I. Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen-thawed sorted X- or Y- chromosome bearing spermatozoa.

Abstract

The fertility of sex-sorted frozen-thawed ram spermatozoa was assessed after insemination of mature Merino ewes at a synchronised oestrus. Ewes were inseminated into the uterus or utero-tubal junction (UTJ) with a total of 140 x 10^6 unsorted (control) or 2-4 x 10^6 sorted (X or Y) frozen-thawed ram spermatozoa 54 to 57 h after removal of progestagen-impregnated pessaries and an injection of 400 i.u of PMSG (Folligon®, Intervet). The spermatozoa were separated into X- and Y- chromosome bearing spermatozoa after analysis with a modified high-speed cell sorter (SX MoFlo®, Cytomation Inc., Fort Collins, CO, USA). The number of ewes pregnant after insemination with unsorted frozen-thawed spermatozoa was significantly higher (26 / 48; 54.3 %) than for ewes inseminated with either X-(12 / 48; 25.0 %) or Y-sorted spermatozoa (7 / 48; 14.6 %; P< 0.05). Seventeen of the eighteen lambs produced by ewes inseminated with X-sorted spermatozoa were female (94.4 %) and 8 / 8 lambs from ewes inseminated with Y-sorted spermatozoa were male (100 %). The sex ratio of the lambs born to ewes inseminated with sex-sorted spermatozoa was significantly skewed from the 51.3 % male and 48.7 % female ratio in the control group (P<0.05). This study showed for the first time that lambs of predicted sex can be produced after insemination with low numbers of sex-sorted cryopreserved ram spermatozoa.
Introduction

The pre-determination of the sex of offspring using spermatozoa separated by fluorescence differential sorting has been achieved in several species (Johnson, 2000). Successful cryopreservation of sorted spermatozoa and subsequent production of offspring after artificial insemination has only been reported in cattle (Seidel et al., 1999) and horses (Lindsey et al., 2002).

The commercial application of sex predetermination using sorted spermatozoa has been made possible in cattle following the development of methods for successfully freezing and thawing the sorted spermatozoa (Schenk et al., 1999). Similar applications in the sheep industry are yet to take place, since only a few lambs have been produced from fresh (unfrozen) sorted spermatozoa (Catt et al., 1996; Cran et al., 1997).

The aim of the present study was to examine the fertilising ability of sorted frozen-thawed spermatozoa after the insemination of low numbers into either the uterus or UTJ of ewes at a synchronised oestrus.

Materials and methods

Animals and experimental design

One hundred and forty-four mature Merino ewes were randomly allocated into three treatment groups. Inseminations were carried out early in the breeding season in February 2001 at a commercial sheep farm located in the southern highlands of N.S.W.. Ewes were maintained on pasture but were also provided with supplementary feed in the form of lupin grains and hay just prior to insemination and for 4 weeks before and during lambing. The time of oestrus and ovulation was controlled by progestagen-impregnated intra-vaginal
pessaries (40 mg Chronogest®, Intervet, Bendigo, Victoria) inserted for 12 d. At sponge withdrawal, all ewes received a single intramuscular injection of 400 I.U. of pregnant mare serum gonadotrophin (PMSG, Folligon®, Intervet) to enhance their ovulatory response.

Preparation of spermatozoa for sorting

Semen was collected by artificial vagina from two Merino rams (Ram 1, Ram 2) during January and February 2001. The concentration of spermatozoa was determined by haemocytometer and motility was determined by subjective assessment (Evans and Maxwell, 1987). Only ejaculates containing spermatozoa with forward progressive motility greater than 80 % were used. Spermatozoa were diluted to a concentration of 400 \( \times 10^6 \) / ml with a modified TALP medium (XY TALP; Schenk et al., 1999). Each 2 mL sample was incubated with either 355.9 µM (Ram 1) or 311.4 µM (Ram 2) of Hoechst 33342 (H33342; Sigma, St Louis, MO, USA) for 1 h at 34°C. The concentration of dye for each ram was determined by a series of optimisation experiments which examined a range of H33342 concentrations (178 µM/ml to 356 µM/ml) and involved the staining of multiple ejaculates (n= 10 to 60-varied for each ram) collected from each of the rams housed at The University of Sydney, Australia (n= 6) over both the breeding and non-breeding seasons (2000-2001).

After 1 h incubation, stained samples were diluted slowly with 2 ml of filtered XY TALP containing 4 % egg yolk (v/v) and 0.002 % food dye (FD&C #40, Warner Jenkinson Company Inc., St Louis, MO, USA) to 200 x 10^6 spermatozoa /ml. The food dye quenches the intensity of fluorescence of the dead sperm in the sample by penetrating their membranes. Therefore only viable sperm are selected for sorting (Johnson et al., 1999).
Immediately before sorting, samples were filtered through a 40-µm nylon mesh filter to remove any agglutinated spermatozoa or debris.

**Flow Cytometric Sorting**

A high-speed cell sorter (MoFloSX®) modified for sperm sorting (Johnson and Pinkel, 1986; Rens *et al*., 1999) operating at 50 psi with a pre-warmed Tris-based sheath fluid (Schenk *et al*., 1999) was used to analyse and separate the spermatozoa. The fluorescent dye was excited by an argon laser running at 200 mW. Gates were set during sorting so that purities of greater than 90 % X- or Y- chromosome bearing spermatozoa were achieved. Average flow rates during the experiment were 20 000-25 000 events/sec and the average sorting rate for each of X- and Y-bearing was 4 000-5 000 spermatozoa/sec.

During sorting the sort mode was set to “Purify 0.5” in order to achieve maximum purity from each spermatozoa sample sorted. Only one drop was sorted at a time and any drop that had the potential for being contaminated by another drop containing a spermatozoon of the incorrect sex or a non-viable spermatozoon falling into it was deflected into the waste stream. Each spermatozoon also had to be within 1/16th of a drop from the centre of the drop in order to be sorted.

Putative male and female spermatozoa were collected simultaneously into 10-ml centrifuge tubes, pre-soaked with 1 % BSA in TRIS sheath fluid, containing 1 ml of warm, filtered XY TALP and 20 % egg yolk (v/v). Each sample was sorted for 30 to 60 min to achieve a total yield of 16 million (8 million of each sex) spermatozoa in 8 ml of sheath fluid (2.5 % egg yolk, v/v).
Reanalysis of sorted spermatozoa

At the completion of each 30-60 minute sort, 200 000 X- and Y- bearing spermatozoa were collected, processed and analysed as described by Welch and Johnson (1999) to provide an estimate of the purity of each sort and a prediction of the proportion of male and female lamb offspring.

Freezing, thawing and assessment of sorted spermatozoa

Sorted spermatozoa were centrifuged at 700 g for 6 min at 30°C and the 80 µL pellet was resuspended (1:4; sperm pellet: diluent, v/v) in either (i) TEST buffer containing 20 % egg yolk (v/v) (Johnson et al., 1989) and 3 % glycerol (TBY; v/v) or (ii) zwitterion-buffered diluent containing 13.5 % egg yolk and 6 % glycerol (ZWIT; Molinia et al., 1996). Separate ejaculates were collected from each ram as control samples and were diluted 1:4 with TBY or ZWIT. Both sorted (X-sort, Y-sort) and control samples were cooled to 4-5°C in 1.5 h and then frozen as 0.2 ml pellets on dry ice (Evans and Maxwell, 1987) before being transferred to liquid nitrogen for storage until use. Two pellets prepared from the same ejaculate were thawed in a dry test tube shaken in a water bath at 37°C and used for insemination within 10 min of thawing. Motility was immediately assessed and recorded after thawing, and slides were made for the assessment of acrosome integrity by fluorescent isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining as described by Roth et al., 1998.
Artificial insemination

Inseminations were carried out 54 - 57 h after withdrawal of progestagen sponges. All ewes were fasted for 12 h before insemination and local anaesthetic (2 % Lignocaine, Troy Laboratories, Smithfield, N.S.W.) was injected at the site of abdominal puncture.

Intrauterine inseminations (IU) were conducted by laparoscopy in 81 ewes as described by Evans and Maxwell (1987) and 0.1 ml of thawed semen was deposited into the lumen of each uterine horn. Utero-tubal junction (UTJ) inseminations were carried out in 63 ewes using a disposable, 3.5 french, 11.4 cm tom cat catheter (Sovereign®, Sherwood Medical, St Louis; MO, USA); after mini-laparotomy, a small puncture was made in the tip of the uterus, and the catheter passed up to the UTJ where 0.1 ml of thawed semen was deposited into each UTJ. A total of 140 x 10^6 spermatozoa per ewe, a commercial AI dose, was inseminated into 48 ewes in the control group (27 IU and 21 UTJ) and between 2 and 4 x 10^6 total sorted spermatozoa were inseminated into each of the 48 ewes in the X (26 IU and 22 UTJ) and Y (28 IU and 20 UTJ) sort groups.

Pregnancy Diagnosis

Jugular blood samples were collected from each ewe on Day 18 after insemination and progesterone concentrations in blood plasma were determined with a commercially available radioimmunoassay kit (Spectra; Orion Diagnostics, Helsinki, Finland). Ewes with progesterone concentrations greater than 1.2 ng/ml were considered pregnant (Robertson and Sarda, 1971). All ewes were scanned by ultrasound for detection of one or multiple foetuses on Day 60. Pregnancy loss in ewes was calculated as the number of ewes pregnant at Day 18 – the number of ewes pregnant at Day 60 / number of ewes pregnant at Day 18.
**Lambing**

After Day 60, pregnant ewes were placed in sheltered paddocks and put on an increasing plane of nutrition leading up to and during lambing. One week before the commencement of lambing, each ewe that had been inseminated with sex-sorted spermatozoa was housed in a separate pen in the shearing shed and was observed 24 h a day until the end of lambing. Every lamb born was weighed with a set of calibrated hand scales, identified for sex and ear tagged within 6 h of birth.

**Statistical Analysis**

Data on pregnancy loss and pregnancy rate in ewes and the effect of ram and insemination site were analysed by logistic regression using the GENSTAT computer program (Version 4.2). Lamb weight, post thaw motility and acrosome status were analysed by ANOVA and logistic regression using GENSTAT. Proportional data for the sex ratio and re-sort analysis values were analysed by the Chi-square test and the two sample t-test respectively.

**Results**

**Post thaw motility and acrosome integrity of spermatozoa**

There was no significant difference in the post thaw motility of control (unsorted), X-sort and Y-sort samples. The percentage of motile spermatozoa (± S.E.M) after thawing was 46.2 ± 0.8 %, 46.3 ± 1.0 % and 42.3 ± 0.9 % for control, X-sort and Y-sort samples respectively. Freezing diluent and ram also had no effect on the post thaw motility or acrosome integrity of spermatozoa.
Sorted frozen-thawed spermatozoa had significantly more intact acrosomes after thawing than unsorted frozen-thawed (control) spermatozoa (P<0.001). The incidence of intact acrosomes after thawing was 80.6 ± 1.0 %, 92.9 ± 0.5 % and 91.1 ± 0.6 % for control, X-sort and Y-sort samples respectively. Sorted samples from ram 2 had significantly less spermatozoa with intact acrosomes (86.1 ± 0.9 %) after thawing than ram 1 (90.1 ± 0.79 %; P<0.001).

Pregnancy rate

Data on pregnancy at Day 18 and Day 60 are presented in Table 1. Ewes inseminated with 140 $\times$ 10$^6$ unsorted frozen-thawed spermatozoa (control) had a higher pregnancy rate at Day 60 (54.2 %) than ewes inseminated with either 2-4 $\times$ 10$^6$ X- (25.0 %) or Y-sorted spermatozoa (14.6 %; P<0.05). There was no significant difference in pregnancy rate at Day 60 between ewes inseminated with either X- or Y-sorted spermatozoa.

The site of insemination and freezing diluent had no significant effect on the proportion of ewes pregnant and there were no significant interactions between the treatments. The pregnancy rate for Ram 2 (17 / 72) was lower than for Ram 1 (28 / 72), though overall there was no statistically significant difference after analysis by logistic regression.
Table 1: Pregnancy loss between Day 18 and Day 60 after artificial insemination of ewes with control and sex-sorted frozen-thawed spermatozoa.

<table>
<thead>
<tr>
<th>Type of Semen</th>
<th>No. ewes inseminated</th>
<th>No. pregnant at day 18 (%)¹</th>
<th>No. pregnant at day 60 &amp; lambing (%)²</th>
<th>Pregnancy Loss³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48</td>
<td>34 (70.8)a</td>
<td>26 (54.2)a</td>
<td>23.5</td>
</tr>
<tr>
<td>X</td>
<td>48</td>
<td>21 (43.8)b</td>
<td>12 (25.0)b</td>
<td>42.9</td>
</tr>
<tr>
<td>Y</td>
<td>48</td>
<td>15 (31.3)b</td>
<td>7 (14.6)b</td>
<td>53.3</td>
</tr>
</tbody>
</table>

¹ determined by progesterone assay  
² determined by ultrasound  
³ (no. pregnant at Day 18 - no. pregnant at Day 60 / no. pregnant at Day 18) x 100

ab Within columns, values with different superscripts differ (P<0.05)

Pregnancy Loss

The overall pregnancy loss estimated from Day 18 to Day 60 after insemination for the flock was 38.6 ± 9.60 %. There was no difference in pregnancy loss for ewes inseminated with unsorted control spermatozoa compared with X-or Y- sorted spermatozoa (Table 1). There was also no ram effect on the pregnancy rate within the flock.

Lambing rate

The number of ewes lambing, number of lambs born and sex of the lambs are presented in Table 2. All lambs were born between Day 148 and Day 153 of gestation. There was no pregnancy loss between ultrasound pregnancy diagnosis at Day 60 and lambing. The number of lambs born per ewe lambing was similar after insemination with unsorted and sorted frozen-thawed spermatozoa.
**Table 2:** Lambing after insemination of ewes with control and sex-sorted frozen-thawed spermatozoa.

<table>
<thead>
<tr>
<th>Type of semen</th>
<th>Number of ewes</th>
<th>Number of lambs</th>
<th>Sex of lambs</th>
<th>Sex of Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inseminated</td>
<td>Lambed</td>
<td>Born</td>
<td>Born/ewe lambing</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(±s.e.m)</td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(54.2)</td>
<td>(81.3)</td>
<td>(81.3)</td>
<td>(51.3M : 48.7F)</td>
</tr>
<tr>
<td>X</td>
<td>48</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>(25.0)</td>
<td>(37.5)</td>
<td>(37.5)</td>
<td>(94.4 F)</td>
</tr>
<tr>
<td>Y</td>
<td>48</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>(14.6)</td>
<td>(16.7)</td>
<td>(16.7)</td>
<td>(100.0 M)</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Within columns, values with different superscripts differ (P<0.05)

**Sex ratio**

Twenty-five of the 26 lambs born from ewes inseminated with sex-sorted spermatozoa were of the predicted sex. There was a statistically significant difference in the sex of the lambs born from ewes inseminated with sorted X- (94.4 % female) and Y- (100 % male) spermatozoa compared with unsorted spermatozoa (51.3 % male and 48.7 % female; P<0.05). The proportion of male lambs born to ewes inseminated with Y-sorted spermatozoa and the proportion of female lambs born to ewes inseminated with X-sorted spermatozoa was indistinguishable from the re-sort analysis values obtained after each sorting session (Table 2).

**Lamb weights**

The mean birth weights for the lambs in both the sorted and control groups are presented in Table 3. Lambs in the control group had similar birth weights to lambs from ewes inseminated with X- or Y- sorted spermatozoa. Only the number of lambs born per ewe had a significant effect on the birth weights. Triplet or twin-born lambs had a significantly lower birth weights than single lambs (P<0.05).
Table 3: Mean (±S.E.M.) birth weights of lambs and number of lambs born after insemination of ewes with control and sex-sorted frozen-thawed spermatozoa.

<table>
<thead>
<tr>
<th>Type of semen</th>
<th>Single</th>
<th>Twin</th>
<th>Triplet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (No. lambs)</td>
<td>Male (No. lambs)</td>
<td>Female (No. lambs)</td>
</tr>
<tr>
<td>Control</td>
<td>5.0 ± 0.19 kg (9)</td>
<td>4.5 ± 0.25 kg (5)</td>
<td>3.7 ± 0.18 kg (10)</td>
</tr>
<tr>
<td>X</td>
<td>5.4 ± 0.36 kg (6)</td>
<td>4.6 ± 0.00 kg (1)</td>
<td>3.1 ± 0.19 kg (8)</td>
</tr>
<tr>
<td>Y</td>
<td>-</td>
<td>5.1 ± 0.26 kg (6)</td>
<td>-</td>
</tr>
</tbody>
</table>

Discussion

This is the first report of the birth of lambs after insemination with low numbers of sex-sorted frozen-thawed spermatozoa. The present study demonstrates that ram spermatozoa are capable of fertilisation after being sex-sorted and cryopreserved. It also shows that offspring of the predicted sex can be produced after insemination of only 1-2 x10^6 motile sex-sorted spermatozoa (2-4 x10^6 total) into the uterus by a standard commercial artificial insemination technique. The overall pregnancy rates obtained in this study were inexplicably low (54 % control, 25 % X-sort group; 15 % Y-sort group) compared to pregnancy rates achieved by commercial artificial insemination programs, (72 %; Hill et al., 1998) though perhaps related to relatively low fertility of one of the rams used. The low pregnancies obtained for ewes inseminated with sorted frozen-thawed spermatozoa were less than half that of the controls. This may be a result of the low numbers of motile spermatozoa inseminated and the timing of insemination in relation to ovulation.

Fertilisation rates are usually 20-30 % lower after insemination with frozen-thawed than with fresh spermatozoa (Maxwell et al., 1993). The results of the present study suggest
that spermatozoa that have undergone both sorting and cryopreservation may have lower fertilisation rates than those that have been only frozen-thawed. However, it was not possible to distinguish between fertilisation failure and embryonic loss in the present study, as egg fertilisation rates were not determined. The low pregnancy rates obtained in this study may have been due to the low numbers of spermatozoa inseminated, since the post-thaw motility of spermatozoa was similar for all treatment groups and the post-thaw acrosome status was higher for sorted than for unsorted spermatozoa.

There have been limited reports on the minimum numbers of fresh or frozen-thawed sorted spermatozoa required for effective fertilisation after intrauterine or oviducal insemination in sheep. In our study, the overall pregnancy rate for both the X- and Y-sort groups was 20% after insemination of 2-4 x 10⁶ sorted frozen-thawed spermatozoa into either the uterus (26%) or UTJ (12%). Cran et al. (1997) reported the birth of 6 female lambs (1 set of twins) after the insemination of 25 ewes with 1 x 10⁵ fresh X-sorted spermatozoa into the tip of the uterine horn. The minimum intrauterine insemination dose recommended by the sheep industry for frozen-thawed spermatozoa is 20 - 25 x 10⁶ motile spermatozoa (Evans and Maxwell; 1997). Results from several studies investigating the minimum effective intrauterine and / or oviducal insemination dose for fresh or frozen unsorted spermatozoa have been variable (reviewed by Evans, 1988). These reports suggest that the inseminate dose used in the present study was too low and that a dose in excess of 2 - 4 x 10⁶ sex-sorted frozen-thawed spermatozoa might be required to achieve pregnancy rates closer to commercially acceptable levels.

Insemination of spermatozoa closer to the site of fertilisation generally yields higher fertilization / pregnancy rates than inseminations carried out in the lower reproductive tract. This is particularly true when the inseminate dose is low or frozen-thawed spermatozoa is used (Jabbar and Evans, 1991; Maxwell et al., 1993). In the present study,
however, there was no significant difference found between insemination into either the uterus or UTJ with either sorted or unsorted frozen-thawed spermatozoa. Maxwell (1986b) suggested that the manipulation of the reproductive tract, which was necessary to inseminate into either the tip or base of the uterine horns, contributed to lower lambing rates than insemination into the middle of the uterine horn which required minimal manipulation. Thus, pregnancy rates may be compromised in ewes inseminated into the UTJ due to the additional handling and manipulation of the upper reproductive tract compared with the simpler intrauterine insemination procedure.

In the present study ewes were inseminated 54 -57 h after progestagen sponge removal. Previous studies have demonstrated higher egg fertilisation and lambing rates when insemination of frozen-thawed spermatozoa occurred before, rather than after, ovulation (Maxwell, 1986a). The median time of ovulation in ewes after synchronisation of oestrus (progestagen sponge and PMSG) has been reported between 58 h and 61 h (Maxwell, 1986a). It may be that inseminations between 54 h and 57 h were performed too early, relative to ovulation, and that the pregnancy rates for both UTJ and intrauterine inseminations could have been increased by inseminating just after ovulation at 60-64 h post sponge removal. Maxwell (1986a) and Eppleston et al. (1986) reported higher fertilisation and lambing rates in non-superovulated ewes inseminated with frozen-thawed spermatozoa at 60 h compared with 48 h after sponge removal. This timing avoids the manipulation of the tract around the time of ovulation, which might interfere with ova pick up. In addition, the changes that occur in the oviduct after ovulation allow spermatozoa to immediately pass through the UTJ into the isthmus (Hunter et al., 1982). Given adequate capacitation conditions, these spermatozoa would participate in fertilisation earlier than those deposited before ovulation, thus reducing the potential for embryonic loss associated with aged spermatozoa. This is particularly important when using frozen-thawed spermatozoa, which have approximately half the lifespan of fresh
spermatozoa in vivo (reviewed by Salamon and Maxwell, 1995b; Gillan and Maxwell, 1999). The capacitation status of the sorted frozen-thawed spermatozoa could not be determined in this study, due to quenching of chlortetracycline by the Hoechst stain used for determination of DNA content. A high proportion of the sorted spermatozoa were acrosome intact, as determined by FITC-PNA staining, but may have been capacitated, and thus have a short viable life-span in the female reproductive tract. Therefore, timing of insemination relative to ovulation may also be an important influence on pregnancy rate.

For insemination to occur just after ovulation, the average time of ovulation in the flock needs to be determined. Walker et al. (1989a) and Eppleston et al. (1991) reported considerable variation in the time of ovulation in ewes synchronised with progestagen sponges and PMSG, with ovulation reported to vary over more than a 24 h period. However, when gonadotrophin-releasing hormone (GnRH) was incorporated into the synchronisation regime, the period over which ovulation occurred was reduced to less than 12 h, depending on the time of GnRH injection after sponge removal. Despite the increase in synchrony of ovulation following the use of GnRH, there have been few reports of increased fertility after insemination with frozen-thawed semen (Maxwell, 1986a; Walker et al., 1989b; Eppleston et al., 1991). However, an increased precision of the time of ovulation, and of insemination relative to ovulation, may play a more crucial role in obtaining satisfactory fertility after the use of sorted frozen-thawed spermatozoa, as the fertile life-span of these cells in the female reproductive tract may be very short.

Embryonic loss in sheep is high even after natural insemination (Edey, 1976). In the present study the overall pregnancy loss between Day 18- Day 60 (38.6 %) was not greater than that reported in ewes inseminated with fresh or frozen-thawed spermatozoa by other workers (Salamon and Maxwell, 1995b). In contrast to the pregnancy rate findings, there was no difference in pregnancy loss between the unsorted and sorted treatment groups.
However, without fertilisation data, we cannot say that normal embryonic development occurred after fertilisation of oocytes with sorted frozen-thawed spermatozoa. In vitro studies in cattle (Lu et al., 1999) have shown similar fertilisation rates but lower blastocyst rates (70 %) with in vitro matured (IVM) oocytes inseminated with sorted frozen-thawed spermatozoa compared with unsorted frozen-thawed spermatozoa. In trials involving the insemination of 1000 heifers with sorted frozen-thawed spermatozoa and unsorted frozen-thawed spermatozoa (Seidel et al., 1999), the overall pregnancy rate, determined by ultrasound, for heifers inseminated with sex-sorted frozen-thawed spermatozoa was 70-90 % of those inseminated with unsexed frozen-thawed spermatozoa. Similarly, in the present study, there was no significant difference in pregnancy loss between the two insemination groups. Also no fertilisation or early embryonic data was collected. Further in vivo studies would need to be carried out to determine fertilisation rates and early embryonic loss potentially associated with sex-sorted frozen-thawed spermatozoa. It is more likely that the insemination of inadequate numbers of frozen-thawed sorted spermatozoa either too early or late relative to ovulation contributed to a low fertilisation rate.

The number of lambs born per ewe lambing was similar for ewes inseminated with Y- or X-sorted spermatozoa and control unsorted frozen-thawed spermatozoa. All of the lambs born from ewes inseminated with Y-sorted frozen-thawed spermatozoa were male and 94.4 % of the lambs born to ewes inseminated with X-sorted frozen-thawed spermatozoa were female. This skewing of the sex ratio from the control, and expected 50 : 50 ratio, has been previously demonstrated in several species (reviewed by Garner, 2001). The re-sort purity analysis values for spermatozoa obtained at the end of each sorting session were not significantly different from the observed sex proportions of the offspring produced by ewes inseminated with sorted spermatozoa. This supports previous findings by Welch and Johnson (1999) that re-sort analysis is a valuable tool for predicting the sex of offspring or the sex ratio of a given population after insemination with sex sorted semen.
In conclusion, this study has demonstrated it is possible to obtain pregnancies with low numbers of sorted frozen-thawed ram spermatozoa using commercial AI techniques. However, the overall pregnancy rate for ewes inseminated with low numbers of sorted frozen-thawed spermatozoa was less than for ewes inseminated with commercial numbers of unsorted frozen-thawed spermatozoa. Further investigation is needed to determine the minimum effective dose of sorted frozen-thawed spermatozoa required to obtain commercially acceptable pregnancy rates. In addition, the optimum time to inseminate ewes relative to the time of ovulation remains to be determined. Overall fertility results are most likely to be improved by increasing the insemination dose. However, constraints on the number of spermatozoa that can be practically sorted should encourage further research on the control of the time of ovulation.

Acknowledgements

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References


II. In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing.

Abstract

The present study was designed to investigate the effect of sex-sorting by flow cytometry and freeze-thawing on the viability and fertility of ram spermatozoa. This involved in vitro sperm assessments after incubation (1 h, 34°C) with Hoechst 33342 stain, flow cytometric sorting (SX MoFlo®), freeze-thawing and incubation (37°C) of sorted and non-sorted (control) spermatozoa (Experiment 1). The effect of time of insemination relative to ovulation and number of sorted frozen-thawed spermatozoa inseminated on in vivo fertility was then investigated (Experiment 2). In Experiment 1, non-sorted (control) frozen-thawed spermatozoa had higher motility and forward progressive motility (FPM) rating than sorted frozen-thawed spermatozoa (61 ± 2.9 % vs 57 ± 3.3 % and 4.0 ± 0.10 vs 3.5 ± 0.10 FPM rating, respectively; P<0.001) after incubation (6 h at 37°C). Sorted and non-sorted (control) frozen-thawed spermatozoa had similar proportions of intact acrosomes (79 ± 1.4 % and 79 ± 1.4 % respectively) after thawing and incubation (6 h at 37°C). Using chlortetracycline (CTC) staining, a greater proportion of sorted spermatozoa displayed patterns that were characteristic of capacitation (B-pattern: 22 ± 1.9 %; P<0.05) than non-sorted (control) spermatozoa (B pattern: 16 ± 2.4 %) before freezing. Using a modified sperm migration test, spermatozoa were assessed for ability to penetrate an artificial cervical mucus at 0 h post-thaw. Similar numbers of non-sorted (control) and sorted frozen-thawed spermatozoa penetrated the artificial cervical mucus after 1 h incubation (76 ± 11.9 vs 74 ± 11.9 spermatozoa, respectively; P<0.05). The distance traveled by the
vanguard spermatozoon was also similar for both treatments (56 ± 7.8 mm and 40 ± 5.8 mm for control and sorted respectively). Sorted and non-sorted (control) frozen-thawed spermatozoa displayed a similar pattern of binding to, and release from, the oviducal epithelial cell monolayer (OECM), but sorted frozen-thawed spermatozoa were released more rapidly (P<0.05) than non-sorted (control) frozen-thawed spermatozoa. In Experiment 2, two fertility trials were conducted. For the first fertility trial, pregnancy rate was greater (P<0.001) for ewes inseminated with 100 x 10^6 non-sorted (commercial control dose) frozen-thawed spermatozoa (59 %) than with lower doses (5, 10, 20 and 40 x 10^6 total spermatozoa) of sorted frozen-thawed spermatozoa (41 % overall). Insemination of 16 x 10^6 sorted frozen-thawed spermatozoa resulted in a higher (P<0.05) pregnancy rate (31 %) than after insemination with 1 x 10^6 (17 %) but was similar to ewes which received 4 x 10^6 sorted frozen-thawed spermatozoa (24 %). There was no effect of time of insemination (54, 58 and 62 h after sponge removal) on pregnancy rate. In the second fertility trial, the proportion of GnRH-treated ewes pregnant was affected by the number of spermatozoa inseminated (P<0.05) but not sperm type (sorted and non-sorted) or ram. Pregnancy rate was higher (P<0.05) after insemination of 40 x 10^6 than 5 or 20 x 10^6 non-sorted (control) or sorted frozen-thawed spermatozoa (70 %, 33 % and 35 %, respectively). The results suggest that sorted frozen-thawed spermatozoa may have a shorter period of viability within the female reproductive tract (FRT) in which to encounter the oocyte than non-sorted (control) frozen-thawed spermatozoa. Further research is needed into methods of increasing the fertilising lifespan of sorted frozen-thawed spermatozoa in the FRT.
Introduction

The first birth of lambs after insemination with sex-sorted frozen-thawed spermatozoa was achieved using low numbers of spermatozoa ($2-4 \times 10^6$ total spermatozoa, Hollinshead et al., 2002a). However, the overall pregnancy rate for ewes inseminated with sex-sorted frozen-thawed spermatozoa (25%) was half that of a commercial dose ($140 \times 10^6$ total spermatozoa) of non-sorted frozen-thawed spermatozoa. Lower fertility after artificial insemination (AI) with low numbers ($1.5-3 \times 10^6$ total spermatozoa) of sorted frozen-thawed spermatozoa compared to $20 \times 10^6$ non-sorted (control) frozen-thawed spermatozoa has also been reported in cattle (Seidel et al., 1999; Brogliatti et al., 2002).

