*Sexing*

## 304 PRODUCTION OF PORCINE EMBRYOS OF A PREDETERMINED SEX AFTER *IN VITRO* FERTILIZATION OF *IN VITRO*-MATURED OOCYTES WITH SEX-SORTED FROZEN-THAWED BOAR SPERM

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Pre-sexed embryos and offspring have been produced after IVF and embryo transfer (ET) with sex-sorted frozen-thawed sperm in cattle and sheep (Maxwell *et al*. 2004 Anim. Reprod. Sci. 82–83, 79–95). The aims of this study were to demonstrate that sex-sorted frozen-thawed boar sperm could be incorporated into pig IVF for the production of embryos of a predetermined sex and that these embryos could be successfully nonsurgically transferred. Ovaries were collected from abattoir slaughtered gilts ($n = 138$) and selected COCs were matured *in vitro* (Long *et al*. 1999 Theriogenology 51, 1375–1390). Sperm were collected from a mature boar and diluted with Androhep (1:3, semen:Androhep; Minitube, Verona, WI, USA), stained with H33342, and separated into X and Y sperm using a SX MoFlo (Cytomation, Inc., Fort Collins, CO, USA). Sex-sorted sperm were cryopreserved in 0.5 mL straws using the Westendorf protocol modified for sorted sperm (Bathgate, unpublished). Thawed sperm (Y sperm only) were prepared for IVF by centrifugation (300*g*, 10 min) through a Porcipure gradient (Nidacon Int. AB, Gothenburg, Sweden), and washed (centrifugation 300*g*, 10 min) in mTALP-PVA. For IVF, COCs were denuded and groups of 100 oocytes were transferred to 200-μL drops of mTALP-PVA (Long *et al*. 1999) and incubated with 5,000 motile sperm for 4–6 (Short) or 18–20 h (Long) . Presumptive zygotes were washed and transferred to 100-$\mu$L drops of mNCSU-23 (Long *et al*. 1999) and cultured until Day 4 (Day 0 = IVF) in humidified 5% $CO_2$, 6% $O_2$, 89% $N_2$. Oocyte cleavage was assessed 48 h post-insemination, and on Day 4 selected morulae were transferred to recipient sows ($n = 7$ Large White × Landrace; 65 morulae per sow) nonsurgically using a Firflex catheter (Magapor, Zaragoza, Spain). Sex of remaining embryos was confirmed by PCR and restriction analysis (Cong *et al*. 1993 Hum. Mol. Genet. 2 1187–1191). Data from three replicates were arc sin transformed and analyzed by ANOVA. Oocyte cleavage was similar after Short (724/1547; 46.8%) or Long (598/1528; 39.1%) co-incubation. Resort analysis showed sperm to be >91% purity, and all sexed morulae were of the predicted sex (16/16). Delayed return to estrus (>23 days) was observed in 5 recipient sows (71.4%). Fetal sacs were observed by transcutaneous ultrasound in one of these sows. Pre-sexed porcine IVP embryos can be successfully produced using sex-sorted frozen-thawed boar sperm, and these embryos are capable of initiating pregnancies when transferred to recipients. However, further refinement of porcine IVP and ET protocols are required to enable full in vivo development.

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## 305 FIRST REPORT OF THE USE OF SEXED SEMEN BY FLOW CYTOMETRY IN *BOS INDICUS*

