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Effects of ultrasonication on damaged spermatozoa and mitochondrial activity rate

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Abstract: The aim of this study was to describe an optimal sonication procedure for sperm cells. Therefore, we used several parameters such as damaged spermatozoa rate (%), mitochondrial activity rate (%), levels of lipid peroxidation, and total antioxidant potential. Ejaculates were collected from rams (n = 3) and were divided into aliquots and 3-, 6-, and 10-s duration times; 1, 3, 5, and 8 repetitive application groups were established. In the groups with 3-, 6- and 10-s duration times, with the increasing number of repeated applications, damaged spermatozoa rates increased ($P < 0.05$) while mitochondrial activity rates decreased ($P < 0.05$). In relation with sonication duration time, total antioxidant potential levels increased ($P < 0.05$) in single-application groups compared to those in control groups and gradually decreased as the repetitions increased. The most effective results were obtained in the group with 8 repetitions and 10-s duration based on damaged spermatozoa rate and mitochondrial activity rate.

Key words: Sonication, spermatozoa, damaged sperm rate, mitochondrial activity, lipid peroxidation

1. Introduction

The composition and dynamics of spermatozoa content change during sperm cryopreservation. In this regard, protection of spermatozoa from the effects of cryodamage, especially with the ameliorating effects of antioxidant agents, is important. Evaluating the antioxidant enzymes and oxidative stress parameters in addition to sperm quality parameters is crucial in the studies regarding cryopreservation (1,2). Therefore, the importance of disrupting sperm cells and organelle membranes by ultrasonication in optimal conditions and obtaining the complete biochemical content emerges.

Sonochemistry applications include sonic and ultrasonic waves. The chemical and mechanical effects of ultrasonic waves are related to cavitation, which is the formation of microbubbles by negative pressure in an applied liquid. Cavitation produces enough energy to break intermolecular van der Waals bonds or even the covalent bonds within the membranes (3–6). Ultrasonic homogenization with 20–100 MHz frequency ultrasonic waves is widely used in the disruption of the integrity of the cells (7). Sonochemical reaction is related to ambient temperature; that is, heat causes an increase in the equilibrium vapor pressure and the formation of more bubbles. This process reduces the efficiency of cavitation. Therefore, a low ambient temperature is needed for efficient ultrasonication (4,8).

Since free radicals have one or more unpaired electrons, they are very unstable reactive molecules (9) and easily react with other organic molecules such as lipids, proteins, DNA, and carbohydrates, collectively leading to structural deterioration (10). It is reported that free radicals, which induce lipid peroxidation (LPO) of biological membranes, are formed because of the ultrasonic heat in the liquid (11). The cell membrane, which is composed of polyunsaturated fatty acids, is a primary target for a reactive oxygen attack. LPO is considered to be the main molecular mechanism involved in the oxidative damage to cell structures and is an indicator of membrane integrity (12,13). Antioxidant potential (AOP) is the capability of the cell to scavenge free radicals and to repair their damage. Moreover, it reflects the oxidative–antioxidative balance of the cell.

Sonication applications for cell membrane disruption were reported by various researchers. Tateno et al. (14) reported that using sonication at a 5-s duration, more than 95% of mouse spermatozoa had their heads and tails

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separated, and more than 95% of human spermatozoa had broken tails. Normal sperm rate was reduced from 95.5% to 42.8% and the tailless sperm rate was increased from 4.5% to 57.2% by sonication at 6 repetitions with at least 30-s intervals (15). Baker et al. (16) reported broken axonemes and sperm heads by sonication at a 15-s duration repeated 3 times at intervals of 1 min, as determined by light microscopic examination.

Besides the determination of damaged spermatozoa (%) rate as a subjective method for evaluating spermatozoa cytoplasmic membrane integrity, evaluation of mitochondrial function (activity) has been suggested as an objective method for indication of organelle damage. Briefly, by means of fluorescent dyes penetrating sperm cell mitochondria, a gradient occurs at 100–200 mV negative potential in the mitochondrial membrane. Evaluation of this gradient presents an objective determination of organelle integrity of spermatozoa (17–19).

In our hypothesis, the optimal disruption of the cell and organelle membranes is proposed to be at the highest rates of damaged spermatozoa (%) and LPO levels, and at the lowest rates of mitochondrial activity (%) and AOP activity. The aim of this study was, therefore, to describe an optimal sonication procedure for disrupting sperm cell and organelle membranes and releasing complete spermatozoa content according to damaged spermatozoa rate, mitochondrial activity rate, and LPO and AOP levels.

