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Short Communication

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The comparing of homogenization methods for oxidative and antioxidative analyses of sperm

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Abstract: Pretreatment of sperm for oxidative and antioxidative analysis has been shown to be different in studies. These differences can affect the result of analyses. The aim of this study was to compare the analyses of sperm homogenization methods. The semen of four rams were used in the study. Pretreatment procedures included the rotary, bead, and ultrasonic homogenization methods. Oxidative and antioxidative status of sperm samples was assessed by measuring serum lipid hydroperoxide, total oxidant status, free sulfhydryl groups (SH total thiol), ceruloplasmin, and total antioxidant capacity levels. As a result, the homogenization methods before oxidative and antioxidative status analyses significantly affected the result ($P < 0.05$), and the ultrasonic homogenization method was the most effective. It is recommended to use the ultrasonic homogenization as a pretreatment method to determine the oxidative and antioxidative status analyses in sperm.

Key words: Spermatozoa, oxidative analysis, homogenization, methods

The analysis needs mutually accepted validation procedures. For clinical laboratories, ISO 17025 and ISO 15189 certification programs are applied. The guide on bioanalytical method validation was published by the U.S. FDA in 2001 [1] but there is no validated method in pretreatment of oxidative and antioxidative analyses in sperm. Pretreatment procedure of semen before oxidative analyses is contradictory in different studies. Satisfactory homogenization of sperm is a necessary prerequisite for oxidative analyses. Different procedures have been used for oxidative analyses. These techniques are also applicable to semen pretreatment, but verification is required. The pretreatment protocols before oxidative analyses can be categorized as direct oxidative analyses [2-4], washing and chemical treatment[5], rotary homogenization [6], and sonication [7]. Reactive oxygen species (ROS) have a physiological and pathological role in semen. Low-level of ROS assists capacitation, hyperactivation, acrosome reaction, oocyte fusion, and fertilization. However, many studies have revealed that the high level of ROS has a pathological role in male fertility [8]. Atmospheric or molecular oxygen of the environment, leucocytes, dead spermatozoa, immature or abnormal spermatozoa, and microorganisms produce different ROS to which spermatozoa are unavoidably exposed [9–11]. Spermatozoa are protected against ROS by their intracellular defense system and the antioxidant buffer capacity of seminal plasma after ejaculation. The seminal plasma and cellular protective system of semen consists of superoxide dismutase (SOD, glutathione peroxidase, glutathione, vitamin E). Seminal plasma is a rich source of different enzymatic and nonenzymatic antioxidants that protect the spermatozoa against oxidative stress. However, sperm cells have limited cytoplasmic capacity, which leads to small quantities of endogenous antioxidants. Therefore, spermatozoa have deficient ROS defense systems [12]. Although seminal plasma is rich in antioxidants [13], many studies used spermatozoa without seminal plasma, which left spermatozoa defenseless against the ROS, creating a contradiction in sperm studies.

Oxidative status of sperm samples is assessed by measuring the levels of serum lipid hydroperoxide (LOOH) and total oxidant status (TOS). Antioxidative status is assessed by measuring the levels of serum free sulfydryl groups (SH total thiol), ceruloplasmin (CP), and total antioxidant status (TAS). In oxidative status, TOS levels are total indicators of oxidant and lipid hydroperoxide (LOOH) known as main indicators of the lipid peroxidation. Lipids are one of the most susceptible substrates to oxidative damage. Spermatozoa contain high concentrations of polyunsaturated fatty acids (PUFAs), which makes spermatozoa vulnerable to lipid peroxidation. LOOH parameter is also considered to be an important factor of male infertility [14,15]. TAS levels are

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total indicators of antioxidant capacity. Furthermore, CP and SH protect the spermatozoa against toxic substances, provide a suitable environment for the spermatozoa, act as an antioxidant enzyme, and scavenge ROS [16,17].

In this study, the rotary, bead, and ultrasonic homogenization methods were tested in terms of the effectiveness of the analysis of the oxidative and antioxidative status of semen.

The study materials were four healthy and fertile rams. Sperm samples of the rams were obtained by using an artificial vagina and were pooled. Approximately 3 mL of semen was divided equally into three parts. The first part of semen was used as whole semen (control). The second part was centrifuged at 3000 × *g* for 10 min and its pellet was used. The third part was centrifuged at 3000 × *g* for 10 min and its seminal plasma was used. The divided semen parts were again divided into four parts as 100 µL in 2 mL Eppendorf tubes and suspended with 900 µL PBS.

