Effect of cooling mouse spermatozoa to 4°C on fertilization and embryonic development

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Attempts to freeze mouse spermatozoa in liquid nitrogen (−196°C) have met with limited success. In an attempt to identify the factor(s) that damage mouse spermatozoa during cryopreservation, the effect of slow cooling to 4°C was examined. Epididymal spermatozoa were collected into a variety of media at 37°C, cooled slowly to 4°C over 4 h and warmed in a water bath at 37°C for 5 min. Survival of spermatozoa was assessed by motility, membrane integrity and acrosome status. Labelling with chlorotetracycline showed that >80% of spermatozoa were capacitated and had intact acrosomes immediately after warming compared with <20% of freshly collected (control) spermatozoa. The rate of fertilization in vitro was similar using spermatozoa cooled in Dulbecco’s phosphate-buffered saline and then mixed with oocytes immediately after warming and with control spermatozoa incubated for 2 h before mixing with oocytes (85%). Fewer oocytes were fertilized with spermatozoa cooled in either a modified HEPES-buffered Tyrode’s medium or a simple HEPES-buffered medium with a high osmolarity (D3), 63% and 58%, respectively. Two-cell embryos were transferred to the oviducts of pseudopregnant recipients. Implantation was similar in all groups (81–88%) and 54–74% of embryos formed normal late stage fetuses.

Introduction

Spermatozoa were the first cell type to be cryopreserved successfully after the chance discovery that glycerol afforded protection to fowl spermatozoa subjected to temperatures below freezing (Polge et al., 1949). Over the past 40 years this discovery has led to the successful storage of many other cell types and has had a major impact in agriculture, medicine and biology. With the advent of techniques to produce transgenic animals the mouse has become an important model for the analysis of embryonic development and genetic disease. Cryopreservation provides an economical method of conserving these unique animals in addition to other mutant, inbred and congenic stocks. Mouse embryos (Whittingham et al., 1972) and oocytes (Whittingham, 1977) have been successfully cryopreserved; however, until recently cryopreservation of mouse spermatozoa has not been reported. Collection of spermatozoa is quicker and easier than that of embryos and storage of the separate gametes offers the possibility of generating new combinations of genetic material.

Although freezing protocols have been described for the spermatozoa of a variety of stocks obtained from inbred (Tada et al., 1990, 1993; Nakagata and Takeshima, 1993; Penfold and Moore, 1993), outbred (Tada et al., 1990), hybrid (Yokoyama et al., 1990; Nakagata and Takeshima, 1993), transgenic (Nakagata et al., 1992) and wild mice (Nakagata et al., 1995), it has been difficult to repeat published techniques (Penfold and Moore, 1993; S. J. Fuller, unpublished observation). All protocols use media of different chemical composition and different cryoprotectants. Spermatozoa were cooled rapidly to 4°C and viability was not examined before freezing. The overall fertility of the frozen–thawed spermatozoa was variable ranging from <3% to 28% of oocytes becoming fertilized and developing to late stage fetuses (Tada et al., 1990; Yokoyama et al., 1990). Another report claimed that removing glycerol after thawing resulted in a significant increase in the rate of fertilization (35% to 85%) and the embryos developed in vivo after transfer in similar proportions to fresh spermatozoa (Tada et al., 1993).

Cooling spermatozoa rapidly to temperatures above zero can lead to an irreversible loss of motility in some species; a phenomenon referred to as ‘cold shock’ (see review, Watson, 1981). An important factor is believed to be the time between collection and the start of freezing, during which spermatozoa acquire resistance to the freezing process (Jones, 1969). Steps to avoid injury due to cold shock have been incorporated into the successful and repeatable cryopreservation protocols of many species. We have examined the ability of mouse spermatozoa to survive cooling to low temperatures above freezing, i.e. 4°C, because of variability in survival and difficulty in obtaining consistent repeatable results after freezing. A slow cooling rate was chosen to minimize the possibility of cold shock.

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Materials and Methods

Source of gametes

Spermatozoa. Spermatozoa were obtained from fully mature B6CB (C57BL6/Lac x CBA/CaLac) F₂ hybrid males of proven fertility after separation from females for about two weeks. They were killed by cervical dislocation, the cauda epididymides removed and each epididymis placed into 0.5 ml cooling medium at 37°C. Spermatozoa were released by gently squeezing the tissue with two pairs of watchmaker’s forceps and they were allowed to disperse for 10 min before cooling.

