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

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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 with 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 with spermatozoa from all other treatments (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 compared with FSF spermatozoa. However, the 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 and 41.2% vs 29.6, 25.9 and 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 embryo transfer, can be used to efficiently produce offspring of pre-determined sex.

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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 (Hollinshead *et al.* 2002b, 2003, O'Brien *et al.* 2003) or re-cryopreservation for later use would enhance the commercial- and conservation-driven applications of the sexing technology.

The production of offspring using sex-sorted spermatozoa in conjunction with *in vitro* fertilisation (IVF) and embryo transfer (ET) has been reported in cattle (Cran *et al.* 1993, 1994, 1995, Fry *et al.* 2004), pigs (Rath *et al.* 1997, 1999, Abeydeera *et al.* 1998, Johnson *et al.* 2000), humans (Levinson *et al.* 1995, Fugger *et al.* 1998, Fugger

1999) and sheep (intra-cytoplasmic sperm injection; Catt *et al.* 1996). Improvements in sorting efficiency (Rens *et al.* 1998, 1999, Johnson & Welch 1999) and cryopreservation methods (Schenk *et al.* 1999) for sex-sorted spermatozoa facilitated the production of offspring after low-dose artificial insemination (AI) in cattle (Seidel *et al.* 1999), sheep (Hollinshead *et al.* 2002a), horses (Lindsey *et al.* 2002) and elk (Schenk & DeGroff 2003).

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 (Hollinshead *et al.* 2002b, O'Brien *et al.* 2003). In the former studies, motility, acrosome integrity and migration through artificial cervical mucus were similar after sorting of frozen–thawed compared with fresh ram spermatozoa.

The objectives of the present study were to (i) evaluate *in vitro* characteristics (motility, forward progressive motility (FPM), acrosome integrity and migration through an artificial cervical mucus) of frozen–thawed and sorted (FS) and frozen–thawed, sorted, and then 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 (IVM) 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

Procedures herein were approved by the University of Sydney's Animal Ethics Committee.

Experimental design

Ejaculates were collected from two Merino rams, housed at the University of Sydney, during the breeding season (January–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 was re-frozen and thawed (frozen–control–frozen treatment (FCF)). Another third was flow cytometrically sorted and either assessed after sorting (FS) or re-frozen and thawed (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 FPM. Control, FS and FSF spermatozoa were also co-incubated with IVM ovine oocytes for assessment of fertilisation, cleavage and blastocyst development. Each sperm evaluation was replicated three 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) (Molina *et al.* 1996) and frozen and thawed using the pellet method described by Evans & Maxwell (1987).

Preparation of spermatozoa for sorting

Thawed spermatozoa were prepared using multiple two-step Puresperm (Cook, Brisbane, QLD, Australia) gradients (0.2 ml thawed semen on 1 ml 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, 87.9 IU/ml penicillin-G, 35.3 IU/ml streptomycin sulphate 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 400×10^6 spermatozoa/ml with TRIS containing 267–311 µM Hoechst stain (H33342; Sigma, St Louis, MO, USA). These stain concentrations had been previously optimised 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 (MoFlo SX; DakoCytomation, Fort Collins, CO, USA) modified for sperm sorting (Johnson & Pinkel 1986, Rens *et al.* 1998, 1999, Johnson & Welch 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 *in vitro* fertilisation (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 8000–10 000 spermatozoa per second depending on the number of viable cells available for sorting. Average flow rates for both sorting conditions were 18 000–22 000 events per second. All spermatozoa were sorted into 10 ml centrifuge tubes containing 0.25 ml 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 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-chromosome bearing fresh or frozen–thawed spermatozoa were collected and processed as described by Welch & 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) 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 & Radziewicz 1998). The upper 100 µl containing motile spermatozoa were removed and the concentration of spermatozoa determined by haemocytometer. For incubation and sperm quality assessment, an aliquot containing 5×10^6 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 IVM oocytes using the same methods as for the FS spermatozoa.

SMT

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 (10 mg/ml sodium hyaluronate diluted with 25% AH containing 1% w/v BSA; Bioniche, Armidale, NSW, Australia) by an SMT developed for human spermatozoa (Mortimer *et al.* 1990) and further modified for low numbers of sorted ram spermatozoa (Hollinshead *et al.* 2002b, O'Brien *et al.* 2003). Aliquots (50 µl) containing 1×10^6 spermatozoa from each treatment were incubated (37 °C; 1 h) in BEEM capsules (BEEM standard embedding capsules; ProSciTech, Thuringawa, QLD, Australia) containing a vertically placed $0.3 \times 0.3 \times 100$ 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 travelled by the vanguard spermatozoon from each treatment after 1 h incubation (37 °C) were evaluated using phase contrast microscopy ($\times 200$) (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 ($\times 100$, 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 agglutinin staining as described by Roth *et al.* (1998). A total of 200 spermatozoa per treatment were examined by phase contrast microscopy ($\times 200$, Olympus) and were considered acrosome intact if the acrosome stained bright green and acrosome reacted if there was no staining or only a single band of green fluorescence at the equatorial segment.