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The only method of sexing sperm that works in any practical sense is measuring DNA content of individual sperm by flow cytometry, and sorting them based on that information (Schenk and Seidel 2002 J. Anim. Sci. 80 (Suppl. 1), 188). This is, also, the first reference of the use of sexed semen in Brazil. The objective of this experiment was to compare the fertility of sexed and non-sexed conventional semen in *Bos indicus* purebreeds. Ejaculates from eight Nelore bulls were collected by artificial vagina, diluted, and stained with Hoeschst 33342 (Schenk *et al*. 1999 Theriogenology 52, 1375–1391). A high-speed flow cytometer (SX-MOFLO®, DakoCytomation, Inc., Fort Collins, CO, USA) was used for sorting X sperm. Sexed semen was then packaged at 3 million total sperm in 0.25-mL straws and frozen. The average estimated purity obtained by resort analysis of one straw per batch was 91.5%. An aliquot of each ejaculate was frozen unsexed at a concentration of $50 \times 10^6$ sperm/mL in 0.5-mL straws (control). A total of 283 Nelore heifers were synchronized with two doses of $PGF_{2\alpha}$ 12 days apart and inseminated in the body of the uterus 12 h after heat detection. Field trials were carried out in four different states in Brazil: Minas Gerais (MG), Mato Grosso (MT), Mato grosso do Sul (MS), Rondônia (RO). Pregnancy diagnosis and fetal sex determination were performed by ultrasound 30 and 60 days post-AI, respectively. Data were analyzed by chi-square and Monte Carlo methods. There was no significant difference ($P < 0.05$) in pregnancy rate between sexed and non-sexed semen (see Table 1). At the time of abstract submission, of 41 pregnant heifers from sexed semen confirmed by ultrasound, 40 had a female fetus (97%). These results indicate that sexing semen by flow cytometry could be a very important tool to accelerate the genetic gain in *Bos indicus* cattle. Brazil has the largest commercial herd in the world and, therefore, a great potential to incorporate this technology that is now available in the country.

**Table 1.**

| State | Sexed semen | | Non-sexed semen | |
|---|---|---|---|---|
| | *n* | Pregnancy rate | *n* | Pregnancy rate |
| MG | 33 | 42.4 (14/33) | 10 | 60 (6/10) |
| MT | 95 | 42.1 (40/95) | 62 | 40.3 (25/62) |
| MS | 24 | 54.2 (13/24) | 7 | 42.3 (3/7) |
| RO | 28 | 53.6 (15/28) | 24 | 70.8 (17/24) |
| Total | 180 | 45.5 (82/180) | 103 | 49.5 (51/103) |

($P < 0.05$).

## 306 EMBRYO ANALYSIS IN FIELD MOET: HIGH SUCCESS RATE AFTER OVERNIGHT CULTURE OF MICROBLADE-BIOPSIED CATTLE EMBRYOS

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Embryo storage before embryo transfer is a necessity in field MOET to enable complex embryo analysis. Cryopreservation of biopsied embryos may reduce pregnancy rates to unacceptable levels, below 40% (Shea BF 1999 Theriogenology 51, 841–854). Our primary objective was to investigate the effect of short-term storage in overnight culture on the pregnancy rate of microblade-biopsied and sexed cattle embryos. A specific aim was to apply embryo storage in culture to marker-assisted selection of MOET embryos.

Day 6.5 embryos were produced using standard superovulatory, AI, and embryo flushing procedures. Embryo donors were lactating cows of top genetic merit. Embryos were transported in straws in Holding medium (ICPbio, Auckland, New Zealand) to the laboratory and individually biopsied by a microblade. Biopsied embryos were cultured overnight in individual oil-overlaid 20-μL drops of Medium 199 with glutamax-1 (GIBCO$^{TM}$, Baisley, UK) containing 0.25 mM sodium pyruvate, antibiotics, and 4 mg mL$^{-1}$ fatty acid-free albumin. The biopsies were lysed in proteinase K and analyzed for sex with the BOV-Y/kappa-casein PCR method (Peura *et al*. 1991 Theriogenology 35, 547–555). Overnight-cultured grade I-II female embryos were transferred into the uteri of heat-synchronized recipients (Day 7.5). Pregnancy was confirmed by rectal palpation at 2–3 months after ET. Grade I-II male embryos as well as embryos of unknown sex were frozen in ethylene glycol, stored in liquid nitrogen, thawed, and cultured overnight for estimation of re-expansion rate and cell counts.

In total, 74 embryos of eight donors were overnight-cultured and sexed. The success rate of the sexing method was 95%. Of the successfully analyzed embryos, 41% (29 of 70) were females and 59% (59 of 70) males. The survival rate of microblade-biopsied overnight-cultured embryos was 99% (73 of 74); 73% of the surviving embryos were of grade I, 23% of grade II, and 4% of grade III. Sixteen of the 27 (59%) grade I–II female embryos transferred resulted in pregnancy. Forty-two grade I–II embryos were frozen and 36 (86%) re-expanded after thawing and overnight culture. Twenty-four of the re-expanded embryos (67%) were of grade I. The re-expanded embryos had on average 73 cells (range 43–114).

