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RESEARCH ARTICLE

Effect of Shilajit on Freezing Rooster Semen

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ABSTRACT

Reactive oxygen species (ROS) are created in excess during the cryopreservation process, which speeds up the rate of lipid peroxidation (LPO). This negatively impacts spermatozoa functions and reduces their capacity to fertilize. The spermatozoon plasma membrane consists of significant amounts of polyunsaturated fatty acids, which can be easily oxidized by ROS and produce harmful agents that are toxic to cells. The plasma membrane of rooster spermatozoa contains very small amounts of mitochondria, cytoplasm, and cytoplasmic antioxidants. Cryopreservation of rooster semen has been associated with adverse effects, including increased lipid peroxidation, structural damage in the mitochondria and acrosomal area, changes in the integrity and permeability of the spermatozoon plasma membrane, and severe damage to DNA. In the study, semen taken from 20 Plymouth Rock roosters were pooled to eliminate individual differences. By adding 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL and 25 µg/mL shilajit to Beltsville Poultry Semen Extender diluent, 5 experimental and 1 control groups were formed and frozen in 0.25 mL straws. After thawing in a water bath at 37oC, spermatologic parameters were analyzed with the CASA system. Viability evaluations were made with eosin – nigrosin stain and morphological evaluations were made with Hancock method. Sperm DNA integrity was examined with the COMET assay. As a result, it was concluded that the addition of 10, 15, 20 µg/mL shilajit to rooster semen extender improves semen quality parameters and DNA integrity of semen after cryopreservation.
Keywords: Cryopreservation, Rooster, Shilajit, Sperm

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ÖZ

Horoz Spermasının Dondurulmasında Shilajit'in Etkisi

Reaktif oksijen türleri (ROS), kriyoprezervasyon sürecinde aşırı miktarda oluşmakta ve lipid peroksidasyonu (LPO) hızını artırmaktadır. Bu durum, spermatozoa fonksiyonlarını olumsuz etkilemekte ve fertilizasyon yeteneğini azaltmaktadır. Spermatozoon plazma membranı, önemli miktarda çoklu doymamış yağ asidi içermekte ve ROS tarafından kolayca oksitlenebilmekte ve hücrelere toksik olan zararlı maddeler üretebilmektedirler. Horoz spermatozoon plazma membranı, çok az miktarda mitokondri, sitoplazma ve sitoplazmik antioksidan içermektedir. Horoz spermasının kriyoprezervasyonu, artmış lipid peroksidasyonu, mitokondri ve akrozomal bölgede yapısal hasar, spermatozoon plazma membranının bütünlüğü ve geçirgenliğinde değişiklikler ve DNA'da ciddi hasar gibi olumsuz etkilerle ilişkilendirilmiştir. Çalışmada, 20 Plymouth Rock ırkı horozdan alınan sperma bireysel farklılıkları ortadan kaldırmak için bir araya getirildi. Beltsville Poultry Semen Extender (BPSE) Sulandırıcısına 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL ve 25 µg/mL miktarlarda shilajit eklenerek 5 deney ve 1 kontrol grubu oluşturuldu ve 0.25 mL payetler içerisinde donduruldu. 37°C su banyosunda çözdürüldükten sonra spermatolojik parametreler CASA sistemi ile belirlendi. Spermatozoa canlılık oranı eosin – nigrosin boyama metodu ile, morfolojik değerlendirmeler ise Hancock yöntemi ile yapıldı. Spermatozoonn DNA bütünlüğü COMET analiz yöntemi ile değerlendirildi. Sonuç olarak, horoz sperma sulandırıcısına 10, 15, 20 µg/mL shilajit eklenmesinin, kriyoprezervasyon sonrası spermatolojik parametreleri ve DNA bütünlüğünü olumlu yönde etkilediği sonucuna varıldı.
Anahtar Kelimeler: Horoz, Kriyoprezervasyon, Shilajit, Sperma

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INTRODUCTION

With the discovery of the cryoprotective properties of glycerol, sperm cryopreservation has begun to be developed for many species. The first freezing of semen was carried out by Polge in 1951 using rooster semen (Polge, 1951). However, fertility rates with frozen poultry semen are highly variable but not reliable enough for commercial use or preservation of genetic material (Long, 2006).

