*J. Reprod. Fert.* (1990) **89**, 253–259

*Printed in Great Britain*


# The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present

C. Vincent, S. J. Pickering and M. H. Johnson

*Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK*

**Summary.** When mouse ovulated oocytes were exposed to 1·5 M-dimethylsulphoxide (DMSO) the resultant hardening of the zona pellucida was not a direct effect but required the presence of an oocyte. The hardening of the zona pellucida when zonae used were aged *in vitro* was also dependent upon the presence of the oocyte. Protocols of DMSO exposure that induce zona-hardening also caused depletion of the numbers of cortical granules underlying the oocyte surface, whereas protocols without effect on the zona did not reduce significantly the cortical granule count. It is proposed that the effects of DMSO may be mediated by a release of cortical granule contents.

*Keywords:* oocyte; zona pellucida; dimethylsulphoxide; fertilization; cryopreservation; cortical granule; ageing; mouse

## Introduction

The successful cryopreservation of oocytes would mark an important clinical advance in the treatment of infertility (Trounson, 1986; Friedler *et al.*, 1988). However, few live young have been delivered successfully after cryopreservation of mouse (Whittingham, 1977; Glenister *et al.*, 1987) or human (Chen, 1986; Van Uem *et al.*, 1987) oocytes. We have suggested previously that the failure of oocyte cryopreservation might lie just as much in the adverse effects of cooling and/or cryoprotectant during the pre- and post-freeze periods as in damage during the freezing and thawing processes themselves (Johnson, 1989). Oocytes cooled to 4°C in the absence of cryoprotectant show a reduced fertilization rate (Johnson *et al.*, 1988) and disrupted meiotic spindles with a resulting potential for aneuploidy (Magistrini & Szollosi, 1980; Pickering & Johnson, 1987). Similarly, exposure to the cryoprotectant dimethylsulphoxide (DMSO) under non-freezing conditions can increase the resistance of the zona pellucida both to sperm penetration and digestion by chymotrypsin, as well as disturbing the organization of the cytoskeleton and the metaphase plate. The nature, severity and reversibility of these latter effects depended on the protocol of exposure to DMSO (Johnson & Pickering, 1987; Johnson, 1989). As a result of these experiments, an optimal protocol for the addition of DMSO before freezing and for its removal after thawing has been proposed (Johnson, 1989).

In this paper, we describe experiments designed to identify the mechanism underlying the effect of cooling and DMSO exposure on the zona pellucida.

## Materials and Methods

***Oocyte recovery and handling.*** Oocytes were recovered from MFI mice (Cambridge University Laboratory Animal Centre) by induction of superovulation with intraperitoneal injection of 5–10 i.u. PMSG followed 45–50 h later by

5–10 i.u. hCG intraperitoneally (Intervet, Cambridge, UK). Animals were killed, 3–6 at a time, 12·5 h after the hCG injection. The oviducts were transferred immediately to phosphate-buffered saline (pH 7·4) prewarmed to 37°C and then into Medium H6 + BSA (a Hepes-buffered variant of medium T6 as described by Howlett *et al.*, 1987) in which the cumulus mass was released. The cumulus masses were exposed to 0·1 mM-hyaluronidase (Sigma Chemical Co., Poole, Dorset, UK) for 2–5 min, and the freed oocytes were transferred to fresh Medium H6 + BSA while they still had a residual corona of cumulus cells which dispersed subsequently in the washing medium. Oocytes were pooled, counted and distributed among control and experimental groups as indicated. Unless otherwise stated, all manipulative procedures were carried out on the heated stage of a Wild M5 dissecting microscope and dishes were retained between manipulations at 37°C on warming blocks or in an incubator. These procedures were adopted to minimize temperature fluctuations and stress to the oocytes. For experimental treatments, oocytes were transferred at time zero to Medium H6 + BSA in cavity blocks pre-equilibrated at the desired temperature and DMSO concentration according to schedules described in the 'Results' section.

***Isolation of zonae pellucidae.*** It seemed possible that, during the preparation of isolated zonae, leakage of enzymes from the oocyte could lead to premature hardening of the zona pellucida. Accordingly (unless stated otherwise in 'Results') to reduce the activity of any released zona-hardening enzymes, oocytes were cooled to 10°C in Medium H6 × BSA, a temperature which has been shown previously to be without effect on the zona sensitivity to chymotrypsin (Johnson *et al.*, 1988). Half of this group of oocytes was passed rapidly up and down a finely pulled, heat-polished glass micropipette. As each zona cracked open, it was rinsed rapidly free of any contaminating oocyte material and accumulated in a group to one side of the dish of Medium H6 + BSA. The remaining half of the group of zona-enclosed oocytes was transferred intact by a wider bore micropipette to a position adjacent to the isolated zonae. The groups of isolated zonae and zona-enclosed oocytes were then transferred back to 37°C before experimental use.

