



CHARACTERISTICS AND KINEMATICS OF RAM SPERMATOZOA MOVEMENT IN FERTILIZATION MEDIUM USING *SERICIN*

Karakteristik dan Kinematika Pergerakan Spermatozoa Domba dalam Medium Fertilisasi dengan Menggunakan *Sericin*

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ABSTRACT

This study aimed to evaluate the effectiveness of *sericin* on quality and kinematics of spermatozoa movement in fertilization medium and substituted for BSA in PEIV. Spermatozoa preparation was done with and without *swim-up*. Each group was incubated in fertilization medium with BSA, *sericin* 0.05%, 0.1%, 0.5%, and evaluated after addition of fertilization medium, 15, 30, 60 minute. The results showed that the progressive motility values, *sericin* 0,5% group were higher than other groups ($P < 0.05$). Membrane integrity showed differences between treatments. The VCL value showed no difference except for 0 and 30 minute *swim-up* group, *sericin* 0.5% was higher ($P < 0.05$). VAP, VSL, LIN, and STR had no difference. The ALH at 30 minute *swim-up* and 60 minute without *swim-up*, *sericin* 0.5% were higher than others ($P < 0.05$). In conclusion, the addition of *sericin* 0,5% has potential to replace BSA, *sericin* 0.5% can maintain quality and show early signs of hyperactivation of spermatozoa.

Keywords: BSA, Fertilization, In vitro, *Sericin*, Spermatozoa

ABSTRAK

Kualitas spermatozoa merupakan faktor penting dalam mendukung keberhasilan fertilisasi in vitro. Penelitian ini bertujuan mengevaluasi efektivitas *sericin* terhadap kualitas spermatozoa dalam medium fertilisasi dan sebagai pengganti BSA pada teknologi produksi embrio in vitro. Persiapan spermatozoa dilakukan dengan dan tanpa *swim up*. Setiap kelompok diinkubasi dalam media fertilisasi dengan BSA, *sericin* 0,05%, 0,1%, dan 0,5% serta dievaluasi segera setelah ditambahkan media fertilisasi, menit ke-15, 30 dan 60. Setiap akhir masa inkubasi, kualitas dan kinematika spermatozoa dievaluasi sebanyak empat ulangan. Hasil penelitian menunjukkan bahwa nilai motilitas progresif, kelompok *sericin* 0,5% lebih tinggi dibanding kelompok lain ($P < 0,05$). Integritas membran spermatozoa menunjukkan perbedaan antar perlakuan. Nilai VCL menunjukkan tidak terdapat perbedaan kecuali kelompok *swim up* menit ke-0 dan 30 nilai *sericin* 0,5% lebih tinggi ($P < 0,05$). VAP, VSL, LIN dan STR tidak terdapat perbedaan. Nilai ALH kelompok *swim up* menit ke-30 dan menit ke-60 tanpa *swim up sericin* 0,5% lebih tinggi dibanding perlakuan lainnya ($P < 0,05$). Simpulan penelitian ini, penambahan *sericin* 0,5% berpotensi menggantikan BSA, *sericin* 0,5% dapat mempertahankan kualitas serta menunjukkan tanda awal hiperaktivasi motilitas spermatozoa.

Kata kunci: BSA, Fertilisasi, In vitro, *Sericin*, Spermatozoa

INTRODUCTION

In vitro embryo production (PEIV) is an assisted reproductive technology aimed at the mass production of embryos. Important stages contained in PEIV technology include in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) (Gordon 2003). PEIV technology has been widely applied to various types of livestock such as cattle (Currin et al. 2021), buffalo (Harissatria and Hendri 2016), sheep (Zhu et al. 2018), and goats (Souza-Fabjan et al. 2014).

The success of each stage of PEIV depends on several factors, such as the quality of oocytes and spermatozoa. Good sperm quality is characterized by high motility, normal morphology, and an intact plasma membrane (Sukmawati et al. 2015). In addition, fertilization media components also play an important role in the success of PEIV, one component that is often added in culture media including fertilization media is Bovine Serum Albumin (BSA) as a source of protein to improve culture quality (Zhang et al. 2016). Asad et al. (2017) provide results that adding BSA as much as 2 mg/ml can be useful in oocyte maturation, fertilization and culture media to increase the development of oocyte rate. BSA is also able to increase motility and induce the capacitation of spermatozoa in various species, such as rats (Yamaga et al. 2024), hamsters (Stewart-Savage 1993), and pigs (Dudkiewicz et al. 2024). Debates have arisen regarding the use of serum both scientifically and ethically. The addition of serum in the culture medium may carry pathogens such as bovine spongiform encephalopathy (BSE) and viruses, leading to an increased risk of disease spread (Hajarian et al. 2017). Therefore, alternative materials are needed to solve the problem.