IVM, IVF 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 homogeneous ooplasm surrounded by several layers of cumulus cells were selected for IVM ($n = 1047$). Groups of 25–35 cumulus–oocyte complexes were incubated in multi-well dishes (Nunclon; Inter Med, Roskilde, Denmark) containing 450 µl maturation medium overlaid with 400 µl mineral oil (M 8410; Sigma) for 22 h. All stages of *in vitro* culture took place at 38.5 °C 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 Earle's salts, 15 mM L-glutamine, 25 mM sodium bicarbonate, 25 mM Hepes supplemented with 20% (v/v) sheep serum (ThermoTrace), 10 µg/ml follicle-stimulating hormone (Folltropin-V; Bioniche), 10 µg/ml luteinising hormone (Lutropin-V; Bioniche), 124 IU/ml penicillin and 37 IU/ml streptomycin.

Following IVM, 25–30 oocytes were co-incubated with 1×10^6 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 multi-well dishes (Nunclon) containing 400 µl fertilisation medium overlaid with 400 µl oil. The fertilisation medium comprised bicarbonate-buffered synthetic oviduct fluid medium (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 Sydney IVF (SIVF) cleavage medium (Cook) overlaid with oil and cultured for 4 days. After this time, cleaved oocytes were transferred to 10 µl droplets of Sydney IVF (SIVF) blastocyst medium (Cook) overlaid with oil for 3 days. The protein source used in both the SIVF cleavage

and blastocyst media was 200 mg/ml human serum albumin (HSA) (CAF-BCF; Scandinavian IVF Science, Goteborg, Sweden). For quality control, parthenogenic development was assessed by evaluating cleavage and blastocyst development of IVM oocytes incubated without spermatozoa (three replicates, $n = 69$).

Oocyte and embryo assessment

Oocytes were visually assessed for cleavage at 24 and 48 h after insemination ($\times 100$, 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 ($\times 200$, Olympus) to identify those undergoing penetration and monospermic or polyspermic fertilisation. The maturation rate was calculated at 48 h p.i. as the proportion of total oocytes undergoing cleavage and the number of uncleaved oocytes containing two pronuclei. The number of oocytes developing to the blastocyst stage was assessed ($\times 100$, Olympus) on Days 5, 6 and 7 p.i.. 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 (blastocoele beginning to form); blastocyst (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 blastocyst, ExB, HgB and HB stages were graded into three groups based on the development of the inner cell mass and trophectoderm (Gardner & Schoolcraft 1999).

Embryo vitrification and re-warming

Day 6 and 7 blastocysts were cryopreserved using the open-pulled straw 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) (E 9129; Sigma) and 7.5% (v/v) dimethyl sulphoxide (DMSO) (D2650; Sigma) for 3 min. Blastocysts were then transferred to and mixed with cryobuffer medium containing 1 M 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 s. Day 6 blastocysts and early ExBs were re-warmed the day before transfer and Day 6 (ExB 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 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 (blastocysts and early ExBs) were subsequently cultured in SIVF blastocyst medium 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.

ET

Fresh blastocysts forming on Day 6 of culture were transferred into 34 mature Border Leicester $\times$ 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 NSW (ET Trial 1). Blastocysts forming on Day 6 and Day 7 of culture were vitrified and later re-warmed and transferred into 27 mature Merino ewes in April 2003 at The University of Sydney farm, Camden, NSW (ET Trial 2).

Fresh Day 6 embryos were transported and held in Sydney IVF buffer and 2.5% (v/v) HSA at 38.5°C until transfer (2.5–4 h). Re-warmed Day 6 and Day 7 embryos were transported in Sydney IVF buffer and 5% (v/v) HSA and held at 38.5°C until transfer (1.5–3 h). Both fresh and vitrified embryos were graded using light microscopy ($\times 100$, Olympus) immediately prior to transfer. Sixty-eight grade 1 blastocysts at the blastocyst–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 blastocyst or ExB stage and 34 grade 1 ExBs and HgBs produced on Day 6 and Day 7 of culture, respectively, were vitrified and later re-warmed and transferred into 27 synchronised recipient ewes (ET Trial 2).

Oestrus was synchronised in 50 mature BLM crossbred ewes (ET Trial 1; 3 years old, 65–70 kg) and 30 mature Merino ewes (ET Trial 2; 5 years old, 45–50 kg) with progestagen-impregnated intravaginal sponges containing 40 mg fluogestone acetate (40 mg Chronogest; Intervet, Bendigo, VIC, Australia). The sponges were removed after 13 days and the ewes were injected i.m. with 500 IU (ET Trial 1) or 400 IU (ET Trial 2) pregnant mare serum gonadotrophin (Pregnenol; Bioniche).

