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Original article

Influence of cytoplasmic microinjection on meiotic competence in growing pig oocytes

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Summary — It is demonstrated that the microinjection of cytoplasm from competent, fully grown oocytes can induce germinal vesicle breakdown in meiotically incompetent growing oocytes. Injection of cytoplasm from oocytes at metaphase I had a similar effect. The maturation in injected growing oocytes was arrested at the premetaphase or metaphase I stage. Induction of germinal vesicle breakdown by cytoplasm microinjection occurs in a dose-dependent manner.

oocytes / meiotic competence / cytoplasm / microinjection / pig

Résumé — Influence de la micro-injection de cytoplasme sur la compétence méiotique des ovocytes en croissance. *La micro-injection de cytoplasme d'ovocytes obtenus après la phase de croissance peut induire la rupture de la vésicule germinative dans des ovocytes injectés pendant la phase de croissance. L'injection de cytoplasme d'ovocytes en métaphase I a le même effet. La reprise de la méiose dans les ovocytes en croissance injectés s'arrête au stade prémétaphase ou métaphase I. L'induction de la rupture de la vésicule dépend de la quantité de cytoplasme injecté.*

ovocytes / compétence méiotique / cytoplasme / micro-injection / porc

INTRODUCTION

Mammalian fully grown oocytes isolated from antral follicles are able to complete meiosis in a suitable *in vitro* environment (see for example Thibault *et al,* 1987; Wassarman, 1988, for review). In contrast, growing oocytes remain mostly arrested at the prophase of first meiotic division and their ability to resume meiosis *in vitro* increases with their size during the period of growth (Motlík *et al,* 1984; Motlík, 1989).

When mature mouse oocytes and growing mouse oocytes were cultured, the former resumed meiosis while the latter remained unchanged at the germinal vesicle (GV) stage. But when growing oocytes were fused with mature oocytes which had reached the germinal vesicle breakdown (GVBD) stage, immature oocytes resumed meiosis shortly after fusion up to metaphase I (Balakier, 1978). These experiments proved that maturing oocytes, like amphibian oocytes (Masui and Markert, 1971), contain cytoplasmic activity known as maturation promoting factor (MPF) which induces GVBD. Cell fusion in mammalian oocytes and microinjection in amphibian oocytes was largely used to test this activity.

Cell fusion revealed that growing oocytes which are not able to mature sponta-

neously possess an inhibiting activity preventing GVBD in fully grown oocytes in their cytoplasm. After cultivation of giant cells composed of one growing and one fully grown oocyte in GV for 24 h, both GVs remained well conserved (Fulka, 1985; Fulka *et al,* 1985). These observations suggest that, like the cytoplasm of somatic cells in G_1 phase, the cytoplasm of growing oocytes strongly perturbs the processes leading to the synthesis of MPF or else block this activity (Adlakha and Rao, 1987). However, it was observed (Fulka, unpublished results) that after prolongation of the culture interval up to 72 h, a double GVBD occurred and 2 sets of chromosomes condensed in the common cytoplasm.

After oocyte fusion a large volume of cytoplasm is introduced into the recipient cell, and so the aim of this preliminary study was to ascertain whether a lower volume of cytoplasm from fully grown premature or maturing oocytes induces nuclear changes in growing oocytes which are typical for the presence of MPF. For this purpose a microinjection technique was used and recipient oocytes were cultured for up to 6 d. GVBD was used as a test for the presence of MPF.

MATERIALS AND METHODS

Collection of oocytes

Porcine ovaries were obtained from a local slaughterhouse. Ovaries were transported to the laboratory in a saline solution at 37°C. Processing of oocytes was completed within 3 h. Fully grown oocytes were collected by puncturing follicles that were 2–5 mm in diameter with a 20-gauge needle.

Growing oocytes were obtained from thin strips (1 mm width, 10–15 mm length) dissected from the surfaces of the ovaries using a surgical blade. The strips were placed in petri dishes containing modified Tyrode-lactate (TALP) medium for use in an air atmosphere (Bavister *et al,* 1983). Growing oocytes were liberated from their follicles by opening the follicular wall using the tip of a 25-gauge needle. Oocytes were washed twice with TALP and their diameters were measured with an ocular micrometer mounted on a binocular magnifier.

