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Effect of Different Sonication Protocols on Merino Ram Sperm Parameters

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ABSTRACT

The biochemical analysis and the determination of the intracellular-extracellular contents of the sperm cells are important in the spermatological studies. Since homogenization processes reveal the intracellular contents of the sperm cells, we aimed to examine the optimal sonication procedure and the protective effect of the antioxidants against sonication. Four merino rams (2–3 years old) were used for semen collection, and the ejaculates were pooled and divided into five equal aliquots. The samples were diluted with a solution having different additives at 37 °C. The first two groups contained L-Cystine as an additive (2 and 4 mM), the next two groups had methionine as an additive (2 and 4 mM), and the last group had no additives (control). After dilution, the semen samples were cooled at 5 °C for 2 h and then stored in liquid nitrogen. For further evaluation, the samples were thawed at 38 °C for 30 s. A phase-contrast microscope (400x) was used to determine the sperm motility. PNA-FITC staining was used to examine the acrosome integrity under a fluorescence microscope. Total oxidant status (TOS) and total antioxidant status (TAS) were measured using ELISA. The methionine group showed an increase in the post-thaw motility (53.08 ±3.6%) and viability (47.5 ±2.9) percentages compared to the control group (46.68 ±2.9; 40 ±4.08%, $p<0.05$). All the groups with antioxidants showed decreased levels of TOS in all sonication replicates compared to the control group ($p<0.05$). The methionine group showed statistical differences in the TAS level measurements between the 4 and 8-repeat sonication compared to the control group ($p<0.05$). Upon the examination of the damaged sperm rates, no difference was found between 8 and 12-repetitions ($p>0.05$). The result of our study showed that methionine had a cryoprotective effect on motility, acrosome integrity, and TOS levels and was also found to have beneficial effects at TAS levels. Considering the percentage of the damaged sperms, applying 8-repeat (4 seconds) was found to be the most suitable measurement.

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Authors' Contribution
CÖ and MO designed the study and collected the samples. CÖ, MO and NHA did the analysis. CÖ and MO wrote the manuscript.

Key words
Sonication, Sperm viability, Methionine additive, Spermatozoa, TOS, TAS level, Ram serum, PNA-FITC staining

INTRODUCTION

Sperm freezing is an innovative assisted reproductive technology that enables the usage of valuable genetic resources over a long period, which can increase the cross-country exchange of perfect fathers (Awda *et al*., 2009). While studying sperm cryopreservation, it is important to evaluate the oxidative stress parameters to determine the sperm's quality (Akalın *et al*., 2016; Bucak *et al*., 2020). Ultrasonication under optimum conditions is necessary to break down the sperm cells and organelle membranes to obtain the biochemical contents (Tateno *et al*., 2000). It was determined that mouse and human sperm cells subjected to 5 s of sonication could lead to separation of the head and tail of more than 95% of the cells.

Sonication involves the propagation of acoustic waves of a specific frequency. This method is used in the laboratory to separate microbial cells before culture and for further analysis. It is also extremely fast, with the splitting of the cells occurring in a short time (Amalfitano and Fazi, 2008). Separation occurs as the effect of external forces acting on a biofilm surface when the biofilm matrix exceeds the internal cohesion (Hunt *et al*., 2004).

During the homogenization process using sonication, the intensity and duration of ultrasonic waves can release the free oxygen radicals and disrupt the physical and chemical integrity of the sperm cells. The radicals that are released as an effect of high heat and pressure formed during the process can disrupt the integrity of the sperm membrane (Karabiga *et al*., 2007). Due to the free radical damage of purine and pyrimidine bonds, DNA is damaged, which causes low blastocyst formation (Agarwal *et al*., 2014). Sound waves propagate as waves based on the properties of the environment, such as solid, liquid, and gas (Akalın *et al*., 2016). The oxidative status of sperm

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samples is determined by the total oxidant status (TOS), while the antioxidative status is evaluated by measuring the levels of total antioxidant status (TAS).