Sericin is a protein purified from silkworm cocoons and contains 18 types of amino acids (Kunz et al. 2016). *Sericin* and BSA are similar in that they are albuminoid proteins and have similar actions on cells. *Sericin* was reported to

prevent cell death and support cell growth when added to cell culture media in place of BSA (Terada et al. 2002) and stimulate mammalian cell proliferation (Zhang et al. 2019). In PEIV technology, *sericin* was used in sheep maturation media by Yasmin et al. (2015) and in cattle by Satrio et al. (2022). The potential of *sericin* as a protein source in oocyte collection media and bovine oocyte maturation has also been reported by Karja et al. (2024), while the potential of *sericin* as a serum substitute in maturation and culture media has been reported by Hajarian et al. (2017). However, the use of *sericin* in fertilization medium on spermatozoa quality has not been reported to date. The in vitro fertilization stage of PEIV requires spermatozoa preparation, which aims to maintain motility and plasma membrane integrity until spermatozoa fertilize oocytes and can support embryo development (Anbari et al. 2016). One method that is often used for spermatozoa selection is swim up. This separation method is based on the movement of motile spermatozoa toward the surface of the media after incubation (Safitri et al. 2017). Therefore, the purpose of this study is to see the potential of *sericin* in the fertilization medium on the quality and kinematics of spermatozoa movement so that it can add information on the use of *sericin* in replacing BSA as a whole in vitro embryo production technology (PEIV).

MATERIALS AND METHODS

The research has received ethical approval by the Animal Ethics Commission SKHB IPB with certificate number 174/KEH/SKE/1/2024. Spermatozoa used were frozen semen of ram. Sperm treatment was divided into two groups, namely without and with swim-up. Frozen semen was thawed at 37°C for 30 seconds in a waterbath. Preparation of spermatozoa with swim-up method using Gordon's procedure (2003), thawed semen was then put into microtubes containing 1 ml of swim-up media with each treatment of BSA and *sericin*. Semen was placed at the bottom of the

microtube, then the microtube was placed in a tilted position at an angle of 45° and incubated in a 5% CO₂ incubator at 38.5°C for 1 hour. At the end of the swim-up period, the top of the media was transferred into a centrifuge tube and then centrifuged at 1800 rpm for 10 minutes. While the spermatozoa group without swim-up, the thawed semen was immediately put into a centrifuge tube containing fertilization media and centrifuged at 1800 rpm for 10 minutes. After centrifugation of both swim-up and no swim-up groups, the supernatant was discarded and the pellet was diluted with fertilization medium (Suzuki et al. 2000) supplemented with BSA 0.3% (P0), ser 0.05% (P1), ser 0.1% (P2) and ser 0.5% (P3) until the concentration was 5x10⁶ spermatozoa/cell. Each group of spermatozoa was incubated in a 5% CO₂ incubator at 38.5°C, then observed every 0, 15, 30, and 60 minutes to evaluate the quality of spermatozoa such as motility and plasma membrane integrity and kinematics of spermatozoa movement. The study was conducted with four replicates.

Quality and kinematics testing of frozen semen

Spermatozoa motility and kinematics

Progressive motility and kinematics of spermatozoa movement such as velocity curvilinear (VCL: µm/second), velocity straight line (VSL: µm/second) and velocity average path (VAP: µm/second), linearity (LIN: %), straightness (STR: %) and amplitude of lateral head displacement (ALH: µm) were observed using computer-assisted semen analysis (CASA, Sperm Vision™ 3.7 Minitube, Germany) connected to a Zeiss Axio Scope A1 microscope and equipped with a warm stage at 37°C. Frozen semen that has been thawed, taken as much as five µL and dripped on the object glass and covered with cover glass. Spermatozoa as many as ± 500 cells in four fields.

Spermatozoa plasma membrane integrity

Test of spermatozoa membrane integrity using Hypo Osmotic Swelling Test (HOST) method based on (Pardede et al. 2022). The HOST solution was incubated on a waterbath at 37°C. Semen samples as much as 30 µL were put into 300 µL of HOST solution, homogenized and incubated at 37°C. Evaluation of plasma membrane integrity of frozen semen was carried out after 30 minutes of incubation in a dry bath incubator by dripping 5 µL of the solution mixture on an object glass, covered with a cover glass, and observed under a 10x40 magnification microscope. Spermatozoa with intact plasma membrane integrity reacted like a curled tail while a straight tail characterizes spermatozoa with damaged membranes. Reacted and unreacted spermatozoa were counted from at least 500 cells.

Statistical analysis

Data were tabulated and presented as mean and standard deviation. The results of testing the quality and kinematics of spermatozoa movement were statistically analyzed using Analysis of Variance (ANOVA) at an actual level of 95%, if there were significant differences in the treatment then continued with the Duncan test.