***Zona resistance to chymotrypsin.*** Zonae were exposed to freshly prepared alpha-chymotrypsin (Sigma Type II, 0·001% in Medium H6 + BSA; Boldt & Wolf, 1986) at 37°C. Unless otherwise indicated, zonae expanded within 2 min of being placed in the enzyme and within 5 min all zonae could be sorted into those being resistant (non-expanded/non-dissolved) and those being sensitive (expanded/dissolved). These groups remained clearly distinguishable during further exposure to enzyme. Therefore, the proportion of resistant zonae after 5 min exposure to chymotrypsin is recorded in the 'Results'.

***Transmission electron microscopy.*** Zona-intact oocytes, exposed to various experimental conditions as described in the 'Results', were fixed, post-fixed, dehydrated and embedded for transmission electron microscopy (TEM) as described by Pickering *et al.* (1988). Samples were embedded at the base of the tip of a beem capsule in a compact group of 10–20 aligned in the same plane. The resin was allowed to polymerize at 60°C for at least 48 h. A block was prepared with the oocytes just below, and in the same plane as, the block face. Ultrathin sections were sampled from this face on a Reichert Ultracut as follows. First, the knife was advanced in 0·5 μm sections into the block until a grazing section through all the oocytes was achieved. The knife was then advanced 20 μm into the block of 1 μm steps, a procedure shown in pilot studies to reach the middle third of each oocyte (which is 60–70 μm diameter). Ultrathin sections for mounting were taken at this level, the total number taken never exceeding a thickness of 1 μm. One section from this series was then used for cortical granule counts. To ensure that no cortical granule was sampled more than once, the knife was advanced a further 5 μm and a further set of sections sampled. This procedure was repeated once more until ultrathin sections from 3 different levels from the middle third of the oocytes in each block had been collected. The diameters of the sectioned oocytes were all between 60 and 70 μm. Sections were mounted on celloidin-coated slotted copper grids and double stained with lead citrate and uranyl acetate. Sections were viewed on a Philips EM300 transmission electron microscope. The number of cortical granules located beneath the plasmalemma of each oocyte profile was counted in 3 sections at different levels and the mean value calculated. Oocyte profiles under different conditions were compared by Student's *t* test on paired samples using the Statworks program (Cricket Software Inc, Malvern, PA 19355, USA).

## Results

### The oocyte is required for the zona hardening effect of DMSO

A total of 276 isolated zonae and 304 oocyte-enclosing zonae (from 6 separate experiments) were each divided into two approximately equal sized groups. One group of oocytes and isolated zonae was placed into 1·5 M-DMSO in Medium H6 + BSA at 37°C for 30 min, whilst the other group was placed in Medium H6 + BSA alone. All zonae and oocytes were then rinsed thoroughly through 2–3 washes of Medium H6 + BSA before exposure to chymotrypsin. Of the controls, 75% of 140 isolated zonae and 77% of the 145 oocyte-enclosing zonae were sensitive to chymotrypsin. Of the DMSO-exposed zonae, 60% of 136 isolated zonae and 7% of the 159 oocyte-enclosing zonae were sensitive to chymotrypsin.

In a second series of 3 experiments, slightly different protocols were introduced. Some oocytes with intact zonae were placed in DMSO at 37°C and the zonae of approximately 25% of these oocytes were isolated and recovered at 10°C (see 'Materials and Methods') before removal from DMSO and rinsing. The remaining oocytes were removed from the DMSO and a second group of zonae were isolated from some of them. As before, control zonae in Medium H6 + BSA at 37°C were sensitive to chymotrypsin (56% of 117 isolated zonae, 61% of 150 oocyte-enclosing zonae; these values are less than in the first series and lower than usual in controls, suggesting that some effect of lysing oocyte contents on zonae may have occurred). However, the zonae that had been isolated whilst in DMSO were relatively insensitive to chymotrypsin (19% of 91). As expected, those zonae enclosing oocytes after exposure to DMSO and those zonae removed from oocytes after removal from DMSO were also insensitive to chymotrypsin (12% of 217 and 7% of 101 respectively).

