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The distribution and requirements of microtubules and microfilaments in bovine oocytes during *in vitro* maturation

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Summary

Microtubules and microfilaments are major cytoskeletal components and important modulators for chromosomal movement and cellular division in mammalian oocytes. In this study we observed microtubule and microfilament organisation in bovine oocytes by laser scanning confocal microscopy, and determined requirements of their assembly during *in vitro* maturation. After germinal vesicle breakdown, small microtubular asters were observed near the condensed chromatin. The asters appeared to elongate and encompass condensed chromatin particles. At the metaphase stage, microtubules were observed in the second meiotic spindle at the metaphase stage. The meiotic spindle was a symmetrical, barrel-shaped structure containing anastral broad poles, located peripherally and radially oriented. Treatment with nocodazole did not inhibit germinal vesicle breakdown. However, progression to metaphase failed to occur in oocytes treated with nocodazole. In contrast, microfilaments were observed as a relatively thick uniform area around the cell cortex and overlying chromatin following germinal vesicle breakdown. Treatment with cytochalasin B inhibited microfilament polymerisation but did not prevent either germinal vesicle breakdown or metaphase formation. However, movement of chromatin to the proper position was inhibited in oocytes treated with cytochalasin B. These results suggest that both microtubules and microfilaments are closely associated with reconstruction and proper positioning of chromatin during meiotic maturation in bovine oocytes.

Keywords: Bovine oocytes, Meiotic maturation, Microfilaments, Microtubules

Introduction

Microtubules, homologous polymers of α- and β-tubulin, are dynamic and intrinsically polar filaments. The cell cycle transition during meiotic maturation in mammalian oocytes appeared to be accompanied by extensive reorganisation of the microtubule network (Messinger & Albertini, 1991; Kubiac *et al*., 1992; Verlac *et al*., 1994). In mouse oocytes, two discrete populations of centrosomes were observed: one in the meiotic spindle pole and the other in the cytoplasm (Rime *et al*., 1987; Messinger & Albertini, 1991). During meiotic maturation, these two discrete populations of centrosomes coordinately regulate microtubule assembly for both nuclear and cytoplasmic events, such as germinal vesicle breakdown (GVBD), chromosome condensation, polar body extrusion and formation of metaphase structures. Although cytoplasmic microtubules are not well observed in most mammalian oocytes, treatment with taxol, a drug that promotes microtubule assembly, results in the formation of subcortical asters that nucleate microtubules in rabbit (Yllera-Ferandez *et al*., 1992), sheep (Le Guen & Crozet, 1989) and pig (Kim *et al*., 1996*a*).

Microfilaments are also major cytoskeletal components in mammalian oocytes and provide the framework for cell division. It is known that microfilaments are located mainly in the cell cortex overlying the meiotic spindle in mouse (Maro *et al*., 1985; Webb *et al*.,

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1986), pig (Kim *et al*., 1996*a, b*), rat (Albertini 1987; Zernicka-Goetz *et al*., 1993) and human oocytes (Kim *et al*., 1998). This domain rich in microfilaments seems to be responsible for the maintenance of the meiotic spindle, chromosomes in a peripheral position and for extrusion of polar body during maturation and fertilisation (Webb *et al*., 1986; Kim *et al*., 1996*a–c*). The microfilaments appeared to be involved also in pronuclear apposition during fertilisation in the mouse (Maro *et al*., 1984) and pig (Kim *et al*., 1997).