RESULTS AND DISCUSSION

Spermatozoa motility

Sperm quality, such as motility and membrane integrity, have an influence on fertilization success (Tanga et al. 2021). Progressive motility is the active forward movement of sperm. The progressive motility of spermatozoa plays an important role because it can reach and penetrate the zona pellucida and a crucial step in the fertilization process (Li et al. 2014). Spermatozoa with good progressive motility also correlates with embryo quality, such as high blastocyst formation rates (Li et al. 2014). Post-thawing motility values in this study ranged from 53-57% for both groups. In the swim-up group, the motility value of

spermatozoa after incubation was higher than without swim-up because only motile spermatozoa were used to separate spermatozoa that moved actively to the surface of the medium after the incubation process. The percentage value of progressive motility in this study is shown in Figure 1. The motility value of the swim-up group (Figure 1A) after centrifugation and addition of fertilization media (0th minute) showed a range between 26-56%, the highest motility value was with *sericin* 0.5% treatment and the lowest was *sericin* 0.05% and there was no difference between *sericin* 0.1% and BSA group ($P > 0.05$). This condition was the same with the next incubation time, which at the 15th, 30th, and 60th minutes showed a range of 21-51%, 16-42%, and 14-34%, respectively. Progressive motility decreased significantly

in the 0th minute period or after exposure to the fertilization medium, except the *sericin* 0.5% treatment began to decrease at the 30th minute. The group without swim-up (Figure 1B) at minute 0 showed motility values ranging from 34-46%, with the highest motility value being the addition of *sericin* 0.5% ($P < 0.05$). At the 15th minute, the motility value showed a range of 23-33%; there were significant differences between treatments except for adding BSA and *sericin* 0.05% ($P > 0.05$). At the 30th and 60th incubation time, the motility value ranged between 16-29% and 10-19%. The motility value decreased as the incubation time increased except for the treatment of *sericin* 0.1% and 0.5%, which were not significantly different at the 15th and 30th minutes ($P > 0.05$).

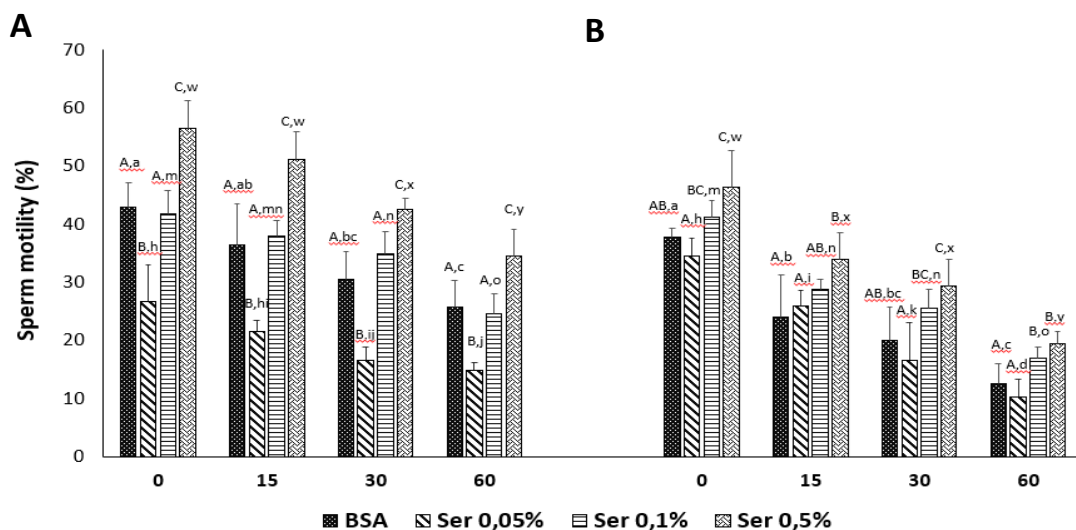


Figure 1 Spermatozoa motility after incubation in fertilization media with BSA and *sericin*. (A. Swim-up; B. Without swim-up). BSA: Fertilization medium added with BSA 0.3%; ser 0.05%: Fertilisation medium added with Ser 0.05%; Ser 0.1%: Fertilisation medium added Ser 0.1%; Ser 0.5%: Fertilisation medium added *sericin* 0.5%. Letters A,B,C, and D in different treatment groups at the same time indicate a difference ($P < 0.05$). The letters a,b,c,d,e; h,i,j,k,l; o,p,q,r,s; u,v,w,x,y in the same treatment group with different incubation times indicate a significant difference ($P < 0.05$).

These results indicate that *sericin* 0.5% can potentially replace BSA in fertilization media. The findings of this study are consistent with the findings of Patel et al. (2019), who reported that adding *sericin* 0.25% and 0.5% in buffalo increased the motility value of spermatozoa. As the incubation time increases,

the metabolic process in spermatozoa will continue to occur, this process will produce free radicals, especially Reactive Oxygen Species (ROS). *Sericin* is a protein reported to have antioxidant activity and high ROS capture ability (Kato et al. 1998). *Sericin* contains hydroxyl amino acids such as serine and

threonine (Yasmin et al. 2015). Kato et al. (1998) hypothesized that hydroxyl groups are responsible for the antioxidant action by binding to reactive metal ions that are catalysts for forming free radicals. Dash et al. (2008) reported that H₂O₂ treated cells, after adding *sericin*, can reduce lactate dehydrogenase, catalase, and thiobarbituric acid reactive substances (all oxidative stress parameters). This study also revealed that *sericin* is an antioxidant that protects sperm from free radicals and oxidative damage. *Sericin* and BSA have similar activities that can act as antioxidants. BSA can protect the sperm surface by scavenging ions and small molecules (Zhang et al. 2015). Ashrafi et al. (2013), have proven that the protective effect of BSA on spermatozoa is associated with increased antioxidant enzyme activity, total antioxidant capacity (TAC) and total thiols (TT).