The overproduction of reactive oxygen species (ROS) during the cryopreservation process accelerates lipid peroxidation (LPO), resulting in harmful effects on spermatozoa functions and a reduction in their fertilization ability. Tissues/cells contain highly oxidizable polyunsaturated fatty acids (PUFA) that are susceptible to lipid peroxidation (Chatterjee et al., 2001; Halliwell & Gutteridge, 2015).

The spermatozoon plasma membrane is rich PUFA, making it highly susceptible to oxidation by ROS, leading to the production of harmful agents that are toxic to cells. On the other hand, spermatozoa are exposed to physical and biochemical stress during the cryopreservation process, which could result in a decline in characteristics such spermatozoa metabolism, motility, plasma membrane integrity, and fertility (Wang et al., 1997).

Oxidative stress is a condition associated with an increased rate of cellular damage caused by oxygen and oxygen-derived oxidants, commonly known as ROS (Sikka et al., 1995). Oxidative stress accelerates a number of pathological conditions that affect fertilization ability (Joyce, 1987; Maneesh et al., 2005; Sharma & Agarwal, 1996).

The main factors hypothesized to potentially play a role in DNA-level damage during the freeze-thaw process are post-thawing osmotic stress and oxidative stress. Spermatozoon remains vulnerable to these stresses as it loses its cytoplasm with its enzymatic defenses (Alvarez & Storey, 1992; Bilodeau et al., 2000) ROS produced in this way can induce DNA damage (Twigg et al., 1998).

Rooster spermatozoa are marked by a relatively low content of cytoplasm, mitochondria, and cytoplasmic antioxidants, along with a high concentration of PUFA in their plasma membranes. These features make rooster spermatozoa especially vulnerable to damage during cryopreservation (Partyka et al., 2010). The cryopreservation of rooster semen has been associated with detrimental effects such as increased lipid peroxidation, structural damage to the mitochondria and acrosomal region, disruptions in plasma membrane integrity and permeability, and considerable DNA fragmentation. (Blesbois et al., 2005; Partyka et al., 2012; Partyka et al., 2010).

Shilajit, also known as shilajatu, mumie or mummiyo, is an exudation of varying consistency, pale brown to blackish-brown in color, found in rocks in many mountain ranges of the world, particularly the Himalayan mountain range in India (Kong et al., 1987; Srivastava et al., 1988).

The main physiological effect of shilajit has been found to be due to the presence of bioactive dibenzo alpha pyrons, together with humic and fulvic acids, which act as carrier molecules for the active ingredients (Ghosal, 1990).

Xiao et al. (2018) has been observed that fulvic acid has a positive effect on sperm morphology, reduces malondialdehyde level, helps to preserve the integrity of the spermatozoon membrane, preserves the integrity of the spermatozoon acrosome and causes an increase in sperm motility.

Sultan et al. (2021), on nili ravi buffaloes, found that after thawing 3% shilajit was added to the semen extender, spermatological parameters such as progressive motility, spermatozoa plasma membrane integrity, viability rate and DNA integrity increased and oxidative stress occurred during cryopreservation decreased.

One of the easiest techniques for identifying single- and double-strand breaks in spermatozoa is the comet test, also known as single-cell gel electrophoresis (McKelvey-Martin et al., 1997). The test's basic idea is that damaged DNA strands can be separated by an electric field while the strands' charge and size assist to make this occur. Following separation, single- and double-stranded broken DNA pieces move to the comet's tail, whereas intact DNA remains in the comet's head (Klaude et al., 1996). As a result, spermatozoa with high DNA strand breakage levels show an ascending comet look (Singh & E. Stephens, 1998) and a dense appearance (Hughes et al., 1996).

To improve the test's efficiency, further metrics such the comet tail moment, olive tail moment, and nucleus diameter were added. The comet test has been applied in numerous investigations, including the assessment of UV radiation, carcinogens, toxicants, and radiotherapy effects, to evaluate DNA damage in a variety of cell types (Singh et al., 1988).

The hypothesis of the study is that the combined effect of fulvic acid and many other components in the content of shilajitin can preserve DNA integrity in frozen rooster semen after thawing. The aim of this study was to examine the effects of five different doses of shilajit, which is an antioxidant, on spermatological parameters and spermatozoon DNA integrity (COMET) in the freezing of rooster semen.

MATERIALS and METHODS

The study was carried out in accordance with the guidelines of the Ethics Committee decision numbered 2020/10 of the Republic of Turkey Ministry of Agriculture and Forestry, Poultry Research Institute.