Oocytes. Immature (21–24-day-old) B6CB F₁ females were induced to superovulate by intraperitoneal injection of 7.5 IU of equine chorionic gonadotrophin (eCG: Foligon; Intervet, Cambridge, UK) followed 48–54 h later by 5.0 IU human chorionic gonadotrophin (hCG: Chorulon; Intervet, Cambridge, UK).

Media

For cooling spermatozoa. Initial experiments screened a variety of media to assess the survival of mouse spermatozoa after slow cooling to 4°C and subsequent warming. The suspending media were based on simple media in which spermatozoa are progressively motile. Components were added to or removed from these media to allow comparisons to be made. The chosen media were: (i) a modified Tyrode’s medium T6 (Quinn et al., 1982) termed HT6 (290 mOsm) with most of the bicarbonate substituted with HEPES buffer to eliminate the need for CO₂ in the atmosphere to maintain the pH. Medium T6 supports mouse sperm capacitation and fertilization in vitro. (ii) Dulbecco’s phosphate-buffered saline (PBS, 280 mOsm): a simple physiological medium in which spermatozoa are progressively motile; (iii) PBS without Ca²⁺ and Mg²⁺ (PBS⁻, 280 mOsm): the lack of extracellular calcium minimizes the possibility of the acrosome reaction occurring (Fraser, 1987); (iv) D3 (450 mOsm): a simple high osmolality medium consisting of 130 mmol NaCl l⁻¹, 5 mmol KCl l⁻¹, 150 mmol glucose l⁻¹ and 35 mmol HEPES l⁻¹. Most recommended diluents for freezing spermatozoa are isotonic with respect to seminal plasma and act to dehydrate cells before freezing reducing the likelihood of intracellular ice formation. D3 was buffered by HEPES as this has a better buffering capacity than phosphate at pH 7.4. (v) PBS with 150 mmol glucose l⁻¹ (GPBS, 445 mOsm): the osmolality of PBS was increased so that it could be compared with D3; (vi) PBS⁻ with 150 mmol glucose l⁻¹ (GPBS⁻, 445 mOsm): to enable PBS⁻ to be compared with D3. The pH of all media was adjusted to 7.4.

Collecting and handling oocytes. Oviducts were removed 14–16 h after hCG and placed in medium M2 (Quinn et al., 1982) containing 4 mg BSA ml⁻¹ (BSA: Crystalline; ICN Flow, High Wycombe) at 37°C.

Fertilization in vitro and embryo culture. Oocytes were fertilized in medium T6 supplemented with 15 mg BSA ml⁻¹ (Fraction V; Sigma, Poole, UK) and the resultant embryos were cultured in medium M16 supplemented with 4 mg crystalline BSA ml⁻¹ (Quinn et al., 1982). The culture media were overlaid with paraffin oil and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cooling and warming

After the spermatozoa had dispersed into the cooling medium 125 µl aliquots of the suspension were dispensed into 4 ml borosilicate glass tubes (1 cm diameter, 7.5 cm long; 2 tubes per cooling medium). The tubes were then placed in a 250 ml glass beaker containing 150 ml water at 37°C which was placed into an empty 1 l glass beaker in a polystyrene box containing ice and water (Fig. 1). The lid was placed on the box and the tubes were allowed to cool to 4°C over 4 h. The temperature was recorded with thermocouples. When samples reached 4°C they were warmed immediately in a water bath at 37°C for 5 min (approximately 30°C min⁻¹).

Assessment of sperm survival

Three parameters were assessed before and after cooling and warming to determine survival.

Motility. A 5 µl sample of the sperm suspension was placed on a glass slide and observed under a light microscope (×100 magnification). A qualitative estimate of the percentage of motile spermatozoa was recorded.

