TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2025, vol. 10, No 2(19): 51–56

TEMPORAL DYNAMICS OF HYPERACTIVATION OF RAM SPERMATOZOA AS A RESULT OF CHANGE IN THE KINEMATIC PARAMETERS

Desislava Abadjieva[1], Tsvetan Tsvetkov[1], Krasimir Kotcev[1], Nikola Metodiev[2]

[1]Institute of biology and immunology of reproduction "Akad. K. Bratanov", Bulgarian Academy of Sciences, Sofia 1113, Bulgaria, 73 "Tsarigradsko shose" Blv
[2]Institute of Animal Sciences, Kostinbrod, sp. Pochivka, Bulgaria
E-mail: dabadjieva@ibir.bas.bg
ORCID: 0000-0002-7869-2022 D.A.; 0000-0002-5838-5244 T.T.; 0000-0002-7922-0860 N.M.
(Submitted: 21 May 2025; Accepted: 29 October 2025; Published: 27 November 2025)

ABSTRACT

The presented research is focused on the kinetic parameters of ram spermatozoa related to hyperactivation after incubation in Bovine serum albumine (BSA) -containing medium for 4 h. Seminal samples were collected from five sexually mature rams using artificial vagina. The ejaculates (n=15) were diluted with extender 6A, then incubated (37°C, 5% CO_2 in air) for 4 hours in the absence (control group) or presence (experimental group) of 4 mg/ml BSA. The hyperactivation of the spermatozoa was detected using the Sperm computer analyser (SCA Microptic). Computer assisted sperm analysis (CASA) was performed for progressive and non-progressive motility and kinematic parameters of the sperm cells. The results demonstrated that incubation with BSA, resulted in a significant increase ($P < 0,05$) in the values of the kinetic parameters of spermatozoa at 4 h of incubation. The parameters that most accurately describe the hyperactivated motility are curvilinear velocity (VCL), percentage of fluctuation (WOB), amplitude of lateral displacement of the head (ALH), beat-cross frequency (BCF), velocity in a straight line (VSL), linearity (LIN) and straightness (STR). Our experiments display a significant decrease of the parameters VSL, LIN and STR ($P < 0,05$), which can also be related to the movement pattern of the cells characterized with hyperactivation. The incubation with BSA, under set conditions, resulted in the hyperactivation of ram spermatozoa with drastic changes in the following CASA parameters: VCL values > 100 µm/s, ALH > 7 µm, LIN < 50%, recorded at 4 h. The changes in sperm kinetics under the examined specific conditions can be used as a marker of hyperactivation of spermatozoa during the preparation process of media and diluents before artificial insemination.

Key words: ram, spermatozoa, hyperactivation, kinematics, parameters.

Introduction

The process of hyperactivation is a part of continuous physiological changes that spermatozoa undergo to become suitable for fertilization. The hyperactivation is presented as changes in the initial motility of ejaculated spermatozoa as vigorous and asymmetrical movement, deviating from the normal straight path and species-specific pattern. This change in the movement profile of the male gametes provides them with the ability to pass through the viscous environment in the oviduct and penetrate the zona pellucida surrounding the egg (Suarez & Ho, 2003). The most indicative signal for the onset of hyperactivation is the increase in Ca^{2+} concentrations, from both intracellular and extracellular sources. This suggests that hyperactivation could be induced in vitro by treating spermatozoa with pharmacological agents that are thought to increase gamete membrane permeability for extracellular Ca^{2+}, as well as induce release of intracellular Ca^{2+} (Suarez & Ho, 2003; Marquez *et al.*, 2007). Bovine serum albumin (BSA) has emerged as a stimulant for the secretion of ions, especially calcium which subsequently activates the adenylyl cyclase, leading to increased

levels of cAMP. Interestingly, BSA also induces rapid phosphorylation of glycogen synthase kinase, suggesting that opposing regulatory mechanisms may fine-tune calcium signaling leading to increased sperm motility (Mohanty *et al.*, 2024). Some authors report that a medium containing heparin-hypotaurin-BSA induced capacitation-related changes in sperm motility and membrane fluidity compared to other treatments, highlighting the crucial contribution of BSA to sperm functionality (García-Álvarez *et al.*, 2015). Furthermore, Masrizal *et al.* (2024) suggest the techniques involving sperm treatment with a BSA could affected sperm quality. These conflicting results highlight the need to understand the precise role of BSA in sperm functionality and to further characterize the conditions that support hyperactivation of ram spermatozoa in in vitro conditions.

The present study aimed to follow the dynamics of hyperactivation in ram spermatozoa after incubation in a BSA- containing medium for 4 h, based on their kinematic parameters.

