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*Abstract-* Sperm sexing represents the most promising approach for production of gender-specific livestock under commercial settings. Flow cytometry is currently the method of choice for sexing sperm cells, but remains costly, has low throughput and lowers fertility in vivo. Thus, the study was aimed to test Percoll density centrifugation for sexing sperm cells in small ruminants. Semen from both rams (n = 4) and bucks (n = 4) was collected by electro-ejaculation, cryopreserved and further used for Percoll density centrifugation (PDC). Sperm sexing was confirmed by conventional PCR by determining the ratio of X and Y-bearing sperm cells. The semen before and after PDC in goats did not alter proportion of X-bearing (30%A vs 28%A; 34%A vs 41%A; 55%A vs 56%A; 42%A vs 40%A) or Y-bearing (70%A vs 72%A; 66%A vs 59%A; 45%A vs 44%A; 58%A vs 60%A).

*Keywords:* *capra hircus, ovis aries, IVF, gender section,sexed semen.*

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# Use of Percoll Density Centrifugation for Sperm Sexing in Small Ruminants

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*Abstract-* Sperm sexing represents the most promising approach for production of gender-specific livestock under commercial settings. Flow cytometry is currently the method of choice for sexing sperm cells, but remains costly, has low throughput and lowers fertility in vivo. Thus, the study was aimed to test Percoll density centrifugation for sexing sperm cells in small ruminants. Semen from both rams (n = 4) and bucks (n = 4) was collected by electro-ejaculation, cryopreserved and further used for Percoll density centrifugation (PDC). Sperm sexing was confirmed by conventional PCR by determining the ratio of X and Y-bearing sperm cells. The semen before and after PDC in goats did not alter proportion of X-bearing (30%[A] vs 28%A; 34%A vs 41%A; 55%A vs 56%A; 42%A vs 40%A) or Y-bearing (70%A vs 72%A; 66%A vs 59%A; 45%A vs 44%A; 58%A vs 60%A). However, semen from one ram was efficiently sexed by PDC for both X-bearing (46%A vs 62%B; 37%A vs 34%A; 52%A vs 54%A; 60%A vs 70%A) or Y-bearing (54%A vs 38%B; 63%A vs 66%A; 48%A vs 46%A; 40%A vs 30%A). In conclusion, Percoll density centrifugation was effective for sexing sheep sperm cells but requires further improvements for its usage for small ruminants.

*Keywords: capra hircus, ovis aries, IVF, gender section, sexed semen.*

## I. Introduction

Sheep production is an economic activity performed on almost all continents under diverse edaphoclimatic conditions for production of meat, skin, wool, and milk for a wide array of applications (Ferreira-Silva et al 2016). Reproduction plays a major role in productivity in most, if not all, sheep and goat production schemes (Oliveira et al 2016; Ferreira-Silva et al 2017abc). Thus, gender selection may contribute to enhance small ruminant production by meeting the demand of each production system.

Gender selection has a great economic impact upon livestock production systems since females are more profitable for dairy farms, males are more attractive for beef farms or other applications with gender-specific demands (Taylor et al 1985; Ruvuna et al 1992; Hohenboken 1999; Rath and Johnson 2008). Endangered species could also benefit from gender selection during programs for reestablishing their populations (Seidel 2003/2007).

The discovery of sex chromosomes in 1910, demonstrated the genetic basis of gender choice in mammals. Sex pre-selection has been pursued more than 70 years now, where multiple methods have been developed to separate sperm cells into two X-specific and Y-specific populations (Johnson 1994). Despite numerous attempts, methods based on differences in DNA content between X and Y sperm cells are the sole option for efficient sexing sperm cells (Johnson 1994; Rath and Johnson 2008).

The difference in DNA content between X and Y bovine sperm cells is of approximately 4% and allows accuracy of 90% in sexing sperm cells (Windsor et al 1993; Chandler et al 1999). The usage of flow cytometry to sexing sperm cells has detrimental drawbacks, since it remains expensive, has low throughput and damages sperm cells, that ultimately leads to lower fertility (Rath and Johnson 2008). Due to the limited availability of this technology, sexing sperm cells has been mostly applied to cattle and much later in sheep (Rath and Johnson 2008). Thus, alternative methods for sexing sperm cells with high accuracy and lower detrimental effects are still on demand (Espinosa-Cervantes and Córdova-Izquierdo 2013).

Percoll density centrifugation (PDC) was described as an alternative to sexing sperm cells (Blottner et al 1993; Resende et al 2009; Malik et al 2011). The difference in density due to DNA content between X and Y in bovine sperm cells is 0,06% and can be captured by this approach (Windsor et al 1993; Chandler et al 1999). In cattle, PDC allows sperm cell sexing with the accuracy of around 70% and high viability for in vitro fertilization (Resende et al 2009).