Membrane integrity. Membrane integrity was assessed by a modification of a method described by Harrison and Vickers (1990). Samples of spermatozoa (about 10⁷ ml⁻¹) were added to the staining solution consisting of 20 µmol
6-carboxyfluorescein diacetate $1^{-1}$ (Calbiochem, Cambridge, UK) and 7.3 µmol propidium iodide $1^{-1}$ (Sigma, Poole). Slides were prepared using 5 µl aliquots of stained suspension, overlaid with a coverslip and examined on a Leitz Ortholux epifluorescent microscope using the standard fluorescein and rhodamine filter sets at $\times 400$ magnification. In each sample, at least 70 spermatozoa were counted from each of two aliquots. The assessment of membrane integrity relies on the different characteristics of 6-carboxyfluorescein diacetate and propidium iodide. 6-Carboxyfluorescein diacetate is permeant and consequently enters cells freely. Intracellularly it is de-esterified by cellular esterases yielding the impermeant fluorescent carboxyfluorescein which accumulates in intact cells causing them to fluoresce green throughout. Propidium iodide is impermeant and therefore only enters damaged cells, where it binds to and stains DNA and fluoroses red. All spermatozoa exhibiting any red fluorescence were classified as damaged, i.e. membrane integrity was incomplete.

**Acrosomal status.** Spermatozoa were fixed with 2% (v/v) glutaraldehyde in phosphate buffered saline supplemented with 1 mg polyvinyl alcohol ml $^{-1}$, placed on clean slides and coverslips added. Spermatozoa were examined under phase contrast illumination ($\times 1000$ magnification) for the presence or absence of an acrosomal cap and 100 spermatozoa were counted from each sample (Fraser, 1987).

**Assessment of capacitation**

After warming, sperm samples that had been cooled in isosmotic medium were mixed with 250 µl of fresh cooling medium and centrifuged at 750 g for 5 min. The supernatant was removed and the cells resuspended in 100 µl T6 (Fraser, 1984). Spermatozoa cooled in hyperosmotic media were diluted with 1 volume of hyperosmotic cooling medium and 1 volume of water to prevent osmotic shock before centrifuging and resuspending in T6. Capacitation was assessed using a chlortetracycline (CTC) fluorescence assay described by Fraser and McDermott (1992). CTC staining was performed on samples before cooling, after cooling and warming and after resuspending in T6 before IVF. Briefly, 45 µl CTC solution was added to an equal volume of sperm suspension in a microcentrifuge tube wrapped in aluminium foil. After careful mixing, the spermatozoa were fixed by adding 8 µl 12.5% (w/v) paraformaldehyde in 0.5 mol Tris HCl $1^{-1}$ (pH 7.4) and placed onto slides. Slides were stored at 4°C in the dark until assessed on a Leitz Ortholux microscope with filter block A. In each sample, 100 spermatozoa were classified according to one of three staining patterns: (1) ‘F’ pattern, with bright fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact spermatozoa, (2) ‘B’ pattern, with a fluorescent free band in the post-acrosomal region characteristic of capacitated, acrosome-intact spermatozoa; and (3) ‘AR’ pattern with dull or absent head fluorescence characteristic of capacitated acrosome-reacted spermatozoa.

**Fertilization in vitro and embryo transfer**

The methods for fertilization in vitro and embryo transfer in the mouse have been described by Wood et al. (1987). After warming and washing, 100 µl of spermatozoa in T6 was placed in a 35 mm Petri dish and overlaid with paraffin oil. Freshly collected spermatozoa preincubated in medium T6 for 2 h were used as controls. Excised oviducts were washed in T6 and then transferred to the 100 µl drops of sperm suspension ($5\times10^5$ spermatozoa ml $^{-1}$) and ruptured to release the cumulus masses. The contents of two oviducts were added to each sperm suspension. After incubation for 4 h, the oocytes were removed, washed through three changes of medium M16 to remove unattached spermatozoa and incubated in M16 for 20 h. After culture the number of oocytes that had developed to the two-cell stage was recorded. They were either left to develop to the blastocyst stage in vitro or transferred to the oviducts (six per oviduct) of pseudopregnant B6CB F1 females (mated with genetically sterile T145/H.Re males) on day 1 of pseudopregnancy. On day 15 of pregnancy, foster mothers were killed by cervical dislocation and the number of implantation sites and fetuses recorded.

**Statistical analysis**

Survival after cooling and warming and CTC data were analysed using paired Student's $t$ test. Data on the fertilization and development to the blastocyst stage in vitro were assessed by two way analysis of variance after angular transformation of the data. The results for development of two-cell embryos in vitro were compared by $\chi^2$ analysis using Yates' correction for continuity. The $\chi^2$ was partitioned into several components to determine where the significant differences lay (Maxwell, 1964).

