Comparative study of extra and intrafollicular hamster oocyte maturation

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Summary. A comparative study of intra- and extra-follicular maturation of hamster oocytes checked by *in vitro* fertilization showed that zona pellucida of extrafollicularly matured oocytes was not penetrated by spermatozoa. Even when gonadotropins (FSH, LH) or ovarian steroids (estradiol and progesterone) were added to the maturation medium they did not render the oocytes fertilizable. Fertilizability was only acquired *in vivo* 6 to 8 hrs after gonadotropinsurge. Complete intrafollicular hamster oocyte maturation has been obtained *in vitro* in Ham medium and under 56 p. 100 O_2, 39 p. 100 N_2, 5 p. 100 CO_2. Gonadotropins were absolutely necessary to complete meiosis up to metaphase II stage. With an optimal dose of 10 µg of FSH and LH/ml, 70 p. 100 of these oocytes reached metaphase II ; in 43 p. 100 of them, fertilization proceeded normally. We advance the hypothesis that zona maturation is necessary for oocyte penetration and fertilization.

Introduction.

The aptitude of the extrafollicular mammalian oocyte to resume meiosis spontaneously when cultured offers many possibilities for *in vitro* studies on fertilization and on their further development.

However, with the exception of the mouse (Mukherjee, 1972), all attempts to obtain litters from such *in vitro*-matured oocytes have been unsuccessful (rabbit : Thibault and Gérard, 1973 ; cow : Hunter *et al.*, 1972 ; sheep : Moor and Trounson, 1977). Histological studies revealed that decondensation of sperm chromatin does not occur or takes place more slowly than in ovulated oocytes. This asynchronism in male and female pronucleus formation may explain abnormal development (rabbit : Thibault and Gérard, 1970 ; Motlik and Fulka, 1974*a* ; pig : Motlik and Fulka, 1974*b* ; cow : Thibault *et al.*, 1975 ; hasmter : Usui and Yanagimachi, 1976). Furthermore, the comparison of protein synthesis during intrafollicular or extrafollicular maturation of sheep oocyte reveals the maintenance of protein pattern synthesis in extrafollicular oocytes as opposed to the switch occurring during normal intrafollicular maturation (Warnes *et al.*, 1977).

The birth of young (rabbit : Thibault et al., 1975 ; sheep : Moor and Trounson, 1977) has shown that it is possible to obtain complete oocyte maturation in vitro when large antral follicles are cultured in presence of gonadotropins.

The ability of hamster sperm to be capacitated in vitro and to fertilize ovulated oocytes (Barros and Austin, 1967), and the obtention of regular nuclear maturation of extrafollicular hamster oocytes in a purely synthetic medium (Haidri and Gwatkin, 1973) encouraged us to reinvestigate the different aspects of oocyte maturation in this species (Mandelbaum and Plachot, 1977 ; Mandelbaum et al., 1977).

Material and methods.

Follicle and oocyte recovery. — Oocytes from preovulatory follicles encompassed in the cumulus cells, or entire preovulatory follicles, have been collected from cyclic female hamsters 48 hrs after the injection of 30 IU of PMSG given in the morning of estrus.

Control tubal oocytes were obtained from females receiving a 30 IU HCG injection 48 hrs after PMSG pretreatment. Oocytes were collected in the tubes 16 hrs later.

Culture system for maturation and fertilization.

— Extrafollicular oocytes were cultured in 0.5 ml of GH_2 medium (Haidri and Gwatkin, 1973) under paraffin oil and a gas mixture containing air (95 p. 100) and CO_2 (5 p. 100) for 12 hrs at 37 °C. Fertilization of these oocytes was attempted in 0.5 ml of GH_2 medium with the same gas mixture.

— Intrafollicular oocytes were cultured in Falcon dishes in 0.5 ml of either Ham F 10 medium or TC 199 medium supplemented or not with fœtal calf serum (15 p. 100), hamster serum (15 p. 100) and chicken embryo extract (20 p. 100).

