ABSTRACTS FOR POSTER PRESENTATION

Sexing

329 PREGNANCY RATE IN EWES INSEMINATED WITH SEXED SEMEN IN ARGENTINA

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Offspring of many species have been born from flow cytometrically sex-sorted sperm since its introduction in 1989 (LA Jonhson *et al*.; 1999, Theriogenology). Births of lambs after insemination of ewes with low doses of sexed semen were first reported by Hollinshead *et al.* (Rep. Fert. Dev. 2002). A field trial was carried out in Patagonia Argentina comparing the use of low doses of sexed and non-sexed frozen semen. This study is aimed at comparing pregnancy rates in ewes inseminated with sorted and nonsorted frozen-thawed sperm. Ejaculates from two Merino rams were diluted with modified tirode solution (Shenk *et al.*, 1999, Theriogenology), stained with Hoescht 33342, and finally incubated at 35°C for 40 minutes. The addition of 10% ram seminal plasma to the sample was done to avoid sperm agglutination caused by dilution effect (Mann, 1964). Sorting was performed with a high speed flow cytometer (MOFLO®). Up to 8 million of sexed spermatozoa were collected in 15-mL conical tubes containing modified tirode solution plus 10% ram seminal plasma, centrifuged at 600*g* 12 min and resuspended with test yolk media plus 11% skimmed milk and 5% glycerol. After being refrigerated at 4°C for at least 1.5 h, sexed and nonsexed semen were packaged in 0.25-mL straws at a concentration of 4 to 5 total million sperm and then frozen in an automatic freezing machine (IMV®). One hundred and eighty-three ewes and hoggets were laparoscopically inseminated by the same technician with eight to ten million sorted and nonsorted frozen-thawed semen 14 h after heat detection. Pregnancy diagnosis was performed by ultrasound at day 30 post-insemination. Data were analized by Chi-square. There was significant difference in the overall pregnancy rate between sorted and nonsorted sperm, although, for one of the rams that difference was not significant. There was, also, no significant difference in pregnancy rate between ram 1 and 2 in ewes inseminated with either sexed or nonsexed semen (Table 1). These results indicate that the different performance of sorted compared to non sorted sperm is mainly explained by the lower results achieved with semen from ram 2. According to this conclusion, further studies should be done in order to evaluate the degree to which the sorting process affects the sperm fertilizing capability in different rams. This research was supported by Fundación Margarita Perez Companc.

Table 1.

	No. of ewes inseminated	Preg. rate sexed	Preg. rate non-sexed
Ram 1	98	44% (32/72)[a]	61% (16/26)[a,b]
Ram 2	85	30% (19/63)[a]	59% (13/22)[b]
Total	183	38% (51/135)[a]	60% (29/48)[b]

[a,b]Values within and between columns with different superscripts differ significantly ($P < 0.05$).

330 THE EFFECT OF SHEATH FLUID ON THE QUALITY OF SEX-SORTED RAM SPERMATOZOA

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The fertility of sexed frozen-thawed ram spermatozoa at low AI doses remains below commercially acceptable levels (Maxwell WMC *et al.* 2003 Theriogenology 59, 511 abst). The aim of the present study was to determine if the use of an artificial seminal plasma sheath fluid could attenuate the stress of dilution during flow cytometry and thus improve the functional capacity of sexed ram spermatozoa. Semen was collected from 3 Merino rams, processed for sex-sorting (Hollinshead FK *et al.* 2002 Reprod. Fertil. Dev. 14, 503–508), and then allocated to 2 treatment groups according to the sheath fluid used within the flow cytometer: (i) a tris-citrate-fructose diluent (TRIS), or (ii) an artificial seminal plasma diluent (ASP) (ODonnell JM 1969 J. Reprod. Fert. 19, 207–209). Spermatozoa were sorted using a modified high speed cell sorter (SX MoFlo®, DakoCytomation, Fort Collins, USA) without separation of X and Y bearing gametes, and then frozen and thawed using established protocols (Hollinshead FK *et al.* 2002). Motility characteristics (HTM-IVOS; Hamilton-Thorne, Beverly, USA) and acrosome integrity (FITC-PNA) were assessed throughout. Statistical