**Results**

**Cooling and warming**

A representative trace of the cooling curve from 37°C to 4°C is given in Fig. 2. All samples reached 4°C between 4 h and...
Table 1. Effects of different media on mouse sperm motility, membrane integrity and acrosomal status after slow cooling to 4°C and warming to 37°C

<table>
<thead>
<tr>
<th>Media for cooling spermatozoa</th>
<th>Percentage motilea</th>
<th>Percentage intacta membranes</th>
<th>Percentage intacta acrosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>HT6</td>
<td>71</td>
<td>57–82</td>
<td>73</td>
</tr>
<tr>
<td>D3</td>
<td>66</td>
<td>53–80</td>
<td>92</td>
</tr>
<tr>
<td>PBS</td>
<td>71</td>
<td>71–79</td>
<td>98</td>
</tr>
<tr>
<td>PBS−</td>
<td>19**</td>
<td>12–28</td>
<td>81</td>
</tr>
<tr>
<td>GPBS</td>
<td>75</td>
<td>67–88</td>
<td>89</td>
</tr>
<tr>
<td>GPBS−</td>
<td>35*</td>
<td>18–36</td>
<td>95</td>
</tr>
</tbody>
</table>

Values are percentage of spermatozoa that survived the cooling and warming process (for example (percentage motility after cooling/percentage motility before cooling) * 100). *P < 0.05 indicates spermatozoa were significantly less motile after cooling to 4°C and warming.

4 h 20 min. Cooling is non-linear, beginning at about 2°C min⁻¹ during the first 20 min and slowing down to about 0.02°C min⁻¹ in the last hour. The effect of the different media on sperm motility, membrane integrity and acrosomal status after cooling slowly to 4°C and warming is shown (Table 1) and is based on data from three replicate experiments. The reduction in motility after cooling and warming spermatozoa in HT6, PBS, D3 and GPBS was similar (about 30%). In all media slow cooling had no significant effect on membrane integrity and the acrosome. The survival of spermatozoa is not affected by the osmolality of the suspending medium during cooling and warming as all three parameters were affected adversely to the same degree for spermatozoa cooled in PBS and GPBS. A significantly greater decrease in motility occurred in phosphate-buffered media lacking calcium (PBS− and GPBS−). Only 12–40% of spermatozoa retained their motility after cooling in these media; therefore, PBS− and GPBS− were eliminated from further examination.

**Assessment of capacitation state**

After incubation for 2 h the proportion of spermatozoa changing from the uncapacitated to capacitated state, that is, from F pattern to B pattern CTC staining, increased significantly (Fig. 3).

Figure 4 shows the percentage of spermatozoa expressing the F, B or AR staining patterns before cooling (control), after slow cooling to 4°C and warming and after cooling, warming, centrifuging and resuspending in T6 prior to IVF. The percentage of spermatozoa exhibiting the three staining patterns was independent of the medium used for cooling; therefore, the data for spermatozoa cooled in PBS, HT6, D3 and GPBS have been combined. Labelling with CTC showed that >80% of cooled spermatozoa were showing the B staining pattern, characteristic of capacitated and acrosome-intact spermatozoa, immediately after warming compared with <20% of freshly collected spermatozoa. Cooling did not induce the acrosome reaction. No further change in the percentage of spermatozoa showing the B staining pattern was observed after centrifuging and resuspending in T6.

**In vitro fertilization**

There were no significant differences in the rate of fertilization or blastocyst development with spermatozoa previously cooled in PBS or HT6 and then mixed with oocytes immediately after warming and freshly collected spermatozoa incubated for 2 h before mixing with oocytes (Table 2). Fewer oocytes were fertilized with spermatozoa cooled in medium D3 (0.01 < P < 0.05), and development to blastocysts was reduced significantly in oocytes fertilized using spermatozoa cooled in GPBS (0.01 < P < 0.05).

**Embryo transfer**

A total of 264 two-cell embryos obtained from in vitro fertilization with spermatozoa cooled in PBS, HT6 and D3 was...
transferred to 25 pseudopregnant recipients. Twenty-four recipients established pregnancy (Table 3). The overall $\chi^2$ value for the combined fertilization data was highly significant indicating that the four groups were heterogeneous ($\chi^2[3] = 63.05, P < 0.0001$). No significant difference in fertilization was observed with spermatozoa cooled in PBS and control spermatozoa. The percentage of two-cell embryos produced from oocytes fertilized by either control spermatozoa or spermatozoa cooled in PBS was significantly higher than that by spermatozoa cooled in HT6 or D3 ($\chi^2[1] = 62.1, P < 0.001$). The rate of fertilization with spermatozoa cooled in HT6 and D3 was similar. The implantation rate was similar for all groups. Partitioning the $\chi^2$ value of the combined data for development to late stage fetuses showed a significantly higher proportion of embryos derived from cooled spermatozoa developed compared with controls ($0.01 > P > 0.001$). No significant variation occurred among the cooled groups.

