Biotechnology in Animal Husbandry 40 (2), 87- 99, 2024
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 636.053.453'62
https://doi.org/10.2298/BAH2402087Y

EFFECT OF COOLING AND FREEZING ON THE KINEMATIC PARAMETERS OF RAM SPERMATOZOA SEXED BY MODIFIED PROTOCOL WITH TLR7/8 LIGAND R848

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Original scientific paper

Abstract: The present study investigated the effect of sex-sorting by TLR7/8 ligand R848, cooling and cryopreservation on the motility and kinematic characteristics of ram spermatozoa for the first time. Four ejaculates per ram (n=2) were collected, pooled and after that were split to 8 equal parts. Four parts were used for sexing and four parts were analyzed as unsexed semen. The sperm sexing was done by application of the TLR7/8 ligand R848 (resiquimod). Diluted with freezing extender unsexed and sex-sorted semen were cooled in a refrigerator at temperature 5°C for 5 hours and after that freeze/thawed. Each part of semen at each step of the experiment was subjected to computer assisted semen analysis for evaluation of motility and speed parameters of spermatozoa. After sexing the significant reducing of kinematic parameters in both fractions were observed. The dilution of sexed spermatozoa with freezing extender before cooling reliably increased the progressive motility and speed parameters as VCL, VAP, and VSL in X- and Y-fractions and kept them during the cooling at relatively high level. Freezing adversely impaired the motility of sexed spermatozoa. The values of speed parameters of unsexed spermatozoa after thawing were significantly higher than those of X and Y spermatozoa. Dilution with an appropriate freezing extender and cooling are most suitable approaches for storing of sexed ram spermatozoa.

Key words: ram semen sexing, TLR7/8 ligand R848, kinematics, cooling, freezing

Introduction

The introduction of sex-sorted sperm for AI in small ruminants is of great importance for increasing the reproductive efficiency, utilizing the genetic resources, and producing the optimal sex-ratio of offspring in production systems (Gonzalez-Marín et al., 2021). Sperm sexing by flow cytometry is considered as an accurate and effective method for offspring sex selection (Vishwanath and Moreno, 2018). This pre-selection of spermatozoa bases on the different DNA amount between the X and Y chromosomes (Johnson, 2000). Recently, the flow cytometric procedure for sorting of ram semen was also commercialized (Gonzalez-Marín et al., 2021). However, the currently available commercial method still presents many limitations like high cost, low sperm number per dose, sperm damage and decreased fertility (Quelhas et al., 2021). Additionally, the use of this technology requires expensive equipment and skilled personnel. Regardless the fact that at this stage only flow cytometry gives a separation with an accuracy of up to 90%, the scientific search for different ways for sexing of ram sperm are still in progress.

The main goal of these studies is to discover an easier method for semen sexing, maintaining good quality of sperm after treatment and to make it accessible to animal husbandry practice. Two methods, adapted to these requirements have been recently tested for ram spermatozoa. One of them uses bovine serum albumin (BSA) gradient for spermatozoa separation (Hadi and Al-Timimi, 2013; Solihati et al., 2019a; Yotov et al., 2021). Another is based on the activation of *Toll- like receptor 7/8 (TLR7/8)* on X- sperm by ligand resiquimod (R848) (Umehara et al., 2020; Ren et al., 2021; Abadjieva et al., 2022; Yotov et al., 2024a). Necessity to inseminate a large flock of ewes after estrus synchronization for 1-2 days provoke the investigations about the appropriate way for storage of the sexing spermatozoa fractions. While there are some data about the effect of cooling and freezing on the motility and survivability of ram spermatozoa sexed by BSA column (Maxwell et al., 1984; Solihati et al., 2019b; Yotov et al., 2024b), information on storage of ram spermatozoa separated by R848 in cooled and frozen state is not available.

The objective of present study was to investigate the possibility of storage of ram spermatozoa, sexed by modified R848 protocol, by cooling and freezing. Given the objective, the effects of freezing extender and the cooling and freezing processes on the motility and different kinematic parameters of non-sexed and sexed ram spermatozoa were analyzed and compared.

Material and Methods

Animals, semen collection, primary assessment and processing

The investigation was performed with four ejaculates per Pleven Blackhead ram (n=2) collected by artificial vagina method. After semen collection

the samples were transported to the laboratory, placed on a water bath at 37°C, and submitted to a primary assessment.

Only ejaculates with normal colour and transparency, volume > 1.5 mL, sperm concentration > 1.5 $\times 10^9$/mL, motile sperm > 70% and lack of agglutination were used in this experiment. To minimize ram individual effect all the ejaculates were pooled and after that were split to 8 equal parts. Four parts were used for sexing and four parts were analyzed as non-sexed semen. Each part of the semen was subjected to computer assisted semen analysis (CASA, Microptic S.L. Barcelona, Spain) after each step of the procedures. The sperm parameters (motility characteristics and average values of speed) were evaluated by three measurements of each part used for sexing and non-sexing by analyzing six areas per samples. The mean values were considered as final.