2. Materials and methods

2.1. Animals and semen collection

Ejaculates from healthy, mature, and fertile Merino rams (n = 3) were used in the study. The rams, belonging to the Experimental and Education Farm of the Selçuk University Faculty of Veterinary Medicine, were maintained under uniform feeding and housing conditions. Ejaculates were collected from the rams with the aid of an artificial vagina twice a week as 8 replicates, according to standard AI procedures during the breeding season (September and October).

2.2. Evaluation and dilution of semen

Only ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 2.0×10^9 spermatozoa/mL were used in the study. Immediately after collection, the ejaculates were pooled and immersed in a warm water bath at 37 °C until their assessment in the laboratory. The pooled ejaculates in each replication were diluted with a final concentration of approximately 1.0×10^9 spermatozoa/mL in Tris-based extender (297.58 mM Tris, 96.32 mM citric acid, 82.66 mM fructose, 55 mM glycerol, and 15% egg yolk) and divided into 4 equal aliquots with groups (n = 8) established as below:

1- 3-s group with 1, 3, 5, and 8 repetitions,
2- 6-s group with 1, 3, 5, and 8 repetitions,
3- 10-s group with 1, 3, 5, and 8 repetitions,
4- Control (only diluted spermatozoa).

2.3. Sonication process

Diluted ejaculate samples were washed with phosphate-buffered saline (PBS) 3 times. Briefly, each sample volume was completed to 1 mL with PBS and centrifuged at 800 × *g* for 10 min at 4 °C. Supernatant was discarded and the pellet was completed to 1 mL with PBS. After reevaluation of the concentration of each group (Tables 1–3), samples were sonicated in 2-mL transparent polyethylene tubes (1.5 cm in diameter, 6 cm in height) on a continuous basis by the Sonics Vibra-Cell (Sonics & Materials, Inc., USA; model: VCX 130, serial no: 45822, net power output: 130 W, frequency: 20 kHz, amplitude: 100%, probe: S&M 630-0422, probe model: CV18, probe serial no: 6837) with a 30-s cooling period (on ice) between each duration time.

Table 1. Effects of 3-s duration and single, 3, 5, and 8 repetitive sonication applications on damaged spermatozoa rate (%), mitochondrial activity (%), and levels of LPO and AOP.

Groups	Concentration (1×10^6/mL)	Damaged spermatozoa (%)	JC1-PI high activity (%)	JC1-PI low activity (%)	LPO (μm/1×10^9 sperm)	AOP (mM/1×10^9 sperm)
Single-term	831.25 ± 66.10^{bc}	23.75 ± 5.23^{b}	3.73 ± 0.48^{b}	56.3 ± 3.05^{b}	23.04 ± 2.17^{b}	125.60 ± 2.40^{d}
3 repetitions	953.125 ± 26.9^{c}	51.25 ± 2.45^{c}	2.86 ± 0.46^{b}	54.3 ± 3.04^{b}	16.85 ± 1.73^{a}	101.10 ± 3.53^{c}
5 repetitions	693.75 ± 61.50^{ab}	81.25 ± 2.05^{d}	2.45 ± 0.49^{a}	1.8 ± 0.57^{a}	23.40 ± 1.97^{b}	67.8 ± 3.46^{b}
8 repetitions	631.25 ± 43.20^{a}	88.12 ± 0.90^{d}	0 ± 0^{a}	0.52 ± 0.28^{a}	23.23 ± 1.75^{b}	56.12 ± 1.68^{a}
Control	946.87 ± 73.80^{c}	0 ± 0^{a}	16.59 ± 1.04^{c}	58.6 ± 2.42^{b}	17.83 ± 1.30^{ab}	77.07 ± 5.70^{b}
P	*	*	*	*	*	*

a, b, c, d - values with different letters in the same column are statistically different (*P < 0.05).

Table 2. Effects of 6-s duration and single, 3, 5, and 8 repetitive sonication applications on damaged spermatozoa rate (%), mitochondrial activity (%), and levels of LPO and AOP.