In conclusion, overnight culture of microblade-biopsied cattle embryos does not compromise embryo viability, resulting in high pregnancy rate and post-thaw re-expansion rate. The method can be utilized as a short-term embryo storage in the field MOET scheme and it will be applied in marker-assisted selection of MOET embryos for genotypes associated with milk production.

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## 307 *IN VITRO* PRODUCTION OF BOVINE EMBRYOS USING FLOW-CYTOMETRICALLY SORTED SPERMATOZOA

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There are two practical ways to predetermine the sex of mammalian offspring: sexing pre-implantation embryos and sexing spermatozoa. The only successful and non-invasive method of sexing spermatozoa is quantifying sperm DNA with fluorescing DNA-binding dye, followed by flow cytometry and cell sorting. Our investigations aimed to develop a technology for *in vitro* embryo production in cattle using fresh and/or frozen-thawed spermatozoa sexed by flow cytometry. Sperm was sorted in a MoFloSX® cytometer using the method of XY, Inc. (Fort Collins, CO, USA; Research Collaboration Agreement). After sorting, the sperm was either used for IVF or frozen and stored in liquid nitrogen. Immature oocytes, recovered from slaughterhouse ovaries, after 22 to 23 h of IVM in TCM-199 containing 20% estrous cow serum and additional granulosa cells (Katska *et al*. 1998, J. Anim. Feed Sci. 7, 353–362), were fertilized *in vitro* with fresh or frozen-thawed X and Y fractions of spermatozoa. Simultaneously control, unsorted, fresh and frozen-thawed sperm was used for IVF. The standard protocol of sperm capacitation (Katska and Rynska 1998 Theriogenology 50, 213–222) was applied for both control sperm and fresh fractions of sexed sperm. Briefly, sperm was separated by Percoll gradient centrifugation, washed, and introduced into drops of Tyrode's albumin-lactate-pyruvate (TALP)-IVF (containing 10 μg heparin mL$^{-1}$ and mixture of penicillamine, hypotaurine, and epinephrine) at a concentration 1 to 2 × 10$^6$ spermatozoa mL$^{-1}$ of medium. Frozen fractions of sorted spermatozoa were centrifuged after thawing (500 *g* for 10 min) and immediately introduced into the IVF drops at 2 to 3 × 10$^6$ spermatozoa mL$^{-1}$ of medium. Embryos resulting after IVF were co-cultured with Vero cells in B2 medium supplemented with 2.5% fetal calf serum for 8 to 10 days, (i.e., to the hatched blastocyst stage). A total of 2074 IVM oocytes were fertilized with both fresh and frozen-thawed sexed and control sperm of 5 bulls. There were significant differences ($P < 0.01$) in cleavage rates among fresh control sperm (120/256; 46.9%), the X fraction (66/254; 26.0%), and the Y fraction (58/230; 25.2%). Similar differences in cleavage rates ($P < 0.01$) were shown for frozen-thawed control sperm (156/335; 46.6%), the X fraction (137/498; 27.5%), and the Y fraction (118/501; 23.6%). No differences were observed in efficiency of embryo development to the blastocyst stage between the fresh control (25.8%) and the Y fraction (25.9%), or among the frozen control (16.7%) and the X fraction (13.1%) or the Y fraction (16.9%). However, significant differences ($P < 0.05$) were shown between blastocyst rates with the fresh X fraction (10.6%) and the control. Our results suggest that there were differences due to sperm sorting but no differences in efficiency of both fresh and frozen-thawed X and Y fractions of spermatozoa.