The motility characteristics included progressive motility (PR, %), non-progressive motility (NP, %) of spermatozoa, immotile sperm (IM, %), total motility (TM, %) velocity (rapid - R and medium - M; %) and velocity and progressivity (slow - S, rapid - RP and medium - MP; %). The registered values of speed of motile spermatozoa were curve linear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) average path velocity (VAP, μm/s), linearity index (LIN, %), straightness (STR, %) and oscillation index (WOB, %). Additionally, amplitude of lateral head movement (ALH, μm), beat frequency (BCF, Hz) and percent of hyperactive spermatozoa (H) were also evaluated. All procedures were in an agreement with the requirements for welfare and animal's protection included in Bulgarian legislation.

Semen sexing procedure

The semen sexing was done by application of the TLR7/8 ligand R848 (resiquimod) by adaptation and modification of the protocols of Umehara et al. (2020) and Yotov et al. (2024a) to the fresh ram semen. As it is known (Umehara et al., 2019), the incubation of sperm in a medium with TLR7/8 ligand led to decreased activity of X-bearing sperm and their sinking to the lower layer of the medium. The activity of Y chromosome-bearing sperm is less likely to be decreased, so that they float in the upper layer of the medium.

After removing of the seminal plasma from pools by centrifugation (400g, 5 min, 37°C) the collected spermatozoa were diluted with modified human tubal fluid (mHTF) medium (Umehara et al., 2020) in concentration 200 × 106 spermatozoa per mL. 3 mL of diluted semen from each pool was treated with 0.3 μM R848 (Sigma-Aldrich, Co, St. Louis, MO, USA) and 0.27μM pyruvate (Institute BCN, Spain) followed by incubation at 37ºC for 60 min, and then the upper layer (1 mL) was collected for the Y-bearing spermatozoa. The same procedure was applied to the other 3 mL of diluted semen from each pool, but without pyruvate and followed incubation was 30 min. The X- bearing spermatozoa were collected from the lower layer (1 mL).

Each sample was washed by centrifugation and spermatozoa were suspended with 1 ml freezing extender (Steridyl one step, Minitube, Germany). In accordance with our previous validation of R848 effect on ram semen sexing (Abadjieva et al., 2022), the upper layer contains 74-78% Y-bearing and 22-26% X-bearing spermatozoa whereas the lower layer contains 64-70% X-bearing and 30-36% Y-bearing sperm cells.

Semen cooling, freezing and thawing

Diluted with freezing extender non-sexed and sex-sorted semen was filled in straws of 0.25 ml which were placed of floating freezing rack and were cooled in a refrigerator at temperature 5°C for 5 hours. After cooling, freezing procedure was done through a special freezing unit (Minitube, Germany). The straws stayed upon liquid nitrogen vapours for 15 minutes, then were stored in a liquid N2 container. After freezing, four straws from each part of semen were thawed by placing in a water bath at 37°C for 60 seconds and subjected to CASA analysis.

Statistical analysis

The results were statistically processes by software product Stat.Soft, v.10 (StatSoft Inc., Tulsa, USA). The data are presented as a mean ± standard deviation (SD). After checking for normal distribution of variances by Kolmogorov-Smirnov test, the mean values of the spermatozoa parameters after cooling and freezing were compared with control by the non-parametric Mann-Witney test. Two-ways ANOVA, especially, main effects analyse, was used for the estimation of the effects of factors "sexing" and "extender" and their interaction on the speed parameters of spermatozoa (VCL, VAP and VSL). The differences were considered significant at $P< 0.05$.

Results

The results about the effects of the used diluting media on non-sexing and sexing sperm kinematics are presented in Table 1. The application of freezing extender and main medium for washing of the spermatozoa before semen sexing not affected considerably sperm motility. More significant changes of the motility parameters were observed after the sexing procedure. "Main effects" ANOVA showed that the simultaneously influence of both factors - "extender" and "sexing procedure" significantly affected spermatozoa speed parameters (observed power 0.999; $P=0.0111$, Table 2). The similar trends were observed for all three parameters VCL, VAP and VSL (Figure 1 A, B show these effect on VCL). However, in both studied groups, the strength of the effect was higher for factor extender, especially clear expressed for the sexed spermatozoa group (Table 2).

Table 1. Changes in the motility and kinematic parameters of non-sexed and sexed ram spermatozoa after dilution with different media.

