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Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes

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Summary. NBD–phallacidin revealed a polymerized actin distribution in the cortical region of the rabbit egg and along junctional feet. Staining with anti-alpha-tubulin antibody showed that the microtubule distribution was restricted to the barrel-shaped spindle. After cryoprotective treatment in the presence of propanediol, cortical polymerized actin was no longer visible within the egg and along junctional feet but filamentous actin was still present after treatment with dimethylsulphoxide. However, exposure to dimethylsulphoxide or propanediol led to the appearance of microtubules in the cytoplasm and to a disassembly of the spindle often associated with anomalies in chromosome position. Cytoplasmic microtubules formed by the action of propanediol were still present after freezing, thawing, and removal of the cryoprotectant, but after recovery of eggs in culture, they disappeared and barrel-shaped spindles were able to reform.

When the effect of propanediol addition on in-vivo fertilization and development of frozen oocytes was examined, 39% (79/200) of frozen oocytes were fertilized and 9% (9/105) developed to normal fetuses, compared to 81% (38/47) and 32% (12/38) respectively for unfrozen control oocytes.

Keywords: cryoprotectants; rabbit; oocytes; microtubules; microfilaments; freezing

Introduction

The survival rate of mammalian embryos after freezing and long-term storage has been improved progressively for many mammalian species (mouse, rabbit, sheep and cow; Heyman, 1986) including man (Testart *et al.*, 1986). However, it would also be useful if cryopreservation of unfertilized ovulated oocytes was possible both for animal breeding and medical applications. Such technology would offer the possibility of establishment of oocyte banks like those already used for spermatozoa. Moreover, storing oocytes offers a unique opportunity of pregnancy to women who undergo ovariectomy at a relatively young age. It also provides a way to avoid some ethical objections raised by embryo freezing. However, very few successes as assessed by live fetuses or birth of young have been reported except for the mouse (Whittingham, 1977). In humans, a twin pregnancy has been achieved after cryopreservation of oocytes using dimethylsulphoxide as a cryoprotectant (Chen, 1986; Van Uem *et al.*, 1987).

High survival rates for most eukaryotic cells frozen to low subzero temperature require the presence of protective compounds which protect cells from solution effects by reducing both the concentration of electrolytes (Lovelock, 1953) and cell dehydration (Meryman *et al.*, 1977). However, the mechanisms by which these compounds protect (and perhaps also damage) cells are still the subject of considerable investigation and may influence the choice of the cryoprotectant depending on the cellular model to be frozen. Since they permeate the cell and change the

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intracellular conditions, it is likely that protective additives may influence cellular structure and function.

There is evidence that organic solvents can influence cytoskeletal protein organization *in vitro* (Shelanski *et al.*, 1973; Lee & Timasheff, 1975; Himes *et al.*, 1976, 1977; Tellam *et al.*, 1983; Stromqvist *et al.*, 1984) and within cells. In fibroblasts and *Dictyostelium* cells, dimethylsulphoxide causes the disorganization of stress fibres and the formation of intranuclear actin bundles (Fukui, 1978; Fukui & Katsumaru, 1980; Sanger *et al.*, 1980a, b). In a rabbit zygote, we demonstrated a depolymerizing action of propanediol contrary to that of dimethylsulphoxide (Vincent *et al.*, 1988).

In the mouse oocyte, cytoskeletal elements are implicated in events occurring at fertilization (Maro *et al.*, 1986a) and perturbing cytoskeletal organization by the addition of cryoprotectant or by freezing may have drastic effects. Johnson & Pickering (1987) have shown that dimethylsulphoxide modifies the microtubule organization of mouse oocytes. Exposure to dimethylsulphoxide leads to progressive disassembly of the spindle that has also been shown to be cold-sensitive (Magistrini & Szollosi, 1980; Pickering & Johnson, 1987), and causes appearance of microtubular asters associated with pericentriolar material. In some oocytes the removal of dimethylsulphoxide resulted in the restoration of a normal spindle.

Propanediol has been shown to exert a good cryoprotective action on 1- and 2-cell rabbit embryos while dimethylsulphoxide does not (Renard *et al.*, 1984; Vincent *et al.*, 1989). In the present study, we therefore examined and compared the effect of 1,2-propanediol and dimethylsulphoxide on microtubule and microfilament organization within the cytoplasm of rabbit ovulated oocytes. We also investigated the effects of propanediol on the in-vivo fertilization and survival rates of frozen rabbit oocytes.

Materials and Methods

Egg recovery. New Zealand White rabbits were induced to superovulate with FSH and LH treatment (Kennelly & Foote, 1965). At 12 h after mating with a vasectomized male, females were killed and ovulated oocytes were recovered from oviducts by flushing with PBS (Tervit *et al.*, 1972). Eggs were removed from cumulus cells by brief exposure to 0·5% hyaluronidase (Sigma, St Louis, MO, USA) and oocytes were washed twice in PBS at room temperature before use.