Zona hardening has also been described after the ageing of oocytes *in vitro* or *in vivo* (Wolf & Hamada, 1976; Defelici & Siracusa, 1982; Gianfortoni & Gulyas, 1985; Johnson, 1989; Dodson *et al.*, 1989). To determine whether the presence of the oocyte was also required in the mediation of this effect, zonae were either isolated as above or, in controls, were left enclosing oocytes, and then cultured *in vitro* for up to 56 h, before exposure to chymotrypsin. The results are shown in Table 1. It is clear that, as the time in culture increases, the percentage of isolated zonae insensitive to chymotrypsin remained the same, whilst zonae that enclosed oocytes showed increasing resistance to the enzyme.

**Table 1.** Hardening of the mouse zona pellucida during ageing *in vitro*

| Time *in vitro* (h) | % Isolated zonae resistant to chymotrypsin (no. examined) | % Oocyte-enclosing zonae resistant to chymotrypsin (no.) |
|---|---|---|
| 4 | 22 (96) | 13 (78) |
| 9 | 30 (53) | 31 (61) |
| 26 | 31 (145) | 68 (145) |
| 32 | 24 (98) | 65 (98) |
| 50 | 28 (47) | 88 (49) |
| 56 | 30 (69) | 88 (100) |

These results indicated that hardening of the zona pellucida, whether by ageing or DMSO exposure, required the presence of an enclosed oocyte. We therefore examined oocytes exposed to DMSO in an attempt to determine what role the oocyte might have in mediating the effect of the cryoprotectant and ageing on the zona.

**Exposure to certain protocols of cooling and DMSO reduces the cortical granule count**

Various protocols for addition of DMSO to and its removal from oocytes were examined previously and some are without apparent effect on the zona sensitivity to chymotrypsin, whilst others reduce it (Johnson, 1989). We compared the cortical granule counts of control oocytes with those in 3 protocols that reduce zona sensitivity and in 3 that do not affect it. Figure 1 shows that a significant reduction ($t$ test) in granule count occurred when oocytes were placed in medium pre-cooled to 4°C for 30 min (M4), in 1·5 M-DMSO at 37°C for 30 min (D37) and in 1·5 M-DMSO at 37°C for 30 min followed by removal from DMSO to medium (D37,M37). Each of these 3 treatments has been shown to remove sensitivity to chymotrypsin and to block fertilization (Johnson *et al.*, 1988; Johnson, 1989). In contrast, no significant reduction in cortical granules occurred

after exposure of oocytes to 1·5 M-DMSO at 4°C for 30 min (D4), to 1·5 M-DMSO at 4°C for 30 min followed by return to DMSO-free medium at 37°C (D4,M37) or to 1·5 M-DMSO at 4°C for 30 min followed by exposure to medium free of DMSO at 4°C and then return to 37°C (D4,M4,M37). None of these 3 treatments reduced the sensitivity of zonae to chymotrypsin (Johnson, 1989).

