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Original article

Microtubule rearrangement during *in vitro* maturation of pig oocytes. Effect of cycloheximide

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Summary — In freshly isolated fully grown pig oocytes at the germinal vesicle (GV) stage, the cytoplasmic microtubules are arranged in a meshwork. This microtubule arrangement is also maintained during the initial phases of meiotic maturation *in vitro*. A perinuclear array of microtubules is formed immediately before germinal vesicle breakdown (GVBD). Short-term treatment of oocytes with taxol when oocytes are at metaphase I stage induced formation of cytoplasmic asters. The oocyte cytoplasm is unable to respond to the taxol treatment at the earlier stages of meiotic maturation. In oocytes cultured with the proteosynthesis inhibitor cycloheximide, meiotic maturation is blocked. Condensation of chromatin occurs but the nuclear envelope is preserved and the microtubule arrangement is unchanged. A perinuclear array of microtubules does not appear and oocyte cytoplasm does not respond to short-term taxol treatment by the formation of cytoplasmic asters. We can conclude that the microtubule rearrangement and the acquisition of competence for tubulin assembly are blocked by cycloheximide and are thus dependent on *de novo* proteosynthesis.

pig oocyte / meiotic maturaton / microtubule / cycloheximide / taxol

Résumé — Réarrangement des microtubules pendant la maturation *in vitro* des ovocytes de truie. Effet de la cycloheximide. *Après achèvement de leur croissance, les ovocoytes porcins au stade de la vésicule germinative contiennent des microtubules en réseau. Cette distribution est maintenue pendant les phases initiales de la maturation méiotique* in vitro. *Un arrangement périnucléaire de microtubules se forme immédiatement avant la rupture de la vésicule germinative. Un traitement court par le taxol des ovocytes au stade métaphase I induit la formation d'asters cytoplasmiques. L'ooplasme n'est pas capable de répondre au traitement par le taxol à des stades antérieurs. La maturation méiotique est bloquée dans des ovocytes cultivés en présence de cycloheximide, un inhibiteur de synthèse protéique ; dans ces conditions, la chromatine se condense mais l'enveloppe nucléaire persiste et la distribution des microtubules n'est pas modifiée. L'arrangement périnucléaire de microtubules ne s'éta-*

blit pas et l'ooplasme ne répond pas à un court traitement par le taxol par la formation d'asters cytoplasmiques. En conclusion, le réarrangement des microtubules et l'acquisition de la compétence de nucléation, bloqués par le cycloheximide, apparaissent dépendant de la synthèse protéique.

ovocyte porcine / maturation méiotique / microtubule / cycloheximide / taxol

INTRODUCTION

After finishing their growth in the follicle, mammalian oocytes are able to resume meiosis spontaneously when they are liberated from their follicles and cultured *in vitro* (see Thibault *et al*, 1987). The ability of oocytes to mature *in vitro* is essential for their further use in various technological methods.

Meiotic maturation is a very complex process during which nuclear membrane breakdown, chromatin condensation and formation of meiotic spindles are observed as the most striking features (Thibault *et al*, 1987; Wassarman, 1988). A key point in meiotic maturation is meiosis resumption in which proteosynthesis plays a very interesting but not yet fully understood role. While in rodents, meiosis resumes even when proteosynthesis is blocked (Wassarman *et al*, 1976; Schultz and Wassarman, 1977; Ekholm and Magnusson, 1979; Hashimoto and Kishimoto, 1988), meiosis resumption, according to above-mentioned criteria, did not occur in oocytes of ungulates when proteosynthesis was inhibited (cattle (Hunter and Moor, 1987; Simon *et al*, 1989), sheep (Moor and Crosby, 1986) and pig (Fulka *et al*, 1986; Mattioli *et al*, 1991)), but the nuclei of these oocytes did not remain intact. The nuclear membrane was preserved but chromatin was condensed (Moor and Crosby, 1986; Kubelka *et al*, 1988; Simon *et al*, 1989; Mattioli *et al*, 1991). Moreover, in pig oocytes where maturation was first inhibited by a proteosynthesis inhibitor, which was subsequently cultured in inhibitor-free medium, the resumption of meiosis is significantly accelerated (Kubelka *et al*, 1988). These phenomena provide evidence that none of the processes involved in meiotic maturation of oocytes are inhibited under the influence of proteosynthesis inhibitors. These processes, which are not blocked by proteosynthesis inhibitors, should also involve changes in the cytoplasm, *eg*, redistribution of organelles or microtubule rearrangement.