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## 308 PREGNANCY RATE AND EMBRYO PRODUCTION AFTER INSEMINATION OF MARES WITH SEXED SORTED SEMEN

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In the equine industry the selection of the desired sex of the offspring can be used as a tool in the improvement of a genetic program with the same advantages as cited in other species. Nevertheless, another advantage can be realized if we consider that there are some sport breeds in which females are better performers than males. As an example in polo ponies nine out of ten ponies are females and most male newborns become an undesired product because of their very low commercial value. To date it has been reported that the use of a low dose of sexed sorted semen ($5 \times 10^6$) resulting in pregnancy rates of 40% after hysteroscopic insemination and 50% using a deep intrauterine insemination pipette with a dose of $25 \times 10^6$ non-sexed semen (Lindsey AC *et al*. 2001 Anim. Reprod. Sci. 68, 279–89). Considering that the hysteroscopic procedure is a time-consuming technique that requires well-trained people and expensive equipment, the aim of this study was to evaluate: (a) if the pregnancy rate obtained after insemination of mares with a low dose of fresh sexed sorted semen using the intrauterine pipette could achieve results similar to those of the hysteroscopic procedure, and (b) the possibility of embryo production and pregnancy rates after transferring those embryos. Twenty-four mares with a dominant follicle more than 35 mm in diameter were injected with 2500 UI of human corionic gonadotrophin (hCG) (Endocorion® Elea, Buenos Aires, Argentina) and inseminated between 30 and 36 h after hCG. A total of $40 \times 10^6$ sexed spermatozoa (45–55% of them were progressively motile) were deposited deeply in the ipsilateral horn to the preovulatory follicle using the deep intrauterine insemination pipette (Minitube®, Verona, WI, USA). Ten of the 24 (40%) were inseminated with a second dose because they didn't ovulate 6 h after the first AI. The semen was provided by our commercial laboratory following the XY, Inc. (Fort Collins, CO, USA) protocol for sexing equine semen. In a subsequent study, 18 donors were inseminated following similar criteria and flushed 7.5 days after ovulation using 3 liters of a flushing medium with bovine serum (1%) and antibiotics. After the embryos were washed through 10 drops of holding medium, a single embryo was transferred into the uterine body of each of 11 recipients which ovulated between one day before to three days after the donor mare. Six days after ET, the diagnosis of pregnancy was carried out by transrectal ultrasonography (ALOKA 500 Scanner®, Aloka Technology, Wallingford, CT, USA) and confirmed by a second scanning 25 days later. Results are shown in Table 1. According to these results, the insemination with a low number of fresh sexed sorted semen using the large intrauterine pipette had results similar to those obtained with the hysteroscopic procedure. Although we performed a low number of flushings, results showed no difference in embryo production and pregnancy rates compared with data from when non-sexed semen was used to inseminate the donors.

**Table 1. Artificial insemination and embryo transfer results with sexed semen**

| | Number | Embryo production | Pregnant mares at 30 days |
|---|---|---|---|
| Mares inseminated | 24 | | 54% (13/24) |
| Mares flushed | 18 | 61% (11/18) | 73% (8/11) |

*Current Equine Embryo Transfer Techniques D. Vanderwall, International Veterinary Information Service, Ithaca, NY, 2000.*

## 309 SEX SORTED BOAR SPERMATOZOA: TIME COURSE AND PROFILE OF THEIR *IN VITRO* PENETRATION ABILITY AFTER STORAGE IN THE PRESENCE OF HOMOLOGOUS SEMINAL PLASMA