Samples (n=4x3 repetition) with diluting media	Motility (%)				Velocity (%)		Velocity and progressivity (%)			Average values of speed of motile spermatozoa (µm/sec)			Indices (%)			Hyperactive spermatozoa (%)	BCF (Hz)	ALH (µm/sec)
	PR	NP	IM	TM	R	M	S	RP	MP	VCL	VAP	VSL	LIN	STR	WOB			
Non-sexed spermatozoa																		
PhS	99.3 ± 0.65^{A}	0.7 ±0.64^{A}	0.01 ± 0.00^{A}	99.9 ±0.01^{A}	99.2 ±0.76^{A}	0.7 ±0.6^{A}	0.1 ±0.1^{A}	9.2 ±2.5^{A}	90.1 ±3.1^{A}	232.9 ±10.1^{A}	120.2 ±7.8^{A}	59.9 ± 2.4^{A}	26.4 ±1.5^{A}	49.3 ±3.4^{A}	52.2 ±1.9^{A}	82.5 ±4.1^{A}	12.1 ±0.6^{A}	5.2 ±0.01^{A}
Fex	99.9 ±0.0^{A}	0.03 ±0.02^{A}	0.01 ±0.02^{A}	100 ±0.0^{A}	99.9 ±0.03^{A}	0.01 ±0.03^{A}	0.02 ±0.03^{A}	7.5 ±0.3^{A}	92.4 ±0.3^{A}	267.7 ±8.3^{B}	132.5 ±4.4^{B}	64.3 ±2.8^{A}	24.3 ±0.5^{A}	47.4 ±0.7^{A}	49.8 ±0.2^{A}	92.8 ±0.6^{B}	11.2 ±0.01^{A}	6.3 ±0.2^{A}
Sexed spermatozoa																		
mHTF	99.7 ±0.2^{a}	0.3 ±0.^{a}	0.005 ±0.01^{a}	99.9^{a} ±0.01	99.6 ±0.2^{a}	0.3 ±0.1^{a}	0.1 ±0.06^{a}	7.1 ±0.9^{a}	92.5 ±0.7^{a}	261.2 ±10.7^{a}	128.2 ±5.1^{a}	62.4 ±3.1^{a}	24.2 ±0.8^{a}	47.6 ±0.6^{a}	49.5 ±0.3^{a}	90.9 ±1.9^{a}	10.6 ±0.2^{a}	6.2 ±0.3^{a}
mHTF+R848 (X after sexing)	34.5 ±7.2^{b}	52.7 ±7.8^{b}	12.8 ±1.5^{b}	87.2 ±1.5^{b}	29.4 ±8.2^{b}	16.8 ±1.5^{b}	41.0 ±7.7^{b}	5.5 ±1.9^{a}	28.9 ±5.4^{b}	69.9 ±6.4^{b}	41.6 ±1.8^{b}	25.7 ±2.2^{b}	42.8 ±4.1^{b}	61.1 ±2.9^{b}	65.1 ±2.7^{b}	10.8 ±4.2^{b}	6.7 ±0.7^{b}	2.1 ±0.02^{b}
mHTF+R848 (Y after sexing)	42.58 ±3.1^{b}	51.6 ±3.5^{b}	5.8 ±0.7^{c}	94.1 ±0.5^{c}	38.3 ±4.1^{b}	17.4 ±0.3^{b}	38.5 ±2.8^{b}	6.9 ±0.7^{a}	35.6 ±1.2^{b}	80.2 ±3.1^{c}	44.7 ±0.9^{b}	26.4 ±1.9^{b}	38.8 ±2.3^{b}	59.7 ±1.1^{b}	61.3 ±3.4^{b}	12.8 ±2.1^{b}	7.0 ±0.1^{b}	2.2 ±0.03^{b}
Fex + X	96.6 ±4.2^{a}	3.4 ±4.1^{c}	0.04 ±0.03^{ab} ^{c}	99.9 ±0.04^{a}	95.3 ±5.2^{a}	3.5 ±3.6^{c}	1.1 ±1.5^{a}	13.5 ±2.3^{b}	83.1 ±5.8^{c}	183.2 ±13.3^{d}	98.1 ±12.9^{c}	51.5 ±5.1^{c}	29.9 ±2.3^{c}	52.7 ±2.1^{c}	54.9 ±1.2^{d}	58.2 ±7.1^{c}	12.8 ±0.6^{c}	4.4 ±0.1^{c}
Fex + Y	97.4 ±3.6^{a}	2.5 ±3.6^{c}	0.1 ±0.09^{a}	99.9 ±0.09^{a}	96.7 ±4.7^{a}	2.5 ±3.6^{c}	0.8 ±1.1^{a}	11.3 ±3.7^{ba}	86.0 ±7.4^{ac}	206.9 ±11.2^{d}	108.5 ±14.8^{c}	55.6 ±6^{ca}	28.4 ±3.5^{c}	51.0 ±3.2^{c}	53.8 ±2.1^{d}	69.4 ±10.1^{c}	12.0 ±0.8^{c}	4.9 ±0.09^{d}

