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Sperm Pre-Incubation Prior to Insemination Affects the Sex Ratio of Bovine Embryos Produced *in vitro*

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Contents

The objective of the present study was to determine whether sperm incubation prior to oocyte insemination *in vitro* affects the sex ratio of resulting blastocyst. Cumulus–oocyte-complexes (COCs) collected from slaughterhouse ovaries were matured *in vitro* and inseminated with frozen-thawed semen of three proven artificial insemination (AI) bulls pre-incubated *in vitro* in Sperm-Talp for 6 and 24 h. On day-9 blastocysts were collected and processed for sex determination. More than 80% of blastocyst were successfully sexed. There were no significant differences in cleavage and blastocyst rates using sperm pre-incubated for 6 h as compared with the 0-h pre-incubation control group. The cleavage and blastocyst rates were significantly lower in the 24-h pre-incubation group. The male to female ratio, when compared with the theoretical 1 : 1, differed significantly in favour of females among hatched (viable) blastocysts derived from sperm pre-incubated for 24 h prior to insemination as well as among all blastocytsts in the 6-h group. Moreover, when the sperm treatment was considered, the sex ratio was affected only among hatched blastocysts in 24-h pre-incubation group. It was concluded that prolonged sperm pre-incubation influences the rate of development and the sex ratio among hatched blastocysts.

Introduction

The sex ratio among newborn calves, and embryos produced *in vivo* is approximately 1 : 1 (King et al., 1991). However, several studies on bovine embryos produced *in vitro* suggest that the sex ratio may differ from 1 : 1, and the rate of development may be influenced by the embryo sex under some *in vitro* production and culture conditions (for review see Kochhar et al. 2001). This phenomenon is not fully understood, however it may be attributed to events that occur before fertilization that favour selection of X- or Y-chromosome-bearing spermatozoa, events that occur after fertilization such as preferential development or survival of embryos of one sex or a combination of both (Kochhar et al. 2001). Moreover, analysis of the X- and Y-sperm populations have shown some morphological and functional differences, although it is not known if X- and Y-bearing spermatozoa have the same viability *in vitro* (Sarkar et al. 1984; Watkins et al. 1996; Penfold et al. 1998; Van Munster et al. 1999).

The fertile lifespan (longevity) of gametes is species-dependent and seems to be one of the major factors regulating the efficiency of the fertilization process. *In vivo*, bovine spermatozoa have a fertile life of 30-48 h while that of ova varies from 8 to 12 h (Bazer et al. 1987). However, oocytes may retain the capacity to be fertilized for a longer period, up to 20–24 h, but the normalcy of fertilization can be compromised (Hunter 1989). It has been shown that some sperm cells reach the oviduct site a few minutes after ejaculation. However their fertilizing potential is very low. Bull sperm cells capable of fertilizing an oocyte appear in the oviduct 8 h after mating and are held in isthmus for several hours until ovulation occurs (Hawk 1987). *In vitro*, the fertilizing capacity of bull spermatozoa may be extended up to 30 h after binding to the oviduct epithelial cells (Pollard et al. 1991).

Previously we have shown that reducing the duration of sperm–oocyte incubation *in vitro* from 18 to 6 h resulted in a significant shift in sex ratio in favour of males among blastocyst on day 7 of *in vitro* development that were derived from 6 h of gamete co-incubation. It was postulated that Y-chromosome-bearing spermatozoa have a selective advantage for early fertilization (Kochhar et al. 2001).

The aim of the present study was to determine whether sperm pre-incubation prior to fertilization *in vitro* (IVF) influences the rate of fertilization, embryo development and the sex ratio among blastocysts.

