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Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an in-vitro fertilization programme

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Mouse oocytes and embryos were obtained following ovulation induction of (C57B16 × CBA) F_1 animals. Zonae pellucidae were exposed to α-chymotrypsin in phosphate-buffered medium (PB1) supplemented with 3 mg/ml bovine serum albumin upon a heated stage (37°C) and were observed constantly through an inverted microscope. The endpoint of the bioassay was the limits of the zona no longer being seen clearly at ×200 magnification, and the time taken for each zona to dissolve was recorded. A dose-dependent response in dissolution time was clearly seen, with 1% α-chymotrypsin being chosen as the routine working solution. Cryopreservation of 2-cell mouse embryos using propanediol did not cause zona hardening but induced a small and significant softening, as gauged by the time taken for zona dissolution (2181 ± 167 versus 1864 ± 82 s). Zona hardening was not suspected to occur after the freezing of human embryos as there was no difference in implantation rates per embryo for in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment cycles between fresh [IVF: 63/644 (9.7%); ICSI: 51/330 (15.5%)] and frozen embryos [IVF: 36/458 (7.9%); ICSI: 18/112 (16.1%)]. Conversely, significant hardening of the zonae of mature oocytes was seen following cryopreservation (747 ± 393 s) compared with freshly ovulated oocytes (151 ± 68 s). It is concluded that (i) the freezing of murine oocytes with propanediol results in zona hardening, implying a possible benefit of ICSI after the cryopreservation of human oocytes, and (ii) the cryopreservation of embryos is not associated with zona hardening or reduced implantation, making microdissection of the zona in such cases generally unwarranted.

Key words: α-chymotrypsin/cryopreservation/human/mouse/zona pellucida

Introduction

Cryopreservation has now become an established procedure in the storage of gametes and embryos for the future treatment of patients (Matson *et al.*, 1995). Unfortunately, the cryopreservation of oocytes has been associated with low success in achieving pregnancies and results have shown reduced fertilization in both the mouse (Carroll *et al.*, 1990) and the human (Gook *et al.*, 1994) presumably due to changes in the zona pellucida (ZP) (Carroll *et al.*, 1990). These changes in the ZP appear to be overcome by micromanipulation techniques (Carroll *et al.*, 1990; Kazem *et al.*, 1995), although a net increase in the number of oocytes fertilizing and dividing is not always seen (Gook *et al.*, 1995). However, much work is still needed in identifying the specific effects of different cryopreservation protocols (Carroll *et al.*, 1993), and an experimental model for systematic evaluations would be invaluable.

Similarly, the cryopreservation of embryos does seem to have an effect as results from the national registers of the UK (Human Fertilisation and Embryology Authority, 1995) and the USA (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine, 1996) for activity in 1994 showed an overall live birth rate per embryo transfer of 21.1% (7462/35285) for fresh embryos but a significantly reduced rate of 14.3% (1386/9694) for frozen embryos. Changes in the ZP would seem an attractive explanation for this since the assisted hatching of thawed embryos has been claimed to improve pregnancy rates (Tucker *et al.*, 1991; Check *et al.*, 1996), although other factors may well be influential.

Methods to investigate changes in the human ZP after cryopreservation are often not practical since they are time-consuming and expensive, such as electron microscopy (Calafell *et al.*, 1992), would use valuable human oocytes and embryos that are much needed clinically within donation programmes (Horne *et al.*, 1993), or are illegal because of the irreversible damage done to the embryo (Yovich and Matson, 1996). The aims of the present study were therefore to (i) establish a bioassay for the digestion of murine zonae as an index of hardness, (ii) determine the effect of cryopreservation upon the ZP of murine oocytes and 2-cell embryos, and (iii) use the information obtained from the animal model to formulate a policy on the need for micromanipulation of the ZP after the cryopreservation of human oocytes and embryos.

