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Investigation of changes in spermatozoa characteristics, chromatin structure, and antioxidant/oxidant parameters after freeze-thawing of hesperidin (vitamin P) doses added to ram semen

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Abstract

We conducted this study to determine the potential cryopreservative effects of different hesperidin (vitamin P; HSP) doses on ram semen after freeze-thawing. Semen samples were obtained from Sönmez rams by an artificial vagina. The samples were divided into six groups: control, 10, 50, 100, 250, and 500 µg/mL HSP (C, HSP10, HSP50, HSP100, HSP250, and HSP500, respectively). At the end of the study, sperm motility and kinetic parameters, plasma

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membrane acrosome integrity (PMAI), high mitochondrial membrane potential (HMMP), viability, lipid peroxidation levels (LPL), chromatin damage, oxidant parameters, and antioxidant parameters were assayed. None of the doses of HSP added to the semen extender showed any enhancing effect on progressive motility compared to C ($p>0.05$). In fact, HSP500 had negative effects ($p<0.05$). Moreover, PMI activities were the highest at the HSP10 dose, while LPL values were the lowest ($p<0.05$). The doses of HSP10 and HSP50 added to the Tris extender medium showed positive effects on spermatozoon chromatin damage. Consequently, we can say that HSP doses used in this study are not effective on semen progressive motility, but the HSP10 dose is effective on PMAI and chromatin damage by reducing LPL.

Keywords: Semen, hesperidin; cryopreservation; oxidative stress; antioxidant; ram

1. Introduction

In Turkey, studies have been conducted on the crossbreeding of suitable genotypes for the breeding of domestic sheep. One of these domestic breeds is Sönmez. The Sönmez breed was obtained by crossing the Chios (25%) and Tahirova (75%) genotypes. To ensure the genetic continuity of these special breeds, the most practical technology for rapid genetic improvement at appropriate times is artificial insemination [1]. The success of this technique depends on the long-term protection of spermatozoa from factors that affect their fertilization capacity negatively [2]. Cryopreservation has been developed with technological advancements and scientific studies and is still widely used today for the optimum preservation of the properties of cells and tissues. During the cryopreservation process, the freeze-thaw process may cause oxidative damage, decreased sperm motility, deterioration of sperm morphology, and consequently, a decrease in fertilization capacity [3]. Adding or removing substances with cryoprotectant properties to the semen may cause disruption in the intracellular osmotic balance

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and damage the genetic material of the sperm [4]. Both seminal plasma and spermatozoa are highly prone to lipid peroxidation (LPO) because they contain rich polyunsaturated fatty acids [5]. LPO triggers many chains of chemical reactions [6] and can damage important biological molecules such as proteins, DNA, and RNA [7]. Various enzymatic and non-enzymatic detoxification mechanisms attempt to eliminate the harmful effects of reactive oxygen species (ROS). These mechanisms are generally referred to as antioxidants [8].

Seminal plasma antioxidants play a crucial role in protecting spermatozoa against oxidative stress [9]. Oxidative stress in semen results from either the depletion of seminal antioxidants or excessive free radical formation by the sperm themselves. Therefore, increasing the antioxidant activity in the seminal plasma is extremely important in the cryopreservation of semen [10]. The application of antioxidants to semen diluents has received great interest in assisted reproductive technology applications. To protect sperm cells against various shocks during processing or storage, different diluents are designed, often on a testable basis. While natural additives added to diluents have been examined in the relevant literature, it was determined that there was no research on hesperidin (vitamin P; HSP) within the scope of this subject. HSP (5,7,3´-trihydroxy-4´-methoxy flavanone), a flavonoid, is naturally found in citrus fruits such as lemons, oranges, and grapefruit [11]. Additionally, the protective effect of HSP on reproductive functions against toxicities caused by different chemicals has been reported by previous studies [12, 13]. It was reported that HSP significantly reduces LPO in testicular tissue, has positive effects on sperm parameters and biochemical parameters, and improves epididymal functions [12]. Several mechanisms emerge to explain the biological effects of HSP on reproductive functions. The first is that it affects the secretion and activity of hormones. HSP was reported to have both estrogenic (at low concentrations) and anti-estrogenic (high concentrations) effects, depending on its concentration in the organism [14]. These hormones modulate the action mechanisms of hormones such as the thyroid hormone [15]. Secondly, it

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was explained that HSP affects reproductive functions by inhibiting enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, Ca^{2+}-ATPase, lipoxygenase, and cyclooxygenase [16]. This study was designed to evaluate the protective effect of HSP on ram semen by reducing the damage that may occur after freeze-thawing and determine oxidative stress, chromatin damage, sperm motility, LPO levels, and certain antioxidant parameters.