The groups were as follows:

C1: Semen - Control
C2: Pellet - Control
C3: Seminal Plasma - Control
B1: Semen - Bead Homogenization
B2: Pellet - Bead Homogenization
B3: Seminal Plasma - Bead Homogenization
R1: Semen - Rotary Homogenization
R2: Pellet - Rotary Homogenization
R3: Seminal Plasma- Rotary Homogenization
U1: Semen - Ultrasonic Homogenization
U2: Pellet - Ultrasonic Homogenization
U3: Seminal Plasma - Ultrasonic Homogenization

In rotary homogenization, the cooled semen, pellet, and seminal plasma were homogenized by a rotary homogenizer (Generator: 6 mm diameter, Heidolph, Co., Kelhein, Germany) in ice water for 1.5 min and this was repeated 5 times at intervals of 30 s [6].

In bead homogenization, the semen, pellet, and seminal plasma were cooled in deep freeze at –20 °C in 2 mL homogenization tubes containing steel beads until iced (Hard Tissue Grinding Mix Tubes, 2.4 mm metal balls, SKU: 19 - 620D, Omni International, USA) and shacked for 1.5 min and this was repeated 5 times at intervals of 30 s.

In ultrasonic homogenization, the cooled whole semen, pellet, and seminal plasma were transferred into a 2 mL beaker in ice water and sonicated with a probe (Bandelin Sonopuls, D - 12207, Gerate - Typ: UW 2070, Berlin) for 10 s and repeated 6 times at intervals of 30 s [7].

2.2. Analyses of oxidant and antioxidant parameters

The specimens were stored at –20 °C in deep freeze and transferred to the laboratory in cold chain for analysis.

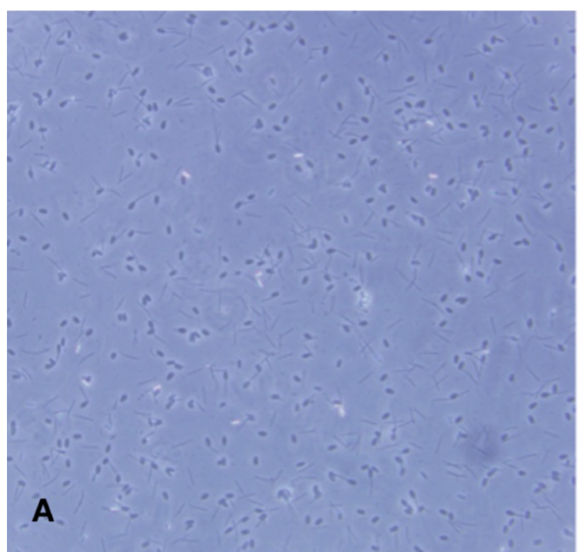
Six parameters, which are lipid hydroperoxide, total oxidant status, oxidative stress index, total antioxidant status, sulfhydryl groups, and ceruloplasmin, were analyzed. Oxidative stress index (OSI) was obtained according to the following formula. OSI = [(TOS, µmol/L)/(TAS, (mmol Trolox Eq/L) × 100]. Oxidative and antioxidative parameters were measured in the tissue samples using the Aeroset automated analyzer (Abbott, IL, USA) and a spectrophotometer (Cecil 3000, Cambridge, UK). The lipid hydroperoxide level (LOOH) was evaluated by the fluorometric method based on the reaction between malondialdehyde (MDA) and thiobarbituric acid [18]. All analyses were made in the Biochemistry Laboratory of the Faculty of Medicine at Harran University.

Statistical analyses were performed using SPSS software (version 23 for Mac; SPSS Inc., Chicago, IL). The data was analyzed to see if there were statistical differences between homogenization methods and oxidative status parameters. Two-way analysis of variance (ANOVA) was used to test group differences in the parametric data. The Duncan test for multiple comparisons was used for post hoc analysis if the groups were statistically different. The nonparametric data were analyzed using the Kruskal–Wallis test and if there were significant differences between groups, the Mann–Whitney test was used to determine the differences between groups after Bonferroni correction. Statistical significance was set at $P < 0.05$. Values were presented as the mean ± standard error of the mean (SEM).