There have been many improvements in the efficiency of the sperm sexing technology (Johnson and Welch, 1999; Rens et al., 1999; Schenk et al., 1999) since the production of the first sexed offspring by Johnson et al. (1989). Despite these improvements, there are still practical limitations on the number of spermatozoa that can be sex-sorted for commercial AI of sheep. In addition, for this technology to be accessible, the sex-sorted spermatozoa need to be cryopreserved to permit transportation from the flow cytometer to the commercial breeding centre. It has been shown that cryopreservation not only reduces the proportion of motile spermatozoa but also causes the spermatozoa that remain to undergo capacitation-like changes (Gillan et al., 1997). Frozen-thawed spermatozoa have more capacitation-like changes than fresh spermatozoa (Watson, 1995; Maxwell and Watson, 1996) and this has been shown to affect the interaction of the spermatozoa with cells of the oviduct (Gillan et al., 2000) and fertilisation rates following intrauterine AI (reviewed by Salamon and Maxwell, 1995b, 2000). Other factors such as the site of insemination (intrauterine or oviductal; Maxwell et al., 1993), dose of semen inseminated (reviewed by Evans, 1988; Maxwell et al., 1993) and time of insemination in relation to
Oestrus (Maxwell, 1986a; Eppleston et al., 1986, 1991; Walker et al., 1989a, 1989b) can also influence pregnancy rates following insemination.

The objective of the present study was to investigate factors responsible for the low pregnancy rates observed after insemination with low numbers of sorted frozen-thawed ram spermatozoa. This involved (i) *in vitro* sperm quality assessments on sorted frozen-thawed spermatozoa in order to further understand the events that take place after intrauterine or UTJ insemination and (ii) examination of the effect on pregnancy rate after inseminating increasing and similar doses of sorted and non-sorted (control) frozen-thawed spermatozoa in ewes synchronized with GnRH. The overall aim of the present study was to improve the fertility of sorted frozen-thawed spermatozoa after low dose insemination of GnRH-treated ewes.

**Materials and Methods**

*Experimental design*

Two experiments were carried out in the present study. In Experiment 1, *in vitro* sperm characteristics (motility, forward progressive motion (FPM), capacitation and acrosomal status, migration ability through an artificial cervical mucus and interaction with epithelial cells of the oviduct) of sorted and non-sorted (control) frozen-thawed spermatozoa were assessed after incubation (4-6 h, 37°C). The experiment was replicated three times. In Experiment 2, two fertility field trials were carried out. The first trial investigated the effect of time of insemination and dose of sorted and non-sorted (commercial control) frozen-thawed spermatozoa on fertility in ewes treated with GnRH and the second trial examined the effect of similar and increasing doses of sorted and non-sorted (control) frozen-thawed spermatozoa on pregnancy rates.
Semen collection, preparation and flow cytometric sorting

Semen was collected by artificial vagina from three Merino rams for Experiment 1 and Trial 1 and from two of the three Merino rams used in Trial 1 for Trial 2. Only ejaculates (n=3 per ram for Experiment 1 and n= 10 for Trial 1 and Trial 2) containing spermatozoa with forward progressive motility greater than 80% were used. Spermatozoa were prepared and processed using a high speed cell sorter (MoFloSX®, DakoCytomation, Fort Collins, CO, USA) modified for sperm sorting (Johnson and Pinkel, 1986; Rens et al., 1999) and operating at 50 psi with an argon laser running at 200 mW as previously described (Hollinshead et al., 2002a).

A sample of spermatozoa from each ejaculate was removed and diluted (400 x 10^6 / ml) with a TRIS-buffered diluent (TRIS; Evans and Maxwell, 1987) and supplemented with 0.3% (v/v) BSA (Sigma A-9647; St Louis, MO, USA), stained with 267 µM - 311 µM of Hoechst 33342 (H33342; Sigma) and incubated (34ºC, 1 h). The composition of the TRIS diluent was 300 mM Tris-(hydroxymethyl) aminomethane, 27.8 mM fructose, 94.7 mM citric acid, 0.15g / l penicillin and 0.25g / l streptomycin. Stained samples were diluted (1:1, sperm sample: diluent, v/v) with TRIS containing 4% (v/v) egg yolk and 0.002% (w/v) food dye (Warner Jenkinson Company Inc., St Louis, MO, USA) and filtered (37 µm) using 5 ml tubes with cell-strainer caps (Falcon 2235, Becton Dickinson, Franklin Lakes, NJ, USA). The food dye quenches the intensity of fluorescence of the dead spermatozoa in the sample by penetrating their membranes, thereby facilitating the sorting of only viable spermatozoa (Johnson et al., 1999).

Spermatozoa were processed at a rate of 15 000-18 000 / sec without sex-sorting into 10-ml centrifuge tubes (pre-soaked with 1% v/v BSA and TRIS sheath fluid) containing 0.25
ml of AndroHep (AH, Minitüb, GmbH, Landshut, Germany) supplemented with 20 % (v/v) egg yolk (pH 7.4). During sorting, “enrich sort mode” was used to maximize spermatozoa recovery. Each spermatozoon was exposed to the same staining, dilution, pressure, laser excitation and physical stress as spermatozoa which are sorted under high purity conditions at much slower rates (approx. 4 000 spermatozoa/sec). After the collection of 2 x 10^6 sorted spermatozoa, a further 0.25 ml of AH and 20 % (v/v) egg yolk was added. A total of 4-5 x 10^6 sorted spermatozoa were collected at a time.

In Experiment 1, two samples from each ejaculate were prepared and sorted as described above, except one of the samples was not stained with H33342 prior to sorting to permit subsequent assessment of capacitation status using CTC staining. X and Y purity and sorting parameters were obtained for each ejaculate using the stained sample.

**Reanalysis of sorted spermatozoa**

Resort analysis was carried out on each sample prepared for sorting to demonstrate that these sperm samples could also be sorted under commercial high purity conditions. After 2 h of sorting each sample, gates were placed not only around the viable but the correctly oriented populations of spermatozoa and 1.3 x 10^6 X- and Y-chromosome bearing spermatozoa were collected and processed for determination of purity (Welch and Johnson, 1999). X and Y purities greater than 90 % were achieved for all samples. Under these high purity sorting conditions the proportion of non-viable spermatozoa was 13 ± 1.3 % and the proportion of oriented spermatozoa 47 ± 1.4 % for each of the fresh samples prepared.
Freezing and thawing of spermatozoa

Four 10-ml tubes containing sorted spermatozoa were centrifuged (700 g; 7 min) simultaneously. Each 50 μl pellet containing either 4 x 10⁶ or 5 x 10⁶ sorted spermatozoa was slowly resuspended (1:4; sperm pellet: diluent, v/v) with a zwitterion buffered diluent containing 13.5 % (v/v) egg yolk and 6 % (v/v) glycerol (ZWIT, Molinia et al., 1996) and frozen as a 250 μl pellet as described by Evans and Maxwell (1987). An aliquot of spermatozoa was removed from each ejaculate and extended to 4 x 10⁶ / 0.25 ml or 5 x 10⁶ / 0.25 ml with ZWIT diluent and also frozen in 250 μl pellets (control). In Experiment 2 the remainder of each ejaculate was diluted (1:4, semen: diluent) with ZWIT diluent and frozen as 250 μl pellets each containing approximately 100 x 10⁶ spermatozoa (commercial control). Two pellets from the same ram, treatment and ejaculate were thawed at 37°C by agitation in a 10-ml dry glass test-tube (Evans and Maxwell, 1987).

Assessment of in vitro sperm characteristics

All spermatozoa concentrations were determined using a hemocytometer as described by Evans and Maxwell (1987).

Motility, forward progressive motion and acrosome integrity

After thawing, pellets were diluted (1:1; pellet: diluent, v/v) with an artificial seminal plasma medium (ASP; Mortimer and Maxwell, 2004) supplemented with 0.3 % (v/v) BSA. Before staining, after staining, before freezing, after thawing (0 h) and at 3 and 6 h or 2 and 4 h after incubation (37°C), subjective assessments of total progressive motility (motility to the nearest 5 %) and forward progressive motion rating (FPM: 0-5; 0 = no movement, 5 = rapid forward progressive motion; modified from Howard et al., 1986) were made by examining several different fields by phase contrast microscopy (100x, Olympus BHS, Tokyo, Japan). Aliquots (10 μl) of spermatozoa were removed from each
treatment at the same time as motility and FPM assessments were made, for evaluation of acrosome integrity by fluorescent isothiocyanate-conjugated peanut agglutin (FITC-PNA) staining as described by Roth et al. (1998). Slides were stored at 4°C, protected from light and assessed within two weeks. A total of 200 spermatozoa per slide were assessed using fluorescent microscopy (400x, Olympus). Spermatozoa were classified as acrosome-intact (AI) if the acrosome stained bright green or acrosome-reacted / damaged (AR) if there was no staining or only a single band of green fluorescence at the equatorial segment.

Capacitation status
Aliquots of non-sorted (control) and sorted spermatozoa were removed before staining and sorting (Pre-sort), after sorting and before freezing (Pre-cool), after thawing (0 h) and at 3 and 6 h after incubation (37°C) to assess the capacitation status of the live spermatozoa using dual CTC-H33258 fluorescence. An aliquot of diluted sperm sample (100 µl) was taken prior to freezing (20 x 10^6 / ml) or after thawing (10 x 10^6 / ml) and incubated with H33258 (1µl; 10mg / ml) at room temperature (25°C) for 2 min. Samples were centrifuged (900 g, 5 min) through 2 ml of 2 % (w/v) polyvinylpyrrolidone (PVP-40; Sigma) in ASP diluent to remove unbound stain, egg yolk and glycerol. The supernatant was discarded and 45 µl from the pellet was stained with CTC staining solution and processed as described by Gillan et al. (1997) for ram spermatozoa.

A total of 100-200 spermatozoa per slide were assessed without prior knowledge of treatment, using fluorescent microscopy (400x, Olympus) within 30 min of preparation. Spermatozoa were classified as “dead” if blue fluorescence covered the head or “live” if no fluorescence or only a narrow band of fluorescence at the posterior margin of the head was evident. Only “live” spermatozoa were assessed for CTC patterns; F, uniform fluorescence of the head (characteristic of uncapacitated, acrosome-intact cells); B, bright
fluorescence over the anterior head region only (characteristic of capacitated, acrosome-intact spermatozoa); and AR, faint or no fluorescence except for a thin bright band in the equatorial area (characteristic of acrosome-reacted spermatozoa).

**Sperm migration test**

Spermatozoa were assessed for their ability to migrate through an artificial cervical mucus (10 mg/ml sodium hyaluronate diluted with 25 % ASP diluent containing 1 % v/v BSA; Bioniche, Armidale, NSW, Australia) by a sperm migration test (SMT) developed for human spermatozoa by Mortimer *et al.* (1990) and then further modified by Hollinshead *et al.* (2002b) for low numbers of sorted ram spermatozoa. Briefly, aliquots (50 µl) containing 1 x 10^6 spermatozoa from each treatment were incubated in a BEEM capsule (ProSci Tech, QLD, Australia) at 37°C. A flat capillary tube (3.0 x 3.0 x 100 mm, Microslide, VitroCom, Mountain Lakes, NJ, USA) filled with artificial cervical mucus was positioned vertically in the BEEM capsule. After 1 h incubation the number of spermatozoa migrating 5 mm in the artificial cervical mucus and the distance traveled by the vanguard spermatozoon was assessed under phase contrast microscopy (200x, Olympus).

**Ovine oviducal epithelial cell monolayers**

Two million sorted frozen-thawed, non-sorted (control) frozen-thawed and fresh (control) spermatozoa were resuspended in 500 µl M199 (GIBCO BRL, Life Technologies, Melbourne, Australia) supplemented with 10 % (v/v) filtered, heat-inactivated foetal bovine serum and 1 % (v/v) antibiotic/antimycotic solution (GIBCO BRL) and were added to a confluent oviduct epithelial cell monolayer (OECM). Oviducal epithelial cell monolayers were prepared using the method described by Gillan *et al.* (2000). At 0.5, 2 and 4 h after the addition of spermatozoa from each treatment group to the confluent
monolayers, a single operator made an estimation using an inverted microscope (200x, Olympus) of the percentage of spermatozoa either attached to the OECM or unattached / released from the OECM after counting 200 spermatozoa over a minimum of 3 fields in each dish. Fresh spermatozoa from the same rams but different ejaculates were used for quality control.

**Animals**

Inseminations were carried out during the breeding season at a commercial sheep farm located in the southern highlands of New South Wales. The time of oestrus was controlled in 375 (Trial 1) and 144 (Trial 2) mature Merino ewes by intra-vaginal progestagen-impregnated sponges (40 mg FGA, Bioniche, A/Asia) inserted for 12 d. At sponge removal (SR) each ewe received an intramuscular (I.M.) injection of 400 I.U. pregnant mare serum gonadotrophin (PMSG, Pregnekol, Bioniche, A/Asia). The time of ovulation was controlled in all ewes except for 15 ewes in Trial 1 and 13 ewes in Trial 2 by an I.M. injection of 40 µg of gonadotrophin-releasing hormone (GnRH, Fertagyl®, Intervet, Bendigo, Victoria) 36 h after SR. Ewes were randomly allocated into treatment groups and ear-tagged accordingly prior to insemination.

**Artificial Insemination**

Intrauterine inseminations were performed by laparoscopy in 375 mature Merino ewes in Trial 1 and 123 mature Merino ewes in Trial 2 as described by Evans and Maxwell (1987). All ewes were fasted for 12 h before insemination and local anaesthetic (2 % lignocaine, Troy Laboratories, Smithfield, N.S.W.) was infused at the site of abdominal puncture.

Of the 375 ewes in Trial 1, groups of 120 ewes were inseminated at 54, 58 or 62 h after SR. Within each insemination time, ewes (n=30) were inseminated with either 1, 4 or 16
x10^6 total sorted frozen-thawed spermatozoa or 100 x 10^6 total non-sorted (commercial control) frozen-thawed spermatozoa from three rams. The total volume of thawed spermatozoa deposited into the lumen of both uterine horns was 0.06, 0.25 and 1 ml for insemination doses of 1, 4, 16 x 10^6 total sorted frozen-thawed spermatozoa respectively and 0.25 ml containing approximately 100 x 10^6 non-sorted (commercial control) total frozen-thawed spermatozoa. The 15 remaining ewes that did not receive GnRH were inseminated with non-sorted (commercial control) frozen-thawed spermatozoa at 54-58 h after SR.

In Trial 2, 110 ewes were inseminated 57-60 h after SR with 5, 10, 20 or 40 x 10^6 sorted (n=56) or non-sorted (control, n=54) total frozen-thawed spermatozoa from two rams contained in total inseminate volumes of 0.25, 0.5, 1 and 2 ml respectively. Thirteen ewes not given GnRH were inseminated with 0.25 ml containing 100 x 10^6 non-sorted (commercial control) frozen-thawed spermatozoa 57-58 h after SR.

Pregnancy diagnosis

Pregnancy was determined by detection of a foetus on Day 57 (Trial 1) or Day 53 (Trial 2) after insemination by real time cutaneous ultrasound. The pregnancy rate in ewes was calculated as the number of ewes pregnant at Day 57 or Day 53 / number of ewes inseminated x 100.

Statistical analysis

Data on pregnancy rate in ewes and the effect of time and dose of insemination were analysed by binomial logistic regression using the GENSTAT computer program (Version 4.2, Numerical Algorithms Group©(NAG) Ltd, Oxford, UK). Motility, FPM rating, sorting data and the distance traveled by the vanguard spermatozoon from each treatment
were subjected to logit transformation and analysed by repeated measures and analysis of variance using GENSTAT. Acrosome integrity and the number of spermatozoa penetrating 5 mm into artificial cervical mucus in the sperm migration test were analysed by binomial and log linear logistic regression respectively using GENSTAT. Capacitation status and proportion of spermatozoa attached to the OECM were analysed using a split-plot analysis of variance with ‘type of spermatozoa’ as the main plot and ‘incubation period’ as the sub-plot. The least significant difference procedure or Student Newman-Keuls test was used to test differences between means within treatments.

Results

Experiment 1

In vitro sperm characteristics

Assessment of motility, forward progressive motion and acrosome integrity of H33342 stained and non-stained spermatozoa after incubation (1 h, 34 °C) and of sorted and non-sorted (control) spermatozoa prior to cooling.

After incubation, spermatozoa stained with H33342 had similar motility, FPM and acrosome integrity to non-stained spermatozoa (84 ± 1.0 % and 84 ± 1.3 % motile spermatozoa, 1.0 ± 0.01 and 1.3 ± 0.31 FPM rating and 53 ± 6.7 % and 62 ± 6.9 % intact acrosomes, respectively). The proportion of intact acrosomes decreased (P<0.01) for both stained and non-stained spermatozoa after 1 h incubation. However, after sorting the number of spermatozoa with intact acrosomes significantly increased (91 ± 1.4 %; P<0.001) and was similar to non-stained, non-sorted (control) spermatozoa (93 ± 0.9 %) prior to cooling.
The FPM rating for stained and non-stained spermatozoa after 1 h incubation was low due to a circular motion. The FPM remained low after sorting and was significantly lower (2.2 ± 0.1 FPM rating, P<0.05) than non-stained, non-sorted (control) spermatozoa (4.3 ± 0.2 FPM rating) prior to cooling.

Assessment of motility, forward progressive motion and acrosome integrity of sorted and non-sorted (control) spermatozoa after thawing and incubation (37 °C, 4-6 h)

Overall, non-sorted (control) spermatozoa had higher motility (P<0.01) and FPM (P<0.001) than sorted spermatozoa after thawing (Fig 1a and 1b). However, spermatozoa from both treatments had similar proportions of intact acrosomes (Fig 1c).

After thawing and 2 h incubation (37°C), sorted spermatozoa decreased (P<0.001) in motility (Fig 1a; P<0.001), whereas non-sorted (control) frozen-thawed spermatozoa did not decrease in motility until 4 h post-incubation (Fig 1a). After 6 h incubation (not illustrated) motility, FPM and acrosomal status of the sorted and non-sorted (control) samples were similar (27 ± 1.9 % and 32 ± 3.3 % motile spermatozoa, 3.3 ± 0.91 and 3.3 ± 0.13 FPM rating and 74 ± 1.8 % and 75 ± 2.1 % intact acrosomes, respectively). The motility, FPM and acrosome integrity of spermatozoa from each of the rams did not differ.
**Fig 1.** Motility (a) and forward progressive motility (b) and acrosomal status (c) of sorted (◆) and non-sorted (■, control) spermatozoa after thawing (0 h) and incubation for a 4 h period at 37°C.

(a)
Assessment of capacitation status of sorted and non-sorted (control) frozen-thawed spermatozoa

There was a significant interaction between the sorted frozen-thawed and non-sorted (control) frozen-thawed spermatozoa with respect to the distribution of B- and AR-patterns over time (P<0.01). Before freezing, a higher proportion of sorted frozen-thawed spermatozoa exhibited the B-pattern than non-sorted (control) frozen-thawed spermatozoa (22 ± 1.9 % and 16 ± 2.4 % respectively; P<0.05). Post-thaw, sorted and non-sorted (control) spermatozoa had similar proportions of B-pattern (85 ± 3.5 %, 89 ± 1.8 %) but, after a 3 h incubation period, a lower proportion of sorted frozen-thawed spermatozoa exhibited the B-pattern (67 ± 2.6 %) and a higher proportion had the AR-pattern (25 ± 3.1 %) than non-sorted (control) frozen-thawed spermatozoa (80 ± 1.2 % and 13 ± 2.4 % for B- and AR-pattern respectively, P<0.01; Fig 2a and 2b).
**Fig 2.** Percentage of (a) non-sorted (control) and (b) sorted frozen-thawed spermatozoa distributed across each of the chlortetracycline staining patterns ('black', F-pattern; 'stripes', B-pattern and 'white', AR-pattern) before sorting (Pre-sort), after sorting and before cooling (Pre-cool), after thawing (0 h) and after a 6 h incubation period at 37°C.

(a)

![Graph showing spermatozoa distribution across different staining patterns at various time points](image)

(b)

![Graph showing spermatozoa distribution across different staining patterns at various time points](image)
Assessment of the migration of sorted and non-sorted (control) frozen-thawed spermatozoa in artificial cervical mucus (Sperm Migration Test)

After thawing, similar numbers of non-sorted (control) and sorted spermatozoa migrated 5 mm into the artificial cervical mucus (76 ± 11.9 and 74 ± 11.9 spermatozoa respectively). There was no difference between treatments in the distance migrated by the vanguard spermatozoon (57 ± 7.8 and 39 ± 5.8 mm respectively). There was no effect of ram on the number of spermatozoa penetrating 5 mm in the artificial cervical mucus. However, the distance reached by the vanguard spermatozoon from Ram 3 (56 ± 8.3 mm) and 5 (66 ± 5.6 mm) was further (P<0.05) than the vanguard spermatozoon from Ram 6 (29 ± 2.7 mm).

Assessment of the binding pattern of fresh, sorted and non-sorted (control) frozen-thawed spermatozoa to oviduct epithelial cell monolayers

The percentage of motile spermatozoa bound to the OECM at each incubation period is shown in Figure 3. Over the 4 h incubation period a greater proportion of fresh (control) spermatozoa attached to the OECM (71 ± 3.1 %) than either non-sorted (control) frozen-thawed (42 ± 3.1 %) and sorted frozen-thawed (32 ± 3.3 %) spermatozoa (P<0.001).

There was a significant interaction between type of spermatozoa and incubation period (P<0.001; Fig 3). Increasing numbers of motile fresh (control) spermatozoa bound to the OECM (P<0.001) reaching a peak at 2 h and then maintaining the same proportion of attached motile spermatozoa for the remainder of the incubation period. Conversely, non-sorted (control) and sorted frozen-thawed spermatozoa were continually released from the OECM during incubation (P<0.001) but sorted frozen-thawed spermatozoa was released more rapidly from the OECM than non-sorted (control) frozen-thawed spermatozoa (P<0.05).
Experiment 2

*Effect of time of insemination and dose of sorted and non-sorted (commercial control) frozen-thawed ram spermatozoa on fertility in ewes (Trial 1)*

There was no difference in motility of sorted (43 ± 0.6 %) or non-sorted (42 ± 0.4 %, commercial control) spermatozoa prior to AI.

The proportion of ewes pregnant was significantly affected by dose and sperm type (P<0.001) but not by time of insemination or ram (Table 1a). The overall pregnancy rate was higher after insemination with non-sorted (commercial control) frozen-thawed spermatozoa than with low doses of sorted frozen-thawed spermatozoa (P<0.05). Of the ewes inseminated with sorted frozen-thawed spermatozoa, the proportion pregnant after insemination with 16 x 10^6 spermatozoa was greater than for ewes inseminated with 1 x 10^6 spermatozoa (P<0.05). A similar number of ewes were pregnant after insemination with 4 or 16 x 10^6 sorted frozen-thawed spermatozoa. Time of insemination did not affect
pregnancy rate. Although not significant, 58 % of GnRH-treated ewes were pregnant 58 h after sponge removal and insemination with 100 x 10^6 non-sorted (commercial control) frozen-thawed spermatozoa compared to 40 % of non-GnRH-treated ewes inseminated with the same dose of control spermatozoa.

Table 1a: The number of GnRH and non-GnRH treated ewes pregnant\(^1\) after insemination (%) with sorted and non-sorted (commercial control) frozen-thawed spermatozoa at 54, 58 and 62 h after sponge removal.

<table>
<thead>
<tr>
<th>Sperm Dose (x10^6)</th>
<th>Sperm Type</th>
<th>Time of Insemination</th>
<th>Total</th>
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<td></td>
<td></td>
<td>54 h (+GnRH)</td>
<td>58 h (+GnRH)</td>
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<tr>
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<td>Sorted</td>
<td>5/29 (17.2)</td>
<td>5/30 (16.7)</td>
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<td></td>
<td>15/89 (16.9)^b</td>
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<tr>
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<td>Sorted</td>
<td>8/30 (26.7)</td>
<td>7/30 (23.3)</td>
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<td></td>
<td></td>
<td>22/90 (24.4)^ab</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Sorted</td>
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<td>12/30 (40.0)</td>
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<tr>
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<td></td>
<td>58/104 (55.8)^c</td>
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</tbody>
</table>

\(^{abc}\) Within column values with different superscripts differ (P<0.05)
\(^{1}\)(Pregnancy determined by ultrasound at Day 60)

Effect of dose of sorted and non-sorted (control) frozen-thawed spermatozoa on pregnancy (Trial 2)

There was no difference in the percentage of motile spermatozoa prior to their use for AI, being 42.9 ± 0.9 % for non-sorted (control) spermatozoa and 37.8 ± 1.8 % for sorted frozen-thawed spermatozoa.

There was no difference in pregnancy rate between ewes that were treated with GnRH (52 / 110, 47 %) and those that were not (7 / 13, 53.8 %). Of the GnRH-treated ewes, the proportion pregnant was affected by the number of spermatozoa inseminated (P<0.05) but
not by sperm type or ram (Table 1b). For ewes inseminated with sorted or non-sorted (control) frozen-thawed spermatozoa, the pregnancy rate was higher for inseminates of 40 x 10^6 than for 5 and 20 x 10^6 spermatozoa (P<0.05; Table 1b).

**Table 1b:** Pregnancy\(^2\) after intrauterine insemination of (i) GnRH-treated ewes with sorted and non-sorted (control) frozen-thawed ram spermatozoa 58 h after progestagen sponge removal and of (ii) non GnRH-treated ewes with non-sorted (commercial control) frozen-thawed ram spermatozoa 54-58 h after progestagen sponge removal.

| Dose (x 10^6 sorted and non-sorted control spermatozoa) | Sperm Type | No. ewes inseminated | No. ewes pregnant (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Sorted</td>
<td>15</td>
<td>3(^a) (20.0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>15</td>
<td>7(^abc) (46.7)</td>
</tr>
<tr>
<td>10</td>
<td>Sorted</td>
<td>14</td>
<td>6(^abc) (42.9)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>10(^c) (71.4)</td>
</tr>
<tr>
<td>20</td>
<td>Sorted</td>
<td>16</td>
<td>5(^ab) (31.3)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>13</td>
<td>5(^ab) (38.5)</td>
</tr>
<tr>
<td>40</td>
<td>Sorted</td>
<td>11</td>
<td>8(^c) (72.7)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12</td>
<td>8(^bc) (66.7)</td>
</tr>
<tr>
<td>100</td>
<td>Commercial Control (- GnRH)</td>
<td>13</td>
<td>7(^abc) (53.8)</td>
</tr>
</tbody>
</table>

\(^{abc}\) Within column values with different superscripts differ (P<0.05)

\(^{2}\)Pregnancy determined by ultrasound at Day 60

**Discussion**

This study demonstrated that the *in vitro* lifespan of sorted frozen-thawed spermatozoa is shorter than non-sorted (control) frozen-thawed spermatozoa, making insemination close to the time and site of ovulation critical for successful fertilisation, especially after low dose insemination. Previously, it was demonstrated that low doses of sorted frozen-thawed
ram spermatozoa inseminated into ewes not synchronised for ovulation resulted in a low pregnancy rate (25%; Hollinshead et al., 2002a). In this study, determination of the optimal time of insemination relative to ovulation and the minimum effective sperm dose resulted in similar fertility of sorted and non-sorted (control) frozen-thawed ram spermatozoa.

Gillan et al. (1997) demonstrated using CTC staining that both the freeze-thaw and incubation processes caused ram spermatozoa to undergo membrane changes similar to capacitation and that further incubation of previously capacitated spermatozoa caused them to acrosome-react. In the present study, spermatozoa were in a more capacitated state directly after sorting than non-sorted (control) spermatozoa (Fig 2a and 2b). After freezing and thawing, sorted spermatozoa progressed more rapidly to an acrosome-reacted state than non-sorted spermatozoa over a 6 h incubation period. Sorted frozen-thawed spermatozoa also had lower motility and FPM than non-sorted (control) frozen-thawed spermatozoa after 6 h incubation. These results suggest that the process of sorting may increase the proportion of capacitated cells. Watson (1995) proposed that capacitated cells have a short life span and if not exposed to oocytes within a short time fertility will not be achieved. Consequently, sorted frozen-thawed spermatozoa would have a reduced lifespan after freeze-thawing and incubation compared to non-sorted (control) frozen-thawed spermatozoa.

The pattern of attachment and detachment to and from the OECM of both controls (fresh and frozen-thawed spermatozoa) were similar to the findings previously reported by Gillan et al. (2000) for fresh and frozen-thawed ram spermatozoa. In the present study, motile sorted frozen-thawed spermatozoa had a similar pattern of attachment and release from the OECM as motile non-sorted (control) frozen-thawed spermatozoa (Fig 3). However,
sorted frozen-thawed spermatozoa attached quickly to the OECM but then immediately started to release and continued to detach from the oviducal cells for the remainder of the incubation period. The transient binding and release of sorted frozen-thawed spermatozoa to the OECM may indicate that they only require a short period of attachment to the oviduct cells to attain the fully capacitated state, even less than non-sorted (control) frozen-thawed spermatozoa.

Similarly, the motility of sorted frozen-thawed spermatozoa significantly decreased after 2 h incubation and continued to decrease for the remainder of the incubation period. Whereas non-sorted (control) frozen-thawed spermatozoa did not decrease in motility until after 4 h incubation (Fig 1a). These findings correlate with the pattern of attachment and release from the OECM after 4 h incubation and the CTC patterns demonstrated after 6 h incubation.

There are many components of the sorting process which may be responsible for these membrane changes. Such components have been investigated previously, including nuclear staining with H33342 (Catt et al., 1997a; Schenk et al., 1999; Seidel and Garner, 2002), dilution during staining and passage through the flow cytometer (Catt et al., 1997b; Maxwell and Johnson, 1999), exposure to a laser (Catt et al., 1997a; Schenk et al., 1999; Guthrie et al., 2002) and mechanical forces during sorting (Suh and Schenk, 2003; Campos-Chillon and De La Torre, 2003). In the present study, the proportion of intact acrosomes (determined by FITC-PNA), motile spermatozoa and FPM were similar for stained and non-stained spermatozoa after incubation (34°C, 1 h), and the proportion of intact acrosomes and motile spermatozoa were similar for sorted and non-sorted (control) fresh spermatozoa before and after freeze-thawing. These results indicate that staining with H33342 has minimal effect on spermatozoa viability and that the sorting process
results in the selection of a viable and acrosome-intact population of spermatozoa. The quality of spermatozoa after sorting of frozen-thawed and liquid stored samples clearly benefited from use of food dye to eliminate non-viable spermatozoa in the sorting process (unpublished data). Nevertheless, the component(s) which contribute the most to the membrane changes observed in the present study and which ultimately result in a reduction of the fertilising life span of sorted ram spermatozoa (Hollinshead et al., 2002a) have yet to be determined.

