The protective action of polyvinylpyrrolidone-Percoll during the cryopreservation of mouse 2-cell embryos and its effect on subsequent developmental potential post-thaw in vitro and in vivo

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The effects of cryopreservation, in media containing (FS3+) or omitting (FS3) polyvinylpyrrolidone (PVP) in the form of Percoll (PVP-Percoll), on the survival of 2-cell mouse embryos was studied. Survival and zona pellucida disruption post-thaw, growth (assessed by in-vitro culture until the blastocyst stage) and development in vivo (assessed by implantation and living fetus rates and the birth of live progeny) were all investigated. Initial post-thaw survival showed no statistically significant difference (P > 0.05) between FS3+ (91.1 ± 9.8%) and FS3 (84.5 ± 6.6%). However, there was a statistically significant (P < 0.05) reduction in the incidence of zona damage when the freezing solution contained PVP-Percoll compared to the control (3.6 ± 1.0 and 8.7 ± 0.6% respectively) and a statistically significant (P < 0.05) greater number of embryos developing in vitro to the blastocyst stage (84.8 ± 7.1 and 72.3 ± 6.1% respectively). The rates of implantation were not significantly different: 72.2 ± 7.0% for FS3+ and 51.2 ± 30.7% for the non-frozen control group. The percentage of live fetuses was also similar between the experimental and control groups: 27.4 ± 10.6 and 24.3 ± 11.3% respectively. We conclude that the presence of polymers can protect embryos against cryoinjury and that PVP in the form of PVP-Percoll provides a non-toxic alternative to PVP in its native form, during the cryopreservation of mouse 2-cell embryos.

Key words: cryopreservation/embryos/mouse/Percoll/polyvinylpyrrolidone

Introduction

The storage of embryos in the majority of in-vitro fertilization (IVF) units is achieved via commercially available computer-controlled cell-freezing systems. Cryopreservation exerts many stresses upon an embryo, of which one important physical manifestation is zona disruption. The prevention of this mechanical breakdown during cryopreservation has been the focus of much work.

The use of external polymeric cryoprotectants was reported in early work by Meryman (1966) on the cryopreservation of human erythrocytes. This suggested that membrane damage due to denaturation during the adverse conditions and osmotic stresses imposed by freezing and thawing may be reduced by the presence of such molecules. This principle has subsequently been applied to embryo freezing, the aim being to protect the zona pellucida against fracture and other damage resulting from osmotic stresses (Dumoulin et al., 1994). These workers investigated the occurrence of cryoinjury due to physical factors, and the protective effects of polymers during the cryopreservation of human and mouse zonae pellucidae and embryos, employing the widely used freezing protocol first described by Testart et al. (1986). They investigated the effect of adding or omitting extracellular polymeric cryoprotectants [dextran, polyvinylpyrrolidone (PVP) and Ficoll] on the incidence of zona damage and assessed the toxicity of these polymers on subsequent embryo growth.

The results presented by Dumoulin et al. (1994) showed that, although the presence of PVP during the freezing and thawing process prevented zona and cell damage as efficiently as the other polymers tested, a relatively short exposure of 2-cell mouse embryos to this agent was clearly deleterious to their subsequent development to the blastocyst stage. The recorded toxicity of PVP (Wilmut, 1972, Fahy et al., 1984; Bergers et al., 1993) has probably contributed to its poor usage in this field.