Swim up is a simple method routinely used to separate motile from non-motile spermatozoa in vitro. Spermatozoa that swim through the medium show increased motility, average velocity, and a higher percentage of normal morphology, producing better fertilization rates in mammals (Magdanz et al. 2019). Rodiah et al. (2015) reported the results of their research, namely an increase in motility rate, percentage of live spermatozoa, spermatozoa concentration, and minimizing mechanical damage. The study also revealed that the technique of spermatozoa preparation in the upper layer has the advantage of avoiding contamination by bacteria or toxins contained in plasma. The results of this study show that spermatozoa exposed to swim-up showed higher motility values than the group without swim-up until the end of the incubation period in the treatment of fertilization medium added with BSA or *sericin*.

The motility value of spermatozoa in the swim-up group when compared with without swim-up at the time of PTM and after exposure to fertilization media (0 minutes) decreased respectively, ranging from (1.74-53.43%) and (12.13-34.80%). The highest decrease was with

the addition of *sericin* 0.05% and the lowest was *sericin* 0.5% treatment. The drastic decrease in the quality of spermatozoa in the fertilization media could be because sheep spermatozoa have a unique lipid composition, including a high proportion of unsaturated fatty acids that affect membrane fluidity and stability in various environmental conditions such as in vitro fertilization media (Díaz et al. 2016). These conditions indicate that *sericin* 0.5% can maintain spermatozoa motility. This study's results indicate that adding *sericin* at a concentration of 0.5% to the fertilization medium is appropriate.

Plasma membrane integrity

Plasma membrane integrity is essential in the assessment of spermatozoa quality. HOST (Hypo Osmotic Swelling Test) is used to assess plasma membrane integrity. Spermatozoa must have an intact membrane to remain viable and motile. The spermatozoa membrane plays a fundamental role in capacitation, acrosome reaction, zona pellucida recognition, and fusion of the spermatozoa and oocyte membranes (Okabe 2013). The plasma membrane integrity values in the swim-up group (Figure 2A) when after the addition of fertilization medium (0 min) and the 15 minute showed no significant difference between treatments ($P < 0.05$) with values ranging from 43-63% and 37-56% respectively, in this case the highest value was with the addition of *sericin* 0.5% treatment and the lowest was *sericin* 0.05%. The subsequent incubation time is the 30 and 60 minutes, with values ranging from 34-51% and 26-47%; there is no significant difference between the addition of *sericin* 0.5% with the addition of BSA and *sericin* 0.1% ($P > 0.05$). In the group without swim-up (Figure 2B) at minute 0, the membrane integrity value ranged from 50-57%; in this case, the value of *sericin* 0.5% showed the highest value compared to other treatments ($P < 0.05$). At the next incubation time, the 15th minute, there was no significant difference, the value of all treatments was 43%. At the 30th minute, the values

ranged from 32-43%, with the highest value being with the addition of *sericin* 0.5% and the lowest being with the addition of BSA and *sericin* 0.1%. At 60

minutes the values ranged from 25-34%, with the highest values being *sericin* 0.5% and 0.1%.