ET was performed using a modified laparoscopic mini-laparotomy technique (O'Brien *et al.* 1996) either 6 days (ET Trial 1) or 7 days (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 Ketamil; Ilium, Troy Laboratories, Smithfield, NSW, Australia); 2 ml diazepam

(Pamlin; Parnell Laboratories, Alexandria, NSW, 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 corpora 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 (blastocyst, ExB, HgB, HB), age (Day 6 or 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 μ l capillary pipette (Unopette; Becton Dickinson). After ET each ewe was given a non-steroidal anti-inflammatory i.m. injection (1 ml Ketoprofen; Nature Vet, Agnes Banks, NSW, 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 RIA (Spectra; Orion Diagnostics, Helsinki, Finland). Ewes with progesterone concentrations greater than 1.2 ng/ml were considered pregnant (Robertson & 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: (Number of ewes pregnant at Day 20 minus Number of ewes pregnant at Day 60)/(Number of ewes pregnant at Day 20). Foetal loss was determined as: (Number of ewes pregnant at Day 60 minus 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 a 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: ((Number of lambs born)/(Number of embryos transferred)) $\times$ 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 5 ml dexamethasone (Dexadren 2 mg/ml; Intervet) and 5 ml oestradiol benzoate (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, UK). Data for sorting, motility, FPM rating and the mean distance travelled by the vanguard spermatozoon were analysed by ANOVA. Motility and FPM rating measurements at 0, 3 and 6 h incubation were subjected to natural log transformation and analysed by repeated-measures ANOVA. 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 (Fig. 1). Re-analysis revealed high levels of purity for X- and Y-sorted samples obtained from fresh (X: $89 \pm 2.0\%$, Y: $88 \pm 1.2\%$) and frozen–thawed (X: $89 \pm 1.0\%$, Y: $89 \pm 1.3\%$) spermatozoa. There was a greater proportion of non-viable sperm (Fig. 1a and b) for frozen–thawed (FS and FSF treatment) compared with fresh samples (25 ± 1.7 and $12 \pm 1.6\%$ respectively; $P < 0.001$). The percentage of oriented spermatozoa (Fig. 1a and b) was higher for fresh ($60 \pm 3.0\%$) than frozen–thawed samples ($47 \pm 1.5\%$; $P < 0.001$). Fresh spermatozoa were sorted at higher rates than frozen–thawed spermatozoa (3540 ± 239.0 vs 1783 ± 151.0 spermatozoa per second respectively; $P < 0.001$). There was no ram effect on any of the sorting parameters.

Figure 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 bivariate histogram. R1 (region 1) represents the correctly orientated sperm population; R2 (region 2) represents non-viable spermatozoa.

SMT

More sorted frozen–thawed spermatozoa derived from frozen–thawed samples (FS) penetrated 5 mm into the artificial cervical mucus compared with 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 travelled by the vanguard spermatozoon was similar for all treatments.

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

Treatment	No. of spermatozoa reaching 5 mm	Vanguard distance (mm)
Control	56 ± 16.4^b	33 ± 0.2
FCF	7 ± 1.3^c	27 ± 0.4
FS	152 ± 3.9^a	25 ± 0.3
FSF	31 ± 9.8^{bc}	29 ± 0.4

^{abc}Within columns, values with different superscripts differ ($P < 0.01$).

Assessment of motility, FPM 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 fewer intact acrosomes after the 6 h incubation period than Control and FS spermatozoa (86 ± 2.6 and $83 \pm 1.6\%$ vs 92 ± 1.4 and $90 \pm 1.3\%$ respectively; $P < 0.05$).

Figure 2 Motility (a) and FPM (b) of FS (▲) and non-sorted Control (■) and FSF (◆) and FCF (—) spermatozoa after thawing (0 h) and incubation for 6 h at 37°C. Values are means $\pm$ S.E.M.

Effect of sperm preparation for IVF on motility, FPM and acrosome integrity

The proportion of motile spermatozoa was higher for frozen-thawed spermatozoa after sorting (FS; $85 \pm 1.5\%$) and thawing (FSF; $71 \pm 5.0\%$) than for non-sorted frozen-thawed spermatozoa (Control; $45 \pm 4.5\%$; Fig. 3a; $P < 0.001$). However, after preparation for IVF, involving a swim-up for Control spermatozoa, and wash and centrifugation for sorted (FS and FSF) spermatozoa, the percentage of motile Control spermatozoa significantly increased ($75 \pm 6.1\%$; $P < 0.001$) but decreased for the FS and FSF treatments (60 ± 8.3 and $32 \pm 4.6\%$ 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).

