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Original article

Inhibition of meiotic maturation in growing pig oocytes by factor(s) from cumulus cells

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Summary — Total inhibition of germinal vesicle breakdown (GVBD) was observed in growing pig oocytes (internal diameter 80, 90 and 100 μm) when they were cultured in a medium conditioned by cumulus oocyte complexes (COCs) of fully grown oocytes. In denuded growing oocytes, only partial inhibition was observed. The inhibitory effect was fully reversible. The addition of heparin (300 IU/ml) could overcome the effect of the conditioned medium. Transient exposure (6 h) of oocytes to dibutyryl cyclic adenosine monophosphate (dbcAMP) (1 mg/ml) could also partly reverse the effect of factor (s) produced by cumulus cells of fully grown oocytes. Follicle-stimulating hormone (5 μg/ml) was able to increase the percentage of maturing oocytes. The addition of luteinizing hormone (5 μg/ml) had no effect on GVBD inhibition by cumulus-conditioned medium.

growing oocyte / maturation / cumulus cells / pig

Résumé — Inhibitition de la maturation méiotique dans les ovocytes en croissance par des facteurs des cellules du cumulus. *La rupture de la vésicule germinative est inhibée dans des ovocytes en croissance de porc (diamètre interne : 80, 90 et 100 μM) cultivés dans un milieu conditionné avec des complexes cumulus/ovocytes après croissance. Dans les ovocytes en croissance dénudés, c'est seulement une inhibition partielle qui se produit. L'inhibition est complètement réversible : l'addition d'héparine (300 UI/ml) peut renverser l'effet du milieu conditionné. Une exposition transitoire (6 h) des ovocytes à 1 mg/ml de dibutyryl AMPc (dibutyryl cyclic adénosine monophosphate) est aussi capable de renverser partiellement l'effet des facteurs produits par les cellules du cumulus d'ovocytes après croissance. L'hormone folliculo-stimulante (5 μg/ml) augmente le taux de maturation des ovocytes. L'addition d'hormone lutéinisante (5 μg/ml) n'a pas d'effet sur l'inhibition de la rupture de la vésicule germinative par le milieu conditionné.*

ovocytes en croissance / maturation / cellules du cumulus / porc

INTRODUCTION

Fully grown nonatretic mammalian oocytes resume meiotic maturation spontaneously after their removal from the follicle. In contrast to this, growing oocytes have limited ability to undergo resumption of meiosis and complete their maturation (see *eg* Thibault *et al,* 1987; Wassarman, 1988, for review). Pig oocytes acquire the competence to resume meiosis *in vitro* when their diameter is about 110 μm, but only those with diameters about 115 μm are able to complete the first meiotic division *in vitro* (Motlík *et al,* 1984).

The germinal vesicle breakdown (GVBD) in fully grown, meiotically competent, follicular oocytes is induced by a preovulatory gonadotropin surge *in vivo* (Thibault *et al,* 1987; Wassarman, 1988). Meiotic maturation can be induced by gonadotropins even in oocytes, which acquired meiotic competence during *in vitro* culture under the inhibitory influence of exogenous hypoxanthine or granulosa cells (Eppig and Downs, 1987; Eppig, 1991).

It is thought that cyclic adenosine monophosphate (cAMP) plays an important role in gonadotropin-induced maturation. On the other hand cAMP is known to be a potent inhibitor of meiosis. Dekel *et al* (1988) have suggested that in the rat cAMP has a dual role in the regulation of oocyte maturation. Lower levels of this nucleotide maintain meiotic arrest, while elevated levels of cAMP mediate LH (luteinizing hormone) action to induce meiosis resumption. A similar dissociation between the inhibitory and the stimulatory action of cAMP on maturation of oocytes was observed in rabbits (Hosoi *et al,* 1989).

LH is also known to stimulate the preovulatory follicle to produce glycosaminoglycans, which accumulate on the surface and in the intracellular spaces of the cumulus cells (Eppig, 1979). Sato *et al* (1986) have demonstrated that of all the glycosaminoglycans, heparin interacts with meiosis inhibiting factor from granulosa cells and is able to nullify its maturation-inhibiting activity.

In our previous studies, we have demonstrated the presence of a factor produced by cumulus cells from fully grown porcine oocytes, which inhibits GVBD in fully grown pig and cattle oocytes (Petr *et al,* 1989). This factor is released into the culture medium and its inhibitory effect can be overcome by the addition of LH, heparin or by the transient exposure of oocytes to dbcAMP (Petr *et al,* 1991).

The aim of the present study is to investigate the effect of meiosis inhibitory factor from cumulus cells of fully grown porcine oocytes on growing pig oocytes with respect to their different abilities to resume meiotic competence. Further experiments were undertaken to clarify the meiotic maturation of growing oocytes under the effect of inhibitory factor interacting with various agents.