Our previous study (Titterington et al., 1995) showed that PVP in the form of Percoll could be used beneficially during a vitrification protocol for 8-cell mouse embryos, when used in a cryosolution also containing glycerol and sucrose. It was also shown that 8-cell mouse embryos equilibrated in Percoll showed no statistically significant difference in survival between solute concentrations of 25 and 75% (v/v) Percoll but did show a significant (P = 0.0286) increase in survival between 25 and 100% Percoll. These results therefore indicated that Percoll has no deleterious effect on 8-cell embryos and, at 100% (v/v), may even facilitate survival. A significant reduction (P = 0.0079) in zona damage was found in the presence of macromolecules and also a significant reduction (P = 0.0079) when Percoll replaced human serum albumin. Unlike the results obtained by Dumoulin et al. (1994), there was no statistically significant difference recorded in subsequent embryo development to the expanded and hatched blastocyst stages. These results supported the hypothesis that PVP in the form of PVP-Percoll retains its protective ability against physical damage to the zona pellucida of mouse 8-cell embryos during cryopreservation. In addition, it was concluded that PVP-Percoll does not appear to adversely affect developmental potential after vitrification.

Although vitrification systems may eventually have a place in the cryostorage of human embryos, most IVF units presently employ conventional freezing systems, generally similar to
of London Ltd., London, UK) containing integral polyvinyl alcohol

straw as shown in Figure 1, before sealing with plastic plugs (Rocket

in Table I. No more than five embryos were loaded into each freezing

plugs. The final compositions of the freezing solutions are summarized

of 10-15 min, at room temperature (~22°C), and then to either FS3

Embryo cryostorage procedure

Embryos were cryopreserved according to the procedure described

by Testart et al. (1986) with and without the addition of Percoll

(Percoll, Gibco). Test embryos were washed in freezing solution 1 [FS1:

PBS + 20% HSA, propylene glycol (PROH, Sigma)] for an equilibration period

PBS + HSA, sucrose (Sigma) containing Ham's F-10 medium

and in-vitro development

Embryo and in-vitro development

Embryo recovery was recorded for each straw and the embryos placed in Ham’s F-10 supplemented with 0.4% BSA at 37°C in a humidified incubator for at least 4 h prior to examination. The embryos were then examined under a phase-contrast microscope at ×300 magnification to assess any damage to the zona pellucida that had occurred during the freezing and thawing processes. The embryos were gently rotated using a mouth pipette during examination and damage assessed as described by Dumoulin et al. (1994). A count of initial survival was also recorded: defined as at least one blastomere of the embryo remaining intact following freezing, thawing and 4 h incubation. Blastocyst formation was evaluated following in-vitro culture in Medicult IVF culture medium (Imperial Laboratories Ltd., Andover, Hants, UK) at 37°C in a humidified atmosphere of 5% CO2. This was termed ‘test survival’ and defined as the percentage of intact 2-cell embryos after freezing and thawing able to develop to the blastocyst stage

In-vivo development

CBA/C57, F1 hybrid, 10 week old female mice weighing ~20 g were used as foster mothers because of their recorded success for establishing pseudopregnancy. Pseudopregnancy was induced by making use of the Whitten effect (Whitten, 1956) and mating the females with vasectomized males overnight. The next morning the females were checked for the presence of vaginal copulation plugs; this day was designated day 1 of pseudopregnancy. A few minutes prior to surgery the recipients were anæsthetized with a 1.2% solution of Avertin (Hogan et al., 1986; Wood et al., 1987), injected i.p. at a dose of 0.02 ml/g of body weight. Thawed test embryos and control, non-frozen embryos were allowed to develop to the expanded or hatched blastocyst stages of development prior to asynchronous transfer to recipients at day 3 of pseudopregnancy. No fewer than five embryos were transferred to each uterine horn of each recipient. Test embryos were always replaced into the right horn and control embryos into the contralateral horn (Wood et al., 1987; Trounson

that described by Testart et al. (1986). The aim of the present study was to investigate the cryoprotective action of PVP-Percoll in these latter freezing regimens.