Recent advances in *in vitro* maturation (IVM) and fertilisation (IVF) systems for livestock animals have markedly increased the availability of embryos for the artificial manipulation of early embryo such as production of clone and transgenic animals. However, the viability of IVM/IVF-derived bovine embryos is still low in comparison with those of *in vivo* matured and fertilised oocytes. Previous reports showed several types of abnormalities of IVM/IVF-derived zygotes, such as polyspermy, polygyny, asynchronous pronuclear development and failure of pronuclear apposition (Xu & Greve, 1988; Chian *et al*., 1992). Unsuitable conditions during IVM/IVF possibly impair the function of cytoplasmic organelles, including microtubule and microfilament assembly, which results in abnormalities following fertilisation and early embryonic development (Hyttel *et al*., 19889; Kim *et al*., 1996*a, b*). Unlike in *in vivo*-derived oocytes, abnormal cytoskeletal assembly and incorrect positioning of metaphase chromatin were frequently observed in *in vitro* matured porcine and bovine oocytes (Long *et al*., 1993, 1994; Kim *et al*., 1996*a, b*). A higher incidence of these abnormalities is observed in incorrectly matured (Funahashi *et al*., 1996), aged (Webb *et al*., 1986; Kim *et al*., 1996*b*) or cryopreserved mammalian oocytes (Carroll *et al*., 1989; Pickering *et al*., 1990; Van der Elst *et al*., 1992).

Despite the importance of both microtubule and microfilament assembly during maturation, systematic examination has not been conducted on this subject in bovine oocytes. In this study we imaged microtubule and microfilament assembly in bovine oocytes during *in vitro* maturation. The effects of two cytoskeletal inhibitors, cytochalasin B and nocodazole, and a microtubule stabiliser, taxol, on their organisation and meiotic maturation were also examined to determine their interactive roles in the maturation processes.

Materials and methods

In vitro *maturation*

Bovine cumulus–oocyte complexes (COC) with uniform ooplasm and compact cumulus cells were prepared in HEPES-buffered Tyrode's albumin lactate pyruvate (TALP) medium containing 0.1% polyvinyl-alcohol. Culture medium for *in vitro* maturation was Tissue Culture Medium 199 (TCM199) supplemented with fetal bovine serum (10%, Gibco), 0.2 mM sodium pyruvate, 1 μg/ml FSH and 1 μg/ml oestradiol-17β. Five to ten COC were cultured in 500 μl of maturation medium at 39 °C in an atmosphere of 5% CO_2 in air. At 0, 6, 9, 12, 15, 18, 21 and 24 h of culture, oocytes were fixed for immunocytochemistry.

Drug treatments

Effects of nocodazole (a microtubule inhibitor), cytochalasin B (a microfilament inhibitor) and taxol (a microtubule stabiliser) on meiotic maturation and cytoskeletal alteration were examined during *in vitro* maturation. Stock solutions of 1 mM nocodazole (Sigma), 5 mM cytochalasin B (Sigma) and 1 mM taxol (Sigma) in dimethyl sulphoxide were used. The stock solutions were stored at –20 °C and diluted to 10 μM nocodazole and 5 μM cytochalasin B in TCM199 prior to treatment of oocytes. Oocytes were treated with the drugs between 0 to 12 and between 12 to 24 h of maturation.

Immunofluorescence microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques described by Kim *et al*. (1996*b*). Briefly, the eggs were permeabilised in a modified Buffer M (Simerly & Schatten, 1993) for 20 min at 39 °C, fixed in methanol at –20 °C for 10 min and stored in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 0.1% bovine serum albumin for 2 to 7 days at 4 °C. Microtubule localisation was performed using α-tubulin monoclonal antibody (Sigma, Sigma Chemicals, St Louis, MO). Fixed oocytes were incubated for 90 min at 39 °C with antibody diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton X-100 and 0.5% bovine serum albumin, oocytes were incubated in a blocking solution (Simerly & Schatten, 1993) at 39 °C for 1 h. The blocking was followed by incubation in fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody (Sigma). DNA was observed by exposure to 10 μg/ml propidium iodide (Sigma). To detect distribution of microfilaments, the oocytes were cultured in FITC-labelled phalloidin (1 μg/ml) for 1 h. Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientific Co., Huntsville, AL) to retard photobleaching. Slides were examined using laser-scanning confocal microscope (Bio-Rad MRC 1024). All images were recorded and archived on an erasable magnetic optical diskette and downloaded to a dye sublimation printer (Sony, Japan) using Adobe Photoshop Software (Adobe, Mountain View, CA).