Material and Methods

Oocyte maturation

To determine the effect of spermatozoa pre-incubation prior to sperm–oocyte co-culture, oocytes were collected from slaughterhouse ovaries, matured and fertilized *in vitro* with spermatozoa pre-incubated in Sperm-Talp (Parrish et al. 1986) for 6 and 24 h. The method for oocyte maturation and fertilization was as described by Xu et al. (1992) with a modified protocol for sperm processing. In brief, oocyte–cumulus-complexes (COC) were aspirated from follicles of 2–5mm in diameter and collected into Hepes-buffered Ham's F-10 (Gibco BRL, Burlington, Ontario, Canada). Only COCs with evenly granulated ooplasm and several (two to six) layers of non-expanded cumulus cells were selected for *in vitro* maturation in Hepes-buffered TCM 199 (Gibco BRL) supplemented with 10% steer serum (SS; Cocalico Biologicals, Ontario, Canada). On average, 100–125 COCs were matured in 750 µl of maturation medium in a four-well plate under silicone oil for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air. After 24 h, COCs were washed three times in Talp-Hepes and once in IVF-Talp and transferred into 500-µl drop of IVF medium supplemented with 20 µg/ml of heparin.

Sperm processing

Frozen-thawed sperm cells from three AI bulls with proven *in vitro* fertility were utilized. After swim-up, the motile fraction of sperm was incubated in 10 ml of Sperm-Talp (no heparin included) in a conical tube for 0, 6 and 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air. Tubes with sperm incubated for 24 h were additionally centrifuged and the medium was replaced after the initial 15 h. The sperm count was carried out in each treatment group and adjusted to a final concentration in IVF droplets of 1×10^6/ml. Sperm motility was also evaluated and the number of motile sperm cells was kept at a similar level in each experimental group. The rate of motile spermatozoa decreased as the co-incubation proceeded and reached on average: 80–85% in the control group, 65–70% in the 6 h pre-incubation group and 45–50% in the 24 h group.

In vitro fertilization, embryo culture and sex determination

After washing, 20–25 matured oocytes were placed into a 95-µl drop with IVF medium (IVF-Talp) and inseminated. After 18 h of co-incubation, cumulus cells were stripped by vortexing for 90 s in warm Talp-Hepes or by vigorous pipetting in IVF drop. After three Talp-Hepes and one *in vitro* culture (IVC)-medium washes, 20–25 presumptive zygotes were transferred into 50-µl IVC drop [TCM199 supplemented with 10% SS and 0.35% bovine serum albumin (BSA)] containing bovine oviductal epithelial cells (BOEC), covered with sterile filtered silicone oil. BOEC had been previously cultured for 24 h in IVC medium. Fresh culture medium (25 µl) was added to each IVC drop on day 4 of culture and embryos were incubated *in vitro* until day 9 after insemination. Embryo cleavage rate was evaluated on day 2 post-insemination and represented the number of early-stage embryos in comparison with the number of inseminated oocytes. Six replicates for each bull were performed. On average, 100 COCs were included in each replicate group.

Expanded and hatched day-9 blastocysts were sexed according to the PCR method described by Bredbacka and Peippo (1992).

Statistical analysis

The cleavage and development data were analysed by SAS mixed procedure (random effects – bull and replicate; fixed effect – pre-incubation group). Data on embryonic sex was analysed using chi-square test for equal proportions and the one-way chi-square test for the expected 1 : 1 ratio (SAS 1989).

Results

Altogether 670 day 9 blastocyst were produced, of which 424 were submitted for sexing (because of the small number of blastocysts produced in 24 h group we did not sex all embryos produced in 0 and 6 h groups). In all, 357 (84.2%) were successfully sexed. No bull effect on the cleavage or development ratios was detected ($p > 0.05$). There were no significant differences in cleavage and blastocyst rates using sperm pre-incubated for 6 h when compared with the 0-h control group however, there was a significant reduction ($p < 0.05$) in the rates of cleaved oocytes and day-9 blastocyst in the 24 h pre-incubation group (Table 1).

The male to female ratio, when compared with the theoretical 1 : 1, differed significantly in favour of females among the combined day-9 blastocysts in the 6-h group and the hatched blastocysts in the 24 h group. Moreover, when comparisons between groups were made and the actual sex ratios were taken into consideration, there were significantly more female hatched blastocysts among the 24 h group than among those of the either the 0- or 6-h pre-incubation groups ($p < 0.01$, Table 2).