Roosters and Semen Collection

In our study, semen was collected from Plymouth Rock (n=20) breed roosters, aged 49 weeks, fed ad

libitum and treated with 16 hours of light and 8 hours of dark photoperiod in individual cages, by dorso-abdominal massage method (Tarif et al., 2013). Roosters with semen motility of 90% and above, which were taken and examined, were included in the study. After the preliminary examination, semen from 20 roosters were pooled and divided into 6 equal parts.

Sperm Prosessing and Cryopreservation

Beltsville poultry semen extender (BPSE) was used as diluent and divided into 6 equal parts by adding 5% glycerol as cryoprotectant. By adding various doses of shilajit (S) to the BPSE diluent, study groups were created given as Control (0 µg/mL), S5 (5 µg/mL), S10 (10 µg/mL), S15 (15 µg/mL), S20 (20 µg/mL) and S25 (25 µg) /mL) and semen was diluted. Diluted semen was cooled for 2 hours at 4oC in the refrigerator. After equilibration, the semen was drawn into 0.25 mL straws and sealed with polyvinyl alcohol. Straws were frozen at a distance of 4 cm from the liquid nitrogen surface in the cryobox for 7 minutes and stored in liquid nitrogen. After being thawed in a water bath for 30 seconds at 37 oC, straws were evaluated.

Sperm Motion Parameters

Sperm motility was performed using the CASA system (SCA, Sperm Class Analyzer, Version 6.5.0.91; Microptic, Barcelona, Spain) and a phase contrast microscope (Eclipse Ci-L, Nikon, Japan) with a heating plate. Thawed semen was transferred to Eppendorf tubes. 7 µL of semen was placed on a slide and examined by covering it with a coverslip. Motility (M, %), progressive motility (PM, %), mean path velocity from kinematic parameters (VAP, µm/s), linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), lateral head displacement width (ALH, µm), straight progression (STR, %), linearity (LIN, %) parameters were examined.

Sperm Morphology

Hancock stain was used to evaluate morphology (Schäfer & Holzmann, 2000). The percentages of head, mid-piece, tail, and total spermatozoa abnormalities were calculated. A droplet of semen was mixed with Hancock's stain and then applied to a slide. Phase-contrast microscopy (Eclipse Ci-L, Nikon, Japan) at an enlargement of 100 was used to analyze any anomalies in the sperm (n = 200/slide).

Sperm Viability

The eosin-nigrosin staining method was used to assess the vitality of spermatozoa (Ommati et al., 2013). After thoroughly mixing two droplets of nigrosin-eosin and semen, the mixture was spread out on a microscope slide and allowed to air dry before being examined at 400X magnification using a phase-contrast microscope (Eclipse Ci-L, Nikon, Japan). From each sample, the number of sperm was assessed to be 200; unstained sperm were classified as viable, whereas stained spermatozoa were classified as nonviable.

Comet Assay

Frozen rooster semen thawed at 37 °C for 30 seconds was transferred to eppendorf tubes. The semen in Eppendorf tubes were diluted 1:1 with phosphate buffer solution (PBS), which does not contain Ca+2 and Mg+2, and washed by centrifugation at +4oC for 10 minutes at 800 rpm and the supernatant was removed. The semen was reconstituted and centrifuged and the washing process was repeated. The supernatant was removed again and the spermatozoa were diluted 1:1 with PBS (Fraser & Strzezek, 2004; Nandre, 2007).

120 µL of 0.75% low-melting agarose (LMA) gel prepared in PBS was dropped on the sandblasted slides and smeared. After smearing, it was left to dry at room temperature and the first agarose layer was formed. 10 µL of semen diluted with PBS and 90 µL of 1% LMA Gel were mixed in an eppendorf tube at 37 °C. The entire prepared 100 µL mixture was spread on the first agarose layer and covered with a 24 x 60 mm coverslip and left on the ice pack until solidified. After solidification, the coverslips were carefully pulled and slide preparation was completed (Hughes et al., 1997; Singh et al., 2003).

Lysis solution is used to lyse the cell and nuclear membranes and to release the DNA helixes in agarose. After the spermatozoa were embedded in the agarose gel on the prepared slide, the slides were incubated at +4 oC for 1 hour in a copplin jar using Comet Assay Lysis Solution (R&D Systems, Comet Assay Lysis Solution, Catalog number: 4250-050-01) containing high concentrations of salt and detergent and 1% Triton X-100. After one hour of incubation, 1 mL of Dithioerithrol (DDT) was added to the prepared lysis solution and incubated at +4 oC for 1 hour. At the end of the incubation, 0.5 mL Proteinase K was added into the Copplin jar and incubated in an incubator at +37 oC overnight (Hughes et al., 1997; Singh et al., 2003).

