

STUDY OF THE RELATIONSHIP BETWEEN THE AGE OF THE RAMS AND THE QUALITY OF THEIR EJACULATES OBTAINED OUTSIDE THE BREEDING SEASON

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Abstract: The present study aims to research the effect of age on the quality of ejaculates of rams from Synthetic population of Bulgarian dairy breed. Rams divided into two age groups were used for the experiment: G1 (2.5 years) versus G2 (6 years). Ejaculates were obtained by the method of artificial vagina and diluted with medium 6A-G. The tested ejaculates were obtained outside the breeding season. A Sperm Class Analyzer (SCA Microptic, Barcelona, Spain) was used to study sperm motility. The biochemical activity of the enzymes lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) was determined using an automatic Mindrai BA88 spectrophotometer. The activity of the enzymes was studied in sperm plasma, in aqueous and tritonic extract. Significant differences between the groups were found in total sperm motility ($P \leq 0.05$) and LDH enzyme activity in sperm plasma ($P \leq 0.01$). Regarding the efficiency of the LDH enzyme in aqueous and tritone extract, no significant differences between the groups was found. No significant differences were found between groups for GGT activity, while age had significant impact on LDH enzyme in sperm plasma.

Keywords: ram, spermatozoa, motility, LDH, GGT

Introduction

In rams, fluctuations in reproductive capacity during different seasons of the year are evaluated (*Boland et al., 1985*). The choice of fertility in sheep can be made by selecting correlated traits in young rams such as age, testicular size, testicular length and others (*Land and Carr, 1975*). In general, the sexual development of rams appears to be more closely related to body growth than to their age (*Dyrmundsson and Lees, 1972*). The qualitative indicators of ejaculate are highest in autumn and winter and low in spring and summer, the same pattern is

observed in quantitative indicators such as volume and concentration of sperm (Hafez, 1952). Sexual activity in rams changes with seasonality, which is influenced by changes in day light periods, age and social hierarchy (Mickelsen et al., 1982). They have shown that both sexual behaviour and sperm quality vary with age and breed (Aisen, 2004; Pelayo, 2019), although other authors believe that libido may be influenced more by season than by age (Aisen and Venturino, 2004). There are scientific reports of a reduction in the quantity and quality of sperm production and sperm fertility in rams during the non-breeding season (Makawi et al., 2007; Azawi and Ismaeel, 2012).

Sperm analysis involves a series of tests that evaluate various factors or functions of the sperm. Evaluation of sperm motility is one of the most widely studied parameters (Gallego et al., 2018), as well as the activity of key enzymes for the reproductive system such as lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) (Stefanov et al., 2013). The activity of certain enzymes varies in seasonally polycyclic animals (sheep, goats, horses) and shows reproductive seasonality (Gündoğan, 2005). The enzyme lactate dehydrogenase (LDH) has been shown to be important for various metabolic processes that provide energy for viability, mobility and sperm fertility (Duan and Goldberg, 2003). The function of the enzyme gamma-glutamyl transferase (GGT) is not well defined. According to some authors, the enzyme is a specific marker of sperm, plays an important role in redox balance and performs a protective function during the transport and storage of sperm in the epididymis (Hinton et al., 1991). Therefore, the evaluation of biochemical components and enzymes in semen plasma can be recommended as biological markers of sperm quality, as their values determine sperm function, integrity and damage (Tvrda et al., 2013; Tejaswi et al., 2016).

The aim of the present study was to investigate the effect of age on the quality of ejaculates from rams of the Synthetic Bulgarian Dairy population.

Material and Methods

Animals

The experiment was conducted with four clinically healthy rams of the breed Synthetic population of Bulgarian dairy, divided into two age groups: Group 1 (2.5 years - young) vs Group 2 (6 years - mature). A total of 16 ejaculates from each group obtained on 8 consecutive days were examined. The studied ejaculates were obtained outside the breed's breeding season. Rams were kept under the same breeding conditions and nutrition.

Sperm production and analysis

Sperm collection is performed by the method of an artificial vagina by an experienced operator. All obtained ejaculates were subjected to an initial macroscopic evaluation and those outside the standard requirements were discarded. Immediately after that, each ejaculate was diluted in a ratio of 1:12 with medium 6A, prepared at the Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov" - BAS. The obtained ejaculates were analyzed by computer sperm analyzer (SCA, Microptic SL, Barcelona, Spain) to determine the total motility of sperm (TM,%), progressively motile sperm (PM,%), sperm with non-progressive movement (NPM). and immotile sperm (Immotile,%).