It is known that, in addition to the above-mentioned changes occur in the oocyte nucleus, changes in the cytoplasm. In particular, the distribution of organelles (Szollosi, 1972; Van Blerkom and Runner, 1984; Szollosi *et al*, 1988) and cytoskeleton (Maro *et al*, 1990; Mattson and Albertini, 1990; Albertini, 1992; Plancha and Albertini, 1992) are changed. There is a distinctive change in the microtubule assembly (Maro *et al*, 1985; Van Blerkom, 1991). This activity is exhibited by pericentriolar material (PCM) in the poles of the meiotic spindle and also by numerous PCM foci in the cytoplasm (Maro *et al*, 1985; Van Blerkom, 1991). The ability to assemble tubulin can be assessed after exposure of the oocytes to taxol, a drug that promotes microtubule assembly by shifting the equilibrium between the tubulin polymer and dimer in favor of microtubule formation (Schiff *et al*, 1979; Horwitz, 1992).

The nature of the events occurring in ungulate oocytes in the presence of proteosynthesis inhibitors is not known in more detail. This study thus investigates the changes in the microtubule arrangement during meiotic maturation and microtubule assembly after taxol treatment. Further, it investigates the influence of the proteosynthesis inhibitor cycloheximide on changes in microtubule arrangement and the ability of

microtubules to assemble in the cytoplasm of pig oocytes cultured *in vitro*.

MATERIALS AND METHODS

Collection and culture of oocytes

Porcine ovaries were obtained from a local slaughterhouse. Ovaries were transported to the laboratory in a saline solution at 39°C. Oocytes were aspirated from follicles that were 2–5 mm in diameter with a syringe with a 20-gauge needle. Only oocytes with compact cumuli were used for the culture.

Before culture, the oocytes were washed 3 times in a culture medium: E199 medium (ÚSOL Praha, Czech Republic) supplemented with 0.039 ml 7.5% solution of sodium bicarbonate per millilitre, calcium lactate (0.6 mg per millilitre of 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). In experiments to ascertain the influence of cycloheximide, the medium was supplemented with 10 µg cycloheximide (Sigma, Saint Louis, USA) per millilitre.

For *in vitro* culture, the oocytes were placed in groups of 20 in 100 µl droplets, covered with a paraffin oil (PhBS CSL4, Spofa Praha, Czech Republic) and cultured for an appropriate amount of time at 39°C in air with 5% CO_2. At the end of the culture, part of the 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 a phase-contrast microscope to check their nuclear maturation. The remaining oocytes were processed for immunochemistry as described below.

Immunofluorescence for tubulin

Zona-free oocytes were washed in a HEPES buffer and then fixed for 60 min at room temperature in a HEPES buffer containing 2.5% formaldehyde, 2.5 mM MgCl, 2.5 mM EGTA and 1 µM taxol. After careful washing in the PBS buffer with 0.1% Triton X 100 and 0.1% of human serum albumin (HSA), the oocytes were incubated with a mouse monoclonal antibody to α-tubulin (Sigma) diluted in PBS supplemented with 0.1% HSA and 0.01% Tween 20 (antibody dilution 1:200) for 13–16 h at 4°C. Following 3 washes in PBS the oocytes were incubated for 1 h with a ovine antibody to mouse IgG (Sigma) conjugated with FITC diluted in PBS supplemented with 0.1% HSA and 0.01% Tween 20 of (antibody dilution 1:50). After careful washing, the oocytes were stained with Hoechst 33 258 (10 µg/ml PBS buffer) for 10 min at room temperature. After washing in a PBS buffer with 0.1% HSA, the oocytes were mounted on slides coated with poly-L-lysine and covered with a cover glass in glycerol with Tris buffer pH 8.5 (1:1 v/v).

