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Effects of follicle-stimulating hormone, bovine somototrophin and okadaic acid on cumulus expansion and nuclear maturation of Blue fox (*Alopex lagopus*) oocytes *in vitro*

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Summary

The meiotic competence and meiosis resumption of Blue fox (*Alopex lagopus*) oocytes from anoestrous animals were followed. Oocyte–cumulus complexes (OCC) were cultured in modified TC 199 medium with or without FSH, recombinant bovine somatotrophin (bST) and okadaic acid (OA). The results showed that oocytes less than 100 µm in diameter did not achieve germinal vesicle breakdown (GFBD) by 72 h of culture, which indicates their meiotic incompetence. Oocytes larger than 100 µm in diameter underwent GVBD after 48 h of culture (27%) and reached metaphase II (MII) after 72 and 96 h (20% and 27%) in control medium. Both bST and OA accelerated resumption of meiosis (bST: 55% GVBD and 42% MII after 48 h; OA: 66% GVBD after 18 h). In contrast, FSH significantly reduced meiosis resumption (only 3% GVBD and MII after 72 h) and induced changes in the shape of cumulus granulosa (CG) cells and F-actin assembly typical for cumulus expansion. However, the innermost layers of CG cells (corona radiata) remained connected with the oocyte via gap junctions until the end of culture. Cumuli of oocytes cultured in control, bST-supplemented or OA-supplemented medium did not expand (changes in cell shape and F-actin redistribution did not occur). Moreover, especially in media with bST and OA an increased detachment and rapid disconnection of their gap junctions with the oocyte were observed. These results suggest that under *in vitro* conditions FSH stimulates expansion of the CG cells and the attached membrana granulosa cells but in contrast it secures heterologous gap junctions between cytoplasmic processes of the corona radiata cells and oolemma during 3 days of culture. Thus, in agreement with the *in vivo* situation in which Canidae oocytes are ovulated in the GV stage, the cumulus, mainly corona radiata cells, controls resumption of meiosis in Blue fox oocytes under *in vitro* conditions also.

Keywords: Blue fox, bST, FSH, Okadaic acid, Oocyte maturation

Introduction

Meiosis in all mammalian species is triggered by the preovulatory LH surge but the timing of nuclear maturation differs substantially according to species. Germinal vesicle breakdown (GVBD) requires 2 h in mice (Donahue, 1968), 3–4 h in rabbits (Thibault, 1972; Motlík *et al.*, 1989), 7–8 h in ruminants (Moor & Trounson, 1977; Motlík *et al.*, 1978; Hyttel *et al.*, 1986) and about 20 h in human, primate and pig oocytes (Edwards, 1975; Shea *et al.*, 1975; Motlík & Fulka, 1976). Oocytes of fox and dog, however, remain in the germinal vesicle (GV) stage under 48 h after the peak of the LH surge (Farstad *et al.*, 1989) or hCG injection (Yamada *et al.*, 1992). These observations indicate that differences in the timing of nuclear maturation of mammalian oocytes are mainly caused by the time required for GVBD, but the underlying reasons for the species differences are not clear.

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Fully grown cumulus-enclosed oocytes resume meiosis spontaneously when removed from the ovarian follicle and placed in culture (Edwards, 1965; Pincus & Enzmann, 1935). However, co-culture of the oocyte–cumulus complex (OCC) with a small piece of membrane granulosa inhibits meiosis resumption of pig oocytes cultured in gonadotropin-free medium (Mattioli *et al.*, 1988; Motlík *et al.*, 1991). These observations suggest that the membrane granulosa plays an important role in maintaining the oocyte in meiotic arrest. The timing of nuclear maturation *in vitro* closely resembles that seen *in vivo* when the medium is supplemented with gonadotropins (Motlík & Fulka, 1976; Sirard *et al.*, 1989; Vanderhyden & Armstrong, 1989; Kastrop *et al.*, 1991). However, FSH alone causes a transient but significant delay in the resumption of meiosis of mouse (Schultz *et al.*, 1983) and bovine (Süss *et al.*, 1988) cumulus-enclosed oocytes. Taken together, these observations suggest that cumulus cells are not able to maintain meiotic arrest without the support of mural granulosa cells. Both cell subpopulations participate in the arrest of meiosis, within the follicle or under suitable culture conditions. The species differences in the timing of GVBD could depend on the ability of these two cell types to provide this function.

To better understand the role of cumulus granulosa cells in maintaining meiotic arrest and the resumption of meiosis, the Blue fox was used as a model animal. In nearly all mammalian species studied so far, meiosis progresses to metaphase II shortly before ovulation and the oocytes are ovulated and fertilised at this stage. Conversely, fresh ovulated dog, Blue fox and Silver fox oocytes are still in GV stage, surrounded with a compact corona radiata and expanded cumulus oophorus (Pearson & Enders, 1943; Phemister *et al.*, 1973; Tsutsui, 1975). During maturation in the oviduct for 48–72 h after ovulation there is a loss of the junctional contacts between cumulus and the oocyte (Holst & Phemister, 1971; Hyttel *et al.*, 1990).

Determination of the meiotic competence of the Blue fox oocyte is of primary interest, and the effects of okadaic acid (OA) were studied for this purpose. This potent inhibitor of protein phosphatases 1 and 2A (Biolojan & Takai, 1988; Haystead *et al.*, 1989) induces hyperphosphorylation and new phosphorylation of several proteins important for the entrance of both somatic (Haystead *et al.*, 1989) and germinal (Schwartz & Schultz, 1992) cells into M-phase. The ability of mouse oocytes to undergo GVBD in the presence of OA reflects their meiotic competence (Gavin *et al.*, 1994; Chesnel *et al.*, 1994).