Discussion

In the present study, the pre-incubation of sperm for 6 h prior to fertilization *in vitro* resulted in significantly more female bastocysts on day 9 of culture *in vitro* but did not affect the cleavage rate or development to blastocyst stage. In contrast, pre-incubation for 24 h resulted in a significantly lower rate of fertilization and development but an overall sex ratio that did not differ from 1 : 1. However, a greater proportion of blastocysts that hatched by day 9 of development were females in the 24-h pre-incubation group than in the control or 6-h pre-incubation groups.

It is well documented, that *in vitro* male embryos reach the more advanced stages earlier than their female counterparts (Xu et al. 1991; Kochhar et al. 2001) and selection of developmentally more advanced embryos in anticipation that they have a greater developmental capacity may be one of the underlying causes of a preponderance of males among IVP calves (Hasler 2000). The overall tendency for more males among developmentally advanced embryos was also noted among hatched blastocysts in the control (0-h pretreatment) group in the present study, however, it was not statistically significant. The significant shift in sex ratio in favour of females among hatched blastocysts on day 9 following prolonged pre-incuba-

Table 1. Blastocyst production from sperm pre-incubated *in vitro* for different periods of time

Timing of pre-incubation(h)	Oocytes for IVM	Oocytes cleaved	% Cleaved/oocytes	% Blastocysts/oocyte	% Blastocysts/cleaved
0	2007	1431	69.8 ± 2.6[a]	20.2 ± 2.1[a]	33.0 ± 3.9[a]
6	2173	1509	67.6 ± 3.1[a]	16.3 ± 1.9[a]	36.4 ± 4.0[a]
24	2114	955	45.7 ± 3.9[b]	8.6 ± 1.9[b]	17.9 ± 4.6[b]

Cleavage and blastocyst rates are expressed as least squares mean ± standard error of the mean.
[a,b]Different superscripts in the same column indicate significant differences ($p < 0.05$).

Table 2. Sperm pre-incubation time in relation to sex and developmental stage of day 9 bovine blastocyst

	Stage of development						Total blastocysts		
	Hatched			Expanded					
Timing of pre-incubation (h)	M	F	Ratio (M : F)	M	F	Ratio (M : F)	M	F	Ratio (M : F)
0	33	21	1.57[a]	50	63	0.79[a]	83	84	0.99[a]
6	25	30	0.84[a]	31	55	0.56[a]	56	85	0.66[Aa]
24	5	15	0.34[Ab]	14	15	0.94[a]	19	30	0.64[a]
Total	63	66	0.95	95	133	0.71	158	199	0.79

[A]Capital letter in superscript indicates significant difference in sex ratio compared with the theoretical 1 : 1 ($p < 0.01$).
[a,b]Small letters in superscript in the same column indicate significant differences in actual sex ratios between groups ($p < 0.01$); different superscripts in the same column indicate significant differences.

tion of sperm suggests either fewer males at the outset of development or a preferential loss of developmental potential of males during culture.

The fertile life of spermatozoa *in vitro* is suggested to be about 3–5 days when stored at ambient temperatures (10–21°C), however its fertility declines earlier at temperatures higher than 25°C (Vishwanath and Shannon 1999). The sperm longevity has been shown to be prolonged to 30 h by binding to epithelial cells *in vitro* at 39°C suggesting that the oviduct may also maintain its viability (Pollard et al. 1991). In the present study, pre-incubation of spermatozoa in the absence of oviductal cells for 24 h reduced the fertilization capacity by about 50%. In the ram, the sperm motility reached 65% after 24-h incubation at 39°C (Smith et al. 1993). The loss of ability to fertilize has been attributed mainly to oxidative stress and free-radicals production that affects the sperm survival potential (Vishwanath and Shannon 1999).