IVF and early embryo development

The overall oocyte maturation rate was 90.6% (949/1047). The proportions of oocytes fertilised and of zygotes that had cleaved 48 h after insemination were higher after

Figure 3 Motility (a) and FPM (b) of FS (▲) and non-sorted Control (■) and FSF (◆) spermatozoa after thawing (Post-thaw) and preparation for IVF (Pre-IVF). Values are means $\pm$ S.E.M.

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 polyspermic fertilisation (20/949; 2.1%) were low.

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

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.

Treatment	No. matured oocytes* fertilised ¹ (%)	No. mature oocytes undergoing cleavage after insemination (%)		No. cleaved oocytes forming blastocysts (%)		
		24 h	48 h	Day 5	Day 6	Day 7
Control	121 (80.1) ^a	79 (52.3)	116 (76.8) ^a	20 (17.2)	42 (36.2)	58 (50.0) ^a
FS	228 (68.5) ^{ab}	134 (40.2)	222 (66.7) ^{ab}	43 (19.4)	75 (33.8)	117 (52.7) ^a
FSF	263 (64.5) ^b	192 (49.3)	251 (61.5) ^b	46 (18.3)	94 (37.5)	156 (62.2) ^b

¹ Monospermic fertilisation.

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

^{ab} Within column, values with different superscripts differ ($P < 0.05$).

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 two 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), seven (25.9%) gave birth to eight 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 with vitrified

(14.8%) embryos survived to lambing ($P < 0.01$). A total of eight neonatal deaths (8/36; 22.2%) were recorded, which were evenly distributed across embryo (fresh vs vitrified) and sperm (Control vs FS vs FSF) treatment groups.

Sex ratio

Overall, 26/30 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 with 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; $89 \pm 1.2\%$).

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

Table 3 Pregnancy rate on Day 20, Day 60 and lambing and *in vivo* embryo survival of fresh and vitrified embryos, produced after IVF of IVM 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.

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

¹ Determined by peripheral plasma progesterone assay.² Determined by ultrasound.³ No. ewes that lambled between Days 149 and 155 (fresh) or Days 145 and 156 (vitrified)/no. recipient ewes $\times 100$.⁴ No. lambs born/no. of transferred embryos $\times 100$.^{ab} Within columns, values with different superscripts differ ($P < 0.05$).

(single vs twin) on the gestation length. However, the mean gestation length of ewes in ET Trial 2 (150 ± 1.5 days) 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 FS and 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 FS and FSF spermatozoa 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 & 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 travelled 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 probably 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 FS spermatozoa penetrated 5 mm into the artificial cervical mucus than for all other treatments (Control, FSF and FCF). However, the distance travelled 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 oviductal 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 (Aitken 1995).

Nevertheless, development of cleaved oocytes to Day 7 blastocysts was higher after insemination with FSF spermatozoa than all for 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.* 2004) 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. ET 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 with 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 & 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 with non-sorted, frozen-thawed or fresh bull spermatozoa. Significant improvements in both biological (bull: Schenk *et al.* 1999, ram: F K Hollinshead, unpublished observations) and instrumental (Rens *et al.* 1999, Campos-Chillon & De La Torre 2003, Suh & 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 & Johnson 1999) and may be contributing to improvements in the IVF parameters found in this study compared with 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 FS and FSF spermatozoa in the present study (Table 3). Vitrification instead of conventional slow freezing was utilised 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 with those reported by other workers after transfer of fresh and vitrified embryos derived from non-sorted ram spermatozoa (fresh: 41–66%, Martinez *et al.* 1997, O'Brien *et al.* 1997, Brown & Radziewicz 1998, Dattena *et al.* 2000, Walmsley *et al.* 2000, Peura *et al.* 2002, vitrified: 33–50%, Dattena *et al.* 2000, Walmsley *et al.* 1999, Fogarty *et al.* 2000, 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. These factors most probably contributed to the lower pregnancy and lambing rate after transfer of vitrified embryos.

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. Secondly, the incubation enabled assessment of re-expansion after re-warming as a measure of viability (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 & Radziewicz 1998) and the lambs born from the same flock of ewes after AI with non-sorted (control) or sorted frozen-thawed spermatozoa (range: 3.1–5.4 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, 1996, Thompson *et al.* 1995, Holm *et al.* 1996, Brown & Radziewicz 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 ET (Wilmot & Sales 1981), particularly after the transfer of vitrified embryos (Naitana *et al.*, 1995, Leoni *et al.* 2003), progesterone treatment of ewes early in gestation (Kleeman *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 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 trophoblast and inner cell mass (Brown & Radziewicz 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 & 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 optimisation of flow cytometer parameters, sperm preparation and cryopreservation methods. Furthermore, future research into improved methods for preparation of FS and 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 offspring. This significant development will help overcome limitations imposed by distance of the sperm sorter from the site of semen collection and ET in both livestock and wildlife species.

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