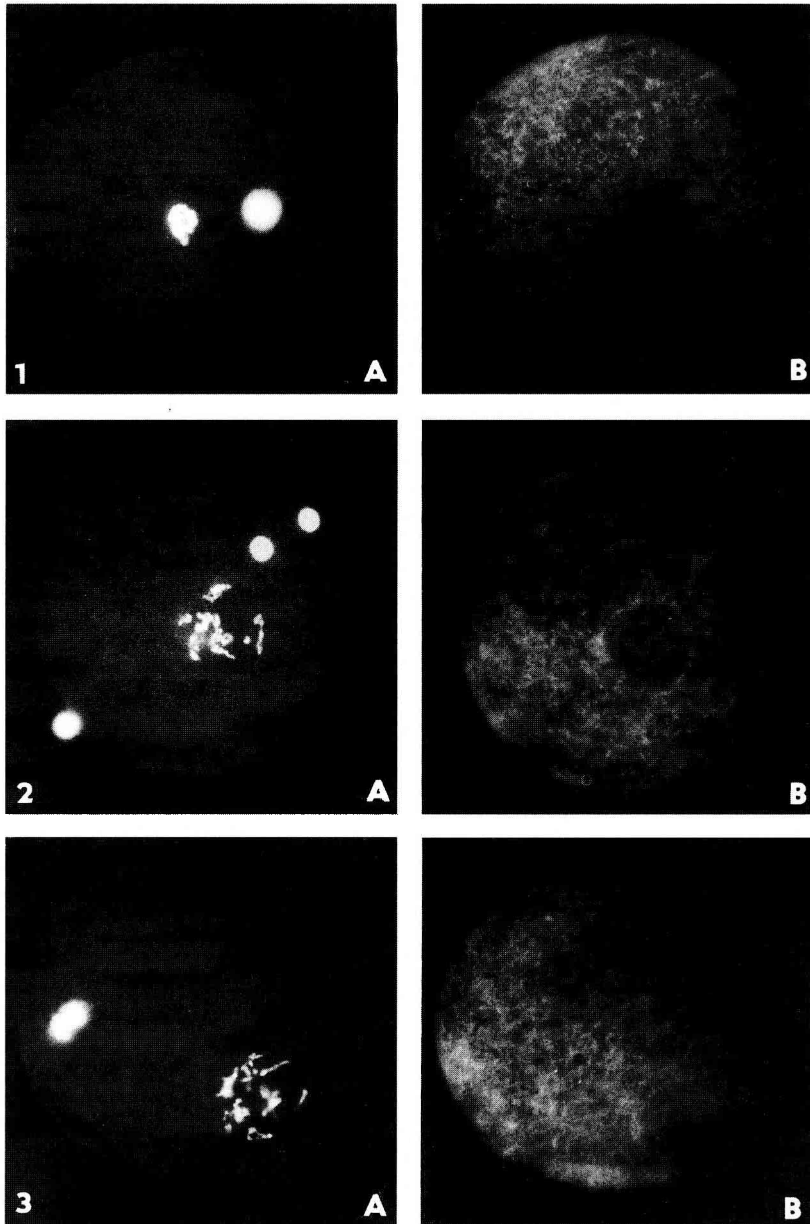
Labelled oocytes were analyzed on fluorescence Jenalumar microscope (510 nm for tubulin labelled with fluorescein and 410 nm for Hoechst).

Taxol experiments

To evaluate the nucleating ability of cytoplasmic microtubule, oocytes were incubated in a culture medium with 10 µM taxol (Sigma) for 20 min at 38.5°C and immediately fixed for immunofluorescent analysis.

RESULTS

In fully grown pig oocyte, freshly isolated from the follicle, the nucleus chromatin is arranged in a ring or horseshoe shape surrounding the nucleolus. The cytoplasmic microtubule formed a meshwork (fig 1A and B). After *in vitro* culture the initial chromatin configuration progressively disappears over several hours (3–8 h) and Hoechst positive foci start to form in the nucleoplasm. The microtubules are still arranged in a meshwork (fig 2A and B). This microtubule arrangement does not change during further *in vitro* culture of the oocytes (8–16 h), when the further chromatin condensation becomes apparent. The number of Hoechst positive foci in the nucleus increases and the ring or horseshoe chromatin arrangement is no longer visible (fig 3A and B). Distinctive changes in the microtubule arrange-