Microinjection and culture of oocytes

Microinjection was carried out at room temperature on an inverted phase-contrast microscope (IMT–2, Olympus, Japan) fitted with 2 mechanical micromanipulators (MN–151, Narishige, Japan) and a microinjector (IM–5, Narishige, Japan).

The micropipettes were prepared with a Narishige micro-puller (PB–7), a Narishige microforge (MF–9) and a Narishige micro-grinder (EG–4). The injection micropipettes had a tip diameter of about 7 μm in order to avoid blocking the micropipette by the large lipid vesicles that are abundant in pig oocyte cytoplasm.

Oocytes were placed in droplets of TALP medium covered with paraffin oil. Freshly isolated, fully grown pig oocytes, which had internal diameter (excluding the zona pellucida) of about 120 μm, were used as cytoplasm donors. Growing pig oocytes surrounded by granulosa cells (internal diameter about 75 μm) were used as recipients. Each recipient was injected with about 10, 30, or 50 pl donor cytoplasm. A control injection was performed using the cytoplasm of growing oocytes or culture medium.

After the procedure, the injected oocytes were cultured in the medium described by Fulka *et al* (1986) in paraffin oil at 37°C, under air with 5% CO_2 for 1, 2, 3, 4, 5 and 6 d after injection, respectively. Control growing and fully grown GV oocytes were exposed to the same culture conditions.

The nuclear changes of each category of growing oocytes were checked at the beginning of culture and every 24 h thereafter up to 7 d. In fully grown oocytes the cultivation was terminated at 24 h and the progression of maturation was controlled. For the examination of nuclear status the oocytes were checked before the culture and part of them 24 h later.

The internal diameter of oocytes (without zona pellucida) was measured every day during culture. At the same time, the integrity of complexes of oocytes with their surrounding cumulus cells was morphologically evaluated.

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

Statistical analysis

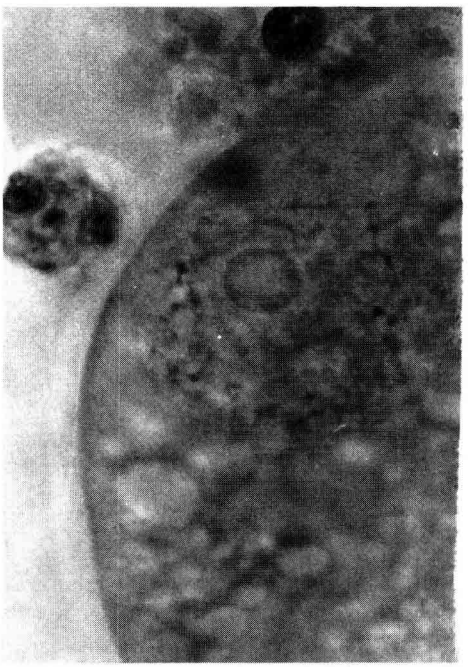
Statistical differences were determined by χ^2 analysis.

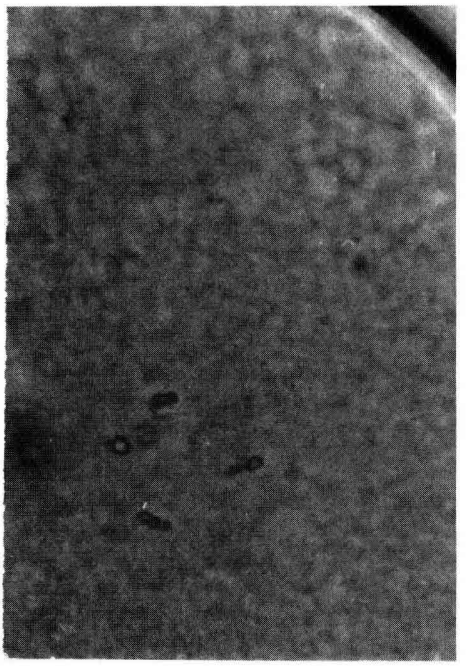
RESULTS

The nuclear status was checked in fully grown pig oocytes immediately after their isolation from the follicles. Almost all of these oocytes (61/63) were observed at the GV stage. Fully grown oocytes resumed meiosis in the culture. After a 24-h culture *in vitro* about 82% of oocytes (98/120) underwent GVBD and they were observed largely at metaphase I or late diakinesis.