Materials and methods

Murine oocyte and embryo recovery

Murine oocytes were recovered by ovulation induction of (C57B16×CBA) (Animal Resource Centre, Murdoch, W.Australia) F_1 mice given 10 IU i.p. of pregnant mare's serum gonadotrophin (PMSG, Folligon; Intervet, Cambridge, UK) followed 50 h later by 10 IU human chorionic gonadotrophin (HCG, Profasi; Serono

Laboratories, Frenchs Forest, NSW, Australia). Animals were killed by cervical dislocation 16 h after the HCG administration and the ampullae transferred immediately into phosphate-buffered medium (PB1) supplemented with 3 mg/ml bovine serum albumin (BSA) for release of the cumulus masses. The masses were exposed to 0.1% hyaluronidase (Sigma Chemical Co., St Louis, MO, USA) for 2–5 min to disperse the cumulus cells, and the denuded oocytes were pooled and cultured in human tubal fluid medium (HTFM; Quinn *et al.*, 1985) containing 3 mg/ml BSA in microdroplets under oil (BDH paraffin oil, 0.22 μm filtered).

Two-cell embryos were collected using the stimulation protocol described above and females were placed with F_1 males overnight immediately following the HCG injection. The males were removed the next morning and the females examined for vaginal plugs. The females were killed by cervical dislocation 40 h after the HCG injection, after which the oviducts were collected into PB1 containing BSA and flushed through with medium. The 2-cell embryos were pooled and cultured in HTFM microdroplets with BSA under oil.

Patient selection and implantation of embryos

Women were treated by conventional IVF for a range of disorders including occlusion of the Fallopian tubes, endometriosis and ovulatory disorders (Yovich *et al.*, 1986). Those with supernumerary embryos worthy of cryopreservation then returned for replacement of thawed embryos. Intracytoplasmic sperm injection (ICSI) was performed in cases of previous failure of oocytes to fertilize, or in cases of severe sperm disorders.

Thawed human embryos were transferred on 207 consecutive occasions in either a natural cycle or one in which the endometrium was primed with exogenous oestradiol valerate (Progynova; Schering Pty Ltd., Alexandria, NSW 2015, Australia) and progesterone (Proluton; Schering Pty Ltd.). Gestational sacs were confirmed at delivery, and the implantation rate calculated by dividing the total number of embryos transferred by the number of sacs present. Cycles were analysed separately depending on whether the original cycle was IVF or ICSI. Pregnancy and implantation rates were compared against the IVF and ICSI cycles undertaken in the same period for which fresh embryos were transferred.

Cryopreservation of oocytes and embryos

The same protocol was used for the murine oocytes and all the human and murine embryos, with cells being frozen in 0.25 ml straws and stored in liquid nitrogen. A modification of the method described by Lassalle *et al.* (1985) was used involving propanediol and sucrose dissolved in PB1 supplemented with 20% human serum. The modifications were that the freeze was done with 0.1 M sucrose exclusively, the straws were taken down only to a temperature of –140°C before plunging into liquid nitrogen, and 0.2 M sucrose was present in all solutions during thawing. Murine embryos were frozen at the 2-cell stage as mentioned above, whereas the human embryos were frozen on day 2 or 3 after oocyte collection, i.e. at the 4–8-cell stage, having even blastomeres and minimal fragmentation. The human embryos had ⩾50% of the blastomeres intact to be said to have survived and to be transferred.

Zona digestion by α-chymotrypsin

Zonae digestions were carried out on an inverted microscope (Diaphot-TDM, Nikon, Japan) fitted with a stage warmer set to 37°C and observed using bright field (×40, ×100) and Hoffman modulation (×200) optics. Zonae were exposed to α-chymotrypsin dissolved in PB1 with BSA, and the endpoint of the assay was when the limits of the zonae were no longer clearly defined under ×200 magnification.