Samples prepared by modifying Shanmugam et al. (2016), were incubated for 20 minutes in a freshly prepared and cooled electrophoresis buffer solution (600 mM NaOH ve 2 mM EDTA, pH 7,3) in an electrophoresis tank for the purpose of separating DNA strands before being carried out in electrophoresis. After the incubation of the spermatozoa embedded in the agarose layer was completed in the electrophoresis buffer solution, they were subjected to electrophoresis in the same buffer solution at 20 volts and 30 mA electrical field for 15 minutes.

After the electrophoresis of the prepared samples, the slides were washed with a freshly prepared Tris buffer solution (0.4 M Tris HCl, pH 7.5) to remove the electrophoresis solution from the samples, and neutralization of the samples was performed (Shanmugam et al., 2016).

After the neutralization process was completed, the DNAs were stained using a fluorescent dye, ethidium bromide (5 µg/mL). For this purpose, a drop of ethidium bromide was dripped onto the samples and

covered with a 24 x 60 mm coverslip and evaluated within 4 hours (Gliozzi et al., 2011).
Samples stained with Ethidium bromide were examined at 400X magnification using a fluorescent attachment phase contrast microscope (Olympus CX-31). 100 comet images from all groups were evaluated (TriTek Comet Score™ Freeware v1.5). All evaluation steps were performed in a dim light environment to avoid further DNA damage (Gundogan et al., 2010). Tail DNA (%), tail Length (μm), Comet Length (μm) and Olive Tail Moment parameters were recorded to define the DNA damage detected as a result of the evaluation.

Statistical analysis

The analyzes in the study were compared and evaluated according to the between-group and in-group analysis. While the one-way ANOVA method was used for in-group analyses, factorial trial design was used for intergroup comparisons. In addition, the differences between the means were investigated with the Duncan Multiple Comparison Test, and the interaction effects were carried out according to the Tukey Multiple Comparison Method. All statistical analyzes and evaluations were made according to the SAS (2009) statistical software. A difference that considered significant was $p<0.05$.

RESULTS

Sperm Motion Parameters

In the statistical evaluation made as a result of the examination of sperm motility and kinematic parameters; It was determined that the differences between the kinematic parameters of VAP, VSL and ALH were significant ($P<0.05$). While no significant difference was detected in motility and progressive motility parameters, the highest motility (77.40 ± 4.34), progressive motility (21.96 ± 1.50), kinematic parameters VAP (33.24 ± 1.04), VSL (18.58) Values ±0.75), VCL (61.09 ±3.28), ALH (1.94 ±0.06), STR (43.89 ±1.54) and LIN (24.45 ±1.60) It was detected in the S20 group (Table 1).

Sperm Morphology and Viability

In the statistical evaluation made as a result of the examination of sperm morphology and viability rate parameters; It was determined that the differences between head, middle part, tail and total parameters of abnormal spermatozoa were significant ($P<0.01$). While there was no significant difference in the ratio of dead/live spermatozoa, the highest survival rate (76.60 ± 3.90%) was found in the S20 group. Among the abnormal spermatozoa values, the lowest head abnormal value (3.50 ± 0.56) was found in the S15 group, the lowest middle part abnormal value (4.40 ± 0.73) was in the S5 group, the lowest tail abnormal value (4.40 ± 0.73) was in the S10 group, and the lowest total abnormal sperm value (26.30 ± 1.54) was in the S5 group (Table 2).

Comet Assay

In the statistical evaluation made as a result of the examination of spermatozoa DNA damage parameters; The differences between the DNA damage parameters Tail DNA ($P<0.0001$), Tail length ($P<0.0001$), Comet length ($P < 0.01$) and Olive tail moment ($P < 0.001$) were found to be significant. The lowest value between groups in Tail DNA value was detected in the S15 group (20.99 ± 0.49), while the highest value was detected in the S25 group (27.52 ± 0.58). The lowest value between the groups in the tail length value was detected in the S15 group (13.20 ± 0.77), while the highest value was detected in the S25 group (19.71 ± 1.00). The lowest value between the groups in olive tail moment value was detected in the S15 group (5.17 ± 0.09), while the highest value was detected in the S25 group (7.04 ± 0.41) (Table 3).