Although there was also no difference in motility before and after incubation (34°C, 1 h), the number of intact acrosomes and FPM decreased for both stained and non-stained samples. After incubation of spermatozoa in TRIS diluent, spermatozoa were vigourous but had low FPM. We have previously made this observation (unpublished data) after incubation of ram spermatozoa in TRIS diluent. However, sorted spermatozoa have the capacity to undergo improved FPM as demonstrated by the sperm migration test in the study.

Despite the capacitation-like changes that sorted spermatozoa have undergone, sorted and non-sorted (control) frozen-thawed spermatozoa traveled similar distances in the SMT and had similar numbers of spermatozoa penetrating 5 mm into the artificial cervical mucus. The artificial cervical mucus is used in the sperm migration test to mimic the penetration of spermatozoa through cervical mucus and has properties comparable with ewe cervical mucus (S. T. Mortimer, pers. comm.). Cox et al. (2002) reported a significant correlation between sperm migration in cervical doe mucus and the ability of buck spermatozoa to colonize the oviduct and fertilise in vivo matured oocytes. These results suggest that sorted frozen-thawed spermatozoa may have a similar capacity to non-sorted (control) frozen-
thawed spermatozoa to migrate to the site of fertilisation after intrauterine or utero-tubal junction insemination.

The *in vitro* assessments in the present study suggest that sorted frozen-thawed spermatozoa may have a reduced lifespan *in vivo* but maintain the ability to migrate to the site of fertilisation. Thus it was hypothesized that treatment of ewes with gonadotrophin-releasing hormone (GnRH) in combination with progestagen sponges and PMSG to reduce the period over which ovulation occurs (Eppleston *et al.*, 1991) and permit insemination close to the time of ovulation would improve the fertility of sorted frozen-thawed spermatozoa. However, pregnancy rates were not different in GnRH-treated ewes after insemination with sorted or non-sorted (commercial control) frozen-thawed spermatozoa 54, 58 or 62 h after progestagen sponge removal and PMSG injection (Table 1a), though there were indications that a larger scale experiment may be warranted to detect a significant benefit for the 54 and 58 AI times over the latter AI time. There was also no difference in pregnancy rate after insemination with frozen-thawed (commercial control) spermatozoa in ewes treated with GnRH or ewes synchronised with only progestagen sponges and PMSG. These findings were similar to previous reports that found no improvement in fertility after treatment with GnRH and insemination of frozen-thawed spermatozoa (Maxwell *et al.*, 1986a; Walker *et al.*, 1989b) and suggest that the number of sorted frozen-thawed spermatozoa, especially after low dose insemination, plays a greater role on fertility in ewes after intrauterine insemination. Smith *et al.* (1986) reported that GnRH treatment facilitated increased pregnancy rates after insemination of low numbers (1 x 10^6) of frozen-thawed ram spermatozoa compared to non-GnRH treated ewes (53 % and 37 %, respectively), but the effect of GnRH was not significant after insemination of commercial doses of frozen-thawed ram spermatozoa. While the present study did not specifically examine the effect of GnRH treatment after low dose insemination of non-
sorted frozen-thawed spermatozoa there was no difference in pregnancy rate after insemination with similar numbers of sorted and non-sorted (control) spermatozoa in GnRH treated ewes (Table 1b). Pregnancy rates in this study were lower than those reported previously after low dose intrauterine insemination with frozen-thawed ram spermatozoa in synchronized ewes (Eppleston et al., 1986; Maxwell et al., 1993). The pregnancy results reported in this study suggest that a minimum of $40 \times 10^6$ total sorted frozen-thawed spermatozoa ($20 \times 10^6$ motile) are required to obtain commercially acceptable pregnancy rates (70 %). This falls within the range of the minimum intrauterine insemination dose recommended by the sheep industry for frozen-thawed (non-sorted) ram spermatozoa (Evans and Maxwell, 1987). However, acceptable pregnancy rates were achieved after insemination of $10 \times 10^6$ sorted or non-sorted (control) frozen-thawed spermatozoa (57 %) but not $20 \times 10^6$ (35 %). Variation in pregnancy rates after intrauterine insemination with low doses of frozen-thawed ram spermatozoa have been reported in many previous studies (reviewed by Evans, 1988).

This study has demonstrated that sorting accelerates the capacitation and acrosome reaction processes of spermatozoa after freeze-thawing and incubation. The rapid release of sorted frozen-thawed spermatozoa from the oviduct cells also suggests that they have a reduced fertilising lifespan in vivo. Future research into sorting and freeze-thawing methods that incorporate strategies to stabilize sperm membranes, such as reduction of sheath fluid pressure and the addition of seminal plasma, may improve their fertilising lifespan. Consequently, an increase in the number of fertile cells in an optimal functional state may reach and establish reservoirs at the utero-tubal junction after intrauterine insemination. Commercially acceptable pregnancy rates may then be achievable with insemination of lower doses of sex-sorted frozen-thawed ram spermatozoa.
Acknowledgements

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References


III. Liquid storage of flow cytometrically sorted ram spermatozoa.

Abstract

This study was designed to investigate the optimum short-term storage conditions for ram sperm before and after flow cytometric sorting. Prior to sorting, semen from 4 rams (n=3 ejaculates per ram) was diluted in either a Tris-based diluent (TRIS) or AndroHep® (AH) and stored at 5, 15 or 21°C for 0, 6 or 24 h. Sperm characteristics (motility and acrosome integrity) were assessed during storage and after sorting, freeze-thawing and incubation (6 h, 37°C). Functional capacity and migration ability in artificial cervical mucus (Sperm migration test; SMT) of stored, sorted and non-sorted (control) spermatozoa were assessed after freeze-thawing. Overall, spermatozoa stored in TRIS were sorted more efficiently (P<0.01), had higher motility after sorting, freezing, thawing and incubation (37 ± 1.4 %; P<0.001) and had greater numbers penetrating 5 mm into the artificial cervical mucus (38 ± 13.7; P<0.01) than spermatozoa stored in AH prior to sorting (15 ± 1.4 % overall motility and 10 ± 2.1 spermatozoa at 5 mm respectively). Spermatozoa stored at 21°C had higher motility (82 ± 4.0 %) than spermatozoa stored at 5°C (64 ± 3.1 %; P<0.001) but there was no difference in sorting efficiency between the three storage temperatures. There was also no difference (P>0.05) in the fertilisation (61 ± 4.3 %, overall), cleavage (49 ± 3.8 %, 48 h overall), blastocyst development rate (54 ± 3.2 %, D 7 overall) or the proportion of grade 1 embryos with > 150 cells (60 ± 6.1 %, overall) after in vitro insemination with spermatozoa liquid stored in TRIS or AH for 24 h prior to sorting and freeze-thawing or non-sorted (control) frozen-thawed spermatozoa. After sorting, semen from 3 rams (n=3 ejaculates per ram) was split and diluted in four different extenders: Ultra-heat-treated (UHT) long life milk, TRIS containing 10 % (v/v) egg yolk (TRIS-EY),
AH (pH 7.4) or TEST buffer containing 10 % (v/v) egg yolk (TYB). Sorted and non-sorted (control) spermatozoa were stored at 15°C for 24 h or 5°C for 6 d. Sperm characteristics (motility and acrosome integrity) were evaluated at 0, 6 and 24 h for samples stored at 15°C and daily for samples stored at 5°C. The SMT was performed on sorted and non-sorted (control) spermatozoa after 6 h and 3 d storage at 15°C and 5°C respectively. Spermatozoa stored in UHT at both temperatures had higher (P<0.001) overall motility, acrosome integrity and traveled greater distances (P<0.01) in the SMT than spermatozoa stored in all other diluents. In summary, the results showed that storage in TRIS at 21°C is optimal for transport of ram spermatozoa to the sorting site, and storage of spermatozoa in UHT diluent after sorting preserved spermatozoa viability and migration ability best at both 15°C and 5°C. These findings indicate that sires for semen collection and females for artificial insemination (AI) can be located at considerable distance from the sperm sorting facility.

Introduction

Cryopreservation of sorted spermatozoa and the production of offspring has been reported in cattle (Seidel et al., 1999), pigs (Johnson et al., 2000) horses (Lindsey et al., 2002), elk (Schenk and DeGrofft, 2003) and sheep (Hollinshead et al., 2002a). Predetermination of sex of livestock via sperm sorting involves use of a cell sorter at a fixed location which may be remote from donor males or females for artificial insemination (AI). The need for transport of semen from the point of collection to the site of sorting requires the preservation of spermatozoa under artificial conditions. There have been previous reports of ram spermatozoa transported to the site of the sperm sorter in a frozen state (Hollinshead et al., 2002b). However, the efficiency of transporting spermatozoa in this form is low as the number of viable sperm available for sorting is reduced after thawing,
generally by 50 %, thus requiring twice the number of frozen-thawed spermatozoa in order
to sort similar numbers as fresh samples. An alternative for shorter periods of time is to
transport spermatozoa in a liquid (unfrozen) state at temperatures and in diluents that not
only maintain viability of spermatozoa but that maintain suitability for staining and
sorting.

Successful liquid storage of ram spermatozoa is dependent on the reversible reduction of
motility and metabolic activity of the spermatozoa, thereby prolonging their fertile life.
This is achieved by storing semen at temperatures lower than body temperature (i.e 15°C
and 5 ºC) and in diluents composed of compounds that help delay the production of
reactive oxygen species. High fertilisation rates (75 %) have been achieved after
intrauterine insemination of ram spermatozoa stored at 5°C for 6 days (Salamon et al.,
1979). It would be commercially advantageous if similar results were possible with sex-
sorted spermatozoa.

The objectives of the of the present study were to (i) evaluate in vitro sperm characteristics
and sorting efficiency of spermatozoa stored for 0, 6 and 24 h in two different diluents and
at three different temperatures (21, 15 and 5°C) prior to sorting, (ii) investigate the in vitro
functional capacity of stored (24 h at 21°C), sorted, frozen-thawed spermatozoa and (iii)
evaluate in vitro sperm characteristics of sorted and non-sorted (control) spermatozoa
liquid stored in four different diluents at 15°C for 24 h and 5°C for 6 d.
Materials and Methods

Experimental design

Semen collection

For both experiments, semen was collected by artificial vagina from mature Merino rams housed at The University of Sydney, Camperdown, N.S.W., during the non-breeding season. Only ejaculates containing spermatozoa with > 80 % forward progressive motility and concentrations more than 2 500 x 10^6 spermatozoa/ml were used.

Experiment 1: Liquid storage prior to sorting

In Experiment 1, two extenders (TRIS and AH) x three storage temperatures (21°C, 15°C and 5°C) x three storage times (0 h, 6 h and 24 h) x four rams (Rams 1-4) were examined before flow cytometric sorting. As a control, an aliquot of semen was removed from each ejaculate and diluted with a zwitterion-buffered diluent containing 13.5 % egg yolk and 6 % glycerol (ZWIT; Molinia et al., 1996) to a concentration of 20 x 10^6 spermatozoa/ml and frozen using the pellet method (Evans and Maxwell, 1987). The remainder of each ejaculate was diluted with either AndroHep (AH; Minitüb, GmbH, Landshut, Germany) adjusted to pH 7.4 or a Tris-buffered diluent (TRIS; pH 7.4; Evans and Maxwell, 1987) modified for both liquid storage and staining of ram spermatozoa (see below). Each sample was then divided into three equal parts and stored at 21°C, 15°C or 5°C. In vitro spermatozoa characteristics were assessed, as described below, at 0, 6 and 24 h during liquid storage for samples stored at 5, 15 and 21°C in both TRIS and AH diluents. After 0, 6 and 24 h of liquid storage at 21°C in both TRIS and AH diluents, samples were sorted by flow cytometry, frozen and subsequently thawed by the methods previously described (Hollinshead et al., 2002a).
Spermatozoa sorted, frozen and thawed after collection (0 h) and after 6 h of liquid storage at 21°C were tested for ability to penetrate an artificial cervical mucus after thawing. At 0, 3 and 6 h during incubation (37°C) after thawing aliquots of spermatozoa were removed for assessment of spermatozoa characteristics (see below). Spermatozoa sorted, frozen and thawed after liquid storage for 24 h at 21°C were evaluated, using *in vitro* matured oocytes, for fertilisation, cleavage and blastocyst development after culture for 7 d. Control spermatozoa were thawed, incubated and assessed at the same time as sorted spermatozoa treatments. The experiment was replicated three times.

**Experiment 2: Liquid storage after sorting**

In Experiment 2, four extenders (UHT, TRIS-EY, AH and TYB) x two storage temperatures (5°C and 15°C) x three rams (Rams 2, 3 and 4) were examined at 24 h and 6 days after flow cytometric sorting. Non-sorted (control) and sorted spermatozoa were extended in either Ultra-heat-treated (UHT) ‘long life’ milk, TRIS containing 10 % (v/v) egg yolk (TRIS-EY), AH or TEST buffer (Johnson *et al*., 1989) containing (v/v) 10 % egg yolk (TYB) and stored at 15°C for 24 h or 5°C for 6 d. The pH of each of the liquid storage diluents was 7.4. Migration through an artificial cervical mucus was assessed for each treatment after 6 h storage at 15°C and 3 d storage at 5°C. In *vitro* spermatozoa assessments (see below) were made at 0, 6 and 24 h for spermatozoa stored at 15°C and daily for spermatozoa samples stored at 5°C. Both non-sorted (control) and sorted spermatozoa came from the same ejaculate for each ram and replicate. The experiment was replicated three times.

**Preparation of spermatozoa for liquid storage prior to sorting**

After removal of an aliquot of spermatozoa from each ejaculate (control) the remainder was halved and slowly diluted 1:4 (semen: diluent, v/v) with either AH or TRIS modified
as follows for both storage and staining of ram spermatozoa. The composition of the modified TRIS diluent was 300 mM Tris-(hydroxymethyl) aminomethane, 27.8 mM fructose, 94.7 mM citric acid, 0.15g / l penicillin, 0.25g / l streptomycin and 0.3 % BSA (w/v; A-9647 Sigma, St Louis, MO, USA). Each diluted sample was then divided into three parts (A, B and C) and placed in 4 ml sterile sealed test-tubes (Falcon 2003, Becton Dickinson and Company, Franklin Lakes, N. J, USA). Part A was cooled in a temperature controlled room to 21ºC and then held in a 21ºC thermos. Part B and C were placed in a temperature controlled cabinet and slowly cooled from 21ºC to 15ºC over 1.5 h. Part B was maintained at 15ºC in a thermos containing 15ºC water. Part C was then transferred to a 5ºC cool-room, cooled to 5ºC over 1.5 hours and then held at 5ºC in a thermos containing 5ºC water. All samples were stored for 24 h. Each thermos was tested for maintenance of temperature over 24 h and none deviated by more than +/- 1ºC from the three temperatures under test. A thermos was used rather than a temperature controlled cabinet, in order to replicate potential transport conditions for spermatozoa samples being sent from the site of collection to the sperm sorting laboratory.

Preparation for sorting

Spermatozoa from samples stored for 0 and 6 h at 21ºC and for 24 h at 5, 15 and 21ºC (Experiment 1) or from fresh ejaculates (Experiment 2) were all prepared using the method described by Hollinshead et al. (2002a). Briefly, spermatozoa were diluted (400 x 10^6/ml) with the same diluent in which they had been stored (Experiment 1) or with TRIS diluent for fresh ejaculates (Experiment 2). Each sample was then incubated with either 267 µM / ml (Ram 1) or 311 µM / ml (Rams 2, 3 and 4) of Hoechst 33342 (H33342; Sigma) at 34ºC. These stain concentrations had been previously optimized for each ram. After 1 h incubation, stained samples were carefully diluted 1:1 (stained sample: diluent, v/v) with the same diluent in which they were incubated during staining (TRIS or AH) and
containing 4 % (v/v) egg yolk and 0.002 % (w/v) food dye (Warner Jenkinson Company Inc., St Louis, MO, USA). Prior to sorting, samples were filtered through a 37-µm nylon mesh filter (Falcon 2235) to remove any large aggregates of agglutinated spermatozoa or debris.

**Flow Cytometric Sorting**

Spermatozoa stored at 21°C and fresh ejaculates from each ram were processed, without separation according to sex, through a high-speed cell sorter (Mo FloSX®, DakoCytomation, CO, USA) modified for sperm sorting (Johnson and Pinkel, 1986; Rens et al., 1999). The sorter was operated at 40 psi with TRIS (without 1 % BSA) sheath fluid and an argon laser running at 200 mW in a temperature controlled laboratory (21°C).

During sorting, “enrich sort mode” was used to maximize spermatozoa recovery thus sacrificing purity. Sort gates were set to include all viable oriented and non-oriented spermatozoa populations. The flow rate during both experiments was 20 000 events/sec and spermatozoa were processed at 5 000-9 000 /sec (Experiment 1) and 11 300-13 000 /sec (Experiment 2). Sort rates for each treatment depended on the number of viable spermatozoa available for sorting.

Sorted spermatozoa were collected into 10-ml centrifuge tubes containing 0.25 ml of either the same diluent in which they had been previously stored (Experiment 1) containing 20 % (v/v) egg yolk or TRIS diluent (Experiment 2) containing 20 % (v/v) egg yolk. After collection of 2 x 10⁶ spermatozoa another 0.25 ml of 20 % (v/v) egg yolk collection medium was added. A total of 5 x 10⁶ spermatozoa in 5 ml of TRIS sheath fluid were collected at a time. The final concentration of egg yolk was 2 % (v/v).
Reanalysis of sorted spermatozoa and sorting parameters

In Experiment 2, an additional 500 000 X- and Y- chromosome bearing spermatozoa were collected from each ejaculate with sort gates placed around only the viable and correctly oriented sperm populations to demonstrate that greater than 85 % purity could be obtained from the spermatozoa samples prepared for storage. The spermatozoa were processed and analysed as described by Welch and Johnson (1999) to determine X and Y purity. Due to the large number of treatments in Experiment 1 resort analysis could not be carried out. However, in both Experiments all samples prepared for sorting were analysed under the same high purity conditions and sorting parameters for each treatment were recorded. These included sort rate, R-value (measurement of the degree of separation between the two peaks in the histogram which represent the X- and Y- chromosome bearing spermatozoa populations), proportion of correctly oriented spermatozoa and percentage of viable spermatozoa.

Preparation of spermatozoa for storage after sorting

After sorting, each sample was centrifuged at 700 g for 8 min leaving a 50 µl pellet containing 5 x 10^6 sorted spermatozoa.

In Experiment 1, each pellet was resuspended 1:4 (sperm pellet: diluent, v/v) in ZWIT diluent and frozen and thawed using the same method as for non-sorted (control) spermatozoa.

In Experiment 2, each 50 µl pellet containing 5 x 10^6 sorted spermatozoa was slowly resuspended 1:9 (sperm pellet: diluent, v/v) with either UHT, TRIS-EY, AH or TYB diluent. Aliquots containing 5 x 10^6 spermatozoa from each ejaculate were extended to 0.5 ml with the same diluents as the sorted samples (control). Both non-sorted (control) and
sorted spermatozoa were stored in 5 ml sterile sealed test-tubes (Falcon 2003) and cooled to 15°C over 1.5 h in a temperature controlled cabinet. Half the samples were then further cooled to 5°C over 1.5 h in a 5°C cool-room. These samples were maintained at 5°C for 6 d. The 15°C samples were held in the temperature controlled cabinet for 24 h.

In vitro spermatozoa characteristics
Subjective assessments of total progressive motility of spermatozoa (motility to the nearest 5 %) were made on heated slides (37°C) by the same observer using phase contrast microscopy (100x, Olympus, Tokyo, Japan). These were done at 0, 6 and 24 h during liquid storage at 5, 15 and 21°C prior to sorting, and after staining, sorting, freeze-thawing and incubation (37°C) for 6 h (Experiment 1) and at 0, 6 and 24 h during liquid storage at 15°C and daily for 6 d during liquid storage at 5°C (Experiment 2).

At the same time dry smears were prepared for the assessment of acrosome integrity of spermatozoa. Within 2 weeks, slides were stained with fluorescent isothiocyanate-conjugated peanut agglutin (FITC-PNA) staining as described by Roth et al. (1998) and evaluated within 1 h of staining. A total of 100-200 spermatozoa per sample were examined under UV light using fluorescent microscopy (400x, Olympus). Spermatozoa were classified as acrosome intact (AI) if the acrosome stained bright green or acrosome-reacted (AR) if there was no staining or only a single band of green fluorescence at the equatorial segment.

A sperm migration test (SMT) developed in humans (Mortimer et al., 1990) and modified for low numbers of sorted ram spermatozoa (Hollinshead et al., 2002b) was used to assess the ability of spermatozoa to migrate through artificial cervical mucus comprising sodium hyaluronate cryopreservative (10mg/ml; Bioniche, Armidale, NSW) diluted with 25 % v/v
TRIS (Experiment 1) or AH (Experiment 2) and supplemented with 1 % v/v BSA. The SMT was performed post-thaw (Experiment 1) and after 6 h storage at 15°C or 3 d storage at 5°C (Experiment 2). Aliquots (50 µl) containing 1 x 10^6 (Experiment 1) or 0.5 x 10^6 (Experiment 2) spermatozoa from each treatment were incubated (37°C, 1 h) in BEEM capsules (BEEM standard embedding capsules, ProSciTech, QLD, Australia) with a vertically placed 0.3 mm x 0.3 mm x 100 mm rectangular capillary tube (Microslides, VitroCom, Mountain Lakes, NJ, USA) filled with artificial cervical mucus. The number of spermatozoa migrating 5 mm into the artificial cervical mucus was determined at an upper and lower field using phase contrast microscopy (200x, Olympus) and the mean distance traveled by the vanguard spermatozoan from each treatment was also determined using phase contrast microscopy (200x, Olympus).

In vitro fertilisation system

The methods of in vitro production of embryos in Experiment 1 have been described previously by O’Brien et al. (1996) and were modified for sorted spermatozoa by Hollinshead et al. (2003). All in vitro culture took place at 38.5ºC in a humidified atmosphere of 5 % O₂: 6 % CO₂: 89 % N₂ (V-MINC-1000, Cook®, QLD, Australia).

Oocytes were aspirated from follicles 2 to 4 mm in diameter and only those with a homogenous ooplasm surrounded by several layers of cumulus cells were selected for IVM. Groups of 25-30 cumulus-oocyte complexes were incubated in multi-well dishes (Nunclon; Inter Med, Roskilde, Denmark) containing 450 µl of maturation medium overlaid with 400 µl mineral oil (M8410, Sigma) for 22 h. The maturation medium contained Medium 199 (Gibco BRL, Grand Isalnd, NY, USA; with Earl’s salts, 15mM L-glutamine, 25mM sodium bicarbonate, 26 mM HEPES) supplemented with 20 % (v/v)
sheep serum (ThermoTrace), 10µg/ml FSH (Folltropin-V; Bioniche), 10µg/ml LH (Lutropin-V; Bioniche), 124 IU/ml penicillin and 37 IU/ml streptomycin.

For IVF, non-sorted (control) spermatozoa and spermatozoa stored for 24 h in either TRIS or AH prior to sorting were slowly diluted in 0.5 ml of warm (37ºC) Sydney IVF SOF bicarbonate buffered medium (SIVF Sperm buffer; Cook®) and centrifuged at 500 g for 5 min in 2 ml centrifuge tubes (ThermoTrace Ltd, Noble Park, Victoria, Australia) after thawing. Following in vitro maturation and removal of cumulous cells by gentle pipetting, 20-25 oocytes were co-incubated with 1 x 10^6 motile spermatozoa / ml from each treatment (n= 3 treatment groups / ram) in multiwell dishes (Nunclon; Inter Med, Roskilde, Denmark) with each well containing 400 µl of fertilisation medium (O’Brien et al., 1996) and 400 µl washed and filtered mineral oil (M8410, Sigma). After 3 h culture in IVF medium, 10-15 presumptive zygotes were transferred to 10 µl droplets of Sydney IVF cleavage medium (Cook®) under mineral oil for 4 d followed by Sydney IVF blastocyst medium (Cook®) for an additional 3 d culture.

For quality control of each IVF replicate, frozen-thawed and fresh spermatozoa pooled from the four rams were prepared for IVF using the swim up technique described by Brown and Radziewic (1998). Briefly, 100 µl of control spermatozoa was placed in a 5 ml test-tube (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) under 500 µl Sydney IVF SOF bicarbonate buffered medium (SIVF Sperm buffer; Cook®). After incubation in a humidified atmosphere of 5 % CO₂ for 1 h in fertilisation medium, motile spermatozoa were removed from the top of the swim up and added at a concentration of 1 x 10^6 /ml to wells containing 20-25 matured oocytes in 400 µl of fertilisation medium and 400µl of mineral oil and incubated for 6 h.
Oocyte and embryo assessment

Oocytes were assessed for cleavage at 24 and 48 h after insemination by light microscopy (100X). At 48 h, uncleaved oocytes were fixed in 2 % paraformaldehyde and phosphate-buffered saline solution (PBS) for 2 h at 37°C; stained with Hoechst 33342 (2 µg/ml, Sigma) and examined using fluorescent microscopy (400x; Olympus).

Due to limitations in ovary availability arising from drought conditions experienced at the time this experiment was carried out, the maturation rate was calculated at 48 h post-insemination (p.i) as the proportion of oocytes undergoing cleavage and the number of uncleaved oocytes with chromosomes at metaphase-II and an extruded polar body and/or containing 2 pro-nuclei. Oocytes containing more than two pronuclei were considered to have undergone polyspermic fertilisation. The monospermic fertilization rate was determined as the proportion of matured oocytes undergoing cleavage and the number of uncleaved oocytes containing 2 pro-nuclei at 48 h pi. The number of cleaving oocytes developing to the blastocyst stage was assessed on Days 5, 6 and 7 after insemination by light microscopy (100x). Criteria for blastocyst formation were the presence of a partially or fully formed blastocoel, a distinguishable inner cell mass and a population of trophoblast cells. Blastocysts were classified into five developmental stages based on their degree of expansion and hatching (O’Brien et al., 1996, 1997): early blastocyst (EB: blastocoele beginning to form); blastocyst (B: blastocoele formed); expanded blastocyst (ExB: large increase in blastocyst diameter and thinning of the zona pellucida); hatching blastocyst (HgB); and hatched blastocyst (HB). A blastocyst scoring system based on the development of the inner cell mass and trophectoderm as described by Gardner and Schoolcraft (1999) was used to classify Day 7 blastocysts into grade 1, 2 or 3. Blastocyst cell number was determined by fixation and staining on day 7 of culture as described for fertilisation assessment and examined using fluorescent microscopy (400x).
Statistical Analysis

Statistical analyses were performed using GENSTAT computer program (Version 4.2, Numerical Algorithms Group©(NAG) Ltd, Oxford, UK). Motility data from Experiments 1 and 2 were subjected to logit and folded power transformation and analysed by repeated measures and analysis of variance. Sorting data and distances traveled by the vanguard spermatozoon from Experiments 1 and 2 were analysed by analysis of variance. Vanguard data from Experiment 1 was subjected to natural log transformation before analysis. The number of spermatozoa liquid stored at 21ºC before sorting (Experiment1), and at 15 and 5ºC after sorting (Experiment 2) that migrated 5 mm into artificial cervical mucus in the SMT were analysed by negative binominal logistic regression and logistic regression respectively. The proportion of intact acrosomes of spermatozoa from Experiments 1 and 2 was analysed by binomial logistic regression. Statistical differences in the proportion of oocytes undergoing fertilisation with spermatozoa liquid stored for 24 h prior to sorting and freeze-thawing and cleaved oocytes developing to the blastocyst stage were determined by binomial logistic regression. The proportion of grade 1 embryos with greater than 250 cells at day 7 of culture was also determined by binomial logistic regression.

Results

Experiment 1: Liquid storage of spermatozoa before sorting

(i) Sorting Efficiency

Sorting parameters for Experiment 1 are presented in Table 1. Overall, sperm samples liquid stored in TRIS before sorting had the highest proportion of viable and correctly oriented spermatozoa (P<0.001). This treatment produced the greatest separation between X- and Y-chromosome bearing spermatozoa populations (lower R
value) and was therefore sorted at a faster rate (P<0.001) than sperm samples diluted in AH. Samples stored at 21°C or 15°C had a greater proportion of viable spermatozoa after 24 h (P<0.05). Sperm samples sorted immediately after dilution (0 h) had a higher proportion of viable and oriented spermatozoa, greater separation between X- and Y-sperm populations, and were able to be sorted at faster rates than after liquid storage for 24 h (P<0.05). There was no interaction between liquid storage duration at 21°C and diluent. Sperm samples prepared for sorting from Ram 3 had significantly less correctly oriented spermatozoa (29 ± 2.6 %) than spermatozoa from Ram 1, 5 and 6 (38 ± 2.0 %, 35 ± 2.3 % and 41 ± 1.9 % respectively) and were sorted at a slower rate (897 ± 104.0 spermatozoa/sec) than samples from Ram 1, 5 and 6 (1358 ± 75.5, 1208 ± 91.8 and 1443 ± 80.5 spermatozoa/sec respectively; P<0.05).

