce oocyte maturation? In the absence of LH receptors on the oocyte (Lawrence *et al.*, 1980) it is accepted that the action of

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Fig. 1. Sections through implantation sites fixed on Days 12–14 presenting normal and resorbed embryos: (a) part of the fetal placenta of an absorbed embryo and some trophoblastic remnants; (b) section through remnants of the fetal placenta; (c) normal embryo; (d) resorbed embryo: E = embryo; A = amnion; G = giant cells; L = labyrinth; T = trophospongium; U = uterine wall; D = decidua; H = heart; B = brain vesicle. H&E, bar = 0·2 μm.

this gonadotrophin is mediated by somatic follicular cells (Dekel, 1988). Binding of LH to specific receptors on cells in the cumulus/granulosa compartment stimulates activation of the cAMP second messenger system and finally leads to oocyte maturation. GnRH also binds to receptors on the granulosa (Jones *et al.*, 1980; Clayton & Catt, 1981) but this interaction does not involve elevation of cAMP (Dekel *et al.*, 1985). Alternatively, in the pituitary, GnRH utilizes the calcium-dependent protein kinase C (PKC) second messenger system (Harris *et al.*, 1985; Hirota *et al.*, 1985; Naor *et al.*, 1985). There is evidence that PKC activators can mimic hormone action to induce oocyte maturation (Aberdam & Dekel, 1985). It is possible that granulosa cells are involved in mediating the action of GnRH on oocyte maturation. Direct interaction of GnRH with the female gamete should not be excluded, since receptors for GnRH have been found on the oocyte (Dekel *et al.*, 1988). To clarify this possibility further, we are currently looking for biochemical markers in oocytes exposed *in vitro* to GnRH.

Two-cell embryos were chosen for transfer to oviducts of hosts because, in the rat, development in culture does not proceed beyond this stage (Toyoda & Chang, 1974; Shalgi, 1984). The comparison of the number of implantation sites at Day 13 of pregnancy with that of normal embryos at term revealed that the major loss of transplanted embryos occurred at the early stages of pregnancy. In rats fewer in-vitro fertilized zygotes will develop into viable embryos than after in-vivo fertilization (Shalgi, 1984). For mouse embryos grown *in vitro*, the rate of successful transfers is related to the duration of the culture period (Hahn & Schneider, 1982). Vanderhyden *et al.* (1986) reported that zygotes resulting from in-vitro fertilization, although being able to develop to the 2-cell stage at a rate similar to that of the controls, suffered a significantly greater embryonic loss beyond this stage of development. Similar to our findings (Shalgi, 1984), they reported that $<20\%$ of the zygotes produced by in-vitro fertilization were recovered as live fetuses. The success rate of development of 2-cell stage embryos originating from GnRH- or hCG-stimulated oocytes in the present study was similar to that of the control group of oocytes obtained from intact animals, and resembled our results obtained previously for embryonic development after in-vitro fertilization (Shalgi, 1984).

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