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Original article

RNA and protein synthesis requirements for the resumption of meiosis in rabbit oocytes : the role of cumulus cells

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Summary — *In vitro* maturation of rabbit cumulus-enclosed oocytes was fully inhibited in α-amanitin- (100 μg/ml) and cycloheximide- (5 μg/ml) supplemented media.The inhibition was reversible and substantially reduced by delaying the addition of α-amanitin (2h) or cycloheximide (3 h).In contrast, both drugs did not inhibit germinal vesicle breakdown in denuded oocytes.Co-culture of granulosa cells (1 x 10^6/ml) with denuded oocytes did not substitute for an intact cumulus.The data presented here suggest that the resumption of meiosis in rabbit cumulus-enclosed oocytes is dependent upon early transcriptional and translational events which probably occur within the cumulus cells.

oocyte maturation — cumulus oophorus — transcription — translation — rabbit

Résumé — Synthèse d'ARN et de protéines nécessaires à la reprise de la méiose : rôle des cellules du cumulus oophorus. *La reprise de la méiose* in vitro *d'ovocytes de lapins inclus dans leur cumulus oophorus est complètement inhibée par l'α-amanitine (100 μg/ml) et le cycloheximide (5 μg/ml).L'inhibition est réversible et fortement diminuée si l'on retarde le traitement par l'α-amanitine (2 h) ou la cycloheximide (3 h).Au contraire, les deux inhibiteurs n'empêchent pas la rupture de la vésicule germinale d'ovocytes dénudés (sans cumulus oophorus).Des cellules de la granulosa (10^6/ml) en coculture avec des ovocytes dénudés ne remplacent pas un cumulus oophorus intact.Les résultats présentés suggèrent que la reprise de la méiose dans des ovocytes de lapins inclus dans le cumulus oophorus est sous la dépendance de phénomènes de transcription et de traduction survenant probablement dans les cellules périovocytaires.*

maturation de l'ovocyte — cumulus oophorus — transcription — traduction — lapin

INTRODUCTION

Although the spontaneous resumption of meiosis in mammalian oocytes under *in vitro* conditions was originally observed in rabbits (Pincus & Enzmann, 1935), the mechanism of oocyte maturation in this species has not been well studied. Only the effects of medium composition, *i.e.* osmolarity and addition of hormones, have been investigated (Thibault & Gérard, 1973; Bae & Foote, 1975a, b; Smith *et al.*,1978; Magnusson *et al.*,1981).

On the other hand, in rodent oocytes the effects of cAMP elevating drugs, RNA and protein synthesis inhibitors have been studied extensively (see for review Thibault *et al.*,1987). Alpha-amanitin, a specific inhibitor of RNA polymerase II involved in the synthesis of hnRNA, does not inhibit the resumption of meiosis in mouse oocytes (Crozet & Szöllösi, 1980). Sheep-denuded oocytes, cultured in α-amanitin supplemented media, also show normal metaphase plates (Osborn & Moor, 1983). However, the maturation rate of sheep and cattle cumulus-enclosed oocytes is substantially reduced in the presence of α-amanitin (Osborn & Moor, 1983; Hunter & Moor, 1987).

Protein synthesis inhibitors, *i.e.* puromycin and cycloheximide, do not prevent the resumption of meiosis in rodent oocytes either; however, the latter cannot proceed beyond the circularly arranged bivalent stage (Schultz & Wassarman, 1977; Ekholm & Magnusson, 1979). In contrast, pig, sheep and cattle oocytes — denuded or not — are very sensitive to protein synthesis inhibition (Fulka Jr. *et al.*, 1986; Moor & Crosby, 1986; Sirard & First,1988).

All these studies indicate substantial differences between species in requirements of *de novo* RNA and protein synthesis for the resumption of meiosis. This study reports the establishment of sensitivity of rabbit oocytes to α-amanitin and cycloheximide under *in vitro* conditions; attention is paid to the site of action of both inhibitors.