MATERIALS AND METHODS

Porcine ovaries were obtained from a local slaughterhouse and were transported to the laboratory in a saline solution at 39°C.

Fully grown oocytes were aspirated from follicles that were 2–5 mm is diameter. Growing oocytes were obtained from thin strips dissected from the surfaces of ovaries using a surgical blade. The strips were transferred to Petri dishes containing a Tyrode lactate (TALP) medium for the work in air (Bavister *et al,* 1983). Growing oocytes were liberated under a dissection stereomicroscope from their follicles by rupture of the follicular wall using the tip of a 25 gauge needle. Only oocytes surrounded by several layers of granulosa cells were used for further studies. The internal diameter of oocytes (without zona pellucida) placed in culture dishes was measured with an ocular micrometer mounted on a binocular magnifier. Oocytes were divided into 4 groups according to their internal diameter: fully

grown oocytes with an internal diameter of ≈ 120 μm; and growing oocytes with an internal diamete of ≈ 80, 90 or 100 μm. Only oocytes with compact cumuli were used.

The oocytes were handled in a box maintained at 39°C. Before culture oocytes were washed 3 times in a culture medium: E 199 medium (USOL Praha, Czech Republic) supplemented with 0.039 ml 7.5% solution of sodium bicarbonate per ml medium, calcium lactate (0.6 mg per ml medium), sodium pyruvate (0.2 mg/ml), gentamycin (0.025 mg/ml), HEPES (1.5 mg/ml) and with 10% bovine serum (ZD Hustopeče, Czech Republic).

To produce medium conditioned from porcine cumulus cell cultures, fully grown oocyte–cumulus complexes (40 COC per 10 μl) were cultured in droplets of culture medium for 24 h. After this interval oocytes were removed from the droplet and checked for their maturation status. Eighty-one percent of oocytes remained at GV stage.

Growing pig oocytes were isolated from their follicles and divided into 3 groups according to their internal diameter (80 μm, 90 μm and 100 μm). The oocytes were placed in droplets of conditioned medium and cultured for 48 h. Control growing oocytes were cultured in the same volume of unconditioned medium.

The effect of heparin was tested upon the inhibitory action of the conditioned medium: growing oocytes within 1 μl of medium containing 3 300 IU/ml of heparin (Spofa, Czechoslovakia) were introduced into the 10 μl droplet of conditioned medium to bring the final heparin concentration to 300 IU/ml. Control oocytes were introduced into the droplet of conditioned medium within 1 μl of media in the absence of heparin.

Similarly, the effects of LH and follicle-stimulating hormone (FSH) (both hormones from National Hormone and Pituitary Program, University of Maryland, USA) were determined. LH and FSH were used at the final concentration of 5 μg/ml in the conditioned medium. Control oocytes were introduced into the conditioned medium within 1 μl of medium without LH or FSH.

The effect of transient exposure of growing oocyte to dbcAMP (Serva, Heidelberg, Germany) on the inhibitory action of conditioned medium was analysed as follows: growing oocytes were placed in a droplet of conditioned medium with 1 μl medium containing 10 mg dbcAMP per ml; the final concentration of dbcAMP in the droplet was 1 mg/ml. Previous studies revealed that this concentration was the most effective. After 6 h, growing oocytes were removed, carefully washed and placed in a new droplet of conditioned medium where they were cultured for 42 h.

For culture all oocytes were placed in the droplets of indicated medium, covered with parafin oil (PhBS CSL 4, Spofa Praha, Czech Republic) and cultured for an appropriate time interval at 39°C, under an atmosphere of 5% CO_2 in air.

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

Statistical analysis

Each experiment was performed 4 times. The results were pooled for presentation, and a χ^2-analysis was used as a test of significance between groups. The mean percentage of GVBD for the 4 trials did not vary from the pooled percentage by more than 2.4%. Moreover, relative differences between groups within an experiment were the same in all trials.

RESULTS

When 40 fully grown COCs were cultured in 10 μl droplets, 16% resume meiosis, whereas 87% meiotic resumption was observed when only 10 COCs were cultured in the same volume. However, when 10 COCs were cultured in 10 μl conditioned medium, only 20% resumed meiosis; these experiments show an inhibitory effect of the conditioned medium. On the contrary, cumulus-free competent oocytes resume meiosis when cultured either with 40 COCs or in conditioned medium (GVBD, 72 or 78%, respectively). The slightly reduced percentage of GVBD in denuded oocytes could be due to the certain damage during the removal of cumulus cells.