1 to 20 μg/ml of ovine FSH and LH or estradiol 17β (0.1 μg/ml) and progesterone (1 μg/ml) were added to the preceding maturation media. Two culture systems were used to avoid granulosa pyknosis :

— in some experiments the increase of oxygen tension was obtained under a pressure of 5 bars, in an atmosphere containing air (95 p. 100) and CO_2 (5 p. 100) according to Thibault and Gérard's method (1973) ;

— other experiments were performed under a gas mixture containing O_2 (56 p. 100), N_2 (39 p. 100) and CO_2 (5 p. 100).

Cultures were limited to 16 hrs at 37 °C. The fertilization attempts of these intrafollicularly matured oocytes were realized in Ham F 10 or GH_2 medium under air (95 p. 100) and CO_2 (5 p. 100).

Method of fertilization.

1. In each fertilization attempt, the caudal epididymal sperm samples were recovered according to Wittingham and Bavister (1974) ; the same sample of epididymal sperm was used for the fertilization of in vivo or in vitro matured oocytes.

2. Tubal or follicular fluid was added to the fertilization medium as shown by Barros (1968) to improve the fertilization rate.

a) tubal fluid was collected by flushing a tube of a preovulatory female hamster with 0.5 ml of medium. This medium containing tubal fluid represented the fertilization medium ;

b) follicular fluid was obtained by opening 10 preovulatory follicles before the LH discharge in 0.5 ml of medium. The follicular walls and the oocytes were removed but the granulosa and cumulus cells were left.

3. The following criteria for egg fertilization were utilized :

— presence of the second polar body ;

— presence of two pronuclei in the oocyte cytoplasm.

We were not able to recognize the sperm tail with its midpiece which soon disappears after fertilization.

Eggs were fixed either according to Tarkowski's method (1966) or in Bouin medium and then cut after a double embedding in gelatin paraffin and stained with hematoxylin-eosin.

Results.

1. *Fertilization rate of freshly ovulated oocytes.* — Oocytes examined 24 hrs after sperm and oocyte mixing are in pronuclear stage.

When tubal or follicular fluid is added to the fertilization medium, respectively 50 p. 100 (7/14) and 80 p. 100 (60/77) of the oocytes are fertilized.

As the highest fertilization rate was obtained with follicular fluid ($p < 0,02$ with X^2 test), this medium was therefore used for all fertilization experiments.

2. *Attempts to fertilize extrafollicularly matured oocytes.* — When extrafollicularly cultured oocytes are examined 12 to 16 hrs later, 90 p. 100 (92/102) have reached metaphsae II stage, but these oocytes are not fertilizable. Cytological examination of hematoxylin-eosin stained serial sections of eggs, show that sperm heads, although in contact with the zona pellucida, never penetrate it.

TABLE 1

In vitro fertilization of extrafollicularly matured oocytes

Maturation medium	Fertilization medium	N⁰ of oocytes	Fertilization p. 100
GH_2	GH_2 + tubal fluid	3	0
GH_2	GH_2 + follicul. fluid	37	0
GH_2 .... + FSH (10 µg/ml) + LH (10 µg/ml)	GH_2 + follicul. fluid	11	0
GH_2 .... + estradiol (0,1 µg/ml) + progesterone (1 µg/ml)	GH_2 + follicul. fluid	21	0

As FSH and perhaps LH show a specific dispersive action on the cumulus cells which elongate at the same time. (Thibault, 1972 ; Thibault et al., 1975) and as cumulus cells have been implicated in the capacitation process, (Gwatkin et al., 1972), we added gonadotropins to the maturation medium. No sperm penetration occurred although cumulus cells were dissociated.

The dissociation of the cumulus cells is called positive (+) when it appears identical to the dispersive aspect of the cumulus surrounding the tubal oocytes recovered early after ovulation.

Fertilization also failed when estrogen and progesterone were added without gonadotropins (table 1).

Thus, zona pellucida penetrability does not seem to depend on the stimulation of cumulus cells either by gonadotropins or follicular steroids.

3. *When does the zona pellucida become penetrable in vivo ?* Oocytes are collected at increasing time intervals after HCG injection and further cultured for 2 to 10 hrs so that all the oocytes have matured for 12 hrs before attempted insemination trials. Sperm penetration as shown in table 2, appears when oocytes remain at least 6 hrs in the follicle after HCG injection ; sperm nucleus decondensation always occurs.

