MONITORING THE QUALITATIVE PARAMETERS OF THE REFRIGERATED RAM SEMEN DURING NON-BREADING SEASON

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Abstract

The association of the biotechnology of artificial insemination with the conservation of the semen allows the use of a small number of males with which a large number of females can be inseminated. Artificial insemination gives numerous genetic and economic benefits for animal production, being the safest method to bring into the herd the superior genes, of valuable individuals. The aim of this study was to evaluate the effect of Tris-based extender containing 2% glycerol and 20% egg yolk on the motility and viability of rams' semen collected in non-breeding season and refrigerated at 4^0C. During 30 days, a total of 16 ejaculates from 6 Texel rams were collected. The semen was collected by artificial vagina during May-June 2019. The main qualitative parameters: motility and viability were evaluated immediately after collection and also at 24, 48, 72, 96 and 120 hours. The results were statistically processed and the extender's influence and storage time were analyzed. In conclusion, the Tris-based extender containing glycerol and egg yolk showed a satisfactory protective effect on rams' semen collected in non-breeding season and refrigerated at 4^0C. Storage time ($P < 0.05$) has significantly affected the qualitative parameters of the semen recording a 10-15% decrease of the qualitative parameters every 24 hours.

Key words: Semen quality; ram; non-breeding season

INTRODUCTION

Artificial insemination with frozen sperm in cattle has been successfully and widely used. Unlike cows, artificial insemination in sheep using frozen sperm is not common due to the difficulty of the method and low fertility rates [12]. Ram semen cryopreservation is of high interest, particularly in European countries, aiming to increase productive parameters by animal genetic improvement in selected flocks. Additionally, the need for widespread performance of sheep artificial insemination (AI) over extended periods or at different times of the year, stimulated more research on semen preservation.

Therefore, in ewes breeding, instead of frozen semen, native or liquid preserved semen has been used and a 60% or higher fertility rate can be achieved [10]. The greatest difficulty with liquid storage is the 10% to 35% loss of sperm fertility if the storage time is over 24 h. Even though semen can remain motile for up to a week, its fertility capacity can decrease [11]. Although successful fertility rates have been reported after a storage period for more than 24 h [10] in some studies, contradictory or low fertility results have also been reported [10]. It is necessary to extend the liquid storage time to benefit from artificial insemination techniques on a wider platform. More research on the subject is required to achieve optimum fertility rates in storage periods over 48 h.

In ewes breeding, due to the large scale of the herd to be inseminated and breeding performed over long distances, sperm must be transported without any problems and loss

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in its fertility capacity. Moreover, to take advantage of the rams for longer periods and in various times of the year, sperm storage technologies should be more advanced. The basic principle of sperm storage is to reduce the spermatozoa metabolism, thus extending its life. For this purpose, sperm is stored at low temperatures (4–22 °C, liquid storage) or frozen (−196 °C, long-term storage) [12]. The success of short- and long-term storage methods is, on the whole, dependent on the storage temperature, cooling rate, chemical composition of the extender, reactive oxygen species (ROS), and seminal plasma composition. The protein and chemical composition of seminal plasma and its effect on sperm function are dependent on the secretory activity of the accessory sex glands, epididymis and testis. This varies with breed, animal and ejaculate, due to numerous sources of intra- and inter-animal variation including climate (high temperatures and humidity negatively effect sperm quality), plane of nutrition, sexual maturity, health status, frequency of collection and ejaculate number [5].

The aim of this study was to evaluate the effect of Tris-based extender containing 2% glycerol and 20% egg yolk on the motility and viability of rams' semen collected in non-breeding season and refrigerated at 4^0 C.

MATERIAL AND METHOD

Six Texel rams were used in the present study. The rams were housed at ICDCOC Palas Constanta. From May to June in the non-breeding season, the semen was collected twice a week using an artificial vagina and was kept in a water bath at 37°C until use. The volume of the ejaculate is appreciated right after collection, by reading the divisions on the graduated test tube of the collecting glass. Raw semen was analyzed and used if the following criteria were met: volume bigger than 0.5 ml, motility and membrane integrity ≥70%, and concentration ≥2.5 × 109 spermatozoa/mL.

In order to assess the semen quality, the physical and morphocytological parameters were evaluated at collection, and after 24, 48, 72, 96 and 120 hours of refrigeration at 4^0 C. The analysis of the morphological parameters was performed by optical microscopy techniques.

Tris-citric-glucose-egg yolk (20%) - glycerol 2% was used as dilution medium. Motility was assessed by manual evaluation technique [13] in wet enviroment, under the optical microscope (Novex, Holland) (x100 magnification) equipped with heating plate maintained at 37° C and camera.The evaluation of motility must take into account the speed, linearity and lateral movements of spermatozoa.