Plasma membrane and acrosome are the main structures in the sperm cell that are affected by a cold shock (Hammerstedt *et al*., 1990). Ram sperm cells are highly sensitive to oxidative stress due to a higher number of polyunsaturated fatty acids in their plasma membranes (Allai *et al*., 2016). Exogenous antioxidants are required to maintain the balance between ROS production and scavenging. Many antioxidants, coenzyme Q10, trehalose, Gallic, and carnosic acids have been extensively added to semen extenders for minimizing the deleterious effect of oxidative stress (Gungor *et al*., 2019; Öztürk *et al*., 2020; de Albuquerque *et al*., 2020).

Methionine is a methyl and thiol provider, and its depletion in the presence of repeated ROS formation causes the pro-oxidant state (Bhardwaj and Yadav, 2013). In the antioxidant defense mechanism, methionine plays an important role because it easily reacts with the oxidants to form methionine sulfoxide (Levine *et al*., 1999). Glutathione synthesis occurs due to the presence of cysteine in the cell (De Matos and Furnus, 2000), and Cysteamine is a thiol compound that reacts with cystine to form a mixed disulfide, which is taken up by the mammalian cell and then cleaved to cysteine in the cytoplasm (Swami *et al*., 2017).

This study aims to determine the effects of antioxidants in semen extender on the oxidative stress parameters and investigate the effects of both additives and different sonication protocols on oxidative stress. Also, our study aimed to reveal the optimal sonication method for other studies as well.

MATERIALS AND METHODS

Animals and semen collection

Four adult merino rams were used for this study, and semen samples were collected via an artificial vagina. Ethics Committee permission was obtained (No 2018/441).

Experimental design

The ejaculates were collected once a week for four weeks, and the ones with more than 80% motility and concentrations higher than $2x10^9$ spermatozoa/mL were used. Tris base extender (TB) was used to dilute the semen (15% egg yolk, 6% glycerol), and the following additives were added based on which the groups were determined: TB + L-Cystine (2 mM), TB + L-Cystine (4 mM), TB + methionine (2mM), TB + methionine (4mM).

Diluted semen samples were loaded into 0.25 mL straws and equilibrated at 5°C for 2 h. Straws were frozen by placing them 5 cm above liquid nitrogen vapor (-110 to -120 °C) for 15 min, which was then stored in liquid nitrogen (-196 °C). The straws were later thawed in a water bath at 37 °C for 30 s for further evaluation.

Sperm motility

Spermatozoa motility was determined subjectively using a phase-contrast microscope (400x) and was estimated in five different microscopic areas. The mean of the five successive estimations was recorded as the final motility score.

Plasma membrane integrity

A modified staining protocol of Garner and Johnson (1995) was used. The semen samples were diluted at a ratio of 1: 3 with the tris stock solution, and then 30 µL of semen was mixed with 6 µL of SYBR-14 and 2.5 µL of propidium iodide (PI). Samples were incubated at 37 °C for 20 min in the dark and then 10 µL of Hancock solution was added to terminate the process.

Acrosome integrity

The acrosome integrity was determined as described by Nagy *et al*. (2003). The thawed straws were diluted with tris buffer at a ratio of 1: 3, then 60 µL semen, 10 µL FITC-PNA, and 2.5 µL of PI were mixed. The samples were incubated at 37 °C for 20 min in the dark, and 10 µL of Hancock solution (Schafer and Holzmann, 2000) was added for fixation.

Semen preparation for the enzymatic assays

To separate the spermatozoa, the diluted ejaculate was centrifuged three times at 4 °C for 15 min at 800 g. The cells were then washed with PBS and finally resuspended in 0.5 mL of PBS. For homogenization, the sperm suspension was placed in a 2 mL chamber of ice water. Each antioxidant group was further divided into four groups; 4- repeat (4 seconds), 8- repeat (4 seconds), 12- repeat (4 seconds) sonication, and the 4th group was the control group, which was sonicated for 4-seconds (no repeat).