To test the hypothesis that cumulus granulosa cells influence the resumption of meiosis, Blue fox oocytes were cultured in medium supplemented with porcine FSH, bovine somatotrophin (bST) or OA. Mechanical removal of cumulus cells without serious damage to the oocyte was found to be impossible, and bST offers an alternative, physiological approach to their removal. Furthermore, it has been suggested that bST can enhance luteinisation of cumulus cells (Jia *et al.*, 1986; Hsu & Hammond, 1987; Langhout *et al.*, 1991) and thereby quickly eliminate their influence on the oocyte. In addition to the effects mentioned above, OA causes rapid detachment of cumulus cells from the oocyte and accelerates meiosis in pig and cattle cumulus-enclosed oocytes (Kalous *et al.*, 1993*a*). The effects of OA, bST and FSH on the timing of nuclear maturation, ultrastructural morphology of oocyte and cumulus cells, and changes in cumulus cell microfilaments were also investigated.

Materials and methods

Source of oocytes and culture procedure

OCC were obtained in the Czech Republic during the pelting season in November and December, largely from prepubertal vixens. The ovaries were transported to the laboratory within 2 h of slaughter in Krebs–Ringer phosphate buffer supplemented with antibiotics, at 25 °C. After two washings, the ovaries were placed in Petri dishes with the Krebs–Ringer buffer under a stereomicroscope and dissected into several parts. The ovarian tissue was sliced to liberate the OCC. Only oocytes surrounded with compact cumulus and possessing a dark, homogeneous cytoplasm were used. The vitelline diameter was measured using a calibrated ocular micrometer, and the OCC divided in two groups: small (S) oocytes with a vitelline diameter < 100 µm and large (L) oocytes with diameter > 100 µm. The OCC were washed three times in culture medium composed of TCM 199 medium (Sevac, Prague, Czech Republic) supplemented with 2.92 mM calcium lactate, 2 mM sodium pyruvate (Serva, Heidelberg, Germany), 4.43 mM Hepes (Sigma, St Louis, MO), antibiotics and 10% (v/v) heat-inactivated bovine serum (Sevac, Prague, Czech Republic).

The OCC were cultured for up to 96 h under paraffin oil at 38 °C in a humidified atmosphere of 5% CO_2 in air in unsupplemented culture medium (controls) or in medium containing 1 µg/ml FSH (UCB-Bioproducts, Belgium), 2.5 mM OA (Moana BioProducts, Hawaii) or 10, 500 and 1000 ng/ml recombinant bST (Elanco, Indianapolis, IL) for between 18 and 72 h. After 24, 48, 72 and 96 h, cumulus expansion was subjectively evaluated and the OCC were prepared for either fluorescence microscopy of the microfilaments and gap junctions, or electron microscopy, or evaluation of nuclear maturation, as described below.

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Fluorescence labelling of microfilaments and gap junctions

To study the microfilaments and gap junctions, the OCC were fixed for 20 min in 2.5% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and processed according to the protocol used by Šutovský *et al.* (1993). The microfilaments were stained with rhodamine-conjugated phalloidin (Sigma, St Louis, MO) diluted 1:50. The gap junctions were labelled using a 1:10 dilution of a mouse monoclonal antibody against connexin 43 (Cx43, Zymed Lab., San Francisco, CA), followed by fluorescein isothyocyanate (FITC)-conjugated anti-mouse IgG (USOL, Prague, Czech Republic), diluted 1:40.

Electron microscopy

For electron microscopy, the OCC were fixed in a mixture of 2.5% glutaraldehyde and 0.6% paraformaldehyde in 0.2 M phosphate buffer pH 7.2 at 4 °C, post-fixed by 1% osmium tetroxide at room temperature and, after dehydration, embedded in Epon. Serial ultrathin sections of OCC stained by uranyl acetate and lead citrate were examined under a transmission electron microscope (JEOL 1200EX).

Evaluation of nuclear maturation

To evaluate nuclear maturation, the OCC were treated for 10 min with hyaluronidase (247 U/ml), and the remaining cumulus cells were then removed by pipetting through a narrow-bore pipette. The denuded oocytes were mounted on slides, fixed in an acetic acid:ethanol (1:3, v/v) mixture for 24 h, stained by aceto-orcein and examined under a phase-contrast microscope to evaluate nuclear maturation, according to the criteria described by Motlík & Fulka (1976).

Statistical analysis

The effects of FSH, bST and OA on the proportions of oocytes reaching various stages of nuclear maturation were evaluated by chi-squared comparisons with the proportions in the corresponding control treatment group.