PhS - physiological solution - 0.9% NaCL; Fex - freezing extender; mHTF- modified human tubal fluid; R848 – resiquimod; PR- progressive motility; NP - non-progressive motility; IM - immotile sperms; R - rapid and M - medium velocity; S - slow, RP - rapid and MP – medium velocity and progressivity; VCL - curve linear velocity; VSL - straight-line velocity; VAP - average path velocity; LIN - linearity; STR - straightness; WOB - oscillation; ALH - amplitude of lateral head displacement ; BCF - beat frequency

$^{A, B}$ - Different letters within the column of non-sexed spermatozoa show the significant difference between the values after dilution with PhS and Fex at P<0.05

$^{a, b, c, d, e,}$ - Different letters within the column of sexed spermatozoa show the significant difference between the values after dilution with mHTF, mHTF+R848 and Fex at P<0.05

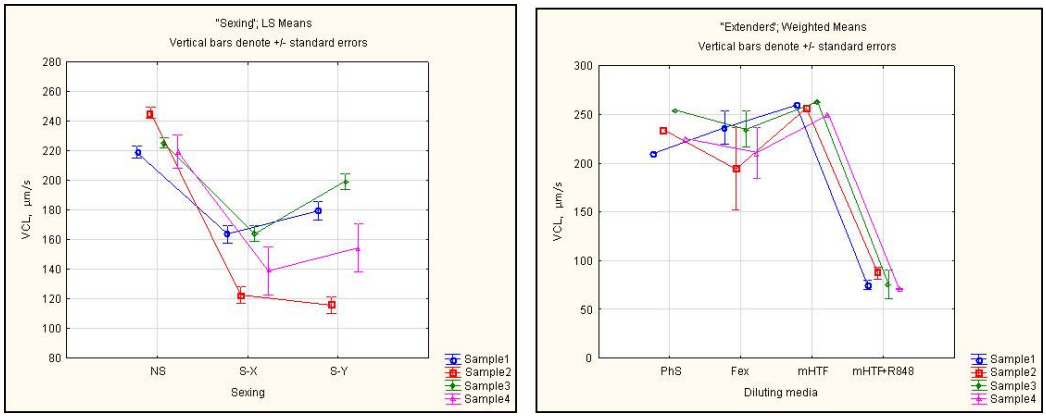

Figure 1. Effects of sexing (A) and diluting media (B) on the VCL of ram spermatozoa
NS - non-sexed spermatozoa, S-X - X bearing sperm fraction; S-Y- Y bearing sperm fraction; PhS - physiological solution 0.9% NaCL; Fex- freezing extender; mHTF- modified human tubal fluid; mHTF+R848 - modified human tubal fluid with R848

Use of Steridyl had the greatest influence, compared to other media (Figure 1B). It affected both non-sexed and sexed spermatozoa, although to varying degrees. In non-sexed spermatozoa, VCL and VAP were significantly higher after dilution with Steridyl, compared to the values obtained in the primary evaluation after dilution with saline (267.7±4.3 vs. 233.1±10.1, P=0.032 and 132.5±4.4 vs. 120.2±7.8, P=0.05, Table 1).

Table 2. Power of extender's effect on the speed parameters of non-sexed and sexed ram spermatozoa (ANOVA main effect analysis)

Effects	Test	Value	P	Observed power (alpha=0.05)
Interaction of Sexing and Extender on the VCL, VAP, VSL for whole group	Wilks	0.000299	0,011011	0.999994
Extender on the VCL for whole group	Wilks	0.001647	0.050166	0.667267
Extender on the VCL for sexed group	Wilks	0.001845	0.027353	0.931995
Extender on the VAP for whole group	Wilks	0.001124	0.042676	0.758951
Extender on the VAP for sexed group	Wilks	0.001194	0.022006	0.976722
Extender on the VSL for whole group	Wilks	0.001735	0.050464	0.654685
Extender on the VSL for sexed group	Wilks	0.002455	0.031554	0.886269