Growing oocytes (internal diameter 75 μm) exhibited intact GV after their liberation from follicles (79/79). Growing oocytes cultured *in vitro* remained at the GV stage up to 7 d of culture (148/148).

Growing oocytes microinjected with 30 pl cytoplasm from fully grown GV oocytes remained at GV for 72 h after microinjection (fig 1, table I). However, when the culture was prolonged beyond this interval,





Figs 1–2. 1. Growing pig oocyte injected with 30 pl cytoplasm from fully grown GV oocyte and subsequently cultured for 3 d remained at the GV stage. Phase contrast; magnification 800 x. **2.** Growing pig oocyte injected with 30 pl cytoplasm from fully grown GV oocyte and cultured for 5 d underwent GVBD. Condensed chromosomes are dispersed in cytoplasm and some of them are out of focus. Phase contrast; magnification 800 x.

Table I. Oocyte maturation in pig oocytes after cytoplasm microinjection.

Days after microinjection	*Type of injected material*												*Not injected*			
	Cytoplasm from fully grown oocyte (30 pl)				*Cytoplasm from growing oocyte (30 pl)*				*Culture medium (30 pl)*							
	GV (%)	*GVBD (%)*	*Deg* [a] *(%)*	n	*GV (%)*	*GVBD (%)*	*Deg* [a] *(%)*	n	*GV (%)*	*GVBD (%)*	*Deg* [a] *(%)*	n	*GV (%)*	*GVBD (%)*	*Deg* [a] *(%)*	n
3	93	0	7	30	80	0	20	20	83	0	17	70	–	–	–	–
4	33	60	7	30	85	0	15	20	84	0	16	63	–	–	–	–
5	23	68	9	54	72	0	28	20	80	0	20	78	–	–	–	–
6	20	53	27	30	–	–	–	–	–	–	–	–	100	–	–	148

[a] Deg: degenerated.

some oocytes clearly underwent GVBD. These oocytes were observed at stages from late diakinesis to metaphase I; metaphase chromosomes were dispersed in the cytoplasm (fig 2). Even after further prolongation of culture, oocytes did not continue maturation beyond the metaphase I stage and an increased proportion degenerated. Controlled injection of growing oocytes with 30 pl cytoplasm from growing oocytes, or with culture medium had no comparable effect (statistical significance of difference $P < 0.01$). Ultrastructural studies using electron microscopy did not reveal any signs of degeneration in any experimental group of oocytes at the end of their culture.

Further experiments (table II) revealed that the effect of cytoplasm from fully grown GV oocytes to induce GVBD in meiotically incompetent growing oocytes was dose dependent, since an increased volume of injected cytoplasm induces a decreased percentage of GV remaining after injection.

Microinjection of cytoplasm from maturing (metaphase I, MI) oocytes was demonstrated to possess the same effect as the microinjection of cytoplasm from fully grown GV oocytes (table III). The effect was obtained after 3 d culture.

Table II. The effect of different doses of injected cytoplasm from fully grown oocytes at GV stage on the maturation of growing oocytes 5 d after injection.

Volume of injected cytoplasm (pl)	*GV (%)*	*GVBD (%)*	*Degenerated (%)*	n
10	34	47	19	52
30	23	68	9	54
50	4	71	25	48

Table III. Maturation of growing pig oocytes after their microinjection with 30 pl cytoplasm from fully grown metaphase I pig oocytes.

Days after microinjection	*GV (%)*	*GVBD (%)*	*Degenerated (%)*	n
1	100	0	0	24
2	100	0	0	30
3	44	54	2	46

DISCUSSION

In the present study, we have observed GVBD in meiotically incompetent pig oocytes after their microinjection with cytoplasm from fully grown homologous GV oocytes or MI oocytes and subsequent prolonged culture. The results are different from those demonstrated by Procházka *et al* (1989) in fully grown pig oocytes. In meiotically fully competent oocytes, premature chromosome condensation and accelerated GVBD can be induced after injection of 14% of maturing cytoplasm (Procházka *et al*, 1989). The microinjection of cytoplasm from immature oocytes has no effect (Procházka *et al*, 1989).

