

MORPHOLOGICAL EVALUATION OF RAM SEMEN RELATED TO THE COLLECTION METHOD

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Abstract

The study was carried out to perform a morphological evaluation of ram semen related to the collection method. A total of 20 Turcana Alba rams aged 3 to 6 years old were used in the study. The study was conducted in a farm located in Cluj County. Semen samples were collected from each animal using two collection methods: the artificial vagina (AV) and the electrostimulation (E). The average variation of normal spermatozoa (%) for the artificial vagina method was $X \pm S = 94.31 \pm 2.07$, and by electrostimulation $X \pm S = 93.62 \pm 3.19$. Regarding the percentage of primary anomalies recorded, no significant changes were found: $X \pm S = 2.27 \pm 1.05$ in the case of AV and $X \pm S = 2.83 \pm 1.61$ for E. The dynamic of secondary anomalies (%) present in the analyzed semen was as follows: for AV it was $X \pm S = 2.94 \pm 1.14$, while when collected by electrostimulation an average of $X \pm S = 3.04 \pm 1.36$ was found. The percentages of immature spermatozoa were approximately equal: $X \pm S = 0.57 \pm 0.25$ for AV and $X \pm S = 0.59 \pm 0.40$ for the electrostimulation method. The results showed that both sperm collection methods can be used in field conditions, with the mention that the values obtained were superior for the artificial vagina method.

Key words: artificial vagina, electrostimulation, ram, semen

The first step for having good quality sperm is the use of an effective method for the collection of the ejaculates. Artificial vaginas (AV) are widely used for semen collection from ruminants (Leboeuf B. *et al*, 2000), but this technique requires a previous training period (Wulster-Radcliffe M.C. *et al*, 2001). This is a practical method and use of this technique does not lead to alterations in semen quality compared to that when there is natural mating. Another method of collection such as the electroejaculation (EE) involves an alternative when males are not trained to AV or for wild species, and may be a viable method of repeatedly collecting ejaculates from individual specimens without causing death (Santiago-Moreno *et al*, 2009; Abril-Sánchez S. *et al*, 2019).

Motility and morphology of spermatozoa are accepted as markers of fertility for a long time. It has been shown that these parameters of semen are strongly associated with successful conception *in vivo* (Davis R.O., Siemers R.J., 1995; Abadieva D. *et al*, 2014). Additionally, the authors indicated a

high correlation coefficient between the morphology and fertilization capacity of the spermatozoa (Zhang B.R. *et al*, 1998; Bohlooli S.H. *et al*, 2012). Also poor morphology has been associated with deviant kinematic and inefficient penetration of both cervical mucus and the zona pellucida (Morales P.K., Overstreet D.R., 1998).

Evaluation of sperm morphology is part of the assessment of fertility in animal reproduction. Sperm morphological evaluation determines the percentage of normal and abnormal sperm (Koziol J.H., Armstrong C.L., 2018; Barth A.D., Oko R.J., 1989). Microscopic examination of ejaculates indicated that sperm morphological assessment has discrepancies, even within the same ejaculate, and these discrepancies create difficulties in determining bull fertility potential (Barth A.D., Oko R.J., 1989; Auger J., 2010). Various stains and methods were used for sperm morphological analysis, resulting in ambiguous outcomes (Gatimel N. *et al*, 2017a; Gatimel N. *et al*, 2017b). In this regard, eosin-nigrosin (ENS) staining has remained the most

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commonly used technique for detecting sperm morphological abnormalities (Koziol J.H., Armstrong C.L., 2018; Barth A.D., Oko R.J., 1989). However, it should be noted that sperm morphological evaluation methods can critically affect sperm morphology outcomes (Freneau G.E. *et al*, 2010; Brito L.F. *et al*, 2011; Brito L.F., 2016). The Spermac staining method, on the other hand, was first used for seminal evaluations in domestic animals and has since been applied to a range of species including goats, horses, bulls, dogs, boars, and humans (Chan P.J. *et al*, 1999; Schaäfer S., Holzmann A., 2000). A study on cat spermatozoa demonstrated that this method provides a clear view of cell morphology, particularly the acrosome (Schaäfer S., Holzmann A., 2000; Agarwal A. *et al*, 2022). The study was carried out to perform a morphological evaluation of ram semen using the Spermac stain.

MATERIAL AND METHOD

Animals: The study was conducted in a farm located in Cluj County, on 20 rams of the breed Turcana Alba divided equally into two batches according to the collection method: BAV (n= 10 rams) and BE (n=10 rams).

The including criteria of the rams in the study were: males, sexual mature, clinically healthy.

The excluding criteria from the study were: sexual imature rams or at andropause.