Materials and methods

Source and collection of embryos

Female CBA/C57 F1 hybrid mice, 6-8 weeks old, were injected i.p

with 7 IU pregnant mares’ serum gonadotrophin (PMSG; Intervet,

UK Ltd., Cambridge, UK), stimulating follicle development. Approximately 48 h later they were injected, again i.p., with 7 IU human chorionic gonadotrophin (HCG, Chorulon; Intervet), inducing ovulation. They were then paired overnight with proven males of the strain CBA/C57 and examined the next morning for the presence of a vaginal copulation plug. Exactly 48 h after the HCG injection, the animals were killed. The oviducts were removed and 2-cell embryos flushed and handled in HEPES-buffered Ham’s F-10 medium (Gibco, Paisley, Scotland) and cultured at 37°C in four-well plates (Nunclon, Life Technologies, Paisley, Scotland) containing Ham’s F-10 medium supplemented with 0.4% (w/v) bovine serum albumin (BSA, Fraction V powder, embryo culture tested; Sigma Chemical Company Ltd., Poole, Dorset, UK) in an humidified atmosphere of 5% CO2 in air.
Table II. Protection provided by polyvinylpyrrolidone–Percoll (in solution FS3+) during the cryopreservation of mouse 2-cell embryos and its effect on subsequent embryonic developmental potential after thawing

<table>
<thead>
<tr>
<th>Test solution*</th>
<th>Recovery</th>
<th>Initial survivalb</th>
<th>DZ</th>
<th>Test survivalc</th>
<th>Control survivald</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>100</td>
<td>50</td>
<td>D</td>
</tr>
<tr>
<td>FS3+</td>
<td>104/105</td>
<td>99.0</td>
<td>94</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>98/101</td>
<td>97.0</td>
<td>63</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>100/100</td>
<td>100.0</td>
<td>88</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>122/126</td>
<td>96.8</td>
<td>108</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>98.2 ± 1.6</td>
<td>91.1</td>
<td>± 9</td>
<td>36</td>
<td>± 9</td>
</tr>
</tbody>
</table>

HB = hatched blastocyst; EB = expanded blastocyst; PEB = partial expanded blastocyst; D = degenerate

*FS3+ contains PVP-Percoll; FS3 does not. See Table I for composition of solutions.

bThe number and percentage of embryos with at least one intact blastomere, 100 and 50 indicate the presence of the total or half the blastomere complement respectively.

cThe number and percentage of intact 2-cell embryos after freezing and thawing in either test solution developing to the blastocyst stage.

dThe number and percentage of untreated embryos developing to the blastocyst stage.

*P < 0.05 (Mann-Whitney) comparing FS3+ with control (FS3) group.

et al., 1988, Wilson and Quinn, 1989; Chedid et al., 1992, Ishmon et al., 1992, Vasuthevan et al., 1992) to eliminate the possibility that a failure to become pregnant could be attributable to a maternal factor (Trounson et al., 1988). Recipients were killed at day 15 of gestation and the number of implantation sites and viable fetuses for each horn was recorded (Whittingham, 1979; Massip et al., 1984; Scheffen et al., 1986, Rail et al., 1987, Wilson and Quinn, 1989, Krzyminska and O'Neill, 1991; Chedid et al., 1992; Goto et al., 1992; Ishmon et al., 1992; Vasuthevan et al., 1992; Ishimori et al., 1993). The fetuses were dissected from the gestational sacs and their morphological aspects recorded before being weighed individually on an analytical balance (Chedid et al., 1992). Certain recipients received experimental transfer of frozen-thawed embryos into both uterine horns and these pregnancies were allowed to continue to term. After delivery the offspring were assessed for morphological anomalies and their reproductive potential was investigated further by mating them (Chedid et al., 1992), breeding being stopped after one litter. This was carried out because chromosomal anomalies occurring after manipulation are known to impair fertility, particularly in males (Gordon et al., 1989).

Statistics
Differences between treatment groups were analysed for significance using the statistics package SPSS for Microsoft Windows, release 6.0. No assumptions of normality were made regarding the data. Because data were presented as percentages, the non-parametric Mann-Whitney U-test for comparisons was used when comparing independent samples, and the non-parametric Wilcoxon signed rank test when comparing the distribution of related samples. The level of statistical significance was set at P < 0.05.