Oocytes with an internal diameter of 80, 90 or 100 μm were used in further studies. In standard medium the percentage of

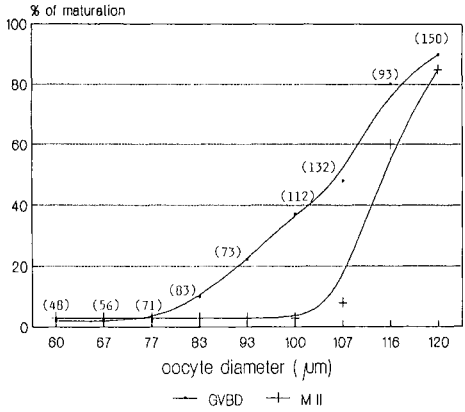

Fig 1. Nuclear status of growing pig oocytes after 48 h culture. The percentage of GVBD is indicated by solid circles; the percentage of oocytes reaching the metaphase II is indicated by crosses. Numbers in parentheses indicate the number of examined oocytes.

GVBD depends on oocyte size (fig 1). Whatever the size, conditioned medium always inhibits meiotic resumption. This effect is reversible (table I).

When growing oocytes with an internal diameter of 80, 90 or 100 μm were cultured for 48 h in conditioned medium, washed, and subsequently cultured for another 48 h in fresh medium, GVBD occurred in 20, 33 or 53%, respectively. The GVBD percentage after reversal was significantly higher than that obtained in controls in all the size categories of oocytes (table I). The percentage of growing oocytes which reached the stage of metaphase II after the reversal remained unchanged.

Various substances were tested for their ability to overcome the inhibitory effect of medium conditioned with fully grown COCs (table II).

The inhibitory effect was observed to be fully reversible by the addition of heparin (300 IU/ml). In the presence of heparin in the conditioned medium, GVBD occurrence was the same as controls cultured in unconditioned medium. Transient exposure to dbcAMP (1 000 μg/ml) for 6 h, which should mimic the stimulatory effect of gonadotropins on COCs, induces a significant increase in GVBD in oocytes with diameter of 90 and 100 μm (GVBD 11 or 18%, respectively) but not in the smallest category of oocytes with diameter of 80 μm (GVBD 0%). How-

Table I. The inhibitory effect of medium conditioned by fully grown pig COCs in growing pig oocytes and its reversibility.

Type of culture	*Internal oocyte diameter (μm)*					
	80		*90*		*100*	
	% GVBD	*n*	*% GVBD*	*n*	*% GVBD*	*n*
Control medium (48 h)	10[a]	81	24[a]	64	36[a]	73
Conditioned medium (48 h)	0[b]	63	0[b]	72	0[b]	79
Conditioned medium (48 h) + control medium (48 h)	20[c]	53	33[c]	56	53[c]	61

Statistically significant differences ($P < 0.02$) within the group of oocytes of the same diameter are indicated by different superscripts. The number of examined oocytes is represented as n.

Table II. The effect of gonadotropins, heparin and transient exposure to dbcAMP on the inhibitory effect of medium conditioned by fully grown pig COCs.

Type of culture	*Internal oocyte diameter (μm)*					
	80		*90*		*100*	
	% GVBD	*n*	*% GVBD*	*n*	*% GVBD*	*n*
Control medium (48 h)	9[a]	66	24[a]	71	34[a]	64
Conditioned medium (48 h)	0[b]	81	0[b]	92	0[b]	53
Conditioned medium with LH (5 μg/ml) (48 h)	0[b]	56	2[b]	72	3[b]	78
Conditioned medium with FSH (5 μg/ml) (48 h)	8[a]	73	10[c]	75	18[c]	72
Conditioned medium with heparin (300 IU/ml)	14[a]	48	24[ba]	80	33[a]	65
Conditioned medium with dbcAMP (1 mg/ml) (6 h) + conditioned medium (42 h)	0[b]	70	11[c]	61	18[c]	66

Statistically significant differences ($P < 0.02$) within the group of oocytes of the same diameter are indicated by different superscripts. The number of examined oocytes is represented as n.

ever, GVBD induction by transient exposure to dbcAMP did not attain the level of GVBD in controls. The addition of LH has no influence on the inhibitory effect of cumulus cells. GVBD was observed in 0,2 and 3% of oocytes with a diameter 80, 90 and 100 μm, respectively. However, the addition of FSH-induced maturation in all size categories of oocytes (8, 10 and 18%, respectively).

Maturation in denuded growing oocytes was similar to controls. However, when these oocytes were cultured for 48 h in conditioned medium, the percentage of GVBD was significantly decreased, but not to the level observed in oocytes surrounded by somatic cells (table III).

The portion of oocytes which degenerate during the culture was very low in each trial and did not exceed 3% in any experiment.