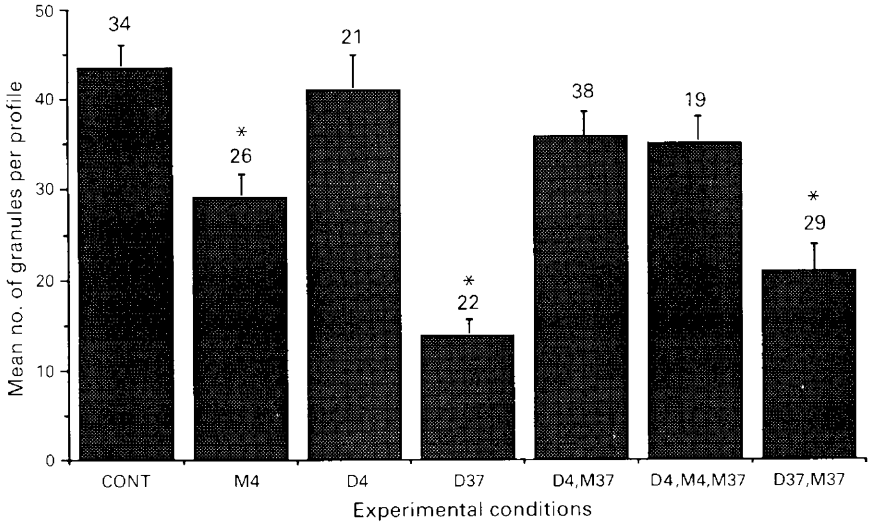


**Fig. 1.** Mean ± s.e.m. numbers of granules per oocyte profile under different conditions. The numbers above each bar represent the numbers of oocytes sampled. Cont = control; M4 = 30 min at 4°C; D4 = 30 min in 1·5 M-DMSO at 4°C; D37 = 30 min in 1·5 M-DMSO at 37°C; D4,M37 = 30 min in 1·5 M-DMSO at 4°C followed by transfer to rinsing medium at 37°C for 30 min; D4,M4,M37 = 30 min in 1·5 M-DMSO at 4°C followed by transfer to rinsing medium for 30 min at 4°C followed by transfer to medium at 37°C for 30 min; D37,M37 = 30 min in 1·5 M-DMSO at 37°C followed by transfer to rinsing medium at 37°C for 30 min. $*P \leqslant 0{\cdot}001$ compared with control.

These results indicate a relative reduction of cortical granules in the cortex of oocytes exposed to DMSO at 37°C. There was no clear evidence for the central migration of cortical granules away from the cortex. However, we did observe ultrastructural evidence of cortical granules and their contents in the subzonal space of DMSO-treated oocytes, whereas in control oocytes this was a rare occurrence (compare Figs 2a & b). These cortical granules in DMSO-treated samples were often intact and surrounded by a layer of oolemma (Fig. 2b); less frequently, evidence of the more conventional process of fusion and extrusion of granules was seen (Fig. 2c). It was not possible to estimate the proportion of granules extruded by each mechanism, since cortical granules extruded by fusion are not readily identifiable ultrastructurally when the fusion process has been completed.

## Discussion

The results of our previous studies on the effect of cooling and DMSO on the cytoskeletal organization, zona properties and fertilization rates of oocytes (Johnson & Pickering, 1987; Pickering & Johnson, 1987; Johnson *et al.*, 1988; Johnson, 1989) have led us to the conclusion that the schedule with the least deleterious consequences for cryopreservation of mouse oocytes is to equilibrate oocytes in 1·5 M-DMSO precooled to 4°C before freezing and then to remove DMSO at 4°C after thawing before restoring the oocyte to 37°C (Johnson, 1989). Our results indicated that