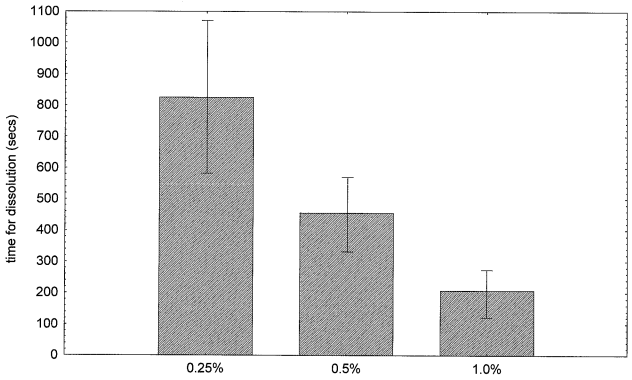

Figure 1. The time taken for zonae pellucidae of freshly ovulated oocytes to dissolve in 0.25% ($n = 4$), 0.5% ($n = 5$) and 1.0% ($n = 4$) solutions of α-chymotrypsin.

Table I. The time taken to dissolve totally the zonae pellucidae of murine oocytes and 2-cell embryos

Cryopreservation of mouse cells	Dissolution time (s)	
	Control	Frozen
Unfertilized oocytes	151 + 68 ($n = 35$)	747 + 393 ($n = 17$)**
2-cell embryos	2181 + 167 ($n = 10$)	1864 + 82 ($n = 9$)*

*$P < 0.002$; **$P < 0.00005$.

Statistics

Dissolution times were recorded as mean ± 1 SD, and groups compared using Student's *t*-test. The proportion of human embryos implanting and the pregnancy rates were compared using the χ^2-test. Differences were considered significant if $P < 0.05$.

Results

Optimal enzyme concentration

In an attempt to identify the optimal working concentration, mature ovulated murine oocytes were incubated in 0.25, 0.5 and 1% chymotrypsin solution. The results are shown in Figure 1. A working solution of 1% was chosen for future experiments, to keep the dissolution time within practical limits.

Cryopreservation of murine oocytes and embryos

The effect of cryopreservation upon the time for digestion of the murine zonae pellucidae is given in Table I. The cryopreservation of oocytes was associated with a significant 5-fold increase in digestion time. However, the cryopreservation of murine 2-cell embryos resulted in a small but significant reduction in dissolution time.

There was wide variation in the digestion times for each control group, confirming the need to minimize batch variation and to use appropriate controls in each experiment.

Implantation of human frozen–thawed embryos

The proportion of embryo transfers resulting in a viable pregnancy, and the rate of implantation of individual embryos,

Table II. The implantation of fresh and frozen–thawed human embryos in an in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) programme

Treatment	Birth rate (%)	Implantation rate (%)
IVF		
Fresh	47/234 (20)	63/644 (9.7)
Frozen	37/166 (22)	36/458 (7.9)
ICSI		
Fresh	38/118 (32)	51/330 (15.5)
Frozen	14/41 (34)	18/112 (16.1)

Birth rate = births/no. of transfer procedures; implantation rate = embryos implanted/total no. of embryos transferred.

are given in Table II. It can be seen that there was no difference in either the pregnancy rate or implantation rate when comparing the fresh or frozen embryos for IVF and ICSI treatment cycles. There were significant differences for both parameters between the IVF and ICSI cycles, although the role of patient selection giving an advantage to the ICSI group of women cannot be excluded.

Discussion

Enzymatic digestion of the zona pellucida has been used in the past to identify the presence of various substrates and to elucidate the chemical composition of the ZP before and after fertilization (Smithberg, 1953; Gwatkin, 1964) and between different strains of mice (Krzanowska, 1972). However, conflicting information has occasionally been found regarding the potency of different enzymes to digest the zonae, suggesting differences in the purity and properties of individual enzyme preparations. For instance, early work found trypsin and chymotrypsin to be effective in dissolving the murine ZP but not papain (Smithberg, 1953), whereas a later report found exactly the reverse situation (Gwatkin, 1964). Nevertheless, more recent reports have shown α-chymotrypsin to be effective and of use as a bioassay of zona hardness (Johnson *et al.*, 1988).