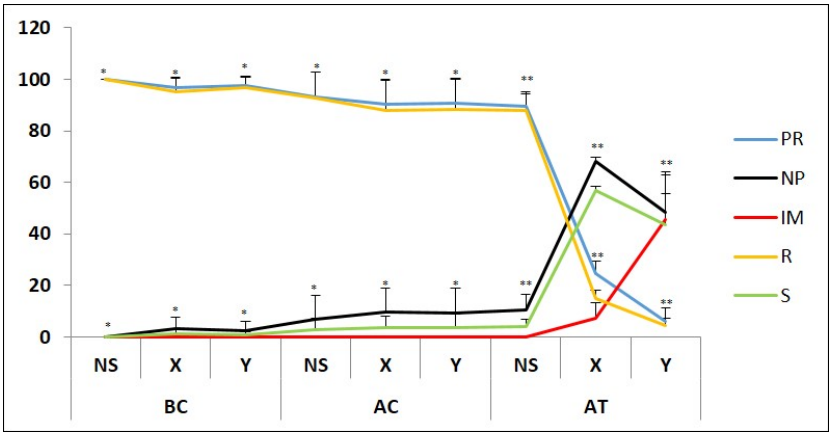

Figure 2. Effect of cooling and freezing on the motility and velocity of non-sexed and sexed ram spermatozoa

NS - non-sexed spermatozoa; X and Y - fractions of sexed spermatozoa; BC - before cooling; AC - after cooling; AT - after thawing; PR - progressive motility; NP - non-progressive motility; IM - immotile spermatozoa; R - rapid velocity; S - slow velocity and progressivity

Values of PR, NP, R and S for the same spermatozoa (NS, X, Y) marked with two asterisks differ at $P<0.05$ according to type of process (BC, AC and AT).

In the sexed spermatozoa, significant changes were observed in the percentages of PR, NP, S and H. Also, in both fractions X- and Y, the speed parameters such as VCL, VAP and VSL increased after dilution with freezing extender. The values of these parameters were close to those recorded for non-sexed spermatozoa (Table 1). During the storage of non-sexed and sexed sperm cells in cooled and frozen condition, the trends for motility changes were similar (Figure 2). Significant decrease in PR and R with increase of NP and S were determined after thawing only, but a percentage of these changes was different. Comparative analysis between both fractions showed significant ($P<0.05$) elevation in the values of PR and R for non-sexed spermatozoa and decrease of NP and S in comparison between X and Y fractions.

The main changes in VCL, VAP и VSL of non-sexed sperms were detected after thawing whereas the indices of movement (LIN, STR and WOB) and ALH started to change with cooling (Figure 3 A, B, C, $P<0.05$). In the X fraction VCL, VAP and VSL were also significantly decreased after thawing, and remaining parameters were changed after cooling (increase of LIN, STR and WOB and decrease of H, ALH и BCF, $P<0.05$) keeping a similar tendency until thawing. In the Y fraction VSL significantly decreased after cooling, while at the same time in VAP, H, ALH и BCF there was only trend to decrease ($P=0.058$ and $P=0.07$). However, after thawing procedure the values of all abovementioned parameters were reduced. There was no change in the movement indices (LIN, STR, WOB)

for cooled or frozen Y fractions. The mean values of speed parameters between non sexed and sexed spermatozoa after thawing differed significantly (P<0.05). At the same time in sex-sorted semen there was an increase of PR, VAP and VSL in X-, compared to Y spermatozoa. Only the value of VCL was significantly (P<0.05) lower in Y than in X fraction.