Table 1. Motility and kinematic parameters after thawing of rooster semen.

Parameters	SK	S5	S10	S15	S20	S25	P Value
	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
MOT(%)	73.41 ± 3.00	75.46 ± 3.47	73.33 ± 3.28	74.84 ± 3.61	77.40 ± 4.34	74.58 ± 3.89	> 0.05
PM (%)	17.74 ± 1.50	20.52 ± 1.14	18.56 ± 1.12	19.23 ± 0.99	21.96 ± 1.50	18.83 ± 1.54	> 0.05
VAP (μm/s)	27.89 ± 1.34^{ab}	31.19 ± 0.99^{ab}	29.14 ± 1.03^{ab}	30.48 ± 1.13^{ab}	33.24 ± 1.04^{a}	27.20 ± 3.87^{b}	< 0.05
VSL (μm/s)	15.60 ± 0.93^{b}	17.41 ± 0.68^{ab}	16.44 ± 0.73^{ab}	17.03 ± 0.75^{ab}	18.58 ± 0.75^{a}	16.58 ± 0.86^{ab}	< 0.05
VCL (μm/s)	54.49 ± 2.16	60.23 ± 1.65	57.46 ± 1.67	58.15 ± 1.65	61.09 ± 3.28	58.27 ± 2.40	> 0.05
ALH (μm)	1.65 ± 0.06^{b}	1.82 ± 0.04^{ab}	1.75 ± 0.04^{b}	1.77 ± 0.04^{ab}	1.94 ± 0.06^{a}	1.76 ± 0.06^{b}	< 0.05
STR (%)	43.50 ± 1.34	43.76 ± 0.77	42.83 ± 1.18	43.89 ± 1.21	43.89 ± 1.54	43.09 ± 1.61	> 0.05
LIN (%)	23.29 ± 1.31	23.76 ± 0.90	22.83 ± 0.95	24.33 ± 1.39	24.45 ± 1.60	22.88 ± 1.59	> 0.05

a, b: Values with different letters in each row are statistically significant.
MOT (%): Motility. PM (%): Progressive Motility, VAP (μm/s): average path velocity, VSL (μm/s): straight-line velocity, VCL (μm/s): curvilinear velocity, ALH (μm): amplitude of lateral head displacement, STR (%): straightness, LIN (%): linearity. SK: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

Table 2. Statistical results of sperm morphology and viability rate after thawing.

Parameters		SK	S5	S10	S15	S20	S25	P Value
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
Sperm Viability (%)		72.70 ± 3.54	75.70 ± 3.97	73.40 ± 3.74	74.70 ± 3.16	76.60 ± 3.90	73.50 ± 3.56	> 0.05
Sperm Morphology (%)	**Head**	7.00 ± 0.80^{a}	4.40 ± 0.52^{b}	4.10 ± 0.40^{b}	3.50 ± 0.56^{b}	4.70 ± 0.57^{b}	4.30 ± 0.57^{b}	< 0.01
	Mid-piece	6.10 ± 0.99^{bc}	4.40 ± 0.73^{c}	8.70 ± 0.95^{a}	9.40 ± 0.76^{a}	7.50 ± 0.95^{ab}	7.60 ± 0.52^{ab}	< 0.01
	Tail	19.50 ± 1.62^{abc}	17.50 ± 1.15^{bc}	14.20 ± 1.09^{c}	17.90 ± 1.53^{bc}	22.40 ± 3.08^{ab}	23.80 ± 1.89^{a}	< 0.01
	Total	32.60 ± 2.14^{ab}	26.30 ± 1.54^{b}	27.00 ± 0.73^{b}	30.80 ± 1.81^{ab}	34.60 ± 3.57^{a}	35.70 ± 1.84^{a}	< 0.01

a, b, c: Values annotated with distinct letters within each row indicate statistically significant differences. SC: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