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analyses were conducted by ANOVA. Results indicate that in all but one instance (VSL pre-freeze), TRIS spermatozoa exhibited higher ($P < 0.05$) total motility (TM), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) compared to ASP spermatozoa (Table 1). Conversely, ASP treatments displayed enhanced straightness (STR) and linearity (LIN) pre-freezing and immediately post-thaw. In addition, the percentage of hyperactivated (HA) spermatozoa prior to freezing (± SEM) was lower ($P < 0.05$) for ASP (3 ± 1.1) than for TRIS (20 ± 3.4), although post-thaw the differences were NS. Acrosome integrity remained similar ($P > 0.05$) among treatments before and after thawing (range: 92.3–95.9% intact). In conclusion, the ASP sheath fluid resulted in decreased TM, velocity and ALH of sex-sorted frozen-thawed ram spermatozoa, a clear indication of the superiority of TRIS sheath fluid. The partial improvement in STR, LIN and HA that the ASP affords sex-sorted ram spermatozoa does not offset the aforementioned negatives, suggesting ASP is unsatisfactory for use as a sheath fluid during sperm sorting. Research supported by XY, Inc.

Table 1. The effect of sheath fluid on motility characteristics (±SEM)

Time	Sheath	TM (%)	VAP ($\mu m\ s^{-1}$)	VSL ($\mu m\ s^{-1}$)	VCL ($\mu m\ s^{-1}$)	ALH (μm)	STR (%)	LIN (%)
Pre-freeze	TRIS	87 ± 5.1^{a}	133 ± 5.5^{a}	93 ± 3.5^{a}	250 ± 13.1^{a}	9 ± 0.4^{a}	71 ± 2.2^{a}	40 ± 1.8^{a}
	ASP	57 ± 6.9^{b}	116 ± 4.3^{b}	101 ± 4.2^{ab}	204 ± 6.8^{b}	7 ± 0.2^{b}	85 ± 1.0^{b}	51 ± 1.1^{b}
Thaw0h	TRIS	68 ± 4.9^{c}	122 ± 4.9^{ab}	109 ± 3.8^{b}	194 ± 10.9^{b}	6 ± 0.5^{c}	88 ± 1.2^{b}	58 ± 2.0^{c}
	ASP	17 ± 3.9^{d}	94 ± 5.0^{c}	90 ± 5.0^{c}	132 ± 7.6^{c}	4 ± 0.3^{d}	93 ± 0.9^{b}	67 ± 2.9^{d}
Thaw6h	TRIS	46 ± 3.0^{e}	91 ± 2.9^{c}	86 ± 3.2^{c}	132 ± 4.0^{c}	4 ± 0.2^{d}	94 ± 0.8^{b}	66 ± 2.4^{d}
	ASP	1 ± 0.3^{f}	12 ± 5.4^{d}	11 ± 4.9^{d}	22 ± 9.3^{d}	0 ± 0.0^{e}	29 ± 12.3^{c}	17 ± 7.4^{e}

Within column, values without common superscripts differ significantly ($P < 0.05$).