Structural integrity of plasma membranes (viability) was assessed by the eosin-nigrosine staining method [4].

The results were statistically processed using IBM SPSS Statistics 20 software.

RESULTS AND DISCUSSION

Several studies have demonstrated that season has an influence on ram's reproductive characteristics [7], [14]. They reported that the standard method of evaluating the fertility of male breeding is the examination of sperm production [3].

The semen collected for dilution came from 6 Texel rams between 2.5 and 3 years old. For each male, the ejaculate was collected using the artificial vagina. The collections were performed during non-breeding season, twice a week. From each ram, an average of 15 ejaculate were collected. The morpho-cytological parameters of the collected semen are shown in Table 1.

Table 1 Morpho-cytological parameters of semen in Texel rams collected during non-breeding season

	Spermatozoa indices	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6
1.	Ejaculates (n)	16	14	15	16	15	16
2.	Volume (ml)	1.21± 0.04	1.89± 0.31	1.34± 0.25	1.31± 0.16	1.56± 0.22	1.17± 0.09
3.	*pH*	7.0± 0.04	7.1± 0.10	6.9± 0.21	6.9± 0.17	7.1± 0.08	7.0± 0.25
4.	Concentration (mild./ ml)	2.99± 0.11	4.12± 0.22	3.01± 0.34	3.23± 0.19	4.62± 0.08	2.86± 0.29

The results are presented as mean ± standard deviation

Semen extenders have been designed to protect and maintain spermatozoa during the processing and storage of semen [9]. Extender as a medium creates optimal conditions for the extension of the life of sperm and, most importantly, preserves the reproductive ability of semen. Extender replaces the seminal plasma and assumes its role. In other words, extender maintains motility and fertilizing capacity, and preserves sperm membrane integrity. It is also necessary to dilute semen to obtain a larger number of doses from the ejaculate.

The extender's composition is critical for the success of ram's liquid semen conservation at low temperature. Tris-based and skimmed-milk extenders are currently the most used for preserving goat semen. In addition to Tris, the extender also contains fructose or glucose, egg yolk, antibiotic and citric acid. Antioxidants can be used to remove reactive oxygen species generated from the intracellular compartments of spermatozoa. In order to be protected against cold shock, in addition to lipids from egg yolk, the dilution environment must contain also other cryoprotectant substances. The medium contains glycerol as cryoprotectant substance. The glycerol concentration in the dilution medium is 2% (v / v) which leads to a final concentration in diluted semen of about 1%. For preparation, the volume of glycerin required is calculated and added to the egg yolk medium (20%), heated to 300ºC, to facilitate homogenization. The glycerol used is of purity p.a. (Sigma, Germany) to prevent the environment's contamination.

The volume of ejaculates collected during non-breeding season from Texel rams varied between 1.17 and 1.89, and the pH of the semen ranged between 6.9 and 7.1. The semen concentration was established by assessing the consistency of the semen. It resulted an average concentration of 4.15 billion spermatozoa / ml. Concentration (density) is an important feature on which the level of dilution and the number of fractions subsequently depend.

Motility was assessed by manual evaluation technique [13] in wet environment, under the optical microscope (Novex, Holland) (x100 magnification) equipped with heating plate maintained at 37°C and camera. An average motility of 91.7% resulted. The minimum permissible motility for the semen to be diluted for refrigeration is of 80%.

Determination of viability is one of the basic elements of semen quality assessment, being of great importance, in order to distinguish between dead and living immotile speramatozoa, especially for the samples in which many immotile spermatozoa are found. In order to assess viability, we used the eosin-nigrosine staining method.

The semen from the 6 rams was diluted with Tris-based dilution medium and then refrigerated at +4ºC. At 24-hour intervals, the motility and viability of the diluted semen were evaluated. The obtained results are presented in Table 2.

Table 2 Variation of motility in relation to storage time

Refrigeration Period (h)	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6
0	92±2.54	93.9±1.49	90.7±2.01	88.1±1.74	90.1±1.54	93.1±1.94
24	80.3±3.17	82.7±2.23	79.2±2.29	75.9±2.26	79.4±1.98	81.9±2.81
48	65.2±2.96	67.8±2.44	65.7±2.94	62±2.01	65.5±1.86	67.5±2.65
72	51.4±2.24	52.8±2.10	51.6±2.84	48.3±1.54	51.7±1.81	52.9±2.30
96	37.1±1.73	38.5±2.20	37.2±1.71	35.1±1.56	37.3±1.49	38.3±1.72
120	22.9±2.20	27.6±1.59	25.3±2.13	20.3±2.37	23.5±1.65	26.8±1.95

The results are presented as mean ± standard deviation

According to these data it can be observed that within the 5 days in which the semen was refrigerated and tested, there was a decrease of motility between 65-70%, from the moment of collection until the day 5, but with significant differences for each male in part. There were no significant differences regarding the rate of decreased motility from 0 to 120 hours, between male 2 and male 5, between male 2 and male 6 and between male 5 and male 6 ($p > 0.05$).