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Statistical analyses

The data were pooled from at least four replications. Differences in the percentages of oocytes developing to a particular stage were determined by chi-square procedures.

Results

Microtubule organisation during meiotic maturation

Meiotic progression of bovine oocytes at 0, 6, 9, 12, 15, 18 and 21 h of maturation is shown in Table 1. Well-organised microtubules were not seen in the cytoplasm in GV stage oocytes (n = 14, Fig. 1*A*). After GVBD, small microtubule asters were produced near the condensed chromatin (2/8, 25% at 6 h; 2/2, 100% at 9h; data are not shown). The microtubule asters elongated, and were observed in association with each chromatin particle during pro-metaphase stage (n = 18, Fig. 1*B*). At metaphase I, the microtubules were observed around the spindle (n = 14, Fig. 1*C*). During anaphase I and telophase I (n = 3), microtubules were found as a midbody in the well-organised spindle (Fig. 1*D*). In oocytes at metaphase II stage (n = 15), microtubules were observed in the second meiotic spindle, and to a lesser degree in the polar body (Fig. 1*E*). The meiotic spindle was symmetrical, barrel-shaped, contained anastral broad poles and was located peripherally and radially. To observe cytoplasmic microtubules, some oocytes at 0, 12 and 24 h of maturation were incubated with 10 µM taxol for 10 min at 39 °C. Treatment with taxol induced a dense microtubule network in GV stage eggs (9/14, 64% at 0 h; Fig. 1*F*). At metaphase I and II, the numerous microtubule foci were observed in some taxol-treated metaphase I and II stage oocytes (Fig. 1*G*; 4/15, 27% at 12 h of maturation; 6/15, 40% at 24 h of maturation).

To determine requirements of microtubule assembly for maturation processes, the oocytes were treated with 10 µg/ml nocodazole between 0 and 12, and 12 and 24 h of maturation. Treatment with nocodazole did not inhibit GVBD (Fig. 3). However, progression to metaphase failed to occur in eggs treated with nocodazole (Figs. 3, 1*H*). When oocytes were cultured with nocodazole between 12 and 24 h of maturation, 47% (7/15) of the oocytes contained dispersed chromatin particles in the cytoplasm (Fig. 1*I*). Treatment with cytochalasin B did not influence other microtubule assembly or formation of a metaphase spindle (Fig. 1*J*). When oocytes were matured with 1 µg/ml taxol between 0 and 12 h of maturation, the relatively long microtubule asters were organised in the condensed chromatin (7/12, 58%). The dispersed chromatin particles were observed in oocytes which were cultured with taxol between 12 and 24 h of maturation (Fig. 1*J*; 13/15, 87%).

Microfilament organisation during meiotic maturation

At GV stage, microfilaments were seen in the cortex where the vesicle was located (Fig. 2*A, B*). Following GVBD, the microfilaments appeared to be concentrated close to the chromatin (Fig. 2*C, D*). At metaphase I, the chromatin was located in the microfilament-rich cortex (Fig. 2*E*). During anaphase I to telophase I, a microfilament furrow was observed between the two sets of chromatin, indicating the role of microfilaments in polar body extrusion (n = 3, Fig. 2*F*). After polar body extrusion microfilament domains were observed around both the metaphase chromatin and polar body (n = 9, Fig. 2*G*).

