PREOVULATORY AND OVULATORY MECHANISMS IN OOCYTE MATURATION

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Summary. Studies of the effect of gonadotrophins on the cumulus and oocyte or on the whole follicle *in vitro* offer a good tool for understanding the mechanisms of oocyte maturation, the ability of follicles of different sizes to respond to gonadotrophins and the processes of atresia. The birth of normal young from intrafollicular oocytes matured *in vitro* furnishes proof that in-vitro techniques are valuable as long as follicular integrity is maintained during the whole culture period.

Three main events occur during the preovulatory period before the ovulation of a normal and fertilizable oocyte: (i) cytoplasmic and nuclear maturation of the oocyte; (ii) disruption of cumulus cell cohesiveness among the cells of the granulosa layer; (iii) thinning and opening of the external follicular wall. Extending these three areas of investigation one may ask: (iv) why do only a very limited number of Graafian follicles *in vivo* respond to gonadotrophins; and (v) why do the other Graafian follicles become atretic?

Since 1967 we have developed in-vitro techniques as tools for the study of these aspects of ovarian physiology. The results thus obtained and others from the literature are mentioned and discussed in this paper.

DISRUPTION OF THE COHESIVENESS OF THE CUMULUS CELLS

In the growing follicles of some species, e.g. rabbit, the oocyte remains in a subcentral position suspended by pillars of cumulus cells which join the granulosa cells at many points. In primates, ruminants and many other mammals, cumulus cells of growing Graafian follicles are cytologically indistinguishable from the granulosa cells, although metabolic exchange with the egg occurs through this area, the discus proligerus, during follicular growth. After cyclic surges of gonadotrophins, cavities appear inside the cumulus mass and the cells become progressively separated from each other. Only the cumulus cells anchored in the zona pellucida remain surrounding the oocyte and forming the corona radiata (Pl. 1, Fig. 1). As soon as separation of the cumulus cells begins, metabolic exchange can occur only through the follicular fluid, and oocyte maturation *in vivo* is accomplished in a type of in-vitro culture system in this medium which is chemically similar to blood serum, although more diluted (Edwards, 1974; Menezo & Testart, 1975).

Metabolic exchange is probably easier in small mammals with small pre-

ovulatory follicles, since the volume of fluid in which the cumulus cells and oocytes are supported is less than that in ruminants or primates. It is not known if there is a relationship between the duration of final maturation and follicle size, but follicle and oocyte maturation does not exceed 12 hr in species with small follicles, and 1–2 days in species with follicles of >10 mm in diameter.

Culture of cumulus cells recovered from Graafian follicles of different sizes in the presence of hormones shows that FSH is more potent than LH for inducing loss of cohesion, and in several species oocytes with a typical corona radiata can regularly be obtained in this way (Pl. 1, Figs 2 and 3). Prolactin is without effect, and no spontaneous loosening of the cumulus cells has been observed without hormones (Thibault, 1972). It is not known if the specific FSH effect is necessary for final capacitation of the fertilizing spermatozoa as well as for oocyte release.

It is unlikely that the FSH effect is mediated by steroids, since cumulus cells do not show the typical cytoplasmic structure of steroid-secreting cells. Granular and agranular endoplasmic reticulum are present in various proportions, but the few cristae of the mitochondria are not of the tubular type (Zamboni, 1974), although the Golgi complex is well developed and seems particularly active in the corona cells after the gonadotrophic surge (rabbit: Moricard *et al.*, 1969; pig: Norberg, 1972).

When the whole follicle is cultured in the presence of FSH or FSH+LH, the cumulus cells do not disperse. A limited cumulus cell response gives a moderate corona radiata, but we have rarely observed release of the oocyte inside the follicle as we have seen in the same species *in vivo*.

FOLLICULAR ENLARGEMENT, THINNING OF THE WALL AND OVULATION

The synthesis of collagenase-like enzymes, probably under the influence of progesterone, seems to play a major role in the distension of the follicular wall and the rupture of the apex *in vivo* (see reviews by Blandau, 1970; Espey, 1974; Rondell, 1974). Enlargement of follicles during culture of follicles for 6–48 hr, or rupture and ovulation, has not been obtained, even with the preovulatory follicles of oestrous rabbits, with any type or dose of hormone in the culture medium. Similar results with hamster, cattle and monkey follicles corroborate the conclusions of Baker & Neal (1972) on mice and Tsafiriri *et al.* (1972) on rats. Modification of collagenous fibres surrounding the follicle does not seem to occur, and there is no increase in fragility of the isolated follicle during culture, as observed *in vivo* by Espey (1974).

During culture of follicles, it is impossible to simulate the ovarian epithelium, including the tunica albuginea, and the blood flow. Since the contents of the follicle are released outside the ovary, the ovarian epithelium is probably involved in the activation of collagenase activity. The rupture of cultured follicles has been described for ovarian fragments (Baker & Neal, 1972) and whole ovaries (Fritz *et al.*, 1965), but *in vivo* the decrease in the blood flow to a circumscribed area on the external surface of the follicle (Blandau, 1970) probably contributes to lysis of the granulosa and theca cells in that area.