Results
Embryo damage and development in vitro
As can be seen from Table II there was no statistically significant difference between the recovery of embryos, or their initial survival, from straws containing the control freezing solution FS3 or the test solution FS3+. There was a significant reduction in zona damage recorded when the freezing solution contained PVP-Percoll, compared to the control solution without it, and the proportion of surviving embryos developing to the blastocyst stage was found to be significantly higher when the freezing solution contained PVP-Percoll. The corrected survival rate (after comparing each treatment group with embryos which had not been frozen) confirmed the above results (Table II).

Embryos in which zona disruption was evident were found to have damaged blastomeres. These embryos were seen to be completely degenerate within 12–24 h of recovery, and none developed to the blastocyst stage. Zona disruption was not experienced in the group of embryos which were cultured without prior freezing.

In-vivo developmental capacity
All fetuses developed from experimental embryos (frozen in FS3+) developed at the same rate as embryos not subjected to freezing and were morphologically indistinguishable from them. No abnormal fetuses were found. Implantation rates and the proportion of viable fetuses, with their mean weight following transfer of the frozen-thawed, experimental, and non-frozen, control embryos, are shown in Table III. A higher rate of implantation was found in the experimental group when compared to the control group (72.2 ± 7.0 and 51.2 ± 30.7% respectively). However, these were not significantly different (P > 0.05). The proportion of viable fetuses was statistically similar for the two groups (27.4 ± 10.6 and 24.3 ± 11.3% respectively). In addition, the mean weight of the fetuses was statistically similar in the two groups and no macro-morphological abnormalities were observed in any of the fetuses recovered.
Figure 2. Twenty-four day old mice developed from 2-cell embryos preserved in solution containing polyvinylpyrrolidone-Percoll (solution FS3+).

Table III. Implantation and fetus formation rates following transfer of embryos frozen using FS3+ solution and control embryos that had not been frozen. Mann–Whitney analysis did not show a statistically significant difference between the treatment groups.

<table>
<thead>
<tr>
<th></th>
<th>No of transfers performed</th>
<th>No of pregnancies</th>
<th>No of embryos transferreda</th>
<th>No of implantation sites</th>
<th>No of viable fetuses</th>
<th>Overall implantation rateb</th>
<th>Mean weight of fetuses (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS3+</td>
<td>8</td>
<td>6</td>
<td>40 (6.7 ± 0.5)</td>
<td>18 (44.8 ± 10.6)</td>
<td>11 (27.4 ± 10.6)</td>
<td>29 (72.2 ± 7.0)</td>
<td>0.345 ± 0.07</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>6</td>
<td>41 (6.8 ± 0.4)</td>
<td>11 (27.0 ± 26.3)</td>
<td>10 (24.3 ± 11.3)</td>
<td>21 (51.2 ± 30.7)</td>
<td>0.5 ± 0.05</td>
</tr>
</tbody>
</table>

aNumber in parentheses is the mean ± SEM of embryos transferred per recipient becoming pregnant.
bNumber in parentheses is the mean % ± SEM.

Table IV. Live birth rate following transfer of embryos frozen using FS3+ solution or control embryos that had not been frozen. Mann–Whitney analysis did not show a statistically significant difference between the treatment groups.

<table>
<thead>
<tr>
<th></th>
<th>No. of transfers performed</th>
<th>No of recipients that delivered live young</th>
<th>No of embryos transferred to pregnant recipientsb</th>
<th>Live birth rateb</th>
<th>Litter size ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS3+</td>
<td>7</td>
<td>5 (71.4%)</td>
<td>68 (13.6 ± 0.9)</td>
<td>18 (26.4 ± 11.8)</td>
<td>3.8 ± 1.7</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>6 (50.0%)</td>
<td>73 (12.2 ± 1.8)</td>
<td>25 (34.1 ± 10.1)</td>
<td>4.2 ± 1.5</td>
</tr>
</tbody>
</table>

aNumber in parentheses is the mean ± SEM of embryos transferred per recipient becoming pregnant.
bNumber in parentheses is the mean % ± SEM.