Microscopic investigation of spermatozoa demonstrated that (Figure) the mechanical homogenization cannot fully break down the wall of spermatozoa.

SH, LOOH, TOS, TAS, and OSI were significantly different ($P < 0.01$) in the groups. The semen LOOH (µmol G^{-1} protein) levels in C1, R1, B1, and U1 were 12.7, 10.6, 6.7, and 17.8, respectively ($P < 0.05$). B1 was significantly lower than that in the control. The LOOH levels of pellets in R2 and B2 groups were significantly lower and U2 higher than that of the control group. The LOOH levels (µmol G^{-1} protein) in C3, R3, B3, and U3 were 5.8, 5.5, 7.5, and 5.8, respectively ($P > 0.05$). The LOOH levels were 12.7 in semen (C1) and 14.2 in the sum of pellet and seminal plasma (C2 + C3) (an increase of 1.5). After ultrasonic homogenization, the lipid peroxidation levels (µmol G^{-1} protein) were 17.8 in semen and 21 in the sum of pellet and seminal plasma (an increase of 3.2). When the LOOH parameter in semen and the sum of pellet and seminal plasma are considered together, the change in rotary and bead homogenization was 1.2 and 6.5, respectively (Table).

When the TAS data was analyzed, the most of antioxidants were observed in the seminal plasma compared with pellet. In the control group, C1, C2, and C3 were 0.09, 0.05, and 0.08, respectively. However, the sum of the pellet and seminal plasma of TAS values were higher than semen. OSI difference was statistically nonsignificant



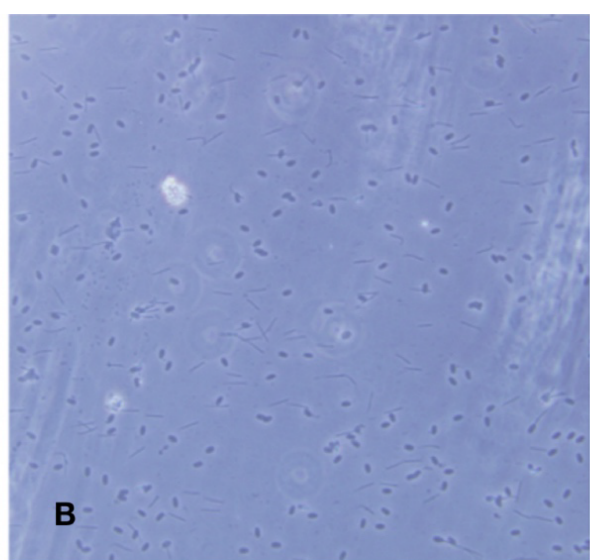


Figure. The picture of semen after 30 (A) and 60 (B) s of sonication.

Table. Some oxidative and antioxidative parameters in control, bead homogenization, rotary homogenization, and ultrasonic homogenization groups.