The sex ratio among *in vitro*-produced bovine embryos varies according to the method of production, culture and analysis (Bernardi and Delouis 1996; Gutierrez-Adan et al. 2001; Kochhar et al. 2001; Larson et al. 2001). In general, distortion in sex ratio among embryos produced *in vitro* has been attributed to media composition. There is no clear evidence as to whether defined medium (SOF) or cell co-culture system leads to a deviation in sex ratio. However, media supplementation with fetal calf serum (FCS) and glucose were shown to influence the sex ratio among *in vitro*-produced blastocysts (Gutierrez-Adan et al. 2001; Larson et al. 2001). Larson et al. (2001) showed that in the presence of glucose, more than 75% of blastocysts produced in SOF medium were males. The SOF medium containing FCS was not only shown to accelerate the rate of development but also to enhance survival of male blastocyst (Gutierrez-Adan et al. 2001). The same authors found that cell co-culture in TCM medium shifted the sex ratio of day-7 blastocysts in favour of males but on day 8 it was in favour of females.

In the present study, the TCM199 medium supplemented with SS and BOEC was used for IVC. We cannot exclude that culture conditions could also affect the observed deviation in the sex ratio although all embryos were cultured under the same conditions. This complicating factor might be avoided by comparing the sex ratio of day-9 blastocysts to sex ratio at the time of fertilization (e.g. zygote stage) within the same treatment. This approach could provide an indication as to whether survival is influenced by the sex of the embryo. However, this was beyond the sensitivity of the sexing assay and scope of the present study.

It has recently been reported that timing of sperm–oocyte interaction is also a factor in determining the sex ratio in pools of *in vitro*-produced embryos (Kochhar et al. 2001). The predominance of females following the pre-incubation of spermatozoa in fertilization medium suggests a greater longevity of the X-chromosome bearing spermatozoa. The similar rate of embryo loss between cleavage and blastocyst for the 0-, 6- and 24-h pre-treatment (49, 51 and 37%) would argue against preferential loss of male embryos in the later two groups.

The timing of insemination, in relation to the maturation state of an oocyte has also shown to influence the sex ratio of bovine pre-attachment embryos (Dominko and First 1997; Gutierrez-Adan et al. 1999; Rorie 1999). Oocytes inseminated immediately after the first polar body (pb) extrusion or a few hours before ovulation *in vivo*, tend to develop into female embryos. However, a majority oocytes inseminated 8 h after extrusion of the first pb or a few hours after ovulation developed into males (Dominko and First, 1997). These findings suggest the preferential processing of X- and Y-chromosome-bearing sperm by oocyte cytoplasm depending on the maturation state and support the hypothesis that X sperm show greater longevity and penetrate the majority of oocytes when they become ready for fertilization. Watkins et al. (1996) revealed a tendency for X-chromosome-bearing sperm after 24 h of incubation to show higher longevity in motility, higher progression and hyperactivation when compared with Y-chromosome-bearing spermatozoa. It is not known whether this influences their longevity *in vitro*. However, in the present study, the oocytes matured *in vitro* were randomly assigned to the treatment groups to eliminate maturational biases on fertilization.

In conclusion, under the conditions described here, prolonged incubation of sperm for 24 h prior to insemination creates conditions that cause a reduction in fertilization and development but give rise to more female-hatched blastocysts on day 9 of development possibly through a fertilization advantage to X-chromosome-bearing sperm.

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References

Bazer FW, Geisert RD, Zavy MT, 1987: Fertilization, cleavage and implantation. In: Hafez ESE (ed.), Reproduction in farm animals. Lea & Febiger: Philadelphia, pp. 210–228.

Bernardi ML, Delouis C, 1996: Sex-related differences in the developmental rate of *in-vitro* matured/*in-vitro* fertilized ovine embryos. Hum Reprod **11,** 621–626.

Bredbacka P, Peippo J, 1992: Sex diagnosis of ovine and bovine embryos by enzymatic amplification and digestion of DNA from the ZFY/ZFX locus. Agric Sci Fin **2,** 233–238.