**Discussion**

The fertilizing capacity of mouse spermatozoa was retained after slow cooling to 4°C in Dulbecco’s phosphate-buffered saline. Although fertilization was reduced after cooling in HT6 and D3 but not in PBS, the resulting two-cell embryos implanted normally. A significantly higher proportion of embryos derived from cooled spermatozoa developed to late stage fetuses but this was not considered to be related to the cooling of spermatozoa per se. Previously it has been shown that the outcome of embryo transfers can be influenced by factors unrelated to cryopreservation (Bos-Mikich et al., 1995). Membrane changes equivalent to capacitation occurred during cooling or warming; however the acrosome remained intact.

It is generally accepted that frozen–thawed spermatozoa are less fertile than freshly collected samples owing to a reduction in the number of progressively motile cells and poorer fertility of those that survive. Recent evidence suggests that after cryopreservation the surviving population of spermatozoa differs from the pre-freeze condition (reviewed by Watson, 1995). The use of a chlortetracycline assay has demonstrated that after slow cooling to 4°C and warming, > 80% of mouse spermatozoa exhibited the B staining pattern characteristic of capacitated, acrosome-intact cells compared with < 20% before cooling. These spermatozoa are competent for fertilization without the 2 h incubation in medium T6, that is required by freshly collected spermatozoa. Biological membranes have a liquid-like character and are often described as being fluid. A reduction in temperature causes the phospholipid bilayers to undergo a reversible transition to a more rigid gel (Melchior and Steim, 1976). The capacitation-like membrane changes observed after cooling mouse spermatozoa were irreversible suggesting that they were not due to the lipid phase transition. Changes in the selective permeability of the sperm plasma membrane occur during cooling, in particular calcium is seen to accumulate intracellularly (reviewed by Watson, 1981). Current evidence suggests that capacitation involves a rise in the concentration of intracellular calcium (Fraser, 1987; Adeyowa-Osiguwa and Fraser, 1993). The change in membrane permeability during cooling may cause an increase in the concentration of intracellular calcium which initiates the capacitation-like changes seen in mouse spermatozoa after cooling. In other species, fertility has been increased by reducing the interval between thawing and inseminations. Frozen–thawed human (Critser et al., 1987), boar (Clarke and Johnson, 1987; Wang et al., 1991), Siberian tiger (Byers et al., 1989) and gorilla (Lambert et al., 1991) spermatozoa penetrated a higher proportion of zona-free hamster oocytes than did freshly collected spermatozoa when mixed with oocytes immediately after thawing. High fertility can be achieved when cryopreserved ram spermatozoa are inseminated, immediately

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**Table 2.** A comparison of the effect of cooling mouse spermatozoa to 4°C in various media on fertilization in vitro and preimplantation development

<table>
<thead>
<tr>
<th>Medium</th>
<th>Treatment</th>
<th>Number of oocytes inseminated</th>
<th>Percentage of two-cell embryos</th>
<th>Percentage of blastocysts$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Control</td>
<td>250</td>
<td>73.1</td>
<td>63.8</td>
</tr>
<tr>
<td></td>
<td>Cooled</td>
<td>219</td>
<td>89.2</td>
<td>67.3</td>
</tr>
<tr>
<td>HT6</td>
<td>Control</td>
<td>229</td>
<td>72.3</td>
<td>70.6</td>
</tr>
<tr>
<td></td>
<td>Cooled</td>
<td>231</td>
<td>53.7</td>
<td>76.4</td>
</tr>
<tr>
<td>D3</td>
<td>Control</td>
<td>216</td>
<td>68.3</td>
<td>64.3</td>
</tr>
<tr>
<td></td>
<td>Cooled</td>
<td>205</td>
<td>47.2$^b$</td>
<td>63.7</td>
</tr>
<tr>
<td>GPBS</td>
<td>Control</td>
<td>255</td>
<td>65.0</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>Cooled</td>
<td>243</td>
<td>49.8</td>
<td>68.8$^b$</td>
</tr>
</tbody>
</table>

$^a$Expressed as a percentage of two-cell embryos; $^b$significantly different from fresh uncooled spermatozoa (0.01 < $P < 0.05$). All cooled samples had their own controls. Control spermatozoa were incubated in T6 with 151 mg BSA ml$^{-1}$ (fraction V) for 2 h before mixing with oocytes.
after thawing, into the uterine horn at the time of ovulation (Maxwell, 1986a, b). Furthermore, the fertility of cryopreserved rabbit spermatozoa improved when the time from insemination to ovulation was reduced (Parrish and Foote, 1986; Chen et al., 1989). These studies suggest that cryopreservation reduces the period required for capacitation.