Figs 1–3. Sequence of nuclear and microtubule configurations from time of collection to 48 h *in vitro* culture. Correlative Hoechst 33258 (**A**) and anti-tubulin (**B**) labelling patterns in whole-mount preparations from pig oocytes. At time of collection the oocytes present a chromatin arranged in a ring shape (**1A**) and microtubule formed a meshwork (**1B**). After 6 h of culture Hoechst positive foci appeared within GV (**2A**), microtubule meshwork remained unchanged (**2B**). After 12 h of culture further chromatin condensation is seen (**3A**) but no changes in tubulin arrangement are detected (**3B**). Magnification: 450 x.

ment are only seen immediately before the germinal vesicle breakdown (GVBD), *ie* 16–19 h from the beginning of the *in vitro* culture, when a marked perinuclear array of microtubules appeared (fig 4A and B).

Pig oocytes that had not undergone GVBD did not respond to taxol treatment by the formation of cytoplasmic asters. However, the oocytes that had entered the metaphase I stage after GVBD (see fig 10A and B, below) responded to a 30 min exposure to taxol by the formation of numerous cytoplasmic asters (fig 5A and B) and the same situation was observed when matured oocytes reaching the metaphase II stage were exposed to taxol (fig 6A and B).

When pig oocytes were cultured immediately after isolation in a medium supplemented with cycloheximide, a gradual chromatin condensation was observed. In addition to chromatin surrounding the nucleolus, Hoechst positive foci start to appear in the nucleus (fig 7A). The number of these foci progressively increased and the ring or horseshoe chromatin arrangement disappeared (fig 8A). These changes in chromatin configuration were not followed by changes in the microtubule arrangement, which maintained a meshwork structure (figs 7B and 8B). After a 24 h culture interval in the presence of cycloheximide condensed chromosomes appeared within the nucleus (fig 9A) but the microtubules still retained their arrangement in a meshwork. We did not observe the formation of a perinuclear array of microtubule in oocytes cultured with cycloheximide (fig 9B).

Oocytes cultured in a medium with cycloheximide did not respond to an exposure to taxol by the formation of cytoplasmic asters even if they exhibited highly condensed chromosomes in their nuclei.

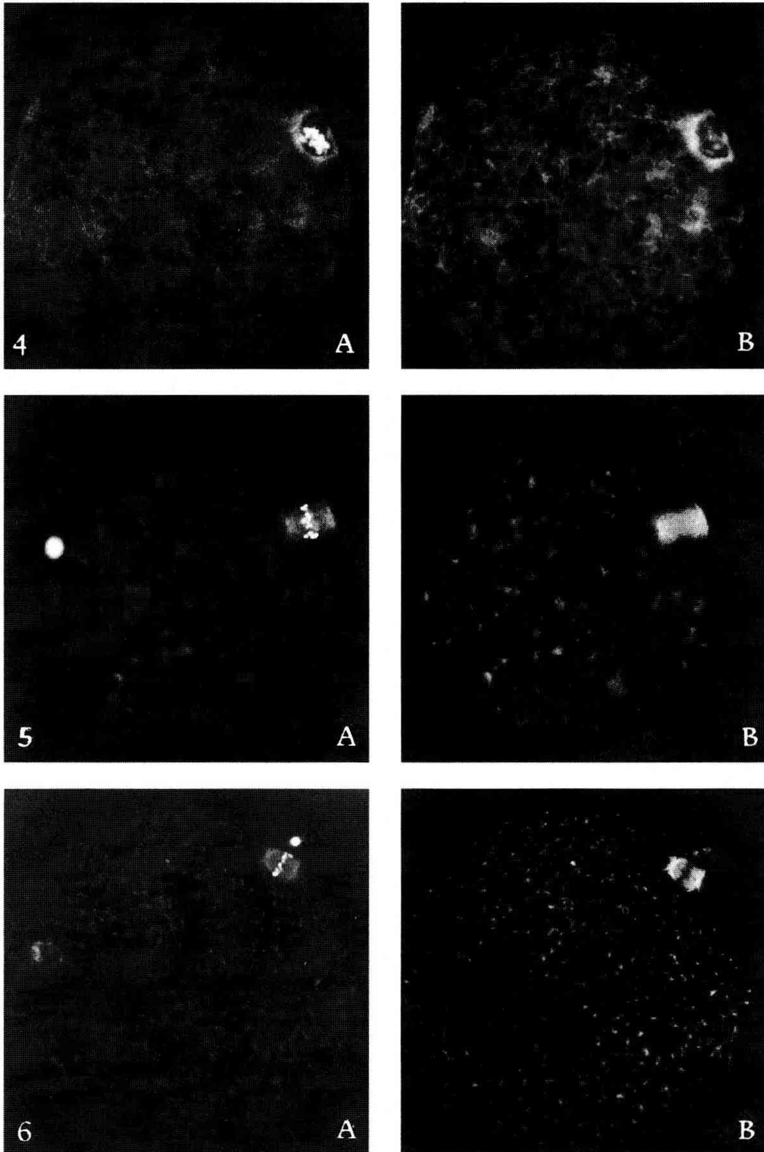
We cannot exclude the possibility that the most labile microtubules may not have been preserved and/or detected by immunofluorescence microscopy.