Cryoprotective treatment. The cryoprotectants used were 1,2-propanediol (mol. wt 76·1) (propanediol; Prolabo, Paris, France) and dimethylsulphoxide (mol. wt 78·1) (Merck, Rahway, NJ, USA). Eggs were equilibrated in 1·5 M cryoprotectant at room temperature for 30 min. Cells shrank initially but returned to isotonic volume when equilibration was complete. Then, cells were partly dehydrated in a solution containing 2 M cryoprotectant and 0·5 M-sucrose (Prolabo, Paris, France) (Renard *et al.*, 1984). Sucrose does not permeate the cell and allows partial dehydration, maximum shrinkage occurring within 5 min. Some eggs were exposed only to 0·5 M-sucrose. All compounds were diluted in PBS containing 10% fetal calf serum (FCS).

Freezing and thawing. The freezing and thawing of eggs was carried out as described by Renard *et al.* (1984). After cryoprotective treatment, eggs were frozen directly to −25°C in precooled straws and kept at this temperature for 90 min before plunging into liquid nitrogen. Rapid thawing was achieved by plunging the straws from −196°C into a +37°C water bath.

Removal of cryoprotectant. The content of each frozen–thawed straw was expelled into a Petri dish, and the eggs were transferred successively to sucrose solutions of 0·5 M (10 min), 0·25 M (5 min) and 0·05 M (5 min) to remove the cryoprotectant (Leibo & Mazur, 1978; Renard & Babinet, 1984). They were then washed in PBS. Eggs were examined morphologically under a stereomicroscope (×40) to assess the percentage of eggs apparently intact.

Fertilization in vivo *and assessment of viability*. Because of the variability of success during in-vitro fertilization for rabbit eggs, assessment of viability was done using in-vivo fertilization. New Zealand White recipients were mated with a normal male 9 h before transfer. Only intact frozen–thawed eggs were used for transfer. At the time of transfer, we confirmed that ovulation had not yet taken place. In each recipient female, the ovary on one side was left intact, providing a control measure of the success of in-vivo fertilization. On the side of transfer, the ovary was removed. Control untreated and frozen–thawed eggs were held at 37°C in B2 medium (Menezo, 1976) for a maximum of 1 h before transfer. Then they were aspirated in a catheter with 12 µl B2 medium and injected into the oviduct 2 cm from the infundibulum. All recipients were killed either the following day to assess fertilization rate (calculated as the

percentage of eggs that formed at least 2 pronuclei) or on the 25th day of pregnancy to assess the number of viable fetuses.

Comparisons between treatment groups were made by χ^2 tests.

Fixation and staining. For actin staining, rabbit eggs were fixed in 4% formaldehyde in PBS at room temperature for 30 min and then washed in PBS. For microtubule visualization, cells were fixed for 30 min in 0·3% acetic acid in 70% ethanol at room temperature and then washed successively in 50% ethanol, 30% ethanol and finally PBS.

After fixation, eggs were embedded in 10% gelatin in PBS before inclusion in 15% gelatin blocks and cooling in isopentane chilled in liquid nitrogen to −130°C. Thick (10 μm) cryosections (Frigocut 2700, Reichert Jung, Heidelberg, West Germany) were laid on slides precoated with albumin, then hydrated and permeabilized for 30 min at room temperature in PBS containing 0·03% saponin. The sections were then washed in PBS before incubation for 1 h with NBD–phallacidin (Molecular Probes, Inc., Eugene, OR, USA) at a concentration of 5 i.u./ml to stain the polymerized actin (Barak *et al.*, 1980) or with a monoclonal anti-alpha-tubulin antibody (N.356, Amersham, Bucks, UK) (dilution in PBS: 1/300) followed by an incubation of 1 h in FITC-labelled anti mouse IgG and IgM (Biosys, Paris, France) (dilution in PBS: 1/500) to visualize tubulin. Visualization of chromosomes was achieved by incubating sections briefly in Hoechst 33342 dye (125 μg/ml). After washing in PBS, sections were mounted in a PBS/glycerol mixture (Citifluor, City University, London, UK) with a coverslip. A Reichert microscope was used for interference and fluorescence observations. Micrographs were taken using Ilford HP5 films (400 ASA).

Results

Microfilament organization

Control oocytes. After collection from the oviduct and extraction from the cumulus mass, oocytes were fixed immediately. A continuous uniform submembranous layer of microfilaments was visible in all oocytes (Fig. 1a). A submembranous layer of F-actin was also observed in the first polar body (not shown). The cytoplasmic foot processes of the corona cells which connect with the oocyte through the zona pellucida were also stained with NBD–phallacidin, indicating the presence of microfilaments. Corona cells around the zona pellucida were also stained intensely.

Effects of cryoprotective treatment at room temperature. After cryoprotective treatment at room temperature, eggs were fixed immediately without removal from cryoprotectant. In the presence of propanediol, polymerized actin was no longer evident in the oocyte, while microfilaments were still present in the polar body and corona cells (Fig. 1b). Only a few connections between oocyte and corona cells were stained. This change in polymerized actin staining observed after propanediol exposure shows that this cryoprotectant acts as a depolymerizing agent in rabbit oocytes and in the foot connections.