2. Materials and Methods

2.1. Animals and general experimental procedure

In the study, semen samples were taken from six Sönmez breed rams (2-3 years old; Kapucuoğlu sheep farm, Afyonkarahisar, Turkey) using an artificial vagina. These procedures were carried out in the same breeding season and at different times as ten replications for each group. The ejaculates were used individually for each replication to eliminate the effects caused by different rams. A volumetric cup and a photometer were used to determine semen volume and concentrations. The mass activity (≥ +++ 3 [scale 1-5]), sperm concentrations ($\geq 0.8\times10^9$/mL, volume ≥0.8 mL), and motility (80%) of the ejaculates were used to qualify the sources of semen. Tris (3.63 g Tris, 0.5 g fructose, 1.82 g citric acid, 100 ml double-distilled water, 7% glycerol (v/v) and 15% egg yolk) was used as the main diluent, and HSP was dissolved in dimethyl sulfoxide (DMSO; 1 g HSP, 1 mL DMSO). The extender osmolarity of the solutions was adjusted to 310 mOsm. The semen samples were divided into six groups, and 0, 10, 50, 100, 250, and 500 µg/mL HSP were added to the groups (control (C), HSP10, HSP50, HSP100, HSP250, and HSP500, respectively). After these procedures, the samples that were injected into straws were stored until they reached an equilibrium temperature (approximately 2 hours at 4°C). After providing the optimum cooling conditions, all samples were frozen at -196°C for 6 months. Ethics committee approval of the study was given by Afyon Kocatepe

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University Faculty of Veterinary Medicine Animal Care Committee with the decision numbered 49533702/333.

2.2. Sperm motility and kinetic parameters

The motility kinetic parameters of ram semen were evaluated with a computer-assisted semen analyzer (CASA) in a phase contrast microscope using special software (Sperm Class Analyzer). Characteristic analyses of motility were carried out with the aid of green filtered negative phase contrast (100x magnification). Curvilinear velocity variations for motile spermatozoa were categorized as fast (>75 µm/s), medium (45-75 µm/s), slow (10-45 µm/s), and static (<10 µm/s). Sperm with ≥75% flatness were considered progressive. In addition to these parameters, the values for kinetic calculations were quantified as follows: total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), amplitude lateral head displacement (ALH, µm/s), beat cross frequency (BCF, Hz), linearity (LIN %) [(VSL/VCL) × 100], yaw (WOB %) [(VAP/VCL) × 100], and straightness (STR %) [(VS /VAP) × 100]. In the evaluation of these parameters, a total of 200-400 sperm were recorded per sample in six microscopic zones.

2.3. Chromatin damage

Chromatin damage in semen was analyzed using the single cell gel electrophoresis (COMET) assay method under intensively alkaline conditions. The samples on the slide were visualized with a fluorescence microscope (Olympus CX31) and scored using the Comet Score software (TriTek, V.1.5). To make the assessment more accurate, a total of at least one hundred sperm cells were selected from six different zone in each sample [17].

2.4. Flow cytometric evaluations

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The flow cytometry analyses were performed by using a CytoFLEX flow cytometer (Beckman Coulter, CA, USA) equipped with 525±40 nm, 585±42 nm, and 610±20 nm emission filters and a 50-mW laser output (488 nm laser beam). In each analysis, ~10,000 events were examined. A side scatter area (SSC-A) versus forward scatter area (FSC-A) pseudo plot was used to exclude debris from the analysis, and duplicates were excluded by using forward scatter height (FSC-H) and forward scatter area (FSC-A) [18]. Working solutions of fluorescein isothiocyanate-conjugate peanut agglutinin (FITC-PNA) [100 µg/mL, Sigma, L7381], Sybr-14 and propidium iodide (PI) solution [1:10 Sybr-14, 2.99 mM PI, L7011, molecular probes, Invitrogen], 5,5',6,6'-tetrachloro-1,1'3,3'-tetramethylbenzimidazolyl-carbocyanine iodide (JC-1) [0.153 mM T3198, molecular probes, Invitrogen], and BODIPY (5 µM, D38611, molecular probes, Invitrogen) were prepared with DMSO, divided into 50 µL portions, and stored at -20°C until use.

