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Effects of different doses and sizes of BSA nanoparticles on cryopreservation of angora buck semen: motility, functional membrane ıntegrity and, viability

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Abstract

Purpose: BSA is a highly soluble substance consisting of 585 amino acids, which has been successfully used for the cryopreservation of sperm from various species for a long time. However, the nanoforms of BSA (BSA NPs) have been utilized in drug delivery applications in different cell types in recent years due to their high bioavailability and increased surface areas. In this study, we investigate the effects of BSA NPs on the cryopreservability of Angora buck sperm.
Method: Sperm samples from five different Ankara rams were collected in six replicates and divided into five groups: control, BSA 20-5, BSA 20-10, BSA 40-5, and BSA 40-10. The sperm, frozen in liquid nitrogen vapor, were thawed at 37 ºC, and motility, functional membrane integrity (HOST), and live/dead ratios were assessed.
Findings: The data obtained showed no statistically significant difference among the groups in terms of motility and live/dead ratios. However, in the HOST test, the control group and the BSA 20-5 group had higher resistance capabilities compared to the other groups, while BSA 20-10, BSA 40-5, and BSA 40-10 groups reduced membrane resistance capability. This situation is considered to result from the removal of cholesterol from the membrane by BSA nanoparticles.
Conclusion: Consequently, the study concludes that BSA NPs used at low doses and sizes do not harm sperm and hold promise for use in drug delivery systems.

Keywords: BSA, nanoparticle, sperm, freezing, membrane, drug delivery

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Farklı doz ve boyuttaki BSA nanopartiküllerinin ankara tekesi spermasının kriyoprezervasyonu üzerindeki etkileri: motilite, membranın fonksiyonel bütünlüğü ve canlılık

Özet
Amaç: BSA, 585 amino asit içeren ve yüksek oranda çözünebilir bir madde olup, uzun zamandır farklı türlerde spermanın dondurulmasında başarıyla kullanılmaktadır. Ancak, BSA'nın nanoformları (BSA NPs), yüksek biyoyararlanım kapasiteleri ve artan yüzey alanları sayesinde son yıllarda farklı hücrelerde ilaç taşıma uygulamalarında

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kullanılmaktadır. Bu çalışmada, BSA NPs'ın Ankara tekesi spermasının dondurulabilirliği üzerindeki etkileri incelenmiştir.
Metod: Beş farklı Ankara tekesi sperması, 6 tekrar halinde toplanmış ve kontrol, BSA 20-5, BSA 20-10, BSA 40-5 ve BSA 40-10 olacak şekilde 5 gruba ayrılmıştır. Sıvı azot buharında dondurulan spermalar, 37 ºC'de çözdürülmüş ve motilite, membranın fonksiyonel bütünlüğü (HOST) ve canlı/ölü oranları değerlendirilmiştir.
Bulgular: Elde edilen veriler, motilite ve canlı/ölü oranı açısından gruplar arasında istatistiksel olarak anlamlı bir fark olmadığını göstermektedir. Ancak, HOST testi sonucunda, kontrol grubu ve BSA 20-5 grubu diğer gruplara oranla daha yüksek membran dayanım kabiliyetine sahip bulunurken, BSA 20-10, BSA 40-5 ve BSA 40-10 gruplarındaki spermaların membran dayanım kabiliyeti düşmüştür. Bu durumun, BSA NPs'ın membrandaki kolesterolü delokalize etmesinden kaynaklandığı düşünülmektedir.
Sonuç: Sonuç olarak, düşük doz ve boyuttaki BSA NPs'ın spermaya zarar vermediği ve ilaç taşıma sistemlerinde kullanım potansiyeli taşıdığı sonucuna varılmıştır.

Anahtar kelimeler: BSA, nanoparçacık, sperm, dondurma, membran, ilaç taşınımı

Introduction

Free radicals are a normal component of mammalian cellular oxygen metabolism. However, in many pathological processes, the damage caused by free radicals constitutes a significant factor. Glycation and oxidative stress lead to protein modifications and, consequently, various forms of cellular damage [1].