Small ruminants should benefit from sperm cell sexing both for in vivo or in vitro fertilization schemes. However, few reports have described the usage of sexed semen for these purposes, primarily in sheep (Rath and Johnson 2008). The development of an efficient PDC approach would circumvent most of the limitations found using sexed semen by flow cytometry. The work was aimed to test the accuracy and

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reproducibility of a discontinuous Percoll density gradient for sexing sperm cells of small ruminants.

## II. Material and Methods

*a) Semen Collection and Evaluation*

Semen was collected by rectal electroejaculation from four Anglo-Nubian bucks and four Santa Inês rams and used after cryopreservation as previously described by Arruda et al. (2014). All animals were of proven fertility and subject to andrology exam. Only ejaculates carrying 80% motility, vigor >3, >80% of normal sperm cells, and <10% abnormal morphology after collection were used for cryopreservation, as suggested by CBRA (2013). Only frozen-thawed semen was further used for sperm sexing. Frozen-Thawed semen that had >30% motility, vigor >3, >80% of normal sperm cells, and <10% abnormal morphology were used for sperm sexing using Percoll density gradient.

*b) Preparation of Percoll density gradient*

Percoll working solution was prepared by serial dilutions using a stock solution (Percoll 90%, pH 7.4 and 280-290 mOsm/kg H2O), em DMEM medium containing 0.3% BSA pH 7.4, to obtain a density of 1.123g mL-1. A continuos Percoll density gradient was prepared by layering each working solution in polystyrene conic tubes and freezing and thawing to make continuous layers in the gradient.

*c) Density gradient centrifugation and recovery of sperm cells*

Pools of 100 to 200 x 106 sperm cells mL-1 were layered on each Percoll density gradient (one sample for each male was prepared by mixing frozen-thawed semen from three different ejaculates). Both rams and bucks were considered independent replicates (n = 4 for each species). Gradients were centrifuged from 550 to 1,500g in horizontal rotors, for 10-30 minutes at 4 to 25 oC. The supernatant of each tube was discarded. Sperm pellets were recovered and used for sexing efficiency and cell viability assays.

*d) DNA Extraction*

Fresh semen samples or after cryopreservation of each species were subjected to DNA extraction using a protocol with 10% Cetyl Trimethyl Ammonium Bromide (CTAB), as previously described by Solléro et al. (2004). Samples were treated with 500µL of 10% CTAB at 65 oC for one hour. Samples were further centrifuged at 15,800g for two minutes, and the supernatant was transferred to a conical tube containing a 24:1 chloroform of alcohol isoamyl alcohol solution. Moreover, samples were centrifuged at 15,800g for 15 minutes, supernatant was transferred to a conical tube containing cold isopropyl alcohol and kept at -20 oC for 30 minutes. Finally, samples were further centrifuged at 15,800g for 30 minutes, but supernatant was discarded and pellet was washed in 75% ethanol and followed by 100% ethanol. The pellet was stored in ultra-pure water at -20 oC.

*e) Real Time PCR*

Genomic DNA was used for PCR reactions in order to detect SRY and Aml-X genes, as described by Phua et al (2003). Multiplex PCR reactions were performed containing 100ng of DNA from each sample, 1.5mM MgCl2, 10pmoles of each primer, 200µM dNTP, 1IU Taq polymerase, 1X PCR buffer (20mM Tris-HCl pH 8.4, 50mM KCl, 2mM MgCl2), and final volume of 25µL. The PCR reaction had an initial denaturation step at 94 oC for 5 minutes, followed by 34 cycles of 94 oC for 45 seconds, 58 oC for 45 seconds, 72 oC for one minute and a final extension at 72 oC for 7 minutes. Amplicons were detected by electrophoresis in 2% agarose gels stained with ethidium bromite, visualized with UV transilluminator and compared with a 50pb DNA ladder (Invitrogen, USA).Primers used in the experiment are described in Table 1.

*Table 1:* Primers used in the Experiment

| Gene | Primers | Amplicon (bp) | Access Number (Genbank) |
|---|---|---|---|
| Aml-X | F - CAGTAGCTCCAGCTCCAGC<br>R - TGTGCATCCCTTCATTGGC | 300 | AF215887.1 (Capra hircus) |
| SRY | F - ATGAATAGAACGGTGCAATC<br>R - GGAAGAGGTTTTCCCAAAGGC | 116 | Z30646 (Capra hircus) |

*f) Statistical Analysis*

The data obtained from sperm sexing conventional PCR was analyzed by the chi-square test. To determine if sex ratio was skewed after PDC, percentages were compared with their standard means and the non-sexed control. Differences with a probability of 5% were considered significant.