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Addition of seminal plasma (SP) to the collection medium has been shown to be beneficial for motility and viability of sex-sorted and stored spermatozoa. However, SP could not only delay but also decrease the *in vitro* fertilization rates of IVM pig oocytes. In the present study, the time-course of IVM pig oocyte penetration of sex sorted boar spermatozoa stored in the presence or absence of SP was evaluated. Spermatozoa were sex-sorted following the Beltsville sperm sexing technology (Johnson and Welch 1999 Theriogenology 52, 1323–1342) and collected in TEST-egg yolk buffer (2%) with (10%) or without (control) SP. Sex-sorted spermatozoa were stored at 20°C during 0, 2, 5, and 10 h after sorting. Oocytes were matured *in vitro* in NCSU23 (Peters and Wells 1663 J. Reprod. Fertil. 48, 61–73) for 44 h in 5% $CO_2$ in air at 39°C. The *in vitro* penetration time-course was determined by co-incubating the sex-sorted and stored spermatozoa with IVM oocytes during 3, 6, and 18 h in modified TRIS-buffered medium (mTBM) (Abeydeera and Day 1997 Theriogenology 48, 537–544) at 39°C in an atmosphere of 5% $CO_2$ in air. Penetration rates and number of spermatozoa per oocyte were assessed after fixation and staining of the oocytes. Statistical analyses were conducted by ANOVA. Presence of SP did not delay the onset of the oocyte penetration. Moreover, at 3 h of co-incubation, SP increased ($P < 0.05$) both penetration rates and mean number of spermatozoa per oocyte in sorted and stored boar spermatozoa when compared with control (45 vs. 20, 50 vs. 32, 38 vs. 23, 15 vs. 8, at 0, 2, 5, and 10 h of storage with SP and control, respectively). High penetration rates were reached after 6 h of co-incubation (82 vs. 51, 96 vs. 76, 83 vs. 48, 31 vs. 24, at 0, 2, 5, and 10 h of storage with SP and control, respectively) in sorted and stored samples, with no further increase at 18 h (70 vs. 63, 92 vs. 79, 87 vs. 53, 55 vs. 40, at 0, 2, 5, and 10 h of storage with SP and control, respectively). Spermatozoa stored 2 h in the presence of SP showed the best penetration rate and highest mean number of spermatozoa per oocyte. The mean number of spermatozoa per oocyte increased as the co-incubation time increased (ranging from 2.1 to 5.8 for sorted spermatozoa stored 2 h in the presence of SP at 3 h and 18 h of co-incubation, respectively). In conclusion, the presence of SP during the storage of sex-sorted spermatozoa improves their *in vitro* fertilizing ability without affecting the onset of the oocyte penetration time.

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## 310 USE OF COMPETITIVE FERTILIZATION TO EVALUATE A SIMPLER LASER FOR FLOW CYTOMETRIC SEXING OF BOVINE SPERM

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Statistically significant correlations between laboratory assays of sperm quality and fertility require large sample populations. Experimental differences can be assessed accurately using limited observations with competitive fertilization and fetal sexing, as clearly demonstrated previously (Seidel GE Jr. *et al*. 2003 Theriogenology 59, 515 abst). Our objectives were to compare pregnancy rates in Holstein heifers inseminated with: (1) $2 \times 10^6$ hetero-sex-selected sperm interrogated with different light sources or, (2) $2 \times 10^6$ or $10 \times 10^6$ X-chromosome bearing sperm using a continuous wave (CW) laser, to those of unsexed inseminates containing $10 \times 10^6$ total sperm. Sperm were sexed by flow cytometry/cell sorting at 40 psi with the aid of light produced from either a continuous wave (CW) or a quasi-cw (PULSED; Vanguard 350-HDM, Spectra-Physics, Mountain View, CA, USA) laser operating at 150 mW during flow cytometric/cell sorting. Subsequent fertility was evaluated by competitive fertilization with fetal sex as the genetic marker. Sperm from Holstein bulls were sorted into X- and Y-chromosome populations at >90% accuracy using either the CW or the PULSED laser. After concentration of sperm post-sorting by centrifugation, an equal number of X-sorted sperm illuminated with the CW laser were pooled with Y-sorted sperm illuminated by the PULSED laser within each bull, as well as the converse. Total sorted sperm ($2 \times 10^6$) were placed in 0.25 mL straws. In addition to these sperm, homospermic inseminates containing 2 or $10 \times 10^6$ total sperm, sorted using the CW laser, and unsorted controls ($10 \times 10^6$ total sperm) were then frozen. Holstein heifers ($n = 763$) were synchronized for estrus in five groups (July–December) with a CIDR in place for 7 days followed by 25 mg PGF-2α i.m. Heifers ($n = 626$) were body inseminated either 12 or 24 h after observed estrus. Sexed and unsorted inseminates were balanced across sperm from three bulls and two inseminators. Two months post-insemination, pregnancy and fetal sex were determined using ultrasound. Data were subjected to ANOVA. Pregnancies marked by fetal sex achieved with competitive fertilization resulted in a 52 (PULSED):48 (CW) ratio, which is not different from the expected 50:50 ratio ($P > 0.05$) if neither laser was more or less damaging to the fertilizing potential of sperm. Actual pregnancy rates for the competitive $2 \times 10^6$, homospermic $2 \times 10^6$, and $10 \times 10^6$ sexed inseminates were 54, 56, and 62%, respectively, with $n = 179$, 179, and 180, and were similar to unsorted controls (61%, $n = 88$) ($P > 0.05$). This study demonstrated no deleterious effects, in terms of pregnancy rate, to sperm illuminated with a PULSED laser during sorting when compared to conventional instrumentation. Also, pregnancy rates with sexed sperm were similar to those of unsexed controls. The use of the PULSED laser for sperm sorting has economic advantages because it requires less energy, is air-cooled, and has a longer operating life than the water cooled CW laser.