Albumin is a highly soluble substance composed of 585 amino acids and plays a role in the transport of metals, fatty acids, cholesterol, bile pigments, and drugs. Additionally, it has important physiological and pharmacological functions, such as regulating osmotic pressure [2]. It is the most important antioxidant in human blood plasma and is known to play a major role in the antioxidative defense system against oxidative stress occurring in plasma [3]. Albumin is known as the primary antioxidant circulating in the blood, and it exerts this antioxidative effect either by directly scavenging and neutralizing free oxygen radicals formed after lipid peroxidation (LPO) in cells or by preventing the interaction of metal chelates with cell membranes [4].

Polyunsaturated fatty acids (PUFAs) constitute a significant portion of oxidizable biological compounds in plasma. Serum albumin binds to long-chain fatty acids and PUFAs with high affinity. There is no definitive evidence regarding whether PUFAs are protected from lipid peroxidation (LPO) when bound to albumin. However, antioxidants that are effective in the lipid phase, such as bilirubin and nitric oxide (NO), can bind to albumin. This indirectly suggests that PUFAs bound to albumin may be protected from oxidative stress-induced damage [5].

In their study, Fukuzawa et al. (2005) investigated the role of bovine serum albumin (BSA) in preventing membrane lipid peroxidation. For this purpose, lipid peroxidation was induced by exposing egg yolk phosphatidylcholine liposomes, which contained lipids with different charges, to enzymatically generated superoxide radicals and chelated iron catalysts. The study concluded that BSA inhibited lipid peroxidation both by preventing the interaction of iron chelates with membranes and by scavenging free oxygen radicals [6].

The cryopreservation of sperm is a revolutionary technique in animal husbandry, capable of extending the fertile lifespan of a bull by several decades. For sperm freezing, both permeable (glycerol, dimethyl sulfoxide, ethylene glycol, methanol, and amides) and non-permeable (egg yolk, sucrose, BSA, monosaccharide and disaccharide sugars, polyvinylpyrrolidone, and hydroxyethyl starch) cryoprotectant agents are used [7]. It has been reported that fertilization is negatively affected by uterine infections that occur following artificial insemination with sperm frozen using permeable cryoprotectants [8]. However, due to the improved spermatological parameters obtained after freezing and thawing with permeable cryoprotectants, it has been reported that combining them with non-permeable cryoprotectants such as BSA yields more successful results [9].

The cryoprotective effect of BSA has been tested in many species, including bull, ram, buffalo and, dog sperm. It has been shown to have positive effects on parameters such as sperm viability, membrane integrity, motility, and lipid peroxidation (LPO). However, BSA nanoparticles (NPs) typically have a larger surface area, better bioavailability, and higher stability. This could enable more efficient use of BSA, particularly in drug delivery systems or biological applications. Based on this concept, in our study, the effects of BSA nanoparticles of different doses and sizes on the semen of Angora buck were investigated

2. Materials and methods

2.1. Synthesis of BSA NPs

One gram of Bovine Serum Albumin (BSA) was added to 20 mL of buffer solution and stirred at 500 rpm using a magnetic stirrer until complete dissolution was achieved. Subsequently, ethanol was introduced dropwise to the mixture at a rate of 4 mL every 4 minutes. Following this, 30 µL/mL of glutaraldehyde was incorporated into the solution. The mixture continued to be stirred at 500 rpm for a duration of 8 hours. After this period, the solution was centrifuged at 14,000 rpm for 30 minutes at 20°C. This step was repeated three times to effectively remove any undissolved BSA, ethanol, glutaraldehyde,

and other auxiliary intermediates. The resulting pellets were then dried in an oven at 50°C for 6-8 hours and subsequently re-dispersed in deionized distilled water to restore the original volume using an ultrasonic bath. The produced BSA nanoparticles (NPs) were characterized utilizing scanning transmission electron microscopy (STEM) for morphological analysis, dynamic light scattering (DLS) to determine effective diameter, Zeta potential for assessing surface charge, and Fourier-transform infrared spectroscopy (FTIR) to evaluate binding vibrations [10,11].