DISCUSSION

As we described previously (Petr *et al,* 1989; Petr *et al,* 1991) cumulus cells from fully grown pig oocytes produce factor(s) which are able to inhibit GVBD in fully grown pig and cattle oocytes. The preliminary studies revealed that the inhibitory factor(s) could be clearly attributed to cumulus cells because in these studies neither denuded oocytes nor fibroblasts (Vero cells) at an

Table III. The influence of cumulus cells connected with growing pig oocytes on their maturation in the medium conditioned by fully grown COCs.

Type of culture	*Internal oocyte diameter (μm)*					
	80		*90*		*100*	
	% GVBD	*n*	*% GVBD*	*n*	*% GVBD*	*n*
Oocytes with their somatic cells, control medium	9[a]	56	24[a]	98	37[a]	79
Denuded oocytes, control medium	8[a]	82	19[a]	64	35[a]	56
Oocytes with their somatic cells, conditioned medium	0[b]	62	0[b]	43	0[b]	51
Denuded oocytes, conditioned medium	5[ab]	59	10[c]	48	22[c]	57

Statistically significant differences ($P < 0.02$) within the group of oocytes of the same diameter are indicated by different superscripts. The number of examined oocytes is represented as n.

appropriate concentration were unable to inhibit GVBD. This factor is released in the culture medium, acts on oocytes through their cumulus cells and its effect is reversible by the addition of heparin, LH or by the transient exposure of COCs to dbcAMP (Petr *et al,* 1989; Petr *et al,* 1991).

In this study we have demonstrated that the factor from cumulus cells is able to completely inhibit GVBD in growing pig oocytes. As on fully grown oocytes, the inhibitory effect is reversible. Moreover, after reversal, growing oocytes resumed meiosis at a significantly higher rate than in control oocytes. Since fully grown pig oocytes significantly accelerate their maturation during the reversal (Petr *et al,* 1989), it is thought that the inhibitory factor from pig cumulus cells does not inhibit all processes involved in GVBD. Some of these processes may continue even under the inhibitory effect of cumulus cells without performing any change in germinal vesicle morphology. This is probably also the case in growing oocytes where it can cause increased GVBD percentage during the reversal. Another possibility is that the medium conditioned with fully grown porcine COCs contains, besides meiosis inhibitory factor(s), substances which can improve meiotic competence in growing oocytes.

Contrary to the situation described in fully grown pig oocytes (Petr *et al,* 1989) and to the results published by Hillensjö *et al* (1979) and Tsafriri and Bar-Ami (1982), the inhibitory effect of cumulus cell conditioned medium in growing oocytes is partially performed even when they were denuded from their granulosa cells. This should indicate a pathway of inhibitory factor(s) other than through processes of granulosa cells in contact with the oocyte periphery through the zona pellucida.

Heparin, which was described to bind and inactivate a granulosa cell factor purified from bovine granulosa cells (Sato *et al,*

1986), is able to completely overcome the inhibitory action of factor(s) from cumulus cells. We can suggest that heparin also binds this inhibitory factor and inactivates it.

On the other hand, the addition of gonadotropins and the transient exposure of oocytes to dbcAMP acts at the level of somatic cells surrounding the oocyte (Dekel and Sherizly, 1983; Dekel *et al,* 1988). It is suggested that there are FSH but not LH receptors on granulosa cells surrounding growing oocytes (Richards and Midgley, 1976; Oxberry *et al,* 1982) and the appearance of LH receptors is correlated with the prolonged effect of FSH during follicle and oocyte growth (Nimrod *et al,* 1977; Erickson *et al,* 1979; Sanders and Midgley, 1982; Eppig, 1991). This result is in agreement with our observation that LH had very limited effect on growing pig oocytes in which GVBD was inhibited by medium conditioned by cumulus cells. Without the presence of LH receptors, this hormone cannot induce an increase in cAMP levels in COCs and subsequently breakdown of cytoplasmic processed anchored on the oocyte through the zona pellucida. On the other hand, FSH, whose specific effect on cumulus cells has been shown on mouse (Eppig, 1979), rabbit and cow (Thibault, 1972), was able to induce maturation in growing pig oocytes that were cultured in conditioned medium. The percentage of GVBD was comparable with that after dbcAMP transient exposure and is almost the same as in denuded oocytes cultured in conditioned medium. This result supports the suggestion that after the addition of FSH or transient exposure to dbcAMP but not after LH addition, the level of cAMP in granulosa–oocyte complexes increases and causes the interuption of connections between growing oocyte and somatic cells. This decreases the input of inhibitory factor to the growing oocyte. However, a certain amount of the factor is still able to enter them through an alternative pathway, *eg* by simple diffusion. This decreased influx of inhibitory factor is able to lower GVBD percentage in growing pig oocytes significantly.

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