Results

Morphology of OCC before and during *in vitro* culture

The freshly isolated L and S oocytes surrounded by compact cumulus were ultrastructurally similar to preovulatory follicular oocytes before the LH peak as described by Hyttel *et al.* (1990). The cumulus and corona radiata (CR) cells had an orthogonal shape with F-actin-containing microfilaments homogeneously distributed in the cytoplasm (Fig. 1*a*). They were interconnected by an extensive network of large longitudinal gap junctions (GJs) built by connexin 43 (Fig. 1*b*). The numerous cytoplasmic projections of CR cells traversed zona pellucida and were anchored by GJs in deeply invaginated oolemma (Fig. 2*d*). The oocytes possessed a spherical, centrally localised nucleus and vacuolised nucleolus (Fig. 3*a*). The cumulus granulosa (CG) cells did not expand during the culture in control medium; they only spread out stepwise around the oocyte on the bottom of the culture plate. The remaining CR cells were easily removed at the end of culture. According to the ultrastructural observations and immunofluorescence the cell shape and microfilament distribution did not change over time (Fig. 1*c*). The first indications of CR cell detachment (withdrawal of cell projections, gradual termination of heterologus GJs and perivitelline space creation) occurred after 24 h of culture (Fig. 2*c*), but up to 48 h the cells kept intimate contacts with the oocyte. The homologous cell contacts (contacts between CG cells), characterised by the immunofluorescence pattern of connexin 43, showed a reduction in size of GJs between CG cells, and a typical punctate pattern identical for all treatments was developed by 72 h of culture (Figs. 1*d*, *f*, *h*).

The cumuli of oocytes cultured in medium supplemented with FSH expanded. The expansion started from the outer CG cells as early as 24 h and was well expressed after 48 h of culture. The expansion pattern was very unusual. While the outer layers of CG and membrane granulosa cells expanded, the CR cells looked compact and tightly packed even after 72 h. The ultrastructural studies, however, documented changes in CR cells shape and creation of foot-like projections towards the zona pellucida (Fig. 2*a*) typical also for expanded cells of other mammalian species (Szöllösi & Gerard, 1983). Also actin localisation showed redistribution of microfilaments, and large bundles, similar to those observed in cattle, were assembled (Fig. 1*g*). Even though the number of zona-spanning projections was slightly reduced during culture, communication in the whole OCC was secured via homologous (Fig. 1*h*) and heterologous GJs (3 ± 1 GJ per ultrastructural section; Fig. 2*e*) still after 72 h.

The bST medium did not cause cumulus expansion. During 48 h of culture CG cells detached from the oocyte and spread out on the bottom of culture plate. The oocytes after 72 h of culture were surrounded by a monolayer of CG cells with only a few CR cells attached. As shown by fluorescence and ultrastructural observations the CG cells did not change their orthogonal shape (Fig. 2*b*) and the F-actin microfilaments stayed dispersed throughout the cytoplasm

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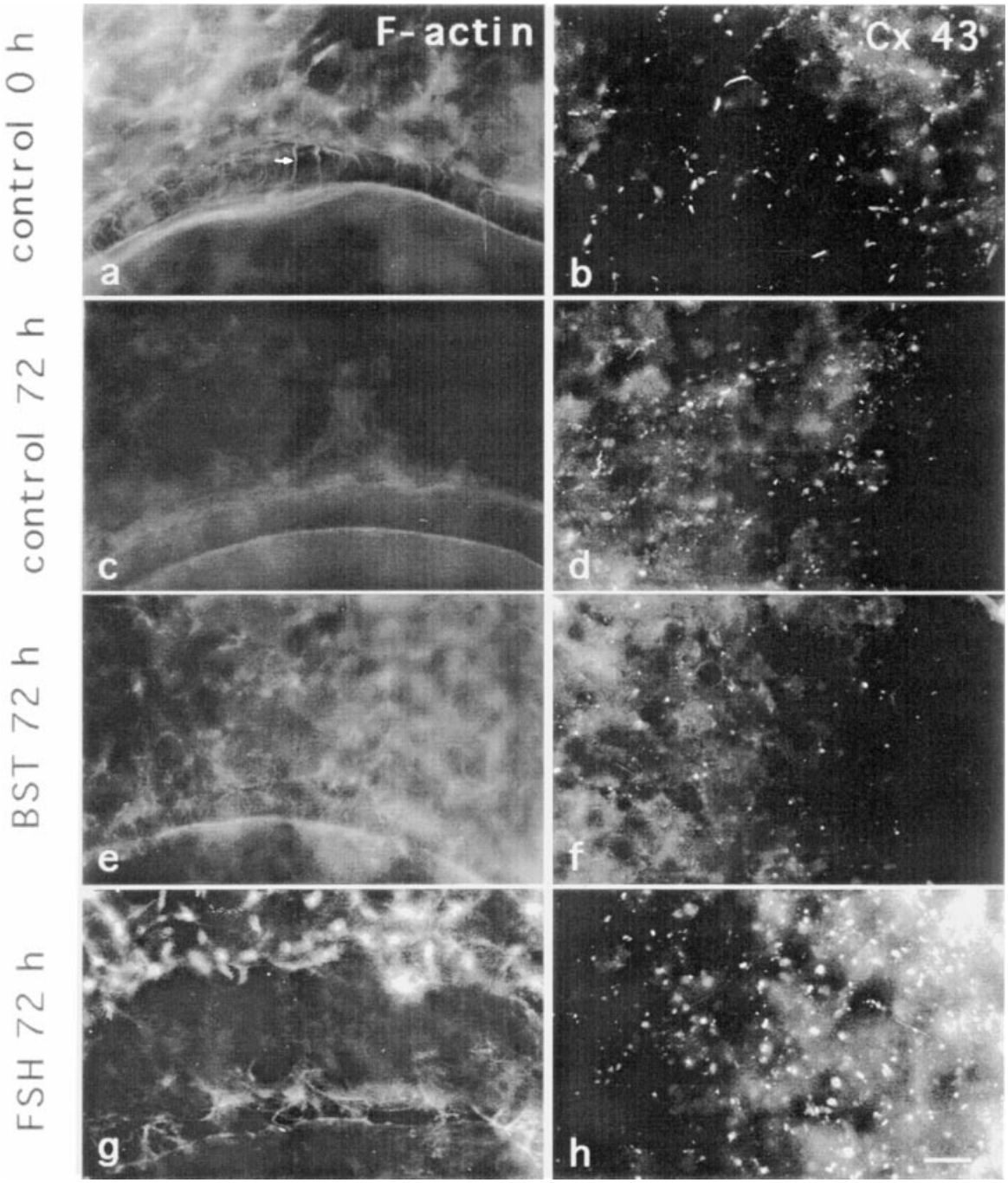