2.2. Animals, Semen Collection, Freezing and Thawing

In the study, 5 Angora goats (aged 3-5 years) housed at Selçuk University Hümeyra Özgen Research and Application Farm were used. The animals were fed ad libitum a few months before the study, and sperm was collected using an electroejaculator twice a week during the breeding season, for a total of 6 times. The collected semen was analyzed for mass movement and motility. Semen samples with a mass movement score of 4 or higher and a motility rate above 80% were pooled and mixed, then divided into the following experimental groups;

1- Control
2- 20nm and 5 mg/mL BSA NPs (BSA20-5)
3- 20nm and 10 mg/mL BSA NPs (BSA20-10)
4- 40nm and 5 mg/mL BSA NPs (BSA40-5)
5- 40nm and 10 mg/mL BSA NPs (BSA40-10)

Each group was supplemented with a Tris-based extender as the basic diluent (27.1 g/L Trizma, 10 g/L fructose, 14 g/L citric acid, 5% glycerol, 18% egg yolk, and 0.1% penicillin-streptomycin-amphotericin B combination), followed by the addition of BSA nanoparticles of varying doses and sizes. All chemicals used in the study were obtained from Sigma-Aldrich.

The diluted semen was drawn into 0.25 mL straws, and the ends were sealed with heat press. Prior to freezing, the straws were left for 3 hours to equilibrate at 4°C and were then frozen in liquid nitrogen vapor for 20 minutes. The frozen semen was poured into liquid nitrogen and stored at -196°C in a liquid nitrogen tank until analyses were performed. When spermatological analyses were to be conducted, the straws taken from the liquid nitrogen tank were thawed in a water bath at 37°C.

2.3. Motility Analysis

The thawed straw was cut in half, and 5 µL of sperm was dropped onto a pre-warmed slide on a heating plate. The slide was then covered with a coverslip. Sperm motility was examined at 400x magnification on a heating plate (37°C) using a phase contrast microscope. Five different areas were analyzed, and the average motility data were recorded subjectively as a percentage [12].

2.4. Assessment of Functional Integrity of Membrane (Hypoosmotic Swelling Test)

A 300 µL aliquot of the prepared HOST solution (0.735 g sodium citrate + 1 g fructose + 100 mL distilled water) was mixed with 30 µL of sperm and incubated at 37°C for 1 hour. A 20 µL sample of the mixture was taken, and a total of 300 spermatozoa were counted under a phase contrast microscope at 400x magnification. The number of swollen and coiled-tail spermatozoa was counted, and the percentage of membrane integrity was determined. Swollen and coiled-tail spermatozoa were classified as HOST+ due to their intact membrane, while spermatozoa with non-coiled tails were classified as HOST- due to membrane damage [13].

2.5. Live/Dead Sperm Viability Rate

The Eosin-Nigrosin staining method was used to determine the live-dead sperm ratio. One drop of sperm was placed on a slide and mixed with two drops of Eosin-Nigrosin stain to prepare smears. The prepared smears were examined under a phase contrast microscope at 400x magnification. At least 300 spermatozoa were counted, and those with red-stained heads were classified as dead, while those without staining (white) were classified as live. The results were recorded as a percentage [14].

2.6. Statistical Analysis

The statistical analysis of the data obtained in the study was performed using IBM SPSS Statistics 25 software. The means of the results in the spermatological parameters and imaging were determined using one-way ANOVA. For comparisons of groups with significant differences within the same parameters, multiple comparison tests were conducted using the Duncan test. A p-value of <0.05 was considered statistically significant when evaluating the results.