## 311 CATTLE SEX REGULATION BY SEPARATION OF X AND Y SPERMATOZOA – PRELIMINARY RESULTS OF FIELD EXPERIMENT IN POLAND

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The only reliable and relatively fast method of sex regulation in cattle is X and Y sperm high-speed sorting by flow cytometry. In October 2003 a field experiment started in Poland. The aim of the experiment was to examine the efficiency of sperm sexing and the fertility rate in field conditions. The semen of three Holstein bulls was used for the experiment. The semen was sexed and frozen according to modified XY, Inc. (Fort Collins, CO, USA) method. Only the X-fraction of spermatozoa was sorted and frozen. Sexing was performed at a speed of $15–20 \times 10^6$ spermatozoa/h and frozen in doses of $2.5 \times 10^6$ spermatozoa/straw. Progressive motility of spermatozoa was 90–95% immediately after sorting and 50–70% after freezeing/thawing for all three bulls. The X-fraction sorting purity was checked by re-analysis and it ranged from 90% to 96%. In total, 316 inseminations at 10 farms were performed up to June 2004. Fertility data of 178 inseminations were collected by ultrasonographic (USG) examination during the same time. Average fertility rate was 37.08%. However, significant variations of fertility rates were observed between farms: it ranged from 22.22% to 84.21%. Four calves, all females, were born after insemination with the X-fraction until June 2004. The experiment will continue.

## 312 BLASTOCYST DEVELOPMENT OF MALE AND FEMALE BOVINE EMBRYOS PRODUCED BY IVF WITH FLOW CYTOMETRICALLY-SORTED SPERM

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Several studies have demonstrated that male bovine embryos produced *in vitro* develop faster than female embryos produced *in vitro*, which results in more male than female blastocysts. The objective of this study was to compare the rate of blastocyst development of male and female bovine embryos derived from sexed sperm and cultured in a chemically defined medium + fatty acid-free (FAF)-BSA. Bovine oocytes ($n = 1364$) were fertilized with two types of frozen-thawed sperm (X- or Y-chromosome-bearing sperm sorted at 90% accuracy). Oocytes, aspirated from slaughterhouse ovaries, were matured in TCM199 supplemented with 10% fetal calf serum plus hormone additives (15 ng FSH, 1 mg LH, 1 mg 17 β-estradiol $mL^{-1}$) for 22–24 h at 39°C, 5% $CO_2$ in air with maximum humidity. Semen from one bull was sorted by flow cytometry into X- and Y-chromosome bearing sperm and frozen for later use with IVF. The procedures for IVF and IVC have been previously described (Lu KH, *et al*. 1999 Theriogenology 52, 1393–1405). Presumptive zygotes were removed from culture and placed in chemically defined medium (CDM-1 [Zhang M *et al*. 2003 Theriogenology

60, 1657–1663]) 6–7 h after insemination and cultured for 65–66 h. Embryos which had cleaved by 72 h post insemination were further cultured 96 h in CDM-2 (Zhang M *et al*. 2003 Theriogenology 60, 1657–1663) containing 0.12 IU insulin $mL^{-1}$. Cleavage and blastocyst rates per oocyte inseminated were recorded on Day 3 and Days 7–8 after insemination, respectively. Data were analyzed by ANOVA procedures with replicates and treatments in the model. There was no significant difference in cleavage rate or blastocyst rate between X and Y sperm treatments. These results indicate that embryos produced with Y sperm do not reach the blastocyst stage in significantly higher proportions than embryos produced with X sperm in this chemically defined medium + FAF-BSA. Apparently, this IVC system leads to a more synchronous development of male and female embryos than other methods of producing bovine embryos *in vitro*.