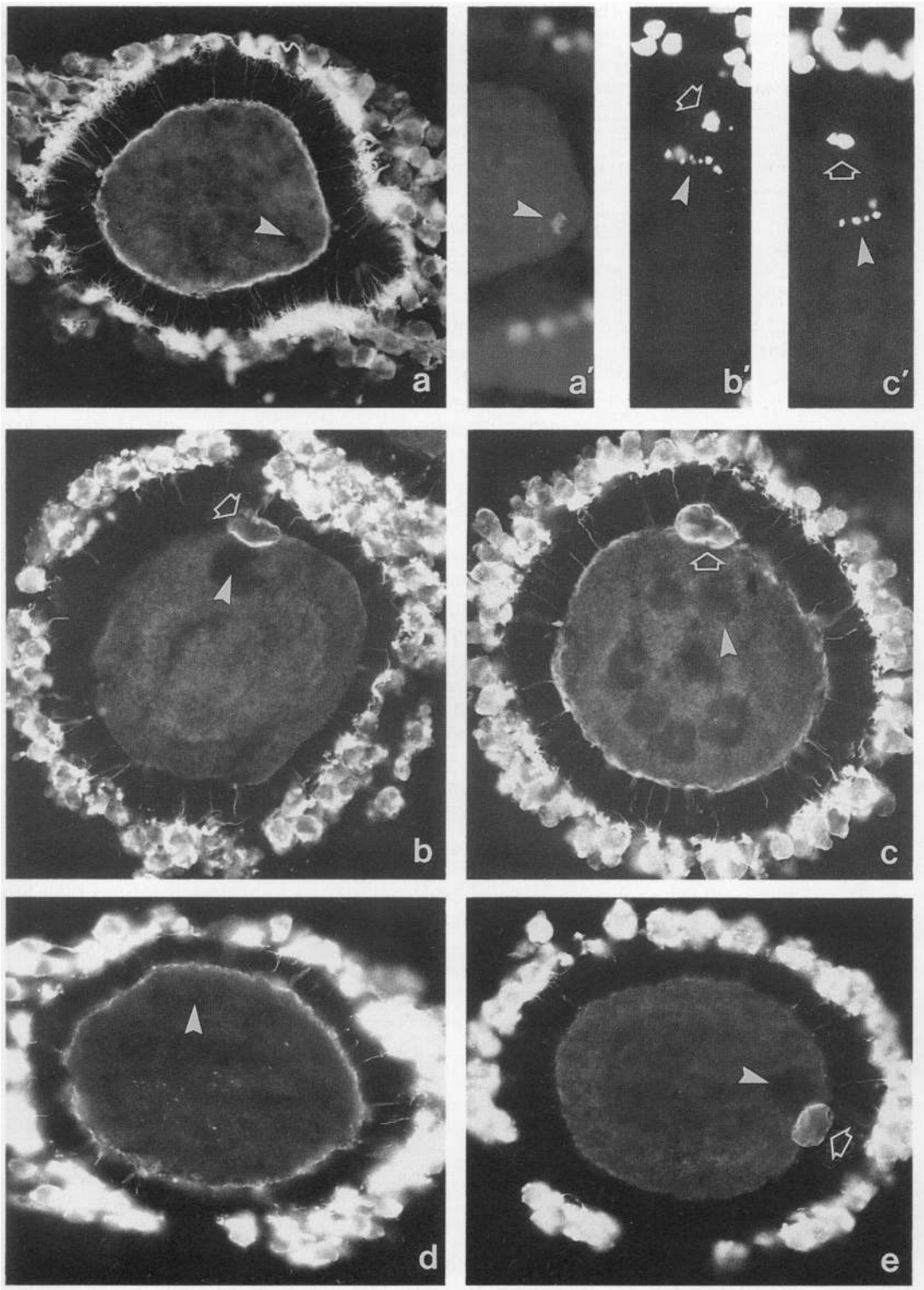
In the presence of dimethylsulphoxide (Fig. 1c) a polymerized actin band was still visible in the cortex. Although cortical focal areas appeared occasionally to be less intensely stained than the cortex of the control oocytes, clear evidence of dimethylsulphoxide action on F-actin distribution was never found.

Effect of freezing and thawing. After dehydration, freezing and thawing in the presence of sucrose without any permeating cryoprotectant (Fig. 1d), the cortical staining appeared to be less uniform than in control oocytes, a more intense, punctate staining pattern being observed. It therefore appears that cortical filamentous actin remains in oocytes which have been frozen without the addition of a permeating cryoprotectant.

After cryoprotective treatment and freezing in the presence of propanediol (Fig. 1e) no polymerized actin staining was visible in the cortex of oocytes frozen, but microfilaments were still present in the polar body.

Microtubule organization

Control oocytes. Oocytes were collected from the oviducts and extracted from the cumulus mass. They were either fixed immediately or they were stored in PBS + 10% FCS at room temperature for 30 min before fixation.