3. Results

3.1. Characterisation of BSA NPs

The STEM analysis of BSA nanoparticles revealed that nanoparticles with sizes of 20 nm (Figure 1A) and 40 nm (Figure 1B) exhibited a predominantly spherical and elliptical morphology. Observations indicated a tendency for BSA

nanoparticles to undergo aggregation. Dynamic light scattering (DLS) gave hydrodynamic diameters to be ~1500 nm and 2300 nm for 40 nm and 20 nm, respectively (Figure 1E and F). The hydrodynamic sizes were measured in solution, and the large sizes were attributed to sticky intrinsic properties of BSA molecules in BSA NPs. The surface charge (Zeta potential) measurements were recorded as -43 mV for 20 nm BSA nanoparticles and -38.5 mV for 40 nm BSA nanoparticles. For FT-IR results, while the aliphatic C-H stretching bonds were recorded between 2970-2820 cm-1, the C=O stretching bonds were seen at around 1700 cm-1. The broad band at around 630 cm−1 can be attributed to −NH2 and −NH oscillation.

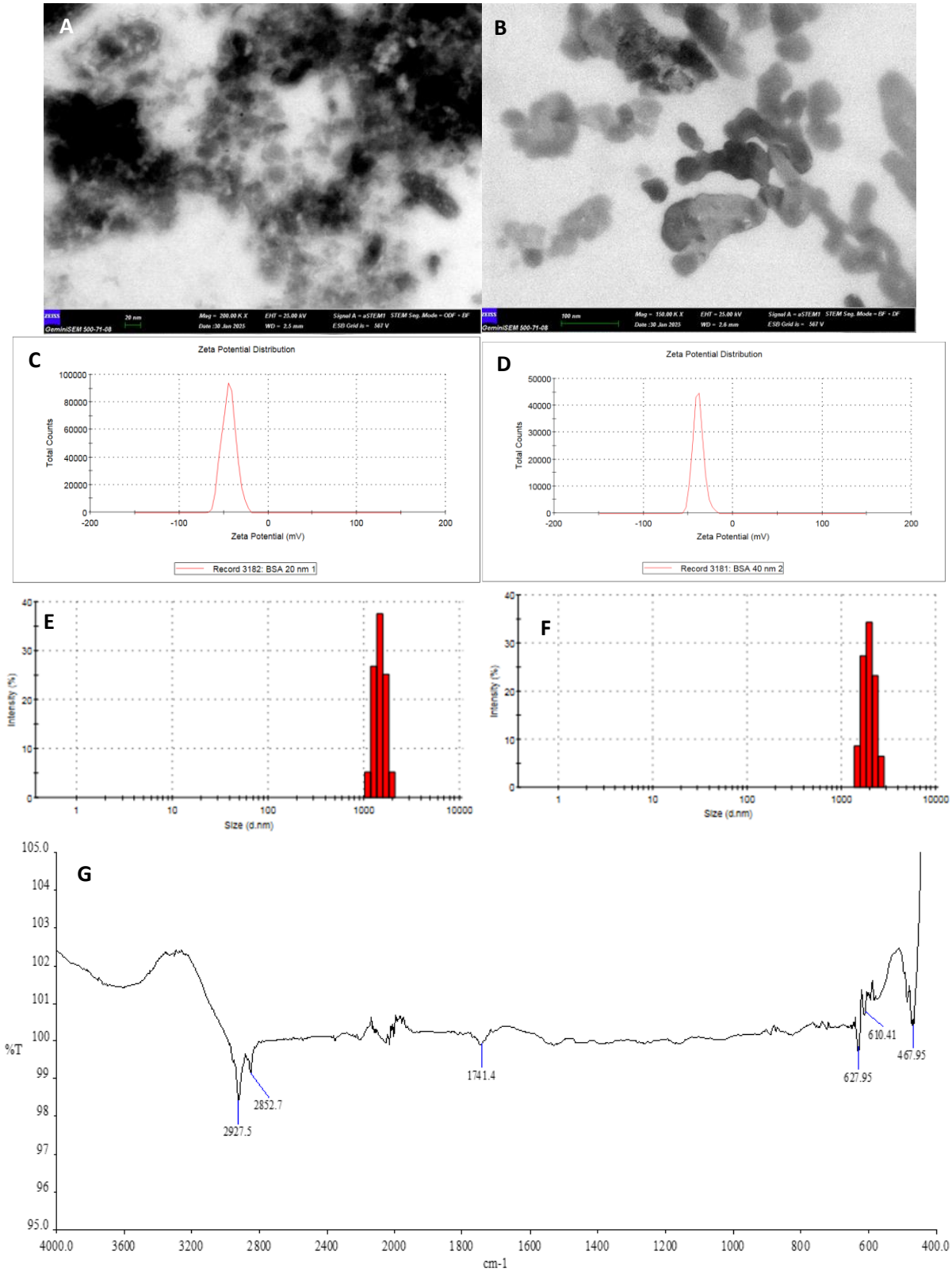

Figure 1. BSA NPs produced in ≈ 20nm (A) and ≈ 40nm size (B). Zeta potential diagram of ≈ 20nm (C) and ≈ 40nm (D). Dynamic light scattering diagram of ≈ 20nm (E) and ≈ 40nm (F) and FT-IR results of BSA NPs (G)