Biochemical analysis

The extracellular activity of the enzymes lactate dehydrogenases isoenzyme C4 (LDH-C4, U / L) and gamma-glutamyl transferase (GGT, U / L) was determined in the sperm plasma. Aqueous and newt extracts were prepared to determine the intracellular activity of both enzymes. After dilution of the ejaculate, the seminal plasma was removed by centrifugation at 3500 rpm at 37 ° C for 15 minutes. The resulting sperm plasma from each tube was carefully aspirated with a micropipette into sterile Eppendorf tubes and the extracellular activity of the enzymes was determined. Distilled water (1ml) was added to the resulting precipitate and frozen at -20 ° C. After 24 h, the samples were thawed and sonicated (150 W MSE ultrasonic disintegrator) by ultrasound three times for 10 s. The tubes were then centrifuged at 12,000 rpm for 15 min. In this way, an aqueous extract is obtained, in which the sperm cells are destroyed and the intracellular enzymes are extracted. Triton extract was also obtained to completely destroy the sperm cells. To obtain it, the resulting precipitate was resuspended with 1% Triton X-100 and centrifuged under the same conditions. In this way, the intracellular activity of the enzymes in the sperm was determined. The activity of the enzymes LDH-C4 and GGT was determined by a semi-automatic spectrophotometer for clinical chemistry BA-88 (Mindray, Medical Germany GmbH, Bensheim, Germany) using a set of reagents manufactured by Via Campania - Italy.

Statistical analysis

The analysis of all results was performed with a specialized statistical analysis package IBM SPSS Statistics 23 (SPSS Inc., Chicago, USA). Comparison of sperm characteristics was performed by ANOVA statistical tests. Significance of group differences was assessed with One Sample T-Test. Results are presented as mean $\pm$ standard error (SE).

Results

Results of sperm motility analysis in both groups of rams are presented in Table 1.

Table 1. Sperm motility

Group	N	TM, % Mean $\pm$ SE	PM, % Mean $\pm$ SE	NPM, % Mean $\pm$ SE	Immotile, % Mean $\pm$ SE
G1	16	99.60 $\pm$ 0.11*	75.82 $\pm$ 2.59 ^{ns}	23.78 $\pm$ 2.50 ^{ns}	0.39 $\pm$ 0.11*
G2	16	98.08 $\pm$ 0.53*	72.05 $\pm$ 4.19 ^{ns}	26.03 $\pm$ 3.89 ^{ns}	1.91 $\pm$ 0.53*

Note: * Significant differences at $P \leq 0.05$; ^{ns} – non significant

The effect of age on sperm motility for semen obtained outside the breeding season of the breed was found to be significant only for the total motility ($P \leq 0.05$). Regarding progressively motile (PM) and non-progressively motile (NPM) sperm, no significant differences between groups were found.

The extracellular and intracellular activity of the enzyme lactate dehydrogenase (LDH-C4) is presented in Table 2. The activity of the enzyme in sperm plasma in young rams (G1) is almost twice lower than in mature rams (G2) ($P \leq 0.01$). Regarding the activity of the enzyme in aqueous and newt x100 extract, the values obtained in both groups are similar.

Table 2. Enzymatic activity of LDH-C4

Group	N	LDH-C4(U/L)		
		Sperm plasma Mean $\pm$ SE	Water extract Mean $\pm$ SE	Triton extract Mean $\pm$ SE
G1	16	6.75 $\pm$ 0.85**	6.75 $\pm$ 1.65 ^{ns}	8.25 $\pm$ 1.25 ^{ns}
G2	16	11.25 $\pm$ 1.03**	6.50 $\pm$ 2.32 ^{ns}	8.00 $\pm$ 1.41 ^{ns}

Note: ** Significant difference at $P \leq 0.01$; ns – non significant

The extracellular and intracellular activity of the enzyme gamma-glutamyl transferase (GGT) is presented in Table 3.

Table 3. Enzymatic activity of GGT in sperm plasma, water and Triton extract within age groups

Group	N	GGT (U/L)		
		Sperm plasma Mean $\pm$ SE	Water extract Mean $\pm$ SE	Triton extract Mean $\pm$ SE
G1	16	41.50 $\pm$ 5.23 ^{ns}	4.25 $\pm$ 0.47 ^{ns}	4.75 $\pm$ 0.47 ^{ns}
G2	16	42.75 $\pm$ 4.55 ^{ns}	3.75 $\pm$ 0.47 ^{ns}	4.50 $\pm$ 0.28 ^{ns}

Note: ^{ns} – non significant

The effect of ram's age was not significant on the activity of the enzyme in sperm plasma or any of studied extracts.