2.5. Spermatozoon plasma membrane acrosome integrity (PMAI)

Spermatozoon PMAI values were determined using the FITC/PNA-PI staining kit according to the method described by [19]. The frozen-thawed sperm sample was diluted to 5×10^6 sperm in 492 µL of PBS. Subsequently, 5 µL of FITC and 3 µL of PI were added, followed by incubation in a water bath at 37°C for 15 min in a dark room. After incubation, the debris (non-sperm) was gated out, and sperm PMAI (FITC/PNA-PI-) analyses were performed using the CytExpert 2.3 software.

2.6. Spermatozoon lipid peroxidation level (LPL)

Spermatozoon LPL values were determined using BODIPY-SYBR staining according to the method reported by [20] with some modification. The frozen-thawed sperm sample was diluted to 5×10^6 sperm in 492 µL of PBS. Subsequently, 5 µL of BODIPY and 3 µL of SYBR

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were added, followed by incubation in a water bath at 37°C for 15 min in a dark room. After incubation, the debris (non-sperm) was gated out, and spermatozoon LPL (BODIPY) analyses were performed using the CytExpert 2.3 software.

2.7. High Mitochondrial membrane potential (HMMP)

Sperm cell HMMP was determined using 5,5′,6,6′ tetrachloro-1,1′3,3′-tetramethyl benzimidazolyl-carbocyanine iodide (JC-1). The frozen-thawed sperm sample was diluted to a concentration of 5×10^6 sperm in 495 µL of PBS. Subsequently, 5 µL of JC-1 was added to the sample, followed by incubation in a water bath at 37°C for 15 min in a dark room. After incubation, the debris (non-sperm) was gated out, and high mitochondrial membrane potential (HMMP) analyses were performed using the CytExpert 2.3 software [21].

2.8. Spermatozoon viability

Sperm viability was determined using the SYBR and PI protocol of [19] with some modification. The frozen-thawed sperm sample was diluted to 5×10^6 sperm in 492 µL of PBS. Subsequently, 5 µL of SYBR-14 and 3 µL of PI were added to the sperm sample, followed by incubation in a water bath at 37°C for 15 min in a dark room. After incubation, the debris (non-sperm) was gated out, and sperm viability analyses were performed using the CytExpert 2.3 software.

2.9. Oxidant and antioxidant parameters

Malondialdehyde (MDA)

The level of MDA, indicative of lipid peroxidation, was measured in accordance with a previously reported method [22]. In this method, lipid peroxides react with thiobarbituric acid and absorb at 532 nm. The amount of MDA was calculated as nmol/mL.

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Glutathione (GSH)

According to Ellman's method, GSH was measured spectrophotometrically at 412 nm, and the amount was calculated as mg/dL [23].

Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI)

We used a colorimetric test kit (Rel Assay Diagnostics, Gaziantep, TR) for the measurement of TAS (as mmol/L in 660 nm) [24] and TOS (as μmol/L in 660 nm) [24]. OSI was calculated according to the formula OSI = [(TOS)/(TAS x 100)].

2.10. Statistical analysis

The number of repetitions in the study was ten. The homogeneity of variances was determined by using the obtained numeric data, Shapiro-Wilk normality, and Levene's tests. The results are expressed in tables as mean (X) ± standard deviation (SD). Spermatological parameters were modulated to the GLM procedure of SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Post hoc testing was performed for identifying the sources of significant interactions between parameters. All statistical analysis results were interpreted with a minimum margin of error of 5% ($p<0.05$), and this rate was considered statistically significant.

3. Results

3.1. Sperm motility and kinetic parameters

Although the highest total motility results were obtained in HSP10 and HSP50, it was determined that the doses of HSP added to the sperm extender did not have a preservation effect on progressive motility in comparison to the control group (Table 1; $p>0.05$). Additionally, we can say that the dose of HSP500 had a negative effect on motility. Except for STR, spermatozoon kinetic parameters were found to be significantly different in all treatment groups ($p<0.05$), but an advantageous result was not obtained compared to the control group (Table 1).