Semen collection and processing: Semen samples were collected from each animal using two collection methods: the artificial vagina (AV) and the electrostimulation (E) according to methodology proposed by Bogdan *et al*, 2020 and Garde J.J. *et al*, 2003 with several adaptations. For EE, we used a manual electroejaculator standardized for small ruminants. The electrical stimulation is made by the user and not by a program, like in electronic electroejaculators. A local toiled was performed by shaving and washing the prepuce with sodium bicarbonate 3% followed by the drying of the area. An enema was performed to eliminate the feces and for a better conductivity. The electrode of the electroejaculator (30 cm long and 2 cm in diameter) was inserted into rectum after the lubrication, for about 15 cm. The EE regime consisted of consecutive series of 5 seconds pulses of similar voltage, each separated by 10 seconds break. The semen was collected after 3-5 stimuli, and the process occurs without erection.

All obtained ejaculates were subjected to an initial macroscopic and microscopic evaluation and those outside the standard requirements were discarded. From each ram 4 ejaculates were

collected and a total of 68 ejaculates were included in the study.

Morphological parameters: In our study, the morphology of ram sperm was evaluated using the Spermac stain. In this way 200 spermatozoa/smear were evaluated, and the staining allowed the observation of spermatozoa with primary and secondary morphological changes represented by: lack of head, double head, lack of tail, bifid tail, twisted tail, proximally or distally flexed tail. The presence of the protoplasmic drop, which denotes the immaturity of the spermatozoa, was also determined.

Spermac®, which is a metachromatic stain, is a rapid, easy and reliable staining technique and is used to observe different levels of acrosome defects (Oettle E.E., 1986). For the Spermac method, slides were air-dried at room temperature, then fixed by immersing in a formaldehyde solution for 5 min. The slides were air-dried, then stained by immersing for 1 min in solutions A, B, and C. Solution A was composed of ultrapure water, ethyl alcohol, rose Bengal, and neutral red. Solution B was composed of ultrapure water, ethyl alcohol, pyronin Y, orange G, and Phosphomolybdic acid. Solution C was composed of ultrapure water, janus green, and fast green FCF. The slides were washed in distilled water between each staining process (7 times). Finally, the slides were washed again and air-dried at room temperature. The results from the semen samples collected by the two different methods were compared using one-way ANOVA procedures.

RESULTS AND DISCUSSIONS

The results revealed that there are differences in the number of ejaculates obtained from a ram, depending on the sperm collection method. In all the rams included in the study, the average volume of ejaculates obtained was higher for the artificial vagina method than with the electrostimulation method. Analyzing the average obtained during the study, it was found that in the case of the BAV group, $X \pm S = 1.8 \pm 0.83$ ejaculates were obtained, which resulted in $X \pm S = 2.58 \pm 0.29$ ml of sperm, while for the BE group there were collected $X \pm S = 2.8 \pm 0.44$ ejaculates from which $X \pm S = 1.2 \pm 0.18$ ml of semen were obtained. The evolution of the averages of microscopic parameters (mobility, concentration, viability, redox test, sperm resistance test), reveals that the results obtained are influenced by the collection method (*table 1*). The data distribution indicates that for all the studied variables the recorded results are in favor of the artificial vagina collection method.

Table 1

Characteristics of ram semen collected by electrostimulation and artificial vagina

Parameter	Collection method	Min.	Max.	AVERAGE STDEV
Mobility (%)	VA	83	94	89.2±3.90 ^a
	E	83	92	87.7±3.85 ^a
Concentration (x10 ⁹ spz/ml)	VA	2.5	3.5	2.79±0.33 ^a
	E	2.1	2.8	2.46±0.25 ^b
Viability (%)	VA	94	98	95.5±1.27 ^a
	E	89	94	91.8±1.87 ^b
Redox (min.)	VA	5.7	7	6.54±0.48 ^a
	E	6.2	7.8	6.87±0.44 ^a
Resistance test	VA	6800	7300	7020±172.04 ^a
	E	6700	7200	6960±142.82 ^a

^{a, b}Row means with different superscripts, differ significantly ($P < 0.05$)

In a study conducted by Matthews N. *et al*, 2003, regarding the comparison of the two methods of semen collection in rams, it was found that both collection methods were effective. However, the semen collected through the artificial vagina has a significantly ($P < 0.05$) better concentration and viability than through the electrostimulation method. Analyzing the results obtained in this study, it can be assumed that the electroejaculation method can be used in satisfactorily in order to increase viability in the breeding season, but with some reservations related to sperm concentration and viability. That is why the artificial vagina method is preferred because it leads to higher concentrations (more insemination doses) and a higher percentage of live

spermatozoa (better sperm quality). The research carried out by Gordon I., 1983, supports the same recommendations offered by these researchers. In other studies, a larger volume of semen was obtained by the electroejaculation method, but with a lower concentration than that collected with the artificial vagina (Mattner P.E., Voglmayr J.K., 1962; Salamon S., Marrant A.J., 1963; Memon M.A., Ott R.S., 1981). In his research, Bertschinger H.J., 1995 claims that the application of the electrostimulation method leads to acceptable semen, but it can rarely be compared to that collected through the artificial vagina. He also supports the same ideas promoted by his predecessors, regarding obtaining a larger volume of sperm, but of a questionable concentration.