DISCUSSION

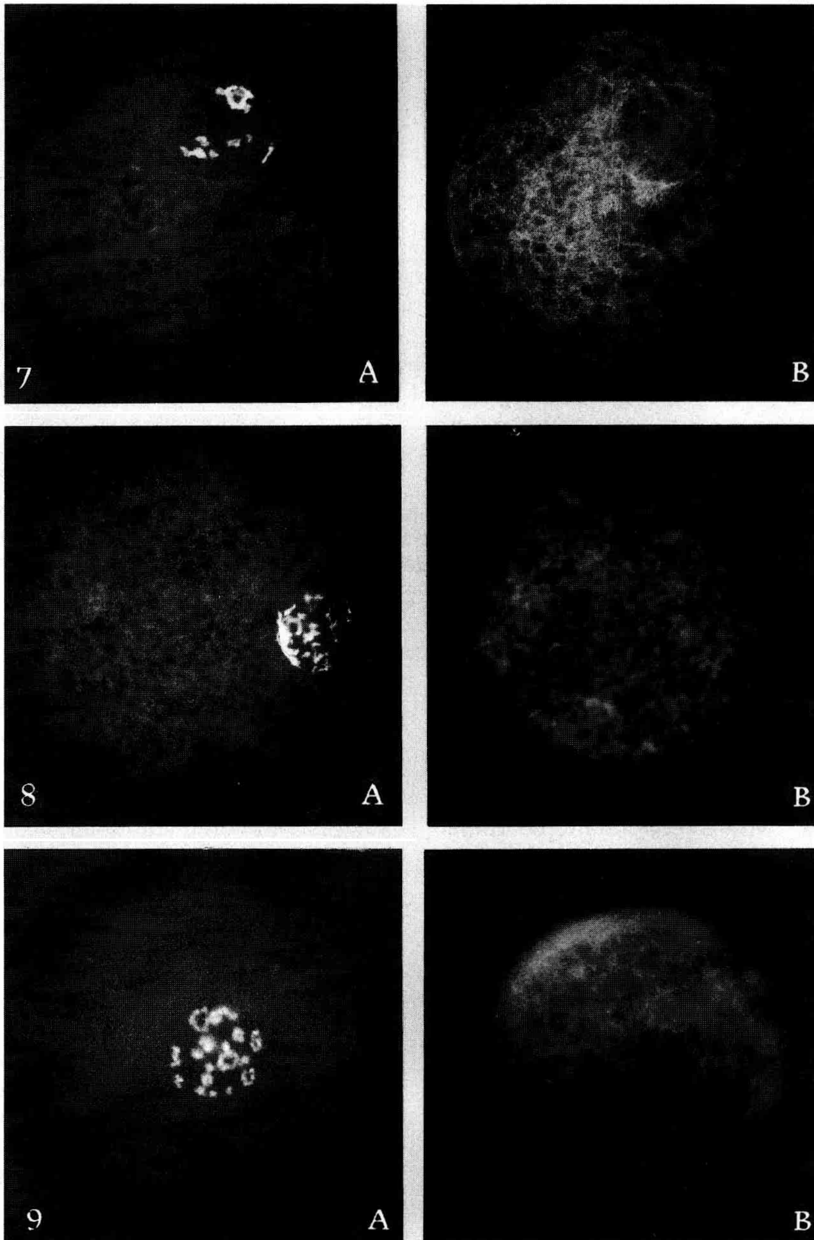
In our study we observed that cytoplasmic microtubules are arranged in a meshwork in fully grown pig oocytes freshly isolated from the follicles. This agrees with observations in GV mouse (Rime *et al,* 1987; Maro *et al,* 1990; Van Blerkom, 1991; Van Cauwenberge and Alexandre, 1992) and hamster (Plancha and Albertini, 1992) oocytes, where similar microtubule networks were demonstrated. On the other hand, these observations sharply contrast with the situation described in the mouse by Mattson and Albertini (1990), where the GV oocytes exhibit an M-phase-like microtubule network and it is thought that this microtubule arrangement is developed in mouse oocytes during oogenesis and accompanied the acquisition of meiotic competence (Wickramasinghe *et al,* 1991).