The oocytes were cultured in 10 µM cytochalasin B between 0 and 12, and 12 and 24 h of maturation to determine the requirements for microfilament assembly during *in vitro* maturation. Treatment with cytochalasin B inhibited microfilament polymerisation but did not influence GVBD (Fig. 1*H*). However, chromatin was not located at the cortex in cytochalasin B

Table 1 Meiotic progression of bovine oocytes during *in vitro* maturation

Time (h)	No. of oocytes examined	No. (%) of oocytes assessed to:					
		GV	GVBD	pro-MI	MI	AI–TI	MII
0	26	24(92)	2(8)	0	0	0	0
6	15	7(47)	8(5)	0(0)	0	0	0
9	17	1(6)	2(12)	15(88)	0	0	0
12	19	2(11)	2(11)	8(42)	7(27)	0	0
15	21	0	2(10)	2(10)	13(62)	1(5)	2(10)
18	24	1(4)	0(4)	1(4)	4(17)	5(21)	13(54)
21	23	1(4)	0(4)	1(4)	1(4)	0	20(87)

GV, germinal vesicle; MI, metaphase I; AI–TI, anaphase to telophase I; MII, metaphase II.

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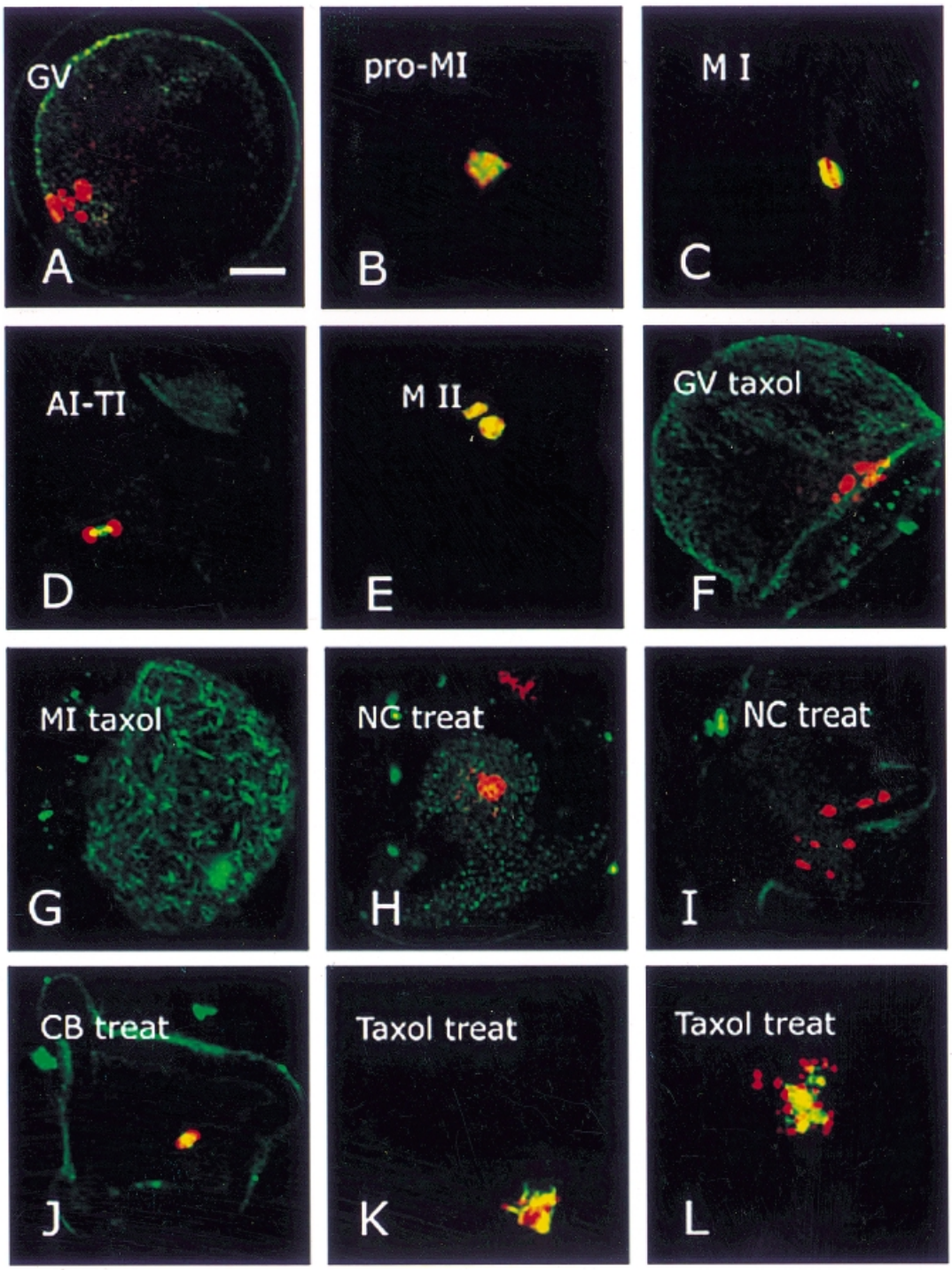