3.2. Motility

Motility data are presented in Figure 2. Upon examining Figure 2, it can be observed that BSA nanoparticles (NPs) at both 20 nm and 40 nm sizes, at a concentration of 5 mg/mL, produced more positive results compared to the control group. However, at the 10 mg/mL concentration of BSA NPs, similar results to the control group were observed for both sizes. Although both sizes of 5 mg/mL BSA NPs showed more favorable results than the control group, these results were not statistically significant (P>0.05).

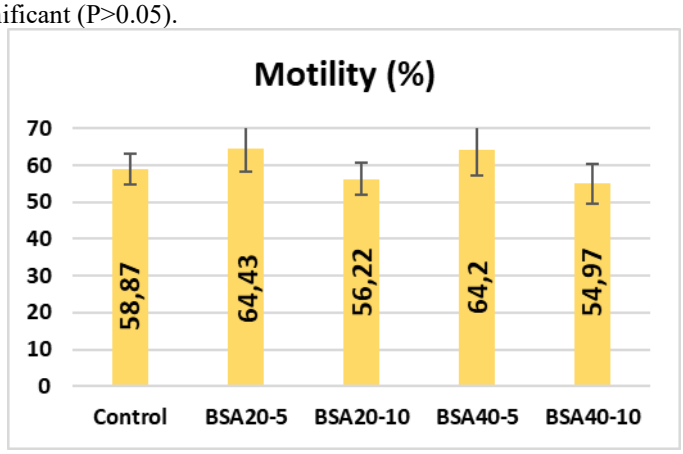

Figure 2. Motility results for BSA NPs added Angora Goat spermatozoa after the freezing-thawing process (P>0.05).

3.3. Functional Membrane Integrity

When assessing the functional integrity of the membrane, it was observed that as the size and dose of BSA nanoparticles (NPs) increased, the membrane resistance capacity decreased (Figure 3). Similarly, an increase in the proportion of membrane-damaged spermatozoa was observed with the increase in both the size and dose of BSA NPs. The results obtained were statistically significant (P<0.05).