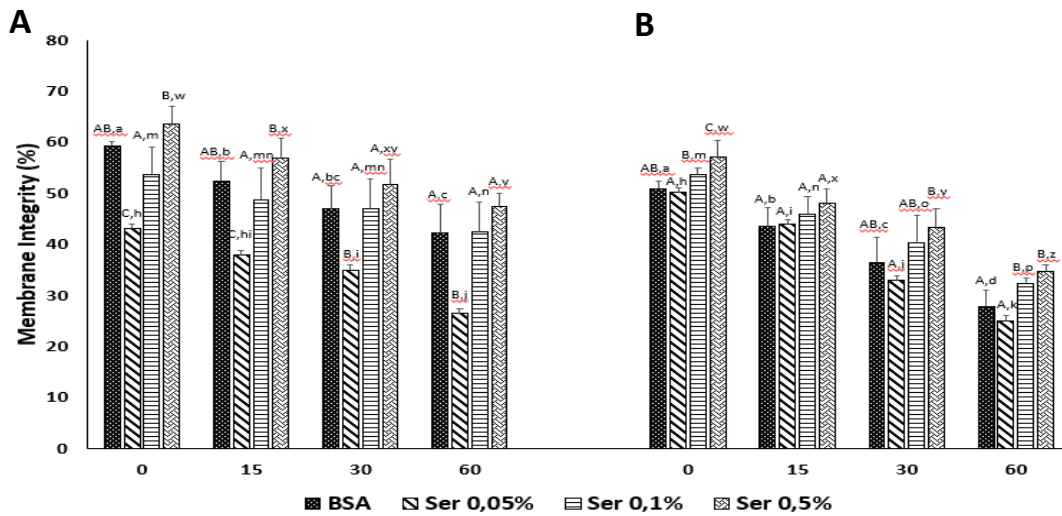


Figure 2 Spermatozoa membrane integrity after incubation in fertilization media with BSA and *sericin*. (A. Swim-up; B. Without swim-up). BSA: Fertilization medium added with BSA 0.3%; Ser 0.05%: Fertilization medium added with *Sericin* 0.05%; Ser 0.1%: Fertilization medium added *Sericin* 0.1%; Ser 0.5%: Fertilization medium added *Sericin* 0.5%. Letters A,B,C, and D in different treatment groups at the same time indicate a difference ($P < 0.05$). The letters a,b,c,d,e; h,i,j,k,l; o,p,q,r,s; u,v,w,x,y in the same treatment group with different incubation times indicate a significant difference ($P < 0.05$).

The plasma membrane value decreased significantly along with the addition of incubation time because the main target of damage to spermatozoa cells is the plasma membrane (Kumar et al. 2015). This condition can be influenced by oxygen consumption and free radical production. Increased oxygen consumption during incubation produces ROS such as hydrogen peroxide (H_2O_2) and superoxide, which can cause damage to the plasma membrane of spermatozoa. Studies conducted by Bravo et al. (2020) showed that exposure of spermatozoa to H_2O_2 caused a decrease in mitochondrial membrane potential and an increase in phosphatidylserine externalization. This externalization is an early sign of cell damage, followed by increased plasma membrane permeability and loss of plasma membrane integrity. Research by Gürlér et al. (2015) found that increased H_2O_2 synthesis was associated with decreased DNA and plasma membrane integrity, indicating that ROS resulting from oxygen

consumption can cause structural damage to the plasma membrane and disrupt cell function. Observations on plasma membrane integrity showed that in vitro fertilization media with *sericin* 0.5% supplementation has the highest plasma membrane integrity value compared to other treatments and can potentially replace BSA. This study's results align with the findings of Kumar et al. (2015), who reported that using extenders with *sericin* 0.25% and 0.5% in cattle produced higher spermatozoa motility and plasma membrane integrity. *Sericin* is an effective antioxidant in inhibiting the lipid peroxidation process and reducing H_2O_2 levels to prevent oxidative stress (Satrio et al. 2022). Ratchamak et al. (2020) also reported that *sericin* with a 0.25-0.75% concentration could suppress the lipid peroxidation index increase. The antioxidant system controls the balance between the production and neutralization of ROS and protects spermatozoa from peroxidative damage (Griveau and Le Lannou

1997). *Sericin* can interact directly with the cell membrane, forming a protective layer that helps maintain the stability of the membrane structure. This condition is in line with the research conducted by Li et al. (2017), who mentioned that *sericin* can form a glass matrix around cells that can help maintain membrane integrity during the freezing process. This is supported by the hypothesis of (Kumar et al. 2015), where *sericin* can protect spermatozoa cells during dilution, cooling, and cryoprotectant toxicity.

Spermatozoa movement kinematics

Spermatozoa kinematics parameters provide an objective picture of motility, a key indicator of sperm quality and highly influential in determining fertility (Maylem et al. 2018). The movement pattern of spermatozoa after insertion into the fertilization medium added with BSA and *sericin* is shown in Table 1, it

can be seen that the VCL parameter in the group without swim-up showed no significant difference between treatments and time ($P > 0.05$), but in the swim up group at minute 0 (immediately before incubation) and minute 30 there was a significant difference between treatments where *sericin* 0.5% was higher than other treatments ($P < 0.05$). Curvilinear velocity (VCL) affects fertilization ability, this parameter indicates the ability of spermatozoa to move efficiently towards the oocyte (Bag et al. 2004). VCL value categories are divided into fast ($> 90 \mu\text{m/s}$), medium ($45\text{-}90 \mu\text{m/s}$), slow ($10\text{-}45 \mu\text{m/s}$), and static or immotile ($10 \mu\text{m/s}$) (Křížková et al. 2017). Spermatozoa incubated with *sericin* 0.5%, both with the additional swim-up method and without swim-up on average showed higher swimming speed compared to other groups.