Figure 1 Arrangement of microfilaments (left-hand column) and distribution of cumulus cell gap junctions (right-hand column) in the Blue fox oocyte–cumulus cell complexes (OCC) cultured *in vitro*. The actin was labelled by a rhodamine-conjugated phalloidin (*a*, *c*, *e*, *g*) and gap junctions by anti-connexin 43 antibody (*b*, *d*, *f*, *h*) either uncultured (*a*, *b*), after 72 h of culture in hormone-free control medium (*c*, *d*), or after 72 h of culture in medium with bST (*e*, *f*) or FSH (*g*, *h*). Arrows point to the zona-spanning projections of corona radiata cells. Scale bar represents 5 µm.

(Fig. 1*e*). The number of zona-spanning projections was rapidly reduced during culture and cumulus cell-to-oocyte GJs were broken down. Some of CR cells, however, exhibited chromatin condensation (Fig. 2*b*).

OA also induced rapid chromatin condensation in CG cells. The cells did not spread out and were easily removable by pipetting. By the end of the culture period (20 h) the oocytes were naked or enclosed in one layer of dark CR cells.

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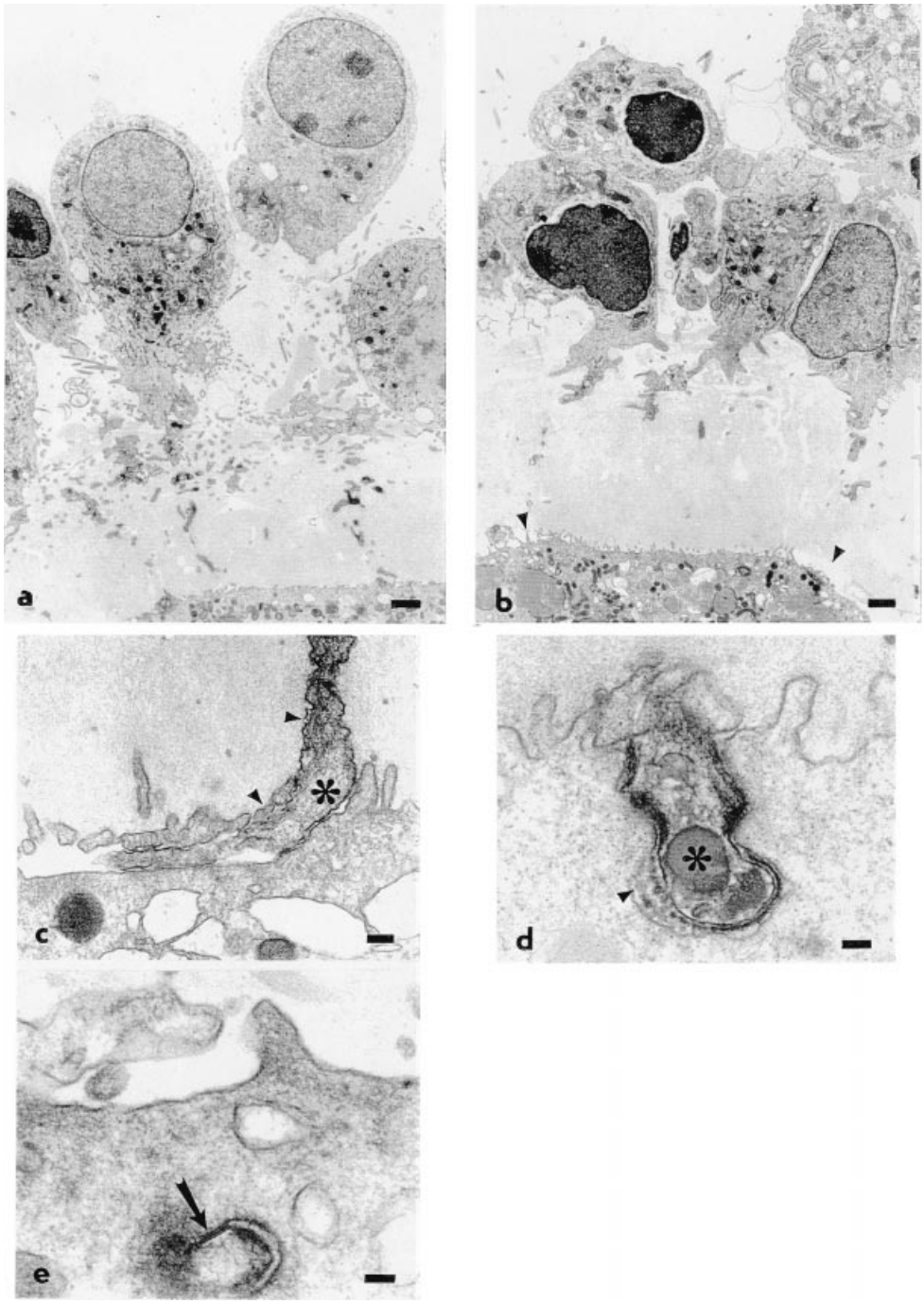