TABLE 2

In vitro fertilization after combined in vivo and in vitro oocyte maturation

Time of oocyte recovery after HCG injection (in hrs)	Additional culture time (in hrs) in GH_2 medium	Dissociation of cumulus cells	N° of oocytes	Fertilized oocytes (p. 100) in GH_2 medium + follicular fluid
2	10	—	26	0
4	8	—	49	6
6	6	+	56	27
8-10	4-2	+	8	50
12	0	+	7	86

The longer the oocyte remains in the follicle, the greater the fertilization rate.

The follicle thus seems necessary for complete oocyte maturation *in vitro*, leading to a normal sperm penetration.

4. *In vitro fertilization of intrafollicularly matured hamster oocytes.* — As in other species (rat : Tsafriri et al., 1972 ; rabbit : Thibault and Gérard, 1973 ; sheep : Moore and Trounson, 1977) nuclear oocyte maturation is only observed when gonadotropins are added to the maturation medium (table 3). The number of matured oocytes increases with the amount of FSH and LH until an optimal dose of 10 $\mu g/ml$ is reached.

Several culture systems were used combining :

— 3 maturation media containing gonadotropins : Ham F 10 ; Ham F 10 supplemented with fœtal calf serum (15 p. 100), hamster serum (15 p. 100) and chicken embryo extract (20 p. 100), and TC 199 supplemented in the same way ;

— 2 oxygenation systems : air-CO_2 under 5 bars or gas containing 56 p. 100 O_2, 39 p. 100 N_2 and 5 p. 100 CO_2 under atmospheric pressure.

TABLE 3

Influence of FSH and LH on maturation rate of follicular-enclosed oocytes
This table represents all the results obtained either in pressure gas or under 56 p. 100 of oxygen.

Media	FSH and LH (μg/ml)	Oocyte maturation (Meta. II) p. 100
Ham	0	13 (1/8)
	1	8 (1/12)
	5	50 (9/18)
	10	50 (56/115)
Ham + fœtal calf serum 15 p. 100 + hamster serum 15 p. 100 + chicken embryo extract 20 p. 100	0 1 5 10	0 (0/8) 17 (2/12) 12 (1/9) 30 (2/7)
199 + fœtal calf serum 15 p. 100 + hamster serum 15 p. 100 + chicken embryo extract 20 p. 100	5 10 20	22 (2/9) 44 (14/32) 41 (7/17)

The best *in vitro* maturation rate is obtained with Ham medium containing 10 μg/ml of FSH and LH under a gas mixture containing 56 p. 100 of O_2. After 16 hrs of culture, 70 p. 100 (27/40) of the oocytes are in metaphase II stage. The other culture systems show maturation rate from 30 to 47 p. 100. At the end of the maturation, oocytes are removed from the follicle, transferred in fertilization medium and then inseminated. 36 p. 100 to 62 p. 100 of these *in vitro* intrafollicularly matured oocytes are penetrated and fertilized. They reach pronuclear or 2-cell stages 16 to 24 hrs later (table 4).

TABLE 4

In vitro fertilization of intrafolliculary matured oocytes

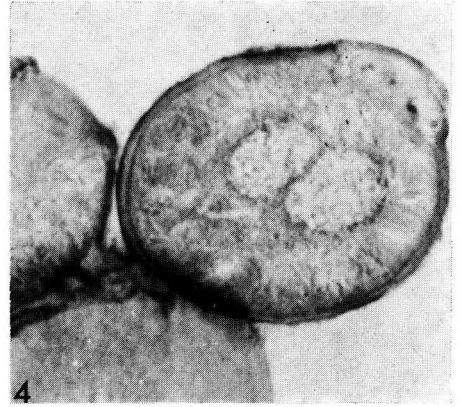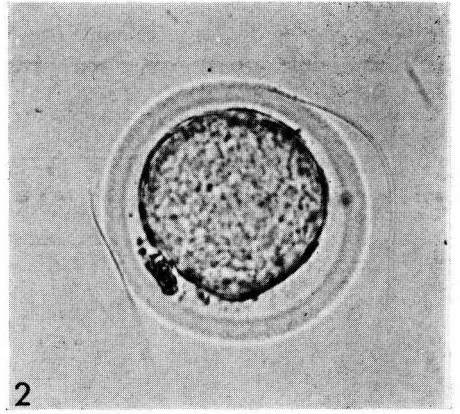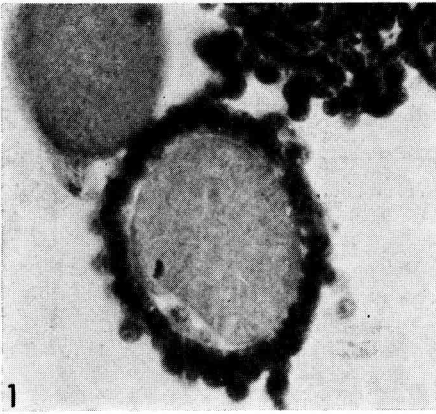| Maturation medium	Fertilization medium under air (95 p. 100) and CO_2 (5 p. 100)	Fertilization p. 100
Ham + FSH (10 μg/ml) + LH (10 μg/ml) under 56 p. 100 of oxygen	Ham + follicular fluid	36 (8/22)
	GH_2 + follicular fluid	62 (5/8)