Measurement of total antioxidant capacity

Total antioxidant status (Relassay, Turkey) was measured using the method of Erel (2004), which is based on bleaching of the characteristic color of a more stable ABTS (2,2 ′- Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. This test has excellent sensitivity values of less than 3%, and the results are expressed as mmol Trolox equivalent/L.

Measurement of total oxidant status

TOS levels were measured using commercially available kits (Relassay, Turkey). The oxidants present in the sample oxidizes the ferric ion-o-dianisidine complex to the ferric ion, and this ferric ion produces a colored complex with xylenol orange in an acidic environment. The intensity of the color is measured spectrophotometrically, which is related to the total amount of oxidant molecules in the sample. The assay was calibrated with hydrogen peroxide, and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H2O2 equivalent/L) (Erel, 2005).

Statistical analysis

The normality and homogeneity of the variances were confirmed by the Shapiro-Wilk test. The results of post-thaw sperm samples were expressed as mean±standard deviation with a One-way analysis of variance (ANOVA) used for evaluation. Analysis of variance was followed by Duncan's post hoc test to determine the differences between the groups. Differences with values of $P<0.05$ were considered to be statistically significant. Analyses were performed using the SPSS 21 package program.

RESULTS

As shown in Table I, the freezing extender having methionine led to higher percentages of sperm viability in comparison to the control group ($p<0.05$). Sperm acrosome integrity did not show any significant differences compared to the control group ($p<0.05$). The number of repetitions increased the level of the total oxidant status (TOS) with statistical differences between the groups with additives and the control group in all the repetitions ($p<0.05$) (Table II). The highest TAS levels were achieved in the group having 4 mM methionine with a statistically significant difference observed between the control, 4- repeat (4 seconds), and 8- repeat (4 seconds) groups whereas, no significant differences were observed in the 12- repeat (4 seconds) group (Table II). As a result of sonication, the sperm damage percentage rates were found to be statistically different between the groups and the control ($p<0.05$), but there was no difference between the 8 and 12- repetition groups ($p>0.05$) (Table II).

DISCUSSION

This study was conducted to define an optimal sonication time for sperm cells and to evaluate the protective effects of antioxidants on both thawed and sonicated cells (Akalın *et al*., 2016). Sperm cells are sensitive to the damage caused by high levels of ROS. Seminal plasma is an important source of antioxidants in semen (Agarwal *et al*., 2014). However, the existing antioxidant defense system is disrupted by the freeze-thawing procedure, which causes oxidative damage, imparting the toxic effects of free radicals (Bucak *et al*., 2015).

Table I. Flourescent staining parameters(Mean±SEM) in frozen–thawed Merino ram semen.

Groups	Sperm viability (%)	Acrosome integrity (%)	Motility (%)
Cystine 2mM	47.28±2.2^{b}	48.80±2.1	42.50±2.8^{ab}
Cystine 4mM	50.13±4 ^{ab}	49.85±3.4	43.75±2.5^{ab}
Methionine 2mM	51.28±4.2^{ab}	54.90±3.9	45±4.1^{ab}
Methionine 4mM	53.08±3.6^{a}	53.18±2.4	47.5±2.9^{a}
Control	46.68±2.9^{b}	49.15±4	40±4.08^{b}
p	*	-	*

(*p < 0.05).

Table II. TOS levels, TAS levels and % damaged sperm rate (Mean±SEM) in thawed ram semen.