Fewer live young were born in the experimental group (26.4 ± 11.8%) compared to the control group (34.1 ± 10.1%) (Table IV), but this difference was not found to be statistically significant (P > 0.05). One pup was found dead the day after birth (mortality 5.6%), but this and all the other pups, both test and control, showed no morphological abnormalities (Figure 2). The progeny of the live offspring all manifested normal reproductive performance in terms of litter size, and their sex ratio was ~50:50.

Discussion

Rall and Polge (1984) made cryomicroscopical observations of 8-cell mouse embryos frozen and thawed in glycerol. They observed that a series of interconnecting ‘fracture-planes’ formed in the extracellular ice matrix during the freezing process and that, when these planes passed through an embryo, cracked or broken zonae were found upon thawing. They therefore postulated that these fracture planes may be responsible for observed zona damage. They also suggested that fracturing may result from mechanical stresses caused by a temperature gradient within the ice crystals, or by differences in the thermal expansion of adjacent materials such as ice crystals and container walls.

This damage may be alleviated by the inclusion of high molecular weight polymeric cryoprotectants. Franks et al. (1977) reported the maintenance of biological ultrastructure when these molecules were used at low concentrations in the freeze-preservation of various biological materials. Three polymers, PVP, hydroxyethyl starch (HES) and dextran were used...
chosen for investigation of the nature of freezing, vitrification, recrystallization and melting. The polymers were found to inactivate ice nuclei in the extracellular solution, limiting the size of ice crystals formed, which may go some way towards explaining the action of polymers in embryo cryopreservation. Echlin et al. (1977) and Skaaer et al. (1977) investigated the physiological effects of these non-penetrating cryoprotectants on plant and animal cells, during ultrastructural and analytical studies of frozen tissues. The polymers were generally found to show no substantial interference with cell function, and it was concluded that they can play an important role in the preservation of cell ultrastructure during morphological and analytical studies.

Whittingham (1971) reported the first successful use of PVP for mouse 8-cell and early blastocyst embryo freezing, but found that embryos did not survive longer than 30 min and that 2-cell embryos did not survive at all. Unpublished results (cited in Whittingham, 1971) showed that if a concentration of PVP >1% was adopted, further embryo development was greatly affected. As a consequence, use of PVP was subsequently avoided in favour of dimethysulphoxide and glycerol (Whittingham et al., 1972).

Leibo and Oda (1993) found that PVP [7.5 or 15% (w/v)] enhanced the cryoprotective properties of ethylene glycol at varying concentrations. Survival rates of up to 95% were reported for mouse zygotes and 8-cell embryos frozen rapidly in these solutions. The PVP could be used as supplied commercially, as exposure to ova and embryos, for up to 60 min, provided no evidence of toxicity and made purification by dialysis unnecessary. These results are in contrast to work reported initially in an abstract by Bergers et al. (1993) and later more extensively by Dumoulin et al. (1994). This latter study investigated the action of polymers as cryoprotective agents as assessed by both embryo survival (2-cell) and zona damage (ZD). The action of three polymeric cryoprotectants was assessed: dextran 70 (survival, 90%; ZD, 5 ± 2%), Ficoll (survival, 82%; ZD, 9 ± 4%) and PVP (survival, 12%; ZD, 11 ± 4%), and compared to a control with no polymer (survival, 80%; ZD, 25 ± 9%). This clearly demonstrated a protective action against physical zona pellucida damage during cryopreservation by each polymer investigated. Although PVP was found to prevent ZD as well as the other polymers tested during freezing and thawing, it was found to be deleterious to the subsequent development of mouse 2-cell embryos after a relatively short exposure time.