Figure 1 Immunofluorescence localisation of microtubules in bovine oocytes. Green, microtubules; red, chromatin; yellow, area of microtubules and DNA overlapping. Scale bar represents 25 mm. (*A*) At germinal vesicle stage, microtubules were not detected. (*B*) After germinal vesicle breakdown, microtubules were produced near the condensed chromatin and they elongated and encompassed the condensed chromatin during pro-metaphase I. (*C*) At metaphase I, microtubules were seen in the meiotic spindle. (*D*) In telophase, microtubules were found in a midbody. (*E*) Metaphase plate and polar body at metaphase II stage. Microtubules were detected in the spindle and some around the polar body. (*F*) Taxol induced a microtubule network at the cortex in the germinal vesicle stage oocyte. (*G*) Treatment of the prometaphase stage oocyte with nocodazole for 10 min induced numerous microtubule foci in the cytoplasm. (*H*) Treatment with nocodazole between 0 and 12 h inhibited microtubule assembly but did not influence germinal vesicle breakdown. (*I*) Treatment with nocodazole between 12 and 24 h of maturation separated chromatin particles. (*J*) Treatment with cytochalasin B between 12 and 24 h did not prevent microtubule assembly. The metaphase chromatin was not located in the cortex of oocyte. (*K*) Microtubules were elongated in taxol-treated oocytes between 0 and 12 h. (*L*) Treatment with taxol between 12 and 24 h of maturation separated chromatin particles.