At the time of ovulation, rabbit oocytes are arrested at metaphase of the second meiotic division. If oocytes were fixed immediately, microtubules were located exclusively within the spindle (Fig. 2a) and chromosomes were arranged at the equatorial plate (Fig. 2a, inset). The meiotic spindle was located at the periphery of the oocyte and orientated radially with its long axis perpendicular to the plasma membrane. A similar microtubule distribution and spindle shape were obtained if oocytes were left at room temperaure for 30 min before fixation (not shown). This last group represents the real control for cryoprotectant-treated eggs as the period of 30 min corresponds to the duration of the cryoprotective treatment.

Effects of cryoprotective treatment at room temperature. In the presence of dimethylsulphoxide, microtubule organization was altered (Fig. 2b). The spindle shape was no longer defined, although microtubules were present around the chromosomes. Loss of an identifiable spindle was sometimes associated with the disorganization of chromosomes from the equatorial plate (Fig. 2b, inset) compared to control. Secondly, microtubules also appeared in the cytoplasm, in the form of multiple asters (Fig. 2b).

In the presence of propanediol (Fig. 2c), the spindle appeared to be expanded and was often associated with disorganization of chromosomes from the equatorial plate (Fig. 2c inset). A network of microtubules also appeared in the cytoplasm. However, unlike the situation after dimethylsulphoxide treatment, the microtubules never appeared organized as asters, but were distributed as a meshwork in the oocyte.

Comparing with the arrangement of chromosomes in the control oocytes, the displacement of chromosomes noted in most of the oocytes treated in the presence of propanediol or dimethylsulphoxide was never extensive. In fact, chromosomes appeared distributed around the initial metaphase plate.

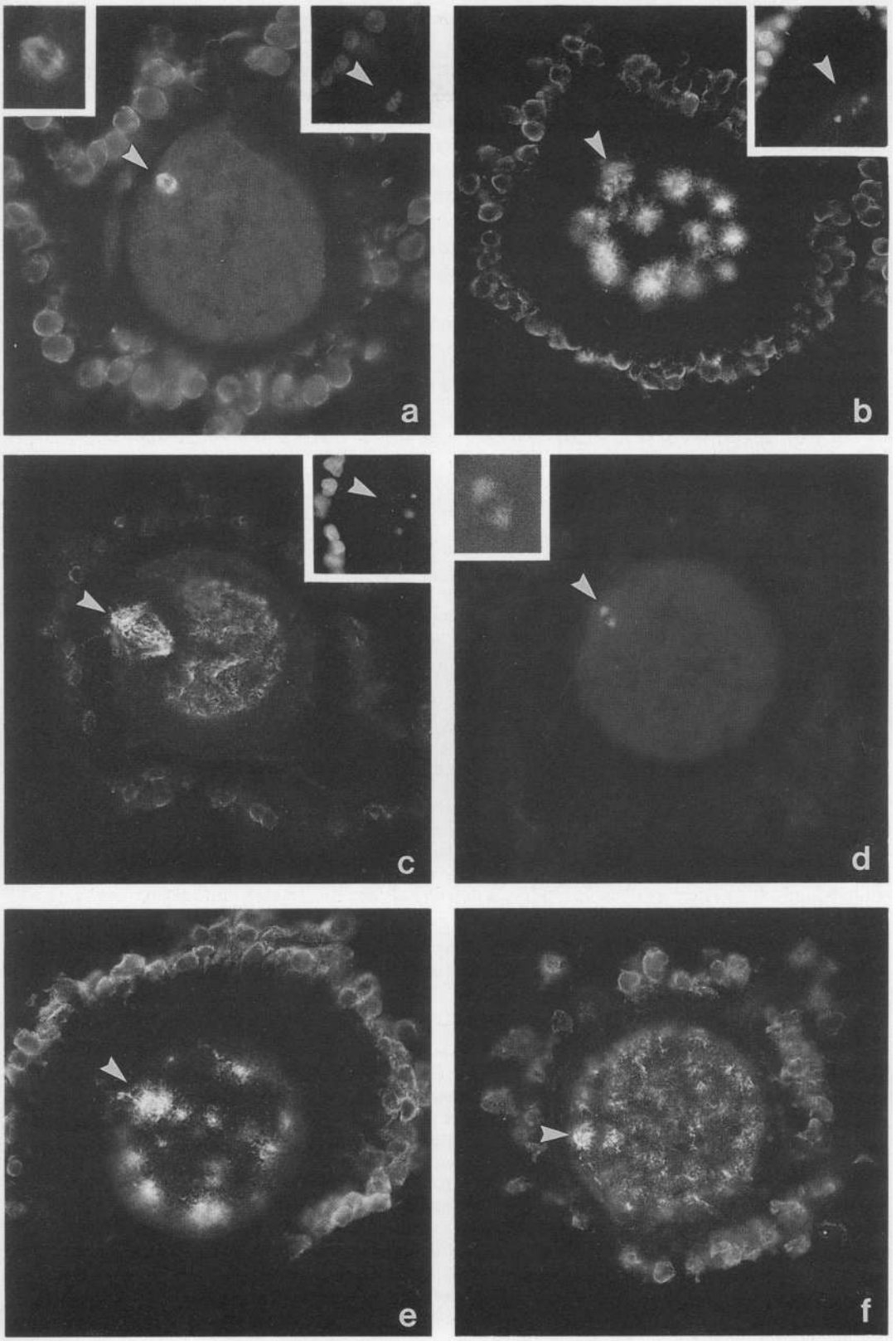
Effect of freezing and thawing. Microtubule organization was studied after cryoprotective treatment and a freezing–thawing procedure. Eggs were fixed immediately after thawing without removal of cryoprotectant.

