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Research Article

Evaluation of Embryonic Sex Ratio in the Left and Right Uterine Horns of Super Ovulated Goats

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Abstract

The objectives of this study are to determine whether there are differences in the number of ovulations between the left and right ovaries, and the male embryo implantation between the left and right uterine horn in goats. Twenty-four multiparous goats were superovulated and used as donors. An intra-vaginal progesterone-impregnated CIDR device was inserted to the estrous synchronization donors for 13 days during the early breeding season. Super ovulations were induced by eight doses of Folltropin with its concentrations decreasing starting at days 10-13 of the estrus cycle. All goats were inseminated with 6×10^8 sperm twice via artificial insemination 42 h and 50 h after removal of CIDR. Semen from three goats, that on average sired an even sex ratio (1:1) for X and Y sperm, were mixed and deposited into the cervix. The embryos from the left and right uterine horn were collected by surgery on the sixth day following insemination and evaluated based on morphological evaluation of developmental stage and quality. The numbers of total corpora lutea, eggs, and embryos per right ovary (6.3 ± 1.2, 5.7 ± 1.7, 5.1 ± 2.1) was significantly greater than the left ovary (5.1 ± 1.4, 4.3 ± 2.0, 4.0 ± 1.9). One hundred ninety-eight embryos (95 from the left uterine horn and 103 from the right uterine horn) were sex-typed by DNA analyses for the amelogenin gene. There were significant differences ($p<0.05$) in the sex ratio of embryos between the right uterine (69M / 34F) and the left uterine (45M / 50F) horns. In conclusion, the male ratio of embryos harvested from the right uterine horn of superovulated goats by insemination with 50% X- and 50% Y-bearing sperm was significantly higher than the left uterine horn. The ovulation capacity of the right ovary was more than the left ovary.

Keywords: Goat; Ovary; Oocyte; Sperm; Embryo; Sex ratio

Introduction

Sex determination is important for reproduction of all mammals. The determination of gender for an offspring depends on whether an X- or a Y - bearing sperm fertilizes the oocyte. While based on a random event, the X- or Y- bearing sperm are equally capable of fertilizing the mammalian oocytes [1]. The sex-determining role of the X- and Y- bearing sperm may be preceded by maternal factors which regulate the sperm penetration of oocytes. The maternal dominance hypothesis demonstrates that females who are more dominant than others are more likely to conceive sons [2], revealing selective differences for X- or Y- bearing sperm in different species. However, it remains to be seen if these selective differences are due to the differences of left and right uterine horns, or the number of oocytes. Furthermore, this theory has yet to be evaluated in goats. Previous studies in mice indicated that ovulation rate, number of fetuses, and total fetal and placental weight were higher in the right than left ovary [3], and ovarian and uterine function is asymmetrical [4]. Improved maternal health may provide optimal conditions for ovum of the right ovary [5]. This might then regulate the development of the zona pellucida, which subsequently increases the likelihood of fertilization by a Y- bearing sperm. Overall, these results suggest that an imbalance between the left and right uterine horns might affect embryonic gender and development.

Recently, Hylan [6] found that bovine embryos originating from the left and right ovary were more likely to be female and male, respectively. More males were produced in the right uterine horn than in the left horn when both ovaries of female gerbils were removed and then returned to their original locations [7]. PCR of each single pronuclear present within the ooplasm can determine gender [1]. Altogether, these observations indicate that mammalian oocytes might not play a role in gender selection since both X- and Y-bearing sperm might simultaneously fertilize a single oocyte. To our knowledge, there are limited studies on the in vivo selection of X- or Y-bearing sperm from the oocyte in left or right ovary in goats. Whether gender selection in goats is possible and whether the maternal dominance hypothesis is plausible requires further studies. In this study, we seek to produce embryos with multiple ovulation and insemination of both 50% X- and 50% Y-bearing sperm in goats. The total numbers of corpora lutea, eggs and embryos per ovary from the right and the left uterine horns will be assessed. Finally, PCR for Amelogenin (AML) will be performed to determine the sex and ratio of embryos from the left or right uterus [8].