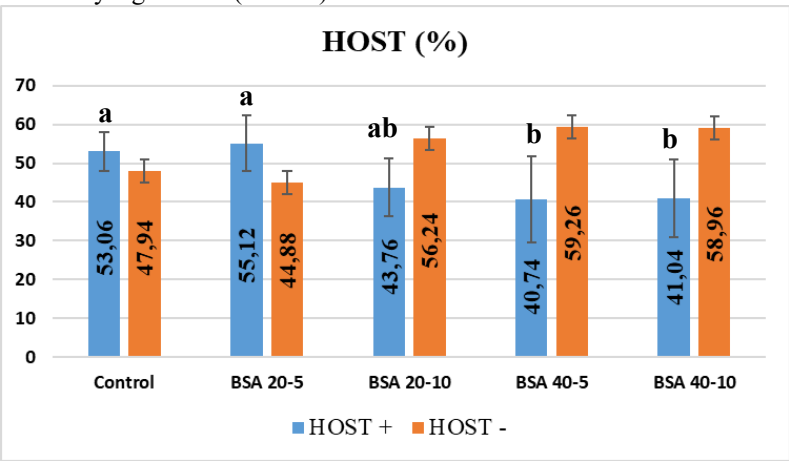

Figure 3. The bars shown in blue represent spermatozoa with intact membrane integrity (HOST+), while the bars shown in orange represent spermatozoa with damaged membranes (HOST-). In the labeling, those marked with "a" represent the statistically most positive results, while those marked with "b" represent the most negative results

3.4. Live&Dead Spermatozoa Rate

When examining the Live&Dead spermatozoa data, all groups containing BSA nanoparticles (NPs) showed similar results to the control group, with no statistically significant differences. Although the BSA20-10 group exhibited a lower viability rate, this difference was not statistically significant (P>0.05).

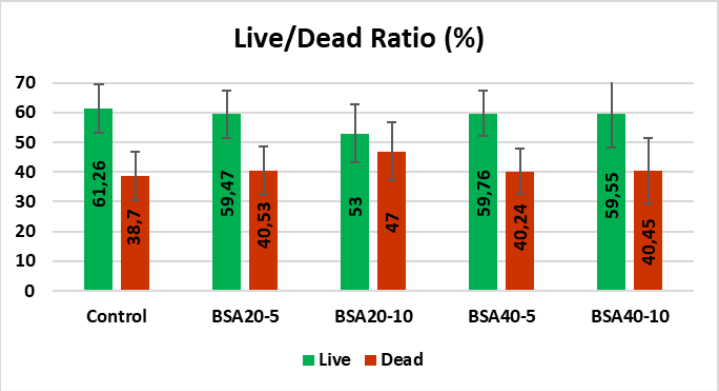

Figure 4. The green bars represent the proportion of live spermatozoa, while the brown bars represent the proportion of dead spermatozoa. No statistically significant differences were found between the groups for either of these data points (P>0.05)

4. Conclusions and discussion

BSA nanoparticles (NPs) have been increasingly used in recent years as drug delivery agents in various tissues and cells [15]. However, no studies have been found that investigate the effect of BSA nanoparticles (NPs) on sperm cryopreservation in animals. In this study, the effects of BSA NPs at two different doses and sizes on the cryopreservation of Angora buck sperm were examined, with a focus on their impact on motility, membrane functional integrity, and live & dead viability rate.

When examining the characterization results of BSA nanoparticles, STEM images and DLS results indicate that BSA nanoparticles tend to form aggregation. When evaluating the zeta potential and FT-IR data, the observation of the amide I (C=O stretching, ~1700 cm^{-1}) and amine ($-NH_2$, ~630 cm^{-1}) bands in the FT-IR spectrum aligns with protein structures. Meanwhile, the zeta potential values of -43 mV (20 nm) and -38.5 mV (40 nm) appear to be consistent with the expected negative charge distribution of BSA.

Regarding motility data, BSA nanoparticles (NPs) at both 20 nm and 40 nm sizes and a 5 mg/mL dose increased motility. However, when a 10 mg/mL dose was used, results were similar to the control group. When evaluating membrane functional integrity, 20 nm BSA NPs at 5 mg/mL positively affected membrane resistance, while all other doses and sizes decreased membrane integrity. No significant differences were observed in the Live&Dead spermatozoa ratio between the groups.