Most oocytes frozen without permeating cryoprotectant but in the presence of sucrose had spindles which were reduced in size (Fig. 2d) and occasionally the spindle disappeared entirely (not shown). Similarly, no microtubules were observed in the corona cells.

In the presence of dimethylsulphoxide (Fig. 2e), microtubules were detected in large numbers around chromosomes, but as in eggs treated with dimethylsulphoxide at room temperature the spindle shape was no longer defined. Asters appearing after exposure to dimethylsulphoxide at room temperature seemed to be disrupted by freezing, but their presence was still obvious. Similarly, microtubules could be detected in corona cells.

In oocytes frozen in the presence of propanediol (Fig. 2f), the spindle was always still clearly visible, but it no longer had the shape of those observed in the control oocytes. Furthermore, many microtubule foci were well distributed all over the cytoplasm. Microtubules were also visualized in the corona cells.

Fig. 1. Rabbit oocytes examined for the distribution of microfilaments after staining with NBD–phallacidin (a, b, c, d, e) and staining of chromatin by Hoechst 33342 dye (a′, b′, c′). × 360. **(a,a′)** Control oocyte. Solid arrow indicates the position of the chromosomes organized on the second metaphase plate. **(b,b′)** Oocyte after propanediol treatment at room temperature. Solid arrow indicates the position of oocyte chromosomes. Open arrow indicates the position of the first polar body. **(c,c′)** Oocyte after dimethylsulphoxide treatment at room temperature. Solid arrow indicates the position of oocyte chromosomes. Open arrow indicates the position of the first polar body. **(d)** Oocyte after dehydration, freezing and thawing in the presence of sucrose without any permeating cryoprotectant. Solid arrow indicates the position of oocyte chromosomes (not shown). **(e)** Oocyte after freezing in the presence of propanediol. Solid arrow indicates the position of oocyte chromosomes (not shown). Open arrow indicates the position of the first polar body.



Reversibility of propanediol action. After freezing and thawing, propanediol was removed at room temperature (Fig. 3a). Immediate fixation revealed that microtubules were still present in the cytoplasm but much reduced in extent to those observed in oocytes fixed without propanediol removal (Fig. 2f). There was a reformation of microtubules (Fig. 3a) associated with the clusters of chromosomes of the oocyte and polar body (Fig. 3a').

Maintenance of oocytes at 37°C for 3 h after propanediol removal resulted in a complete disappearance of microtubules in the cytoplasm in all oocytes, and the restoration of a normal spindle in some of them (Fig. 3b, b'). However, when the chromosomes were not located in an equatorial plate, an abnormal spindle reformed (not shown).

In-vivo fertilization and development of frozen oocytes

After freezing and thawing, 91% of the eggs appeared morphologically normal and 83% of zonae pellucidae were intact. Only normal eggs with an intact zona were transferred to recipients and their development compared to that of control eggs transferred in the same conditions.

In Exp. 1, recipients were killed 24 h after transfer to evaluate the fertilization rate and the recovered embryos were cultured *in vitro* (Table 1). In each of the 10 females used, the oocytes produced by the remaining ovary were fertilized and all of them cleaved *in vitro* to the morula stage. Of 218 frozen oocytes transferred to the ovariectomized side, 200 were recovered of which only 39% ($n = 79$) had been fertilized *in vivo*. This percentage is significantly lower than that obtained with transferred control oocytes (81%). In-vitro development to the morula stage was also significantly lower for fertilized eggs obtained from frozen transferred oocytes compared to the development obtained with transferred control oocytes (31% *versus* 75%).

In Exp. 2, recipients were killed on Day 25 of gestation (Table 2). The presence of living fetuses in the horn ipsilateral to the remaining ovary was used to determine the number of pregnant recipients. Out of 18 females used, 14 became pregnant. Since all ovulated oocytes from the remaining ovary should be fertilized, we infer that some failure of conception or pregnancy occurred for reasons independent of transfer. Survival rate was therefore calculated only on pregnant recipients. For the 14 pregnant females, we assessed the survival rate (not shown) of oocytes from the remaining ovary as the number of live fetuses ($n = 34$) in the uterine ipsilateral horn divided by the number of mature follicles noted at the time of transfer ($n = 93$), i.e. 35%. Our transfer technique appears adequate as the survival rate of transferred control oocytes (32%, 12/38) (Table 2) was not different from that for oocytes from the remaining ovary. However, only 9% (9/105) (Table 2) of frozen oocytes developed into live fetuses and this survival rate was significantly lower than that of control oocytes.