Table 2

Dispersal of the average values of morphological parameters according to sperm collection method

Parameter	Collection method	Min	Max	AVERAGE STDEV
Normal spermatozoa (%)	VA	90	98	96.4±1.62 ^a
	E	88	97	93.9±3.04 ^b
Primary abnormalities (%)	VA	0.7	1.9	1.47±0.4 ^a
	E	0.9	2.4	1.69±0.49 ^a
Secondary abnormalities (%)	VA	1.3	2.7	1.99±0.33 ^a
	E	1.5	2.3	2.14±0.37 ^a
Immature spermatozoa (%)	VA	0	0.9	0.36±0.33 ^a
	E	0	0.9	0.48±0.30 ^a

^{a, b}Row means with different superscripts, differ significantly ($P < 0.05$)

The results of the morphological exam are presented in table 2. In the morphological examination of the semen, the results showed that there are no major differences in the studied parameters depending on the collection method. The morphologically appropriate spermatozoa had:

a well-defined head, with the acrosome colored in dark green, the nuclear portion of the sperm head in red, and the equatorial zone in pale green. The intermediate part and the tail correctly attached, in a straight position, colored in dark green (*figure 1, a -b*).

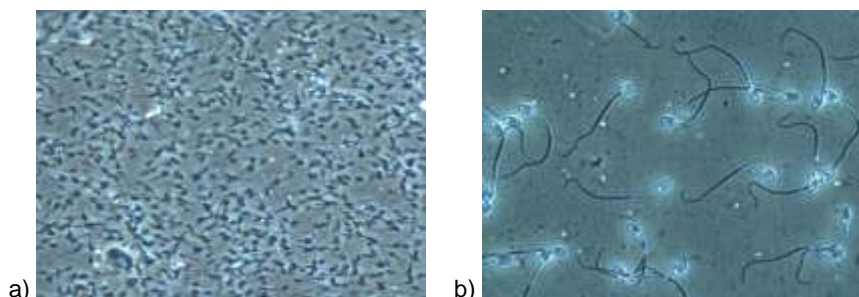


Figure 1 (a, b). **Morphologically appropriate spermatozoa**

The primary morphological changes identified by this staining were represented by the appearance of the double head, detached head,

while the secondary anomalies identified were detached tail and tail flexion (*figure 2, a-f*).

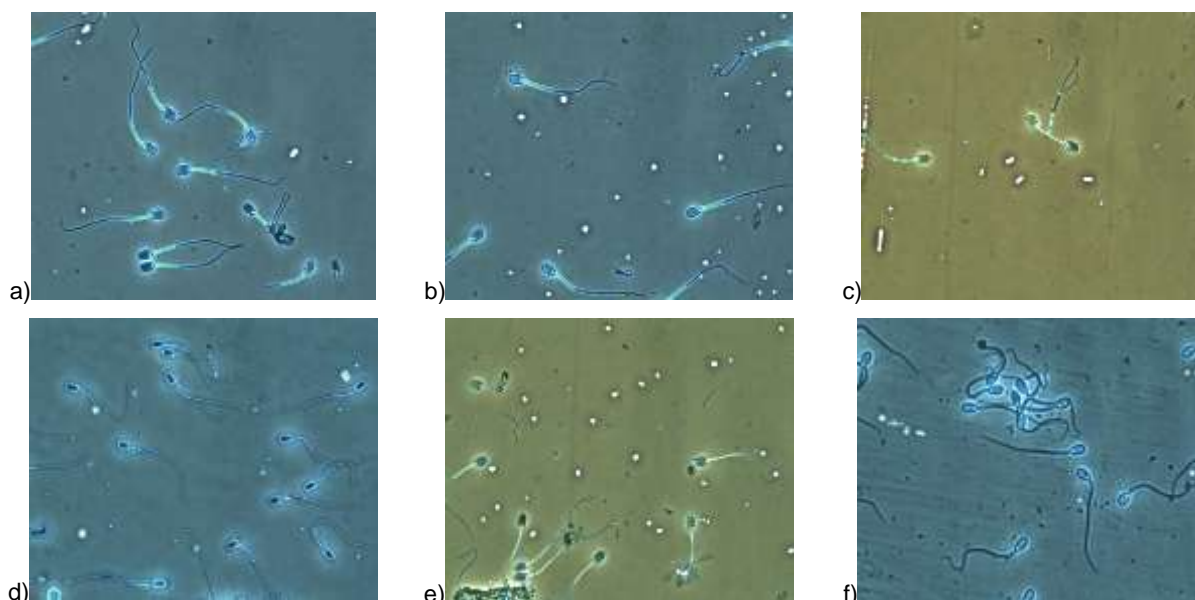


Figure 2 (a-f). **Spermatozoa with abnormalities appearance**

CONCLUSIONS

The results showed that both sperm collection methods can be used in field conditions, with the mention that the values obtained in our study were superior for the artificial vagina method.

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