Table 1 Spermatozoa movement kinematics

Group	Protein	Minute	VCL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	LIN (%)	STR (%)	ALH ($\mu\text{m/s}$)
Swim up	BSA	0	81.9 \pm 10.3a,AB	71.3 \pm 9.6a,AB	64.6 \pm 12.4a,AB	0.7 \pm 0.04a,A	0.9 \pm 0.03a,A	2.3 \pm 0.1a,A
		15	83.7 \pm 0.8a,A	72.2 \pm 5.7a,A	66.6 \pm 5.7a,A	0.7 \pm 0.07a,A	0.9 \pm 0.04a,A	2.3 \pm 0.2a,A
		30	64.0 \pm 2.4a,AB	54.5 \pm 8.7a,A	49.5 \pm 6.0a,A	0.7 \pm 0.07a,A	0.9 \pm 0.03a,A	1.2 \pm 0.3a,A
		60	73.4 \pm 0.6a,A	58.9 \pm 3.8a,A	52.6 \pm 11.5a,A	0.7 \pm 0.07a,A	0.8 \pm 0.11a,A	1.5 \pm 0.1a,A
	Ser 0.05%	0	69.6 \pm 11.8ab,A	56.4 \pm 7.9ab,A	52.5 \pm 5.9ab,A	0.7 \pm 0.14a,A	0.9 \pm 0.04a,A	1.4 \pm 0.3a,A
		15	100.6 \pm 9.6b,A	84.5 \pm 13.9b,A	78.3 \pm 4.6b,A	0.7 \pm 0.13a,A	0.9 \pm 0.09a,A	2.1 \pm 0.2a,A
		30	54.4 \pm 5.4a,A	50.7 \pm 4.5a,A	45.8 \pm 3.7a,A	0.8 \pm 0.04a,A	0.8 \pm 0.03a,A	1.3 \pm 0.04a,A
		60	77.4 \pm 8.9ab,A	61.4 \pm 11.3ab,A	54.0 \pm 5.5ab,A	0.7 \pm 0.06a,A	0.8 \pm 0.06a,A	2.1 \pm 0.2,A
	Ser 0.1%	0	108.6 \pm 5.3a,BC	85.3 \pm 4.6a,AB	75.5 \pm 2.0a,AB	0.6 \pm 0.16a,A	0.8 \pm 0.05a,A	1.8 \pm 0.26a,A
		15	103.1 \pm 14.2a,A	84.6 \pm 5.4a,A	77.6 \pm 0.7a,A	0.7 \pm 0.09a,A	0.9 \pm 0.04a,A	2.3 \pm 0.04a,A
		30	95.5 \pm 5.7a,BC	77.8 \pm 2.8a,AB	73.3 \pm 2.8a,AB	0.7 \pm 0.06a,A	0.9 \pm 0.03a,A	2.1 \pm 0.28a,AB
		60	96.6 \pm 7.5a,A	79.1 \pm 8.1a,AB	72.8 \pm 7.7a,AB	0.7 \pm 0.07a,A	0.9 \pm 0.05a,A	2.1 \pm 0.18a,A
Ser 0.5%	0	119.6 \pm 6.6a,C	97.7 \pm 12.6a,B	88.4 \pm 14.1a,B	0.7 \pm 0.09a,A	0.9 \pm 0.05a,A	2.0 \pm 0.26a,A	
	15	117.5 \pm 7.2a,A	87.9 \pm 8.5a,A	78.6 \pm 11.9a,A	0.6 \pm 0.06a,A	0.8 \pm 0.07a,A	2.2 \pm 0.03a,A	
	30	131.1 \pm 6.6a,C	92.7 \pm 14.7a,B	85.5 \pm 5.2a,B	0.6 \pm 0.09a,B	0.9 \pm 0.03a,A	2.5 \pm 0.38a,B	
	60	111.0 \pm 3.2a,A	95.3 \pm 6.6a,B	89.5 \pm 15.0a,B	0.8 \pm 0.07a,A	0.9 \pm 0.02a,A	2.6 \pm 0.40a,A	
Tanpa Swim up	BSA	0	126.4 \pm 7.4a,A	84.3 \pm 7.7a,A	64.4 \pm 10.4a,A	0.5 \pm 0.03a,A	0.7 \pm 0.03a,A	4.4 \pm 0.27a,A
		15	107.0 \pm 4.7a,A	74.3 \pm 6.7a,A	58.6 \pm 7.7a,A	0.5 \pm 0.05a,A	0.7 \pm 0.03a,A	4.1 \pm 0.35ab,A
		30	90.0 \pm 8.0a,A	63.6 \pm 10.2a,A	47.5 \pm 5.7a,A	0.5 \pm 0.09a,A	0.7 \pm 0.04a,A	4.3 \pm 0.47ab,A
		60	108.9 \pm 8.8a,A	76.5 \pm 11.7a,A	61.5 \pm 7.9a,A	0.5 \pm 0.09a,A	0.81 \pm 0.05a,A	3.8 \pm 0.11b,A
	Ser 0.05%	0	100.4 \pm 6.8a,A	66.0 \pm 8.0a,A	48.9 \pm 8.2a,A	0.4 \pm 0.02a,A	0.7 \pm 0.03a,A	4.6 \pm 0.35a,A
		15	113.2 \pm 10.9a,A	74.3 \pm 6.2a,A	55.6 \pm 4.1a,A	0.4 \pm 0.04a,A	0.7 \pm 0.03a,A	4.6 \pm 0.39a,A
		30	92.7 \pm 1.6a,A	63.6 \pm 3.2a,A	49.2 \pm 10.0a,A	0.5 \pm 0.05a,A	0.7 \pm 0.07a,A	4.1 \pm 0.69a,A
		60	80.8 \pm 10.6a,A	55.4 \pm 9.2a,A	42.1 \pm 10.8a,A	0.5 \pm 0.10a,A	0.7 \pm 0.08a,A	4.0 \pm 0.28a,AB
		0	115.5 \pm 4.4a,A	72.9 \pm 5.2a,A	53.5 \pm 6.0a,A	0.4 \pm 0.07a,A	0.7 \pm 0.04a,A	4.2 \pm 0.31a,A

