

Sperm Motility and Viability of Chilled Ram Semen Collected by Artificial Vagina and Electroejaculation

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Abstract. The current study aimed to evaluate motility and viability of chilled ram semen collected by artificial vagina and electroejaculation during the non-breeding season. A total of 18 ejaculates from clinically healthy rams in a non-breeding season were collected by artificial vagina (AV; $n = 9$) and electroejaculation (EE; $n = 9$) and submitted to preliminary evaluation. After that all ejaculates were diluted through a Tris-based extender containing low concentration (5%) of glycerol and egg yolk and stored at 5°C for 48 hours. Motility and viability of semen samples were evaluated at 0, 6, 24 and 48 h of storage. Estimation of motility was carried out by a microscopic digital system, and viability was assessed by the one-step eosin-nigrosin staining technique. Until 6 h of storage, the differences between motility and viability of semen collected by AV or EE were non-significant, while at 24 and 48 h the parameters were higher ($P < 0.05$) in semen collected by AV. The increasing time of storage correlated negatively with the evaluated parameters ($P < 0.05$). In conclusion, the chilled semen from ram in the non-breeding season collected by AV demonstrated better motility and viability until 48 h, compared with the semen collected by EE, and could be recommended for artificial insemination up to 24 h after storage at 5°C. The time of storage had a negative effect on sperm motility and viability ($P < 0.05$).

Introduction

The intensive sheep farming requires artificial insemination of animals with liquid semen preserved at 0–5°C for an extended period or use of frozen semen (Salmon and Maxwell, 2000; Joshi et al., 2001; Abulizi et al., 2012). Maintenance of the spermatozoa biological potential and their genetic information after storage in low temperatures is crucial for sheep breeding practice (Gundogan, 2009). Many reasons can affect the quality of ejaculates intended to chilling or freezing but one of the most important is the method of semen collection (Maxwell and Watson, 1996; Maxwell et al., 2007; Jimenez-Rabadan et al., 2012; Maksimović et al., 2018).

In ram, the main semen collection techniques are artificial vagina (AV) and electroejaculation (EE). The semen collection with AV is similar to a natural service and is easy to apply, but requires an extended training period of rams and not all animals can be successfully trained (Wulster-Radcliffe et al., 2001). Electroejaculation has some advantages because it is a quick method, appropriate for use in non-trained males or those with problematic sexual behaviour, and the collected semen has an increased volume compared with ejaculates collected by AV (Mattner and Voglmayr, 1962; Marco-Jimenez et al., 2005; Jimenez-Rabadan et al., 2012).

Different studies have presented the effects of both methods on small ruminant semen quality (Matthews et al., 2003; Marco-Jimenez et al., 2005; Ledesma et al., 2015). Electroejaculation is very variable and the collected semen is often contaminated with urine, and thus had poor motility (Fennessy et al., 1990). In a study by Marco-Jimenez et al. (2005), electroejaculation resulted in a lower recovery efficiency, as a consequence of a lack of response to the electrical stimulation and the fresh semen quality was not significantly different between recovery methods, except for the concentration of spermatozoa. However, a higher number of stable and functional spermatozoa were found for frozen-thawed spermatozoa collected by electroejaculation than by artificial vagina. In contrast, other authors (Matthews et al., 2003; Bopape et al., 2015) recorded better percentage of motile and live sperm cell semen collected by AV compared with EE, and Jiménez-Rabadán et al. (2012) indicated a higher sperm quality after thawing of cryopreserved semen obtained by artificial vagina.

Ram spermatozoa are sensitive to extreme temperature changes during cooling and freezing which induce damage to the sperm plasma membrane or structural changes leading to a capacitation process (Hammerstedt et al., 1990; Salamon and Maxwell, 1995; Watson, 1990). The cryopreserved sperm cells, used for artificial insemination, have to be eight times increased to achieve normal fertilization rates because of the lower viability, reduced motility and increased abnormal apical ridge (Shannon and Vishwanath, 1995; Gillan et al., 1997). Also, a significant seasonal

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variation in the semen quality of small ruminants has been reported in different studies (Ritar, 1993; Roca et al., 1992; Maxwell et al., 2007). In spite of all abovementioned, the data for an effect of a semen collection method on the sperm quality during storage at low temperature regimens is still debatable.