Figure 2 Ultrastructure of corona radiata cells and cumulus granulosa (CG) cells-to-oocyte gap junctions in the cultured Blue fox OCC. (*a*) Electron micrograph of corona radiata cells from OCC cultured for 72 h in medium supplemented with FSH. The cells have an elongated shape and possess numerous zona-spanning foot-like projections, typical of expanded cumuli, but they are still tightly packed around the oocyte. Scale bar represents 2 µm. (*b*) The cells from OCC cultured for 72 h in medium supplemented with bST have an unchanged orthogonal shape. The nuclei of some of them express signs of shrinkage and pyknotic degeneration. Note the fully developed perivitelline space (arrowheads). Scale bar represents 2 µm. (*c*) Corona radiata cell projection in the process of termination (asterisk) and perivitelline space development after 48 h of culture in control medium. Note the presence of F-actin microfilaments (arrowheads). Scale bar represents 250 nm. (*d*) Electron micrograph of the bulbous ending of a CG cell projection deeply anchored in ooplasm after 72 h of culture in FSH-supplemented medium. The terminal part of the projection contains a large secretory granule or lyosome (asterisk) and small secretory vesicles (arrowhead). Scale bar represents 50 nm. (*e*) Detail of corona radiata cell projection ending anchored in cytoplasm of an oocyte cultured for 72 h in FSH-supplemented medium. Arrow points to a small GJ at the connection of this projection with oolemma. Scale bar represents 50 nm.

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Table 1 The stage of nuclear maturation of Blue fox oocytes after incubation for 20 h in okadaic acid (OA) and for 48 h in control medium or in medium containing 1 µg FSH ml^{-1} or 10, 500 or 1000 ng bovine somatotrophin (bST) ml^{-1}

Treatment	No. of oocytes	Nuclear stage (%)			
		GV	D–MI	MII	Degenerated
Control	63	57.1	12.7	14.3	15.9
FSH	33	87.9[c]	0.0[a]	0.0[b]	12.1
bST 10	27	59.3	7.4	22.2	11.1
bST 500	20	40.0	15.0	25.0	20.0
bST 1000	31	22.6	12.9	41.9[d]	22.6
OA (20 h)	58	25.0	66.0[c]	0.0[b]	8.0

GV, germinal vesicle; D–MI, diakinesis to metaphase I; MII, metaphase II.
[a]$p<0.05$ compared with the control treatment.
[b]$p<0.025$ compared with the control treatment.
[c]$p<0.01$ compared with the control treatment.
[d]$p<0.005$ compared with the control treatment.

Table 2 The stage of nuclear maturation of Blue fox oocytes after incubation for 72 h in control medium or in medium containing 1 µg FSH ml^{-1} or 10, 500 or 1000 ng bovine somatotrophin (bST) ml^{-1}

Treatment	No. of oocytes	Nuclear stage (%)			
		GV	D–MI	MII	Degenerated
Control	89	51.7	12.4	20.2	15.7
FSH	39	74.4[b]	0.0[b]	2.6[c]	23.1
bST 10	36	25.0[c]	11.1	33.3	30.6
bST 500	24	29.2[a]	8.3	33.3	29.2
bST 1000	37	21.6[d]	13.5	40.5[b]	24.3

GV, germinal vesicle; D–MI, diakinesis to metaphase I; MII, metaphase II.
[a]$p<0.05$ compared with the control treatment.
[b]$p<0.025$ compared with the control treatment.
[c]$p<0.01$ compared with the control treatment.
[d]$p<0.005$ compared with the control treatment.

Oocyte meiotic competence and nuclear maturation *in vitro*

The S oocytes with a vitelline diameter of less than 100 µm cultured in control medium did not undergo GVBD even after 72 h of culture (data not shown). Only gradual movement of their nuclei towards the periphery and advanced stages of chromatin condensation were observed. In the majority of L oocytes (diameter > 100 µm) the nucleus moved towards the oocyte periphery during the first 24 h of culture. The results of oocyte cultures are summarised in Tables 1 and 2. In all 11% and 27% of oocytes underwent GVBD (Fig. 3*b*) after 24 and 48 h of culture respectively. Some oocytes (5%) reached metaphase I (MI; Fig. 3*c* and 14% of them metaphase II (MII; Fig. 3*d*) after 48 h. The oocytes that remained in GV stage were characterised by peripheral nuclei with a deeply undulated membrane and highly condensed chromatin. Prolonged culture (72 and 96 h) did not increase the rate of GVBD dramatically (33% vs 37%), but the number of oocytes in MII increased from 20% to 27% (Table 2). The morphology of MII oocytes did not differ from that of other mammalian species. The extruded first polar body was in close apposition to the barrel-shaped and longitudinally localised MII meiotic spindle.