MATERIALS AND METHODS

Pseudopregnancy was induced in 40 sexually mature female rabbits of different breeds with 100 IU hCG (Praedyn, Spofa, Czechoslovakia).On the 17th, 18th and 19th day of pseudopregnancy, females were injected twice daily with 0.4 mg of FSH (Folicotropin, Spofa). One day later, the does were killed and oocyte cumulus complexes were isolated under dissection microscope by rupture of the follicular wall of the preovulatory follicles.Oocytes cumulus complexes from 3 does were always pooled.Cumulus-enclosed or cumulus-free oocytes (for description of cumulus cell removal, see below) were cultured in 0.1 ml-medium droplets under paraffin oil at 38°C under 5% CO_2 in air. The culture medium contained : isotonic TC199 (Usol, Prague, Czechoslovakia), 72 ml; 1.45% $NaHCO_3$+0.002% phenol red, 18 ml; 5.5% glucose solution, 10 ml; sodium pyruvate, 0.004 g; freeze-dried calf serum growth proteins (Usol), 15 mg/ml; penicillin, 50 IU/ml; streptomycin, 5 mg/ml. The control culture medium was supplemented either with α-amanitin at concentrations of 10, 50 and 100 μg/ml, or cycloheximide (Serva) at concentrations of 1, 5 or 10 μg/ml. In the first series of experiments, all concentrations of the drugs were tested simultaneously. As 100 μg/ml of α-amanitin and 5 μg/ml of cycloheximide proved optimal for our experimental design, only these concentrations were subsequently used. To test the reversibility of α-amanitin and cycloheximide inhibition 4h after culturing in the drug, oocytes were thoroughly washed 5 times in the control medium and subsequently cultured in the same medium for another 4h.

Denuded rabbit oocytes were also co-cultured with 1 x 10^6 parietal granulosa cells/ml. Granulosa cells from the cavity of preovulatory follicles were obtained by pressing a preparation needle gently against the follicle wall. The

granulosa cells were pooled and mixed thoroughly by sucking the suspension in and out of a wide-bore pipette. The concentration of the granulosa cells in the suspension containing 0.06% Trypan blue was determined by counting in a haemocytometer. The cells were then diluted in α-amanitin- or cycloheximide-supplemented media to give the desired concentration.

Finally the cumulus cells were removed by hyaluronidase (Hyasa, Sevac) treatment (247 TRU/ml), and the corona radiata by fine pipettes. Denuded oocytes were mounted on slides, fixed in acetic alcohol (1 : 3 v/v) for 24 h, stained with 1% orcein and examined under the phase-contrast microscope. Germinal vesicles of freshly-isolated rabbit oocytes were characterized by a distinct nuclear membrane, a compact nucleolus or nucleoli and highly condensed bivalents in nucleoplasm, mainly around the nucleolus. After the disappearance of the nuclear envelope, late diakinesis chromatin was visible in the form of one or several orcein-positive lumps. The frequency of GVBD in Table I and II was compared on the basis of chi-square analysis.

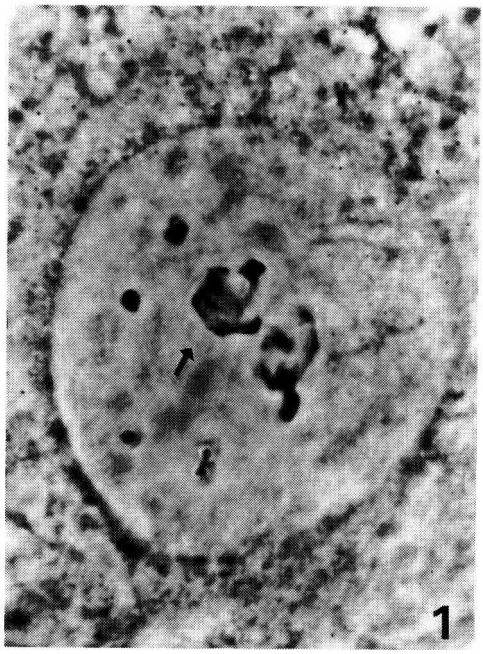
RESULTS

First, the time-sequence of germinal vesicle breakdown (GVBD) under the described conditions was tested. While after 2 h of culture all cumulus-enclosed oocytes remained at the GV stage, after 3 and 4 h of culture 66% and 97% of oocytes respectively reached the late diakinesis stage (Table I). Accordingly, the 4-h cul-

Table I. The effect of α-amanitin on resumption of meiosis in rabbit cumulus-enclosed and cumulus-free oocytes.