The aim of this study was to evaluate the motility and viability of chilled semen from Pleven Blackhead rams collected by artificial vagina and electroejaculation during the non-breeding season.

Material and Methods

Experimental animals and management

The study was carried out in 18 Pleven Blackhead rams with an average age of 1.8 ± 0.9 years, body weight 65 ± 4.9 kg, reared in individual boxes at a small ruminant unit, located at N 42.25 and E 25.38. The animals were housed in the uniform technology, feeding, immunoprophylaxis regimen and drinking of water *at libitum*. Investigation was conducted during the non-breeding season. The study was performed in accordance with the recommendations of Animal Ethics Committee and regulations for human attitude and animal protection.

Semen collection, processing and evaluation

Prior to the semen collection, an abstinence period of 30 days was provided and a physical examination was performed. The rams were separated in two groups according to the semen collection method: group I ($n = 9$, AV) and group II ($n = 9$, EE). A total of 18 ejaculates were used for this study. In group I, semen was collected by the artificial vagina method in presence of a teaser sheep, while in group II the ejaculates were collected by electro-ejaculator for small ruminants (Minitübe, Germany). All semen samples were obtained between 9.00 and 10.00 a.m., then transported to the laboratory within 5 minutes, placed on a water bath at 35°C and submitted to a primary assessment. The volume was measured by a graduated pipette and mass motility was evaluated on the base of wave motion observed under the microscope at 10× magnification (scale 0–5, Evans and Maxwell, 1987). The sperm concentration ($\times 10^9$ /mL) was determined by a Photometer SpermaCue (Minitüb, Germany), calibrated for small ruminant semen. Only semen with a normal color and transparency, volume > 1 mL, concentration $> 1 \times 10^9$ /mL and wave motion > 3.5 and abnormal sperms were included in the experiment.

The semen was diluted with a Tris-glucose-glycerol-egg yolk (TGGY) extender, adapted to Evans and Maxwell's (1987) prescription. All chemicals were purchased from *Alfa Aesar (Thermo Fisher Scientific GmbH, Germany)*. TGGY stock solution included Tris-hydroxymethyl aminomethane 3.63 g, glucose 0.5 g, citric acid 1.99 g, glycerol 5 mL, gentamycin 50 μ g/mL and aqua bidestillata up to 100 mL. The stock solution was prepared one day before semen

collection and stored at 5°C. The completed extender included adding egg-yolk 5% (v/v) to a stock solution before semen collection and the extenders were placed in a water bath at 35°C.

After the primary assessment, each ejaculate was diluted at a ration 1:1 and kept on a water bath 5 minutes for adaptation of semen to the extender. Additional dilution until adjustment of the sperm concentration to 200×10^6 cells per mL was performed. The three samples of equal amounts of diluted semen of each ejaculate were stored at 5°C in a refrigerator for 48 hours.

Semen evaluation

The motility and viability of the spermatozoa were evaluated at 0, 6, 24 and 48 hours after storage at 5°C in a refrigerator.

Motility evaluation

The motility was estimated by microscopic examination using Motic Image Plus Digital System (Motic China Group Ltd, 2001–2004), including a microscope, objectives with different magnification, a digital camera and relevant software. Immediately before examination, the semen samples were gently mixed and a 5 μ L drop was placed on a slide warmed at 37°C, covered with a 20 mm \times 20 mm cover slip and observed at 200–400× by a qualified operator. The average value of three consecutive observations at least of five different microscopic fields was calculated as a final motility (Ax et al., 2000).

Viability evaluation

The sperm viability was assessed by one-step eosin-nigrosin staining technique (Mortimer, 1994). The smear was prepared by mixing 2 equal drops of semen and staining solution (0.67% eosin -Y and 10% nigrosin dissolved in 0.9% sodium chloride in distilled water). After incubation of the mixture at room temperature (20 degrees Celsius) for 30 seconds, it was placed on a warm slide, spreading with a second slide and dried on air. The viability was assessed by counting 200 cells under a microscope at magnification of 400×. Sperm cells that were unstained (white) were accepted as alive, whereas stained (pink or red coloration) were considered to be dead.