The present study has used the enzymatic digestion of the murine ZP to help determine changes taking place after cryopreservation, with a view to formulating and justifying treatment strategies to be introduced in the IVF programme. As with many groups around the world, we are under increasing pressure to seek approval from overseeing bodies before any modifications to our basic protocols can be made; presently we are accountable to our Institutional Ethics Committee, the State regulatory body (the Reproductive Technology Council) and then the national regulatory body (the Reproductive Accreditation Committee) (Yovich and Matson, 1996). The first stage of assay optimization was to find a suitable concentration of α-chymotrypsin that would result in digestion within a reasonable time. There was a dose-dependent reduction in total dissolution time, but a concentration of 1% w/v was found to dissolve unfertilized oocytes in a matter of minutes (Figure 1), which was felt to be a manageable time. This is a different approach to that taken elsewhere (Johnson *et al.*, 1988) in which oocytes were placed in a much lower α-chymotrypsin concentration of 0.001% and oocytes were examined after only 5 min for an initial expansion; the results in that study were then presented as the proportion of oocytes becoming expanded after the 5 min.

The cryopreservation of oocytes offers tremendous ethical advantages over embryos, although much work is still needed in this area to improve the results. The achievement of good fertilization rates is the first hurdle, and some workers have taken the step of performing ICSI on thawed human oocytes (Kazem *et al.*, 1995) albeit based on very little factual evidence. The present study has shown a clear hardening of the ZP after freezing with the standard propanediol/sucrose protocol used routinely with embryos (Table I). The fertilization rate of oocytes has remained low in other reports, even in cases in which survival is good and the cryopreservation method has been optimized mathematically (Karlsson *et al.*, 1996). The cryopreservation of pre-antral follicles with the subsequent in-vitro maturation of the enclosed oocytes appears to be a promising approach (Cortvrindt *et al.*, 1996), although much work is still needed in this area. The mechanism of zona hardening of cryopreserved oocytes seems uncertain, with some believing that premature cortical granule release is responsible (Vincent *et al.*, 1990) while others disagree (Wood *et al.*, 1992). Nevertheless, the hardening effect can be prevented by the use of fetal bovine serum (George *et al.*, 1992) with fetuin appearing to be the active component (Schroeder *et al.*, 1990), although human serum, as used in the present study, is ineffective (George and Johnson, 1993). The effects of cryoprotectants *per se* and cooling have been reviewed by Bernard and Fuller (1996). Interestingly, propanediol is becoming increasingly popular for freezing oocytes because of its reduced toxicity (Gook, *et al.*, 1993) even though dimethylsulphoxide (DMSO) was the cryoprotectant used most widely in the early studies (Chen, 1988). It is therefore proposed that the use of ICSI to achieve fertilization within an oocyte cryopreservation programme using propanediol would be justified in view of the increased zona hardness.

The cryopreservation of embryos has now been a successful part of assisted reproductive technology. Looking at results globally, there appears to be a signficant reduction in the pregnancy rates following transfers of thawed embryos compared to fresh embryos in the USA (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine, 1996) and UK (Human Fertilisation and Embryology Authority, 1995) although this is not seen in Australia (Lancaster *et al.*, 1995). Reports have appeared suggesting that partial zona dissection or assisted hatching may be beneficial in improving pregnancy rates following the transfer of frozen–thawed embryos (Tucker *et al.*, 1991; Check *et al.*, 1996), presumably based on the notion that cryopreservation had some effect upon the ZP. The results of the present study have not shown any hardening of the ZP of murine frozen 2-cell embryos, and even a slight softening. This would indicate that there is no further effect of the cryopreservation process over and above those changes which take place at fertilization, and that micromanipulation of thawed embryos as a blanket policy is unwarranted. Examination of the results from our IVF programme (Table II) has confirmed that surviving frozen embryos from both IVF and ICSI have a similar implantation potential to their fresh counterparts, and

that any attempts by laboratories to improve results after cryopreservation should be directed towards an improvement in the basic technique rather than using methods to attempt to circumvent a fundamental problem.

In conclusion, the present study has shown the value of an animal model in acquiring information upon which clinical strategies can be based. Specifically, the cryopreservation of oocytes was associated with zona hardening, making ICSI a logical addition to the IVF protocol for these oocytes, whereas no hardening of the ZP was seen after embryo cryopreservation, thereby implying assisted hatching procedures to be unwarranted.

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