Type[a] of oocyte	*Time of culture in control medium (h)*	*Time of culture in α-amanitin[b] (h)*	*Time of culture in control medium (h)*	*No. of oocytes*	*Nuclear configuration*		*% GVBD*
					GV	*LD*	
			2	27	27		0
			3	30	10	20	66.6
			4	34	1	33	97.0 •[d]
C+		4		39	37	2	5.1 •+
		4	4	42	15	27	64.2
	1	3		38	34	4	10.5
	2	2		36	9	27	75.0 +
			4	32	1	31	96.8
C—		4		42	4	38	90.4 o
	GR[c]	4		35	8	27	77.1 o

[a] C+ cumulus-enclosed oocytes; C— denuded oocytes.
[b] The culture medium was supplemented with 100 μg α-amanitin/ml.
[c] Denuded oocytes were co-cultured with 1 x 10^6 parietal granulosa cells(GR)/ml.
[d] • • These values are significantly different (α=0.001).
+ +
o o These values are not significantly different.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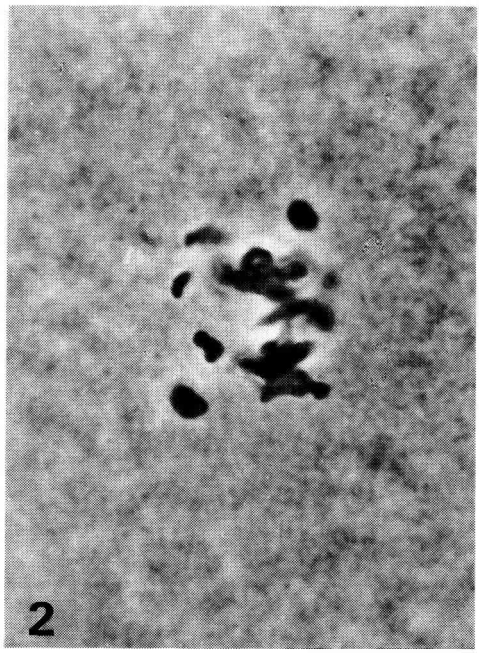
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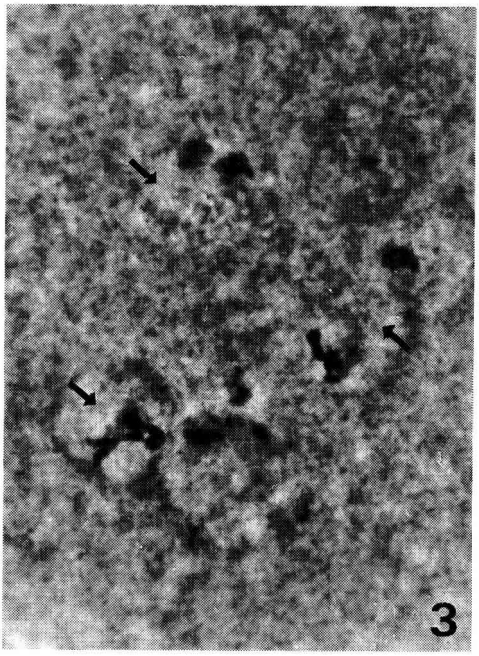
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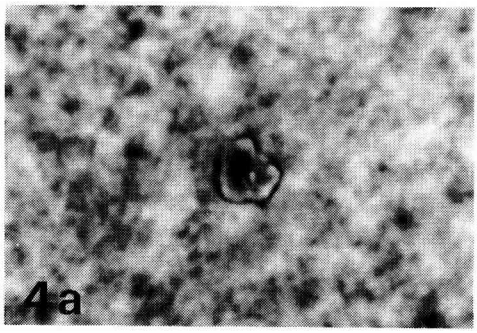
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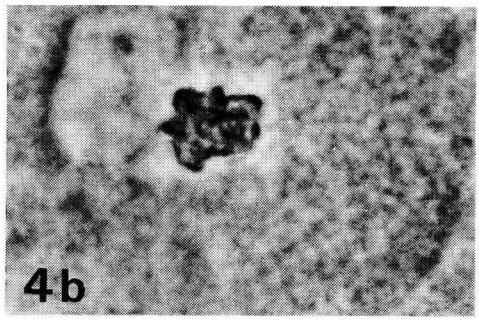
4b

ture interval was applied in all following experiments.

The sensitivity of rabbit cumulus-enclosed oocytes to α-amanitin was tested at concentrations of 10, 50 and 100 μg/ml. As the experiment proved, only the highest concentration fully inhibited GVBD (33, 21 and 3%, respectively) (Fig. 1). The inhibition was reversible after subsequent culture (4 h) in the control medium (64%) (Fig. 2). A greater reversibility of the α-amanitin block was observed on removal of cumulus cells after incubation in the drug (82%, data not shown). The inhibitory effect was greatly reduced when α-amanitin was added 2 h after the onset of culture (75% of GVBD).