Statistical analysis

The results were processed by statistical program Statistica version 7.0 (Stat-Soft., 1984–2000 Inc., Tulsa, OK, USA). The semen motility and viability were expressed as mean \pm standard deviation (Mean \pm SD). Analysis of variance (ANOVA) and Tukey's post hoc test were used for comparison of the motility and viability for the different methods of semen collection. The effect of time of storage on evaluated parameters was determined by correlation analysis. Statistical significance was accepted at $P < 0.05$.

Results and Discussion

The primary evaluation showed a significantly greater volume of ejaculate after using electroejaculation ($P < 0.05$) which confirmed the results of previous research in this topic (Table 1). The sperm concentration and wave motion of spermatozoa for both methods were close. There was no significance between motility and viability of the spermatozoa at 0 h after dilution (Fig. 1). The primary motility and viability of semen collected by AV ($78 \pm 2.9\%$ and $80.5 \pm 2.1\%$) and EE ($72 \pm 6.1\%$ and $76.3 \pm 4.5\%$) decreased slowly until 6 h of storage. A similarity in sperm motility for fresh semen collected by these methods was also stated by Marco-Jimenez et al. (2005) and Jimenez-Rabadan et al. (2012). However, the evaluated parameters negatively correlated with the increased time of storage in both groups. All correlation coefficients for motility ($R = -0.97$ (AV); $R = -0.99$ (EE) and viability ($R = -0.98$ (AV); $R = -0.95$ (EE) were highly negative ($P < 0.05$). It was in accordance with other reports for a decreased motility in ram semen during liquid storage (Kheradmand et al., 2006; Azizunnesa et al., 2014). Significant differences ($P < 0.05$) were detected among the values registered at 24 and 48 h of storage. Between abovementioned intervals, the sperm motility of spermatozoa collected by artificial vagina decreased by 8%, but still was in an acceptable range, whereas in semen collected by EE, this reduction was 21%. A similar relationship was registered for the viability (Fig. 2). The values of this parameter also differed significantly ($P < 0.05$), at 24 h and 48 h of storage at 5°C in a refrigerator (AV – $76.4 \pm 2.4\%$ and $73.4 \pm 3.4\%$ vs. EE – $69.2 \pm 6.4\%$ and $65.1 \pm 5.8\%$). In this aspect, Maksimović et al. (2018) observed that some of the ram semen samples collected by EE almost completely lost sperm cell activity at 24 h after storage. Semen samples from

Table 1. Parameters of ejaculates recorded in primary evaluation according to the method of semen collection

Method of semen collection	Volume (mL)	Concentration ($\times 10^9/\text{mL}$)	Wave motion (Scale 0–5)
	Mean \pm SD	Mean \pm SD	Mean \pm SD
Electroejaculation (n = 9)	1.8 ± 0.35^a	2.36 ± 0.64^a	3.64 ± 0.12^a
Artificial vagina (n = 9)	1.5 ± 0.24^a	2.84 ± 0.42^a	3.72 ± 0.20^a

The values in a column marked with a different superscript differ at $P < 0.05$.

buck ejaculates collected by AV with greater values for most assessed sperm parameters than those obtained by EE have been revealed by Jimenez-Rabadan et al. (2012) and Bopape et al. (2015). In contrast, Ledesma et al. (2015) reported for more resistant sperm cells to cryodamage in ram semen collected by EE compared with sperm collected by AV. It was evidenced by the higher percentage of sperm with intact and functional plasma membrane, intact acrosome and a greater in vitro fertilizing potential observed after thawing. The determined slower reduction of the values of semen collected by AV was in support of the assertion for better resistance of spermatozoa to a low temperature.