## III. Results

Semen from both ram and bucks displayed acceptable seminal parameters for cryopreservation (Table 2). Semen was further cryopreserved and subject to the PDC.

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*Table 2:* Sperm parameters (mean+SD)after collection for Anglo-Nubian bucks and Santa Inês rams

| Species | Animals (n) | Ejaculate Volume (mL) | Progressive Motility (%) | Sperm Vigor (1-5 scale) | Sperm Stirring (1-5 scale) | Sperm Concentration ($10^6$ x $mL^{-1}$) |
|---|---|---|---|---|---|---|
| Caprine | 4 | 0.70+0.08 | 85.0+3.5 | 3.5+0.3 | 4.5+0.4 | 4,023+931 |
| Ovine | 4 | 0.71+0.14 | 82.5+7.5 | 3.6+0.4 | 4.1+0.2 | 4,136+841 |

SD: standard deviation.

The ratio of X and Y-bearing sperm cells in non-sexed samples demonstrated that there is substantial variation among males of both species (Table 3). The PDC did not allow sexing of goat sperm cells since it did not skew ratios of X and Y sperm cells. However, the semen of a single ram was successfully sexed, with a significant ($P < 0.05$) increase in X-bearing sperm cells (Table 3). Despite this latter result, the semen subject to the PDC displayed detrimental effects, where motile sperm cells could not be recovered.

*Table 3:* Sperm sexing efficiency after Percoll density centrifugation determined by Real Time PCR (RT-qPCR)

| Species | Animal (Sample) | X-bearing Sperm cells (%) | | Y-bearing Sperm cells (%) | |
|---|---|---|---|---|---|
| | | Control | Sexed | Control | Sexed |
| Caprine | 1 | $30^A$ | $28^A$ | $70^A$ | $72^A$ |
| | 2 | $34^A$ | $41^A$ | $66^A$ | $59^A$ |
| | 3 | $55^A$ | $56^A$ | $45^A$ | $44^A$ |
| | 4 | $42^A$ | $40^A$ | $58^A$ | $60^A$ |
| Ovine | 1 | $46^A$ | $62^B$ | $54^A$ | $38^B$ |
| | 2 | $37^A$ | $34^A$ | $63^A$ | $66^A$ |
| | 3 | $52^A$ | $54^A$ | $48^A$ | $46^A$ |
| | 4 | $60^A$ | $70^A$ | $40^A$ | $30^A$ |

Different letters on same line for two consecutive columns (e.g. X or Y-bearing sperm cells) indicate statistical significance by the chi-square test ($P<0.05$).

## IV. Discussion

Gender selection remains attractive due to its potential for economic impact upon livestock production systems, since skewed gender ratios may yield different probabilities for both meat and production systems (Taylor et al 1985; Ruvuna et al 1992; Hohenboken 1999; Rath and Johnson 2008). Endangered species could also benefit from sperm sexing technologies for gender selection, to meet specific demands in preservation programs (Seidel 2003/2007).

The sperm sexing by the Percoll density centrifugation was efficient for only one sheep semen sample. Sperm sexing for commercial purposes is currently performed by flow cytometry, which is based on DNA content variation between X and Y-bearing cells, but remains expensive and reduces both fertility in vitro and in vivo (Rath and Johnson 2008), possibly due to DNA damage during the process. The PDC is an alternative approach that distinguishes X and Y cells by their difference in density, since X-bearing sperm cells



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hold a small but yet reproducible difference in sex chromosome size (Resende et al 2009). Moreover, Percoll density centrifugation may be particularly attractive to small ruminant species which have less access to flow cytometry-based sperm cell sexing (Rath and Johnson 2008). Despite the more recent potential for bovine sperm cells (Resende et al 2009), previous reports using Percoll density centrifugation for sperm sexing did not achieve such results (Lisuka et al 1987; Iwasaki et al 1988). Thus, Percoll density centrifugation needs further improvement and may require additional adaptations for small ruminants. Factors such as usage of fresh semen for sexing, variations in gradient preparation, sperm cell density variation and their interaction in sperm sexing are currently under investigation.

The factors that have caused impairment of sperm cell motility after Percoll density centrifugation are currently unknown. Percoll density centrifugation was initially described as a method for sperm selection in sheep and goats before in vitro fertilization (Palomo et al 1999; Morris et al 2003), ruling out possible species-specific factors as the source for this impairment. The effect of different batches of Percoll is under current investigation. Further research is needed to identify these negative factors on sperm motility or to describe approaches to circumvent them.

## V. Conclusion

Sperm sexing by Percoll gradient density centrifugation was effective for sexing sheep sperm cells but requires further improvements for its usage in small ruminants.

*Conflict of Interest*

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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