Groups	Control	4 replicates	8 replicates	12 replicates
TOS(µmol/L)				
Cystine 2mM	8.24±0.5^{b}	8.39±0.6^{b}	10.21±1.2^{b}	11.60±1.0^{b}
Cystine 4mM	7.23±1.3^{bc}	8.28±0.5^{b}	8.73±0.6^{b}	11.56±1.4^{b}
Methionine 2mM	6.45±1.7^{c}	7.26±1.1^{b}	8.49±1.6^{b}	11.00±1.7^{b}
Metiyonin 4mM	5.62±0.5^{c}	7.93±3.02^{b}	8.44±2.0^{b}	10.54±1.3^{b}
Control	9.96±0.9^{a}	11.15±0.7^{a}	13.26±2.3^{a}	14.88±1.5^{a}
p	*	*	*	*
TAS (mmol/L)				
Cystine 2mM	10.88±2.9^{ab}	10.60±1.0^{b}	9.70±1.5^{b}	9.97±4.1^{a}
Cystine 4mM	14.61±3.5^{a}	11.23±1.3^{b}	10.07±0.9^{b}	10.54±2.9^{a}
Methionine 2mM	9.70±1.9^{ab}	10.43±1.6^{b}	9.53±1.3^{b}	9.51±2.2^{a}
Methionine 4mM	14.73±4.2^{a}	14.01±2.6^{a}	12.90±2.2^{a}	11.85±1.4^{a}
Control	9.27±3.4^{b}	9.44±0.8^{b}	8.41±1.1^{b}	7.3±0.8^{a}
p	*	*	*	*
Damaged sperm rates				
Sonication 4 second 4 Repetitions			66.0 ±2.2^{b}	
Sonication 4 second 8 Repetitions			84.0±4.1^{a}	
Sonication 4 second 12 Repetitions			88.5±2.9^{a}	
Control 4 second			44.3±0.8^{c}	
p			*	

(*p < 0.05).

In this study, a repeated sonications lead to an increase in the AOP levels of all the groups as the number of repeats increased. The application of antioxidants showed a protective effect when compared to the control group. Sonication is an easy method used to separate the head and tail of the sperm cells. However, due to the high temperature during the sonication, undesirable free radical molecules are released from the cells that cause lipid peroxidation (Akalın *et al*., 2016). Researchers have observed pronuclear formation in their injection using sperm heads separated by sonication but have not shown the developmental competence of the zygotes (Yanagida *et al*., 1991; Katayose *et al*., 1992). Baker *et al*. (2002) reported that the membrane proteins of mouse sperm were damaged and detached by sonication of 15-seconds, followed by 1-minute cooling with three repetitions. Considering the TOS values and sperm damage percentage, our results determined that 3-repetitive application for 8s was the most suitable method.

Antioxidants added to the extender in the cryopreservation of semen have beneficial effects on the preservation of sperm motility and acrosome integrity (Salamon and Maxwell, 2000). Methionine and thiol-containing antioxidants are used to protect spermatozoa from oxidative damage, thus, providing detoxification (Caylak *et al*., 2008). The claim that methionine has the property of clearing ROS makes it an important antioxidant (Levine *et al*., 1999). Methionine added to sperm extender showed an increase in the motility of sperm cells along with statistically non-significant differences in the LPO levels between the groups (Çoyan *et al*., 2010). In the present study, significant differences were observed in the TOS levels between the methionine and the control group. Similar to our study Omur and Çoyan (2016) reported that semen extender supplemented with methionine preserved the integrity of plasma membrane and acrosome.

It is reported that thiol compounds such as cysteamine and cystine stimulate oocyte GSH synthesis until the highest GSH levels are reached in the oocytes. Low molecular weight thiol compounds such as cysteamine reduce cystine to cysteine, promoting cysteine uptake in the cells while increasing the synthesis of GSH (Gasparrini *et al*., 2006). Pradieé *et al*. (2016) had reported that cysteine did not affect the post-thaw viability and oxidative activity of ram sperm, which was similar to our study, where the spermatological parameters showed a differently positive effect on the TOS level.

CONCLUSION

We observed that the additives added to the semen extender had positive effects on the spermatological parameters and as a result, the antioxidant substances that resulted from sonication decreased the TOS values. Also, TAS values showed a decrease with an increase in the number of repetitions of sonication, but the antioxidants provided a protective effect. There was no difference observed in the percentage of damaged semen in the number of repetitions of sonication between the 8 and 12 repetition. Thus, we concluded that 8 repetition was sufficient.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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