Table 1: Sorting parameters means (± S.E.M.) from Experiment 1 after liquid storage of spermatozoa in TRIS or AH diluent at 21, 15 or 5°C for 0, 6 or 24 h prior to sorting.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sort Rate (spermatozoa / sec)</th>
<th>R-Value</th>
<th>Oriented Spermatozoa (%)</th>
<th>Viable Spermatozoa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>1570 ± 48.3 a</td>
<td>12.8 ± 0.15 a</td>
<td>45 ± 1.1 a</td>
<td>73 ± 1.1 a</td>
</tr>
<tr>
<td>AH</td>
<td>960 ± 54.4 b</td>
<td>14.1 ± 0.18 b</td>
<td>28 ± 1.3 b</td>
<td>56 ± 1.9 b</td>
</tr>
<tr>
<td>21°C**</td>
<td>1333 ± 116.0</td>
<td>13.6 ± 0.31</td>
<td>36 ± 3.1</td>
<td>62 ± 3.1 d</td>
</tr>
<tr>
<td>15°C**</td>
<td>1352 ± 92.6</td>
<td>13.7 ± 0.29</td>
<td>38 ± 2.3</td>
<td>65 ± 2.7 d</td>
</tr>
<tr>
<td>5°C**</td>
<td>1091 ± 86.2</td>
<td>14.3 ± 0.37</td>
<td>31 ± 2.3</td>
<td>56 ± 2.5 c</td>
</tr>
<tr>
<td>0 h*</td>
<td>1454 ± 109 c</td>
<td>12.8 ± 0.17 c</td>
<td>41 ± 2.8 c</td>
<td>69 ± 3.0 c</td>
</tr>
<tr>
<td>6 h*</td>
<td>1196 ±109 f</td>
<td>13.0 ± 0.34 f</td>
<td>38 ± 2.4 f</td>
<td>69 ± 3.2 e</td>
</tr>
<tr>
<td>24 h*</td>
<td>1210.4 ± 99.3 f</td>
<td>13.7 ± 0.17 f</td>
<td>34 ± 2.5 f</td>
<td>63 ± 2.8 f</td>
</tr>
</tbody>
</table>

* Only sperm samples liquid stored at 21°C. ** Only sperm samples liquid stored for 24 h
a, b, c, d, e, f Within column, values with different superscripts within the same sub-group are different P<0.05

Sorting parameters were pooled for presentation as there was no significant time and treatment interaction
(ii) In vitro sperm characteristics

Assessment of motility of spermatozoa during storage before sorting and after staining, sorting, freeze-thawing and incubation (6h, 37°C)

Spermatozoa stored at 21°C in either diluent had greater motility than spermatozoa stored at 5°C (P<0.001). At each storage temperature (5, 15 and 21°C) there was no difference in motility between spermatozoa diluted in TRIS or AH after storage for 6 and 24 h (Fig 1). The motility of spermatozoa from all treatments decreased (P<0.01) over the first 6 h of storage except for spermatozoa stored at 21°C (Fig 1). Spermatozoa stored at 21°C in TRIS maintained a similar motility over the 24 h storage period but spermatozoa diluted in AH showed decreased motility after 24 h.

After storage for 0, 6 or 24 h, spermatozoa stored at 21°C in both TRIS and AH were sorted. Overall, the motility of spermatozoa diluted in TRIS was greater (P<0.001) than motility of spermatozoa that were diluted in AH after sorting, cooling, and thawing (Fig 2). For the duration of staining (1 h; 34°C) motility remained the same within treatment. After sorting, the proportion of motile spermatozoa diluted in either diluent and stored for 6 or 24 h prior to sorting increased so that the motility from all treatments was similar (84 ± 1.4 %). After cooling and thawing the motility of all treatments decreased (P<0.001; Fig 2). However, the rate of decline in motility after thawing was greater (P<0.001) for spermatozoa diluted in AH (Fig 2). Within each diluent, spermatozoa that had been stored for 24 h prior to sorting had a higher motility post-thaw (63 ± 5.3 %) than spermatozoa that had been liquid stored at 0 or 6 h (46 ± 6.0 % and 48 ± 6.7 % respectively; P<0.001).

At 0, 3 and 6 h after thawing, spermatozoa diluted in TRIS prior to, and during, sorting had a higher motility than spermatozoa diluted in AH (P<0.001; Fig 3). Thawed non-sorted (control) spermatozoa had higher motility (P<0.001) over the 6 h incubation period.
(37°C) than spermatozoa from all other treatments except TRIS-6 h. With incubation spermatozoa from all treatments decreased (P<0.001) in motility with the greatest decline in motility for all treatments occurring after 3 h incubation (Fig 3).

**Fig 1:** Total progressive motility of ram spermatozoa diluted in TRIS or AH diluent and stored at 5°C (♦), 15°C (■) and 21°C (▲) for 0, 6 or 24 h before flow cytometric sorting. (Diluent data pooled for each diluent.)

**Fig 2:** Total progressive motility of ram spermatozoa stored in TRIS (▲) or AH (■) diluent at 21°C prior to staining with H33342 (Pre-stain), after staining with H33342 and 1 h incubation at 34°C (Post-stain), after flow cytometric sorting (Post-sort), after cooling to 5°C (Post-cool) and after freeze-thawing (0 h). (Data pooled for 0, 6 and 24 h treatments)
**Fig 3:** Total progressive motility after thawing (0 h) and incubation (37°C, 6 h) of non-sorted (control) frozen-thawed ram spermatozoa (♦) and ram spermatozoa stored in TRIS (▲) or AH (■) at 21°C prior to flow cytometric sorting and freeze-thawing. (Data pooled for 0 and 6 h treatments.)

(a)

**Assessment of acrosome integrity of spermatozoa during storage before sorting and after staining, sorting and freeze-thawing**

Overall, spermatozoa diluted in AH and stored at 21°C had a higher proportion of intact acrosomes (86 ± 2.4 %) than spermatozoa stored in TRIS at 21, 15 and 5°C (75 ± 4.4 %, 62 ± 10.0 % and 57 ± 9.6 % respectively, P<0.05). For spermatozoa stored at 21°C there was no interaction between time and treatment over the 24 h storage period. With storage, the proportion of intact acrosomes decreased (P<0.01) for spermatozoa stored in both diluents. Spermatozoa from Rams 1 and 6 had a lower proportion of acrosome reacted spermatozoa than Ram 5 (15.9 ± 3.2 %, 15.9 ± 3 % and 21.4 ± 5.0 % respectively; P<0.05) after storage at 21°C for 24 h.

Prior to staining (Pre-stain) with H33342, spermatozoa stored in AH diluent for 0 h had a greater proportion of intact acrosomes (94 ± 1.2 %) than all other treatments (87 ± 2.5 %,
77 ± 4.6 %, 85 ± 2.9 %, 62 ± 10.9 % and 80 ± 5.8 % for TRIS-0 h, TRIS-6 h, AH-6 h, TRIS-24 h and AH-24 h, respectively; P<0.05). However, after sorting and thawing all treatments had similar proportions of spermatozoa with intact acrosomes (90.1 ± 1.8 %). A significant proportion (P<0.05) of spermatozoa from the AH-0 h treatment underwent the acrosome reaction during freezing and thawing, resulting in a lower proportion of intact acrosomes post-thaw (87 ± 10.0 %; P<0.01).

Assessment of the migration of stored, sorted frozen-thawed spermatozoa and non-stored, non-sorted (control) frozen-thawed spermatozoa in artificial cervical mucus

The results of the SMT of spermatozoa liquid stored at 21°C for 0 and 6 h in TRIS and AH diluent before sorting and freezing, and of non-sorted (control) frozen-thawed spermatozoa are illustrated in Figure 4. Higher numbers (P<0.01) of frozen-thawed spermatozoa penetrated 5 mm in the artificial cervical mucus for TRIS liquid-stored and sorted spermatozoa and non-sorted (control) spermatozoa (29 ± 6.0 and 38 ± 13.7 spermatozoa respectively) than AH liquid-stored and sorted spermatozoa (10 ± 2.1 spermatozoa).

Overall, the non-sorted (control) vanguard spermatozoon traveled further than the sorted frozen-thawed vanguard spermatozoon diluted in TRIS or AH prior to sorting (33 ± 2.4 mm, 27 ± 2.1 mm and 25 ± 2.8 mm respectively; P<0.05). However, the sorted frozen-thawed spermatozoon diluted in TRIS for 6 h before sorting traveled a similar mean distance to the non-sorted (control) spermatozoon. There was no difference between rams in the mean distance migrated by the vanguard spermatozoon or the number of spermatozoa migrating 5 mm.
**Fig 4**: Mean number (±S.E.M.) of liquid stored-sorted and non-sorted (control), frozen-thawed ram spermatozoa migrating 5 mm (a) and the mean distance (± S.E.M.) traveled by the vanguard spermatozoon in artificial cervical mucus (b) after storage in TRIS or AH diluent. (Data pooled for 0 and 6 h liquid storage at 21°C prior to flow cytometric sorting and freezing) (a) *P<0.01, (b) *P<0.05
(iii) *In vitro fertilisation and early embryonic development*

There was no difference in the fertilisation, cleavage or blastocyst development rate after insemination with spermatozoa liquid stored in either TRIS or AH for 24 h prior to sorting and freezing or non-sorted (control) frozen-thawed spermatozoa. The overall maturation, monospermic fertilisation, cleavage (evaluated separately at 24 and 48 hp.i.) and blastocyst rate (proportion of late cleaving oocytes developing to blastocysts on Day 6 and 7) was 61 ± 4.3 %, 31.3 ± 3.0 %, 49 ± 3.8 %, 43 ± 2.7 % and 54 ± 3.2 % respectively. The proportion of matured oocytes that had cleaved by 48 h after insemination with pooled fresh (control) spermatozoa and frozen-thawed (control) spermatozoa and the proportion of cleaved oocytes that developed into blastocysts by Day 7 was 51 ± 8.1 %, 78 ± 4.1 %, 79 ± 3.7 % and 59 ± 3.2 % respectively. A lower proportion of matured oocytes underwent fertilisation and cleavage (24 and 48 h after insemination) after insemination with spermatozoa from Ram 3 (31 ± 4.7 %, 16 ± 3.8 % and 23 ± 3.1 % respectively; P<0.01) than Ram 1 (69 ± 4.7 %, 36 ± 4.4 % and 50 ± 4.3 % respectively), Ram 5 (69 ± 4.7 %, 36 ± 4.1 % and 57 ± 5.0 % respectively) and Ram 6 (78 ± 7.5 %, 40 ± 7.9 % and 66 ± 7.4 % respectively). However, the proportion of cleaved oocytes developing to the blastocyst stage did not differ among rams.

The proportion of Grade 1 embryos with 150 or more cells developing on Day 7 of culture did not differ between treatments or rams. Overall, a higher proportion (P<0.001) of embryos classified as Grade 1 (59.7 ± 6.1 %) with a minimum of 150 cells (44.9 ± 5.2 % and 36.9 ± 3.9 % containing >250 cells and 150-250 cells respectively) developed by day 7 of culture than lower grade embryos (24.6 ± 4.7 % and 16.2 ± 3.7 % classified Grade 2 and 3 respectively) with less than 150 cells (18.2 ± 3.5 %).
**Experiment 2: Liquid storage after sorting**

*(i) Sorting Efficiency*

Only fresh non-stored ejaculates were used for sorting; the proportion of viable spermatozoa was low for Merino rams (82 ± 1.2 %). The mean sort rate was only 1 344 ± 62.6 spermatozoa/sec. High resolution between the X and Y sperm populations was achieved (51 ± 2.3 % of the total population were correctly oriented spermatozoa), and samples could be sorted at greater than 85 % X and Y purity (X, 88 ± 1.0 % and Y, 86 ± 0.8 %). There was no effect of ram or replicate on any of the sorting parameters.

*(ii) In vitro sperm characteristics*

Assessment of motility of sorted and non-sorted (control) liquid stored spermatozoa after 24 h at 15 °C and 6 d at 5 °C

After 24 h storage at 15°C, non-sorted (control) spermatozoa diluted in TYB had lower motility than non-sorted (control) and sorted spermatozoa diluted in UHT and non-sorted (control) spermatozoa stored in TRIS (Fig 5a and 5b; P<0.001). The motility of sorted and non-sorted (control) spermatozoa diluted in AH declined steeply (P<0.001) over the 6 days storage period at 5°C and had lower motility (P<0.001) than all other treatments (Fig 6a and b).
Fig 5: Total progressive motility of non-sorted (control) ram spermatozoa (a) and flow cytometrically sorted ram spermatozoa (b) after storage at 15°C in UHT (♦), TRIS-EY (■), AH (▲) or TYB (x) diluent for 24 h.

(a)

(b)
**Fig 6:** Total progressive motility of non-sorted (control) ram spermatozoa (a) and flow cytometrically sorted ram spermatozoa (b) after storage at 5°C in UHT (♦), TRIS-EY (■), AH (▲) or TYB (x) diluent for 6 d.

(a)

![Graph showing percent motility over storage time for non-sorted spermatozoa.](image)

(b)

![Graph showing percent motility over storage time for flow cytometrically sorted spermatozoa.](image)
Assessment of acrosome integrity of sorted and non-sorted (control) liquid stored spermatozoa after 6 h at 15 °C and 3 d at 5 °C

Prior to liquid storage (0 h) sorted spermatozoa diluted in TRIS-EY had a lower proportion of intact acrosomes (83 ± 2.6 %) than spermatozoa from all other treatments (96 ± 0.5 %, 90 ± 1.1 % and 88 ± 2.0 % of intact acrosomes for spermatozoa diluted in UHT, AH and TYB, respectively; P<0.001). After storage for 6 h at 15°C, sorted and non-sorted (control) spermatozoa diluted in UHT and TYB had higher proportions of intact acrosomes (96 ± 0.8 % and 95 ± 0.9 %, respectively) than those diluted in TRIS-EY or AH (88 ± 2.7 % and 92 ± 1.6%, respectively; P<0.001). After storage for 3 d at 5°C, only sorted and non-sorted (control) spermatozoa diluted in UHT had higher proportions of intact acrosomes (95 ± 0.7 % and 97 ± 0.4 %, respectively) than all other treatments (82 ± 3.2 %, 89 ± 1.4 % and 81 ± 3.0 % intact acrosomes for spermatozoa diluted in TRIS, AH and TYB, respectively; P<0.001).

Assessment of the migration of sorted and non-sorted liquid stored spermatozoa in artificial cervical mucus after 6 h at 15 °C and 3 d at 5 °C

The number of spermatozoa that penetrated 5 mm in the artificial cervical mucus and the mean distance traveled by the vanguard spermatozoon after liquid storage in UHT, TRIS-EY, AH or TYB diluent for 6 h at 15°C and 3 d at 5°C is illustrated in Figure 7a and 7b respectively.

After liquid storage at 15°C, greater numbers of both non-sorted (control) and sorted spermatozoa diluted in UHT migrated 5 mm than spermatozoa from all other treatments (P<0.01). After liquid storage at 5°C, sorted and non-sorted (control) spermatozoa diluted in UHT and non-sorted (control) spermatozoa diluted in TYB had a higher proportion of spermatozoa reaching 5 mm in the capillary tube than all other treatments (P<0.05).
Overall, greater numbers (P<0.01) of spermatozoa liquid stored at 15°C penetrated 5 mm than spermatozoa liquid stored at 5°C (110 ± 13.1 and 60 ± 8.2 spermatozoa respectively). Similarly, spermatozoa stored at 15°C migrated greater mean distances (P<0.05) than spermatozoa stored at 5°C (19 ± 1.0 and 17 ± 1.0 mm respectively). The mean distance reached by the sorted and non-sorted (control) vanguard spermatozoon liquid stored in UHT at both temperatures was further than the mean distance of the vanguard spermatozoon from all other treatments (P<0.01). However, spermatozoa stored in UHT at 15°C and 5°C after sorting reached greater mean distances (P<0.01) in the artificial cervical mucus than non-sorted (control) spermatozoa stored in UHT (32 ± 1.5 and 25 ± 1.2 mm respectively). The mean distance the vanguard spermatozoon reached from all other treatments was similar.

Greater numbers (P<0.05) of spermatozoa from Ram 5 reached 5 mm than from Rams 3 and 6 (104 ± 14.3, 80 ± 13.2 and 71 ± 13.7 spermatozoa respectively). Spermatozoa from Ram 6 were not able to penetrate as far in the artificial mucus (P<0.01) as spermatozoa from Rams 3 and 5 (16 ± 1.2, 18 ± 1.3 and 19 ± 1.2 mm respectively) after storage at 15 and 5°C.
**Fig 7:** Mean number (± S.E.M) of flow cytometrically sorted and non-sorted (control) ram spermatozoa migrating 5 mm (a) and the mean (± S.E.M.) distance traveled by the vanguard spermatozoon in artificial cervical mucus (b) after liquid storage in UHT, TRIS-EY, AH or TYB diluent at 5°C ('black' column; ■) for 3 d or 15°C ('white' column; □) for 6 h.
Discussion

Extensive literature exists on the function and fertility of liquid stored ram spermatozoa (reviewed by Maxwell and Salamon, 1993; Maxwell and Watson, 1996; Salamon and Maxwell, 2000) but there are no reports of functional assessment of the effect of liquid storage on ram spermatozoa prior to sorting after sorting. The present study investigated the optimal liquid storage conditions for transporting fresh ram semen to the site of the flow cytometer for sex-sorting and freezing (Experiment 1) and the transportation of sorted ram spermatozoa from the sperm-sorting site to the recipient females for insemination without freezing (Experiment 2). The results indicate that ram spermatozoa can be effectively stored and transported in liquid state for up to 24 h prior to sorting and, after sorting, can retain viability for up to 3 d.

Spermatozoa liquid stored in TRIS diluent for 24 h prior to sorting were sorted more efficiently, had higher motility after sorting, thawing and incubation (37°C, 6 h) and penetrated the artificial cervical mucus in greater numbers than spermatozoa stored in AH diluent prior to sorting. Paulenz et al. (2002) also reported that ram spermatozoa diluted in Tris-based extenders maintained high motility after storage for 30 h. Studies carried out on the short-term storage (3-7 h) of bull spermatozoa prior to sorting found that holding semen neat (undiluted) rather than diluted in the staining media (TALP) resulted in higher post-thaw motility or after sorting (Shenck et al., 1999). In the present study, preliminary investigations found that ram spermatozoa maintained higher motility after a four-fold dilution compared to being stored neat or diluted ten-fold with staining media (TRIS). These results suggest that TRIS is a more suitable diluent than AH for the transportation of ram spermatozoa to the sperm sexing centre.
Similar to the findings of Schenk et al. (1999) and the storage of bull spermatozoa prior to sorting, the optimal temperature at which to storerram spermatozoa for sorting appears to be 21°C. Spermatozoa stored at 21°C for 24 h had higher motility and acrosome integrity than spermatozoa stored at 15 or 5°C prior to sorting. However, there was no difference in sorting efficiency between spermatozoa stored at each of the three temperatures. In contrast, Paulenz et al. (2002) reported that storage of ram spermatozoa at 5°C maintained greater spermatozoa viability than 20°C. However, the TRIS diluent used herein for storage of spermatozoa prior to sorting was not supplemented with 20 % (v/v) egg yolk. The spermatozoa did not therefore benefit from the phospholipid fraction of the egg yolk which provides protection against sperm membrane damage during cooling to 5°C (Watson, 1981) and reduces the loss of acrosome integrity (Maxwell and Salamon, 1993).

For the purpose of sorting, egg yolk was not added to the storage diluents used to transport spermatozoa to the sorter, as high levels interfere with the uptake of H33342 by spermatozoa (Johnson and Welch, 1999), reducing staining uniformity and causing lower resolution between the X- and Y-chromosome bearing sperm populations, slower sort rates and lower X and Y purities (Hollinshead et al., 2002b).

Liquid storage of bull spermatozoa prior to sorting has been suggested to have adverse effects on H33342 DNA-staining properties (Seidel and Garner, 2002). This does not appear to be the case for ram spermatozoa, as sperm samples liquid stored at 21°C for 24 h prior to sorting had a similar proportion of correctly-oriented spermatozoa and were sorted at a similar rate to spermatozoa that were only stored for 6 h (Table 1). Maxwell and Watson (1996) reported that the membranes of spermatozoa destabilise during storage. This destabilisation of stored spermatozoa may have enhanced staining uniformity of the remaining viable sperm population, resulting in the high resolution seen in the present
study between X- and Y- chromosome-bearing sperm populations of samples which had been stored for 24 h prior to sorting.

Sperm motility and normal morphology decreases during storage irrespective of the diluent, dilution rate, temperature or conditions of storage and this decline is mainly due to the accumulation of toxic products of metabolism (Salamon and Maxwell, 2000). This deterioration of stored spermatozoa was demonstrated in Experiment 1 and 2 by a decrease in the proportion of motile and acrosome intact spermatozoa over time. However, this adverse effect of liquid storage was overcome in Experiment 1 by the use of fluorescence-quenching food dye which eliminates non-viable spermatozoa from the population to be sorted. Thus, equine spermatozoa stored between 5 and 15°C for 18 h prior to sorting has been reported to have high fertility after sorting (Lindsey et al., 2002) but, unlike ram spermatozoa, do not tolerate further processing such as cryopreservation (Lindsey et al., 2003). In the present study, similar numbers of ram spermatozoa liquid-stored in TRIS for 6 h before sorting and freeze-thawing penetrated 5 mm into the artificial cervical mucus as non-stored, non-sorted (control) frozen-thawed spermatozoa. Similarly, the vanguard spermatozoon from each of these treatments traveled similar distances up the capillary tube in the SMT. Furthermore, the rates of in vitro fertilisation, cleavage, and blastocyst formation also did not differ between frozen-thawed spermatozoa stored in TRIS or AH for 24 h before sorting and non-stored, non-sorted (control) frozen-thawed spermatozoa and a similar proportion of grade 1 embryos were produced after IVF with both stored, sorted and non-sorted (control) frozen-thawed spermatozoa. Not only may the sorting process be selecting viable spermatozoa that are capable of migrating to the site of fertilisation, but the additional cryopreservation step after sorting may facilitate another selection process (Maxwell and Watson, 1996).
The overall fertilisation rates after insemination with both stored, sorted and control spermatozoa were low in this study. Lower oocyte maturation rates (81 %) may be the result of drought conditions experienced in the sheep farms during this experiment and may have impacted on fertilisation. Variability in fertilisation rate between sires is a well documented phenomenon (Fukui et al., 1988). After IVF with Ram 3, a lower proportion of oocytes underwent fertilisation (31 %) and cleavage (23 %) which may have contributed to the overall low fertilisation and cleavage rates for both control and liquid stored treatments.

There are many conflicting reports on the fertility of liquid stored ram semen after dilution in either milk diluents (UHT) or organic buffered (Tris, HEPES and TES) diluents, although this is the first report of liquid storage of flow cytometrically sorted ram spermatozoa. Spermatozoa stored in UHT regardless of storage temperature or length of time had higher motility, acrosome integrity and migrated in greater numbers and distance through the artificial cervical mucus in the SMT compared to spermatozoa stored in all other diluents. The protein fraction in the UHT diluent which acts as a buffer against changes in pH and protects spermatozoa during the dilution and cooling process (Maxwell and Salamon, 1993) is likely to be a contributing factor to the success of UHT in this experiment.

It is interesting that temperatures at which spermatozoa were stored did not appear to be critical to the maintenance of spermatozoa viability. Both 10-15ºC and 0-5ºC have been reported as optimal temperatures at which to store ram semen for various periods of time (reviewed by Maxwell and Salamon, 1993). Spermatozoa (sorted and control) stored at 15ºC for 6 h penetrated in greater numbers and further into the artificial cervical mucus than spermatozoa stored at 5ºC for 3 d. In the present study it is difficult to determine the
effect temperature had on spermatozoa viability because 15°C was used for short-term storage and 5°C for long-term storage. However, pregnancy rates after intrauterine insemination with sex-sorted bull spermatozoa stored short term (5-9 h) at 18°C or 5°C did not differ (Seidel et al., 1999).

Sorted spermatozoa stored in UHT had similar numbers of spermatozoa migrating 5 mm into the artificial cervical mucus as non-sorted (control) spermatozoa stored in UHT. However, the sorted vanguard spermatozoon traveled further in the capillary tube than the non-sorted (control) vanguard spermatozoon after incubation of 0.5 x 10^6 spermatozoa (1 h, 37°C). It has been demonstrated in several species that sodium hyaluronate solution resembles cervical mucus in terms of penetrability (human: Tang et al., 1999; Mortimer et al., 1990; Neuwinger et al., 1991; ram: Maxwell et al., 1999). The sperm migration test modified for low numbers of sorted and non-sorted (control) ram spermatozoa (Hollinshead et al., 2002b) has not yet been correlated to fertility. However, Cox et al. (2002) reported a significant correlation between sperm migration in goat cervical mucus and the ability of goat spermatozoa (15 x 10^6/sperm migration test) to colonize the oviduct and to fertilise in vivo matured oocytes. These results suggest that sorted liquid stored spermatozoa may have similar fertility to non-sorted (control) stored spermatozoa after insemination close to the site of fertilisation and time of ovulation.

In vitro production of embryos of a pre-determined sex may be a more efficient use of low numbers of sorted liquid stored ram spermatozoa. Stojanov et al. (1994) reported fertilisation rates of 32% and 44% after IVF with ram spermatozoa stored for 7 d at 5°C. After 14 d storage at 5°C spermatozoa still maintained their fertilising capacity as 21% of in vitro matured oocytes were fertilised. Although IVF studies were not carried out with sorted liquid stored spermatozoa, the results after IVF with liquid stored, sorted, frozen-
thawed ram spermatozoa in Experiment 1 suggest that high *in vitro* production rates of grade 1 blastocysts would be achieved after insemination with sorted liquid stored ram spermatozoa.

In *vivo* fertility trials are yet to be carried out with stored, sorted and frozen-thawed ram spermatozoa and sorted, stored ram spermatozoa. However, this study has demonstrated that the production of grade 1 blastocysts of a pre-determined sex is possible after IVF with sorted, frozen-thawed spermatozoa derived from sperm samples collected from rams up to 24 h before sorting, and hence possibly from a site remote from the flow cytometer. Additionally, sorted ram spermatozoa stored for 24 h at 15°C and 6 d at 5°C in UHT diluent maintain a high level of motility and acrosome integrity and have similar migration ability in artificial cervical mucus to non-sorted (control) frozen-thawed spermatozoa at the recommended insemination times for liquid stored ram spermatozoa. Further research to determine the effects of liquid storage prior to sorting in combination with sorting, freezing and thawing processes and after sorting on the functional status of ram spermatozoa and fertility after both *in vitro* and *in vivo* insemination is required.

**Acknowledgements**

This research was supported by XY Inc, CO, U.S.A and The Australian Research Council. The researchers would like to thank Bioniche Animal Health, Australia for supplying sodium hyaluronate for the SMTs carried out in this study. Ovaries were kindly supplied by Wollondilly Abattoir, Picton and Southern Meats Pty, Ltd, Goulborn, N.S.W..
References


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Abstract

Research was conducted in sheep to determine an effective preparation method for high purity sorting of frozen-thawed ram spermatozoa. The efficacy of sorting frozen-thawed spermatozoa was then investigated in several non-human primate species. An aliquot of each ejaculate (3 rams, 3 ejaculates per ram) was processed as a fresh control (FRESH). Frozen spermatozoa were thawed and prepared for sorting by no further processing (FT-NEAT), washing (FT-WASH) or gradient centrifugation (FT-GRADIENT), and evaluated for motility at 1 h post-staining, and motility and acrosomal status at 0 and 4 h post-sorting. Samples were analysed using a high-speed cell sorter. High levels of purity for X- and Y-enriched samples were achieved for all treatments (range: 85–92 %). The percentage of motile spermatozoa before sorting was lower (P<0.05) for frozen-thawed samples (FT-GRADIENT: 73.9 ± 3.7 %; FT-WASH: 32.2 ± 3.3 %; FT-NEAT: 32.7 ± 2.5%) compared to FRESH (83.3 ± 1.2 %). Post-sorting, the percentage of motile spermatozoa before and after incubation for FT-NEAT (0h: 60.0 ± 5.1 %; 4h: 27.2 ± 6.1 %) was lower (P<0.05) than that for FRESH (0h: 87.8±0.9%; 4h: 83.3 ± 1.2 %), FT-WASH (0h: 80.0 ±2.4 %; 4h: 71.7 ± 3.6%) and FT-GRADIENT (0h: 84.4 ± 1.3 %; 4h: 77.2 ± 1.7%). Vanguard sperm migration distance through artificial cervical mucus was lower (P<0.05) for FT-NEAT (17.7±1.7 mm) compared to FT-WASH (29.1 ± 3.8 mm) and FT-GRADIENT (28.4 ± 2.0 mm) and similar (P>0.05) to FRESH (23.7 ± 1.8 mm). Sample preparation using a modified wash method enabled high purity sorting (range: 86-97 % purity) of frozen-thawed epididymal spermatozoa in the baboon (Papio hamadryas), common marmoset (Callithrix jacchus) and common chimpanzee (Pan troglodytes). For
all non-human primate species, sorted spermatozoa were progressively motile (marmoset: 68.5 ± 7.5%; baboon: 37.5 ± 2.5%; chimpanzee: 73.0 ± 2.0%) acrosome intact (marmoset: 68.5 ± 7.5%; baboon: 89.5 ± 1.5%; chimpanzee: 84.0 ± 1.0%) and able to penetrate an artificial mucus. In summary, high-purity sorting of frozen-thawed ram and non-human primate spermatozoa with recovery of progressively motile, acrosome-intact spermatozoa was possible after processing to remove cryodiluent.

Introduction

Sex pre-determination using sperm sorting (Johnson et al., 1989) and assisted reproductive technology (ART) has been applied to several livestock species (Schenk et al., 1999; Hollinshead et al., 2002) and is under development for wildlife species (O’Brien et al., 2001, 2002; Schenk and DeGroff, 2003). The combined approach of sperm sexing and ART has great potential as a captive population management strategy for livestock and wildlife species, particularly those with single-sex dominated social structures. Endangered species that reproduce slowly could also benefit from these combined technologies through fast re-population by the preferential production of female offspring. Application of sperm sorting technology to species management is limited in situations where the sperm sorter is located a distance from the animal housing facility. Consequently, procedures for the sorting of frozen-thawed spermatozoa and associated ART would facilitate the practical and conservation-driven applications of the technology.