Although not in its nanoparticle form, BSA has long been used in sperm cryopreservation studies. Therefore, the discussion will focus on studies involving the use of BSA in sperm freezing rather than those involving BSA nanoparticles (NPs). First, the dose-dependent increase observed in the motility data suggests that the effect is dose-dependent rather than dependent on the size of the nanomaterial. The literature reports studies where BSA showed a high motility value at specific doses, while in some doses, it was ineffective. Naijian et al. [16] applied BSA to Mahabadi goat sperm at doses of 2.5, 5, 10, 15, and 20 mg/mL and observed the highest motility value at a dose of 10 mg/mL. Rayan et al. [17] stated that the addition of 1% BSA to bull semen resulted in more positive motility outcomes compared to the 0.5%, 1.5%, and 2% BSA concentrations. It is known that BSA, by being present in seminal plasma fluid, helps preserve motility and acrosomal integrity [18]. The reason why certain doses of BSA show positive effects while others do not is believed to be due to variations in the seminal plasma composition across breeds or species.

The Live&Dead spermatozoa rate and membrane functional integrity data are related to cell membrane durability. The Live&Dead spermatozoa rate reflects the sperm's condition in a balanced osmotic environment after thawing, while membrane functional integrity reflects the membrane's resistance to osmotic pressure in a hypo-osmotic environment. In our study, no statistically significant differences were found between the groups in terms of the Live&Dead spermatozoa rate. However, in the literature, there are studies that report BSA preserves membrane integrity in dog [19], ram and rabbit sperm [20,21]. In the current study, similar results were obtained for the Live&Dead spermatozoa ratio. However, in our study, BSA NPs at lower doses and sizes preserved membrane functional integrity, whereas as the dose and size increased, membrane integrity decreased. The mechanism behind this is not difficult to explain. Larger doses and sizes of BSA NPs may induce more stress on the membrane, potentially leading to destabilization, while smaller doses and sizes might be more effective in maintaining membrane stability. El-Kon [22] reported that during the cryopreservation of buffalo sperm, the BSA used showed a protective effect by reducing the degree of damage to spermatozoa, due to its specific interaction with sperm plasma membrane phospholipids. This effect was attributed to the removal of cholesterol from the sperm cell membrane by BSA, which led to increased membrane fluidity before freezing and enhanced post-thaw sperm viability. Cholesterol plays a role in the stability of the cell membrane. It acts like the cement holding bricks together in a wall, and when its levels increase, the membrane becomes more fragile, whereas a reduction in cholesterol increases membrane fluidity [23, 24]. BSA causes the destabilization of cellular cholesterol and promotes its removal from the membrane [25]. This process

contributes to changes in membrane fluidity, which can have implications for sperm membrane stability during cryopreservation. Through this mechanism, BSA increases the fluidity of the cell membrane and protects the sperm from the effects of cold shock during the cryopreservation process.

However, when it comes to the functional durability of the membrane in our study, it is believed that the protective effect of BSA may have turned into a disadvantage. The membrane, which becomes more fluid and loses its cholesterol structure, may not have been able to withstand osmotic pressure. Therefore, it is suggested that as the size and dose of BSA NPs increase, the membrane's resilience decreases.

In conclusion, BSA NPs, like standard BSA, showed a dose-dependent effect on spermatozoa. However, transforming BSA into nanoparticle form yielded similar results to standard BSA. What differentiates BSA NPs from standard BSA is that BSA NPs have the advantage of cellular drug delivery. Therefore, the fact that BSA NPs did not show any negative effects on spermatozoa in this study holds promise for future drug delivery applications using BSA NPs on spermatozoa.

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Conflicts of interest: The authors of this study declare that they have no conflicts of interest with any institutions or organizations.

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Ethical statement: The ethical approval for this study was granted by the Ethics Committee of Selçuk University, Faculty of Veterinary Medicine, Animal Research and Production Center, with decision number 2022/40 dated 06.05.2022.

Author contributions: Ali Erdem ÖZTÜRK, Yunus Emre ATAY and Mustafa BODU designed the research study. Ali Erdem ÖZTÜRK, Mustafa BODU and Zeliha KILINÇ performed the research. Serpil SARIÖZKAN collected and analyzed the data. İsmail ÖÇSOY has been involved in drafting the manuscript. All authors give final approval of the version to be published.

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