331 INSEMINATION OF OOCYTES WITH AGED SEMEN ALTERS SEX RATIO OF BOVINE EMBRYOS PRODUCED IN VITRO

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Preincubation of semen before insemination may alter the sex ratio of resulting embryos (Lechniak, D. *et al.*, 2003 Reprod. Dom. Anim. 38, 224–227). The overall objective of this study was to evaluate effects of aging sperm before insemination for altering the sex ratio of bovine embryos. In the first experiment oocytes presumed mature were inseminated with frozen semen within minutes post-thaw and percoll-preparation (control) or after aging for 14 h post-thaw in a 34.4°C water bath, or after aging for 23 h post-thaw at 4°C. Sperm from 4 different bulls, representing 3 different breeds, were utilized (1 bull per experimental replicate). Sperm motility was assessed after aging and percoll preparation. Zona pellucidae were removed from cleaved embryos (43–68 h post-insemination; hpi) using 0.5% pronase. Blastomeres were dissociated and counted before transfer to PCR tubes. Sex of cleavage-stage embryos was determined using Ampli-Y™(Bredbacka, P. 1998 Reprod. Nutr. Dev. 38, 605–613). Data were analyzed using a randomized block design with mixed models of SAS (2000) after testing for normality. Aging semen for 14 h post-thaw reduced the proportion of motile sperm and compromised the ability of presumptive zygotes to cleave after insemination (Table 1). However, ability of cleaved embryos to develop to the 8–16-cell stage was not affected. Number of blastomeres comprising cleavage-stage embryos that resulted from insemination of oocytes with aged semen was similar to that for controls. Insemination of oocytes with semen aged 14 h in a water bath increased the proportion of female embryos (Table 1).To determine if effects of aging semen on altering the sex ratio of bovine embryos was time dependent, oocytes were inseminated with frozen semen within minutes post-thaw (control) or after aging for 8 or 14 h post-thaw in a 34.4°C water bath. Sperm from 3 different bulls, representing 2 different breeds were utilized (one bull per experimental replicate). The experiment was replicated 3 times with 195–233 oocytes inseminated within each treatment. Sperm motility averaged 72.7, 65.7 and 45.0% for control and semen aged for 8 and 14 h, respectively (SEM = 10). Cleavage of inseminated oocytes (67 hpi) was similar regardless of sperm treatment (68.5, 70.2 and 70.1%; SEM 14.6, for control semen or after aging for 8 or 14 h post-thaw, respectively). Insemination of oocytes with semen aged for 14 h tended to increase the proportion of female embryos (51.6 v. 38.3 and 39.1%; sperm aged for 14 h v. 8 h or control, respectively; $P = 0.08$; SEM = 9.7). Within the seven replicates across both experiments, the differences in percent female for control v. aged were 5.2, 6.3, 14.9, 18.5, 20.0, 29.7 and 60%, with the highest three being significantly different ($P < 0.05$) by Fisher's Exact test. Bull or replicate variation was noted but the direction of aging for increasing proportion of females was consistent. Preliminary observations suggest that biological differences between X- and Y-bearing sperm may exist such that alternative strategies for altering sex ratio in livestock species may be possible.

Sperm treatments	Sperm motility (%)	No.	Cleaved (%)	8–16 cell (%)	Blastomeres	Total no. sexed	Females (%)
Control	67.5^{a}	125	72.0^{a}	63.8	6.7	91	50.5
Aged 14 h; 34.4°C	37.1^{b}	162	37.9^{b}	46.8	6.2	62	81.7
Aged 23 h; 4°C	61.9^{a}	114	62.4^{a}	61.1	7.0	73	50.1
P-value	0.003		0.003	0.17	0.5607		0.05
SEM	5.4		5.8	11.8	1.1		6.6

[a,b]Values with the same superscript within a column are not significantly different.

332 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 and 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 abst). 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 nonviable 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 support 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 and sorted (Froz-Sort) and frozen-thawed, sorted, then frozen-thawed (Froz-Sort-Froz) ram sperm.

Sperm treatment	No. recipients	No. Pregnant on Day 20 (%)[a]	No. Pregnant on Day 60 (%)[b]	No. Pregnancies lost between Day 20 and Day 60 (%)	No. Lambs born/ no. of transferred embryos (%)
Control	8	6 (75.0)	5 (62.5)	1 (16.7)	5/16 (31.3)
Froz-Sort	9	7 (77.8)	5 (55.6)	2 (28.6)	6/18 (33.3)
Froz-Sort-Froz	17	14 (82.4)	14 (82.4)	0 (0)	17/34 (50.0)

[a]Diagnosed by blood progesterone assay. [b]Diagnosed by ultrasonography.

333 PREGNANCY RATE OBTAINED WITH EMBRYOS COLLECTED AFTER INSEMINATION OF SUPEROVULATED COWS WITH SEXED SORTED SEMEN

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According to the production objectives of beef or dairy commercial farms, the possibility of gender selection of the offspring is an important tool to optimize the use of genetic and economical resources. The use of sexed sorted semen by flow cytometry to inseminate donors in a MOET program has successfully demonstrated a normal embryo production per cow (Theriogenology 59: 513). The aim of this study was to evaluate the pregnancy rate and sex accuracy obtained after transferring embryos collected using sexed semen to inseminate the donors in MOET programs. The semen was provided by our commercial laboratory. Eighteen cows were inseminated with frozen female sexed semen and 22 with frozen male sexed semen. Embryos were collected using a commercial flushing medium with BSA and antibiotics. After the embryos were washed through 10 drops of holding medium, 50 embryos were transferred fresh; 150 were frozen in 1.5 M ethylene glycol, thawed 10 seconds in air; and held for 30 seconds in a water bath at 35°C before transferring them. Fresh embryo stage and quality rates were: morula grade 1: 49%, grade 2: 13%; blastocyst grade 1: 34%, grade 2: 4%. Frozen embryo stage and quality rates were: morulae grade 1: 28%, grade 2: 18%; blastocyst grade 1: 47%, grade 2: 7%. The embryo transfers (ET) were performed in previously synchronized recipients injected with two doses of prostaglandin 12 days apart. Thirty days after ET, the diagnosis of pregnancy was done by transrectal ultrasonography (ALOKA 500 Scanner) to asses the pregnancy rate. Sixty days after the ET, fetal sex determination was done. The results are shown in the table 1 below. The pregnancy rates in both fresh and frozen-thawed preselected sex embryos are similar to those obtained with nonpreselected sex embryos (Theriogenology 56: 1401). The high rate of sex accuracy shows the reliability of the technique of sexed sorted semen by flow cytometry to produce preselected sex embryos in superovulated cows. This research was supported by Fundación Margarita Perez Companc.