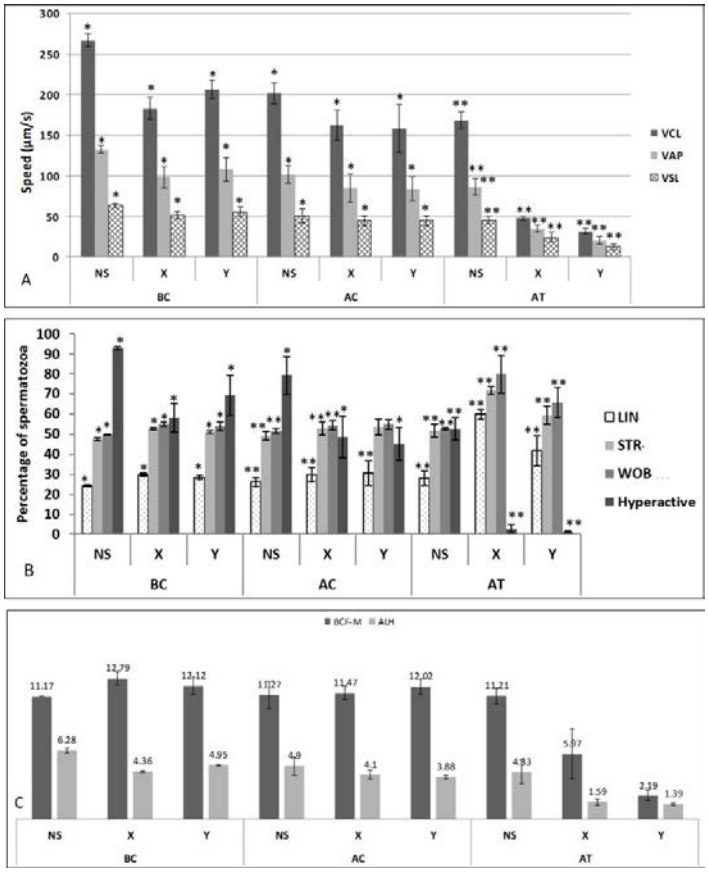

Figure 3. Effect of cooling and freezing on average speed parameters (A), movement indices and hyperactive spermatozoa (B) and amplitude lateral head and beat frequency (C) of non-sexed and sexed ram spermatozoa

NS - non-sexed spermatozoa; X and Y - fractions of sexed spermatozoa; BC - before cooling; AC - after cooling; AT - after thawing; PR - progressive motility; NP - non-progressive motility; VCL - curve linear velocity; VSL - straight-line velocity; VAP - average path velocity; LIN - linearity; STR - straightness; WOB - oscillation; ALH - amplitude lateral head (µm/sec); BCF - beat frequency (Hz) Values for the same spermatozoa (NS, X, Y) marked with two asterisks differ at P<0.05 according to type of process (BC, AC and AT).

Discussion

In the past, assessment of ram sperm quality had been based mainly on subjective evaluation of parameters, such as mass and individual motility, which had proved relationship with semen fertility (David et al., 2015). Computer assisted sperm analysis has allowed an objective and precise evaluation of, not only motility characteristics like progressive and non-progressive motility, rapid and slow spermatozoa, but also the speed parameters, movements indexes, amplitude of lateral head displacement during the movement of motile spermatozoa and number of hyperactive spermatozoa (Valverde et al., 2020). These parameters became important for the ram semen assessment due to numerous last decade investigations, confirming their close relationship with fertility in laboratory (Robayo et al., 2008; Rodriguez- Martinez and Vega, 2013) and in field conditions (Del Olmo et al., 2013; Sinapov and Yotov, 2023).

This study presents for the first time the speed parameters of ram spermatozoa sexed by TLR7/8 ligand R848 and their changes during cooling and freezing of X-and Y-fractions. It was established that interaction of two factors - sexing procedure and use of extenders influences kinematic parameters of ram spermatozoa. In accordance with specifics of R848 sexing protocol, TLR7/8 ligand resiquimod (R848) activates TLR7/8 in X- sperm, resulting in decreased glycolytic activity and ATP production, with a consequent reduction in X sperm motility (Umehara et al., 2019). In confirmation of those are the results of the present study, showing the significant decrease in progressive motility, VCL, VAP, and VSL of X-bearing spermatozoa after sexing. The higher values of these parameters, including TM, for Y-bearing spermatozoa after sexing could be explained by presence of pyruvate in medium for Y-spermatozoa that is a source of energy and an antioxidative agent (Van de Hoek et al., 2022). In contrast, our previous separation of Y-spermatozoa with ligand R848 and creatine showed significant decrease in the total motility for the Y-fraction compared to the whole ejaculate (Yotov et al., 2021). We assume that use of pyruvate is more appropriate than creatine for obtaining the Y-fraction, but future investigations could clarify this question. The suppression of X-spermatozoa motility by TLR7/8 ligand R848 is not stably and can be reversed after removing of R848. In our study the dilution of the sexed spermatozoa with freezing extender "Steridyl" led to significant increase of the motility and speed parameters in both X- and Y-fractions. Moreover, the values became comparable to those of unsexed spermatozoa.

The data about dependence of speed parameters like VCL, VAP, and VSL of ram spermatozoa on the extenders used for dilution has also been reported by Mostafapor and Ardebili (2014). It should be underlined the valuable protective effect of "Steridyl" for unsexed and sexed spermatozoa during the cooling storage. Despite the noticeable trend to decrease the motility and speed parameters in unsexed group, the significant reduction was not observed. However, cooling for 5

hours significantly affected LIN, STR, WOB and number of hyperactive spermatozoa in X-fraction and VSL in Y-fraction, but in the range not more than 10-15%. Important is that cooling did not decrease VAP in either fractions. VAP is the parameter that shows the highest correlation with fertility, and it may be the most useful sperm speed parameter, which can be relied upon for the estimation of sperm fertility (Nagy et al., 2015). The most drastical changes in the kinematic sperm parameters of sexed group appear after freezing. The PR reduced to 24% in X- and 6% in Y-fractions. VCL, VAP, and VSL were reduced by 3-4 times compared to the parameters observed after cooling. The reason for that is a combination of two procedures stressful for sperm - sexing and freezing. The sex-sorting protocol, involving multiple steps and changes in the environment as well as cryopreservation may together lead to motility impairment and oxidative damage of sperm (Vishwanath and Moreno, 2018). The value of PR, speed parameters and number of hyperactive spermatozoa are lower in the post-thawing Y- fraction compared to the X one. These results are in agreement with data reported for the post-thawing Y- fraction separated by BSA column (Yotov et al., 2024b). The mechanism of different cryotolerance of X- and Y-bearing spermatozoa is not clear and has to be investigated in the future.