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Table 1. Mean (±SE) sperm motility and kinetic prameters of freeze-thawing ram semen

Parameters	C	HSP10	HSP50	HSP100	H250	H500	P
Prog. mot. (%)	13.73±1.80^{a}	16.99±3.87^{a}	16.08±0.96^{a}	13.13±1.25^{a}	11.35±1.85^{ab}	7.34±0.83^{b}	*
Total mot. (%)	51.92±3.68^{c}	67.70±1.32^{a}	63.59±3.23^{ab}	55.85±3.72^{bc}	53.85±4.86^{bc}	38.14±3.80^{d}	*
VAP (µm/s)	44.31±4.89^{a}	36.52±0.99^{b}	34.85±2.07^{b}	33.94±1.63^{b}	38.51±1.67^{ab}	31.96±1.44^{b}	*
VSL (µm/s)	26.85±3.16^{a}	22.24±0.85^{b}	20.39±1.32^{b}	20.87±1.31^{b}	23.94±0.82^{ab}	19.99±1.54^{b}	*
VCL (µm/s)	64.45±4.42^{a}	58.21±0.98^{b}	56.97±2.12^{bc}	53.17±1.63^{bc}	57.59±1.89^{bc}	51.47±1.21^{c}	*
ALH (µm/s)	2.37±0.11^{ab}	2.43±0.07^{a}	2.43± 0.08^{a}	2.15±0.03^{b}	2.19±0.06^{ab}	2.18±0.10^{ab}	*
BCF (Hz)	8.76±0.99^{ab}	8.19±0.26^{ab}	8.04±0.22^{ab}	8.51±0.29^{ab}	9.14±0.32^{a}	7.73±0.40^{b}	*
LIN (%)	41.71±2.63^{a}	37.26±1.17^{ab}	35.57±1.37^{b}	37.81±1.48^{ab}	40.58±0.83^{ab}	38.41±2.79^{ab}	*
STR (%)	59.20±1.37	57.71±0.73	56.84±0.54	58.20±0.93	59.38±0.83	59.15±2.19	-
WOB (µm s^{-1})	66.71±2.99^{a}	61.67±1.23^{ab}	59.97±1.86^{b}	61.85±1.54^{ab}	65.11±0.83^{ab}	60.86±2.28^{b}	*

$^{a\ .b.\ c.\ d}$ Different superscripts within the same row demonstrate significant differences (*P<0.05)

$^{-.}$ No significant difference (P>0.05)

3.2. Evaluation of PMI, HMMP, viability, and LPL

Table 2 shows the results of the application of different HSP doses on PMI, HMMP, viability, and LPL after freeze-thawing in ram semen. These data indicated that PMAI and LPL were positively affected by the HSP10 dose ($p<0.05$). No dose of HSP caused a positive change in HMMP ($p<0.05$). Additionally, the viability parameter indicated that the highest dose of HSP500 caused toxic effects (Table 2).

Table 2. Mean (±SE) plasma membrane acrosome integrity (PMAI), high mitochondrial membrane potential (HMMP), viability and lipid peroxidation level (LPL) activities in freeze-thawing ram semen

Parameters	C	HSP10	HSP50	HSP100	HSP250	HSP500	P
PMAI (%)	26.20± 2.36^{b}	32.78± 1.82^{c}	23.56± 1.43^{ab}	25.94± 1.10^{b}	20.67± 0.43^{b}	13.66±0.99^{a}	*
HMMP (%)	30.11±2.88^{c}	34.45±1.36^{c}	20.30±2.16^{ab}	15.17±3.70^{ab}	21.64±2.60^{b}	13.27±0.21^{a}	*
Viability (%)	62.50±3.07^{b}	66.39±0.97^{b}	61.52±1.46^{b}	60.74±1.03^{b}	57.12±2.70^{b}	43.92±8.72^{a}	*
LPL (%)	77.97±7.91^{bc}	64.95±2.78^{a}	71.21±3.23^{b}	73.64±6.21^{b}	85.09±9.85^{bc}	94.29±1.58^{c}	*

$^{a\ .\ b.\ c.}$ Different superscripts within the same row demonstrate significant differences (*P<0.05)

3.3. Evaluation of chromatin damage

According to the data, sperm tail length, tail DNA, and tail moment were preserved at the HSP10 and HSP50 doses compared to the control group. It was determined that chromatin damage increased at high doses of HSP (Table 3; $p<0.05$).