Table 1.

Fresh embryo pregnancy rate	Frozen-thawed embryo pregnancy rate	Fetal sex determination males (% of accuracy)	Fetal sex determination females (% of accuracy)
(31/50) 62%	(83/150) 55%	(52/54) 96%	(58/60) 97%

334 INTRACYTOPLASMIC SPERM INJECTION (ICSI) OF BOVINE OOCYTES WITH SEXED SPERM SORTED AT DIFFERENT PRESSURES

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Fertility of sorted sperm has been low compared to unsorted control sperm, due partly to mechanical damage during sperm sorting by flow cytometry. Lowering system pressure improved both sperm quality and fertility in IVF. The present study evaluated the effect of system pressure during sperm sorting and extended maturation of oocytes on development of embryos after ICSI. Sperm from each of three bulls were stained with 125 μM Hoechst 33342 for 45 min at 34°C, sorted into X- and Y-chromosome bearing populations at 95% accuracy with the pressure of SX MoFlo® sorters at 40 or 50 psi, and then cryopreserved. Fifty bovine oocytes obtained from slaughterhouse ovaries were placed per well with 1 mL of CDM1 supplemented with 0.5% FAF-BSA, 2 mM glucose, 50 ng mL^{-1} EGV, 15 ng mL^{-1} NIDDK-0FSH-20, 1 μg mL^{-1} USDA-LH-B-5, 1 μg mL^{-1} E2 and 0.1 mM cysteamine, and then matured for 24 or 30 h at 38.5°C, 5% CO_2 in air. Cumulus cells of matured oocytes were removed by vortexing, and oocytes with a polar body were selected. Motile sperm from sorted frozen-thawed semen were recovered by centrifugation through 2 mL each of 45 and 90% Percoll, and the concentration was adjusted to 4×10^6 mL^{-1}. Matured oocytes were divided into two injection groups, ICSI and sham injection, using a Piezo injection system. The outer diameter of the sperm injection pipette was 8–10 μm. All manipulations were performed at room temperature (24–25°C). After injection, oocytes were activated with 5 μM ionomycin for 4 min, cultured in 50 μL of CDM1 at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2, and assessed for degeneration/cleavage at 72 h post-injection. Uncleaved oocytes from ICSI and sham-injected groups were stained with orcein and evaluated for fertilization status. Cleaved embryos were further cultured, and blastocyst development was evaluated on Day 7.5 after injection. Data were subjected to ANOVA; the arcsin transformation was used for percentage data, and main effect means are presented. With 24 h matured oocytes, there were no differences ($P > 0.1$) between sperm sorted at 40 v. 50 psi for degeneration, cleavage or blastocyst rates, nor was there pressure × bull interaction. There were significant effects of bulls for all responses studied ($P < 0.05$). When injected with sperm sorted at 40 psi, oocytes matured for 30 h resulted in higher cleavage and lower degeneration rates than 24 h-matured oocytes (22.9 and 13.7% v. 12.2 and 22.0%, respectively, $P < 0.05$), with no difference ($P > 0.1$) in blastocyst rate. Overall blastocyst development was higher in ICSI than in sham injection (7.5 v. 1.3%, $P < 0.05$). When uncleaved oocytes from 24 h maturation were evaluated for fertilization status, ICSI showed a higher percentage with two polar bodies and/or decondensed sperm compared to sham injection (15.7 v. 1.7%, $P < 0.05$). With 30 h matured oocytes there was no difference in fertilization status between those groups. We conclude that there was no difference in cleavage or development to blastocysts after ICSI using motile sperm that had been sorted at 40 or 50 psi.