Group	Protein	Minute	VCL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	LIN (%)	STR (%)	ALH ($\mu\text{m/s}$)
Seri 0.1%		15	115.9 \pm 4.2a,A	78.4 \pm 8.7a,A	61.8 \pm 9.1a,A	0.5 \pm 0.04a,A	0.7 \pm 0.04a,A	4.2 \pm 0.24a,A
		30	102.6 \pm 8.5a,A	69.9 \pm 9.5a,A	53.9 \pm 8.5a,A	0.5 \pm 0.05a,A	0.7 \pm 0.05a,A	4.4 \pm 0.38a,A
		60	112.0 \pm 8.3a,A	72.4 \pm 7.2a,A	55.6 \pm 8.6a,A	0.4 \pm 0.04a,A	0.7 \pm 0.03a,A	4.2 \pm 0.26a,BC
Ser 0.5%		0	128.9 \pm 9.6a,A	81.6 \pm 6.8a,A	60.2 \pm 9.4a,A	0.4 \pm 0.05a,A	0.7 \pm 0.06a,A	4.6 \pm 0.49a,A
		15	120.6 \pm 12.5a,A	83.1 \pm 6.1a,A	66.7 \pm 5.7a,A	0.5 \pm 0.05a,A	0.8 \pm 0.05a,A	4.0 \pm 0.56a,A
		30	120.3 \pm 11.4a,A	81.8 \pm 8.1a,A	64.4 \pm 7.4a,A	0.5 \pm 0.09a,A	0.7 \pm 0.05a,A	4.2 \pm 0.70a,A
		60	116.8 \pm 0.4wa,A	75.5 \pm 8.5a,A	56.8 \pm 5.7a,A	0.4 \pm 0.04a,A	0.7 \pm 0.03a,A	4.4 \pm 0.18a,C

BSA: Fertilization medium added with 0.3% BSA; 0.05% Ser: Fertilization medium added with 0.05% *Sericin*; Ser 0.1%: Fertilization medium added 0.1% *Sericin*; Ser 0.5%: Fertilization medium added 0.5% *Sericin*. Letters a,b,c,d,e in the same treatment group with different incubation times indicate significant differences ($P < 0.05$). Letters A,B,C,D in different treatment groups with the same time indicates a difference ($P < 0.05$).

VAP and VSL values in the group without swim-up had no difference between time and treatment ($P > 0.05$). The swim-up group at minutes 0, 30, and 60 showed a significant difference ($P < 0.05$), the value of *sericin* 0.5% treatment was higher than other treatments and at minute 0 the value of *sericin* 0.05% was found to be lower than other treatments ($P < 0.05$), at minutes 30 and 60 the value of *sericin* 0.05% was not significantly different from BSA ($P > 0.05$).

Linearity (LIN) is a parameter used to measure how straight the trajectory of spermatozoa movement is during swimming. LIN is calculated as the ratio between straight line velocity (VSL, Straight Line Velocity) and curvilinear velocity (VCL, Curvilinear Velocity) (Singh et al. 2017). Straightness (STR) indicates the average trajectory's straightness (Ratnawati et al. 2019). High STR values indicate sperm are moving on a straight and efficient trajectory toward the egg. In contrast, low STR values indicate a more random or circular movement, which may indicate less than optimal motility (Ratnawati et al. 2020). LIN and STR values at different times and treatments showed no significant differences between treatments in the swim-up group and without swim-up ($P > 0.05$), except at minute 30 of the swim-up group it was found that the LIN and STR values in the *sericin* 0.05% treatment were lower than the other treatments ($P < 0.05$). These results indicate that in the swim-up group all

treatments showed a linear swimming pattern with a range of LIN values of 69-84%, while without swim-up ranged from 48-58%. Spermatozoa swim nonlinearly when the LIN value is $< 35\%$ (Susilawati 2011). A high LIN indicates efficient movement toward the oocyte, which is crucial for increasing the chances of fertilization (Mujitaba et al. 2023).