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Table 3. Mean (±SE) chromatin damage values in freeze-thawing ram semen

Parameters	C	HSP10	HSP50	HSP100	HSP250	HSP500	P
Tail lenght (µm/s)	25.23±0.92^{b}	14.00±0.99^{d}	14.07±0.83^{d}	18.46±1.29^{c}	25.12±1.12^{b}	35.03±1.16^{a}	*
Tail DNA (%)	36.67±3.13^{b}	23.75±2.17^{a}	22.72±2.40^{a}	34.70±0.96^{b}	39.00±2.01^{b}	58.42±3.57^{a}	*
Tail moment (µm/s)	20.66±1.86^{b}	15.51±1.78^{cd}	12.36±1.05^{d}	18.91±0.75^{bc}	23.22±1.18^{b}	35.67±2.06^{a}	*

$^{a\ .b.\ c.\ d}$ Different superscripts within the same row demonstrate significant differences (*P<0.05)

3.4. Oxidant and antioxidant parameters

As seen in Table 4, there were no significant changes in TOS and OSI (p>0.05). While it was expected that the high GSH activity at the HSP500 dose and the low MDA level at the HSP100 and HSP500 doses would positively affect the quality of spermatozoa in these groups, this expectation did not materialize.

Table 4. Mean (±SE) glutathione (GSH) and malondialdehyde (MDA) activities, total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) in freeze-thawing ram semen

Parameters	C	HSP10	HSP50	HSP100	HSP250	HSP500	P
GSH (mg/dL)	37.30±0.83^{ab}	35.47±0.45^{a}	37.46±2.11^{ab}	36.26±0.45^{ab}	37.30±0.48^{ab}	39.36±1.17^{b}	*
MDA (nmol/mL)	4.63±0.08^{a}	4.42±0.12^{ab}	4.45±0.11^{ab}	4.15±0.10^{b}	4.39±0.10^{ab}	4.25±0.06^{b}	*
TAS (mmol/L)	17.02±0.54^{ab}	17.20±2.19^{ab}	17.24±1.35^{ab}	16.22±0.51^{a}	19.61±1.28^{b}	17.09±1.10^{ab}	*
TOS (µmol/L)	8.66±0.31	9.32±0.31	9.07±0.22	9.40±0.24	9.17±0.13	9.35±0.18	-
OSI	5.12±0.29	5.49±0.35	5.52±0.66	5.81±0.17	4.74±0.21	5.62±0.48	-

$^{a\ .\ b.}$ Different superscripts within the same row demonstrate significant differences (*P<0.05)

$^{-.}$ No significant difference(P>0.05)

4. Discussion

During the long- or short-term storage of semen, temperature, cooling rate, the chemical contents of diluents, the cryoprotectant ratio, ROS, and seminal plasma components are the main determinants affecting the viability of spermatozoa [25]. In previous studies, it has been reported that sperm viability and motility, as well as the integrity of both the plasma membrane and acrosome, are adversely affected after thawing during the cryopreservation process [26]. The motility and kinetic parameters of the ram semen samples containing HSP doses added to Tris-egg yolk extender that were thawed are given in Table 1. In our study, HSP10 and HSP50 had higher percentages of total motility (67.70±1.32% and 63.59±3.23%, respectively) compared to other groups, while HSP did not have a preservation effect on progressive motility