**Table 1. Cleavage and blastocyst rates with X and Y sperm**

| Sperm type | Oocytes (*n*) | Cleaved[a] (%) | % Blastocysts/ oocyte[a] | % becoming blastocysts[a] Day 7 | Day 8 |
|---|---|---|---|---|---|
| X | 611 | 50.3 ± 4.3 | 19.9 ± 4.3 | 76.5 ± 5.9 | 23.5 ± 5.9 |
| Y | 753 | 57.6 ± 7.6 | 21.0 ± 2.5 | 77.1 ± 2.6 | 22.9 ± 2.6 |

[a] No significant difference, $P > 0.05$: LS means ± SE.

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*Sperm Injection*

## 313 EFFECTS OF ETHANOL TREATMENT AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) ON SPERM ASTER FORMATION AND THE MICROTUBULE ORGANIZATION OF BOVINE OOCYTES

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The cleavage rate of bovine embryos is very low without activation of oocytes after intracytoplasmic sperm injection (ICSI), although both male and female pronuclei are formed. We previously reported that the stimulus due to the injected sperm alone was sufficient to lower the MPF activity of bovine oocytes after ICSI, and the activation treatment of oocytes with ethanol at 4 h after ICSI served to maintain the low levels of MPF activity until the next cell cycle started (Fujinami *et al*. 2004 J. Reprod. Dev. 50, 171–178). These results suggested that activation treatment is necessary to improve the embryonic development after bovine ICSI. In bovine fertilization, the sperm introduces the centrosome into the oocyte. The centrosome acts as the microtubule-organizing center and microtubules are organized within the oocyte. It is reported that the sperm aster is important for the normal fertilization process. Therefore, failure of sperm aster formation possibly causes the failure of cleavage following fertilization. To investigate the reason of the low cleavage rate after bovine ICSI without artificial activation treatment, we examined sperm aster formation and the microtubule organization in bovine oocytes with or without activation treatment after ICSI. Bull spermatozoa immobilized by piezopulse was injected into bovine oocytes matured *in vitro*. At 4 h after ICSI, oocytes were treated with 7% ethanol in TCM199 for 5 min for activation. Oocytes were fixed at 6 and 12 h after ICSI, and the microtubule organization was examined by using specific antibodies and immunofluorescence microscopy. The cleavage rate (51% vs. 15%) and the developmental rate to the blastocyst stage (13% vs. 3%) were increased by ethanol treatment after ICSI (with or without ethanol treatment, respectively, $P < 0.05$). In oocytes activated with ethanol after ICSI, both the sperm aster formation rate at 6 h and the microtubule organization rate at 12 h after ICSI were significantly higher than in oocytes without activation treatment (58%, 80% vs. 12%, 26%, $P < 0.05$). It was reported that the sperm aster has an important role for the pronuclear movement to make the male and female pronuclei come into close apposition. From these results, it was concluded that oocyte activation after bovine ICSI promoted sperm aster formation and microtubule organization, and was effective to improve embryonic development.

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## 314 FULL-TERM DEVELOPMENT OF RAT OOCYTES MICROINSEMINATED WITH FREEZE-DRIED SPERMATOZOA

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Since freeze-dried spermatozoa can be stored at ambient or refrigerated temperature, the costs required for maintenance and shipping of spermatozoa can be reduced. To date, viable offspring in mice (Wakayama and Yanagimachi 1998 Nat. Biotech. 16, 639) and rabbits (Liu *et al*. 2004 Biol. Reprod. 70, 1776) have been produced by intracytoplasmic sperm injection (ICSI) using freeze-dried samples. The objectives of the present study were to