Groups	Concentration (1×10^6/mL)	Damaged spermatozoa (%)	JC1-PI high activity (%)	JC1-PI low activity (%)	LPO (μm/1 × 10^9 sperm)	AOP (mM/1 × 10^9 sperm)
Single-term	787.50 ± 100.77^{ab}	35.6 ± 6.50^{b}	6.30 ± 1.25^{b}	37.14 ± 5.50^{b}	26.90 ± 2.55^{c}	128.60 ± 2.68^{d}
3 repetitions	971.87 ± 69.34^{b}	59.3 ± 4.30^{c}	1.60 ± 0.82^{a}	29.51 ± 2.20^{b}	15.05 ± 2.18^{a}	105.30 ± 3.78^{c}
5 repetitions	743.75 ± 79.30^{ab}	83.7 ± 2.05^{d}	0.26 ± 0.26^{a}	3.18 ± 2.02^{a}	26.32 ± 2.41^{bc}	79.10 ± 9.87^{b}
8 repetitions	675.00 ± 40.08^{a}	94.3 ± 1.70^{d}	0.00 ± 0.00^{a}	0.71 ± 0.55^{a}	22.14 ± 4.77^{ab}	57.20 ± 1.87^{a}
Control	946.87 ± 73.80^{b}	0 ± 0^{a}	16.50 ± 1.04^{c}	58.60 ± 2.42^{c}	17.80 ± 1.30^{ab}	77.00 ± 5.76^{b}
P	*	*	*	*	*	*

a, b, c, d - values with different letters in the same column are statistically different (*P < 0.05).

Table 3. Effects of 10-s duration and single, 3, 5, and 8 repetitive sonication applications on damaged spermatozoa rate (%), mitochondrial activity (%), and levels of LPO and AOP.

Groups	Concentration (1×10^6/mL)	Damaged spermatozoa (%)	JC1-PI high activity (%)	JC1-PI low activity (%)	LPO (μm/1 × 10^9 sperm)	AOP (mM/1 × 10^9 sperm)
Single-term	865.62 ± 54.47^{b}	45.6 ± 5.12^{b}	4.8 ± 1.20^{b}	34.2 ± 5.90^{b}	28.2 ± 5.4^{b}	129.9 ± 4.42^{d}
3 repetitions	915.62 ± 81.68^{b}	64.3 ± 2.90^{c}	2.9 ± 1.70^{ab}	23.2 ± 4.40^{b}	16.9 ± 4.2^{ab}	108.8 ± 3.92^{c}
5 repetitions	640.62 ± 45.79^{a}	88.7 ± 3.50^{d}	0.00 ± 0.00^{a}	24.9 ± 1.40^{b}	27.8 ± 2.3^{b}	80.8 ± 8.77^{b}
8 repetitions	606.25 ± 44.75^{a}	100 ± 0.00^{e}	0.00 ± 0.00^{a}	0.4 ± 0.20^{a}	13.9 ± 1.7^{a}	56.3 ± 2.08^{a}
Control	946.87 ± 73.80^{b}	0.00 ± 0.00^{a}	13.7 ± 2.10^{c}	48.6 ± 5.10^{c}	19.9 ± 3.1^{ab}	74.3 ± 9.14^{ab}
P	*	*	*	*	*	*

a, b, c, d, e - values with different letters in the same column are statistically different (*P < 0.05).

2.4. Evaluation of damaged spermatozoa and mitochondrial activity rates (%)

After sonication of spermatozoa, the number of cells with intact head was evaluated by the hemocytometric method in a Thoma chamber. Mitochondrial activity was evaluated by JC-1/PI fluorescent dye (18). Briefly, 2 μL of JC-1 dye (in 1.53 mM DMSO) was added into 12 μL of PI (2.99 mM PI-modified Tyrode solution) and 0.7 μL of SYBR-14 into 1 mL of HEPES/BSA buffer solution. Next, 110 μL of this prepared solution was added into 390 μL of sperm suspension and the mixture was incubated in a water bath at 37 °C. Mitochondrial activity was evaluated in 2 μL of the sample prepared under a coverslip with fluorescent microscope.

2.5. Evaluation of LPO and AOP levels

LPO levels were determined in control group homogenates and sonicated homogenates using LPO-586 Oxis Research kits (Oxis Research, USA). For determination of AOP levels (AOP-490 Oxis Research kits), homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C to remove the cell debris and the supernatant was collected. LPO and AOP levels were determined spectrophotometrically (UV 2100 UV-VIS Recording Spectrophotometer, Shimadzu, Tokyo, Japan).

2.6. Statistics

Results were expressed as mean ± SE. Means were analyzed by analysis of variance (ANOVA), followed by Duncan's post hoc test to determine significant differences in all the parameters between groups using SPSS for PC (version 12.0, SPSS Inc., Chicago, IL, USA). Differences with values of P < 0.05 were considered to be statistically significant.