Materials and Methods

Experimental design

The experiment was carried out in the animal facility of the Institute of Animal Science – Kostinbrod with five sexually mature, clinically healthy rams from Synthetic Population Bulgarian Milk Sheep breed. Each of the rams included in the present study was fed under uniform nutritional conditions. The ejaculates (n = 15) were collected by using artificial vagina. The experiment has been performed in two replications. Each seminal sample was diluted with ram semen extender 6A to a final concentration of 50×10^6 sperm cells/ml in the laboratory of the Institute of biology and immunology of reproduction "Akad. K. Bratanov ", Bulgarian Academy of Sciences. 500 µL semen samples were incubated (37°C, 5% CO_2 in air) for 4 hours in the absence (control group) or presence (experimental group) of 4 mg/ml Bovine serum albumin (BSA, Sigma Aldrich).

Computer assisted sperm analysis

The prepared semen samples were loaded into Leja 20 chambers (Leja, The Netherlands) and examined using a microscope with a thermal plate at 36°C (Nikon, Japan), part of Sperm Class Analyzer (SCA, Microptic, Spain). 10 µL aliquots of ram spermatozoa were examined at the beginning (0 h), at the middle (2 h), and at the end (4 h) of the incubation to evaluate the following motility and kinetic parameters of the spermatozoa: progressive and non-progressive motility; kinematic parameters as the curvilinear velocity (VCL, mm/s); the average path velocity (VAP, mm/s); the straight-line velocity (VSL, mm/s); straightness of trajectory (STR, %); the percentage of linearity (LIN, %); the width of the sperm head's trajectory was recorded as the mean amplitude of lateral head displacement (ALH, mm); the wobble (WOB, %), which demonstrates the measure of oscillation of the actual path about the average path; the beat-cross frequency of the change in direction of the sperm head (BCF, Hz), and hyperactivity (%). A sperm cell is considered hyperactivated if it meets all of the following CASA-based kinematic criteria: curvilinear velocity (VCL) > 100 µm/s, lateral head displacement (ALH) > 7 µm, low linearity (LIN < 50%). At each time point (0 min, 2h, 4h) the semen samples were evaluated and recorded on the basis of total sperm cells counted (N-_total) and a number of hyperactivated sperm cells (N-_hyper). The percentage of hyperactivated spermatozoa was calculated using the following formula:

$$\text{Hyperactivation percentage} = (\text{N hyper}/\text{ N total}) \times 100$$

Statistical Analysis

The statistical program SPSS 13.0 for Windows was used, and the results were presented as mean ± standard error. The students' t-test evaluated the significance of the differences between the groups. Pearson's test evaluated the correlation. Results are considered statistically significant if P<0,05.

Results

Motility parameters of ram semen samples were measured at the beginning of the experiment (0 h) and after incubation (4 h) with 4 mg/ml BSA. The percentage of motile spermatozoa decreased insignificantly compared to the initial values at 0 h. However, compared to the control group after 4 h of incubation, the values of the experimental group were twice as high for the percentage of total motility, progressive, rapidly progressive, medium progressive, slow and immotile ram spermatozoa (P<0,05; Fig.1).

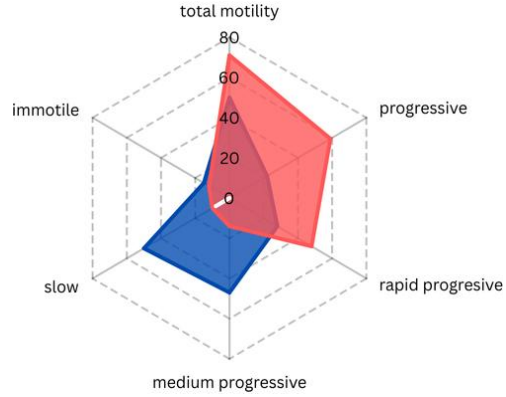

Figure 1: Radar plot of the mean sperm motility (%) measurements for comparison of experimental (red zone) and control (blue zone) groups.

The mean values of the kinematic parameters changed as follows: the viability parameters VCL, WOB, ALH and BCF increased significantly (P < 0,05), while the progression parameters (VSL, STR and LIN) significantly decreased (P < 0,05) in the experimental group compared to the control after 4 h (Table 1).