Conclusions

The present results show that freezing extender "Steridyl one step" can restore the total motility and speed parameters of X- and Y-spermatozoa, reducing after sexing with TLR7/8 ligand R848, close to the primary values and keep them during the cooling at relatively high level comparable with this of unsexed spermatozoa. Regardless protective effect of freezing extender, cryopreservation is very damaging for sexed spermatozoa. In both X- and Y- fractions, the progressive motility, VCL, VAP, VSL and number of hyperactive spermatozoa were adversely affected by freezing. Comparison between fractions allows to conclude that X-spermatozoa are more cryotolerant than Y ones, because they kept higher motility and better kinematic parameters after thawing. Cooling is the better way for short term storage of ram spermatozoa sexed by R848.

Cooled storage can be more successful when sexed fractions are diluted in the appropriate extender that ensures the restoration of the motile and kinematic parameters of spermatozoa after removing of substance R848.

Uticaj hlađenja i smrzavanja na kinematičke parametre spermatozoida ovnova seksiranih modifikovanim protokolom sa TLR7/8 ligandom R848

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Rezime

U okviru ove studije je po prvi put istraživan uticaj sortiranja po polu pomoću TLR7/8 liganda R848, hlađenja i krioprezervacije na pokretljivost i kinematičke karakteristike spermatozoida ovnova. Četiri ejakulata po ovnu (n=2) su sakupljena, spojena i nakon toga podeljena na 8 jednakih delova. Četiri dela su korišćena za određivanje pola, a četiri dela su analizirana kao sperma bez spola. Određivanje pola sperme je urađeno primenom TLR7/8 liganda R848 (resiquimod). Sperma, neseksirana i seksirana po polu, razređena sredstvom za zamrzavanje, hlađena je u frižideru na temperaturi od 5°C tokom 5 sati i nakon toga zamrznuta/odmrznuta. Svaki deo sperme u svakom koraku eksperimenta je podvrgnut kompjuterski potpomognutoj analizi sperme za procenu pokretljivosti i parametara brzine spermatozoida. Nakon seksiranja uočeno je značajno smanjenje kinematičkih parametara u obe frakcije. Razređivanje seksiranih spermatozoida sa sredstvom za zamrzavanje pre hlađenja pouzdano je povećalo progresivnu pokretljivost i parametre brzine kao VCL, VAP i VSL u X- i Y frakcijama i zadržalo ih tokom hlađenja na relativno visokom nivou. Smrzavanje je negativno uticalo na pokretljivost seksiranih spermatozoida. Vrednosti parametara brzine neseksiranih spermatozoida nakon odmrzavanja bile su značajno veće od vrednosti X i Y spermatozoida. Razređivanje odgovarajućim sredstvom za zamrzavanje i hlađenje su najpogodniji pristupi za čuvanje seksiranih spermatozoida ovnova.

Ključne reči: seksiranje sperme ovnova, TLR7/8 ligand R848, kinematika, hlađenje, zamrzavanje

Acknowledgments
This study was supported by the National Scientific Program "INTELLIGENT ANIMAL HUSBANDRY" under Bulgarian Ministry of education and science, grant agreement No. Д01–62/18.03.2021.

Conflict of interest
The authors declare that they have no conflict of interest.

References

Abadjieva D., Kistanova E., Yotov S., Atanasov A., Ivanova B., Yarkov D., Sinapov B. 2022. Sexing of fresh ram semen by activation of the toll-like receptors 7/8 (TLR7/8). *Reproduction of Domestic Animals*, 57, S4, 72.

David I., Kohnke P., Lagriffoul G., Praud O., Plouarboué F., Degond P., Druart X. 2015. Mass sperm motility is associated with fertility in sheep. *Animal Reproduction Science*, 161, 75-81.

Del Olmo E., Bisbal A., Maroto-Morales A., García-Alvarez O., Ramon M., Jimenez-Rabadan P., Martínez-Pastor F., Soler A. J., Garde J. J., Fernandez Santos, M.R. 2013. Fertility of cryopreserved ovine semen is determined by sperm velocity. *Animal Reproduction Science*, 138, 102-109.