Table 3: COMET results after post-thawing rooster sperm

Parameters	SK	S5	S10	S15	S20	S25	P Value
	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
Tail DNA (%)	26.08 ± 0.55^{ab}	23.45 ± 1.06^{bc}	20.93 ± 1.59^{c}	20.99 ± 0.49^{c}	24.93 ± 0.46^{ab}	27.52 ± 0.58^{a}	< 0.0001
Tail length (μm)	15.87 ± 0.66^{b}	15.62 ± 1.07^{b}	13.66 ± 0.71^{b}	13.20 ± 0.77^{b}	15.57 ± 0.78^{b}	19.71 ± 1.00^{a}	< 0.0001
Comet length (μm)	45.86 ± 2.17^{b}	44.68 ± 1.58^{b}	44.23 ± 0.74^{b}	42.67 ± 0.38^{b}	47.33 ± 2.04^{b}	51.91 ± 1.82^{a}	< 0.01
Olive tail moment	6.74 ± 0.48^{ab}	5.71 ± 0.34^{bc}	5.00 ± 0.36^{c}	5.17 ± 0.09^{c}	6.48 ± 0.37^{ab}	7.04 ± 0.41^{a}	< 0.001

a, b, c: Values annotated with distinct letters within each row indicate statistically significant differences. SC: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

DISCUSSION

The data in the studies in which the motility and progressive motility of rooster semen frozen by adding antioxidants to the semen were evaluated with the CASA system after thawing were examined. The values of the S20 group in our study were higher than the values in the study in which Safa et al. (2016) used nano-Selenium and Vitamin E as antioxidants, similar to the values in the study in which Najafi et al. (2020) used astaxanthin and lower than the values in the study in which Najafi et al. (2021) used alpha lipolic acid Interestingly, the differences observed between the findings of the mean values of motility and progressive motility findings determined by using the CASA system after thawing of frozen rooster semen with various doses of shilajit added to the BPSE extender obtained in the study and the findings reported in other studies are interestingly due to the reason that shilajit supplementation to thex rooster semen extender increases semen motility. It was concluded that the semiquinone-hydroquinone complex structure resulted in improvement in spermatozoa motility at all stages of cryopreservation, attributable to the radical scavenging effect of dibenzo-pyrones and fulvic acid.
Calculation of sperm kinematic parameters is based on the principle of functional performance of axonemes and membranes of spermatozoa, metric measurements of spermatozoon motility. The definitions of spermatozoa kinematic parameters are based on different measurements of the central positions of the two-dimensional spermatozoon head per unit time. One of the kinematic parameters, VAP (μm/s) value expresses the average velocity of the sperm head per unit time along the mean trajectory of the sperm. The data in the studies in which the kinematic parameters were evaluated with the CASA system after thawing of rooster semen frozen by adding antioxidants to the semen were examined. The VAP value in our study was observed to be higher than the VAP value of the resveratrol group in Rezaie et al. (2021) in parallel with the value of the astaxanthin group in Najafi et al. (2020) and in parallel with values of the crocin and naringenin groups in Mehdipour et al. (2020).
The VSL (μm/s) value, one of the kinematic parameters, expresses the average velocity of the spermatozoon head per unit time along a straight line from its initial position to its final position. The data in the studies in which the kinematic parameters were evaluated with the CASA system after the thawing of the frozen rooster semen by adding antioxidants to the semen were examined. The VSL value in our study was

observed to be lower than the VSL value of the querectin-loaded nano-structured lipid carrier group in A. Najafi et al. (2020), in parallel with the VSL values of the crocin and naringenin groups in Mehdipour et al. (2020) and higher than the VSL value of the resveratrol used groups in Rezaie et al. (2021).

The ALH (μm) value, one of the kinematic parameters, expresses the amplitude of variations of the spermatozoon head orbit relative to the average orbit. The data from the studies in which kinematic parameters of frozen rooster sperm were evaluated with CASA system at the end of thawing by adding antioxidants to the sperm were examined. The ALH (μm) value in our study was observed to be higher than the ALH values belonging to the nano selenium group used in Safa et al. (2016), lower than the ALH values belonging to the groups in which ellagic acid-loaded liposomes were used in Najafi et al. (2019), lower than the ALH values belonging to the querectin-loaded nanostructured lipid carrier group used in A. Najafi et al. (2020), lower than the ALH values belonging to the astaxanthin group used in Najafi et al. (2020), lower than the ALH (μm) values of crocin and naringenin group used in Mehdipour et al. (2020)(Mehdipour et al., 2020), lower than the ALH (μm) values of the resveratrol-used groups in Rezaie et al. (2021).

The fact that some of the values obtained as a result of examining the kinematic parameters made after thawing in frozen rooster semen with the CASA system show parallelism is a sign that our thesis study is in harmony with similar studies. The differences observed in the kinematic parameters after thawing may be due to the modifications made in the components of the semen extender, the differences in the percentage values of the cryoprotectant used, and the efficacy of the antioxidant substances, as well as the rooster breed. In addition, it is thought that these differences may be caused by differences between different CASA software used in Deciphering kinematic parameters and the image and measurement settings of the poultry module in the same software.