Table 1: Mean SCA values of ram spermatozoa incubated for 4 hours in the absence (control group) or presence (experimental group) of 4 mg/ml BSA

Group	Incub. period	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	STR (%)	LIN (%)	WOB (%)	ALH (µm)	BCF (Hz)	Hyper-activ. sp (%)
Contr. gr. (n=15)	4 h	41,81 ± 1,10	13,43 ± 2,11	39,28 ± 1,90	49,52 ± 2,73	33,46 ± 0,65	49,69 ± 1,71	2,54 ±0,15	1,94 ±0,23	0,304 ±0,08
Exper. gr. (n=15)	4 h	141,34 ± 7,31	8,97 ± 0,70	31,88 ± 0,40	29,63 ± 0,54	20,62 ± 0,39	62,23 ± 2,14	8,06 ±0,30	4,92 ±0,21	4,59 ±0.23
Control / Exper.gr	P	0,001 ***	0,217 n.s.	0,177 *	0,006 ***	0,0001 ***	0,032 *	0,001 ***	0,001 ***	0,001 ***

*Significant differences: * P < 0,05; ** P < 0,01; *** P < 0,001;*

In order to investigate the relationship between quantitative data, Pearson correlation analysis was applied between the percentage of hyperactivated spermatozoa with progressive motility, VCL, VSL, STR, LIN, WOB, ALH, and BCF. A positive correlation ($P < 0,05$) was determined of the already hyperactivated spermatozoa from the experimental group after the incubation period with all SCA parameters (Table 2).

Table 2: Pearson coefficient between % of hyperactivated spermatozoa and SCA parameters

(n=45)		Progressive	VCL	VSL	STR	LIN	WOB	ALH	BCF
Hyperactive sp.	Coefficient	0.771**	0.765**	0.729**	0.596**	0.555**	0.716**	0.777**	0.846**
	p Value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	n	45	45	45	45	45	45	45	45

*Significant differences: * P < 0,05; ** P < 0,01*

Discussion

The hyperactivation of spermatozoa is a crucial point of their capacitation and involves a cascade of changes regarding the movement pattern and the functionality of the gametes. Hyperactivation can be described as a specific and drastic changes in sperms motility and kinetics, exhibiting higher amplitude and asymmetric beating of the sperm's flagellum, which provides them the ability to move through the female reproductive tract and to reach the oocyte. According to Câmara *et al.* (2016), for the identification of spermatozoa's hyperactivation through CASA a minimal value of the following parameters should be set: curvilinear velocity (VCL) > 100 μm/s, lateral head displacement (ALH) > 7 μm, maximum path linearity threshold (LIN < 50%).

In the presented study, a decrease in the values for VSL, STR and LIN, as well as higher values for VCL, WOB, ALH and BCF were determined. Therefore, ram sperm cells, which generally move quickly and follow a linear trajectory compared to other ruminants (Robayo *et al.*, 2008), in the presented study are provoked to deviate from their initial straight movement pattern in conditions with added BSA in the media during incubation for 4h. The kinetic profile becomes circular with high amplitude asymmetrical beating of the flagellum. Similar experiments were conducted to describe the hyperactivation process of spermatozoa in other animal species (Hinrichs & Loux, 2012; Sharif *et al.*, 2022).

The basic mechanisms characterizing the process of hyperactivation involve complex signal pathways initiated by the rise of the cellular Ca^{2+} as a result of the inflow from the plasma membrane and probably also from Ca^{2+} release from the nuclear membrane. This process requires higher pH levels and elevated ATP production. The significant changes in the movement pattern of the gametes in the presented experiments can be related to the 6A extender and the influx HCO_3 from BSA, which stimulates the soluble adenylyl cyclase (sAC). This in turn leads to the production of cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). These physiological signals regulate the protein phosphorylation involved directly in sperm motility, leading to the hyperactivation of the male gametes (Suarez, 2008; Tourmente *et al.*, 2022).

Some studies highlight the role of some extracellular factors in the modulation of these pathways. BSA plays a key role in the hyperactivation of sperm cells by acting as a lipid and cholesterol acceptor, facilitating essential biochemical changes, leading to drastic changes in the motility profile. Some other factors, including steroid hormones have been shown to influence hyperactivation

through their receptors, affecting protein phosphorylation and intracellular calcium levels, critical for initiating hyperactivation in spermatozoa (Gimeno-Martos *et al.*, 2021).

In addition, the use of BSA has been found to promote antioxidant properties, improving sperm motility parameters and signaling mechanisms that mediate hyperactivation, creating an environment that supports optimal sperm vitality and progressiveness (Baumber *et al.*, 2013).

The presented experiments demonstrated that ram semen incubated in a BSA medium for a period of 4 hours alters sperm functionality, highlighting the importance of occurring hyperactivation for the preparation of the samples before artificial insemination of the sheep (Diansyah et al., 2025).

The presented experiment provides valuable information for the importance of BSA, its effective applications in reproductive biotechnology and the potential improvements in ram semen processing protocols.

Conclusion

Capacitation and hyperactivation of the sperm cells are crucial processes which enable male gametes to successfully fertilize the egg. BSA can be successfully used to initiate and regulate these processes in ram sperm cultivation media *in vitro*. This in turn would lead to an increase in fertilization success after artificial insemination. The demonstrated results provide a solid basis to consider that the incorporation of BSA into ram ejaculates would lead to an increase in fertilization success during the artificial insemination process.