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($p>0.05$). It was even determined that the HSP250 and HSP500 doses had a negative effect on progressive motility in comparison to C ($p<0.05$). The percentages of VAP, VSL, and VCL were lower ($p<0.05$) in the extenders containing all HSP doses compared to C. There was no significant difference ($p>0.05$) in the ALH parameter among the C, HSP250, and HSP500 groups (Table 1). However, HSP100 had the highest ALH values compared to C and other treatment groups. In studies conducted in different species, it has been revealed that HPS improved some parameters, which supports our findings. Samie et al. [27] reported that the dose of HSP (50 mg/kg/day) administered to rats contributed to an increase in sperm counts and motility. In another study, 20 µM hesperidin treatment during the cryopreservation of human sperm significantly improved the motility and morphology ratios of spermatozoa after cryopreservation [28]. Aksu et al. [29] also showed that the application of HSP (300 mg/kg/day) for 7 days against reproductive damage in male rats caused an increase in the percentage of total sperm motility. In another study compatible with our results, it was proven that a 200 mg/kg/day dose of HSP had a protective effect on the reproductive system by reducing degenerative changes in sperm motility and density in male rats treated with methotrexate [30]. As a result of an investigation of the effects of HSP on the toxicity of an anticancer drug, cisplatin, in the reproductive system, it was reported that a dose of 7 mg/kg/day may be beneficial for reproductive functions against cisplatin-induced toxicity by causing an increase in sperm motility [12]. The common result of studies in which positive effects on motility have been determined is that the negative effects of various sources of toxicity on reproductive cells can be eliminated by dose-dependent supplements of HSP. The main reason for the decrease in sperm motility was thought to be increased lipid peroxidation. The result in our study can be explained by that HSP at high doses (HSP250 and HSP500) damages the sperm cell membrane's integrity by disrupting the lipids and proteins in the membrane. The results showed

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the protective effects of HSP at low doses (HSP10 and HSP50) on ram semen, which can be attributed to its evident antioxidant potential.

The application of HSP10 showed a significant effect on PMAI. This finding was supported by the low LPL levels at the same dose, which showed that the mitochondrial structure was preserved at the HSP10 dose of semen post-thawing. LPL is an important parameter in determining mitochondrial oxidative damage. The experiment results showed that the HSP10 dose was effective in maintaining sperm viability by reducing mitochondrial LPL after thawing. This was probably because low doses (such as HSP10 and HSP50) reduced lipid peroxidation, and high doses added to the diluent failed to maintain the pH and osmotic pressure balance. Although the HSP10 dose showed protective effects on acrosome membrane defects, cell death could not be prevented at high doses (HSP250 and HSP500). The energy needed by the spermatozoa for their motility can be provided by high rates of glycolysis or oxidative phosphorylation [31]. HSP can be used as a good exogenous antioxidant agent for spermatozoa because it appears to provide low PMAI and high HMMP ratios for the protection of the plasma membrane and acrosome of spermatozoa. It was reported that toxic agents, which have negative effects on sperm functions, disrupt mitochondrial function and cause the depolarization of the mitochondrial membrane [32]. This situation in the membrane potential causes the opening of the cytochrome gates escaping into the mitochondrial pores and cytoplasm, and thus, the initiation of apoptosis [33]. Studies have shown that the correlation between HMMP and plasma membrane integrity is important in the preservation of frozen human [34], bovine [35] and ram [21] semen. In this study, the numerical decrease in HMMP in the frozen-thawed ram semen samples at doses other than HSP10 compared to group C showed parallelism with the results of other studies. It was stated that a decrease in temperature experienced during the freezing process of sperm causes some functional and structural damage in viability, reducing fertility in rams [36] and bulls [37]. İsmail et al. [38] reported that curcumin nanoparticles (100 μg)

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added to goat semen extender preserved sperm viability and plasma membrane integrity. In another study, Valipour et al. [28] found that 20 µM HSP treatment during the cryopreservation of human sperm significantly improved the viability of the sperm after thawing and reduced LPL. The results of our study showed that HSP kept the viability of spermatozoa stable after cryopreservation in the HSP10, HSP50, and HSP100 dose groups compared to C, but it reduced viability at the HSP250 and HSP500 doses.

Scientific data have shown that excessive ROS production leads to DNA mutations and reduces ATP production, resulting in slowing sperm motility [39]. The preservation of the chromatin integrity of spermatozoa is one of the success criteria in transferring genetic information to the next generations [40]. Sperm chromatin is very easily affected by external factors, so it must be packaged in a very dense state to protect DNA. In this process, oxidative stress caused by ROS production may prevent chromatin packaging and cause the peroxidation of the sperm plasma membrane [41]. Similar to our study, there are some studies reporting that chromatin damage is prevented by antioxidant agents in ram sperm to ensure cryopreservation after thawing [40, 42]. In accordance with conclusions reached here, Trivedi et al. [43] determined that HSP doses of 25, 50, and 100 mg/kg daily had a protective effect on tail length, tail moment, and tail DNA in rats in a testicular toxicity model. Vijaya Bharathi et al. [44] explained that glucosyl HSP, a modified form of HSP, had a protective effect against DNA damage in the spermatozoa of rats, in which reproductive toxicity was created by increasing cellular antioxidant levels. Oxidative degradation can cause DNA base sequence degradation, fragmentation, and cross-link of proteins [45]. Structural damage to spermatozoon DNA adversely affects oocyte penetration and fertilization ability [46]. The HSP10 and HSP50 doses can prevent the formation of ROS and contribute to the protection of polyunsaturated oxidized fatty acids in sperm against oxidation, thus increasing the efficiency of living cells and fertilization. Additionally, the ability of HPS to stabilize the mitochondrial membrane was