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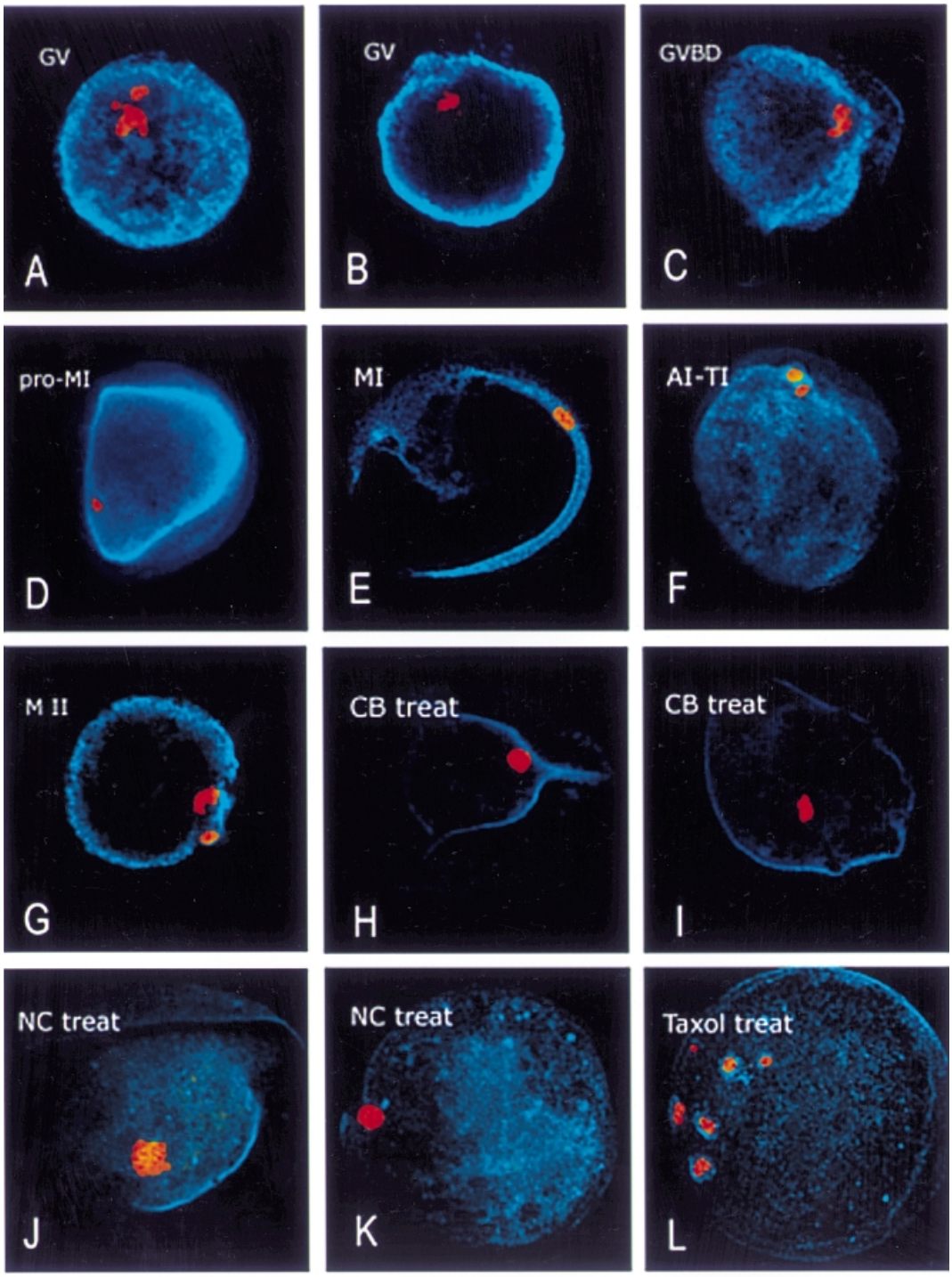

Figure 2 Immunofluorescence localisation of microfilaments in maturing bovine oocytes. Blue, microfilaments; red, DNA. Scale bar represents 25 µm. (*A*), (*B*) Same oocyte at different focal points. (*A*) At germinal vesicle stage, microfilaments were observed in the cell cortex and around the germinal vesicle. (*B*) When focused on the centre of oocytes (about 20 µm from the surface), microfilaments were observed in the cortex. (*C*) After germinal vesicle breakdown, microfilaments were concentrated to the chromatin. (*D*), (*E*) At pro-metaphase (*D*) and metaphase I (*E*) stage microfilaments overload chromatin. (*F*) During anaphase to telophase, microfilament furrows were observed between chromatin. (*G*) In metaphase II stage eggs, microfilament domains were observed around metaphase chromatin and polar body. (*H*), (*I*) Treatment with cytochalasin B between 0 and 12 h (*H*) and 12 to 24 h (*I*) inhibited microfilament assembly and progress to metaphase I. (*J*), (*K*) Treatment with nocodazole did not inhibit either microfilament assembly or germinal vesicle breakdown, but did progress to metaphase. (*L*) Treatment with taxol between 12 and 24 h of maturation separated chromatin particles. Microfilaments were seen around each chromatin particle.

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treated oocytes between 0 and 12 h (23/32, 72%; Fig. 2*I*). Treatments of oocytes with 10 µg/ml nocodazole or taxol did not affect microfilament assembly (Fig. 3); however progression to metaphase or polar body extrusion was inhibited (Fig. 2*J–L*). Microfilaments were seen around condensed chromatin or scattered chromatin particles in oocytes treated with nocodazole or taxol (Fig. 2*K, L*).