The gas supply to a cultured follicle is evenly distributed through the fol-

llicular wall, except in the limited area on the grid where the follicle is in contact with the culture medium, and necrosis of this area has been reported (Moor *et al.*, 1973).

The in-vitro system does not therefore provide valuable information on the ovulatory process since many factors, such as blood flow, presence of ovarian epithelium and ovarian contractility, are missing.

CYTOPLASMIC AND NUCLEAR MATURATION OF THE OOCYTE

Culture of oocyte and cumulus

When rabbit oocytes from preovulatory follicles are cultured with the cumulus cells in a suitable medium, they resume meiosis in a manner similar to that observed *in vivo* in preovulatory follicles after the gonadotrophic surge; the chronology of first polar body formation is the same, and the second maturation spindle usually appears quite normal. When such oocytes are cooled for 24 hr at 10°C and then returned to 37°C, they are parthenogenetically activated and cleave regularly (Thibault, 1973a). Fertilization of these oocytes has been obtained *in vivo* after transfer into a female mated 12 hr earlier, and *in vitro* according to our routine procedure, but the fertilization process is abnormal. It begins as usual, but the sperm head remains morphologically unchanged, although migrating to the centre of the egg. The sperm nucleus does not swell immediately after penetration of the sperm head into the egg cytoplasm. The two pronuclei normally come into contact 3 to 5 hr after penetration in the rabbit, but in the culture system the female pronucleus joins to an unchanged sperm head, suggesting that a factor, not synthesized *in vitro*, appears in the egg cytoplasm during natural oocyte maturation *in vivo*. We call this factor(s) the male pronucleus growth factor (MPGF).

The MPGF in the rabbit appears only in the oocyte 6 to 7 hr after coitus, i.e. 3 to 4 hr after the rupture of the germinal vesicle. Only testosterone and prolactin stimulate the synthesis of MPGF *in vitro* (Thibault *et al.*, 1975). However, the normal development of the male pronucleus in human oocytes, cultured first in the presence of oestradiol for 4 hr, and then with 17 α -hydroxyprogesterone for 40 hr (Soupart, 1973), seems to indicate that this steroid sequence, which normally occurs *in vivo* after the gonadotrophic surge, may induce the synthesis of MPGF by the oocyte itself.

Culture of the Graafian follicle

Absolutely normal oocytes can be produced by culture of whole preovulatory follicles of rabbits in TCM 199+15% fetal calf serum+15% normal rabbit serum+20% chick embryo extract, pH 7.3–7.4, gassed with air and 0.5% CO₂, and at 5 to 10 bars. In the presence of 1 or 10 μ g FSH/ml and/or 1 or 10 μ g LH/ml, meiosis resumes, 8 hr after the beginning of culture the first polar body is formed, and the second maturation spindle is present in all oocytes after 11 hr (Thibault & Gerard, 1973). These oocytes have been recovered inside the follicle after 12 hr of culture and transferred into one oviduct of a doe mated 9 hr previously with a normal buck. Just before transfer, all the follicles in the ovary adjacent to the oviduct receiving the cultured oocytes are

destroyed. Oocytes of the contralateral ovary, shed in the corresponding tube, serve as controls. Experimental oocytes show that MPGF is synthesized *in vitro* in the follicle since the male pronucleus swelling proceeds exactly as in the control eggs (Thibault & Gerard, 1973).

Oocytes were transferred to the oviducts of two rabbits after intrafollicular maturation *in vitro* and permitted to develop to term. Normal young of both sexes were produced from 60% of the transferred oocytes and were themselves fertile. Similar experiments conducted with 60- to 82-day-old immature rabbits, or adult hamsters and monkeys, showed that oocytes with the second metaphase spindle may be obtained in Graafian follicles cultured in the presence of gonadotrophins.

ATRESIA *in vivo* AND PYKNOSIS *in vitro*

The maturation of follicular oocytes *in vitro* needs culture conditions in which the different layers of the follicle (theca, granulosa and cumulus cells) do not show signs of pyknosis, because it is known that the oocyte is able to resume meiosis spontaneously when granulosa cells degenerate *in vivo* or *in vitro* (Foote & Thibault, 1969). Different techniques were used to increase the oxygen available inside the follicle or to impoverish the culture medium.

Table 1. The evolution of nuclear maturation *in vitro* of intrafollicular oocytes of *Macaca fascicularis* (from Thibault, 1973b)

Nuclear stages	In ovary at Day 7 of the cycle	After 24 hr of culture	After 48 hr of culture
Dictyate	9	0	0
Prometaphase	45*	8	14
Metaphase I	2*	5	2
Metaphase II	9*	0	4

Culture conditions were TCM 199+20% fetal calf serum +20% monkey serum; HMG+HCG; air+0.5% CO₂; and 10 bars pressure.

* In pyknotic follicles.