Dominko T, First NL, 1997: Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. Theriogenology **47,** 1041–1050.

Gutierrez-Adan A, Perez-Garnelo S, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J, 1999: Relationship between sex ratio and time of insemination according to both time of ovulation and maturation state of oocyte. Zygote **7,** 37–43.

Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, la Fuente de J, 2001: Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology **55,** 1117–1126.

Hasler JF, 2000: *In-vitro* production of cattle embryos: problems with pregnancies and parturition. Hum Reprod **15** (Suppl 5), 47–58.

Hawk HW, 1987: Transport and fate of spermatozoa after insemination of cattle. J Dairy Sci **70,** 1487–1500.

Hunter RHF, 1989: Aging of the unfertilized cow egg *in vivo*: How soon is fertility compromised? Vet Record **124,** 489–490.

King WA, Yadav BR, Xu KP, Picard L, Sirard MA, Vernini Supplizi A, Betteridge KJ, 1991: The sex ratios of bovine embryos produced *in vivo* and *in vitro*. Theriogenology **36,** 779–788.

Kochhar HPS, Peippo J, King WA, 2001: Sex related embryo development. Theriogenology **55,** 3–14.

Larson AM, Kimura K, Kubisch HM, Roberts RM, 2001: Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ. Proc Natl Acad Sci USA **98,** 9677–9682.

Parrish JJ, Susko-Parrish JL, Leibfried-Ruthledge ML, Crister ES, Eyestone WL, First NL, 1986: Bovine *in vitro* fertilization with frozen thawed semen. Theriogenology **25,** 591–600.

Penfold LM, Holt C, Holt WV, Welch GR, Cran DG, Johnson LA, 1998: Comparative motility of X and Y chromosome bearing bovine sperm on the basis of DNA content by flow sorting. Mol Reprod Dev **50,** 323–327.

Pollard JW, Plante C, King WA, Hansen PJ, Betteridge KJ, Suarez SS, 1991: Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells. Biol Reprod **44,** 102–107.

Rorie RW, 1999: Effect of timing of artificial insemination on sex ratio. Theriogenology **52,** 1273–1280.

SAS, 1989: User's guide: Statistics (Version 5 Ed.). SAS Institute Inc., Cary, NC, USA.

Sarkar S, Jolly DJ, Friedmann T, Jones OW, 1984: Swimming behavior of X and Y human sperm. Differentiation **27,** 120–125.

Smith JF, Asher GW, Briggs RM, Morrow CJ, Murray GR, Oliver JE, Parr J, Valdhuizen FA, Upreti GC, 1993: Effect of diluent storage time on pregnancy rate in ewes after intra uterine insemination. Proc NZ Soc Anim Prod **53,** 295–302.

Watkins AM, Chan PJ, Patton WC, Jacobson JD, King A, 1996: Sperm kinetics and morphology before and after fractionation on discontinuous Percoll gradient for sex preselection: computerized analyses. Arch Androl **37,** 1–5.

Van Muster EB, Stap J, Hoebe RA, te Meerman GJ, Aten JA, 1999: Differences in volume of X- and Y-chromosome bearing bovine sperm heads matches difference in DNA content. Cytometry **35,** 125–128.

Vishwanath R, Shannon P, 1999: Do sperm cell age? A review of the physiological changes in sperm during storage at ambient temperature. Reprod Fertil Dev **9,** 321–331.

Xu KP, Yadav BR, King WA, Betteridge KJ, 1991: Sex-related differences in developmental rates of bovine embryos produced *in vitro*. Mol Reprod Dev **31,** 249–252.

Xu KP, Yadav BR, Rorie RW, Plante L, Betteridge KJ, King WA, 1992: Development and viability of bovine embryos derived from oocytes matured and fertilized *in vitro* and co-cultured with bovine oviductal cells. J Reprod Fertil **94,** 33–43.

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