Discussion

Microtubules and microfilaments are the major cytoskeletal components in mammalian oocytes that provide the framework for chromosomal movement and cellular division. In this study we demonstrated an integral distribution of cytoskeletal components and chromatin, and determined requirements of their assembly for maturation in bovine oocytes. We observed that small microtubule asters were produced in chromatin particles following GVBD, which organised the spindle in the metaphase chromatin. As in the rat (Plancha & Albertini, 1992) and pig (Kim *et al.*, 1996*a*), treatment with taxol induced microtubule foci in oocytes after GVBD. Taxol is an antitumour agent isolated from the bark of the yew, *Taxol brevifolia*. It has been shown to promote microtubule polymerisation by decreasing the critical tubulin concentration (Albertini *et al.*, 1984; Maro *et al.*, 1985). The appearance of cytoplasmic microtubules in bovine oocytes after taxol treatment may be due to the concentration of cytoplasmic centrosomal material. Although it is not known how chromosomes induce microtubule assembly, the condensed chromatin seems to be important in regulating patterns of microtubule assembly in mammalian oocytes during maturation. It is known that under the influence of the chromosomes, maternal centrosomal material determines the organisation and shape of the spindle by nucleation of microtubules in the mouse (Howlett *et al.*, 1985; Maro *et al.*, 1986). Karsenti *et al.* (1984) reported a decrease in the apparent critical concentration for tubulin polymerisation in the vicinity of the chromosomes in meiotic *Xenopus* oocytes. Maro *et al.* (1986) also showed that condensed chromosomes promote tubulin polymerisation, thereby forming a microtubular aster in the cytoplasm of unfertilised mouse oocytes.

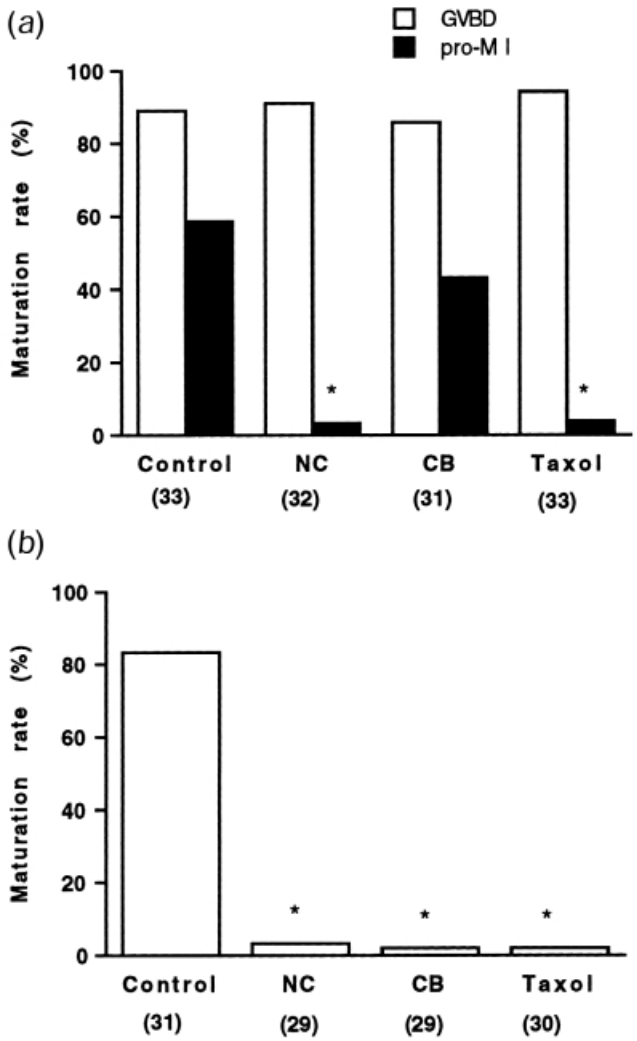

Figure 3. Effects of nocodazole (NC), cytochalasin B (CB) and taxol on germinal vesicle breakdown (GVBD) and the formation of pro-metaphase (PRO-MI) during 0 to 12 h of maturation (*A*), and on the progress to metaphase II during 12 to 24 h of maturation (*B*). The number of oocytes examined in each experimental group is given in parentheses. *Significant difference ($p < 0.05$).