Our present research was undertaken to assess whether PVP in the form of PVP-Percoll (Titterington et al., 1995) could be used in a conventional freezing protocol. Analyses were performed to determine its effect on both embryo survival and damage to the zona pellucida post-thaw and subsequent embryo survival during in-vitro culture, as compared to controls frozen in the absence of extracellular polymer (FS3), and in-vivo developmental capacity as compared to that of controls which had not undergone freezing.

Recovery rates from freezing straws and initial survival rates were not significantly different when FS3+ (98.2 ± 1.6 and 91.1 ± 9.8% respectively) was compared to the control solution FS3 (98.2 ± 1.4 and 84.5 ± 6.6% respectively). The use of PVP-Percoll resulted in significantly less zona damage when compared to the standard freezing solution (3.6 ± 1.0 and 8.7 ± 0.6% respectively, $P < 0.05$) and resulted in a significantly increased rate of corrected survival when compared with the control, no polymer, group (84.8 ± 7.1% and 72.3 ± 6.1% respectively, $P < 0.05$). These results for zona damage are considerably lower than those reported by Dumoulin et al. (1994). This may be attributable to slight differences either in the method of damage assessment or in the cryopreservation procedure utilized by Dumoulin et al. (1994), who used PBS supplemented with 10% human inactivated serum, direct immersion into liquid nitrogen when a chamber temperature of 30°C was attained and performed thawing by holding the straws at room temperature for 1 min.

The rates of implantation and development of embryos frozen using FS3+ reported here are comparable with those of Chedid et al. (1992), who also transferred embryos at the early blastocyst stage of development after post-thaw in-vitro culture. The rates of implantation are also comparable with those of Nowshari et al. (1995), who transferred pronuclear-stage embryos at the 2-cell stage of development to the oviducts on day 1 of pseudopregnancy, although these workers reported higher developmental rates. A similar comparison can be made with the results produced by Shaw et al. (1995), who transferred frozen-thawed expanded or hatched blastocysts to pseudopregnant recipients after ~2 h culture in vitro.

The implantation rates, number of viable fetuses and their mean weights were similar for the test and control groups discussed, showing that embryos frozen in FS3+ displayed normal development in vivo. This survival may be improved by transfer at an earlier stage of development, so that less time is passed in in-vitro culture before transfer to recipients at an earlier stage of pseudopregnancy. We can draw no firm conclusions about the one pup from the test group that died. However, the mother showed no tendency to nurture it after birth and it showed no macro-morphological abnormalities. Rejection by the mother is occasionally encountered in our breeding stock and so is not attributable to the fact that this pup was developed from a frozen-thawed embryo.

Our results demonstrate that PVP-Percoll may be advantageous to mouse embryo survival during a conventional freezing programme, by reducing the extent of physical cryoinjury such as zona disruption. Furthermore, PVP-Percoll was shown to have no deleterious effect on the subsequent pre-implantation in-vitro development, or in-vivo development, of the frozen-thawed mouse 2-cell embryos. We conclude that PVP in the form PVP-Percoll provides a non-toxic alternative to PVP in its native form. However, this study provided no evidence to suggest that the behaviour of PVP-Percoll is identical to that of PVP in its native form. It is possible that the cryoprotective action can be attributed to a separate, unknown characteristic of the Percoll solution.

In conclusion, the present study has shown that it is possible to cryopreserve 2-cell mouse embryos using a conventional preservation regime with the inclusion of PVP-Percoll in the freezing medium. This method provides a significantly reduced incidence of zona damage and increased corrected survival following in-vitro culture after thawing, when compared to...
control embryos frozen in the absence of PVP-Percoll. The in-vivo developmental potential of both frozen and non-frozen control embryos was found to be impaired due to in-vitro culture before transfer. However, all embryo transfers resulted in morphologically normal offspring, which after further mating manifested normal reproductive performance both in terms of litter size and sex ratio. Application of this technique may be beneficial for the successful cryopreservation of human embryos, providing a greater recovery of viable embryos compared with conventional freezing.

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