During the relatively long time interval necessary for *in vitro* and *in vivo,* germinal vesicle breakdown in pig oocytes, a typical pattern of chromatin condensation is observed (Motlík and Fulka, 1976; this work), which, according to our data, is not followed by distinctive changes in the microtubule arrangement. These changes in microtubule configuration only started to become obvious immediately before germinal vesicle breakdown, when the formation of a perinuclear microtubule array was seen. A similar situation was observed during the meiotic maturation of hamster oocytes where germinal vesicle breakdown is initially preceded by the disappearance of the interphase-like microtubule network and then by the formation of a microtubule perinuclear array (Plancha and Albertini, 1992).

The competence for tubulin assembly was investigated after the short-term treatment of oocytes with taxol. Our observations indicate that, in pig oocytes, the response to taxol treatment depends on the stage of meiosis. Oocytes that did not



Figs 4–6. Sequence of nuclear and microtubule configurations from time of collection to 48 h *in vitro* culture (continuation from figs 1–3). Correlative Hoechst 33258 (**A**) and anti-tubulin (**B**) labelling patterns in whole-mount preparations from pig oocytes. After 16 h of culture chromatin is condensed (**4A**) and a perinuclear array of microtubule can be seen (**4B**). After GVBD the oocytes respond to taxol treatment **(5–6).** After 22 h culture chromosome are arranged into the metaphase I plate (**5A**) microtubules are arranged into a meiotic spindle and taxol induced the formation of cytoplasmic asters (**5B**). After 48 h culture the first polar body is extruded and the chromosomes are at the metaphase II plate (**6A**). Taxol treatment induces the formation of cytoplasmic asters (**6B**). Magnification: 450 x.



Figs 7–9. Correlative Hoechst 33285 (**A**) and anti-tubulin (**B**) labelling patterns in cycloheximide-treated oocytes for 24 h. After 10 h of culture Hoechst positive foci appeared within GV (**7A**) but the microtubule meshwork is not changed (**7B**). After 14 h of culture chromatin within GV further condensed (**8A**) but no changes in microtubule arrangement are seen (**8B**). After 24 h of culture condensed chromosomes are seen within GV (**9A**) but no changes occurred in the microtubule arrangement and a perinuclear array is not detected (**9B**). Taxol treatment did not induce the formation of cytoplasmic asters in these oocytes. Magnification 450 x.