Maturation of competent oocytes in media supplemented by FSH, bST and OA

S oocytes did not respond to any of the three treatments. The observations summarised in Tables 1 and 2 pertain to L oocytes only. Surprisingly, FSH significantly reduced GVBD of Blue fox oocytes in culture (Tables 1, 2). Almost all oocytes remained at GV stage after 48 and 72 h. Morphologically these oocytes had undergone nuclear movement towards the periphery, the nuclear membrane was deeply undulated and the chromatin expressed a high degree of condensation, but GVBD did not occur.

Treatment with bST accelerated nuclear maturation compared with the control medium (Table 1). The greatest response was noted at a concentration of 1000 ng/ml, when 55% of oocytes underwent GVBD and 42% reached MII after 48 h. These data are significantly ($p \leq 0.01$) different from those obtained in control medium. Further culture (72 h, Table 2) did not increase the rate of GVBD and MII oocytes. Morphologically the oocytes cultured in bST-supplemented medium did not differ from oocytes cultured in control medium. Similarly, chromatin condensation and meiotic spindle assembly did not show any abnormality.

Sixty-six per cent of oocytes in OA-supplemented culture medium underwent GVBD ($p \leq 0.01$) after only 20 h of culture (Table 1). However, the chromatin was abnormally condensed with orcein-positive clumps or isolated bivalents scattered in the cytoplasm. The meiotic spindles, if present, were irregularly assembled.

Discussion

Nuclear maturation

Our studies were performed on ovaries originating largely from prepubertal anoestrous Blue fox vixens. The results showed that oocytes with diameter up to 100 µm (type S) are meiotically incompetent, and unable

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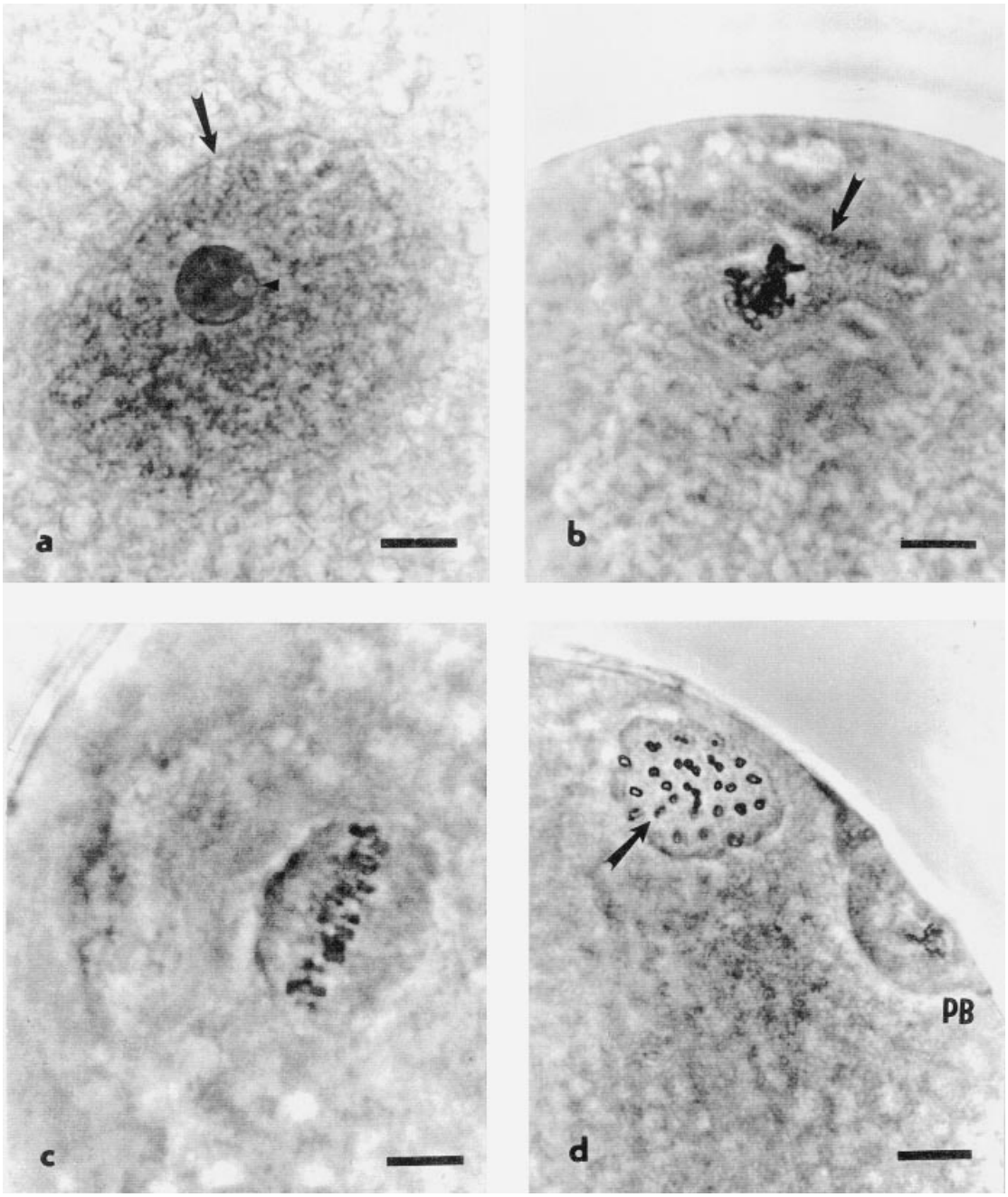