The fertilization rate and the development rate as expressed by the number of live fetuses were reduced significantly in oocytes previously stored at −196°C.

Fig. 2. Rabbit oocytes examined for the distribution of microtubules after staining with anti alpha-tubulin (a, b, c, d, e, f). × 360. **(a)** Control oocyte. Right inset: chromosomes of the same egg stained by Hoechst 33342 dye. Left inset: magnification of the spindle (× 720). Solid arrow indicates the position of the chromosomes on the second metaphase plate. **(b)** Oocyte after dimethylsulphoxide treatment at room temperature. Inset: chromosomes of the same egg stained by Hoechst 33342 dye. Solid arrow indicates the position of the chromosomes. **(c)** Oocyte after propanediol treatment at room temperature. Inset: chromosomes of the same egg stained by Hoechst 33342 dye. Solid arrow indicates the position of the chromosomes. **(d)** Oocyte after dehydration, freezing and thawing in the presence of sucrose without any permeate cryoprotectant. Inset: magnification of the spindle. × 720. Solid arrow indicates the position of the chromosomes (not shown). **(e)** Oocyte after freezing in the presence of dimethylsulphoxide. Solid arrow indicates the position of the chromosomes (not shown). **(f)** Oocyte after freezing in the presence of propanediol. Solid arrow indicates the position of the chromosomes (not shown).

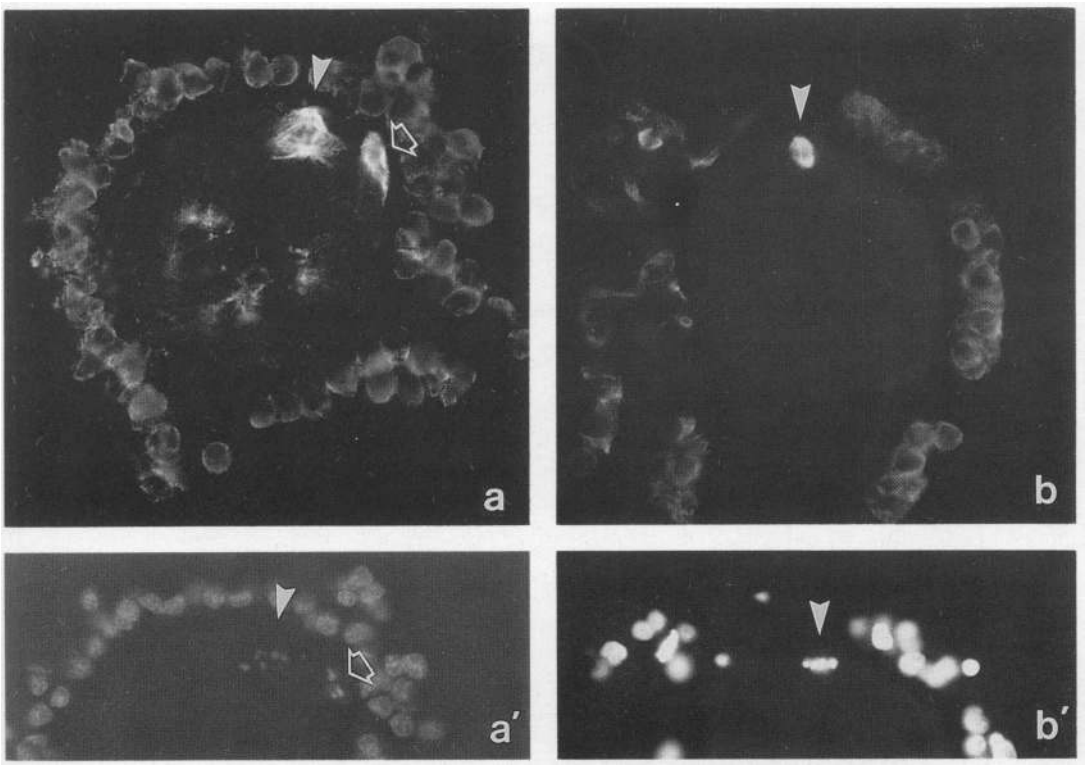


Fig. 3. Rabbit oocytes examined for the distribution of microtubules after staining with anti-alpha-tubulin **(a, b)** and staining of chromatin by Hoechst 33342 dye **(a′, b′)**. × 360. **(a,a′)** Oocyte frozen in the presence of propanediol and fixed immediately after thawing and propanediol removal. Solid arrow indicates the position of the chromosomes. Open arrow indicates the position of the first polar body. **(b,b′)** Oocyte frozen in the presence of propanediol and fixed after thawing, propanediol removal and 3 h of culture. Solid arrow indicates the position of the chromosomes on the second metaphase plate.

