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Original article

Quantitative inhibitory influence of porcine cumulus cells upon the maturation of pig and cattle oocytes *in vitro*

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Summary — Porcine cumulus oocyte complexes (COCs) were cultured together in 10-µl droplets of culture medium. When 10 COCs were cultured for 24 h, germinal vesicle breakdown (GVBD) occurred in 81% of them. When more COCs (20 or 40) were put into the same volume of medium the frequency of GVBD gradually decreased. This inhibition was not observed in denuded oocytes. The process of GVBD was adversely influenced when 10 COCs were cultured in cumulus-preconditioned medium. It is concluded that porcine cumulus cells produced a factor inhibiting GVBD. After removing the inhibitory block and extensive washing, GVBD of arrested oocytes was significantly accelerated. The addition of LH or heparin only partially overcame the inhibitory action. This factor produced by porcine cumulus cells negatively influenced maturation of bovine oocytes; however, a similar effect was not demonstrated in the mouse.

Our results suggest that a high concentration of porcine cumulus cells exerts a quantitative inhibitory effect upon GVBD of porcine and cattle oocytes cultured *in vitro*.

oocyte — GVBD inhibition — cumulus cells — pig

Résumé — Influence inhibitrice quantitative des cellules du cumulus oophorus porcines sur la maturation *in vitro* d'ovocytes de truie et de vache. *Des complexes ovocytes-cumuli (COC) porcins sont cultivés dans une goutte de milieu de culture de 10 µl. Quand 10 COC sont cultivés ensemble pendant 24 h, on observe la rupture de la vésicule germinative (RVG) dans 81% des ovocytes. Quand plus de COC (20 à 40) sont placés dans le même volume de milieu, la fréquence de la RVG diminue graduellement. Cette inhibition n'est pas observée dans des ovocytes dénudés (sans cumulus). Le processus de RVG est inhibé quand 10 COC sont cultivés dans du milieu préconditionné par des cumuli. La conclusion de ces travaux est que les cellules du cumulus produiraient un facteur inhibiteur de la RVG. Si l'on élève l'inhibition et si l'on procède à un lavage extensif, la RVG d'ovocytes bloqués est accélérée significativement. L'addition de LH ou d'héparine ne lève l'inhibition que partiellement. Le facteur inhibiteur produit par les cellules du cumulus porcin agit de la même manière sur la maturation d'ovocytes bovins, mais non sur les ovocytes murins.*

Les résultats obtenus suggèrent qu'une grande concentration de cellules du cumulus porcin exerce un effet inhibiteur quantitatif sur la RVG d'ovocytes de truie et de vache cultivés in vitro.

ovocyte — rupture de vésicule germinative — inhibition — cellules du cumulus — porc

INTRODUCTION

In vivo fully-grown oocytes resume meiosis after the LH surge. However, when competent oocytes with or without cumulus cells are cultured outside their follicle they resume meiosis spontaneously. This observation indicates that follicular cells are responsible for meiotic arrest (for review, see Thibault *et al.*, 1987; Sato & Koide, 1987). This idea originated in the study of Foote & Thibault (1969), who grafted cumulus oocyte complexes (COCs) *in vitro* on the granulosa of immature follicles. Meiosis did not resume spontaneously under these conditions. This demonstrate that granulosa cells are responsible for the inhibition of meiosis in follicle-enclosed COCs. Many studies based on co-culture of oocytes with various follicular components were subsequently performed and further corroborated the concept of follicular inhibitory action (Tsafriri & Channing, 1975; Dekel & Beers, 1978, 1980; Liebfried & First, 1980a, b; Dekel *et al.*, 1981; Downs & Eppig, 1984).

Similarly, numerous attempts have been made to isolate the inhibitor and determine its chemical nature. Up to date, several potential inhibitors have been described : cyclic adenosine monophosphate (cAMP), oocyte maturation inhibitor (OMI), granulosa cell factor (GCF) and lately, purine nucleotides (Sato & Koide, 1987).

It is not known whether the cumulus cells are also the source of the oocyte maturation inhibitor(s). Since COCs are usually cultured *in vitro* in low COC : medium volume ratio, the potential inhibitor may not inhibit oocyte maturation due to its low concentration. The purpose of this study was to determine whether this potential inhibitor from porcine cumulus cells could exert an effect in a high COC : medium volume ratio.