Preservation of granulosa and cumulus cells was obtained in rabbit, monkey and hamster when the gas pressure (air and 0.5% CO₂) was increased to 5–10 kg/cm². In such conditions, all follicular layers remained absolutely normal after 15–24 hr of culture and nuclear maturation occurred in practically all the oocytes in the presence of FSH, LH or FSH+LH (Thibault & Gerard, 1973). The results were similar for oocytes of large preovulatory follicles of unmated oestrous rabbits, and for oocytes from small follicles (<1 mm) from 60–82-day-old rabbits which had been treated 24 hr before explantation with crude horse pituitary extract, a potent stimulant of follicular growth in mature rabbits (Pl. 1, Fig. 4). Meiotic division was also observed in the macaque when small follicles 1.0–2.0 mm in diameter were cultured under 10 kg/cm² pressure (Table 1). The same technique was also satisfac-

PLATE 1

FIG. 1. Follicle of a cow just before ovulation showing the oocyte and its corona free in the follicular fluid and the loosened granulosa cells. The oocyte is at metaphase II and has produced the first polar body.

FIG. 2. Cumulus cells and oocyte from a medium-sized calf follicle after 24 hr of culture in the presence of ovine FSH. The oocyte is in metaphase I and has a typical corona radiata, an aspect similar to that shown in Fig. 1.

FIG. 3. The cumulus cells and oocyte from a medium-sized sheep follicle after 24 hr of culture in the presence of ovine LH. The oocyte is in metaphase II, but there is no loosening of the cumulus cells.

FIG. 4. A small follicle, from an immature rabbit, cultured in the presence of FSH and LH under 10 kg/cm² gas pressure. Meiosis has resumed and has reached metaphase II. There are no signs of pyknosis and early luteinization of the granulosa cells can be seen.

(Facing p. 608)

FIG. 5. Calf follicle cultured in the presence of bovine FSH showing strong secretory activity of the granulosa cells.

FIG. 6. Calf follicle cultured at pH 7.3 for 24 hr showing complete pyknosis of the granulosa cells and loosening of the granulosa and theca layers.

FIG. 7. Calf follicle cultured for 12 hr at pH 7.8 in a medium containing 40% calf serum in the presence of FSH. There is a large area of pyknosis in the cumulus, but other cells are normal.

FIG. 8. Calf follicle cultured in the same conditions as those for Fig. 7 but with a reduced quantity of serum, resulting in a limited number of pyknotic cumulus cells.

tory for the hamster. These results agree well with those of Baker & Neal (1972) for the mouse. Thus, normal maturation under the influence of gonadotrophins can be easily obtained *in vitro* if the oxygen available inside the follicle for the granulosa and cumulus cells is increased (Thibault *et al.*, 1975).

The relationship between gonadotrophic stimulation of granulosa and cumulus cells and their metabolic requirements has been investigated by culture of follicles from 3-month-old calves. Follicles were collected from calves pretreated with fluorogestone acetate and PMSG in order to obtain an homogenous population of non-atretic follicles 2–4 mm in diameter (Arrau, 1974). As indicated above, FSH loosens the cumulus cells very rapidly. Moreover, FSH stimulates the secretory activity of granulosa cells throughout the follicle (Pl. 2, Fig. 5). LH has less effect on cumulus cells and does not enhance the secretory activity of the granulosa layer. If the culture medium is at pH 7.3–7.4, the cumulus cells become pyknotic in a few hours, and pyknosis appears in the granulosa and theca interna cells, which become dissociated (Pl. 2, Fig. 6). Granulosa and theca cells can be preserved by increasing the pH to 7.8 and even to 8.0, but necrosis of the cumulus cells is not prevented (Pl. 2, Fig. 7). If LH or PMSG is used instead of FSH, pyknosis is localized to small areas of the cumulus mass, generally proximal to the oocyte. Normal metaphase II has been obtained after 30 hr of culture.

Complete preservation of these cells, however, is obtained only after impoverishment of the culture medium. When the serum content is limited to 20%, there is no sign of pyknosis in the presence of PMSG or LH, and necrosis is limited when FSH is present (Pl. 2, Fig. 8).

It seems possible to conclude from these experiments that atresia *in vivo* may result from the inability of the ovary to respond to increases in gonadotrophin levels with the right changes of metabolism in the follicle. The decrease in the number of atretic follicles *in vivo* in calf ovaries during progestagen treatment, and the pronounced increase after either the endogenous surge of LH or PMSG+HCG treatment, show clearly that the gonadotrophin levels are important in the process of atresia.

RESPONSE OF SMALL- OR MEDIUM-SIZED FOLLICLES TO GONADOTROPHINS *in vivo* AND *in vitro*

In the experiments described above, resumption of meiosis has been obtained in small Graafian follicles of the macaque (1.0–2.0 mm) and immature rabbit (<1 mm), and in medium-sized follicles of calf and sheep (2–6 mm), and yet these follicles do not respond *in vivo* during the oestrous or menstrual cycle. As long as these follicles remain healthy, the oocyte stays in the dictyate stage, even if the gonadotrophin level increases. This difference in behaviour *in vivo* and *in vitro* suggests internal regulation by the ovary of follicular growth, as suggested by Peters *et al.* (1973), and of follicular sensitivity to gonadotrophins.

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