Material and Methods

Multiple ovulations

Multiple ovulations in 24 farm goats (XinJiang Province, China) were performed during mid- August of 2010 based on the mediated method as described by Taneja et al. [9] and McNatty et al. [10]. The goats were maintained on natural pastures and were provided with supplementary feeds in carrot just prior to multiple ovulations. The time of estrus and ovulation was controlled by a progesterone-releasing intra-vaginal device (EAZI-BREERPCIDR, InterAg, Hamilton, New Zealand) for 13d. Multiple ovulations were conducted with a total dose of 200 mg of Folltropin (400 mg, 20 mL for each phial, Bioniche Animal Health Canada Inc, Belleville, ON, Canada), given i.m. in eight doses every 12 h with decreasing

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concentrations (i.e.40, 40; 30, 30; 20, 20; 10, 10 mg, from days 10 to 13 of the estrus cycle). The animal handling methodology was approved by the Chinese Agricultural Association.

Preparation of sperm

Semen was collected in an artificial vagina from three goats of proven fertility at the end of August. Ejaculates were diluted to a concentration of 10^9 sperm/mL with a modified TRIS medium [11], cooled to 4°C for 1.5 h and maintained in a refrigerator until further use. The semen were evaluated as described by Nadir [12], and re-analyzed to estimate the X-and Y-purity using a high-speed cell sorter modified for sperm (SX MoFlo®, Dako Inc., Beckman Coulter, Fullerton, CA, USA) as described by Welch and Johnson [13] and to predict the proportion of male and female in the resulting embryos.

Artificial insemination

Artificial inseminations were carried out using the method of Baril and Vallet [14] and Baril et al. [15]. Briefly, twenty-four goats were inseminated with 0.6 mL mixed semen (about 6×10^8 sperm per donor) and the semen were deposited twice into the external of the cervix (0.3 mL semen per insemination from the three goats), respectively at 42 h and 50 h after the removal of the CIDR.

Recover and evaluation of embryos

The embryos from the left and right uterine horns were recovered using the surgical uterine flushing techniques 6 d after AI. The Corpora Lutea (CL) and eggs per ovary were observed and counted. The anticipated stage of embryonic development was a compact morula. Quality of viable embryos was scored by a single observer based on the classification of Lindner and Wright [16] that considered compactness and homogeneity of the cell mass. Each viable embryo was then assigned a quality grade of excellent, good, fair, or poor. An Unfertilized Ovum (UFO) was designated to an embryo when there was no sign of cleavage or when two to six possible blastomeres or cytoplasmic fragments failed to reveal nuclei under examination of smears at 400× under differential interference contrast optics (Nikon, Japan). Embryos that had blastomeres with nuclei but were too underdeveloped or retarded to be considered viable embryos were designated as degenerate. All embryos with grades of excellent, good, and degenerate without UFOs were taken by straws (one embryo one straw), and then transferred to liquid nitrogen for storage until use.

Primer design and sequencing

The flanking nested PCR primers were designed using the ClustalW program and the BLAST program according to the bovine amelogenin cDNA sequences (AMELX, GenBank accession no. NM_001014984; AMELY, GenBank accession no. NM_174240). The outer nested primers (amelF1: 5'-CATGGTGCCAGCTCAGCAG-3' and amelR1: 5'-CCGCTTGGTCTTGTCTGTTGC-3') produced 367 and 304 bp X- and Y-amplicons, respectively. The inner nested primers (amelF2: 5'-CAGCAACCAATGATGCCAGTTC-3' and amelR2: 5'-GTCTTGTCTGTTGCTGGCCA-3') produced 329 and 266 bp X- and Y-amplicons, respectively. Primer specificity was verified using the genomic DNA (5ng / reaction) extracted from the blood of male and female goats using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). PCR-products were purified using the QIAEX II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturers' instructions. Cloning was performed using Teasy vector Kit (Applied Biosystems, Weiterstadt, Germany) and Sequenced in Tarkala Company.