MATERIAL AND METHODS

Pig oocytes were aspirated from follicles of ≈ 3—5 mm in diameter. Cattle oocytes were collected from follicles of ≈ 5 mm in diameter. Only those oocytes with compact cumuli were chosen for further experiments. Mouse oocytes were isolated from late antral follicles of sexually mature females (Balb/c) and only those containing a germinal vesicle (GV) were used.

Oocytes with compact cumuli were washed three times in medium and then cultured in 10-µl droplets of medium under paraffin oil at 38 °C under air with 5% CO_2. Pig oocytes were cultured for 24 h and those of cattle and mice for 8 or 4 h, respectively. Preliminary studies showed that after these intervals GVBD occurred under our culture conditions. The composition of the medium was the same as described by Fulka Jr *et al.* (1986). Denuded oocytes were prepared by repeated pippetting of cumulus-enclosed oocytes through a narrow pipette and cultured in the same way.

When used, LH (bLH 13 VO5u-BIO-USDA, National Hormone and Pituitary Program, University of Maryland, USA was diluted in culture medium at a concentration of 5 µg/ml. Heparin (Spofa, Czechoslovakia) was used when oocytes were cultured for 24 h in 10 µl droplets of medium containing 30, 150 or 300 IU of heparin per ml, respectively.

At the end of culture oocytes were mounted on slides, fixed in acetic alcohol (1 : 3, v/v) for at least 24 h, stained with 1% orcein and examined under the phase contrast microscope.

The percentage of inhibition of oocytes was calculated according to the formula described by Sato and Koide (1984) :

% inhibition = (% oocyte GVBD (control) – % oocyte GVBD (expt.)) / % oocyte GVBD (control).

Results were compared with the Chi-square analysis.

RESULTS

To test the effect of cumulus cells on resumption of meiosis, different numbers of

both cumulus-enclosed and denuded porcine oocytes were cultured in 10-µl droplets of culture medium (Table I). When 1, 5, or 10 porcine COCs were cultured, germinal vesicle breakdown (GVBD) was observed in ≈ 80% of them after 24-h culture. However, a significant inhibition of oocyte maturation was demonstrated when 20 or 40 COCs were cultured in the same volume of medium. In the former group GVBD was observed in 61% of oocytes and the latter in 20%. This inhibition was not observed in denuded oocytes. The maturation of denuded oocytes was not inhibited even when 10 denuded oocytes were cocultured with 40 COCs in the same 10µl droplet for 24 h. These results indicated that porcine cumulus cells can quantitatively inhibit the resumption of oocyte maturation, and that their effect is mediated through the cumulus cells.

To test the reversibility of this cumulus cell inhibitory effect, 40 COCs were cultured for 24 h in a 10-µl droplet; the COCs were then washed and divided into 5 groups (Fig. 1). Some COCs were immediately fixed and evaluated. In this group GVDB did not exceed 20%. The remaining groups were cultured separately in 10-µl droplets of fresh medium for 4, 6, 12 or 24 hrs. GVDB was observed in 20, 68, 76 or 85% of oocytes, respectively. Control oocytes, cultured in the same manner immediately after their aspiration from follicles underwent GVBD in 6, 2, 7 or 84%, respectively. This indicates that maturation in oocytes precultured under the influence of cumulus cells was significantly increased and accelerated. Indeed, 24 h after washing, 47% of precultured oocytes were observed in the second metaphase (M II).

LH was used to overcome the effect of eventual follicular inhibitors (Tsafriri, 1988). When 40 porcine COCs were cultured in LH-enriched medium for 24 h the occurrence of GVBD was significantly higher than in those cultured without LH (47% vs 23% of GVBD) (Fig. 2). Significantly more (92.9 %) control oocytes resumed maturation.