Gonzalez-Marín C., Gongora C.E., Moreno J.F., Vishwanath R. 2021. Small ruminant SexedULTRA™ sperm sex-sorting: status report and recent developments. *Theriogenology*, 162, 67-73.

Hadi S., Al-Timimi I.H. 2013. Separation of Y-chromosome bearing ram's sperms using an albumin gradient technique and identification of embryos by PCR. AL-Qadisiyah *Journal of Veterinary Medicine Sciences*, 12, 144-151.

Johnson L. 2000. Sexing mammalian sperm for production of offspring: The state-of-the-art. *Animal Reproduction Science*, 60-61, 93-107.

Maxwell W.M.C., Meddoa G., Shite I.G. 1984. Post thawing survival of motile ram sperm after isolation by layering on protein columns. *Theriogenology*, 21, 601-606.

Mostafapor S., Farrokhi Ardebili F. 2014. Effects of diluting medium and holding time on sperm motility analysis by CASA in ram. *Veterinary Research Forum*, 5, 101-105.

Nagy Á., Polichronopoulos T., Gáspárdy A., Solti L., Cseh S. 2015. Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. *Acta Veteterinaria Hungarica*, 63, 370-81.

Quelhas J., Santiago J., Matos B., Rocha A., Lopes G., Fardilha M. 2021. Bovine semen sexing: Sperm membrane proteomics as candidates for immunological selection of X- and Y- chromosome- bearing sperm. *Veterinary Medicine and Science*, 7, 1633-1641.

Ren F., Xi H., Ren Y., Li Y., Wen F., Xian M., Zhao M., Zhu D., Wang L., Lei A., Hu J. 2021. TLR7/8 signalling affects X-sperm motility via the GSK3 α/β-hexokinase pathway for the efficient production of sexed dairy goat embryos. *Journal of Animal Scienece and Biotechnology*, 12, 89.

Robayo I., Montenegro V., Valdés C., Cox J.F. 2008. CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. *Reproduction in Domestic Animals*, 43, 393-399.

Rodríguez-Martínez H., Vega F.P. 2013. Semen technologies in domestic animal species. *Animals Frontiers*, 3, 26-33.

Sinapov B., Yotov S. 2023. Relationship between some kinematic parameters of ram semen and reproductive performance of dairy sheep after application of assisted reproductive technologies. *International Journal of Current Microbiology and Applied Sciences*, 12, 307-317.

Solihati N., Rasad S.D., Yusrina A., Winangun K., Toha. 2019a. Quality and longevity of local ram's sexed sperm with albumin column. *Jurnal Ilmu Ternak*, 19, 113-121.

Solihati N., Rasad, S.D., Winangun K., Toha, Yusrina A., Noviyana C. 2019b. Viability of ram's X-Y sperm after sexing with bovine serume albumin at different incubation time. *IOP Conference Series: Earth and Environmental Science*, 247, 012031.

Valverde Barquero V., Sole, C. 2020. The application of computer-assisted semen analysis (CASA) technology to optimise semen evaluation. A review. *Journal of Animal and Feed Sciences*, 29, 189-198.

Van de Hoek M., Rickard,] J.P., de Graaf S.P. 2022. Motility assessment of ram spermatozoa. *Biology*, 11, 1715.

Vishwanath R, Moreno JF. 2018. Review: Semen sexing – Current state of the art with emphasis on bovine species. *Animal*, 12, 85-96.

Umehara T., Tsujita N., Shimada M. 2019. Activation of toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. *Plos Biology*, 17, e3000398.

Umehara T., Tsujita N., Zhu Z., Ikedo M., Shimada M. 2020. A simple sperm-sexing method that activates TLR7/8 on X sperm for the efficient production of sexed mouse or cattle embryos. *Nature Protocols*, 15, 2645-2667.

Yotov S., Kistanova E., Abadjieva D., Karadaev M., Ivanova B., Bonev G., Yarkov D., Sinapov B. 2021. Comparative analysis of motility characteristics and kinematic parameters of fresh, chilled and sexed ram semen - preliminary study. *Veterinaria ir Zootechnika*, 79, 37-42.

Yotov S., Abadjieva D., Atanasov A., Ivanova I., Taushanova P., Fasulkov I., Kistanova E. 2024a. In vitro characteristics of X- and Y-bearing ram spermatozoa sorted by bovine serum albumin (BSA) column and TLR7/8 ligand R848. *Animal Reproduction Science*, 263.

Yotov S., Atanasov A., Ivanova B. 2024b. Changes in the kinematic parameters of ram spermatozoa in production of frozen sex-sorted semen by bovine serum albumin column. *Intelligent Animal Husbandry*, 1, 1-11.

Received 14 October 2024; accepted for publication 4 December 2024