DNA sample extracted and amplification

DNA samples of the embryos from the left and from right uterine horns that were flushed three times with double distilled water to remove contaminants of other cells were amplified with the outer and inner nested primers, as previously described [17]. Thawing straws were gently shaken in a water bath at 37°C and each embryo was manually isolated using an inverter microscope (Nikon, Japan). Single embryo was dissolved directly on the slide using 20 μL of lysis buffer (200 mM KOH, 50 mM DTT) for 10 min at room temperature, then incubated at 65°C for 10 min prior to amplification (to dissolve cell components). Four microlitres (about 20-30 cells) lysis solution were transferred to the PCR tube. Nested PCR was conducted in 25 μL reactions containing 12 μL Taq Mix (50 mmol/L KCl, 20 mmol/L Tris–HCl, 3 mmol/L $MgSO_4$, 400 mmol/L dNTPs, 0.1 U/μL Taq polymerase), 1.5 μL of each outer nested primer (0.4 μmol/L), 1 μL glycerol and 5 μL ddH_2O. Both rounds of nested amplification were carried out in a Biometra TGradient thermocycler with one cycle of 95°C for 4 min, 25 cycles of 95°C for 30s, 62°C for 30s, and 72°C for 30s, and a final extension step at 72°C for 5 min, with 2μL of the primary reaction used as template for the nested PCR. A 5°C/s ramp speed was used in the inner nested primer PCR to reduce the total reaction time that the Taq polymerase was exposed to the elevated pH caused by the lysis buffer. The PCR products were separated by 1% agarose gel electrophoresis, stained with EB and analyzed using Typhoon Trio system with Image Quant TL v2005 software (Amersham Biosciences, Piscataway, NJ, USA). The female embryo produced an X band for the inner nested primer, and the male produced both an X and a Y band for the inner nested primer.

Statistical analysis

Data from the corpora lutea, eggs and embryos per ovary were analyzed using One-Way ANOVA implementing the Compare Means program of the SPSS Software (Version11.5, SPSS Inc. San Rafael, CA, USA). Proportional data for the sex ratio of embryos were analyzed by a Chi-Square test using the Nonparametric Test program of the SPSS Software. A confidence level of $p<0.05$ was used for statistical significance.

Results

The PCR for amelogenin to determine sex is shown in Figure 1. The band patterns between male and female were distinct, and sex determination was conducted based on electrophoresis. The sequencing results showed that the outer nested primers produced

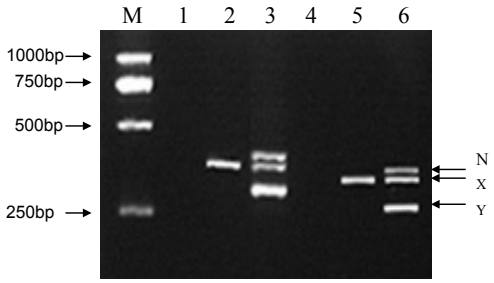

Figure 1: Sex determination by nested amelogenin PCR for goats.

Note: M: TaKaRa DL-2000 DNA marker; 1:a no-template negative control for outer primer (−);2: female produced a X band for outer primer; 3: male produced both a X , a Y and a non specific band for outer primer; 4: a no-template negative control for inner primer (−);5:female produced a X band for inner primer; 6: male produced both a X , a Y and a non specific band for inner primer. The X indicated the AMELX band, and the Y indicated the AMELY band. The N indicated a non specific band.

349 and 289 bp X- and Y-amplicons, respectively, and the inner nested primers produced 311 and 251 bp X- and Y-amplicons, respectively. Amelogenin is located on both sex chromosomes with two diagnostic ins/del of 60 bp. The results also showed a single band for females and a double band for males. Although an additional non-specific band was observed in males, it did not interfere with determining the sex based on differential banding patterns between males and females (Figure 2).

The number of total corpora lutea, eggs, embryos per ovary, and the sex ratio of embryos for the right or left uterine were harvested by in vivo insemination. The semen from these three goats of proven fertility by theoretical sex ratio (1:1) for X and Y sperm are presented in Table 1. The number of total corpora lutea, eggs, and embryos for the right ovary (6.3 ± 1.2, 5.7 ± 1.7, 5.1 ± 2.1) were significantly higher than that of the left ovary (5.1 ± 1.4, 4.3 ± 2.0, 4.0 ± 1.9 respectively) ($p<0.05$). There was a significant difference in the sex ratio of embryos between the right (69M / 34F) and left uterine horn (45M / 50F) ($p<0.05$).