The current study is in agreement with the hypothesis of other investigations for the influence of the semen collection method on resistance of sperm to low temperatures for an extended period. Jimenez-Rabadan et al. (2012) accepted that the EE procedure changes the chemical composition of the seminal plasma connected with sperm cell freezing capacity. Marco-Jiménez et al. (2008) showed differences in the protein profile in samples obtained by both methods, which can

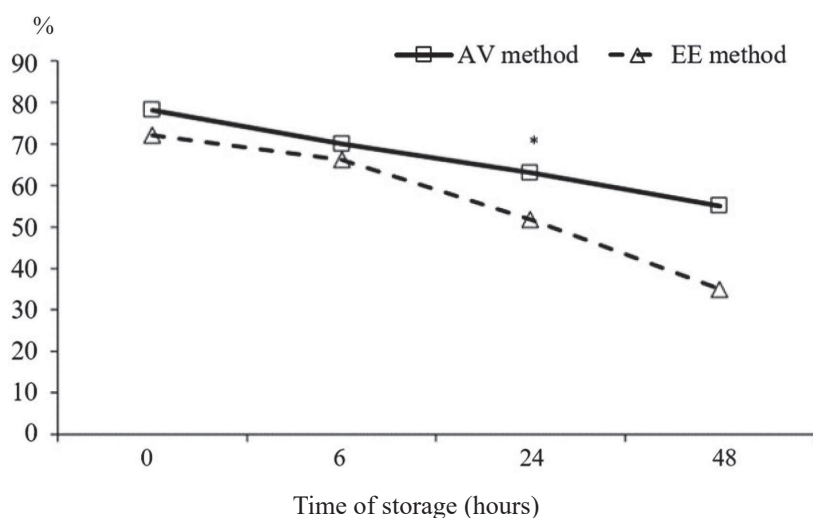


Fig. 1. Sperm motility of chilled semen during storage at a temperature of 5°C for 48 hours according to the method of semen collection

The values in the intervals marked with an asterisk differ at $P < 0.05$.

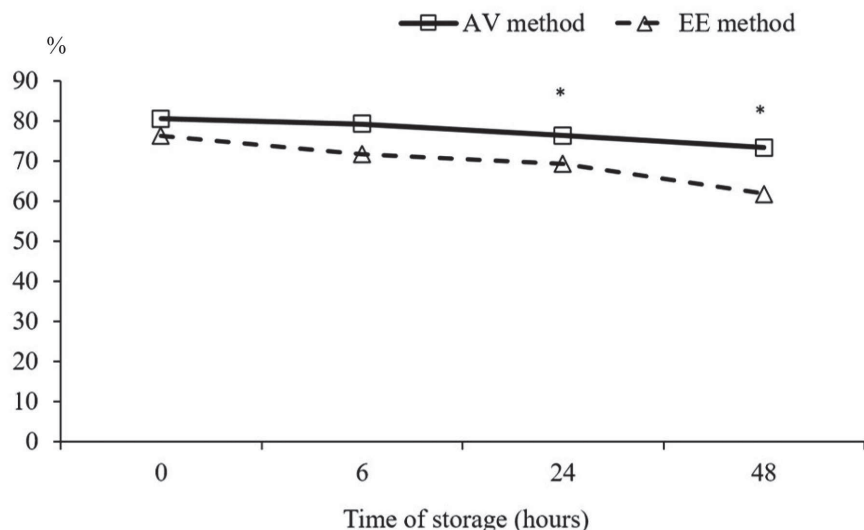


Fig. 2. Sperm viability of chilled semen during storage at a temperature of 5°C for 48 hours according to the method of semen collection

The values in the intervals marked with an asterisk differ at $P < 0.05$.

be attributed to prevention of the sperm against cold-shock damage (Barrios et al., 2005). In addition, the better motility and viability of spermatozoa collected by AV than EE at 24 of storage indicated that it can be recommended for artificial insemination.

In conclusion, the chilled semen from ram in the non-breeding season collected by artificial vagina and diluted through the abovementioned semen extender demonstrated better motility and viability until 48 h, compared with semen collected by electroejaculation, and could be recommended for artificial insemination

up to 24 h after storage at 5°C. The time of storage had a negative effect on sperm motility and viability ($P < 0.05$). This data will be useful when ram semen has to be stored for a short time in a refrigerator or transported in a longer distance.

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