It has been reported that high VCL and ALH values and low LIN values in cattle are observed in hyperactive spermatozoa, and also in terms of fertility parameters, the VCL value in cattle should be higher than 70, and the ALH value should be higher than 7 (Kathiravan et al., 2011). As a result of literature researches conducted in terms of kinematic parameters in poultry spermatozoa, no such information has been found. For this reason, it is thought that further studies should be carried out in order to better interpret the kinematic parameters in poultry.

The data in the studies in which the viability rates of rooster semen frozen by adding antioxidants to the semen were evaluated after thawing with 2% eosin-nigrosin were examined. According to the results of the study in which A. Najafi et al. (2020), used querectin as an antioxidant, the values in our study were higher than the viability rate in the groups using querectin, querectin-loaded nanoliposomes and querectin-loaded nano-structured lipid carriers. On the other hand According to the results of the study in which Mehdipour et al. (2020), used crocin and naringenin as antioxidants, the vitality rate was higher than the groups using crocin and naringenin and according to the results of the study where Masoudi et al. (2020), used glutathione as an antioxidant it was observed that the vitality rate was lower.

The data in the studies on the ratios of abnormal spermatozoa with Hancock stain after thawing of rooster semen frozen by adding antioxidants to the semen were examined. The values we found in our study were observed to be lower than the rate of total abnormal spermatozoa after thawing, according to the results of the study in which Lotfi et al. (2017), used hyaluronic acid as an antioxidant. In addition, according to the results of the study in which Najafi et al. (2021) used alpha lipolic acid and alpha lipolic acid nanostructured lipid carrier as antioxidants, it was higher than the post-thawed groups and according to the results of the study, in which Siari et al. (2022) used querectin as an antioxidant, the abnormal spermatozoa rates were higher in the post-thawed group was observed.

The variations observed between the statistical findings of the mean values obtained through manipulations and those reported in other studies are attributed to differences in the effectiveness of antioxidants and the presence of compounds such as fulvic acids, humic acids, humins, fatty acids, triterpenes, selenium, phospholipids, resins, latex, gums, albumins, and selenium. It has been concluded that shilajit, which consists of approximately 80-85% humic substances, including triterpenes, sterols, and aromatics, has the potential to enhance cellular metabolism and improve cell viability.

When the findings obtained as a result of examining the DNA damage of rooster semen after thawing were examined, it was observed that the values of some researchers (Gliozzi et al., 2017; Gliozzi et al., 2011) were lower. Although it is thought that the reason why DNA damage parameters were found lower than other studies with limited literature information is the effectiveness of the antioxidant we used in our research, it was determined that DNA damage decreased in the S10 and S15 groups, increased DNA damage in the S25 group, and there was no difference in DNA damage between the control group and the control group. It has been concluded that fulvic acids, which are intensely found in shilajit used in our research, show antioxidant activity at the cellular level by neutralizing the effects of free radicals, and may reduce the amount of damage by protecting the cell nucleus and mitochondria. It is thought that factors such as the differences in the procedures applied in the COMET technique used in the evaluation of DNA damage in our study, minor modifications made in the technique, imaging, evaluation software and the person performing the analysis may also be effective in the formation of the differences.

CONCLUSION

As a result, in addition to the positive effects of the use of shilajit in freezing rooster sperm in terms of spermatological parameters and DNA damage, it was found that high doses negatively affect spermatological and DNA damage parameters. It has been concluded that the addition of 10, 15, 20 μg/mL shilajit to rooster sperm diluent improves sperm quality parameters such as movement parameters of sperm, viability and DNA integrity after cryopreservation. It is thought that the effect of shilajit on freezing by adding it to rooster semen should be supported by more comprehensive studies by adding in vivo parameters.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MYN and MS contributed to the project idea, design and execution of the study. MYN contributed to the acquisition of data. MYN and MS analysed the data. MYN drafted and wrote the manuscript. MYN and MS reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Republic of Turkey Ministry of Agriculture and Forestry, Poultry Research Institute and Ondokuz Mayis University Faculty of Veterinary Medicine. This research was approved by The Ethics Committee of the Republic of Turkey Ministry of Agriculture and Forestry (Ref No: 2020/10, Tarih: 12/2020).

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