Similarly heparin was used to inhibit the effect of granulosa cell factor (GCF) (Sato *et al.*, 1986). Forty porcine COCs were cultured in a droplet of medium containing 30, 150 or 300 IU of heparin per ml for 24 h (Fig. 3). GVBD was observed in 12, 25 or

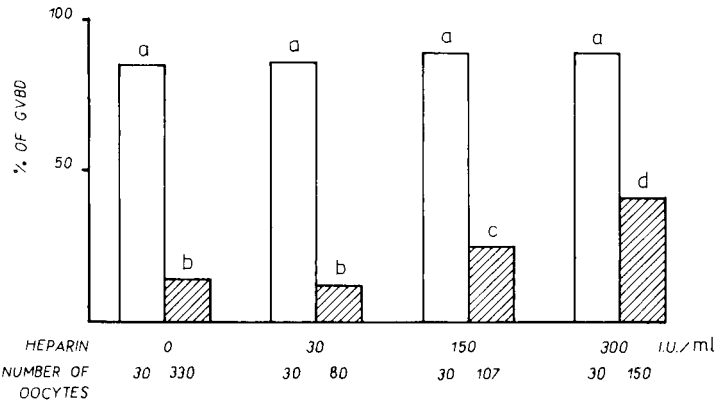

Fig. 1. The reversibility of the inhibitory activity of cumulus cells. The oocytes were either precultured for 24 h under the influence of the factor produced by cumulus cells (hatched columns) or cultured immediately after their aspiration from follicles (open columns). The black column represents oocytes observed in metaphase II. Statistical differences ($P < 0.01$) are indicated by different superscripts.

Table I. The influence of cumulus cells on pig oocyte maturation.

No. of oocytes per droplet	*Type of oocytes*[a]	*No. of oocytes*	*Stage of maturation*			*% of GVBD*	*% of inhibition*
			GV	*GVBD*	*Undefined*[b]		
1	C+	60	7	50	3	87[d]	0
5	C+	60	8	48	4	85[d]	2
10	C+	179	31	140	8	81[d]	7
20	C+	105	38	60	7	61[e]	29
40	C+	396	304	76	16	20[f]	77
10	C–	30	1	27	2	96[d]	0
40	C–	120	16	100	4	86[d]	1
10[c]	C–	67	17	49	1	74[d]	14
40[c]	C+	200	134	62	4	32[g]	64

[a] Oocytes were cultured with compact cumull (C+) or cumulus cells were removed mechanically before culture (C–).

[b] Oocytes in which the stage of maturation could not be recognized.

[c] Oocytes C+ and C– were co-cultured together in the same droplet.

[d—g] Statistical differences ($P < 0.01$) are indicated by different superscripts.

41% of them, respectively. However, even at the highest concentration of heparin the percentage of GVBD was significantly lower than in control oocytes (41% *vs* 85 of GVBD).

To test whether the potential inhibitor from cumulus cells was released into the medium, 40 porcine COCs were cultured in a 10-µl droplet for 24 h. After this interval COCs were removed from the droplet and replaced by 10 freshly isolated porcine COCs (Table II). Only 11% of these COCs underwent GVBD 24 h later.

To assess whether the inhibitor was species-specific, 40 porcine COCs were cultured in 10-µl droplets for 24 h. The porcine COCs were then removed and replaced by 10 freshly isolated murine or bovine COCs and cultured for 4 or 8 h, respectively (Table II). Only 26% of bovine oocytes resumed maturation under these conditions. In control bovine oocytes a significantly higher number (69%) of GVBD was observed. However, the maturation of the murine oocytes was not inhibited significantly and GVBD occurred in 85% of

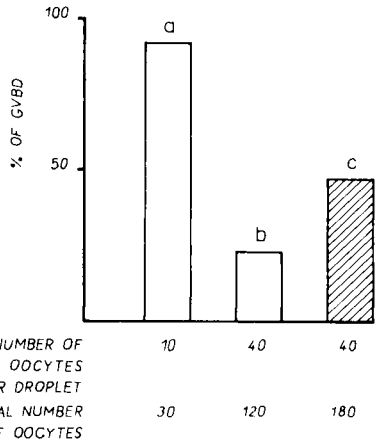

Fig. 2. The influence of LH upon the inhibitory activity of cumulus cells. COCs were cultured with (hatched columns) or without (open columns) 5 μg of LH per ml. Statistical differences ($P < 0.01$) are indicated by different superscripts.

them, compared to 92% of GVBD in controls.