Discussion

The present study showed a high percentage of motile sperm in both young rams (G1) and mature rams (G2). Our results for total sperm motility are higher than those obtained by *Abadjieva et al. (2014)* (97-97.5%), who also studied sperm motility in rams outside their breeding season. High total sperm motility in rams outside the breeding season was also found by *Farshad et al. (2012)*. They have examined seasonal changes in serum testosterone concentration of LDH and characteristics of sperm in other ruminants. In their research, however, total sperm motility (85.90%) was lower than our results, but the percentage of progressively motile sperm was slightly higher (78.69%).

We also found a significant difference between G1 and G2, similar to *Ntemka et al. (2019)*, which also reveal significant differences between age groups of the rams. According to *Stefanov et al. (2013)* the activity of the androgen-dependent enzymes LDH and GGT are influenced by seasonal change. These changes also affect sperm function. The increased percentage of vital and morphologically normal sperm corresponds to increased LDH activity in the sperm. This enzyme has been found to play a significant metabolic role in sperm capacitation and fertility (*Zamiri and Khodaei, 2005*).

Despite the obtained high percentage of motile sperm, in our study we found extremely low extracellular and intracellular activity of the enzymes LDH and GGT. Low LDH activity is one of the main causes of low fertility in ram sperm (*Brooks, 2001*). According to *Duan and Goldberg (2003)*, complete inhibition of LDH activity blocks capacitance. Despite the low activity of LDH, we found that age had some effect on enzyme activity. Different from our results were results obtained by *Atroshchenko et al. (2019)*, who examined the age factor in stallions and didn't found significant differences between the groups.

In their research, *Zakrzewska et al. (2002)* obtained GGT enzyme values several times higher than ours (430.9 U/L) during the breeding season. The epidermal origin of GGT suggests the involvement of this enzyme in sperm maturation (*Kohdaira et al., 1986*). The metabolism of germ cells and the secretory glands are an important expression of GGT. This could explain why the enzyme appears as a sperm marker. In our studies, we did not find significant differences between age groups in terms of GGT activity. Like us, *Viudes-de-Castro et al. (2015)* didn't found significant correlation between the levels of GGT and sperm motility.

From the results obtained for the activity of the two enzymes during the different seasons, we believe that the activity of LDH and GGT is influenced by the breeding season of the animals and the seasons, and less by age.

Conclusions

- The age of rams affects sperm motility in ejaculates obtained outside the breeding season.
- Significant differences between age groups in LDH-C4 activity were found only in the extracellular activity of the enzyme in sperm plasma.
- No significant differences were found between groups for GGT activity.

Studija povezanosti starosti ovnova i kvaliteta njihovih ejakulata dobijenih van sezone parenja

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Rezime

Cilj ove studije bio je da se istraži uticaj starosti na kvalitet ejakulata ovnova iz sintetičke populacije bugarske mlečne rase. Za eksperiment su korišćeni ovnovi podeljeni u dve starosne grupe: G1 (2,5 godine) i G2 (6 godina). Ejakulati su dobijeni metodom veštačke vagine i razblaženi korišćenjem medijuma 6A-G. Testirani ejakulati dobijeni su van sezone parenja. Analizator klase sperme (Sperm Class Analyzer, SCA Microptic, Barselona, Španija) korišćen je za proučavanje pokretljivosti sperme. Biohemijska aktivnost enzima laktat dehidrogenaze (LDH) i gama-glutamil transferaze (GGT) određena je pomoću automatskog spektrofotometra Mindrai BA88. Aktivnost enzima proučavana je u plazmi sperme, u vodenom i tritonskom ekstraktu. Pronađene su značajne razlike između grupa u ukupnoj pokretljivosti sperme ($P \leq 0,05$) i aktivnosti enzima LDH u plazmi sperme ($P \leq 0,01$). Što se tiče efikasnosti LDH enzima u vodenom ekstraktu i ekstraktu tritona, nisu pronađene značajne razlike između grupa. Nisu pronađene značajne razlike između grupa za aktivnost GGT, dok je starost imala značajan uticaj na LDH enzim u plazmi sperme.

Ključne reči: ovan, spermatozoidi, pokretljivost, LDH, GGT

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