Discussion

In this study, a significant difference in sex ratio of embryos between the left and right uterine horns was observed in superovulated goats. The embryos collected in the right uterine horns beared a higher proportion of males, whereas the embryos from the left uterine horns beared both males and females. These observations were consistent with previous reports in bovine [6] and Mongolian gerbil [7]. However, mouse uterine beared a higher proportion of females [1]. These differences suggest that there may be different mechanisms that alter the birth sex ratio among goat, bovine, gerbil and mice. In ancient times, Hippocrates' prophesy predicted that the male and female embryos were typically on the right and left uterine horns, respectively. This suggests that mammalian mothers may have some influence over the gender selection of their offspring [2]. In this study, the Y to X-sperm ratio was approximately equal, but the number of male embryos was significantly higher in the right uterine horn. These findings strongly support the idea that oocytes or environmental factors from the right uterine horn are more conducive to fertilization by the Y sperm than the X sperm. Based on our findings, the number of corpora lutea and total eggs in the right ovary was significantly higher than those of the left ovary. Therefore, in goats, the ovulation capacity of the right ovary is significantly higher than that of left ovary. Similar observations were seen in the hamster [4]. In Swiss-Webster and hybrid mice, Chiroptera, the right and left uterine horns exhibited differential function [3,18]. In cattle, the right ovary exhibited a higher normal ovulation cycle compared to the left ovary [19]. Altogether, these results indicate that the right ovarian follicles might maturely develop and ovulate at a higher rate than the left ovary. With over 30 years of studies, it has only been recently appreciated of the functional differences between the right and left uterine horn. However, the mechanism and context contributing to this observation remains unclear and further studies are required.

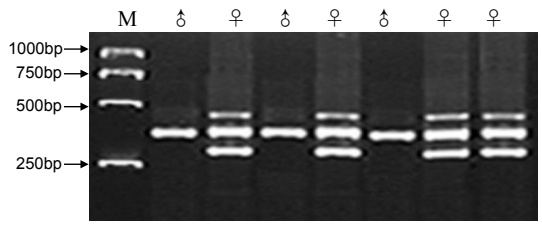

Figure 2: A part of results of sex determination by nested amelogenin PCR for goat embryos.

Note: M: TaKaRa DL-2000 DNA marker; ♀: female embryos produced a X band; ♂: male embryos produced both a X, a Y and a non specific band. Detail of an ethidiume bromide stained agarose gel showed amplicons of the amelogenin gene fragments of lane 2-8 embryo samples from left and right uterine horn.

Recovery of embryos from goat uterine showed intrauterine embryo migration. However, little is known about the timing of this process [20,21]. Intrauterine migration of embryos can occur within a week after embryonic recovery in ewes and heifers [22]. Whether this also occurs in goats is unknown. Embryos enter the uterus from the fallopian tubes about 3 to 4 days after fertilization [23]. At this time, the embryos begin to migrate and mature in the uterine lumen [22]. In ruminants with a single ovulation, intrauterine migration of embryos rarely occurs. However, intrauterine migration could be observed in about 4% of pregnancies [20,24]. An increase in ovulation rate has been associated with migration [20,23,25], thus the multiple ovulation procedures used in this experiment could result in a higher migration. This might create a deviation in embryo numbers collected from each horn. However, this does not alter our conclusion that ovulation and fertilization might occur at a higher rate in the right uterine than the left uterine in goats.

In addition, the amelogenin gene in goat is located on both the sex chromosomes, with two diagnostic insertions and deletions found within the amplified region of the Y-specific gene. In this study, we have established a simple and accurate method for determining the sex of goats based on DNA analyses of the amelogenin gene. In most mammals, the SRY (sex-determining region Y) gene determines the male sex. Amelogenin, which exists on both X and Y chromosomes, has also been used to determine sex in cattle and deer [26,27]. In all, using amelogenin amplification for sex determination could be use for goat embryo sexing, as well as for other mammals.

Conclusion

In conclusion, our findings demonstrate that the ratio of male embryos harvested from the right uterine horns of superovulated goats by in vivo insemination was significantly higher than the left uterine. Furthermore, compared to the left, the right ovary exhibited greater ovulation capacity. The goat oocytes or uterine and oviductal environment of the right uterine can select for Y-bearing sperm to increase the likelihood of bearing a male goat.

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Type of oocyte	Orpora lutea per ovary	Total eggs per ovary	Total embryos per ovary	Sex ratio of sperm (Y/X)	Sex ratio of embryos (M/F)
Left ovary	5.1 ± 1.4[a] N = 24	4.3 ± 2.0[a] N = 24	4.0 ± 1.9[a] N = 24	50.8/49.2[a] N = 3	45/50[a] N = 95
Right ovary	6.3 ± 1.2[b] N = 24	5.7 ± 1.7[b] N = 24	5.1 ± 2.1[b] N = 24	50.8/49.2[a] N = 3	69/34[b] N = 103

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

Table 1: Total corpora lutea, eggs and embryos per ovary and sex ratio of embryos harvested from the left and right uterine horns of superovulated goats by in vivo insemination with 50% X- and 50% Y-bearing sperm.

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