Based on these results it can be concluded that porcine cumulus cells release a factor able to quantitatively inhibit GVBD in porcine oocytes. The effect of this factor was fully reversible and could be partially overcome by LH of heparin.

It could exert its effect on bovine but not murine oocytes

DISCUSSION

The finding that oocytes removed from the follicle mature spontaneously in culture, whereas maturation of follicle-enclosed oocytes occurs only after hormonal stimulation, suggested that, within the follicle, meiosis is prevented through some follicular inhibitory action. It has been found that granulosa cells in contact with cultured oocytes suppress spontanous maturation (Tsafriri & Channing, 1975; Eppig & Downs, 1984; Anderson *et al.*, 1985). These studies indicated that granulosa cells produce factors that sustain meiotic arrest.

Table II. The species specificity of inhibitory activity from cumulus cells.

Species	*Time of culture*	*Type of culture* [a]	*No. of oocytes*	*Stage of maturation*			*% of GVBD*	*% of inhibition*
				GV	*GVBD*	*Undefined* [b]		
Pig	24	control	40	4	34	2	89[c]	0
		X	79	65	8	6	11[d]	88
Cattle	8	control	42	13	29	0	69[c]	0
		X	61	45	16	0	26[d]	62
Mouse	4	control	72	6	66	0	92	0
		X	60	9	51	0	85	8

[a] Ten bovine and murine oocytes were cultured in 10 μl of fresh medium (control) or in 10 μl fo medium where 40 procine COCs were cultured previously for 24 h (X), respectively.

[b] Oocytes in which the stage of maturation could not be recognized.

[c, d] Statistical differences within species ($P < 0.01$) are indicated by different superscripts.

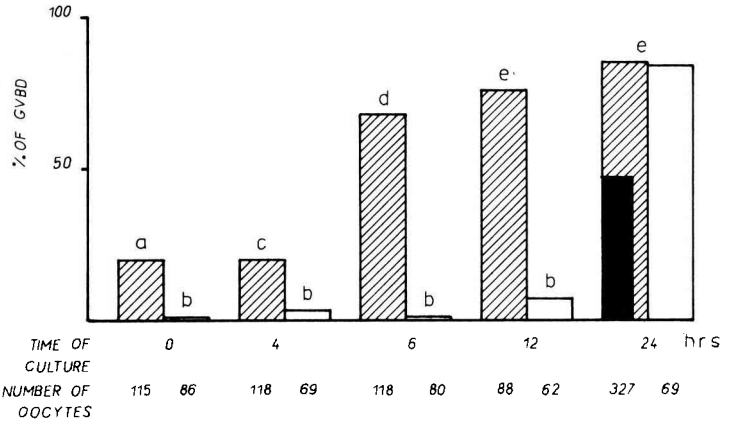

Fig. 3. The influence of heparin upon the inhibitory activity of cumulus cells. Ten (open columns) or 40 (hatched columns) COCs were cultured per droplet. Statistical differences ($P < 0.025$) are indicated by different superscripts.

We report that pig oocytes do not resume meiosis spontaneously when COC : medium volume ratio is increased by culturing more than 10 COCs in a 10-μl droplet of medium. One possible explanation is that the pH of culture medium could influence maturation. Sato & Koide (1987) demonstrated in porcine oocytes that at pH 6.8—7.0 the time required to progress to the second metaphase was delayed by several hours compared to pH 7.2—7.4. However, such pH decrease was not observed in our culture conditions, so that other factor(s) must be involved. The maturation of denuded oocytes was not inhibited by an increasing oocyte : medium volume ratio, even when they were cultured in an environment where COC : medium volume ratio was high enough to inhibit resumption of meiosis in intact oocytes. It is therefore unlikely that exhaustion of some nutritional components in the medium caused the inhibition. These data support the idea that porcine cumulus cells produce at least one factor inhibiting oocyte maturation. They further suggest that this factor exerts its inhibitory action not directly on oocytes, but through the mediation of cumulus cells. This agrees with the observation that cumulus cells are required for the inhibitory action of oocyte maturation inhibitor (OMI) in pig (Hillensjö *et al.*, 1979) and rat oocytes (Tsafriri & Bar-Ami, 1982). In contrast, an inhibitory fraction of human follicular fluid prevented maturation of denuded rat oocytes (Chari *et al.*, 1983), and the low molecular weight fraction of porcine follicular fluid inhibited the maturation of mouse cumulus-free ova (Downs & Eppig, 1984).