Surprisingly, α-amanitin did not exert any inhibitory effect on denuded oocytes (Table I). The rate of GVBD in the control and α-amanitin-supplemented medium was quite comparable (96 and 90%, respectively). The culture of denuded oocytes with α-amanitin in a suspension of granulosa cells did not significantly reduce GVBD (77%); however, the GVBD sequence was slower. Oocytes with remains of nucleoplasm and highly condensed bivalents were included in the GV category (about 10%) (Fig. 3).

The first series of experiments documented the high sensitivity of rabbit cumulus-enclosed oocytes to 1, 5 and 10 μg of cycloheximide/ml (< 5% of GVBD). Accordingly, 5 μg cycloheximide/ml was applied in all further experiments (see Table II). The inhibitory effect of cycloheximide was fully reversible since more than 86% of oocytes reached late diakinesis, when cycloheximide treatment was followed by 4 h culture in the control medium. When cycloheximide was added 2 h after initiation of culture, only 8.8% of oocytes did not keep GV. However, the addition of cycloheximide 2.5 and 3 h after explantation resulted in GVBD in as many as 65% and 89% of oocytes, respectively.

In a similar manner to α-amanitin, the inhibitory action of cycloheximide on rabbit oocytes maturation was dependent upon the presence of intact cumulus. GVBD occurred in 85% of denuded oocytes in cycloheximide supplemented medium (Table II). Co-culture of denuded oocytes with the granulosa cells did not substitute for intact cumulus, since more than 75% of GVs were broken down after 4h of culture (Fig. 4a, b).

DISCUSSION

Under our culture conditions, GVBD occurred in 66% and 97% of rabbit cumulus-enclosed oocytes after 3 and 4 h of culture, respectively. Thibault (1972) observed a somewhat faster maturation sequence, since all oocytes reached prometaphase or metaphase I after 3.5 h of culture.

Fig. 1. The germinal vesicle of a rabbit oocyte cultured as oocyte cumulus complex in α-amanitin-supplemented medium for 4 h. The highly condensed bivalents are localized mainly around the nucleolus (arrow). x 800.
Fig. 2. An oocyte in the late diakinesis stage. The 4-h α-amanitin treatment was followed by 4 h culture in the control medium. x 1 000.
Fig. 3. The rest of the germinal vesicle of an oocyte cultured for 4 h in a suspension of granulosa cells in α-amanitin-supplemented medium. The highly condensed bivalents are still localized in an area of the GV and are surrounded by nucleoplasm (arrow). x 800.
Fig. 4. a) A denuded oocyte cultured 4 h in cycloheximide supplemented medium. The highly condensed bivalents clumped in a orcein-positive spot. x 800. **b)** The same phenomenon was observed when the cycloheximide medium was supplemented with granulosa cells. x 800.

Table II. The effect of cycloheximide on resumption of meiosis of rabbit cumulus-enclosed and denuded oocytes.

Type[a] *of oocyte*	*Time of culture in control medium (h)*	*Time of culture in cycloheximide*[b] *(h)*	*Time of culture in control medium (h)*	*No. of oocytes*	*Nuclear configuration*		*%*	
					GV	*LD*	*GVBD*	
			4	28	1	27	96.4	•[d]
		4		32	31	1	3.2	•+
C+		4	4	30	4	26	86.6	
	2	2		34	31	3	8.8	
	2.5	1.5		38	13	25	65.7	
	3	1		39	4	35	89.7	+
			4	34	2	32	94.1	
C—		4		40	6	34	85.0	o
	GR[c]	4		26	6	20	76.9	o

[a] C+ cumulus-enclosed; C— denuded oocytes.
[b] The culture medium was supplemented with 5 μg cycloheximide/ml.
[c] Denuded oocytes were co-cultured with 1 x 10^6 parietal granulosa cells(GR)/ml.
[d] • • These values are significantly different ($\alpha = 0.001$).
+ +
o o These values are not significantly different.