Birth of offspring of pre-determined sex using flow cytometrically sorted fresh spermatozoa was first performed in rabbits (Johnson et al., 1989). Since then offspring have been produced using fresh (non-frozen) sorted spermatozoa and ART from several species including pigs (Johnson 1991; Rath et al., 1996; 1997; 1999; Vazquez et al.,
2003), cattle (Cran et al., 1993; 1995; Seidel et al., 1997), humans (Levinson et al., 1995; Fugger et al., 1998; Fugger 1999), sheep (Catt et al., 1996; Cran et al., 1997) and horses (Buchanan et al., 2000; Lindsey et al., 2001; 2002a). Development of high-speed cell sorters (Johnson and Welch, 1999) and cryopreservation methods for sorted spermatozoa (Schenk et al., 1999) has enabled the commercial application of sperm sorting in dairy cattle (Garner, 2001; Seidel and Garner, 2002; Seidel, 2003). The advent of successful cryopreservation techniques for sorted spermatozoa has also led to the production of offspring in pigs (Johnson et al., 2000), sheep (Hollinshead et al., 2002) and horses (Lindsey et al., 2002b).

The efficacy of sorting spermatozoa after cryopreservation and thawing has been investigated previously in cattle. Preparation of frozen-thawed bull spermatozoa by washing prior to staining resulted in short-term resolution of X and Y chromosome-bearing spermatozoa populations (Stap et al., 1998). In that study, resolution and separation of X and Y spermatozoa was not achieved after processing of frozen-thawed samples by density gradient centrifugation before or after staining, but the importance of separating non-viable and viable populations during sorting was noted. Lu et al. (1999) reported that the time required for staining of frozen-thawed bull spermatozoa was approximately three times that required for fresh spermatozoa, and sorting rate was low using a standard speed cell sorter (60 spermatozoa/sec). A ten-fold increase in H33342 concentration reduced the staining time to a standard 60 min and high quality blastocysts (23 % of total oocytes cultured) were produced following the use of such sorted spermatozoa in an IVF system. While these studies indicate the potential for sorting frozen-thawed spermatozoa, research is required using current high-speed cell sorting technology to optimise the efficiency of sorting and to examine sperm quality after freeze-thawing and sorting.
The specific objectives of the present study were to: (i) determine the most effective preparation method for high purity sorting of frozen-thawed ram spermatozoa; (ii) evaluate the characteristics (motility, acrosome integrity and ability to migrate through an artificial mucus) of frozen-thawed, sorted ram spermatozoa; and (iii) evaluate the characteristics of spermatozoa from three non-human primates species after cryopreservation, thawing and sorting.

Materials and methods

Procedures described herein were approved by The University of Sydney’s Animal ethics Committee.

Study design

Two studies were performed. In Study 1, frozen-thawed semen from 3 rams (n=3 ejaculates per male; *Ovis aries*) was used to compare the effect of three pre-sorting processing methods on sperm characteristics and sorting parameters (rate and purity of sorting). Semen was split into fresh and frozen aliquots and the fresh treatment was processed immediately for sorting. The remaining semen was frozen, thawed then processed by three methods for sorting on the same day. The study was replicated 3 times using ejaculates from the same 3 rams.

In Study 2, frozen-thawed epididymal spermatozoa from 5 non-human primates representing 3 species (n=2 common marmosets, *Callithrix jacchus*; n=2 hamadryas baboons, *Papio hamadryas*; n=1 common chimpanzee, *Pan troglodytes*) were evaluated. Sperm characteristics and sorting parameters were determined following preparation using a wash method which utilized primate-specific media. One replicate per male was
performed for baboon and marmoset samples and 2 replicates were performed for the chimpanzee sample.

Collection of spermatozoa

In Study 1, semen was collected from 3 Merino rams (aged 3-14 years) by artificial vagina. Only ejaculates containing spermatozoa with forward progressive motility greater than 80% were used.

Epididymal spermatozoa were opportunistically obtained within 4 h post-mortem from 2 common marmosets (aged 2 and 9 years) euthanised as part of another study (Animal Ethics Approval from the University of Sydney, Medicine Faculty), 2 hamadryas baboons (aged 11 and 20 years) and a common chimpanzee (aged 13 years) that died of natural causes unrelated to their reproductive organs. The testes and epididymides were dissected from the animals and held at 21°C during sperm collection. Vas deferens and cauda epididymides were dissected from surrounding connective tissue and blood vessels. For marmoset and baboon samples, epididymal spermatozoa were collected by retrograde flush of the distal caudal epididymis via the vas deferens. For the chimpanzee sample, epididymal tubules were pierced repeatedly by fine forceps and the sperm suspension was extruded using forceps. For all species, spermatozoa were collected in cryopreservation medium (modified by omitting the cryoprotectant) and the final sperm sample was filtered using 5 mL tubes with cell-strainer caps (37 µm, Falcon 2235, Becton Dickinson, Franklin Lakes, NJ, USA).

Reagents and media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma (St Louis, MO, USA), and were prepared with embryo grade
water (ThermoTrace, Noble Park, Victoria). All freezing and sorting diluents containing egg yolk (free range eggs ≤ 48 h old) were prepared by ultracentrifugation of the complete diluent for 20 min at 10 000 g. The supernatant was filtered (0.22 µm; Millipore, North Ryde, NSW) and frozen at -20°C for a maximum of 3 months.

Processing of fresh and frozen-thawed spermatozoa

In Study 1, semen was divided into fresh and frozen treatment groups within 30 min of collection. The fresh treatment (FRESH) was prepared immediately for sorting by dilution to 400 x 10^6 spermatozoa / ml (approximate 1:10 dilution, semen:diluent, v/v) with a medium containing 300.0 mM Tris (hydroxymethyl)aminomethane, 27.8 mM fructose, 94.7 mM citrate (monohydrate), 87.9 I.U. / ml penicillin-G and 35.3 I.U. / ml streptomycin sulfate (TFC medium; modified from Evans and Maxwell, 1987) supplemented with 0.3 % w/v BSA (Sigma A-9647). Processing for freezing commenced with dilution (1:4, semen: diluent, v/v) with a TEST yolk freezing medium (TYB; modified from Graham et al., 1972; 189 mM TES, 85 mM Tris, 11 mM D-glucose, 20 % v/v egg yolk, 6 % v/v glycerol, 97 I.U. / ml penicillin-G, 39 I.U. / ml streptomycin sulfate, pH 7.2 ± 0.2, 1200 ± 50 mosm / kg). The sperm suspension (600-900 x 10^6 spermatozoa / ml) was cooled to 5°C over 1.5 h, frozen as 0.2 ml pellets (10-15 pellets/ejaculate) on dry ice for 3 min then transferred to liquid nitrogen (Evans and Maxwell, 1987).

Pellets were thawed in a 10-ml dry glass test tube shaken in a waterbath at 37°C. Frozen-thawed semen was either used without further processing (FT-NEAT) or processing by 2 methods: standard wash (FT-WASH) or density gradient centrifugation (FT-GRADIENT). All media were warmed to 34°C prior to use. For FT-WASH, 1 mL of thawed semen was diluted with 3 mL of TFC medium and centrifuged at 750 g for 8 min. The supernatant was removed and the sperm pellet re-suspended in an appropriate volume of TFC medium
that resulted in a final concentration of $400 \times 10^6$ spermatozoa / ml. For FT-GRADIENT, a total of 1.2 ml of thawed semen was layered onto two-step Puresperm® (NidaCon International AB, Gothenburg, Sweden) gradients prepared by dilution with TFC medium and 0.3 % BSA (w/v) (0.2 ml thawed semen per gradient; 1 ml of 45 % Puresperm® on 1 ml of 90 % Puresperm®). The tubes were centrifuged at 1000 g for 10 min at room temperature. The 45 % Puresperm® fraction and the bilayer containing debris and dead spermatozoa were removed and discarded. A clean pipette tip was then carefully placed at the bottom of the tube and the sperm pellet (approximately 0.5 ml) was aspirated and transferred to a clean tube for dilution (1:4, pellet:diluent, v/v) with TFC and centrifugation (750 g, 8 min). The supernatant was discarded and the sperm pellet re-suspended in an appropriate volume of TFC that resulted in a final concentration of $400 \times 10^6$ spermatozoa / ml.

In Study 2, epididymal spermatozoa were collected in 0.5 ml aliquots of TYB without glycerol ($345 \pm 5$ mosm / kg) and cooled from 21°C to 5°C over 1 h (-0.27°C / min). At 5°C the sample was diluted (1:1, v/v) in a step-wise fashion (25 % then 25 % then 50 % of the diluent) at 15 min intervals with TYB containing 6 % glycerol (Sigma G-2025; final cryoprotectant concentration of 3 %). After equilibration for 30 min, sperm suspensions were frozen as 0.2 ml pellets on dry ice as described for Study 1. Pellets were thawed at 37°C for 2 min. All frozen-thawed samples were prepared using the wash method as described for Study 1 but modified for use with non-human primate spermatozoa. The wash method was used because a higher recovery rate of non-human primate spermatozoa (70%- 90% of total spermatozoa recovered) could be achieved compared with the gradient centrifugation method (10%- 25% of spermatozoa recovered; recovery rate influenced by sperm viability and morphology; data not shown). Pilot studies demonstrated that a HEPES-buffered HTF medium (Sage Biopharma, San Clemente, CA, USA) supplemented
with 5% HSA (Irvine Scientific, Santa Ana, CA, USA; mHTF) was a suitable medium for
dilution and incubation of spermatozoa from the three non-human primate species
examined (data not shown). Accordingly, frozen-thawed samples were diluted (1:3; semediluent, v/v) with mHTF and centrifuged at 300 g for 6 min. The supernatant was
discarded and the sperm pellet re-suspended in a volume of mHTF that resulted in a final
concentration of 10 x 10^6 spermatozoa / ml. An aliquot of this sample was removed for
assessment as a control (FT-Control).

Preparation of spermatozoa for sorting
In study 1, each 1 ml sample of the FRESH group from each ram was incubated with
either 311.4 μM, 289.3 μM or 222.5 μM of Hoechst 33342 (H33342; Sigma) for 1 hour at
34°C. The concentration of H33342 for each ram was determined by a series of
optimization experiments (data not shown). Frozen-thawed samples (FT-NEAT, FT-
WASH and FT-GRADIENT) were incubated with the same concentration of H33342 as
determined for staining of FRESH spermatozoa since a preliminary experiment indicated
no benefit of reducing H33342 concentration. Immediately before sorting, stained samples
were diluted (1:1, v/v) with TFC medium containing 0.3% BSA, 4% egg yolk and 0.002%
(w/v) food dye (FD&C #40, Warner Jenkinson Company Inc., St Louis, MO, USA) to
200 x 10^6 spermatozoa / ml. The food dye penetrates the membranes of non-viable
spermatozoa and reduces the intensity of H33342 fluorescence. Consequently, only viable
spermatozoa (plasma membrane intact) are selected for sorting (Johnson et al., 1999,
Johnson and Welch, 1999). Immediately before sorting, samples were filtered (37 μm;
Falcon 2235).

In Study 2, frozen-thawed spermatozoa prepared by a wash method were stained with 15
μM H33342 and processed for sorting as in Study 1, except that stained samples were
diluted (2:1, sperm sample: diluent, v/v) with mHTF containing 4 % egg yolk. Hoechst 33342 concentrations for frozen-thawed non-human primate species were derived from previous studies on primate spermatozoa (O’Brien et al., 2001 and 2002).

*Flow cytometric sorting*

A high-speed cell sorter (MoFloSX®, DakoCytomation, Fort Collins, CO, USA) modified for sperm sorting (Johnson and Pinkel, 1986; Johnson and Welch, 1999; Rens et al., 1999) operating at 50 psi was used to analyse and separate spermatozoa. For both studies, sheath fluid was TFC medium (pH 7.2 ± 0.2; 300 ± 10 mosm / kg). H33342 was excited by an argon laser running at 200 mW.

Spermatozoa were sorted into 10-ml centrifuge tubes (pre-soaked overnight with TFC medium with 0.1 % BSA, w/v) containing 1 mL of TFC medium with 0.3 % BSA and 20 % egg yolk (v/v, Study 1) or into 2 mL microcentrifuge tubes (ThermoTrace; pre-soaked overnight with mHTF) containing 0.2 ml mHTF with 20 % egg yolk (v/v, Study 2). For both studies, two separate sorts were performed on each sample. The first sort type, termed “All-sort”, contained oriented and non-oriented viable spermatozoa and enabled sperm sorting rates of 2 000-6 000 spermatozoa / s (Study 1; 5 x 10^6 spermatozoa collected / treatment) and 200-500 spermatozoa / s (Study 2; 2 x 10^6 spermatozoa collected). The second sort type, a high purity sort, was obtained by placing sort gates on the oriented, viable population to achieve purities of greater than 90 % X or Y chromosome-bearing spermatozoa (0.5 x 10^6 and 0.2 x 10^6 spermatozoa collected for Study 1 and 2, respectively). The high purity sort was re-stained, sonicated and re-analysed by flow cytometry to determine the proportions of X- and Y-bearing spermatozoa (Welch and Johnson, 1999).
**Sperm evaluations**

All-sort samples were centrifuged at 700 g (Study 1) or 300 g (Study 2) for 6 min (21°C). Sperm pellets were re-suspended in either 1 mL of TFC containing 20 % egg yolk (v/v) and centrifuged at 750 g for 8 min (Study 1) or 0.5 ml mHTF with 20 % egg yolk (v/v) and centrifuged at 300 g for 8 min (Study 2). Supernatants were removed to leave a 100-150 µl sperm pellet. Sperm concentration was determined and a sub-sample was removed for evaluation of ability to migrate through an artificial cervical mucus (sperm migration test; 0h post-sorting); this test was performed on control (Study 1: fresh sorted; Study 2: frozen-thawed non-sorted) and All-sort samples. For incubation, the same medium used to previously re-suspend the sperm pellet was added to the remaining pellet to give a total volume of 0.5 ml (final concentration: 10 x 10⁶ and 2 x 10⁶ spermatozoa/ml for Study 1 and Study 2, respectively). Total progressive motility (motility), forward progressive motility rating (FPM; 0-5, 0 = no movement, 5 = rapid, forward progressive movement; modified from Howard *et al.*, 1986) and acrosome integrity were determined for control and All-sort samples during a 4 h (Study 1) or 6 h (Study 2) incubation period (30°C.).

The sperm migration test was a modification of that developed for human (Mortimer *et al.*, 1990) and ram spermatozoa (Maxwell *et al.*, 1999). Preliminary experiments with frozen-thawed ram spermatozoa demonstrated good repeatability of the test using as low as 1 x 10⁶ spermatozoa. Aliquots containing either 3 x 10⁶ (Study 1) or 1 x 10⁶ (Study 2) spermatozoa in 50 µl were placed in a BEEM capsule (ProSciTech, Thuringowa, Queensland). A flat capillary tube (3.0 x 0.3 x 100 mm, VitroCom Inc., Mountain Lakes, NJ, USA) was filled with the artificial cervical mucus medium, sodium hyaluronate (Bioniche, Armidale, NSW, 10mg mL⁻¹), diluted to 7.5 mg / ml with TFC medium containing 1 % w/v BSA, sealed with haematocrit sealant at one end (Critoseal, Cope Laboratories, Newcastle, NSW) then positioned vertically through slits cut into the lids of
BEEM capsules. After 1 h incubation at 37°C, the number of spermatozoa migrating 5 mm from the open end of the capillary tube and the distance traveled by the vanguard spermatozoon (vanguard sperm migration distance) were assessed by phase contrast microscopy (200x).

The percentage of motile spermatozoa and FPM were estimated subjectively to the nearest 5 % by examining 4-5 fields of sperm sample (placed on heated slides, 37°C). Acrosomal status was assessed by fluorescent isothiocynate-conjugated peanut agglutinin (FITC-PNA) staining (Roth et al., 1998). Slides were stained within 2 weeks and evaluated within 1 h of staining. Spermatozoa were considered acrosome intact if the acrosome stained bright green, whereas those with no staining or a single band of green fluorescence at the equatorial segment were classified as acrosome reacted. A total of 200 spermatozoa per sample were examined using phase contrast microscopy (400x Study 1; 1000x Study 2).

Data on plasma membrane integrity (viability) were obtained during sorting by determination of the proportions of spermatozoa with normal H33342 fluorescence intensity (viable, intact plasma membrane) and those with reduced H33342 fluorescence due to penetration by food dye (non-viable, non-intact plasma membrane). In addition, the percentage of correctly oriented viable spermatozoa during sorting (i.e. sperm with their edge facing the laser beam) was compared across treatments in Study 1.

Statistical analysis

Percentage data for motility (subjected to arcsine transformation), correct orientation during sorting and sperm migration test data were analysed by analysis of variance (GENSTAT, version 4.2, Numerical Algorithms Group© (NAG) Ltd, Oxford UK).
for FPM and acrosomal status were analysed by logistic regression. Means were compared using the least significant difference procedure. P < 0.05 was considered significant.

Results

Study 1: Flow cytometric sorting of frozen-thawed ram spermatozoa: effect of preparation method on sorting and quality of frozen-thawed ram spermatozoa

Based on subjective assessment of flow cytometric dotplot and histogram outputs (Fig. 1), resolution of X and Y sperm populations for FT-GRADIENT and FT-WASH was similar to that for FRESH, and poorest for FT-NEAT compared to other treatments. The percentage of correctly-oriented viable spermatozoa was lower for FT-NEAT and FT-WASH compared with FT-GRADIENT and was highest for FRESH (15.9 ± 3.6 %, 12.8 ± 1.1 %, 25.6 ± 1.5 % and 44.0 ± 4.6 %, respectively, P<0.05). For high purity samples, sorting rate was lower for FT-NEAT and FT-WASH compared with FT-GRADIENT and was highest for FRESH (674 ± 136, 487 ± 89.1, 1457 ± 132 and 1883 ± 182 spermatozoa / s, respectively, P<0.05). Reanalysis of sorted spermatozoa revealed high levels of purity for X- and Y-enriched samples for all treatments (range: 85–92 %).
Fig. 1: Flow cytometric dotplot and histogram outputs of a) fresh (FRESH) and frozen-thawed samples prepared for sorting by (b) no further processing (FT-NEAT), (c) washing (FT-WASH) or (d) gradient centrifugation (FT-GRADIENT) showing fluorescent signals generated by ram spermatozoa. Region 1 (R1) represents fluorescent signals generated by correctly oriented, viable spermatozoa and region 2 (R2) represents non-viable (plasma membrane non-intact) spermatozoa, which have reduced fluorescence because of the penetration of food dye. The difference in DNA content between X and Y chromosome-bearing ram spermatozoa is 4.2%.
After preparation of frozen-thawed samples, the percentage of motile spermatozoa was higher for FT-GRADIENT compared with NEAT and FT-WASH samples (Table 1, P<0.05). Prior to sorting, motility was lower for frozen-thawed samples compared to FRESH (P<0.05) but FPM was similar for all treatments. At 0 and 4 h post-sorting, percentage of motile spermatozoa was lower for FT-NEAT than for FRESH, FT-WASH and FT-GRADIENT (P<0.05). During the 4 h incubation post-sorting, motility decreased for FT-NEAT (P<0.05) and remained unchanged for FRESH, FT-WASH and FT-GRADIENT. At 0 h post-sorting FPM was similar for all treatments but decreased during the 4 h incubation (P<0.05). FPM at 4 h post-sorting was lower for FT-NEAT compared to other treatments (P<0.05).

The number of spermatozoa reaching 5 mm in the sperm migration test was lower for FT-NEAT compared to FT-WASH, FT-GRADIENT and FRESH (P<0.05). Vanguard sperm migration distance was lower for FT-NEAT compared with FT-WASH and FT-GRADIENT (P<0.05) and similar to FRESH.

**Table 1** Effect of post-thaw preparation method on quality of frozen-thawed (FT) ram spermatozoa during and after flow cytometric sorting (n=3 rams, 3 ejaculates / ram). Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Sperm characteristic</th>
<th>FRESH</th>
<th>FT-NEAT</th>
<th>FT-WASH</th>
<th>FT-GRADIENT</th>
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<tr>
<td><strong>Post-preparation (pre-staining)</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>84.4 ± 1.0^d</td>
<td>32.2 ± 2.8^e</td>
<td>33.9 ± 3.3^e</td>
<td>73.9 ± 3.7^f</td>
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<tr>
<td>FPM (0-5)</td>
<td>3.1 ± 0.07</td>
<td>2.9 ± 0.06</td>
<td>3.0 ± 0.0</td>
<td>2.9 ± 0.06</td>
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<td><strong>Post-staining</strong></td>
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<tr>
<td>Motility (%)</td>
<td>83.3 ± 0.8^d</td>
<td>31.7 ± 2.0^e</td>
<td>33.3 ± 3.0^e</td>
<td>73.9 ± 3.7^f</td>
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<tr>
<td>FPM (0-5)</td>
<td>3.1 ± 0.06</td>
<td>2.8 ± 0.09</td>
<td>2.8 ± 0.08</td>
<td>2.9 ± 0.1</td>
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<td>Viability (%)</td>
<td>88.6 ± 2.6^d</td>
<td>42.7 ± 2.0^e</td>
<td>45.3 ± 4.8^e</td>
<td>61.9 ± 3.6^f</td>
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<tr>
<td><strong>0 h post-sort/incubation</strong></td>
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<tr>
<td>Motility (%)</td>
<td>87.8 ± 0.9^d</td>
<td>59.9 ± 5.1^e</td>
<td>80.0 ± 2.4^d</td>
<td>84.4 ± 1.3^d</td>
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<tr>
<td>FPM (0-5)</td>
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<td>1.7 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>1.9 ± 0.5</td>
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<tr>
<td>Acrosome intact (%)</td>
<td>85.1 ± 6.8</td>
<td>84.8 ± 5.3</td>
<td>82.1 ± 3.7</td>
<td>87.4 ± 4.9</td>
</tr>
<tr>
<td>No. spermatozoa @ 5 mm^2</td>
<td>219.6 ± 40.2^d</td>
<td>109.1 ± 28.5^e</td>
<td>240.7 ± 45.8^d</td>
<td>231.1 ± 21.5^d</td>
</tr>
<tr>
<td>VSM (mm^2)</td>
<td>23.7 ± 1.8^d</td>
<td>17.7 ± 1.7^d</td>
<td>29.1 ± 3.8^e</td>
<td>28.4 ± 2.0^f</td>
</tr>
<tr>
<td><strong>4 h post-sort/incubation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>83.3 ± 1.2^d</td>
<td>27.2 ± 6.1^e</td>
<td>71.7 ± 3.6^df</td>
<td>77.2 ± 1.7^d</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>0.9 ± 0.2^d</td>
<td>0.4 ± 0.2^e</td>
<td>0.9 ± 0.2^d</td>
<td>1.2 ± 0.2^d</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>85.3 ± 2.9</td>
<td>89.2 ± 12.6</td>
<td>81.3 ± 3.1</td>
<td>88.4 ± 2.4</td>
</tr>
</tbody>
</table>
a Forward progressive motility, graded subjectively: 0 = no movement, 5 = rapid, steady forward progression.
b Viability determined during analysis of stained sample by flow cytometry. Viable spermatozoa were classified as those cells which were not penetrated by food dye.
c Sperm migration test: number of spermatozoa migrating 5 mm; VSM: vanguard spermatozoon migration distance.
d,e,f Values with different superscripts within the same row are different (P < 0.05).

Study 2: Flow cytometric sorting of frozen-thawed non-human primate spermatozoa

Good resolution of X and Y sperm populations was achieved in frozen-thawed samples for the baboon (Fig. 2b). Resolution of the two populations was achieved for frozen-thawed epididymal marmoset (Fig 2a) and chimpanzee (Fig 2c). However, there was a high proportion of non-viable marmoset spermatozoa and poor staining uniformity of the chimpanzee spermatozoa. Due to the high numbers of non-viable spermatozoa after washing and staining (Table 2), the percentage of correctly oriented viable spermatozoa was low across species (marmoset: 15 ± 3 %; baboon: 27 ± 1 %; chimpanzee: 27 ± 5 %). Sperm sorting rate during collection of high purity samples for non-human primate species was low compared to that obtained for ram spermatozoa in Study 1 (marmoset: 60-75 spermatozoa / s, baboon: 50-60 spermatozoa / s, chimpanzee: 20-30 spermatozoa / s). The purity of X- and Y-enriched samples was high for all species (range, marmoset: 92-93 %; baboon: 89-97 %; chimpanzee: 86-94 %).

Sperm characteristics after thawing and sorting of frozen spermatozoa are shown in Table 2. Due to the low number of replicates within each species (n=2), statistical analyses were not performed. Percentage of motile spermatozoa and forward progressive motility after post-thaw preparation and staining varied across species but was low for marmoset samples. During the 6 h incubation, sperm characteristics were similar for FT-Control (non-sorted) and All-sort (sorted) samples. For all species, progressively motile (range: 5-
38% of spermatozoa) and acrosome intact (range: 25-90% of spermatozoa) cells were observed in sorted samples after the 6 h incubation.

For all non-human primates species, the number of spermatozoa reaching 5 mm and the vanguard sperm migration distance tended to be similar for FT-Control and All-sort treatments. However, sperm migration test parameters in the marmoset (Control and All-sort) tended to be lower compared to those in the baboon and chimpanzee.
Fig. 2. Flow cytometric dotplot and histogram outputs of frozen-thawed epididymal samples from (a) marmoset, (b) baboon and (c) chimpanzee showing fluorescent signals generated by spermatozoa. Region 1 (R1) represents correctly oriented, viable spermatozoa and region 2 (R2) represents non-viable (plasma membrane non-intact) spermatozoa, which have reduced fluorescence because of the penetration of food dye. The difference in DNA content between X and Y chromosome-bearing spermatozoa is 4.1 %, 4.2 % and 3.3 % for the marmoset, baboon and chimpanzee respectively.
<table>
<thead>
<tr>
<th>Sperm characteristic</th>
<th>Marmoset</th>
<th>Baboon</th>
<th>Chimpanzee</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post-thaw</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Motility (%)</td>
<td>43.5 ± 21.5</td>
<td>42.5 ± 7.5</td>
<td>82.5 ± 2.5</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>3.8 ± 0.3</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>74.5 ± 13.5</td>
<td>3.0 ± 0.5</td>
<td>79.0 ± 1.0</td>
</tr>
<tr>
<td><strong>Post-wash (pre-staining)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>18.0 ± 0.0</td>
<td>40.0 ± 10.0</td>
<td>77.5 ± 2.5</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>3.3 ± 0.8</td>
<td>2.5 ± 1.0</td>
<td>4.5 ± 0.0</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>68.5 ± 7.5</td>
<td>2.5 ± 1.0</td>
<td>70.5 ± 1.5</td>
</tr>
<tr>
<td>Viability</td>
<td>33.5 ± 1.5</td>
<td>28.0 ± 12.0</td>
<td>46.5 ± 1.5</td>
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<tr>
<td><strong>Post-staining</strong></td>
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</tr>
<tr>
<td>Motility (%)</td>
<td>18.0 ± 4.0</td>
<td>39.5 ± 9.5</td>
<td>67.5 ± 2.5</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>2.8 ± 1.3</td>
<td>2.5 ± 1.0</td>
<td>4.3 ± 0.3</td>
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<tr>
<td>Acrosome intact (%)</td>
<td>55.5 ± 12.5</td>
<td>66.0 ± 8.0</td>
<td>65.5 ± 0.5</td>
</tr>
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<td>Sample type</td>
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<tr>
<td>0 h post-sort/incubation</td>
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<tr>
<td>Motility (%)</td>
<td>18.0 ± 0.0</td>
<td>20.5 ± 5.5</td>
<td>77.5 ± 2.5</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>3.3 ± 0.8</td>
<td>3.0 ± 1.0</td>
<td>4.5 ± 0.0</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>68.5 ± 7.5</td>
<td>7.5 ± 16.5</td>
<td>70.5 ± 1.5</td>
</tr>
<tr>
<td>No. spermatozoa @ 5 mm³</td>
<td>2.0 ± 0.0</td>
<td>1.5 ± 0.5</td>
<td>5.5 ± 4.5</td>
</tr>
<tr>
<td>VSM (mm³)</td>
<td>5.0 ± 0.0</td>
<td>13.0 ± 2.0</td>
<td>14.5 ± 2.5</td>
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<td>3 h post-sort/incubation</td>
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<tr>
<td>Motility (%)</td>
<td>9.0 ± 1.0</td>
<td>13.0 ± 2.0</td>
<td>23.5 ± 3.5</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>2.8 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>57.5 ± 18.54</td>
<td>2.0 ± 14.0</td>
<td>55.5 ± 5.5</td>
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<td>6 h post-sort/incubation</td>
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<tr>
<td>Motility (%)</td>
<td>5.0 ± 0.0</td>
<td>7.5 ± 2.5</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td>FPM (0-5)</td>
<td>2.3 ± 0.3</td>
<td>1.5 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Acrosome intact (%)</td>
<td>55.5 ± 12.53</td>
<td>7.5 ± 12.5</td>
<td>76.0 ± 14.0</td>
</tr>
</tbody>
</table>
aForward progressive motility, graded subjectively: 0 = no movement, 5 = rapid, steady forward progression.
bViability determined during analysis of stained sample by flow cytometry. Viable spermatozoa were classified as those cells which were not penetrated by food dye.
cSperm migration test: number of spermatozoa migrating 5 mm; VSM: vanguard spermatozoon migration distance.

Discussion

The development of high speed cell sorters in recent years has resulted in significant increases in efficiency of sorting and quality of sorted spermatozoa (Rens et al., 1999; Johnson and Welch, 1999; Johnson et al., 1999), thereby opening the way for the sorting of frozen-thawed spermatozoa. Our results demonstrate in sheep and three non-human primate species that high purity sorting of frozen-thawed spermatozoa is possible after processing to remove cryodiluent components, and sorted spermatozoa are of similar quality to the study control treatment (Study 1: non-frozen sorted; Study 2: frozen-thawed non-sorted spermatozoa).

The rate of sorting is mainly dependent on sperm orientation, difference in DNA content between X and Y spermatozoa, the proportion of viable spermatozoa, staining uniformity and the concentration of the sperm sample to be sorted. During sorting of ram spermatozoa, the percentage of viable cells was highest for fresh spermatozoa and lowest for frozen-thawed samples undergoing no processing or washing. Consequently, the percentage of correctly oriented viable spermatozoa and sorting rate was significantly lower for frozen-thawed compared with fresh ram spermatozoa. Due to the post-thaw removal of non-viable spermatozoa, the sorting rate of samples receiving density gradient processing was 75-80 % of that obtained with fresh spermatozoa. In contrast, the sorting rate of frozen-thawed samples receiving no processing or washing was only 25-35 % of that achieved with fresh ram spermatozoa.
Although X and Y sperm populations were observed using all ram sperm preparation methods, improved resolution was achieved with samples processed by density gradient centrifugation to remove cryodiluent components. Removal of egg yolk is probably the reason for improved resolution since high levels of egg yolk during staining may impede uniform penetration of spermatozoa with H33342 (Johnson and Welch, 1999). Removal of particulate matter derived from egg yolk by ultracentrifugation and filtering was performed prior to use of the cryodiluents. This practice may also be important for uniform H33342 staining of spermatozoa since dense particulate matter will remain in samples after washing and/or density gradient centrifugation.