Determination of viability is one of the basic elements of semen quality assessment, being of great importance, in order to distinguish between dead and living immotile speramatozoa, especially for the samples in which many immotile spermatozoa are found. In order to assess viability, we used the eosin-nigrosine staining method. The viability of the diluted semen was evaluated at 24-hrs intervals. The obtained results are presented in Table 3.

Table 3 Variation of viability in relation to storage time

Refrigeration Period (h)	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6
0	93.4±1.34	94.7±1.32	91.8±1.40	89.2±1.68	91.5±1.28	94.4±1.65
24	81.7±2.43	82.8±1.96	80±2.35	78.4±1.78	80.3±1.39	83.3±2.13
48	67.2±2.32	68.5±2.138	66.2±2.01	64.6±2.23	66.1±1.70	68.5±1.87
72	52.5±1.82	53.7±1.93	52.3±1.90	50.6±2.13	51.7±2.08	53.7±1.52
96	38.2±1.42	38.5±1.22	37.8±1.70	36.9±2.01	37.5±1.39	38.7±1.68
120	24.5±1.55	28.3±1.64	26.5±1.65	21.7±2.36	25.2±1.25	28.1±2.26

The results are presented as mean ± standard deviation

According to these data it can be observed that within the 5 days in which the semen was refrigerated and tested, there was a decrease of motility between 65-70%, from the moment of collection until the day 5, but with significant differences for each male in part. There were no significant differences regarding the rate of decreased viability from 0 to 120 hours, between male 2 and male 5, between male 2 and male 6 and between male 5 and male 6 ($p > 0.05$).

The most commonly used non-penetrating cryoprotective for freezing ram semen is egg yolk due to its protective effect on plasma and acrosomal membranes [12]. Due to the interactions between this cryoprotective and semen plasma enzymes, such as egg yolk coagulation enzyme (EYCE), it was concluded that a very important factor is the percentage of egg yolk in the extender A variety of results were observed regarding concentrations between 2% and 20% of egg yolk in extender [1]. Glycerol is the most widely used permeable cryoprotectant for the preservation of farm animals semen because it prevents phase changes of the extender when added to the medium in concentrations below 3% [6].

Concentrations greater than 3% lead to decreased survival during preservation and deterioration of acrosomes, resulting in decreased fertility [6]. Glycerol has an osmotic effect and has a direct effect on the plasma membrane by binding to membrane phospholipids. Although the main cryoprotective effect of glycerol is expressed at the extracellular level, it can enter the cell and remain bound to the plasma membrane or cytoplasm [2].

Liquid storage of sperm up to 2–4 days is the main goal of artificial insemination in sheep breeding programs. However, decrease in sperm fertility in durations longer than 24 h of liquid storage is the most important problem. For this purpose, improvements in both the artificial insemination techniques and the storage techniques are required. Mitochondria of spermatozoa are different from those of the somatic cells in terms of morphology and biochemistry. Mitochondrial energy metabolism plays a vital role in the continuation of sperm functions. In liquid storage,

spermatozoa need to be able to maintain their energy reserves and their mitochondria function fully to survive for an extended period of time without losing their motility.

In a study conducted by Maxwell and Salamon [8], it was reported that more than 24 h of storage rapidly decreased fertility. The rate of decline in fertility was between 10% and 15% per day. In our study there was recorded a decrease rate between 10% and 15%. Researches will continue with the quality assessment of semen during breeding season (August-September).

CONCLUSIONS

The Tris-based extender containing glycerol and egg yolk showed a satisfactory protective effect on rams' semen collected in non-breeding season and refrigerated at 4^0 C. The volume of ejaculates collected during non-breeding season from Texel rams varied between 1.17 and 1.89, and the pH of the semen ranged between 6.9 and 7.1. The average semen concentration was 3.47 billion spermatozoa / ml, and the average motility was 91.3%, with variations between 88.1 and 93.9. After 5 days of preservation by refrigeration at 4^0 C, there was a decrease of viability between 60-70%, and a decrease of motility between 60-70% with a daily rate of decrease between 10-15%. The decrease in motility and viability depends on the individual, in our study there were significant differences ($p < 0.01$) between the studied rams.

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