Ideally a medium for cryopreservation should maintain viability without inducing capacitation or the acrosome reaction which reduces the functional life of the spermatozoa. As the mature spermatozoon is a complex motile cell consisting of several components, all essential for fertilization, initial survival was assessed using three parameters: motility, membrane integrity and acrosomal status. The ultimate test of functional integrity is the ability of the spermatozoon to fertilize the oocyte and sustain development to birth. PBS is a simple physiological medium which retained cell function during cooling to 4°C. Removing the calcium and magnesium was detrimental, producing large decreases in motility for spermatozoa cooled in PBS− and GPBS−; however, this did not occur in spermatozoa cooled in D3. Exogenous calcium is required to maintain sperm motility. Heffner and Storey (1981) showed that motility of epididymal mouse spermatozoa can be restored after incubating spermatozoa in calcium-free medium by adding calcium. When calcium was added to spermatozoa cooled in PBS− and GPBS− before or after warming, some spermatozoa regained their motility (S. J. Fuller, unpublished observation); however, they were still inferior to cooling in PBS, HT6, D3 or GPBS. Osmolality did not affect survival during slow cooling to 4°C (PBS versus GPBS and PBS− versus GPBS−).

Cooling spermatozoa rapidly to temperatures above zero can lead to an irreversible loss of motility in some species; this phenomenon is termed ‘cold shock’ (reviewed by Watson, 1981). Bull and ram spermatozoa are particularly susceptible and are affected by temperatures below 15°C, but epididymal mouse spermatozoa cooled rapidly to 4°C appear relatively unaffected (S. J. Fuller, unpublished observation). This finding differs from observations by Tao et al. (1995) who reported a significant irreversible loss of motility when epididymy mouse spermatozoa were abruptly cooled to near 0°C. The change in sensitivity between 4°C and 0°C may be the result of a membrane phase transition to a more brittle structure (Noiles et al., 1995).

In conclusion, this study shows that mouse spermatozoa suspended in various media undergo capacitation-like membrane changes when cooled to 4°C resulting in cells that are competent for fertilization immediately after warming. Spermatozoa cooled in PBS are highly fertile and the resultant embryos were capable of further development. These findings are an important advance in determining the stages of the cryopreservation process that are detrimental to mouse spermatozoa and will allow a more detailed study to proceed to identify where cold injury may occur.

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Table 3. Implantation and fetal development after ovudctal transfer of two-cell mice embryos derived from the fertilization in vitro of oocytes with freshly collected or cooled spermatozoa

<table>
<thead>
<tr>
<th>Medium for cooling spermatozoa</th>
<th>Number of oocytes inseminated</th>
<th>Percentage of two-cell embryos</th>
<th>Number of two-cell embryos transferred</th>
<th>Percentage implantation</th>
<th>Percentage fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>376</td>
<td>85.1</td>
<td>150</td>
<td>80.7</td>
<td>53.8</td>
</tr>
<tr>
<td>PBS</td>
<td>141</td>
<td>85.1</td>
<td>72</td>
<td>81.9</td>
<td>63.9b</td>
</tr>
<tr>
<td>HT6</td>
<td>172</td>
<td>62.8b</td>
<td>60</td>
<td>88.3</td>
<td>71.7b</td>
</tr>
<tr>
<td>D3</td>
<td>112</td>
<td>58.0b</td>
<td>54</td>
<td>87.0</td>
<td>74.1b</td>
</tr>
</tbody>
</table>

*Significantly different from sperm cooled in PBS and control sperm (P < 0.001).

bSignificantly different from control (0.01 > P > 0.001).

Freshly collected spermatozoa were incubated in T6 with 15 mg BSA ml−1 (fraction V) for 2 h.
Mouse spermatozoa cooled to 4°C


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