Most primate species including baboons and marmosets produce significantly lower numbers of spermatozoa (per ejaculate) than with livestock species such as sheep. Consequently, lower sperm concentrations were used during staining and sorting for these species compared to the sheep. The wash method was selected for processing of frozen-thawed non-human primate spermatozoa to provide adequate numbers of spermatozoa for staining (minimum of 10 x 10^6 spermatozoa / ml) and sorting. Despite high numbers of spermatozoa present in ejaculated semen and epididymal samples of the chimpanzee, the wash method was applied, as the number of pellets available for use in this study was limited. Even though the difference in DNA content between X and Y spermatozoa in the marmoset and baboon (4.1 %, 4.2 %, respectively; O’Brien et al., 2001) is similar to sheep (4.2 %, Johnson, 1992), lower total numbers of viable marmoset and baboon sperm at sorting precluded a similar sorting rate. Prior to the addition of food dye for identification of non-viable cells, the percentage of correctly oriented spermatozoa was high for all primate species (range 45-65 %). Thus, the sorting rate of primate spermatozoa could be optimized through an increase in the total number of viable spermatozoa at sorting. This could be achieved by routine collection and banking of ejaculated spermatozoa, followed
by bulk thawing and processing by the density gradient method to improve the efficiency of sorting as demonstrated for ram spermatozoa. The smaller difference in DNA content between X and Y chimpanzee spermatozoa (3.2 %, J. K. O’Brien, unpublished) would have an impact on sorting rate as demonstrated by the lower sorting rates achieved in Study 2 for the chimpanzee sample compared to the marmoset and baboon. Nevertheless, higher sorting rates (500-600 spermatozoa / s) can be achieved in the chimpanzee using fresh spermatozoa (sorting performed 24 h after collection and transport to laboratory at 15°C) incubated for staining at 100 x 10⁶ spermatozoa / ml (J. K. O’Brien and E. G. Crichton, unpublished).

Evaluation of *in vitro* sperm characteristics demonstrated that frozen-thawed ram spermatozoa have decreased motility and viability compared to fresh spermatozoa. However, following processing to remove cryodiluent components (by density gradient centrifugation or wash methods), frozen-thawed ram spermatozoa exhibited similar sperm characteristics (motility, acrosome integrity and ability to migrate 5 mm in an artificial cervical mucus) to fresh spermatozoa after sorting. Similarly, the sorting process did not negatively impact on *in vitro* sperm characteristics of primate spermatozoa. For all primate species examined, sorted sperm numbers and quality appeared to be potentially suitable for use in IVF. However, further research is required to examine the effect of sorting of both epididymal and ejaculated spermatozoa in non-human primates.

The low quality of frozen-thawed sorted ram spermatozoa receiving no processing prior to staining and sorting is possibly due to exposure of spermatozoa to glycerol during H33342 staining (Murdoch and Jones, 1978). Alternatively, the poor sperm quality may be associated with the interaction of glycerol and H33342 during staining.
Subjective assessment of forward progressive motility as a measure of sperm quality could be misleading for ram samples undergoing dilution with the Tris fructose citrate medium. After sorting, motion of ram spermatozoa appeared vigorous but was characterized by low forward progressive motility. Regardless of the low forward progressive motility ratings, sorted spermatozoa were able to undergo forward progressive motion as demonstrated by the sperm migration test. Furthermore, sorted ram spermatozoa exhibit increased forward progressive motility after dilution with complex media (F. K. Hollinshead and J. K. O’Brien, unpublished). These observations suggest that the Tris fructose citrate medium used throughout the sorting process may be beneficial to sperm longevity, possibly by modifying and/or reducing the metabolic activity of spermatozoa. Objective assessment of sperm kinematics using computer assisted sperm motion analyzers (Mortimer, 2000) and of sperm metabolism (Garner et al., 1997) would be useful to include in future studies on sorted spermatozoa.

The modified sperm migration test used in the present study was useful in providing additional parameters of sperm quality. This is particularly important for wildlife species where homologous oocytes, required for testing sperm quality in an IVF system, are scarce or unavailable. For all species, acrosome integrity of frozen-thawed spermatozoa was higher after sorting than before sorting, reflecting the preferential selection of membrane intact spermatozoa during the sorting process. In addition, the percentage of sorted spermatozoa with intact acrosomes remained high over time, providing further support for a high functional capacity of frozen-thawed sorted spermatozoa.

In addition to the economic benefits for livestock species, structuring the sex ratio through the application of sperm sexing has the potential to improve management of captive populations of numerous wildlife species. By overcoming limitations imposed by distance
of the sperm sorter from the animal facility, the findings of the present study may facilitate wider application of sperm-sorting technology in livestock and wildlife species.

**Acknowledgements**

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**References**


Evans, G. and Maxwell, W. M. C. (1987). Salamon's Artificial Insemination of Sheep and Goats. Butterworths; Sydney, Australia


V. Birth of lambs of a pre-determined sex after *in vitro* production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa.

Abstract

The characteristics and functional capacity of ram spermatozoa frozen-thawed prior to and after flow cytometric sorting was assessed after incubation (37°C; 6 h), *in vitro* fertilisation (IVF), and transfer of fresh and vitrified *in vitro* produced embryos. Frozen-thawed spermatozoa from two rams were allocated to four treatment groups:-(i) non-sorted (Control); (ii) sorted (FS); (iii) sorted then re-frozen (FSF) and (iv) re-frozen control (FCF). Frozen-thawed samples were separated into X- and Y- chromosome bearing spermatozoa using a high-speed sperm sorter after density gradient centrifugation (X: 88 ± 1.5 % and Y: 87 ± 1.1 % purity). After 6 h incubation (37°C), the percentage of motile spermatozoa was higher (P<0.001) for FS (84 ± 2.0 %) compared to all other treatments (Control: 36 ± 3.3 %, FSF: 28 ± 3.1 %, FCF: 20 ± 2.0 %). In a sperm migration test greater numbers of FS spermatozoa penetrated 5 mm into the artificial cervical mucus compared to spermatozoa from all other treatements (152 ± 39.4 vs 31 ± 9.2 spermatozoa, respectively; P<0.05). Fertilisation and cleavage rates were higher (P<0.05) for in vitro matured oocytes inseminated with Control (80.1 %) compared to FS or FSF spermatozoa (68.5 % and 64.5 % respectively; P<0.05). However, Day 7 blastocyst development rate was higher for oocytes inseminated with FSF (62.2%) than FS and Control spermatozoa (52.7 % and 50.0 %; P<0.05). The number of ewes pregnant (Day 60), lambing and the *in vivo* embryo survival rate was greater (P<0.01) after the transfer of fresh embryos rather than vitrified embryos derived from X- and Y- spermatozoa (67.6 %, 64.7 %, 41.2 % vs 29.6 %, 25.9 %, 14.8 %, respectively). Twenty-six of the 30 (86.7 %) lambs derived from
sex-sorted spermatozoa were of the correct sex. These results demonstrate that frozen-thawed ram spermatozoa can be sex-sorted for immediate or future use after re-cryopreservation and, in conjunction with IVF and ET, can be used to efficiently produce offspring of pre-determined sex.

**Introduction**

The application of sex pre-determination using flow cytometric sorting of spermatozoa (Johnson *et al.*, 1989) to the breeding of livestock and wildlife is limited when the sorter is located a long distance from the male(s) and / or female(s). High purity sorting of frozen-thawed spermatozoa for immediate use (O’Brien *et al.*, 2003, Hollinshead *et al.*, 2002b, 2003) or re-cryopreservation for later use would enhance the commercial and conservation-driven applications of the sexing technology.


The efficacy of sex-sorting frozen-thawed bull spermatozoa was previously investigated by Stap *et al.* (1998) and Lu *et al.* (1999). Poor resolution of the X and Y populations, low
sorting rates and a low blastocyst development rate after IVF were reported (Lu et al., 1999). Improved preparation methods, involving the removal of the cryo-diluent from frozen-thawed samples before staining and sorting have resulted in high resolution of X and Y populations and improved sorting rates (O’Brien et al., 2003; Hollinshead et al., 2002b). In the former studies, motility, acrosome integrity and migration through artificial cervical mucus were similar after sorting of frozen-thawed compared to fresh ram spermatozoa.

The objectives of the present study were to (i) evaluate in vitro characteristics (motility, forward progressive motion, acrosome integrity and migration through an artificial cervical mucus) of frozen-thawed sorted (FS) and frozen-thawed, sorted, and re-frozen and thawed (FSF) ram spermatozoa after 6 h incubation at 37°C; (ii) investigate the ability of FS and FSF spermatozoa to fertilise in vitro matured oocytes and produce blastocysts and (iii) produce offspring after the transfer of fresh and vitrified embryos derived from FS and FSF spermatozoa.

**Materials and methods**

*Experimental Design*

Ejaculates were collected from two Merino rams during the breeding season (January to May). Sorting parameters from each ejaculate were recorded (Fresh treatment) and the ejaculates were frozen and thawed (Control treatment). One third of the thawed control pellets were re-frozen and thawed (Froz-Control-Froz treatment; FCF). Another third were flow cytometrically sorted and either assessed after sorting (Froz-Sort treatment, FS) or re-frozen and thawed (Froz-Sort-Froz treatment; FSF).

Thawed spermatozoa from Control, FCF, FS and FSF treatments were assessed for migration ability through artificial cervical mucus in a modified sperm migration test
(SMT) after thawing (0 h) and for sperm characteristics after thawing (0 h) and incubation (6 h; 37°C). The latter involved assessment of acrosome status, motility and forward progressive motility (FPM). Control, FS and FSF spermatozoa were also co-incubated with in vitro matured ovine oocytes for assessment of fertilisation, cleavage and blastocyst development. Each sperm evaluation was replicated 3 times.

Semen collection and freezing

Ejaculates were collected by artificial vagina from two Merino rams (Ram 1, Ram 2). Semen was diluted 1:4 (semen : diluent, v/v) using a zwitterion-buffered diluent containing 13.5 % egg yolk and 6 % glycerol (ZWIT, Molinia et al., 1996) and frozen and thawed using the pellet method described by Evans and Maxwell (1987).

Preparation of spermatozoa for sorting

Thawed spermatozoa were prepared using multiple two-step Puresperm® (Cook, QLD, Australia) gradients (0.2 ml thawed semen on 1 ml of 45 % Puresperm® on 1 ml 90 % Puresperm®) centrifuged at 1000 g for 10 min. Pellets containing motile spermatozoa were pooled after centrifugation and immediately diluted 1:4 (sperm pellet: diluent, v/v) with a modified TRIS-buffered diluent (TRIS; 300 mM Tris-(hydroxymethyl) aminomethane, 27.8 mM fructose, 94.7 mM citric acid, 87.0 I.U. / ml penicillin-G, 35.3 g / l streptomycin sulfate and 0.3 % w/v BSA ) and centrifuged again at 600 g for 5 min. Both the neat fresh semen and the thawed prepared sperm pellets were diluted to 400 x 10⁶ spermatozoa/ml with TRIS containing 267-311 µM of Hoechst stain (H33342; Sigma, St Louis, MO, USA). These stain concentrations had been previously optimized for each ram (data not shown). The samples were incubated at 34°C and rotated every 15 min to improve staining uniformity. After 1 h incubation the stained samples were extended 1:1 (sperm sample: diluent, v/v) with TRIS (34°C) containing 4 % (v/v) egg yolk and 0.002 % (w/v) food dye.
(Warner Jenkinson Company Inc., St Louis, MO, USA) and were then filtered (37 µm, Falcon 2235, Becton Dickinson, Franklin Lakes, NJ, USA).

Flow cytometric sorting

X and Y chromosome bearing spermatozoa were separated using a high speed cell sorter (MoFloSX®, DakoCytomation, CO, USA) modified for sperm sorting (Johnson and Pinkel, 1986; Johnson and Welch, 1999; Rens et al., 1998, 1999) operating at 40 psi with a TRIS sheath fluid and running at 200 mW with an argon laser. Two types of sorting were used. For IVF samples, gates were placed around the viable and correctly oriented population during sorting to obtain purities greater than 85 % for X and Y chromosome-bearing sperm populations. Spermatozoa required for all other sperm quality tests were processed through the sperm sorter but with the gates set during sorting to include the entire viable (oriented and non-oriented) sperm population. The average sorting rate of spermatozoa for the sperm quality assessments was 8 000-10 000 spermatozoa / s depending on the number of viable cells available for sorting. Average flow rates for both sorting conditions were 18 000 - 22 000 events / s. All spermatozoa were sorted into 10-ml centrifuge tubes containing 0.25 ml of Androhep (AH; Minitüb, GmbH, Landshut, Germany) supplemented with 20 % egg yolk (v/v) adjusted to pH 7.4. Five million spermatozoa were collected for each treatment in approximately 5 ml of TRIS sheath fluid (final concentration of egg yolk 2 %, v/v).

Reanalysis of sorted spermatozoa

At the completion of sorting of each sample, 500 000 X- and Y- bearing fresh or frozen-thawed spermatozoa were collected and processed as described by Welch and Johnson (1999), to estimate the X and Y purity and to predict the proportion of male and female offspring. The proportion of correctly oriented spermatozoa, percentage of non-viable
spermatozoa, sort rate, R-value (histogram measurement of the separation between the X and Y sperm populations) and purity were recorded for every fresh and frozen-thawed sample prepared.

**Preparation of spermatozoa for post-sort and post-thaw assessments**

Sorted spermatozoa (FS) were centrifuged at 700 g for 6 min at room temperature (21°C). Each 50 µl pellet was slowly re-suspended 1:4 (sperm pellet: diluent, v/v) with either ZWIT diluent and re-frozen (FSF treatment) or Sydney IVF sperm buffer (Sperm buffer; Cook®, QLD, Australia) and re-centrifuged in 2 ml centrifuge tubes (ThermoTrace Ltd, Noble Park, VIC, Australia) at 500 g for 3 min in preparation for IVF (FS treatment).

Spermatozoa sorted but not sexed were resuspended 1:8 (50 µl pellet:diluent, v/v) with AH containing 20 % (v/v) egg yolk in preparation for incubation (37°C; 6 h) and sperm quality evaluation. In preparation for IVF, a thawed pellet of Control spermatozoa was carefully placed under 0.5 ml of sperm buffer (Cook®) and incubated for 1 h at 37°C (Brown and Radziewic, 1998). The upper 100 µl containing motile spermatozoa was removed and the concentration of spermatozoa determined by haemocytometer. For incubation and sperm quality assessment, an aliquot containing 5 x 10^6 thawed control spermatozoa was removed prior to preparation for sorting and was either (i) extended to 0.25 ml with AH containing 20 % (v/v) egg yolk (Control treatment) or (ii) re-frozen in 0.25 ml of ZWIT diluent (FCF treatment). FCF and FSF spermatozoa were thawed at 37°C by agitation in 10-ml dry glass test tube and prepared for incubation (37°C, 6 h) or co-incubation with *in vitro* matured (IVM) oocytes using the same methods as for the FS spermatozoa.

**Sperm migration test**

Spermatozoa from each treatment were assessed at 0 h after thawing (Control, FSF and FCF) or sorting (FS) for their ability to migrate through an artificial cervical mucus
(10mg/ml sodium hyaluronate diluted with 25 % AH containing 1 % w/v BSA; Bioniche, Armidale, Australia) by a sperm migration test (SMT) developed for human spermatozoa (Mortimer et al., 1990) and further modified for low numbers of sorted ram spermatozoa (J. K. O’Brien unpublished; Hollinshead et al., 2002b). Aliquots (50 µl) containing 1 x 10^6 spermatozoa from each treatment were incubated (37°C; 1 h) in BEEM capsules (BEEM standard embedding capsules, ProSciTech, QLD, Australia) containing a vertically placed 0.3 mm x 0.3 mm x 100 mm rectangular capillary tube (Microslides, VitroCom, Mountain Lakes, NJ, USA) filled with artificial cervical mucus. Evaluations of the number of spermatozoa from each treatment migrating 5 mm into artificial mucus and the distance traveled by the vanguard spermatozoon from each treatment after 1 h incubation (37°C) were evaluated using phase contrast microscopy (200x, Olympus, Tokyo, Japan).

Motility and acrosome integrity
The remainder of each sperm sample was incubated at 37°C for 6 h. Assessments of percentage motile spermatozoa (to the nearest 5 %) and FPM rating (FPM: 0-5 scale; 0 = no movement, 5 = rapid forward progression; modified from Howard et al., 1986) were made by examining several different fields by phase contrast microscopy (100x; Olympus) after thawing (Control, FCF and FSF) or sorting (FS) and 3 and 6 h incubation. Dry smears were made after thawing or sorting and 3 h incubation for the assessment of acrosome integrity by fluorescent isothiocyanate-conjugated peanut agglutin (FITC-PNA) staining as described by Roth et al. (1998). A total of 200 spermatozoa per treatment were examined by phase contrast microscopy (200x; Olympus) and were considered acrosome intact (AI) if the acrosome stained bright green and acrosome reacted (AR) if there was no staining or only a single band of green fluorescence at the equatorial segment.
In vitro maturation, fertilisation and culture of oocytes

Methods of in vitro production of embryos in the present study have been described previously (O’Brien et al., 1996). Briefly, ovaries were collected from 407 mature Merino ewes at two local abattoirs during the period December, 2002 to January, 2003. Oocytes were aspirated from follicles 2-4 mm in diameter and only those with a homogenous ooplasm surrounded by several layers of cumulus cells were selected for IVM (n = 1047). Groups of 25-35 cumulus-oocyte complexes (COCs) were incubated in multi-well dishes (Nunclon; Inter Med, Roskilde, Denmark) containing 450 µl of maturation medium overlaid with 400 µl mineral oil (M 8410, Sigma) for 22 h. All stages of in vitro culture took place at 38.5ºC in a humidified atmosphere of 5 % O₂ : 6 % CO₂ ; 89 % N₂ (V-MINC-1000, Cook®). The maturation medium contained Medium 199 (Gibco BRL, Grand island, NY, USA; with Earl’s salts, 15mM L-glutamine, 25 mM sodium bicarbonate, 25 mM HEPES) supplemented with 20 % (v/v) sheep serum (ThermoTrace), 10µg / ml FSH (Folltropin-V; Bioniche), 10µg /ml LH (Lutropin-V; Bioniche), 124 I.U. / ml penicillin and 37 I.U. / ml streptomycin (H199+).

Following IVM, COCs were transferred into H199+ and denuded by gentle pipetting. Oocytes (25-30 per well) were then co-incubated with 1 x 10⁶ motile spermatozoa / ml from each treatment (Control, FS and FSF X-and Y- chromosome bearing spermatozoa; n = 5 treatment groups / ram) for 3 h in multiwell dishes (Nunclon) containing 400 µl of fertilisation medium overlaid with 400 µl of oil. The fertilisation medium comprised bicarbonate-buffered synthetic oviduct fluid medium (SOF; Tervit et al., 1972) supplemented with 2 % (v/v) heat-inactivated and filtered sheep serum (ThermoTrace). Presumptive zygotes were then transferred to 10 µl droplets of SIVF cleavage medium (Cook®) overlaid with oil and cultured for 4 d. After this time cleaved oocytes were transferred to 10 µl droplets of SIVF blastocyst medium (Cook®) overlaid with oil for 3 d.
The protein source used in both the SIVF cleavage and blastocyst media was human serum albumin (200mg/ml, HSA; CAF-BCF, Belgium Red Cross, Belgium). For quality control, parthenogenic development was assessed by evaluating cleavage and blastocyst development of IVM oocytes incubated without spermatozoa (3 replicates, n = 69).

*Oocyte and embryo assessment*

Oocytes were visually assessed for cleavage at 24 h and 48 h after insemination (100x; Olympus). At 48 h post insemination (p.i) uncleaved oocytes were fixed in ethanol acetic acid : chloroform (6:3:1) for 24 h, stained with 1 % orcein (w/v) and examined using phase contrast microscopy (200x; Olympus). The maturation rate was calculated at 48 h post-insemination (p.i) as the proportion of oocytes undergoing cleavage and the number of uncleaved oocytes with chromosomes at metaphase-II and an extruded polar body and/or containing 2 pronuclei. Oocytes containing more than two pronuclei were considered to have undergone polyspermic fertilisation. The monospermic fertilisation rate was determined as the proportion of matured oocytes undergoing cleavage and the number of uncleaved oocytes containing only 2 pronuclei at 48 h p.i. The number of oocytes developing to the blastocyst stage was assessed (100x; Olympus) on Days 5, 6 and 7 post-insemination. The criteria for blastocyst formation were the presence of a partially or fully formed blastocoele and a distinguishable inner cell mass and trophoblast population. Blastocysts were further categorised into five developmental stages based on their degree of expansion and hatching (O’Brien *et al.*, 1997); early blastocyst (EB: blastocoele beginning to form); blastocyst (B: blastocoele formed); expanded blastocyst (ExB: large increase in blastocyst diameter and thinning of the zona pellucida); hatching blastocyst (HgB); and hatched blastocyst (HB). Blastocysts at the B, ExB, HgB and HB stages were graded into three groups based on the development of the inner cell mass and trophectoderm (Gardner and Schoolcraft, 1999).
Embryo Vitrification and re-warming

Day 6 and 7 blastocysts were cryopreserved using the Open Pulled Straw (OPS) vitrification method (Vajta et al., 1998). Briefly, embryos were first washed in a Hepes-buffered M91 medium (Sydney IVF cryobuffer, Cook®) supplemented with 6% (v/v) HSA. This cryobuffer medium was used as the basic holding medium for both vitrification and thawing. All media were warmed to 38.5°C and all manipulations were carried out on a heated stage (38.5°C) in a warm room (26-28°C). The blastocysts were then exposed to cryobuffer medium containing 7.5% (v/v) ethylene glycol (EG; Sigma E 9129) and 7.5% (v/v) dimethyl sulfoxide (DMSO; Sigma D2650) for 3 min. Blastocysts were then transferred to and mixed with cryobuffer medium containing 1M sucrose, 16.5% EG and 16.5% DMSO, and loaded by capillary action into open-pulled straws before plunging into liquid nitrogen all within 25 sec. Day 6 B and early ExB were rewarmed the day before transfer and Day 6 (Ex B and HgB) and all Day 7 blastocysts were re-warmed on the morning they were to be transferred. All blastocysts were warmed by holding each straw for 3 s in air and then immersing the end containing the blastocysts in 1 ml of cryobuffer medium containing 0.33 M sucrose (38.5°C). After 5 min, blastocysts were transferred to cryobuffer medium containing 0.16 M sucrose and held for 5 min, and finally they were moved into cryobuffer medium for a further 5 min. Day 6 blastocysts (B and early ExB) were subsequently cultured in SIVF blastocyst medium (Cook®) for 18 h prior to transport. Late Day 6 and Day 7 blastocysts were placed immediately into transport medium (see below) and transferred into recipients within 4 h of re-warming. Only early Day 6 embryos were assessed for re-expansion of the blastocoele prior to transfer since late Day 6 and Day 7 embryos were not cultured after re-warming.
Embryo Transfer

Fresh blastocysts forming on Day 6 of culture were transferred into 34 mature Border Leicester x Merino (BLM) crossbred ewes early in the breeding season (February, 2003). The transfers were performed at a commercial sheep farm located in the southern highlands of N.S.W. (ET Trial 1). Blastocysts forming on Day 6 and Day 7 of culture were vitrified and later thawed and transferred into 27 mature Merino ewes in April, 2003 at The University of Sydney farm, Camden, N.S.W. (ET Trial 2).

Fresh Day 6 embryos were transported and held in Sydney IVF buffer (Cook®) and 2.5 % (v/v) HSA (CAF-BCF) at 38.5°C until transfer (2.5 – 4 h). Re-warmed Day 6 and Day 7 embryos were transported in Sydney IVF buffer (Cook®) and 5 % (v/v) HSA (CAF-BCF) and held at 38.5°C until transfer (1.5 - 3 h). Both fresh embryos and vitrified embryos re-warmed and cultured for 18 h prior to transfer were graded using light microscopy (100x; Olympus) immediately prior to transfer. Sixty-eight grade 1 blastocysts at the B-ExB, ExB and HgB developmental stages produced on Day 6 of culture were transferred into 34 synchronised recipients (ET Trial 1). Twenty grade 1 blastocysts at the B or ExB stage and 34 grade 1 ExB and HgB produced on Day 6 and Day 7 of culture respectively, were vitrified and later thawed and transferred into 27 synchronised recipient ewes (ET Trial 2).

Oestrus was synchronised in 50 mature BLM crossbred ewes (ET Trial 1; 3 years, 65-70 kg) and 30 mature Merino ewes (ET Trial 2; 5 years., 45-50 kg) with progestagen-impregnated intravaginal sponges containing 40 mg fluogestone acetate (40 mg Chronogest®, Intervet, Bendigo, VIC). The sponges were removed after 13 d and the ewes were injected intramuscularly (I.M.) with 500 I.U. (ET Trial 1) or 400 I.U. (ET Trial 2) of pregnant mare serum gonadotrophin (PMSG, Pregnecol, Bioniche).
Embryo transfer was performed using a modified laparoscopic mini-laparotomy technique (O’Brien et al., 1996) either 6 d (ET Trial 1) or 7 d (ET Trial 2) after the onset of oestrus. Recipient ewes were treated 20 min before surgery with a combination of I.M. sedatives (1 ml ketamine; Ketamil, Ilium, Troy Laboratories, Smithfield, N.S.W., Australia: 2 ml diazepam; Pamlin, Parnell Laboratories, Alexandria, N.S.W., Australia: and 1 ml buprenorphine hydrochloride; Temgesic, Reckitt Benckiser, Auckland, New Zealand) and local anaesthetic (4 ml Lignocaine; Ilium, Troy Laboratories). Before transfer, both the ovaries were visualised by manipulation with a probe to check for the presence of copora lutea. Ewes which had failed to ovulate were excluded from the study (n=16 in ET Trial 1; n=3 in ET Trial 2). The tip of the uterine horn ipsilateral to an ovary with at least one corpus luteum was exteriorised through a 1-2 cm mid ventral incision. A small opening was made in the tip of the uterine horn and two blastocysts of the same developmental stage (B, ExB, HgB, HB), age (Day 6 or Day 7), grade (1 or 2) and from the same sperm treatment (Control, FS and FSF X or Y) and embryo treatment (fresh, vitrified, thawed and incubated or vitrified and thawed) were transferred into each recipient using a 10 µl capillary pipette (Unopette; Becton Dickinson, Rutherford, NJ). After embryo transfer each ewe was given a non-steroidal anti-inflammatory I.M. injection (1 ml Ketoprofen, Nature Vet®, Agnes Banks, N.S.W., Australia).

During transfer of the fresh embryos (ET Trial 1) a poor ovulatory response was noted in 88 % (30 / 34) of recipients. Progesterone sponges were placed in each of the recipient ewes from Day 12 to Day 20 of gestation to help maintain pregnancy (Pearce et al., 1984; Scaramuzzi et al., 1988).
**Pregnancy Diagnosis**

Jugular blood samples were collected from each recipient ewe on Day 20 after oocyte insemination and plasma progesterone concentrations were determined with a commercially available radiommmunoassay kit (Spectra; Orion Diagnostics, Helsinki, Finland). Progesterone concentrations greater than 1.2 ng/ml were considered pregnant (Robertson and Sarda, 1971). At Day 60 of gestation all recipient ewes were scanned by ultrasound for detection of foetuses. Pregnancy loss in ewes was calculated as the number of ewes pregnant at Day 20 - the number of ewes pregnant at Day 60 / number of ewes pregnant at Day 20 x 100. Foetal loss was determined as the number of ewes pregnant at Day 60 - the number of ewes lambing / number of ewes pregnant at Day 60.

**Lambing**

At the start of the third trimester of gestation, pregnant ewes were separated from the flock and placed in sheltered paddocks and provided with supplementary feed in the form of lupin grains. Several days before the commencement of lambing, ewes were placed in small sheltered paddocks and observed 24 h per day until the end of lambing. Lambs were weighed, measured (crown-rump length and head width), identified for sex and ear-tagged within 6 h of birth. *In vitro* embryo survival rate was calculated as the number of lambs born / number of embryos transferred x 100.

Due to the large size of a proportion of the lambs and associated birthing difficulties and ewe and lamb mortality experienced during the lambing of recipient crossbred ewes in ET Trial 1, a combination of dexamethasone (5 ml; Dexadreson 2 mg/ml; Intervet) and oestradiol benzoate (5 ml; Mesalin 0.2 mg/ml, Intervet) was given at Day 143 of gestation to each of the Merino recipient ewes in ET Trial 2 to induce earlier births and smaller offspring (Ptak et al., 2002).
Statistical Analysis

Statistical analyses were performed using GENSTAT computer program (Version 4.2, Numerical Algorithms Group (NAG©) Ltd, Oxford, U.K.). Data for sorting, motility, FPM rating and the mean distance traveled by the vanguard spermatozoon were analysed by ANOVA. Motility and FPM rating measurements at 0 h, 3 h and 6 h incubation were subjected to natural log transformation and analysed by repeated measures analysis of variance. The number of spermatozoa migrating 5 mm into the artificial cervical mucus was analysed by log linear regression and the acrosomal status of spermatozoa from each treatment was determined by binomial logistic regression. Statistical differences in the proportion of oocytes undergoing fertilisation and cleaved oocytes developing to the blastocyst stage and the proportion of grade 1 embryos developing on Day 6 or Day 7 were analysed by binomial logistic regression. The pregnancy and lambing rate, and pregnancy and foetal loss after the transfer of fresh and vitrified embryos into recipient ewes, were also analysed by binomial logistic regression. Lamb weight, body length, head width and gestation length were analysed by ANOVA. Proportional data for the sex ratio of offspring and in vivo embryo survival were analysed by Chi-square analysis and Fisher’s Exact Test using the SigmaStat® computer program (Version 2.0. SPSS Inc., San Rafael, CA, USA).