The role of microtubules during meiotic maturation was documented by using a microtubule inhibitor, nocodazole. Treatment with nocodazole did not inhibit GVBD but did inhibit metaphase formation in bovine oocytes. This result is similar to previous results in the mouse (Simerly *et al.*, 1990). Our study also showed that long-term treatment with nocodazole or taxol following GVBD induced separation of chromosome particles. Previous results have indicated that the kinetochore, the centromeric region of the chromosome in mitotic and meiotic cells, seems to be responsible for capturing and stabilising microtubules which consequently align chromosomes at the spindle equator during prometaphase (Mitchison & Kirschner, 1985). Simerley *et al.* (1990) reported that microinjection of kinetochore antibodies interfered with chromosome movement and segregation of chromatin in either meiotic and mitotic mouse oocytes. Collectively, our study and previous studies indicate that following GVBD the kinetochore may regulate microtubule assembly, which is important in aligning chromatin for forming a metaphase spindle.

The microfilaments were seen in the cortex and around GV in bovine oocytes. Treatment with cytocha-

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lasin B, a microfilament disrupting agent, did not inhibit GVBD. However, the meiotic spindle with chromatin did not remain in the cortex of oocytes. These results suggest that microfilaments are involved in the peripheral location of chromatin, which seems to be important to the further maturation processes. At metaphase, the thick microfilaments were organised near the metaphase chromatin, which possibly induced polar body extrusion. This result is similar to that shown in the mouse (Longo & Chen, 1985). In the present study we also demonstrated microfilament furrows between chromatin during polar body extrusion. The microfilaments were also seen in the chromatin particles following treatment of oocytes with taxol or nocodazole. Van Blerkom & Bell (1986) have shown that in the mouse chromosomes gain the capacity to modify the organisation of microfilaments in their vicinity during specific stages of the cell cycles. Although the mechanism controlling microfilament polymerisation during the cell cycle is elusive at present, microfilament furrows may be regulated by kinase activity during the cell cycle. The cell cycle during oocyte maturation is controlled by a kinase activity called maturation or M-phase promoting factor (MPF; Masui & Markert, 1971). In *Xenopus* oocytes, the translation of mRNA encoding the Mos kinase indirectly triggers MAP kinase activation which sequentially influences the activation of MPF in metaphase II arrested oocytes (Sagata *et al*., 1989; O'Keefe *et al*., 1991; Haccard *et al*., 1993). It is also known that the Mos and MAP kinases are involved in cytoskeletal organisation during the transition between the two meiotic metaphases (Verlhac *et al*., 1994, 1996). Therefore, both microtubule and microfilament assembly in maturing bovine oocytes may be controlled by Mos and MAP kinase activity, but this is still poorly understood.

In summary, we have described the integrated organisation between cytoskeletal components and chromatin in bovine oocytes during meiotic maturation. The results suggest that microtubule and microfilament dynamics are integrated and interact with chromosomal changes during oocyte maturation. Two sources of microtubules were observed in bovine maturing oocytes, which is certainly involved in chromatin reconstruction during meiotic maturation. Microfilaments are involved in chromosomal movement to a peripheral position after GVBD, which may be important for continued embryonic development after fertilisation. The experiments using cytoskeletal inhibitors revealed that microtubules and microfilaments are not required for GVBD but are required for metaphase formation and polar body extrusion.

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