Declaration of conflicting interests

The authors declare no conflicts of interest for the current publication.

References

1. Baumber, B., Donoghue, Fialkow, Gillan, R., Sikka. (2013). *Antioxidant effects of bovine serum albumin on kinetics, microscopic and oxidative characters of cryopreserved bull spermatozoa*. 'Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA), https://core.ac.uk/download/229477190.pdf.
2. Câmara, D. R., L. C. Pinto, M. M. C. M. Pintoq, J.P., Kastelic, J.F., Nunes, J.M.P., Barbosa, M.M.P., Guerra. (2016). *Influence of catalase and pre-freezing equilibration on post-thaw semen quality and conception rate in ewes laparoscopically inseminated*. Anim Reprod. vol.13, n. 1, p. 21–27.
3. Diansyah, A.M., A., Nurlatifah, C., Damaris, R., Rahmat, T., Saili, M.F., Amrullah, A.M., Alfian, A.A.S., Adam, E.M., Kaiin, E., Damaynti. (2025*). Evaluation of sperm sexing technique using BSA column medium in Garut rams: Enhancing sperm quality, kinematics, and fertility performance for improved breeding efficiency*. Open Vet J., 15(8), 3727–3736. doi: 10.5455/OVJ.2025.v15.i8.37.
4. García-Álvarez, O., A., Maroto-Morales, P., Jiménez-Rabadán, M., Ramón, E., del Olmo, M., Iniesta-Cuerda, L., Anel-López, M.R., Fernández-Santos, J.J., Garde, A.J. Soler. (2015). *Effect of different media additives on capacitation of frozen-thawed ram spermatozoa as a potential replacement for estrous sheep serum*. Theriogenology,84(6), 948–955.

5. Mohanty, G., C., Sanchez-Cardenas, B., Paudel, D.A., Tourzani, A.M., Salicioni, C.M., Santi, M.G., Gervasi, J.R., Pilsner, A., Darszon, P.E., Visconti. (2024). *Differential role of bovine serum albumin and HCO3- in the regulation of GSK3 alpha during mouse sperm capacitation*. Mol Hum Reproduction, 30(3):gaae007. doi: 10.1093/molehr/gaae007.
6. Gimeno-Martos, S., M., Santorromán-Nuez, J.A., Cebrián-Pérez, T., Muiño-Blanco, R., Pérez-Pé, A., Casao. (2021). *Involvement of progesterone and estrogen receptors in the ram sperm acrosome reaction*. Domest Anim Endocrinol., 74, 106527.
7. Hinrichs, Katrin, Shavahn C. Loux. (2012). *Hyperactivated Sperm Motility: Are Equine Sperm Different?* Journal of Equine Veterinary Science, Vol. 32, Iss. 8, p. 441–444, https://doi.org/10.1016/j.jevs.2012.05.070.
8. Marquez, B., G.G., Ignotz, S.S., Suarez. (2007). *Contributions of extracellular and intracellular Ca^{2+} to regulation of sperm motility: release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm*. Developmental Biology, 303, p. 214–221.
9. Masrizal, M., T., Afriani, D., Wahyudi, S., Saharani, A., Ananda. (2024). *The Quality of Frozen Buffalo Sperm Following Sexing using Bovine Serum Albumin (BSA) Column and Swim-Up (SU) Methods*. Buletin Peternakan, vol. 48, n. 1, https://doi.org/10.21059/buletinpeternak.v48i1.89394.
10. Robayo, I., V., Montenegro, C., Valdés, J.F., Cox. (2008). *CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus*. Reprod Domest Animal, 43(4), 393-399. doi: 10.1111/j.1439-0531.2007.00920.x.
11. Sharif, M., V., Hickl, G., Juarez,, X. Di, K. Kerns, P. Sutovky, N. Bovin, D.J. Miller. (2022). *Hyperactivation is sufficient to release porcine sperm from immobilized oviduct glycans*. Sci Rep., 12, 6446. https://doi.org/10.1038/s41598-022-10390-x.
12. Suarez, S.S. (2008). *Control of hyperactivation in sperm*. Hum Reprod Update, 14(6), 647–657. doi: 10.1093/humupd/dmn029.
13. Suarez, S.S., H.C., Ho. (2003). *Hyperactivated motility in sperm*. Reprod Domest Anim., 38(2), 119–124. doi: 10.1046/j.1439-0531.2003.00397.x.
14. Tourmente, M., E., Sansegundo, E., Rial, E.R.S., Roldan. (2022) *Capacitation promotes a shift in energy metabolism in murine sperm*. Front. Cell Dev. Biol. doi: 10.3389/fcell.2022.950979.