Figure 3 Stages of Blue fox oocyte nuclear maturation in control culture. The oocytes were fixed in ethanol:acetic acid and stained by aceto-orcein. (*a*) Oocyte at the start of the culture at germinal vesicle (GV) stage (the nuclear membrane is arrowed) with vacuolised nucleolus (arrowhead). Scale bar represents 10 µm. (*b*) Germinal vesicle breakdown (GVBD) stage after 48 h of culture. Note the deeply undulated nuclear membrane (arrow) and highly condensed chromatin. Scale bar represents 10 µm. (*c*) Oocyte in metaphase I (MI) stage with meiotic spindle formed after 48 h of culture. Scale bar represents 10 µm. (*d*) Oocyte that had reached metaphase II (MII) stage after 72 h of culture. The chromosomes are arranged on a meiotic plate (arrow). The first polar body (PB) is extruded. Scale bar represents 10 µm.

to resume meiosis in control or in OA-supplemented medium. Similarly, growing mouse oocytes with a diameter of less than 55 µm are insensitive to *in vitro* culture with or without OA supplementation (Gavin *et al.*, 1991; Chesnel *et al.*, 1994). FSH in the culture medium did not improve resumption of meiosis or stimulate cumulus expansion in S oocytes. This agrees with previous findings suggesting that meiotically incompetent oocytes do not produce enough cumulus expansion enabling factor (Vanderhyden *et al.*, 1990; Epigg *et al.*, 1993). The present experiments demonstrate that Blue fox oocytes do acquire meiotic competence

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when their diameter exceeds 100 µm. In control medium, meiosis resumed in a majority of oocytes after 24–48 h of culture with one-third reaching MII after 72–96 h of culture. A similar rate of GVBD and time sequence of nuclear maturation has been observed in dog oocytes originating from anoestrous animals (Mahi & Yanagimachi, 1978: Cinone *et al.*, 1992). After bitches were superovulated, there was a slight increase in the proportion of oocytes that resumed meiosis (Yamada *et al.*, 1992). In contrast, Blue fox oocytes isolated during proestrus had a substantially higher rates of meiotic resumption and maturation than oocytes isolated during anoestrus; however, the time sequence of nuclear maturation was similar for oocytes from anoestrus or proestrous vixens (Krogenes, 1993). One possible source of the difference in maturation potential between dog and fox might be that the oocytes in the present study were isolated from prepubertal vixens. However, in our experience maturation rates of oocytes from prepubertal and breeding vixens taken during the pelting season did not differ significantly (data not shown). While still prepubertal, vixens were about to cycle as were the breeding ones, with both groups in the same stage of the preparation for oestrus. The breeding season in young and mature animals differs by only 10–14 days.

The low maturation rates in Blue fox could also be caused partly by the quality of oocytes, as many expressed morphological abnormalities when examined by electron microscopy. It is also possible that a proportion of the oocyte population taken for these experiments was not fully competent. Even though the diameter of the oocytes was the same or little smaller than the ovulated ones (Hyttell *et al.*, 1990), none of the L oocytes examined by electron microscopy possessed a compact nucleolus, which is considered a definitive sign of meiotic competence in other species (Crozet *et al.*, 1981). On the other hand, an uncompact fibrillogranular nucleolus was found in oocytes from preovulatory Blue fox follicles during oestrus (Hytell *et al.*, 1990), suggesting that compaction of the oocyte nucleolus in Blue fox resembles that of human (Tesařik *et al.*, 1983) and takes place much later during follicular development. Despite the morphological observations, two-thirds of oocytes cultured in OA-supplemented medium did resume meiosis during the first 20 h of culture, confirming the meiotic competence of L oocytes. These observations, together with recently published data reporting that about two-thirds dog oocytes isolated from preantral follicles in anoestrus, resumed meiosis (Bolamba *et al.*, 1996), suggests that nucleolus compaction in Blue fox oocytes is not necessarily associated with meiotic competence.

Interestingly, a few Blue fox cumulus-enclosed oocytes resumed meiosis and matured in FSH-supplemented medium. Because in OCC only CG cells have been shown to possess FSH receptors and its mRNA (van Tol *et al.*, 1996), this meiotic block is suggested to be a result of the influence of FSH. Similarly FSH in the culture medium caused a significant but only transient delay in meiotic resumption of *in vitro* cultured murine (Schultz *et al.*, 1983) and bovine (Süss *et al.*, 1988) oocytes. The possible role of cumulus cells in Blue fox meiotic maturation is further discussed. Both OA and bST showed an accelerating effect consistent with results obtained in other mammalian species. OA has been found to accelerate meiotic resumption in pig and cattle cumulus-enclosed oocytes *in vitro* (Kalous *et al.*, 1993*a*). Presumably by overcoming an inhibitory effect of the cAMP-dependent protein kinase A or protein kinase C (Goris *et al.*, 1989; Rime & Ozon, 1990), OA induces activation of histone H1 kinase. The high rate of GVBD in Blue fox oocytes cultured in OA-supplemented medium could be explained by the activation of both main M-phase kinases (H1 kinase and MAP kinase) that independently could induce GVBD (Gavin *et al.*, 1994). While OA is proposed to have an influence on both CG cells and oocyte, the bST acts via CG cells (Izadyar *et al.*, 1996*b*). The acceleration of oocyte nuclear maturation has been observed in rat (Apa *et al.*, 1994), pig (Hagen & Graboski, 1990) and bovine (Izadyar *et al.*, 1996*a*) oocytes.