Results

Sorting Efficiency

High resolution of X and Y sperm populations was achieved for both fresh and frozen-thawed samples (Figure 1). Re-analysis revealed high levels of purity for X- and Y- sorted samples obtained from fresh (X: 89 ± 2.0 %, Y: 88 ± 1.2 %) and frozen-thawed (X: 89 ± 1.0 %, Y: 89 ± 1.3 %) spermatozoa. There was a greater proportion of non-viable sperm
(Figures 1a and 1b) for frozen-thawed (FS and FSF treatment) compared to fresh samples (25 ± 1.7 % and 12 ± 1.6 %, respectively; P<0.001). The percentage of oriented spermatozoa (Figures 1a and 1b) was higher for fresh (60 ± 3.0 %) than frozen-thawed samples (47 ± 1.5 %; P<0.001). Fresh spermatozoa were sorted at higher rates than frozen-thawed spermatozoa (3540 ± 239.0 vs 1783 ± 151.0 spermatozoa / s respectively; P<0.001). There was no ram effect on any of the sorting parameters.

Sperm migration test

More sorted frozen-thawed spermatozoa derived from frozen-thawed samples (FS) penetrated 5 mm into the artificial cervical mucus compared to Control, FCF and FSF spermatozoa (P<0.01) and more Control spermatozoa migrated 5 mm in the capillary tube than spermatozoa that had been frozen and thawed twice (FCF; Table 1; P<0.01). Higher numbers of spermatozoa reached 5 mm for Ram 1 than Ram 2 (82 ± 26.7 vs 40 ± 9.0; P<0.01). The mean distance traveled by the vanguard spermatozoon was similar for all treatments.

Table 1: Mean (± S.E.M.) number of spermatozoa migrating 5 mm into the artificial cervical mucus and the mean (± S.E.M.) distance traveled by the vanguard spermatozoon from the frozen-thawed non-sorted (Control), frozen-thawed and sorted (FS), frozen-thawed and re-frozen and thawed (FCF), frozen-thawed, sorted and re-frozen and thawed (FSF) treatment after 1 h incubation (37°C).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of spermatozoa reaching 5 mm</th>
<th>Vanguard distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56 ± 16.4 b</td>
<td>33 ± 0.2</td>
</tr>
<tr>
<td>FCF</td>
<td>7 ± 1.3 c</td>
<td>27 ± 0.4</td>
</tr>
<tr>
<td>FS</td>
<td>152 ± 3.9 a</td>
<td>25 ± 0.3</td>
</tr>
<tr>
<td>FSF</td>
<td>31 ± 9.8 bc</td>
<td>29 ± 0.4</td>
</tr>
</tbody>
</table>

abc Within columns, values with different superscripts differ (P<0.01)
Fig 1: Flow cytometric histograms and dot plots derived from fresh (a) and frozen-thawed (b) spermatozoa from the same ejaculate. The arrows indicate that the information from the spermatozoa gated in R1 is then illustrated as a univariate histogram.

**R1-Region 1:** represents the correctly oriented sperm population  
**R2-Region 2:** represents non-viable spermatozoa
Assessment of motility, forward progressive motion and acrosome integrity

After thawing, FS spermatozoa had higher motility than spermatozoa from all other treatments (P<0.001). Control and FSF treatments had a similar proportion of motile spermatozoa, which was higher than FCF over the 6 h incubation period (Fig 2a; P<0.001). After 3 h of incubation, motility of spermatozoa declined (P<0.001) for all treatments except for FS which maintained similar motility over the 6 h incubation period (Fig 2a).

The FPM rating of spermatozoa was higher (P<0.001) for the Control than for all other treatments (FS, FSF and FCF; Fig 2b). After 6 h incubation, FS spermatozoa had a higher FPM rating than spermatozoa that had been re-frozen (FCF and FSF; Fig 2b; P<0.001). At 0 h post-thaw, FPM was higher for Control and FCF spermatozoa compared with FS and FSF spermatozoa (P<0.001). The FPM rating of FS, FSF and FCF spermatozoa increased after 1 h incubation (P<0.001) but then gradually declined (Fig 2b; P<0.001). Similar to motility, FPM of FS spermatozoa was maintained throughout the incubation period (Fig 2b).

Spermatozoa that were re-frozen and thawed (FSF and FCF) had less intact acrosomes after the 6 h incubation period than Control and FS spermatozoa (86 ± 2.6 % and 83 ± 1.6 % vs 92 ± 1.4 % and 90 ± 1.3 %, respectively; P<0.05).
**Fig 2:** Motility (a) and FPM (b) of frozen-thawed sorted (▲; FS) and non-sorted (■; Control) and re-frozen and thawed sorted (♦; FSF) and non-sorted (○; FCF) spermatozoa after thawing (0 h) and incubation for 6 h at 37°C.

(a)

![Graph showing motility](image)

(b)

![Graph showing FPM](image)

**Effect of sperm preparation for IVF on motility, forward progressive motion and acrosome integrity**

The proportion of motile spermatozoa was higher for frozen-thawed spermatozoa after sorting (FS; 85 ± 1.5 %) and thawing (FSF; 71 ± 5.0 %) than for non-sorted frozen-thawed spermatozoa (Control; 45 ± 4.5 %; Fig 3a; P<0.001). However, after
preparation for IVF involving a swim-up for Control spermatozoa, and wash and centrifgation for sorted (FS and FSF) spermatozoa, the percentage of motile Control spermatozoa significantly increased (75 ± 6.1 %; P<0.001) but decreased for the FS and FSF treatments (60 ± 8.3 % and 32 ± 4.6 % respectively; Fig 3a). FS spermatozoa maintained a higher motility after IVF preparation than FSF spermatozoa (P<0.001). Spermatozoa from all treatments had higher FPM before than after the preparation for IVF (Fig 3b; P<0.05). Overall, Control spermatozoa had a higher FPM rating than sorted spermatozoa (FS and FSF; Fig 3b; P<0.05).

Spermatozoa from each treatment had a similar percentage of intact acrosomes both before and after preparation for IVF (results not shown).

**Fig 3**: Motility (a) and FPM (b) of frozen-thawed sorted (▲; FS) and non-sorted (■; Control) and re-frozen and thawed sorted (●; FSF) spermatozoa after thawing (Post-thaw) and preparation for IVF (Pre-IVF).
In vitro fertilisation and early embryo development

The overall oocyte maturation rate was 90.6 % (949/1047). The proportions of oocytes fertilised (monospermic) and of zygotes that had cleaved 48 h after insemination were higher after insemination with Control than FSF spermatozoa (Table 2; P<0.05). However, the proportion of cleaved oocytes that developed to blastocysts by Day 7 was significantly higher after co-incubation with FSF than with Control or FS spermatozoa (P<0.05; Table 2). A similar proportion of blastocysts developed on Day 5 and Day 6 of culture after insemination with FS, FSF or Control spermatozoa (Table 2). There was no effect of ram (Ram 1, Ram 2) or sex (X- or Y-chromosome bearing spermatozoa) on fertilisation, cleavage or blastocyst development. Cleavage of oocytes in a parthenogenic control group (3 / 69; 4.3 %) and the proportion of uncleaved oocytes assessed at 48 h p.i that had undergone polyspermic fertilisation (20 / 949; 2.1 %) were low.
Table 2: Fertilisation and early embryonic development of oocytes after incubation with frozen-thawed non-sorted (Control), frozen-thawed and sorted (FS) and frozen-thawed, sorted then frozen-thawed (FSF) ram spermatozoa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. matured oocytes*fertilisedd (%)</th>
<th>No. mature oocytes undergoing cleavage after insemination (%)</th>
<th>No. cleaved oocytes forming blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td>121 (80.1)a</td>
<td>79 (52.3)</td>
<td>20 (17.2)</td>
</tr>
<tr>
<td>FS</td>
<td>228 (68.5)ab</td>
<td>134 (40.2)</td>
<td>43 (19.4)</td>
</tr>
<tr>
<td>FSF</td>
<td>263 (64.5)b</td>
<td>192 (49.3)</td>
<td>46 (18.3)</td>
</tr>
</tbody>
</table>

ab Within column, values with different superscripts differ (P<0.05).

d Monospermic fertilisation.

*Maturation rate = No. oocytes undergoing cleavage at 48 h p.i + No. uncleaved oocytes containing two pro-nuclei at 48 p.i.

Embryo Quality

Overall, embryo quality was high (grade 1: 70.7 %; 215/304) with no difference in the proportion of grade 1 blastocysts developing from oocytes inseminated with Control, FS or FSF spermatozoa. For all treatments, a higher proportion of grade 1 embryos developed on Day 6 (88.3 %; 159 / 180) than Day 7 (52 / 125, 41.6 %; P<0.05). All Day 6 vitrified embryos underwent re-expansion during the 18 h culture after re-warming.

Pregnancy rate and pregnancy loss

Data for the number of recipient ewes pregnant as determined by progesterone assay on Day 20 and by ultrasound on Day 60 are presented in Table 3. A higher proportion of recipient ewes were pregnant on Day 60 after the transfer of fresh (23/34; 68 %) than vitrified embryos (8/27; 30 %; P<0.01). The overall pregnancy loss estimated from Day 20 to Day 60 after transfer did not differ between ewes which received either fresh (4/27; 15
or vitrified (6/14; 43 %) embryos. There was no effect of ram, sperm type or sex of spermatozoa on pregnancy rate or pregnancy loss after the transfer of fresh or vitrified embryos. However, the number of ewes involved in the transfer of fresh and vitrified embryos was low (n=34 and n=27 respectively).

Pregnancy rate on Day 60 did not differ between ewes receiving vitrified embryos that were incubated prior to transfer (4 / 9; 44 %) compared with those transferred immediately after re-warming (4 / 18; 22 %).

Lambing and in vivo embryo survival

Foetal loss between Day 60 of gestation and lambing occurred in 2 recipient ewes (one from each ET Trial). Of the 34 recipient ewes which received fresh embryos (ET Trial 1), 22 (64.7 %) gave birth to 28 lambs and of the 27 recipient ewes which received vitrified embryos (ET Trial 2), 7 (25.9 %) gave birth to 8 lambs. The in vivo survival for both fresh and vitrified embryos was similar across all sperm treatments (Table 3). However, a higher proportion of fresh (41.2 %) compared to vitrified (14.8 %) embryos survived to lambing (P<0.01). A total of 8 neonatal deaths (8 / 36; 22.2 %) were recorded, which were evenly distributed across embryo (fresh v vitrified) and sperm (Control vs FS vs FSF) treatment groups.
Table 3: Pregnancy rate on Day 20, Day 60 and lambing and in vivo embryo survival of fresh and vitrified embryos, produced after in vitro fertilisation of in vitro matured oocytes with frozen-thawed, non-sorted (Control), frozen-thawed and sorted (FS) and frozen-thawed, sorted, re-frozen and thawed (FSF) spermatozoa, into synchronised recipient ewes.

<table>
<thead>
<tr>
<th>Type of semen</th>
<th>Type of embryo</th>
<th>No. of recipient ewes</th>
<th>No. pregnant on Day 20 (%)</th>
<th>No. pregnant on Day 60 (%)</th>
<th>No. Ewes lambing (%)</th>
<th>Embryo survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Fresh</td>
<td>8</td>
<td>6 (75)</td>
<td>5 (62.5)a</td>
<td>4 (50.0)a</td>
<td>5/16 (31.3)a</td>
</tr>
<tr>
<td></td>
<td>Vitrified</td>
<td>6</td>
<td>5 (83.3)</td>
<td>2 (33.3)b</td>
<td>1 (16.7)b</td>
<td>1/12 (8.3)b</td>
</tr>
<tr>
<td>FS</td>
<td>Fresh</td>
<td>9</td>
<td>7 (77.8)</td>
<td>5 (55.6)</td>
<td>5 (55.6)a</td>
<td>6/18 (33.3)a</td>
</tr>
<tr>
<td></td>
<td>Vitrified</td>
<td>8</td>
<td>3 (37.5)</td>
<td>2 (25.0)b</td>
<td>2 (25.0)b</td>
<td>2/16 (12.5)b</td>
</tr>
<tr>
<td>FSF</td>
<td>Fresh</td>
<td>17</td>
<td>14 (82.4)</td>
<td>13 (76.5)a</td>
<td>13 (76.5)a</td>
<td>17/34 (50.0)a</td>
</tr>
<tr>
<td></td>
<td>Vitrified</td>
<td>13</td>
<td>6 (46.2)</td>
<td>4 (30.8)b</td>
<td>4 (30.8)b</td>
<td>5/26 (19.2)</td>
</tr>
</tbody>
</table>

1 determined by peripheral plasma progesterone assay
2 determined by ultrasound
3 no. ewes that lambed between Days 149 and 155 (Fresh) or Days 145 and 156 (Vitrified) / no. recipient ewes x 100
4 no. lambs born / no. of transferred embryos x 100
ab Within columns, values with different superscripts differ (P<0.05)

Sex ratio

Overall, 26 of the 30 lambs (86.7 %) born after transfer of fresh (20/23; 87.0 %) and vitrified (6/7; 85.7 %) embryos derived from sex-sorted spermatozoa were of the predicted sex. There was a significant difference (P<0.05) in the sex of lambs born from ewes receiving embryos derived from sorted X- spermatozoa (12/12; 100 %) compared to ewes that received embryos derived from non-sorted (control) spermatozoa (female: 3/6; 50 %).

Due to low numbers, there was not a significant difference between the sex of lambs born from ewes receiving embryos derived from sorted Y- spermatozoa (14/18; 77.8 %) or control spermatozoa (male: 3/6; 50 %). The proportion of ewes receiving embryos derived
from sex-sorted spermatozoa did not differ from the re-sort analysis values obtained after each sorting session (X and Y data pooled; 89 ± 1.2 %).

**Gestation length and birth weights**

Lambs were born between Day 149 and Day 157 (ET Trial 1), and between Day 145 and Day 156 (ET Trial 2). There was no effect of sex, sperm type, or number of lambs born (single vs twin) on the gestation length. However, the mean gestation length of ewes in ET Trial 2 (150 ± 1.5 d) was shorter than ewes in ET Trial 1 (152 ± 0.4; P<0.05).

Furthermore, the heavier lambs (> 4.0 kg) were born later (after Day 153) in both ET trials. The mean body weight did not differ for lambs born after transfer of either fresh or vitrified embryos (Fresh: 5.2 ± 0.3 kg; Vitrified: 4.4 ± 0.5 kg). However, single lambs were heavier (5.9 ± 0.35 kg) and longer in body length (470 ± 14.4 cm) than twin born lambs (4.2 ± 0.16 kg, 400 ± 10.3 cm, respectively; P<0.01) after the transfer of fresh but not vitrified embryos. There was no difference in body weight or length between male or female lambs after the transfer of fresh or vitrified embryos derived from non-sorted (control) or sex-sorted spermatozoa.

**Discussion**

This is the first report of the production of offspring of a pre-determined sex after transfer of embryos produced *in vitro* with frozen-thawed sorted (FS) and re-frozen and thawed sorted (FSF) spermatozoa. The results from this study indicate that frozen-thawed ram spermatozoa undergoing sex-sorting and a second cryopreservation step are fully functional when used in IVF.
It was previously demonstrated that, after density gradient preparation, it was possible to sort frozen-thawed ram spermatozoa at high X and Y purity and maintain sperm motility, acrosome integrity and migration ability through artificial cervical mucus (Hollinshead et al., 2002b, 2003). The present study progressed this by investigating in vitro functional capacity of both frozen-thawed sorted ram spermatozoa (FS) and frozen-thawed sorted, re-frozen and thawed ram spermatozoa (FSF) resulting in production of live offspring.

High resolution of X and Y sperm populations resulting in X and Y purities greater than 89% was achieved for frozen-thawed spermatozoa. Conversely, Lu et al. (1999) and Cran et al. (1994) reported poor resolution and low purities (X: 82%; Y: 84%) after sorting frozen-thawed samples. However, the frozen-thawed samples in the latter studies were not prepared by gradient density centrifugation prior to sorting. Density gradient preparation plays an important role in removing the non-viable (dead) spermatozoa and the majority of the egg yolk and glycerol that is present in cryopreservation diluents. High concentrations of egg yolk can interfere with staining uniformity and contribute to poor resolution, and non-viable spermatozoa can cause interference to the sorting core. This results in a decreased proportion of correctly oriented sperm and lower sorting rates.

Spermatozoa frozen and thawed prior to sorting (FS) had higher motility than non-sorted (Control) spermatozoa after thawing and incubation. Furthermore, the proportion of motile spermatozoa after freezing, thawing, sorting, re-freezing, thawing and incubation (FSF) was higher than non-sorted spermatozoa that had undergone two cycles of freeze-thawing (FCF) and incubation. These results are most likely attributable to the selection of only viable spermatozoa during the sorting process. The food dye added to the spermatozoa with the Hoechst stain penetrates the nuclear membrane of non-viable spermatozoa, allowing their identification and exclusion by gating from the sorted sperm population.
The additional cryopreservation step after sorting (FSF) may also be a factor in the selection of a more viable sperm population (Maxwell and Watson, 1996).

The FPM of sorted (FS and FSF) spermatozoa was vigorous but lower than non-sorted (Control and FCF) spermatozoa after sorting, thawing and incubation. However, after resuspension of sorted spermatozoa in a more complex medium and 1 h incubation (37°C) FPM significantly increased. The distance traveled by the vanguard spermatozoon from FS and FSF treatments in the SMT also suggests that the Tris-based diluent used for both staining and sorting (sheath fluid) spermatozoa is more likely contributing to the decrease in FPM after sorting than the potential physical damage and dilution involved in the sorting process.

Despite the low FPM rating after sorting, higher numbers of frozen-thawed sorted spermatozoa (FS) penetrated 5 mm into the artificial cervical mucus than for all other treatments (Control, FSF and FCF). However, the distance traveled by the vanguard spermatozoon was similar for all treatments, suggesting that some spermatozoa have a similar ability to migrate to the site of fertilisation. FS spermatozoa had higher motility after sorting than Control, FSF or FCF spermatozoa after thawing, which may have contributed to the greater number of FS spermatozoa migrating through the artificial mucus. The SMT results suggest that successful fertilisation and pregnancy might be achieved after intrauterine or oviducal insemination with FS, FSF or FCF spermatozoa, close to the time of ovulation.

In vitro production of pre-sexed offspring may be a more efficient use of FS and FSF spermatozoa. Fertilisation and cleavage rates were similar for Control and FS spermatozoa. However, FSF spermatozoa were associated with lower fertilisation and
cleavage rates compared with Control, possibly indicating more spermatozoa with impaired function. However, this difference in fertilisation rate may also have resulted from incubation of oocytes with higher numbers of non-motile spermatozoa in the FSF treatment creating increased levels of reactive oxygen species (ROS; Aitken, 1995).

Nevertheless, development of cleaved oocytes to Day 7 blastocysts was higher after insemination with FSF spermatozoa than for all other treatments (FS, Control) and was similar after insemination with FS or Control spermatozoa. Comparable results have been reported after IVF with sorted and non-sorted (control) frozen-thawed bull spermatozoa (Lu et al., 2001; Zhang et al., 2003) but blastocyst rates as high as those achieved in the present study have not been reported. The increased selection pressure on spermatozoa with intact membranes after freeze-thawing, sorting and a second re-cryopreservation may be associated with the isolation of a population of spermatozoa yielding a higher development rate to blastocysts after IVF than Control or FS spermatozoa. Embryo transfer trials incorporating higher numbers of recipients are required, since although statistically non-significant, the pregnancy rate determined by ultrasound on Day 60 (82 %) was higher after transfer of embryos derived from FSF spermatozoa compared to FS (56 %) and Control (62 %) spermatozoa. Pregnancy loss between Day 20 and Day 60, and between Day 60 and lambing did not differ between ewes receiving Control, FS or FSF derived embryos (Table 3). These results and previous reports (reviewed by Seidel and Garner, 2002) suggest that the staining, sorting, cryopreservation and re-cryopreservation processes for FS and FSF spermatozoa do not appear to cause DNA damage that influences embryonic survival.

The majority of blastocysts in the present study developed on Day 6 of culture regardless of the type of spermatozoa used (FS, FSF or Control). This is in contrast to previous
reports of delayed embryonic development after IVF with spermatozoa sorted on either a high speed (Lu et al., 1999) or a standard speed flow cytometer (Cran et al., 1993, 1994; Merton et al., 1997) prior to freeze-thawing compared to non-sorted, frozen-thawed or fresh bull spermatozoa. Significant improvements in both biological (bull: Schenk et al., 1999; ram: F. K. Hollinshead, unpublished) and instrumental (Rens et al., 1999; Campos-Chillon and De La Torre, 2003; Suh and Schenk, 2003) aspects of flow cytometric sperm sorting have occurred since these reports. Such developments have significantly reduced the dilution rate of spermatozoa during staining (Maxwell and Johnson, 1999) and may be contributing to improvements in the IVF parameters found in this study compared to earlier reports.

Transfer of fresh and frozen-thawed (conventional slow freezing) embryos derived from sorted frozen-thawed bull spermatozoa (Cran et al., 1994) resulted in pregnancy rates similar to those observed after the transfer of fresh and vitrified embryos derived from frozen-thawed sorted (FS) and re-frozen and thawed (FSF) spermatozoa in the present study (Table 3). Vitrification instead of conventional slow freezing was utilized as it is a quick, simple and cost-effective method of cryopreserving embryos (Vajta, 2000) making it commercially attractive, especially in combination with in vitro production of ‘pre-sexed’ embryos. The overall pregnancy rates after the transfer of in vitro produced fresh and vitrified embryos in this study were comparable to those reported by other workers after transfer of fresh and vitrified embryos derived from non-sorted ram spermatozoa (Fresh: 41 %-66 %, Brown and Radeziewic, 1998; O’Brien et al., 1997; Walmsley et al., 2000; Martinez et al., 1997; Dattena et al., 1999, Peura et al., 2002; Vitrified: 33 %-50 % Fogarty et al., 2000; Dattena et al., 1999; Walmsley et al., 1999; Peura et al., 2002). In the present study, the pregnancy rate and in vivo embryo survival was higher after the transfer of fresh than vitrified embryos (Table 3). Vitrification eliminates the adverse effects of ice
crystal formation and chilling injury during freezing (Vajta, 2000), but the embryonic cells still undergo membrane damage and osmotic stress due to dehydration and toxicity from the high concentrations of cryoprotectants used. The absence of serum and its membrane protective properties (Vajta et al., 1999) in the vitrification and holding solutions used in this study may also have contributed to the lower pregnancy and lambing rate after transfer of vitrified embryos through reduced blastulation and hatching rates (Vajta et al., 1999; Gardner et al., 1994; Thompson et al., 1998).

Embryos vitrified after 6 days of culture were incubated for 18 h prior to transfer for two purposes. First, so that they would be at the same developmental stage as the embryos vitrified after 7 days and thawed just prior to transfer into recipient ewes. Second, the incubation enabled assessment of re-expansion after re-warming as a measure of viability (Greve et al., 1993; Naitana et al., 1995; Donnay et al., 1998; Dattena et al., 2000). All of the Day 6 vitrified embryos re-expanded after thawing. This re-expansion rate is higher than previously reported for in vitro produced embryos, but is similar to in vivo derived, vitrified ovine embryos (Dattena et al., 2000). As observed in humans (Guerif et al., 2003), the pregnancy rate was similar for embryos transferred into ewes within a few hours of thawing and embryos that were thawed and cultured overnight prior to transfer.

Incidence of lambs with large birth weights (range: 2.5-9.5 kg) was higher than that reported for naturally bred Merino lambs (4 kg; Brown and Radziewic, 1998) and the lambs born from the same flock of ewes after AI with non-sorted (control) or sorted frozen-thawed spermatozoa (range: 3.1 to 5.4 kg; Hollinshead et al., 2002a). Similar findings after the transfer of in vitro produced embryos have been reported previously for sheep (Walker et al., 1992; Thompson et al., 1995; Holm et al., 1996; Walker et al., 1996; Brown and Radziewic, 1998). A number of factors have been implicated in the birth of
large offspring after ET of *in vitro* produced embryos (reviewed by Walker *et al*., 1992, 1996) including asynchronous embryo transfer (Wilmut *et al*., 1981), particularly after the transfer of vitrified embryos (Naitana *et al*., 1995; Leoni *et al*., 2003), progesterone treatment of ewes early in gestation (Kleemann *et al*., 1994) and the use of serum as the source of protein during *in vitro* culture (Thompson *et al*., 1995). Despite substituting serum with a purified, quality controlled HSA in the *in vitro* culture media, the procedures of *in vitro* production of embryos and possibly progesterone supplementation at Day 12 of gestation, may have had a mitogenic effect on embryonic cells or altered the distribution of cells in the trophectoderm and inner cell mass (Brown and Radziewic, 1998), ultimately resulting in enhanced foetal growth and increased birth weights of a proportion of the lambs in this study.

At present, approximately 30 % of fresh and fewer than 10 % of frozen-thawed spermatozoa prepared for sorting can be effectively sex-sorted. This inefficiency is due to both biological and mechanical losses that occur during the sorting processes (reviewed by Seidel and Garner, 2002). Lower numbers of frozen-thawed spermatozoa are sorted because of the increased proportion of spermatozoa with damaged membranes after thawing. Efficiency may be improved by further optimization of flow cytometer parameters, sperm preparation and cryopreservation methods. Furthermore, future research into improved methods for preparation of frozen-thawed sorted (FS) and re-frozen-thawed (FSF) spermatozoa for IVF may help to increase fertilisation and cleavage rates and ultimately the efficiency with which pre-sexed embryos can be produced.

In conclusion, this study has demonstrated that frozen-thawed samples can be sex-sorted at high purities and then re-cryopreserved and successfully used in an IVF system to produce viable embryos. Assisted reproductive techniques (sperm and embryo preservation, IVF,
ET) were successfully combined with sperm sexing technology, resulting in the production of normal offspring. This significant development will help overcome limitations imposed by distance of the sperm sorter from the site of semen collection and embryo transfer in both livestock and wildlife species.

Acknowledgements

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References


Conclusion

Prior to the commencement of this thesis there had been no report of lambs born after AI or IVF with spermatozoa frozen-thawed after sex-sorting. The birth of 25 lambs of predicted sex after intrauterine (IU) insemination of low numbers (2-4 x 10^6 total) of sex-sorted frozen-thawed spermatozoa was achieved after the development of successful sorting and cryopreservation protocols for ram semen. However, further obstacles to the commercialization of sperm sexing in sheep remained, including the number of spermatozoa that could be practically and economically sorted for large scale AI programs in sheep and the fixed location of the sperm sorter. These prompted the development of methods to transport ram semen to the sorting site for sex separation and processing and to transport sorted spermatozoa to the site of either in vivo or in vitro insemination. After sorting both liquid and frozen-stored spermatozoa, sperm samples of high quality were produced, enabling cryopreservation after sorting. The birth of 26 IVP lambs of predicted sex after the transfer of both fresh and vitrified embryos, derived from frozen-thawed sexsorted and frozen-thawed, sex-sorted, re-frozen and thawed spermatozoa, was a further “world-first”.

Despite these major advances, there remain several limitations to the widespread use of sexed spermatozoa. The pregnancy rate of ewes after insemination with sex-sorted frozen-thawed spermatozoa was low. Investigation into factors potentially contributing to this low pregnancy rate associated with sex-sorted spermatozoa was undertaken. Despite an increase in the proportion of motile and acrosome-intact spermatozoa after sorting, the proportion of sorted spermatozoa that had undergone capacitation-like changes was greater than for non-sorted spermatozoa. Cryopreservation of ram spermatozoa has also been
shown to cause sperm membrane changes (Gillan et al., 1997). Collectively, the sorting and freeze-thawing processes accelerated the maturation of sorted frozen-thawed spermatozoa thus reducing their fertilising lifespan. Thus, for use of sex-sorted frozen-thawed ram spermatozoa in AI, insemination close to the site of fertilisation and time of ovulation is critical for successful fertilisation and ongoing pregnancy.

Despite inseminating ewes either just before or after ovulation with sorted frozen-thawed spermatozoa, the pregnancy rate of ewes treated with GnRH did not differ from ewes synchronized with progestagen sponges and PMSG only. However, insemination with low numbers of non-sorted spermatozoa was not tested at the same time. Pregnancy rates were similar after insemination of GnRH-treated ewes with similar doses of sorted and non-sorted frozen-thawed spermatozoa but overall were low. Investigation of the minimum effective dose of sorted frozen-thawed spermatozoa required to obtain commercially acceptable pregnancy rates was inconclusive, although it appears from these trials that 20 x 10^6 motile spermatozoa may be required which falls within the range of the minimum IU AI dose recommended by the sheep industry for non-sorted frozen-thawed spermatozoa (Evans and Maxwell, 1987). Low pregnancy rates obtained in these studies after insemination with sorted frozen-thawed spermatozoa may be due to a number of factors including fertilisation failure, increased early embryonic loss or may be due to inadequate numbers of viable spermatozoa in the inseminate. However, for commercialization of sexed ram spermatozoa, future research into sorting and cryopreservation methods that incorporate strategies to prevent destabilization of sperm membranes, such as a reduction in sheath fluid pressure during sorting, the addition of seminal plasma at various stages during the transporting, sorting and freeze-thawing process and the development of new cryopreservation techniques such as the Multi-Thermal-Gradient freezing technology (Arav et al., 2002) may improve the fertilizing lifespan of the sorted spermatozoa and,
importantly, increase the number of fertile cells in an optimal functional state to establish reservoirs at the utero-tubal junction. These developments, together with continued improvement in sorting instrumentation and biological handling, should improve sorting efficiency sufficiently to achieve commercially acceptable pregnancy rates after conventional laparoscopic IU AI.

While the processes of sorting and storage (liquid or frozen) cause changes to sperm membrane integrity which result in a reduction in their fertilising life, they also result in the spermatozoa being in a physiological state of readiness to participate in fertilisation. The conditions encountered during IVF provide an optimum environment for successful fertilisation using spermatozoa in this state, and therefore a means of producing offspring of a pre-determined sex as demonstrated in this thesis. However, limitations of the associated reproductive technologies used to produce offspring of pre-determined sex, such as the embryo vitrification technique, were also documented. Future research into not only improving the efficiency of existing associated reproductive technologies but optimising the integration of the sexed semen with other ARTs is required in order for the sperm sexing technology to become economically beneficial to mainstream commercial production of sheep and other livestock species.