Table 1. The effect of freezing in the presence of propanediol on in-vivo fertilization and subsequent development *in vitro* of rabbit oocytes

Treatment	No. of oocytes transferred	No. of recipients	No. of oocytes: Found after flushing	No. of oocytes: Fertilized *in vivo* (%)	No. of oocytes: Developing into morulae *in vitro* (%)
Control	52	2	47	38 (81)	35 (75)
Frozen thawed	218	8	200	*79 (39)	†63 (31)

*$P < 0{\cdot}005$ ($\chi^2 = 24{\cdot}5$).
†$P < 0{\cdot}005$ ($\chi^2 = 27{\cdot}6$).

Table 2. The development into fetuses of rabbit oocytes frozen in the presence of propanediol and fertilized *in vivo*

Treatment	No. of oocytes transferred	No. of recipients	No. of recipients becoming pregnant	No. of live fetuses	Survival rate for pregnant females
Control	46	5	4	12	32 (12/38)
Frozen–thawed	136	13	10	9	*9 (9/105)

*$P < 0{\cdot}005$ ($\chi^2 = 10$).

Discussion

In the rabbit oocyte, microfilaments are organized in a uniform submembranous layer, with no increased concentration over the metaphase plate. This distribution has been noted in human (Pickering *et al.*, 1988) and sheep (Le Guen *et al.*, 1989) eggs. By contrast, ovulated mouse oocytes present two domains of microfilaments: in the non-microvillous region overlying the chromosomes there is a higher concentration of actin filaments than in the rest of the cortex (Maro *et al.*, 1984; Longo & Chen, 1985; Longo, 1987). This microfilament concentration is believed to be induced by the influence of the chromosomes (Maro *et al.*, 1986b; Van Blerkom & Bell, 1986).

In the mouse, the spindle is orientated tangentially to the plasma membrane and rotates at fertilization to become radially orientated before extrusion of the second polar body (Schatten *et al.*, 1985). By contrast in the rabbit, as in ruminant species (Thibault *et al.*, 1987; Le Guen *et al.*, 1989) and the human (Pickering *et al.*, 1988), no rotation is required as the spindle is already radially orientated before fertilization. These observations suggest that there is a correlation between the presence of a high concentration of microfilaments over the spindle and its rotation. In fact, in the mouse, intact microfilaments are required for spindle rotation which can be prevented by addition of cytochalasin D (Maro *et al.*, 1984). However, in all the species considered, microfilaments seem to be involved in anchorage of the spindle to the plasma membrane (Webb *et al.*, 1986; Le Guen *et al.*, 1989).

After propanediol treatment no polymerized actin is revealed in the rabbit oocyte. We have reported in a previous study a similar depolymerizing effect of propanediol on rabbit zygotes (Vincent *et al.*, 1988) that has also been confirmed using anti-actin antibody-staining (unpublished data). This effect of propanediol appeared to be rapidly reversible since the cortical microfilament network was reconstituted within 30 min after starting to wash out propanediol (Vincent *et al.*, 1989). The smaller effect of propanediol on polar bodies seen when treating oocytes or zygotes could be related to various factors such as a higher actin concentration or the stabilization of microfilaments by actin binding proteins in polar bodies (Stossel *et al.*, 1985).

Conversely, the microfilaments are still present after dimethylsulphoxide treatment. It has been shown that this solvent at similar concentration can have dramatic effects on actin organization in other cell types (Fukui, 1978; Fukui & Katsumaru, 1980; Sanger *et al.*, 1980a, b; Osborn & Weber, 1980).

Spindle microtubules appear to be less sensitive to low temperature in rabbit than mouse. Indeed, in rabbit oocytes exposed to room temperature (20–22°C) for 30 min, no changes in microtubule organization were detected. By contrast, exposure of mouse oocytes to 20–25°C for the same period produced several spindles of abnormal shape and multiple cortical asters (Pickering & Johnson, 1987; Van der Elst *et al.*, 1988).

At room temperature, addition of cryoprotectant (propanediol or dimethylsulphoxide) to rabbit oocytes leads to disorganization of spindle microtubules. In mouse oocytes, a similar effect of dimethylsulphoxide (Johnson & Pickering, 1987; Van der Elst *et al.*, 1988) and propanediol (Van der Elst *et al.*, 1988) has already been noted. In both species, a resulting dispersal of chromosomes is often seen. Less spindle disassembly is observed in the presence of solvents than is observed in the

presence of nocodazole (a drug which binds to and sequesters free tubulin, reducing the effective free tubulin concentration) (Maro *et al.*, 1986b). With nocodazole, chromosome dispersion around the cortex is microfilament-dependent (Maro *et al.*, 1986b). It seems that, in the presence of solvents, microfilaments do not mediate the observed dispersion of chromosomes for two reasons. First, this dispersion is less severe than that observed in the presence of nocodazole. Secondly, the dispersion takes place even in the presence of propanediol which depolymerizes microfilaments. We conclude that chromosome dispersion takes place solely because of spindle disruption.