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reported to protect sperm cells from apoptosis and increase mitochondrial functions [28]. There is some evidence that HSP has a protective effect against oxidative and nitrosative modifications in DNA induced by endogenous and exogenous ROS production [27, 44, 47]. Our results revealed important data on the effects of adding HSP during semen cryopreservation, such as the reduction or complete elimination of chromatin damage, depending on the doses of HSP.

Polyunsaturated fatty acids in the spermatozoon membrane are exposed to excessive ROS attacks during the freeze-thawing process, and thus, they are oxidized, which results in increased LPO [48]. Contrary to our findings, [U]. Valipour et al. [28]. showed that the level of ROS formation increased during the freeze-thawing process, and 20 μM HSP treatment significantly reduced ROS levels. In another study, the authors observed that testicular damage caused by diabetes increased MDA levels, but with HSP (50 mg/kg/day) treatment, MDA decreased, and GSH increased, and TAS increased simultaneously [27]. Trivedi et al. [43] stated that HSP (25, 50, and 100 mg/kg) treatment applied together with a chemotherapeutic agent, doxorubicin, significantly reduced MDA in rat sperm, increased GSH levels, and thus, significantly reduced oxidative stress. Unlike the findings obtained in this study, Helmy et al. [49] reported that HSP showed positive effects in regulating the antioxidant capacity of testicular tissue and reducing cell death, testicular histology, and oxidative damage indicators. In another study, it was shown that HSP regulated testicular insults and had a protective effect against oxidative stress in an experimental varicocele model [50]. However, our findings revealed that the GSH levels of the semen samples after thawing did not show a significantly effective activity at any dose of HSP. Additionally, we cannot say that the HSP doses that were used in our study had a significant effect on TAS levels compared to the control group. The reason for this may be that these selected doses were not sufficient for an endogenous enzyme activity or in the context of the semen samples of the selected animal species. In line with our

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results, Kaneko et al. [51] reported that low GSH levels may reduce spermatogenesis and TAS levels. Similarly, a study on human semen reported no correlation between seminal parameters and ROS [52]. It was observed that antioxidant molecules, especially low-weight molecules in the seminal plasma, can freeze and preserve semen better [53]. Kovalski et al. [54] emphasized the importance of select substances with low molecular weight in non-enzymatic exogenous antioxidant applications. It was shown that HSP, the exogenous antioxidant agent chosen for this study, plays a relatively minor role in increasing the antioxidant levels of semen in cryopreservation, depending on its molecular weight.

5. Conclusions

The positive and negative effects of many endogenous and exogenous antioxidant substances on cryopreservation have been documented by scientists in many regions of the world. However, some plant extracts used in studies may have harmful effects on reproductive functions, even if they have antioxidant activities. Therefore, it is important to elucidate the bioactive components, detailed chemical structures, animal species differences, and effective doses of such agents. In this study, HSP that was added to ram semen was directly supplied in pure form to determine its cryopreservative effects. For this reason, the results of our study on the cryopreservative effects of HSP at the determined doses revealed clearer information. According to our results, one can say that HSP doses are not very effective on semen progressive motility, but the HSP10 dose is effective in protecting plasma membrane integrity and preventing DNA damage by reducing lipid peroxidation.

Author Contributions: UT, DY, ŞG, FA, MFG and MEİ obtained the ejaculates, worked on semen processing and froze the semen. MEİ and ŞG determined PMAI values and conducted the flow cytometric evaluations. DY and FA examined spermatozoa DNA damage. MTO

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determined spermatozoon motility characteristics. BD specified biochemical alterations. UT designed the study and edited the manuscript. All authors read and approved the manuscript

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