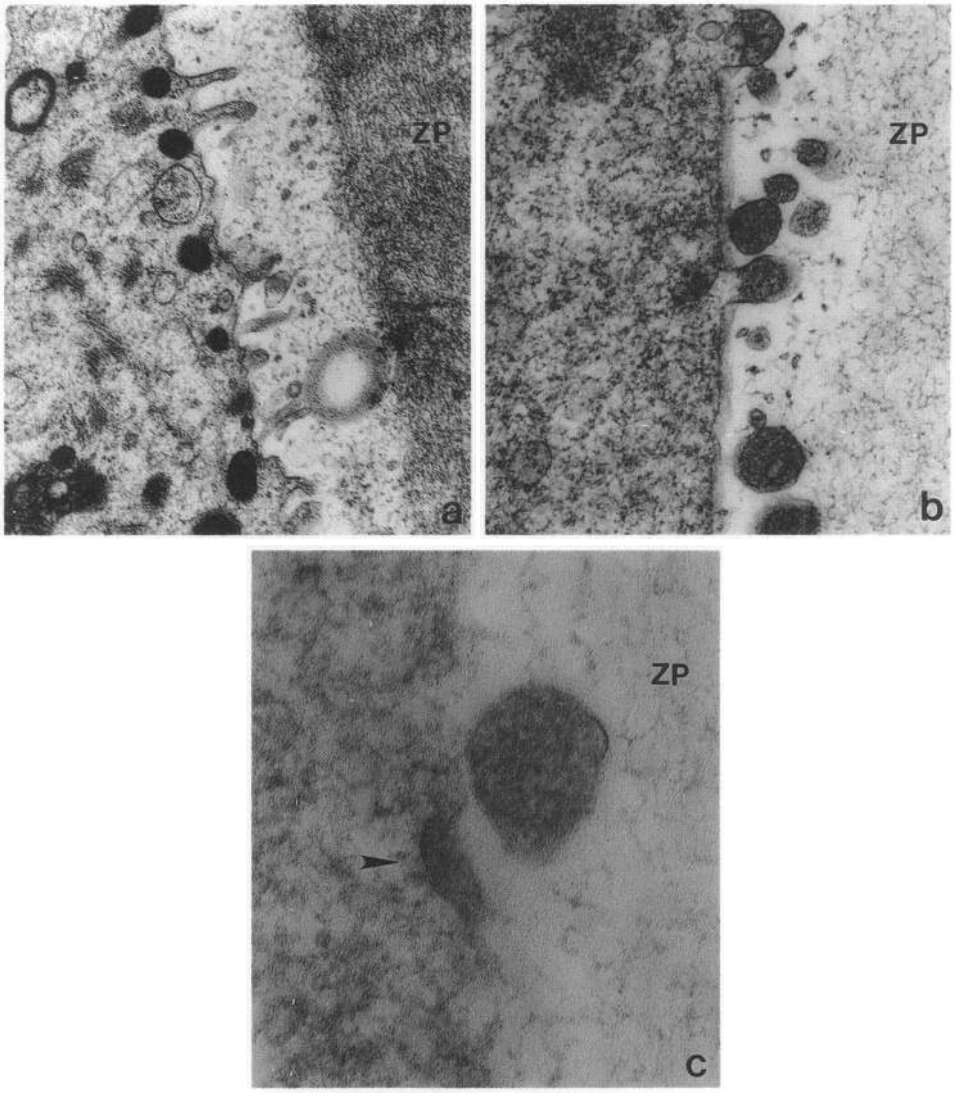


**Fig. 2.** Electron micrographs of oocytes. **(a)** Control oocyte. Cortical granules are subjacent to the oolemma ( × 18 750). **(b, c)** Oocytes treated in 1·5 M-DMSO at 37°C for 30 min. (b) Notice cortical granules surrounded by membrane in the subzonal space ( × 31 000). (c) Recently released cortical granule, showing the characteristic membrane thickening (arrow) in the oolemma at the site of release ( × 138 000). ZP = zona pellucida.

cooling to 4°C in the absence of cryoprotectant and exposure to cryoprotectant at temperatures between 20 and 37°C both produced adverse effects on the zona pellucida, fertilization rate and spindle/chromosome organization.

Our results indicate that the adverse effects of cryoprotectant on the zona are mediated largely if not entirely via the oocyte itself. The development of resistance to chymotrypsin induced by the DMSO in zonae around intact oocytes was almost completely lacking when isolated zonae were exposed to DMSO. Moreover, the results of the experiments in which zonae were removed after being placed in DMSO but before removal from DMSO show that the DMSO–oocyte interaction leading to zona hardening occurs during the addition of the cryoprotectant. Our earlier results had

also suggested that the hardening of the zona occurred during the addition of DMSO (Johnson, 1989). A requirement for the oocyte in the generation of zona hardening is observed not only in the response to DMSO but also after ageing. Moreover, the inhibition of sperm penetration of the zona after exposure to phorbol esters also requires the presence of an oocyte (Endo *et al.*, 1987), suggesting that perhaps a common mechanism might operate in each case.

Zona hardening after fertilization or activation seems to involve release of cortical granules (Wolf & Hamada, 1977; Gulyas & Yuan, 1985). Our results show that the numbers of cortical granules decline at low temperature or after exposure to DMSO at 37°C. Dodson *et al.* (1989) also correlated the zona hardening in ageing mouse oocytes with a reduced cortical granule count. The loss of granules could therefore be implicated in the effects of all these conditions on the zona. In support of this conclusion, the 3 protocols without marked effect on the zona did not reduce the cortical granule count significantly. The reduced cortical granule counts in the cortex of oocytes might be explained by their release into the perivitelline space but could also result from their migration deeper into the cell (Szollosi, 1971; Gulyas, 1974). In our study, no ultrastructural evidence of central migration was found, but evidence of release of cortical granule contents as well as some intact granules (Szollosi, 1967; Longo, 1975) into the subzonal space was seen. However, correlative observations cannot in themselves provide proof of causality and we are at present investigating this question further. Finally, if the release of cortical granule contents is responsible for the zona changes elicited by DMSO or cooling, neither the mechanism of release of granule contents nor the number or subpopulation of granules affected need be identical to those which are involved at fertilization.

We thank Martin George and Brendan Doe for technical assistance and John Bashford and colleagues for photographic work. This work was supported by a Medical Research Council programme grant to M.H.J. and P. R. Braude and by a grant to C.V. from La Fondation de la Recherche Medicale.

## References

**Boldt, J. & Wolf, D.P.** (1986) An improved method for isolation of fertile zona-free mouse eggs. *Gamete Res.* **13,** 213–222.