In our culture system, meiosis resumption in injected growing oocytes was not accompanied by an increase in the internal diameter of oocytes. Moreover, these oocytes lost their granulosa cells and most of them became naked within 48 h. The granulosa cells formed a monolayer and denuded oocytes remained on this during the culture. From this fact could arise the objection that the relatively long cultivation period and the absence of intact granulosa cells may cause the degeneration of oocytes. However, Bachvarova *et al* (1980) observed the growth and acquisition of meiotic competence in naked growing

mouse oocytes cultured on monolayers of ovarian cells even when these oocytes did not attain full size. On the other hand, it is stated that for normal physiological growth, the association with the somatic compartment plays a crucial role; only those mouse oocytes grown *in vitro*, which were surrounded with intact cumulus throughout the whole time of culture, acquired full developmental ability after fertilization (Eppig and Schröder, 1989; Eppig, 1991). Acquisition of meiotic competence was not found to be related to the completion of processes accompanying oocyte growth (Canipari *et al*, 1984). In mouse growing oocytes cultured *in vitro*, the meiotic resumption occurred before the oocytes reached their full size. Some evidence against the objection that the degeneration may occur in injected oocytes that have been cultured for a long time was provided by electron microscopic studies. We observed well-preserved cytoplasmic structures in microinjected and subsequently cultured pig growing oocytes (Rozinek and Petr, unpublished data). We can also eliminate the possibility that GVBD in injected oocytes is due to the dilution of meiosis-inhibiting activity demonstrated by Fulka (1985), because injection of culture medium did not induce meiosis resumption.

In comparison with the results published by Fulka (1985) and Fulka *et al* (1985), which demonstrated a block of maturation of fully grown GV mouse and pig oocytes after fusion with growing homologous oocytes, our results are rather unexpected. These authors concluded that the cytoplasm of growing oocytes inhibits the production of MPF at least for 24 h of culture. In giant cells, 2 GVs were clearly detected. Similar inhibition activity was also reported in extracts of somatic G_1 cells that promptly eliminated MPF in extracts from mitotic cells (Adlakha and Rao, 1987). In this respect growing G_1 oocytes resemble somatic cells. However, in the oocyte model, inhibiting activity may gradually disappear after prolongation of the culture interval. Giant cells, composed of 1 growing oocyte and 1 fully grown oocyte, being in GV stage I according to the classification of Motlík and Fulka (1976), possessed 2 sets of chromosomes 48 h after fusion (Fulka, unpublished data). In these cases, the cytoplasm of growing oocytes represents 30% and the cytoplasm of fully grown oocytes 70% of the total. In our present experiments, the injected cytoplasm of fully grown immature or maturing oocytes did not exceed an average of 10% of the growing oocyte volume. Motlík (1989) also reported similar volumes for the injections into growing oocytes of the same size. For the expression of cytoplasmic activity, the proportion between different types of cytoplasm seems to play an important role. Bulet *et al* (1985) were not able to induce GVBD in growing starfish oocytes after injection of a cytoplasm known to contain MPF while the use of fusion enabled the dilution of the inhibiting activity by the MPF active cytoplasm. The dose-dependent effect on the frequency of GVBD was also reported by Procházka *et al* (1989) who injected 7 or 14% of maturing cytoplasm into immature fully grown pig oocytes. In our experiments the dose effect was also clearly expressed.

Based on these results, it seems that during the interaction of cytoplasm from growing oocyte with cytoplasm from meiotically competent oocytes, meiosis-inhibiting activity can prevail for certain amount of time. However, this activity disappears after a long time and meiosis resumption occurs.

Nuclear changes recorded in growing oocytes cultured after microinjection with the cytoplasm from fully grown oocytes suggest that MPF occurs in the cytoplasm of injected oocyte. This effect is accelerated after the injection of maturing cyto-

plasm, probably due to the content of MPF in injected cytoplasm. As is generally accepted, the origin of this cellular activity is accompanied by complex biochemical events. Our results based on morphological observations permit only a demonstration that MPF activity is acquired in injected growing oocytes. The long delay between the injection and the expression of an effective level of MPF cannot be completely explained until biochemical analyses are available.

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