Expansion

Blue fox cumulus-enclosed oocytes, like many other mammalian oocytes explanted from the follicle and cultured *in vitro* without hormone supplements, mature spontaneously (Edwards, 1965; Pincus & Enzmann, 1935), i.e. cumulus cells themselves are not able to prevent meiotic resumption in such conditions and do not express any sign of expansion. Even a small piece of membrane granulosa attached to OCC is sufficient to inhibit meiosis resumption in pig oocytes (Mattioli *et al.*, 1988; Motlik *et al.*, 1991). The inhibiting influence of mural granulosa has been documented in both homologous co-cultures of membrane granulosa pieces or granulosa cells with sheep and bovine oocytes (Sirard & Bilodeau, 1990; DeSmedt & Szöllösi, 1991), and heterologous co-cultures of bovine oocytes with pieces of porcine mural granulosa (Kalous *et al.*, 1993*b*). In Blue fox, however, the inhibitory role of mural granulosa cells appears not to be preserved in gonadotropin-free medium. The oocytes cultured with an attached piece of membrane granulosa resumed meiosis in a similar manner to cumulus-enclosed oocytes (unpublished results).

In contrast to all other media used, the culture medium supplemented with FSH induces cumulus expansion (mucinification) of *in vitro* cultured Blue fox OCC, which is unexpectedly associated with arrest of meiotic progression in GV stage. The expansion event

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in other mammalian species parallels the meiotic maturation of oocytes and is characterised by elongation of CG cells, polarisation, cytoskeleton rearrangement, redistribution of GJs, secretion of hyaluronic-acid-enriched extracellular matrix and conversion of steroid metabolism to synthesis of progesterone (Šutovský *et al.*, 1993). These processes start in the outer cumulus layers, with the CR being affected last. Expansion is associated with withdrawal of cell projections from ooplasm accompanied by uncoupling of heterologous GJs (Hyttel, 1987). The pig CR remains unexpanded during the first 24 h either *in vivo* after hCG stimulation or *in vitro* after culture in 100 ng / ml of FSH. In Blue fox, however, the corona cells stayed closely attached to zona pellucida and appear unexpanded even at the end of the culture period. Such an unusual expansion pattern was reported when mouse OCC were cultured in medium with high doses of dbcAMP (Eppig *et al.*, 1982). Detailed immunocytochemical and ultrastructural observations of Blue fox *in vitro* cultured OCC revealed that the CR cells express the same signs of expansion as the cells in bovine gonadotropin-stimulated cumuli (Šutovský *et al.*, 1993). However, the GJs between corona cells and oocyte, although reduced in number, were present even after 72 h of culture, which sharply contrasts with the situation in Blue fox ovulated (Hyttel *et al.*, 1990) or bovine cultured OCC (Hyttel, 1987). The reduction but not complete uncoupling was documented also for mouse OCC treated with dbcAMP (Eppig & Ward-Bailey, 1982). In both cases the existence of contacts between the oocyte and CG cells results in an effective block of meiotic resumption.

A further impact of CG cells is the effect of their viability and differentiation on the time required for meiosis resumption documented by treatments with bST and OA. In both cases the cumuli did not expand as in control medium but CG cell detachment from the oocyte was faster. Absence of cumulus expansion in bST-supplemented medium showed that the reaction of CG cells to some medium additives could differ between species, because bovine OCC expanded under the influence of somatotropin (Izadyar *et al.*, 1996*b*). The mechanism of somatotropin action on CG cells is still controversial and could occur directly through growth hormone (GH) receptor or indirectly via stimulation of IGF-I gene expression and synthesis (Bevers *et al.*, 1997). A possible explanation of the bST effect on Blue fox CG cells is the enhancement of GH receptor expression on these cells, their final differentiation (luteinisation; Jia *et al.*, 1986; Hsu & Hammond, 1987; Langhout *et al.*, 1991) and functional isolation from the oocyte, which may also accelerate meiosis resumption. OA influenced CG cells similarly to other cultured mammalian cells and they rapidly lost cell–cell contacts and detached from the zona pellucida (Paulson *et al.*, 1994; Vandré & Wills, 1992).

The presence of cell–cell contacts seems to be an important prerequisite for meiosis resumption, allowing passage of signals between granulosa cells and the oocyte. Under culture conditions preserving the GJs between CR cells and the oocyte (FSH-supplemented medium) a rather small population of cumulus cells (about 10^3; Staigmiller & Moor, 1984) is able to prevent meiosis resumption. This is further supported by the *in vivo* observations that canine and Blue fox oocytes are ovulated in GV stage surrounded with expanded cumulus and closely attached CR cells (Holst & Phemister, 1971; Hyttel *et al.*, 1990). As shown in other animals the complete uncoupling of CR cells from the oocyte in Canidae is not a direct response to the endogenous gonadotropin surge (Hytell, 1987) *in vivo* or to FSH *in vitro*. Meiosis resumption and its time sequence are more a result of CG cell differentiation and signals produced during this process.

In summary, somatic follicle cells are essential both for metabolism and for control of the cell cycle in oocytes. The degree to which granulosa cells control resumption of meiosis in fully grown mammalian oocytes can differ among species. The present study suggests that cumulus granulosa cells participate in meiotic arrest, with their gonadotropin-influenced differentiation inducing a signal(s) controlling resumption of meiosis and the species-specific interval required for GVBD.

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