Amplitude Lateral Head (ALH) describes the average width of the vibration or movement of the spermatozoa head during the swimming process (Kathiravan et al. 2011). An increase in ALH reflects broader flagellum movement, indicating more energetic movement. ALH values in the swim-up group showed no significant differences between treatments and time ($P > 0.05$), except at minute 30, the value of *sericin* 0.5% was higher than other treatments ($P < 0.05$). The group without swim-up showed no difference between treatments, except at 60 minutes, the ALH value in the *sericin* 0.5% treatment was higher than BSA, *sericin* 0.05%, and 0.1%. The increase in incubation time showed no significant difference except for the BSA treatment, which decreased from minute 0 to 60 minutes of the incubation period ($P < 0.05$).

Spermatozoa generally have two motility phases, active and hyperactive (Ngcauzele et al. 2020). Active motility is usually observed immediately after ejaculation or removal from storage media, with coordinated and directed movements. Spermatozoa motility changes significantly when entering the

hyperactive phase after the capacitation process. Capacitation occurs in vivo when sperm are exposed to the female reproductive tract or in vitro after incubation in certain media (Saleh and Abdul-Ameer 2019). Hyperactive motility in sperm is characterized by high amplitude, symmetrical flagellum movement (Gordon 2003). Without capacitation, spermatozoa cannot fertilize oocytes (Macias-Garcia et al. 2015).

In vivo spermatozoa hyperactivity values reported in the study of Akthar et al. (2023) were $VCL \geq 200 \mu\text{m/s}$, $ALH \geq 3 \mu\text{m}$, and $LIN \leq 40\%$. Shojaei et al. (2012) reported that spermatozoa experienced a change in movement to hyperactivity when ALH values $> 7 \mu\text{m}$, $LIN < 65\%$, and $VCL > 80 \mu\text{m}$. VAP, VCL, and ALH parameters are related to the hyperactivation of motility and capacitation and indicate specific aspects of sperm function (Chatiza et al. 2012). Based on this, this study showed that the initial sign of hyperactivation of spermatozoa motility only occurred in the group without swim up for BSA and *sericin* addition treatment. The swim-up group was hyperactivated only when *sericin* 0.1% and 0.5% were added. This condition can be caused because the sperm in the swim-up group was first exposed to the swim-up medium, which in accordance with the Gordon method (2003), the composition of the medium without additional ingredients can trigger hyperactivation. The group without swim-up is directly exposed to the fertilization medium because components such as caffeine can initiate hyperactivation. This condition aligns with research conducted by Gordon (2003), which states that fertilization medium containing caffeine and BSA can capacitate spermatozoa to be exposed to matured oocytes.

Caffeine enhances sperm motility by increasing intracellular calcium through translocation from internal calcium stores or by inhibiting phosphodiesterases, increasing cyclic adenosine monophosphate and protein kinase A (Bender and Beavo 2006). BSA plays an important role in the sperm capacitation by removing cholesterol from

the sperm plasma membrane, which increases membrane fluidity and triggers the necessary biochemical changes. This cholesterol removal is associated with increased protein tyrosine phosphorylation, regulated by signaling pathways involving cAMP and protein kinase A (PKA). By facilitating cholesterol removal, BSA allows sperm to reach the maturation stage required to fertilize oocytes, making it an essential component in vitro capacitation media (Osheroff et al. 1999; Visconti et al. 1999). This study's results indicate that adding *sericin* at concentrations of 0.05%, 0.1%, and 0.5% can cause hyperactivation. This condition contradicts the research conducted by Nasirabadi et al. (2019), where it was stated that *sericin* as an antioxidant could increase the amount of GSH, which results in a decrease in the amount of intracellular Ca^{2+} then results in a decrease in cAMP and tyrosine phosphorylation. Finally, these events reduced the percentage of capacitated sperm in the antioxidant treated group.

Synthetic and Natural Chemicals Used on Spermatozoa

For comparison, another synthetic chemical compound, Pentoxifylline (PTX), was also applied to spermatozoa. PTX is a dimethylxanthine derivative that is widely used in spermatozoa owing to its antioxidant properties and its ability to increase motility. The addition of PTX to the fertilization medium (IVF) with a 30 minute incubation period in bovine spermatozoa at concentrations of 1 mM and 5 mM has been shown to increase sperm motility (Barakat et al. 2015). Furthermore, there are other synthetic chemical compounds, such as Trolox, analogous to vitamin E with antioxidant activity (Minaei et al. 2012). Glutathione, a natural antioxidant, protects spermatozoa from lipid peroxidation (Adeoye et al. 2018). Supplementation with 1 mM glutathione in the washing medium enhances the fertilization capacity of frozen-thawed bovine sperm with low IVF competence and supports normal embryo development (Itahashi et al. 2022).

CONCLUSION

Sericin has the potential to be added to fertilization media for spermatozoa with an optimum concentration of 0.5% and can be an alternative to BSA. *Sericin* 0.5% can maintain quality and show early signs of hyperactivation of spermatozoa. The decrease in motility and plasma membrane integrity values in the swim-up method followed the same pattern as the method without swim-up, the swim-up method showed higher motility and membrane integrity values until the end of the incubation period.

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