In the current economic environment the demand for sexed spermatozoa and associated ARTs would be driven only by a small niche market of top breeders in the Australian sheep industry. On an international level, due to the higher economic value of individual animals in some animal industries, the demand for sexed semen may be much greater. For example, the economic benefits derived from control of the sex ratio of a flock or herd would have to be greater than the premium cost of a straw of sexed semen. This would apply particularly to high value industries such as the dairy, beef and polo pony breeders.
where it has been projected that semen sorting is economically viable (Amann, 1989). However, despite these predictions, simplification of sex-sorting instrumentation, further optimisation and simplification of sperm sorting itself and the vehicles which aid its distribution needs to be carried out to increase the level of efficiency and reliability of the ‘sperm sexing technology’ if it is to be adopted by the standard animal breeding industries.

References


Conference Proceedings


I. PREGNANCIES AFTER INSEMINATION OF EWES WITH LOW NUMBERS OF SEX-SORTED, CRYOPRESERVED RAM SPERMATOZOA

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Introduction: Commercial application of the sex pre-determination technology, “sperm sorting”, has been achieved in cattle following successful cryopreservation protocols for sorted spermatozoa (1). Similar developments in the sheep industry are yet to take place, since lambs have only been produced from fresh (unfrozen) sorted spermatozoa (2, 3). The objective of this study was to examine pregnancy rates after insemination of frozen-thawed sex-sorted spermatozoa.

Semen collection: Semen was collected by artificial vagina from two rams (Ram1, Ram2) during January and February 2001. Only ejaculates with forward progressive motility greater than 70% were used.

Preparation for sorting: Semen was diluted with a modified TALP medium (XY TALP; 1) and incubated with Hoechst 33342 (H33342; Sigma, St Louis, MO, USA) for 1 h at 34°C. The optimum concentration of H33342 was determined by a series of preliminary experiments performed for each ram. After incubation, stained samples were diluted with XY TALP containing 4% egg yolk (v/v) and 0.002% food colouring dye (FD&C #40, Warner Jenkinson Company Inc., St Louis, MO, USA).

Sorting: Samples were analysed using a high speed cell sorter (SX MoFlo®, Cytomation Inc., Fort Collins, CO, USA). Gates were set during sorting so that purities of ≥ 90% were achieved, and reanalysis of sorted samples (10,000 events/sample) confirmed this high purity. Sorted samples were collected into 10ml centrifuge tubes. Each sample was sorted for 30-60 min. to achieve a total yield of 16 x 106 spermatozoa (8 x 106 X-sperm, 8 x 106 Y-sperm).

Cryopreservation and thawing: Sorted spermatozoa were centrifuged and the pellet was resuspended in egg-yolk glycerol diluent (1:4, sperm pellet:diluent) and frozen in pellet form by standard methods (4). A control ejaculate from each ram was diluted(1:4) and frozen without further treatment (standard commercial practice). After storage for at least 1 week, pellets were thawed in a dry test tube shaken in a waterbath at 37°C and inseminated within 10 min. of thawing.

Artificial insemination: Oestrus was synchronised in 160 ewes by treatment with intravaginal progestagen-impregnated sponges (40 mg chrono-gest®, Intervet, Bendigo, VIC) for 12 days. At sponge withdrawal, the ewes each received 400 IU of PMSG (Folligon®, Intervet). At 54-57 h post-sponge removal, 144 ewes were inseminated into either the uterine horns (n = 81) by commercial laparoscopic methods (IUAI; 4) or into the utero-tubal junction (UTJ; n= 63) using a tom-cat catheter after mini-laparotomy. Ewes received 140 x 106 spermatozoa in total (control, n=48) or 2-4 x 106 total spermatozoa (X-sort, n=48; Y-sort, n=48). Pregnancy was diagnosed by ultrasound on day 60. Data were analysed by logistic regression.

Results: The percentage motility of sperm samples after thawing was 51.3±0.5% (control), 46.1±1.0% (X) and 42.0±0.6% (Y). The proportion of ewes pregnant was significantly affected by semen type (p=0.02), but not by the site of insemination (p=0.132) or ram (p=0.09; Table 1). There was no interaction between treatments.

Table 1: Pregnancy after artificial insemination of frozen-thawed control and sex-sorted ram spermatozoa.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. pregnant/no inseminated (%)</th>
<th>Insemination site</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IUAI</td>
<td>UTJ</td>
<td>Ram 1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>16/27 (59.3)</td>
<td>10/21 (47.6)</td>
<td>17/24 (70.8)</td>
</tr>
<tr>
<td>X</td>
<td></td>
<td>10/26 (38.5)</td>
<td>2/22 (9.0)</td>
<td>8/24 (33.3)</td>
</tr>
<tr>
<td>Y</td>
<td></td>
<td>4/28 (14.3)</td>
<td>3/20 (15.0)</td>
<td>3/24 (12.5)</td>
</tr>
</tbody>
</table>
Conclusions: This is the first demonstration that pregnancy can be achieved in sheep with sex-sorted frozen-thawed sperm. Although the results in Table 1 appear to indicate a ram effect on the pregnancy rate, this difference is not statistically significant due to the small numbers involved. Insemination at the UTJ offered no advantage over standard laparoscopic AI. Overall fertility may be improved by further controlling the time of ovulation with respect to time of insemination, and by increasing the number of sorted sperm inseminated.

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References
II. FLOW CYTOMETRIC SORTING OF FROZEN-THAWED RAM SPERMATOZOA

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Sex pre-determination using sperm sorting and assisted reproductive technology (ART) is under development for wildlife species (1). Application is limited in situations where the sperm sorter is located a distance from the male(s). In this study, the quality of frozen-thawed, sorted ram spermatozoa was compared after three post-thaw processing methods. Semen from three rams (n=3 ejaculates per ram) was frozen using standard methods. An aliquot of each ejaculate was processed as a fresh control (FRESH; 2) in parallel with frozen samples. Frozen spermatozoa were thawed, prepared for sorting by no further processing (NEAT), washing (WASH) or gradient centrifugation (GRADIENT) and evaluated for motility at 1 h post-staining and motility and acrosome status at 0 and 4 h post-sorting. Samples were analysed using a high-speed sorter (SX MoFlo®, Cytomation Inc. CO, USA) after incubation with H33342 and food dye (2). Reanalysis of sorted spermatozoa revealed high levels of purity for X- and Y-enriched samples for all treatments (range: 84.7–91.1%). Percentage of motile spermatozoa prior to sorting was lower (P<0.05) for frozen-thawed samples (GRADIENT: 73.9±3.7%; WASH: 32.2±3.3%; NEAT: 32.7±2.5%) compared to FRESH (83.3±1.2%). Post-sorting, percentage of motile spermatozoa for NEAT (0h: 60.0±5.1%; 4h: 27.2±6.1%) was lower (P<0.05) than that for FRESH (0h: 87.8±0.9%; 4h: 83.3±1.2%), WASH (0h: 80.0±2.4%; 4h: 71.7±3.6%) and GRADIENT (0h: 84.4±1.3%; 4h: 77.2±1.7%). There was a male effect (P<0.05) on the percentage of acrosome-intact spermatozoa but no effect (P>0.05) of time or treatment (range: 81.3–89.2%). Using a modified sperm migration test (3), sample aliquots were assessed for ability to penetrate an artificial cervical mucus at 0 h post-sorting. Vanguard sperm migration distance was lower (P<0.05) for NEAT (17.7±1.7 mm) compared to WASH (29.1±3.8 mm) and GRADIENT (28.4±2.0 mm) and similar (P>0.05) to FRESH (23.7±1.8 mm). In summary, high purity sorting of frozen-thawed ram spermatozoa was possible after processing to remove cryodiluent and samples showed only slight reductions in quality after sorting. The model has potential for use in sorting wildlife spermatozoa. (1) O’Brien et al. (2001) Biol. Reprod. (Suppl. 1) 64:158. (2) Hollinshead et al. (2001) Proc. SRB 32: 20. (3) Mortimer et al. (1990) Hum. Reprod. 5:835-841.

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III. EFFECT OF TIME OF INSEMINATION AND DOSE OF SORTED, CRYOPRESERVED RAM SPERM ON FERTILITY IN EWES


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Lambs have been produced after artificial insemination (AI) with cryopreserved sex-sorted sperm in sheep (1). This study aimed to determine the optimum time to inseminate ewes and the minimum effective dose of sorted frozen-thawed sperm to obtain commercially acceptable fertility. Semen samples from 3 rams were prepared for sorting, stained, incubated and analysed using a high speed cell sorter (SX Mo Flo®; 1). Sperm were processed through the sperm sorter at 15,000-18,000/sec without sex-sorting. After each run 1.3 x10^6 sperm were sorted for sex and analysed to determine purity (3). The sperm were frozen using standard techniques (2). A control sample from each ram’s ejaculate was frozen without machine processing. Oestrus was controlled in 360 Merino ewes using progestagen-impregnated intra-vaginal pessaries (FGA, Vetrepfarm) inserted for 12 days and an injection of 400 IU PMSG (Pregnecol, Vetrepharm) at sponge removal (SR). The time of ovulation was controlled by injection of 40µg GnRH (Fertagyl®, Intervet) 36h after SR. Ewes were inseminated with 1, 4 or 16 x 10^6 frozen-thawed processed sperm or 100 x 10^6 control frozen-thawed sperm into the uterus by laparoscopy 54, 58 or 62h after SR. Fifteen ewes not given GnRH were inseminated with control sperm at 54-58h. Pregnancy was diagnosed by ultrasound on d 57 and the data analysed by logistic regression. Motility of sperm after thawing was 42±0.4% (control) and 43±0.6% (machine processed). The numbers of ewes pregnant/inseminated (%) were 15/89  (16.9%), 22/90 (24.4%) and 28/90 (31.1%) for 1, 4 and 16 x 10^6 sperm doses, respectively (p<0.001), but pregnancy was not affected by time of insemination (p=0.347) or ram (p=0.783). There were no interactions between treatments. The results suggest that doses in excess of 16 x 10^6 sorted frozen-thawed sperm, deposited close to the anticipated ovulation time (58h; 12/30; 40.0% pregnant), are required to obtain fertility similar to the control (6/15, 40.0% pregnant).


This research was supported by XY, Inc; Colorado, USA and the Australian Research Council.
IV. SEX-SORTING AND RE-CRYOPRESERVATION OF FROZEN-THAWED RAM SPERM FOR IN VITRO EMBRYO PRODUCTION

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Application of sperm sorting to breeding of livestock and wildlife is limited when the sorter is a long distance from the male(s) but would be facilitated by the sorting of frozen-thawed sperm (Lu KH et al., Theriogenology 1999:52:1393-1405) and re-freezing it. High purity sorting with maintained quality of frozen-thawed ram sperm has been achieved after processing to remove the cryodiluent (Hollinshead FK et al., Proc. Soc. Reprod. Biol. 2002 submitted). The aim of this study was to evaluate the functional capacity of frozen-thawed sperm after sorting and a second cryopreservation/thawing step. Frozen semen from 2 rams (n=2 ejaculates per ram) was used throughout. Post-thaw sperm treatments comprised (i) unsorted (Control); (ii) sorted (Froz-Sort) and (iii) sorted then re-frozen (Froz-Sort-Froz). X and Y sperm were separated using a high-speed sorter (SX MoFlo®, Cytomation, CO, USA) after incubation with Hoechst 33342 and food dye to eliminate non-viable sperm. Reanalysis revealed high levels of purity for X- and Y-enriched samples for all treatments (87.0 ± 4.5%). For IVF, 472 IVM oocytes were inseminated with 1 x 10^6 motile sperm/mL. After 3 h in SOF medium, oocytes were transferred to Sydney IVF cleavage medium (Cook®, QLD, Australia) for 4 d followed by Sydney IVF blastocyst medium (Cook®) for an additional 3 d culture in 5% O₂: 5%CO₂: 90% N₂. Oocytes were assessed for cleavage at 24 and 48 h post-insemination (p.i.). At 52 h p.i., uncleaved oocytes were stained with orcein for assessment of maturation and fertilization. Data from 3 replicates were analyzed by ANOVA, Chi-square and Fisher Exact Test. At insemination, % motile sperm (± SEM) was higher (P<0.001) for Froz-Sort (85.8 ± 2.4%) and Froz-Sort-Froz (66.7 ± 7.7%) than Control (36.7 ± 2.1%). Maturation rate was 95.6% (451/472). Cleavage of oocytes in a parthenogenetic control group (no sperm) was low (2/56; 3.6%). Polyspermic fertilization was low (9/451; 2.0%) and did not differ among treatments.

Table 1. Fertilization & early embryo development of oocytes after incubation with frozen-thawed unsorted (Control), frozen-thawed & sorted (Froz-Sort) & frozen-thawed, sorted then frozen-thawed (Froz-Sort-Froz) ram sperm. Values in parentheses are percentages.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mature oocytes fertilized</th>
<th>No. of mature oocytes undergoing cleavage after insemination</th>
<th>No. of cleaved oocytes forming blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td>40 (67.8)</td>
<td>26 (44.1)</td>
<td>36 (61.0)</td>
</tr>
<tr>
<td>Froz-Sort</td>
<td>110 (63.6)</td>
<td>67 (38.7)</td>
<td>109 (63.0)</td>
</tr>
<tr>
<td>Froz-Sort-Froz</td>
<td>94 (57.7)</td>
<td>71 (43.6)</td>
<td>91 (55.8)</td>
</tr>
</tbody>
</table>

^aMonospermic fertilization. Within column, values with different superscripts differ (P<0.05).

Fertilization and cleavage rates were consistently high across treatments. Blastocyst development rate was higher for oocytes fertilized with Froz-Sort-Froz than with Control sperm. These results demonstrate that frozen-thawed ram sperm can be sex-sorted for either immediate or future use in an IVF system after re-cryopreservation.

V. IN VITRO ASSESSMENT OF FUNCTION OF SEX-SORTED FROZEN-THAWED RAM SPERM


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Lambs of predetermined sex have been produced by laparoscopic AI with low numbers of frozen-thawed sex-sorted sperm. However, sperm that have undergone both sorting and freezing may have lower fertility than those that have only been frozen (1). In the present study, we attempt to evaluate in vitro the altered function of sex-sorted sperm in the female reproductive tract (FRT). Semen (3 rams, 3 ejaculates per ram) was collected and either a) used directly (FRESH) b) frozen by a commercial method (FT Control; 1) or c) Hoechst 33342 stained and sex-sorted using a modified high-speed cell sorter (2) then frozen (FT Sort; 1). Assessments of sperm i) binding to ovine oviduct epithelial cell (OEC) monolayers (3) ii) migration through artificial cervical mucus (HA; 4) iii) acrosomal integrity using FITC-PNA and iv) motility were made over 4 h. FRESH sperm bound to the OEC during incubation (61.6, 74.6 and 75.9% bound at 0.5, 2 and 4 h, SEM = 5.3%) whereas FT Control and FT Sort sperm were released from the OEC with incubation (52.1, 45.6, 29.9% and 45.6, 29.0 and 18.1% bound respectively at 0.5, 2 and 4 h). More FT Sort sperm were released at 2 h than FT Control sperm (p<0.05). More FRESH sperm (241.6 ± 11.9 sperm; p<0.05) migrated 0.5 cm into the HA than either the FT Control (76.4 ± 11.9) or FT Sort (73.9 ± 11.9) samples. There was no difference between treatments in the distance migrated by the vanguard sperm. Overall, more FT Sort sperm (88.6 ± 1.5%, p<0.05) were acrosome-intact than Fresh (84.0 ± 1.5%) and FT Control sperm (81.8 ± 1.6%). Motility of FT Sort (51.1, 39.4 and 23.3% motile, SEM = 2.6%) decreased more rapidly during incubation (p<0.05) than that of both Fresh (89.4, 85.6 and 78.9% motile) and FT Control sperm (67.2, 58.3 and 35.6% motile at 0, 2 and 4 h, respectively). The rapid release of FT Sort sperm from OEC and their decreased longevity may indicate that FT Sort sperm have a shorter period of time within the FRT to encounter the oocyte than FT Control sperm.

VI. EFFECT OF DOSE OF SPERM PROCESSED FOR SEX-SORTING, AND CRYOPRESERVED, ON FERTILITY IN EWES

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Lambs have been produced after artificial insemination (AI) with low numbers (2-4x10^6) of cryopreserved sex-sorted sperm (Hollinshead et al., Reprod. Fertil. Dev. 2002; in press). Fewer ewes were pregnant after AI with X- or Y-sorted frozen-thawed (25%, 15% respectively) than with a commercial dose of unsorted frozen-thawed sperm (54%). The object of the present study was to determine the minimum numbers of sorted frozen-thawed sperm required to obtain pregnancy rates similar to those obtained with unsorted sperm.

A sample of sperm from single ejaculates of 2 rams was stained, incubated, analysed and sorted using a modified high speed cell sorter (MoFlo®, Cytomation, Fort Collins, CO, USA) as previously described (Hollinshead et al., Reprod. Fertil. Dev. 2002; in press). Sperm were processed at 15,000-18,000/sec without sex-sorting into 10ml centrifuge tubes pre-soaked with 1% BSA in sheath fluid containing 0.2ml Tris-buffered medium and 20% egg yolk (v/v). For every sample, 1.3 x 10^6 sperm were sex-sorted and analysed to determine purity (Johnson & Welch, Theriogenology 1999;52: 1323-1341). Sorted and unsorted (control) samples were extended with a zwitterion-buffered diluent containing 13.5% egg yolk and 6% glycerol (Molinia et al., Reprod. Nut. Dev. 1996; 36:21-29) and frozen as 250ul pellets containing 5x10^6 sperm. The time of oestrus was controlled in 144 Merino ewes by progestagen sponges (FGA, Vetrepharm A/Asia, Sydney) inserted intravaginally for 12 days and an injection of 400 I.U of PMSG (Pregnecol, Vetrepharm A/Asia) at sponge removal (SR). Thirty-six h after SR 134 ewes were injected with 40µg GnRH (Fertagyl®, Intervet) to control the time of ovulation. One hundred and eleven ewes were inseminated in the uterus by laparoscopy 57-60h after SR with 5, 10, 20 or 40x10^6 sorted or unsorted frozen-thawed sperm. Thirteen ewes not given GnRH were inseminated with a commercial dose of unsorted frozen-thawed sperm 57-58h after SR. Pregnancy was diagnosed by ultrasound on d53. The data were analysed by Chi-square.

Sperm motility after thawing was 37.8±1.78% (sorted) and 42.9±0.93% (unsorted). Seven of 13 (53.8%) ewes not given GnRH were pregnant. Of the GnRH-treated ewes the proportion pregnant was affected by the number of sperm inseminated (p<0.05) but not by ram or type of sperm (p>0.05). For ewes inseminated with sorted or unsorted (control) frozen-thawed sperm, pregnancy rate was higher for inseminates of 10 and 40x10^6 than for 5 and 20x10^6 sperm (Table 1). The results suggest that a minimum of 40x10^6 sorted frozen-thawed sperm inseminated close to the time of ovulation are required to obtain commercially acceptable pregnancy rates.

<table>
<thead>
<tr>
<th>Dose (x10^6 sperm)</th>
<th>No. ewes inseminated</th>
<th>No. ewes pregnant</th>
<th>% ewes pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>30</td>
<td>10</td>
<td>33.3^a</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>16</td>
<td>57.1^b</td>
</tr>
<tr>
<td>20</td>
<td>29</td>
<td>10</td>
<td>34.5^a</td>
</tr>
<tr>
<td>40</td>
<td>23</td>
<td>16</td>
<td>69.6^b</td>
</tr>
</tbody>
</table>

*This research was supported by XY Inc; CO, USA; The Australian Research Council; Vetrepharm A/Asia.

Within columns different superscripts differ (p<0.05).
Previously we demonstrated that sex-sorted sperm could produce IVF embryos from juvenile and adult cattle at rates similar to those for unsorted sperm (Fry et al., 2003 Theriogenology 52, 198). In this study we investigated the pregnancy rates of recipient cattle following the transfer of frozen/thawed IVF embryos generated from young heifers using sex-sorted and unsorted sperm. COC's collected from FSH-stimulated Senepol or Beefex heifers by TVR were matured, fertilized with either sex-sorted or unsorted Senepol sperm and cultured for 6 days under our standard laboratory conditions (Fry et al., 2003 Theriogenology 59, 446, Earl et al., 1997 Theriogenology 47, 255). Embryos reaching the blastocyst or expanded blastocyst stage of development were frozen by the CL-V method of vitrification. Briefly, embryos were equilibrated for 5-10 min in HEPES-199 media containing 20% FCS (HM), placed in HM containing 10% EG, 10% DMSO for approximately 2 minutes and then in HM containing 20% EG, 20% DMSO for between 20-60 sec (Vatja et al., 1997 Cryoletters 18, 191). Vitrification was achieved by collecting between 5-10 IVF embryos in a 3-µL droplet and securing this droplet to a coded CL-V holder. The droplet was vitrified using the CL-V kit (Lindemans et al., 2004 Theriogenology in press) and then sealed in a precooled "straw" for storage in liquid nitrogen. To thaw, the "straw" with specimen was removed from storage; the specimen droplet was withdrawn from the "straw" and placed directly into HM containing 0.2 M sucrose (SM). After approximately 5-10 min each embryo was assessed, loaded into a tomcat catheter in SM and transferred surgically into a recipient cow within 10-15 min of thaw. Of 129 Brahman and Braham cross cows receiving 2 injections of 125 µg cloprostenol 11 days apart, 60 exhibited oestrus 2-4 days after the second injection and 53 were deemed suitable for embryo transfer. Pregnancy was determined by ultrasound on Day 40. No difference in pregnancy rate was found between treatment groups (P < 0.05; Table 1). The low submission rate (60/129) and pregnancy rate for the in vivo control group indicate that the fertility of the recipient cows may have been compromised by the drought conditions predominating in Central Queensland. Notwithstanding, the CL-V method for the vitrification of IVF embryos produced by either sex-sorted or unsorted sperm gave similar and very promising pregnancy results of around 40%. This provides new opportunities for the rapid banking of large numbers of sexed IVF embryos generated from elite cattle by TVR for user friendly embryo transfer programs.

Table 1: Pregnancies from IVF embryos derived from sex-sorted and unsorted sperm and frozen by the CL-V method of vitrification, or from in vivo embryos frozen in glycerol

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Sex-sorted IVF</th>
<th>Unsorted IVF</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>3/6 (40%)</td>
<td>8/17</td>
<td>3/8</td>
</tr>
<tr>
<td>Grade 2</td>
<td>2/7</td>
<td>1/6</td>
<td>0/0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>3/7</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>8/20 (40%)</td>
<td>9/24 (38%)</td>
<td>3/9 (33%)</td>
</tr>
</tbody>
</table>
VIII. LAMBS BORN AFTER IN VITRO EMBRYO PRODUCTION FROM PREPUBERTAL LAMB OOCYTES AND FROZEN-THAWED UNSORTED AND SEX-SORTED SPERMATOZOA.

K.M. Morton\textsuperscript{a}, S.L. Catt\textsuperscript{b}, F.K. Hollinshead\textsuperscript{a}, W.M.C. Maxwell\textsuperscript{a}, and G. Evans\textsuperscript{a}.

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Developments in sperm sexing technology have resulted in the birth of a number of offspring after IVF of oocytes from adult animals (Johnson LA, 2000 An. Reprod. Sci. \textbf{60-61}, 93-107). The aim of this study was to combine sperm sexing technology with juvenile breeding.

Merino lambs, 2-3 weeks (n=43) were hormone stimulated (Morton KM \textit{et al.}, 2003 Proc. Soc. Reprod. Fert., P18), and COCs matured in TCM-199 (Sigma) with 10 µg mL\textsuperscript{-1} p-FSH (Folltropin-V; Bioniche Animal Health Australasia), 10 µg mL\textsuperscript{-1} pLH (Bioniche) and 20% sheep serum (v/v) in a humidified 6% CO\textsubscript{2}, 5% O\textsubscript{2}, 89% N\textsubscript{2} atmosphere for 22 h. Semen collected from Merino rams was diluted, and frozen as pellets (Unsorted), or stained with H33342 and separated into X and Y sperm using a SX MoFlo (Cytomation Inc., Fort Collins, CO, USA) and frozen as pellets (Sorted). Sperm were prepared for IVF by swim-up under 0.5 mL SOF with 2% sheep serum (v/v; SOF+) for 45 min (Unsorted), or diluted in 0.5 mL Sydney IVF Sperm Buffer (Cook IVF, Brisbane, Australia) and centrifuged at 650g for 3 min (Sorted). After IVM, oocytes were transferred to SOF+, and cultured with 0.5 (Unsorted) or 1.0 x 10\textsuperscript{6} mL\textsuperscript{-1} (Sorted) motile sperm for 18 h. Presumptive zygotes were transferred to Sydney IVF cleavage and blastocyst medium (Cook IVF) for 3 and 5 days, respectively. Oocyte maturation and fertilisation were assessed by orcein staining 18 hours post-insemination (hpi). Two Day-7 blastocysts were transferred to recipient ewes (n=9; 3 per group) and pregnancies diagnosed by ultrasound on Day 57 of gestation. Data were analysed by chi-squared test.

Oocyte maturation was 83.9% (73/87) and monospermic fertilisation was 68.7% (22/32), 42.8% (6/14) and 55.6% (15/27) for Unsorted, X- and Y-sperm groups, respectively (P>0.05). Polyspermic fertilisation was 9.4% (3/32) and 7.4% (2/27) for the Unsorted and Y groups (P>0.05). Cleavage was reduced with X- and Y-sperm, but blastocyst formation (from cleaved oocytes) did not differ (P>0.05; Table 1). Pregnancy diagnosis revealed 3 (100%) 0 (0%) and 1 (33.3%) foetuses from Unsorted, X- and Y-embryos, respectively. Three female (Unsorted) and one male (Y) lamb were born, demonstrating that juvenile breeding can be successfully combined with sperm sexing.

\textbf{Table 1:} Cleavage and blastocyst formation after IVF with Unsorted, X, or Y-sperm. Values in parenthesis are percentages.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. oocytes</th>
<th>No. oocytes cleaving (%)</th>
<th>Blastocyst formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hpi 48 hpi</td>
<td>Day 6 Day 7 Day 8</td>
</tr>
<tr>
<td>Unsorted</td>
<td>632</td>
<td>217 (34.3)\textsuperscript{a} 332 (52.4)\textsuperscript{a}</td>
<td>62 (18.7)\textsuperscript{a} 115 (34.6) 132 (39.8)</td>
</tr>
<tr>
<td>X</td>
<td>556</td>
<td>113 (20.3)\textsuperscript{b} 206 (37.1)\textsuperscript{b}</td>
<td>19 (9.2)\textsuperscript{b} 56 (27.2) 68 (33.0)</td>
</tr>
<tr>
<td>Y</td>
<td>551</td>
<td>88 (16.0)\textsuperscript{b} 171 (31.0)\textsuperscript{b}</td>
<td>31 (18.1)\textsuperscript{b} 68 (39.8) 76 (44.4)</td>
</tr>
</tbody>
</table>
IX. IN VIVO DEVELOPMENTAL CAPACITY OF IN VITRO PRODUCED EMBRYOS DERIVED FROM SEX-SORTED AND RE-CRYOPRESERVED FROZEN-THAWED RAM SPERM

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The ability to sort and re-freeze frozen-thawed sperm would significantly increase the potential application of sperm sexing technology to species management. Frozen-thawed, sorted, re-frozen then thawed ram sperm appear fully functional in vitro with blastocyst production greater than that for frozen-thawed, non-sorted sperm (Hollinshead FK et al. 2003 Theriogenology 59 209). The aim of this study was to evaluate the in vivo capacity of in vitro produced embryos derived from frozen-thawed sperm after sorting and a second cryopreservation/thawing step. Frozen semen from 2 rams (n=3 ejaculates per ram) was used throughout. Post-thaw sperm treatments comprised (i) non-sorted (Control); (ii) sorted (Froz-Sort) and (iii) sorted then re-frozen (Froz-Sort-Froz). X and Y sperm were separated using a high-speed sorter (SX MoFlo®, DakoCytomation, Fort Collins, CO, USA) after incubation with Hoechst 33342 and food dye to eliminate non-viable sperm. Reanalysis revealed high levels (mean ± SEM) of purity for X- and Y-enriched samples for all treatments (89 ± 1.2%). At Day 6 post-insemination, 2 embryos (blastocyst stage or greater) were transferred per recipient. Data were analyzed by Chi-square and Fisher Exact Test. In vivo embryo survival was similar across sperm treatments (28/64, 43.8% overall) and 20 of 23 (87.0%) sexed lambs were of the predicted sex (Table 1). These results demonstrate high in vivo developmental capacity of in vitro produced sexed embryos derived from frozen-thawed ram sperm after sorting and a second cryopreservation/thawing step, and increase the potential application of sperm sexing technology. Research supported by XY, Inc., Australian Research Council and Zoological Parks Board of NSW

Table 1. In vivo survival of transferred in vitro produced embryos derived from frozen-thawed non-sorted (Control), frozen-thawed & sorted (Froz-Sort) & frozen-thawed, sorted then frozen-thawed (Froz-Sort-Froz) ram sperm.

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>No. recipients</th>
<th>No. pregnant on Day 20 (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. pregnant on Day 60 (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. pregnancies lost between Day 20 and Day 60 (%)</th>
<th>No. lambs born/ no. of transferred embryos (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>6 (75.0)</td>
<td>5 (62.5)</td>
<td>1 (16.7)</td>
<td>5/16 (31.3)</td>
</tr>
<tr>
<td>Froz-Sort</td>
<td>9</td>
<td>7 (77.8)</td>
<td>5 (55.6)</td>
<td>2 (28.6)</td>
<td>6/18 (33.3)</td>
</tr>
<tr>
<td>Froz-Sort-Froz</td>
<td>17</td>
<td>14 (82.4)</td>
<td>14 (82.4)</td>
<td>0 (0)</td>
<td>17/34 (50.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Diagnosed by blood progesterone assay. <sup>b</sup>Diagnosed by ultrasonography.