**Chen, C.** (1986) Pregnancy after human oocyte cryopreservation. *The Lancet* **i,** 884–886.

**DeFelici, M. & Siracusa, G.** (1982) Spontaneous hardening of the zona pellucida of mouse oocytes during in vitro culture. *Gamete Res.* **6,** 107–113.

**Dodson, M.G., Minhas, B.S., Curtis, S.K., Palmer, T.V. & Robertson, J.L.** (1989) Spontaneous zona reaction in the mouse as a limiting factor for the time in which an oocyte may be fertilized. *J. In Vitro Fert. Embryo Transfer* **6,** 101–106.

**Endo, Y., Schultz, R.M. & Kopf, G.S.** (1987) Effects of phorbol esters and a diacylglycerol on mouse eggs: inhibition of fertilization and modification of the zona pellucida. *Devl Biol.* **119,** 199–209.

**Friedler, S., Giudice, L.C. & Lamb, E.J.** (1988) Cryopreservation of embryos and oocytes. *Fert. Steril.* **49,** 743–764.

**Gianfortoni, J.G. & Gulyas, B.J.** (1985) The effects of short term incubation (aging) of mouse oocytes on in vitro fertilization, zona solubility and embryonic development. *Gamete Res.* **11,** 59–68.

**Glenister, P.H., Wood, M.J., Kirby, C. & Whittingham, D.G.** (1987) Incidence of chromosome anomalies in first cleavage mouse embryos obtained from frozen-thawed oocytes fertilized in vitro. *Gamete Res.* **16,** 205–216.

**Gulyas, B.J.** (1974) Cortical granules in artificially activated (parthenogenetic) rabbit eggs. *Am. J. Anat.* **140,** 577–582.

**Gulyas, B.J. & Yuan, L.C.** (1985) Cortical reaction and zona hardening in mouse oocytes following exposure to ethanol. *J. exp. Zool.* **233,** 269–276.

**Howlett, S.K., Barton, S.C. & Surani, M.A.H.** (1987) Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. *Development* **101,** 915–924.

**Johnson, M.H.** (1989) The effect on fertilization of exposure of mouse oocytes to dimethylsulfoxide: an optimal protocol. *J. In Vitro Fert. Embryo Transfer* **6,** 168–175.

**Johnson, M.H. & Pickering, S.J.** (1987) The effect of dimethylsulphoxide on the microtubule system of the mouse oocyte. *Development* **100,** 313–324.

**Johnson, M.H., Pickering, S.J. & George, M.A.** (1988) The influence of cooling on the properties of the zona pellucida of the mouse oocyte. *Human Reprod.* **3,** 383–387.

**Longo, F.J.** (1975) Ultrastructural analysis of artificially activated rabbit eggs. *J. exp. Zool.* **192,** 87–112.

**Magistrini, M. & Szollosi, D.** (1980) Effects of cold and isopropyl-N-phenylcarbamate on the second meiotic

spindle of mouse oocytes. *Eur. J. Cell Biol.* **22,** 699–707.

**Pickering, S.J. & Johnson, M.H.** (1987) The influence of cooling on the organisation of the meiotic spindle of the mouse oocyte. *Human Reprod.* **2,** 207–216.

**Pickering, S.J., Maro, B., Johnson, M.H. & Skepper, J.N.** (1988) The influence of cell contact on the division of mouse 8-cell blastomeres. *Development* **103,** 353–363.

**Szollosi, D.** (1967) Development of cortical granules and the cortical reaction in rat and hamster eggs. *Anat. Rec.* **159,** 431–446.

**Szollosi, D.** (1971) Morphological changes in mouse eggs due to aging in the fallopian tube. *Am. J. Anat.* **130,** 209–226.

**Trounson, A.** (1986) Preservation of human eggs and embryos. *Fert. Steril.* **46,** 1–12.

**Van Uem, J.F., Siebzehnrubl, E.R., Schuh, B., Koch, R., Trotnow, S. & Lang, N.** (1987) Birth after cryopreservation of unfertilized oocytes. *The Lancet* **1,** 752–753.

**Whittingham, D.G.** (1977) Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at −196°C. *J. Reprod. Fert.* **49,** 89–94.

**Wolf, D.P. & Hamada, M.** (1976) Age-dependent losses in the penetrability of mouse ova. *J. Reprod. Fert.* **48,** 213–214.

**Wolf, D.P. & Hamada, M.** (1977) Induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs with cortical granule exudate. *Biol. Reprod.* **17,** 350–354.

**Received 5 September 1989**