PLATE I

FIG. 1. — *In vitro extrafollicularly matured hamster oocyte.*
Metaphase II stage. Hematoxylin-eosin.

FIG. 2. — *Extrafollicularly matured hamster oocyte unpenetrated by hamster sperm head in vitro.*
20 hrs after the beginning of the fertilization attempt (phase contrast).

FIG. 3. — *In vitro intrafollicularly matured hamster oocyte. Formation of the first polar body (anaphase I).*
Hematoxylin-eosin.

FIG. 4. — *In vitro fertilization of intrafollicularly matured hamster oocyte.*
Note the polar bodies and two pronuclei in the cytoplasm. Hematoxylin-eosin.

Discussion.

Extrafollicularly matured hamster oocytes could not be fertilized because the spermatozoa never passed through the zona pellucida as shown by cytological examination. This has never been described in other mammalian oocytes. In the hamster, a fertilization block occurs at the zona level since Usui and Yanagimachi (1976) only obtained *in vitro* fertilization of oocytes at different stages of their nuclear maturation by using zona-free oocytes.

It is hardly credible that capacitation should be concerned, as 80 p. 100 of freshly ovulated oocytes are fertilized *in vitro* in the well defined and repeatable conditions of our experiments, except if the final steps of capacitation occurring during the contact between acrosome and zona pellucida are absent. We need ultrastructural studies to obtain a detailed picture of sperm head-zona pellucida connections. If capacitation is not concerned, there may be a blockade at the zona pellucida level.

Two propositions may explain this phenomenon :

1) Complete cortical reaction occurs in extrafollicularly matured oocytes before sperm penetration. However, it seems that only the fertilizing spermatozoon is able to induce a complete cortical reaction and that parthenogenetic activation of oocytes in different species by various stimuli leads to incomplete cortical reaction. Longo (1977) also observes an incomplete cortical reaction by spontaneous activation of unfertilized hamster eggs after a prolonged stay in the oviduct. Culture conditions (pH, temperature, osmolarity) may have activated oocytes with an incomplete cortical reaction sufficient to block the sperm entry. This would not appear in follicle-enclosed oocytes matured *in vitro*. Ultrastructural studies are in progress to verify this hypothesis.

2) The maturation of the zona pellucida may play a role ; the nature of this maturation is unknown but it would be a necessary prerequisite for sperm penetration.

We propose that there is a change in the nature or in the configuration of zona proteins, a sort of mirror image of the change occurring after cortical granule discharge, which renders the zona insensitive to acrosome enzymes. So, hasmter oocyte maturation includes at least three stages: a nuclear maturation leading to metaphase II, a maturation involving zona pellucida that we call « zona maturation », and a cytoplasmic maturation leading to fast sperm nucleus swelling (Usui and Yanagimachi, 1976). Cytoplasmic and zona maturation proceed *in vivo* in the preovulatory follicle approximately 6 hrs after gonadotropin surge. Tubal fluid does not play any role in this process as 86 p. 100 of ovarian oocytes collected 12 hrs after HCG injection are fertilized. The zona maturation is achieved in the follicle 6 to 8 hrs after HCG injection ; 56 p. 100 of the oocytes collected at this time from the ovary and finishing their maturation *in vitro* are fertilized. We observe no abnormal male pronucleus growth as Usui and Yanagimachi did, but we only studied the end phase of fertilization as proved by the presence of the second polar body and two pronuclei in the oocyte cytoplasm. Examination of the early stages after sperm penetration would be necessary to be sure that cytoplasmic maturation is really achieved concomitantly with zona maturation. However, when oocytes can be penetrated, their fertilization also seems to be normal.