3. Results

The effects of sonication on different groups are presented in Tables 1–3. In the 3-, 6-, and 10-s duration time groups, as the number of repetitions increased, the damaged spermatozoa rate increased, while the JCI-PI% high activity and JCI-PI% low activity decreased (P < 0.05). Regarding

the spermatozoa damage and mitochondrial activity rates, the most effective results were obtained in the group with 8 repetitions and 10-s duration. In relation to sonication duration time, LPO ($P > 0.05$) and AOP levels ($P < 0.05$) increased in single-repetition groups compared to the control groups, whereas levels were affected differently during repeated applications (Tables 1–3).

4. Discussion

This study was performed to describe an optimal sonication procedure for sperm cells. Sonochemical reactions lead to an increase in temperature and highly reactive free radicals (20). Peroxidation of the membrane lipids of spermatozoa due to the effects of free radicals deteriorate the membrane integrity (12). In vitro application of ultrasound waves (1 MHz, 0.61–2.44 W/cm^2 intensity) into carp erythrocytes for a 5-min duration increased the LPO and methemoglobin levels in parallel to the increase in intensity (21). In the current study, a single-term application of sonication in the 3-, 6-, and 10-s duration groups increased the LPO levels ($P > 0.05$) as compared to the control group, in parallel to the increase in the duration time (Tables 1–3). As regards the LPO levels in the 3-, 6-, and 10-s duration groups, the levels were lower in groups with 3 repetitions as compared to those in the single-term and 5- and 8-repetition groups ($P < 0.05$; Tables 1–3). This suggests that the sonication process broke down the cell and organelle membranes and increased the LPO levels in the single-term groups. Because the repetition of sonication may lead to the formation of molecules with lower molecular weight, which represents different levels of LPO, the levels of LPO determined in the current study seem incompatible with the applications. We have previously reported (22) that in liver homogenates LPO levels were increased gradually by 2-, 4-, 6-, and 8-s sonication, while levels were reduced ($P > 0.05$) at a 10-s duration. With respect to the AOP, levels increased significantly in single-term groups in all durations, but repetition of sonication gradually reduced the levels. These data may reveal that sonication released cell and organelle content by a single-term application, and repetition of sonication resulted in higher levels of free radicals in the medium, which consumed the AOP (Tables 1–3).

To investigate sperm cell integrity, the effects of sonication on damaged sperm have been established in several studies. A 5-s duration of sonication (Brinwell Scientific, Rochester, NY, USA; model BP-II, with 12-mm diameter horn, 60% output) led to the separation of tails from the heads by 95% in mouse spermatozoa and broken tails by 95% in men (14). Yamamoto et al. (15) also reported that the separation of heads from tails increased as the repetition of sonication (2, 4, and 6 times) increased (Ultrasonic generator, model US150) with a 15-s duration in the spermatozoa of Sprague Dawley rats. By sonication, membrane proteins split off in addition to the separation of heads and tails. Baker et al. (16) reported that with a 15-s duration, 1 min of cooling, and 3 repetitive applications of sonication (VirTis, Gardiner, NY, USA), membranes were damaged and thus membrane proteins were split off. In the current study, different sonication duration times and repetitions induced damage of the spermatozoa, and, as the duration time and repetitions increased, the damaged sperm rate also increased (Tables 1–3).

Mitochondrial activity has been suggested to be an objective parameter for evaluation of spermatozoa (17,19). We observed that with the increased rate of damaged spermatozoa, the mitochondrial activity decreased with the increased duration time and repetitions of sonication application (Tables 1–3). Repetition of sonication increased the damaged sperm rate and decreased the JC1-PI% high activity in all duration groups. The highest percentage of damaged spermatozoa (100%) with the lowest percentage of JC1-PI high activity (0%) and the lowest low activity (0%) were observed in the 8-repetition, 10-s duration group ($P < 0.05$; Table 3). The decrease in mitochondrial activity, with the increase in damaged spermatozoa rate, seemed to be in good agreement and supported the effects of sonication on complete spermatozoa and organelle membrane damage.

In conclusion, in ram spermatozoa, as the duration and repetitions of sonication applications increased, the damaged spermatozoa rate increased while the mitochondrial activity decreased. The most effective application of sonication related to the damaged spermatozoa rate (100%) and mitochondrial activity (0%) was observed in the 8-repetition, 10-s duration group in Merino ram spermatozoa.

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