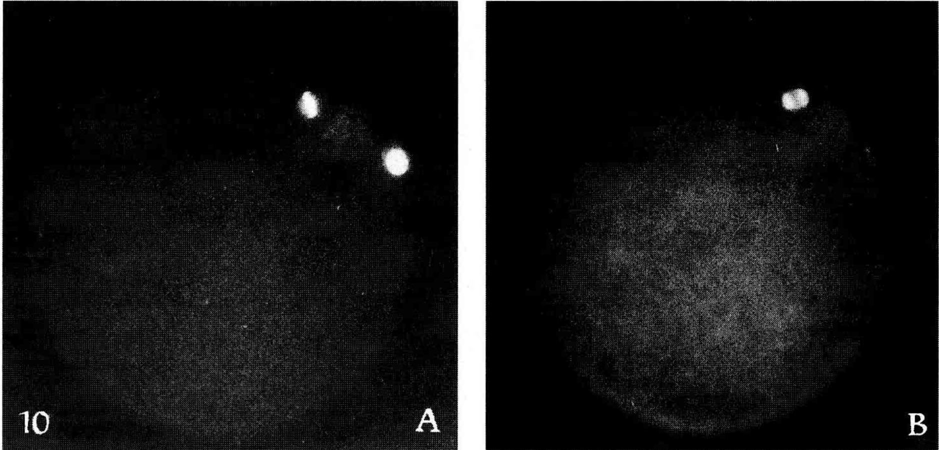


Fig 10. Correlative Hoechst 33258 (**A**) and anti-tubulin (**B**) labelling patterns in whole mount preparation from pig oocyte cultured for 22 h *in vitro.* Oocyte was labelled without the short-term taxol treatment. Chromosomes are arranged into a metaphase plate (**A**) and microtubules are arranged into a meiotic spindle (**B**).

undergo GVBD did not respond to taxol treatment, whereas oocytes entering the M-phase formed numerous cytoplasmic asters after culture with taxol. A similar situation was observed during maturation in rodent oocytes, where response to taxol also appeared after GVBD (rat (Albertini, 1987) and mouse (Maro *et al,* 1985; Rime *et al,* 1987)), although some changes in microtubule arrangement after taxol treatment of mouse GV oocytes were reported (Rime *et al,* 1987).

Pig oocytes cultured with cycloheximide exhibit marked condensation of chromatin, during which the nuclear membrane is well preserved (Kubelka *et al,* 1988; Mattioli *et al,* 1991; this work). However, the cytoplasmic microtubule in pig oocytes cultured in cycloheximide remained in a meshwork arrangement, which is identical to the microtubule arrangement in freshly isolated oocytes before culture. No microtubule perinuclear array was observed in oocytes cultured with cycloheximide. Neither response to taxol treatment nor cytoplasmic aster formation was seen, even in oocytes in which chromatin was markedly condensed after culture with cycloheximide, and competence for tubulin assembly in these oocytes is comparable with freshly isolated oocytes or oocytes which did not undergo GVBD. It is suggested that competence for tubulin assembly appeared in xenopus oocytes when components of germinal vesicle were released into the cytoplasm after GVBD (Heideman and Gallas, 1980; Karsenti *et al,* 1984; Jessus *et al,* 1987, 1988). In mammalian oocytes competence for tubulin assembly is probably not evoked by components of the germinal vesicle but is a result of the maturation of oocyte cytoplasm (Van Cauwenberge and Alexandre, 1992). This is why maintenance of the nuclear envelope in oocytes cultured in cycloheximide should not be the main limiting factor for the appearance of competence for tubulin assembly.

M-phase-promoting factor (MPF) represented by the cdc2/cyclin B or cdc2/cyclin A complexes was identified as a major factor implicated in the control of microtubule arrangement during the cell cycle progres-

sion (Verde *et al,* 1990) and mitogen-activated protein (MAP) kinase was shown to control microtubule dynamics during meiosis (Gotoh *et al,* 1991; Verlhac *et al,* 1993; Verlhac *et al,* 1994). During meiosis resumption in mammalian oocytes MPF (Hashimoto and Kishimoto, 1988) and MAP kinase (Sobajima *et al,* 1993) activities are increased. At present, there are no data available on the role of proteosynthesis in the activation of MAP kinase in pig oocytes. However, it is clear that the active protein synthesis is required for the MPF rise in pig oocytes (Mattioli *et al,* 1991). We can suggest that the M-phase microtubule rearrangement is blocked in cycloheximide-treated pig oocytes through the prevention of an MPF increase, and possibly also the increase in MAP kinase activity.

On the basis of our results we can conclude that microtubules in intact GV pig oocytes are arranged in a meshwork and that this microtubule arrangement is maintained up to the period immediately preceding GVBD, when an apparent perinuclear array of microtubules is formed. Taxol experiments demonstrated that oocytes which have not undergone GVBD are unable to elicit microtubule assembly, whereas in M-phase oocytes short-term treatment with taxol induced the formation of cytoplasmic asters. These asters were not observed after taxol treatment of oocytes cultured with cycloheximide. Consequently, in contrast to the condensation of chromosomes, M-phase microtubule rearrangement and the acquisition of competence for tubulin assembly are processes which are suppressed during the culture of pig oocytes with cycloheximide or are dependent on *de novo* proteosynthesis.

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