Moreover, dimethylsulphoxide induces microtubule asters in the cytoplasm of rabbit oocytes as has been observed for mouse eggs in similar conditions (Johnson & Pickering, 1987; Van der Elst *et al.*, 1988). By contrast, microtubules formed in rabbit oocytes after propanediol treatment do not seem to be organized into asters as observed in mouse oocytes with the same cryoprotectant (Van der Elst *et al.*, 1988). It has already been demonstrated that solvent addition induces tubulin polymerization *in vitro*. Indeed, both glycerol (Lee & Timasheff, 1975) and dimethylsulphoxide (Himes *et al.*, 1977) induce self assembly of tubulin in the absence of associated proteins. Those two cryoprotectants lowered the critical protein concentration for assembly in microtubules (Lee & Timasheff, 1977; Algaier & Himes, 1988). Dimethylsulphoxide preferentially induces the nucleation process and causes the formation of much shorter and more numerous microtubules than those formed in its absence (Algaier & Himes, 1988).

In mouse oocytes, during metaphase the critical concentration for polymerization of tubulin is lower in the vicinity of chromosomes than in the rest of the cytoplasm (Maro *et al.*, 1985; Karsenti & Maro, 1986). It seems likely that, in the cytoplasm, solvents reduce the critical concentration of tubulin, changing transitorily the preferred polymerization from chromosomes to the rest of the cytoplasm. Indeed, microtubule asters appear associated with pericentriolar material in the cytoplasm of mouse oocytes (Maro *et al.*, 1985) and rabbit oocytes (unpublished data) if the critical concentration for tubulin assembly is reduced by taxol addition. Moreover, in mouse oocytes, if the concentration of free tubulin is reduced by nocodazole addition combined with dimethylsulphoxide addition, chromosomal control over polymerization is retained (Johnson & Pickering, 1987). These authors postulate that, when dimethylsulphoxide is added, the continuous turn-over of tubulin leads to the gradual unravelling of the spindle, whilst asters proliferate.

Spindle and cytoplasmic microtubules are still present in the rabbit oocyte which has been frozen in the presence of propanediol or dimethylsulphoxide although their organization seems to be disturbed. We assume that the addition of solvent reduces the deleterious effect of freezing on microtubules. In mouse oocytes, dimethylsulphoxide favours the polymerization of tubulin even at 4°C (Johnson & Pickering, 1987) and can protect the spindle against the depolymerization induced by freezing (Magistrini & Szollosi, 1980). It has been suggested that, once microtubules have been assembled in the presence of dimethylsulphoxide, associated proteins combine with them to protect against cold disruption (Magistrini & Szollosi, 1980; Johnson & Pickering, 1987). Dimethylsulphoxide has also been shown to exert a stabilizing effect at low temperature *in vitro* on microtubules made from purified tubulin (Himes *et al.*, 1976) and to lower their dissociation constant (Algaier & Himes, 1988).

In some mouse oocytes, the effects of dimethylsulphoxide appeared fully reversible because after removal of the solvent the spindle reformed and cytoplasmic asters disappeared (Johnson & Pickering, 1987; Van der Elst *et al.*, 1988). In a similar manner, we have shown that microtubules formed within the cytoplasm of rabbit eggs treated with propanediol progressively disappeared and an apparently normal spindle may be reformed 3 h after propanediol removal. On the contrary, in mouse eggs treated with propanediol, spindles rarely reform after solvent removal (Van der Elst *et al.*, 1988).

In the literature there is only one report of freezing and thawing of rabbit oocytes (Al-Hasani *et al.*, 1986). Using dimethylsulphoxide as cryoprotectant, the survival rate was low (1 blastocyst for 30 oocytes inseminated). It seems clear that the poor rates of fertilization and development of frozen oocytes may result in part from the incidence of abnormal spindles and dispersed

chromosomes. Glenister *et al.* (1987) found that the frequency of polyploid mouse embryos was increased by freezing oocytes in the presence of dimethylsulphoxide, while the incidence of aneuploidy is not increased by the same treatment.

In vivo 39% of frozen oocytes were fertilized compared to 81% for controls. After calculations taking into account the rate of fetal development obtained, we can argue that 22% of the zygotes from the frozen oocytes would develop into fetuses versus 39% for control. The greatest difference between control and frozen oocytes is registered during fertilization, suggesting that most the deleterious effects of freezing influenced this process adversely, as reported by Glenister *et al.* (1987) for mouse oocytes. The failure of oocyte fertilization after cryopreservation could arise from deleterious effects of cryoprotectant on the elements involved in fertilization. Johnson (1989) has shown that dimethylsulphoxide can reduce the sensitivity of the zona pellucida to digestion by chymotrypsin and that this effect is associated with a reduction in the fertilization rate.

Our study shows that cryoprotectants have a considerable effect on cytoskeletal organization in rabbit oocytes. We are convinced that these effects have important practical consequences for the cryopreservation of oocytes.

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