The inhibition of GVDB was reversible. After thorough washing in the medium, the subsequent culture in lower COC : medium volume ratio led to the GVBD after 6 h. This means that oocytes released from the influence of cumulus cell factor completed GVBD much faster that oocytes freshly isolated from the follicle (Motlík and Fulka, 1976; and the present result). This acceler-

ation could not be due to the delayed onset of GVBD occuring under the influence of the cumulus cell factor, because GVBD was not observed and GVs remained intact, even in 40 COCs cultured for 48 h in a 10-µl droplet (data not shown). It therefore appears that the inhibitory factor from porcine cumulus cells did not prevent all events involved in GVBD and only represents a part of the inhibitory influence that is exerted on the oocyte by the somatic components of the follicle. In this connection, a similar acceleration of maturation was observed after the washing away of cycloheximide, a protein synthesis inhibitor, in sheep (Moor & Crosby, 1986) and pig oocytes (Kubelka *et al.*, 1988).

LH induces resumption of meiosis *in vivo* or within the follicle-enclosed oocytes *in vitro*. In addition, LH has been reported to alleviate the inhibitory action of porcine follicular fluid or of its purified fractions (Tsafriri & Channing, 1975), of bovine or hamster follicular fluid (Gwatkin & Andersen, 1976), of rat granulosa cell conditioned medium, as well as that of cocultured with rat granulosa cells (Tsafriri *et al.*, 1977). Hence the ability of LH to partly overcome the inhibition of meiosis by a cumulus cell factor described in the present study lends support to the view that this factor may have a physiological role in the regulation of meiosis.

LH is known to stimulate the preovulatory follicle to produce glycosaminoglycans which accumulate on the surface and in the intercellular spaces of the cumulus cells (Eppig, 1979). Sato *et al.* (1986) have demonstrated that among the glycosaminoglycans, heparin and heparan sulfate interact with the GCF and nullify its maturation inhibitory activity. Based on their experimental results, Sato & Koide (1987) have proposed the hypothesis that glycosaminoglycans can prevent the action of GCF on the oocyte by binding the factor. In this manner oocytes are supposed to be protected from the arresting influence of the maturation inhibitory factor and to resume meiosis. In the present study it is shown that heparin at least partly blocked the activity of cumulus cell factor. It is thus possible that glycosaminoglycans could be involved in the blocking of cumulus cell factor during gonadotropin-induced resumption of maturation.

It is generally accepted that the inhibitory effect of OMI is not species-specific. Thus human follicular fluid inhibits the maturation of pig (Hillensjö *et al.*, 1978), rat (Hillensjö *et al.*, 1981) and *Xeponus* oocytes (Pomerantz & Bilello, 1987); bovine follicular fluid inhibits hamster oocyte maturation (Gwatkin and Andersen, 1976). Under our conditions the inhibitory factor from porcine cumulus cells does not inhibit maturation in mouse oocytes but it is able to exert its effect on cattle oocytes.

The quantitative inhibiton and its incomplete reversibility by LH or heparin may be perhaps related to the nonhomogeneity of the oocyte population aspirated from ovaries (Homa *et al.*, 1988), the impurity of substances, or to culture conditions employed. Another explanation for quantitative inhibition is the interaction of inhibitory factor(s) and maturation-inducing factor(s). Such factors produced by granulosa cells were proposed by Byskov (1979) and detected by use of bioassay in mouse (Byskov, 1974), hamster (Fayer *et al.*, 1979) and human (Yding Andersen *et al.*, 1981). Recently, substances promoting cytoplasmic development of pig follicular oocytes were demonstrated in porcine follicular fluid (Naito *et al.*, 1988) and pig follicular tissue was reported to produce similar factors (Mattiolli *et al.*, 1988a, b).

Our data strongly suggest that pig cumulus cells produce factor(s) quantitatively inhibiting oocyte maturation, but its biochemical nature has yet to be determined.

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