Autoradiographic experiments with ^{3}H-uridine incorporation in cumulus oocyte complexes in α-amanitin supplemented media (Kanka & Motlik, unpublished results) confirmed the conclusion of Kidder *et al.* (1985) that α-amanitin in concentrations < 100 μg/ml did not bring about a quick interruption of heterogeneous nuclear RNA synthesis. As we demonstrate here, only this particular concentration (100 μg/ml) fully inhibited GVBD in cumulus-enclosed rabbit oocytes. In the sheep and the cow respectively, only 71% and 62% of cumulus-enclosed oocytes remained in the GV stage in the presence of 10 μg/ml of α-amanitin throughout culture (Osborn & Moor, 1983; Hunter & Moor, 1987). This inhibitory effect was substantially reduced by delaying the addition of α-amanitin. While 1 and 2 h of preincubation without inhibitor permitted GVBD in 60% and 83% of ovine oocytes respectively (Osborn & Moor, 1983), 10 and 75% of rabbit oocytes attained late diakinesis with the same scheme (the present results). Again, this discrepancy could be explained by using different concentrations of the inhibitor. Probably 10 μg/ml of α-amanitin was unable to effect a sufficiently rapid inhibition of hnRNA synthesis.

The inhibitory effect of α-amanitin in all examined species (the sheep, the cow and the rabbit) was reversible. Moreover, 70%

of denuded sheep oocytes (Osborn & Moor, 1983) and 90% of rabbit oocytes (present results) showed normal late diakinesis or metaphase I in α-amanitin-supplemented media. All these data argue against the possibility that the inhibition of maturation by α-amanitin is due to a secondary effect. Furthermore, the present data support a previous conclusion by Osborn & Moor (1983) that an early transcriptional event is required for the resumption of meiosis in mammalian oocytes.In the present study, we unequivocally demonstrate that this necessary transcriptional event occurs within the cumulus cells.When in some cases (not reported in the *Results*) only corona cells were left around oocytes, GVBD occurred in the presence of α-amanitin. This shows that the drug does not act on the oocytes even in a case where there should be no doubt about its penetration (see Osborn & Moor, 1983), but that an intact cumulus is necessary for inhibition to occur.

The evidence obtained in the present study strongly supports the view that the translation of newly synthesized mRNAs results in the synthesis of proteins which may participate in the resumption of meiosis (Moor & Crosby, 1986). Indeed, GVBD was absolutely and reversibly inhibited by cycloheximide in cumulus-enclosed rabbit oocytes. This block was evident even after 2 h of preincubation in the control medium. However, the addition of the inhibitor 2.5 and 3 h after explantation allowed 65 and 89% oocytes to undergo GVBD, respectively. These results elucidate the effect of RNA and protein synthesis on the timing of meiosis resumption in cumulus-enclosed rabbit oocytes. In fact, an α-amanitin-susceptible period is followed by a cycloheximide-sensitive period for the resumption of meiosis. Both events are localized in the cumulus cells, since cycloheximide did not significantly influence maturation rate in rabbit denuded oocytes, although this drug is able to act on cumulus-free oocytes (at least in other species; see *Introduction*). It should also be stressed that the resumption of meiosis in LH-stimulated explanted antral rat follicles requires an active protein synthesis (Lindner *et al.*, 1974).

Rabbit cumulus-enclosed oocytes mature effectively *in vitro* in a suspension of homologous granulosa cells (Motlik & Fulka, 1981). Co-culture of the granulosa cells with denuded rabbit oocytes in α-amanitin or cycloheximide-supplemented media did not substitute the intact cumulus. This implies that the maintenance of oocyte-cumulus cell contact is necessary for the inhibitory action of α-amanitin and cycloheximide on the resumption of meiosis in the rabbit. The contact is secured by numerous gap junctions between the granulosa cells, the corona radiata cells and the oocyte (Albertini & Anderson, 1974; Szöllösi *et al.*, 1978). Intrafollicularly, these junctions form a kind of functional syncitium which is a morphological prerequisite for the maintenance of the oocytes in dictyate stage in Graafian follicles before LH surge (Moor *et al.*,1980). Since the mitotic index of granula cells is < 1%, fully grown mammalian oocytes are supplied with "interphase factors" described in somatic cells (Rao & Adlakha, 1985). These interphase-specific substances might be a negative signal passing from granulosa cells to the oocyte and preventing activation of maturation-promoting factor (MPF) (Adlakha *et al.*, 1983; Fulka Jr. *et al.*, 1985). The present results obtained under *in vitro* conditions strongly suggest that metabolic changes induced in parietal and cumulus granulosa cells, either *in vivo* by LH or *in vitro* by explantation are key events for the resumption of meiosis in mammals and initiation of a metabolic pathway leading to MPF activation in the

cytoplasm of oocytes (Kubelka *et al.*, 1988).

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