Zona maturation in the hamster does not depend on the direct action of gonado-tropins on the oocyte or on the cells surrounding it. When FSH and LH were added to the maturation medium of extrafollicularly matured oocytes, sperm penetration was not observed although the specific dispersive action of the gonadotropins on cumulus cells, already observed *in vitro* by Thibault (1972), was still present.

As gonadotropins also enhance steroidogenesis by follicular cells it is probable that high steroid levels were responsable for zona and cytoplasmic maturation, and that extrafollicular oocytes could not reproduce this hormonal event even when sur-rounded by cumulus cells.

Our attempts to mature zona by 17β estradiol and progesterone were unsuccess-ful. However, we have not duplicated the steroid sequence found in plasma (estradiol peak followed by an increase of progesterone) and probably have not reached the follicular steroid levels. It would be necessary to know the intrafollicular hormonal sequence in detail to reproduce the true physiological condition *in vitro*.

We cultured PMSG-induced preovulatory hamster follicles. It is interesting to note that nutritional requirements for maturation of extra and intrafollicular oocytes are different. The GH_2 medium described by Haidri and Gwatkin (1973) is the optimal medium for extrafollicular oocyte maturation but does not allow the maturation of follicle-enclosed oocytes. The intrafollicular oocytes probably need more amino acid and nucleic acid precursors as contained in Ham medium (and not in GH_2) during *in vitro* maturation.

As in other mammals (rat : Lindner *et al.*, 1974 ; calf : Thibault *et al.*, 1975 ; rabbit : Thibault and Gérard, 1973 ; macaque : Thibault *et al.*, 1976 ; sheep : Moor and Trounson, 1977) gonadotropins (FSH, LH) are absolutely necessary to reinitiate meiosis, when follicles are cultured *in toto*.

Recently, Gwatkin and Andersen (1976) made the same observation in hamster. The number of oocytes in metaphase II increases with the gonadotropin level until an optimal dose of 10 μg/ml is reached. This dose is usually employed in other mammals, except the sheep where Moor and Trounson have shown that more than 2 μg/ml of FSH and 1 μg/ml of LH depressed blastocyst formation while it significantly increased the fragmentation rate. Intrafollicularly cultured oocytes mature completely since 43 p. 100 of them are fertilized normally, as was also observed by Thibault *et al.* (1975) in the rabbit and Moor and Trounson (1977) in the sheep.

Résumé. Les tentatives de fécondation *in vitro* d'ovocytes de Hamster ayant achevé leur maturation soit hors du follicule, soit à l'intérieur du follicule, montrent que les sper-matozoïdes ne pénètrent pas la membrane pellucide des ovocytes maturés hors du follicule. Ni FSH ou LH, ni l'estradiol et la progestérone ajoutés au milieu de culture pendant la maturation, en rendent la membrane pellucide pénétrable. *In vivo*, cette propriété n'est acquise que 6 à 8 h après la décharge ovulante de gonadotropines. La maturité complète

de l'ovocyte de Hamster a pu être obtenue *in vitro* dans son follicule cultivé dans du milieu de Ham, sous 57 p. 100 d'O_2, 38 p. 100 de N_2 et 5 p. 100 de CO_2. FSH et LH sont absolument indispensables pour obtenir dans de telles conditions la reprise de la méïose jusqu'à la métaphase II. En présence de 10 μg de FSH et de LH par millilitre de milieu, 70 p. 100 des ovocytes intrafolliculaires atteignent la métaphase II et 43 p. 100 d'entre eux sont fécondés normalement (pénétration et formation des pronuclei). Il semble donc que normalement l'ovocyte de Hamster subisse peu avant l'ovulation un changement de sa membrane pellucide, qui la rend pénétrable par le spermatozoïde capacité.

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