Groups	CP* (U/g protein)	SH* (mmol G^{-1} protein)	LOOH* (µmol G^{-1} protein)	TOS* (µmol H_2O_2 Eq G^{-1} protein)	TAS* (mmol Trolox Eq G^{-1} protein)	OSI* (Arbitrary units)
C1	213.8 ± 3.0	0.085 ± 0.002[ab]	12.7 ± 1.1[ab]	20.5 ± 2.1[bcd]	0.09 ± 0.01[abc]	29.5 ± 6.8[a]
C2	210.4 ± 3.4	0.081 ± 0.002[b]	8.4 ± 0.5[cd]	13.8 ± 0.9[d]	0.05 ± 0.00[c]	29.8 ± 3.7[a]
C3	215.5 ± 10.3	0.074 ± 0.001[c]	5.8 ± 0.6[e]	8.4 ± 1.0[e]	0.08 ± 0.02[abc]	12.4 ± 3.0[a]
R1	217.1 ± 9.7	0.069 ± 0.002[c]	10.6 ± 0.9[bc]	18.4 ± 1.7[cd]	0.07 ± 0.00[abc]	27.2 ± 2.2[a]
R2	207.7 ± 4.0	0.071 ± 0.003[c]	6.3 ± 0.5[e]	10.3 ± 0.8[e]	0.05 ± 0.01[c]	19.5 ± 1.5[ab]
R3	208.4 ± 3.4	0.069 ± 0.002[c]	5.5 ± 0.3[e]	9.7 ± 0.7[e]	0.08 ± 0.02[abc]	11.2 ± 0.9[c]
B1	235.7 ± 14.5	0.071 ± 0.001[c]	6.7 ± 0.5[de]	24.7 ± 1.4[abc]	0.09 ± 0.02[abc]	38.9 ± 7.8[a]
B2	212.7 ± 3.4	0.072 ± 0.002[c]	5.7 ± 0.2[e]	17.0 ± 2.4[cd]	0.06 ± 0.02[bc]	27.6 ± 3.2[a]
B3	212.3 ± 1.2	0.072 ± 0.001[c]	7.5 ± 0.5[de]	37.6 ± 5.0[a]	0.11 ± 0.02[a]	42.7 ± 6.1[a]
U1	201.2 ± 1.5	0.087 ± 0.002[a]	17.8 ± 1.6[a]	28.5 ± 2.8[ab]	0.10 ± 0.01[ab]	30.6 ± 3.2[a]
U2	212.6 ± 1.9	0.088 ± 0.002[a]	15.2 ± 2.1[ab]	23.8 ± 2.5[abc]	0.07 ± 0.01[abc]	36.4 ± 4.1[a]
U3	214.5 ± 2.4	0.070 ± 0.002[c]	5.8 ± 0.5[e]	7.9 ± 0.3[e]	0.08 ± 0.02[abc]	12.3 ± 2.1[bc]

*CP (ceruloplasmin), SH (serum free sulfydryl groups), LOOH (lipid hydroperoxide), TOS (total oxidant status), TAS (total antioxidant status), OSI (oxidative stress index).
Values are mean percentages ± SEM (n = 6)
Different superscripts within the same columns denote significant differences (P < 0.05).
No superscripts within the same columns denote nonsignificant differences (P > 0.05).

in the groups without R3 and U3. There was no differences in ceruloplasmin between the groups (Table)

The total oxidant levels (TOS/mmol Trolox Eq G^{-1} protein) in C1, R1, B1, and U1 were 20.5, 18.4, 24.7, and 28.5, respectively (P < 0.05). The semen TOS levels were not different compared with the control group (P > 0.05). The pellet TOS levels in C2, R2, B2, and U2 were 13.8, 10.3, 17.0, and 23.8, respectively (P < 0.05). These data showed that R2 was lower, B2 and U2 were higher than C2 (control group). TOS levels in C3, R3, B3, and

U3 were 8.4, 9.7, 37.6, and 7.9, respectively. B3 level was statistically different when compared within groups and also extremely higher than C3. TOS were 20.5 in semen (C1) and 22.2 in the sum of pellet and seminal plasma (C2 + C3) (an increase of 1.7). When the TOS parameter in semen and the sum of pellet and seminal plasma are considered together, the change in rotary, bead, and ultrasonic homogenization was 1.6, 29.9, and 3.2, respectively. B3 and U2 TOS levels were extremely high compared to C3 and C2 ($P < 0.05$) (Table).

The homogenization methods are expected to contribute to correctly determining the oxidative stress parameters in cells that are not self-harming. The study's theory is that suitable homogenization methods should provide an increase in determination rate of oxidants in pellets. If seminal plasma oxidant levels are higher than the control groups, this shows us that the method produces oxidant itself. Seminal plasma TOS levels in C3, R3, B3, and U3 were 8.4, 9.7, 37.6, and 7.9, respectively. As these data have demonstrated, the ultrasonic homogenization method is closer than other methods to this result. When considered in terms of LOOH and TOS, the differences between C3 and U3 levels were statistically not significant ($P > 0.05$). In addition, U2 levels were higher than C2 ($P < 0.05$). This proves that ultrasonic homogenization increases the analyses power for oxidant and LOOH detection in cells. Compared with the control group, TOS level (B3) was statistically increased in seminal plasma. Moreover, this demonstrated that bead homogenization caused oxidative stress itself to spermatozoa. The LOOH and TOS levels were low or nonsignificantly different from the control groups after rotary homogenization.

Different procedures have been used in analyses for oxidative status, and dissimilar measurement units can also be used at times. Bucak et al. [3], Sarıözkan et al. [19], and Naijan et al. [20], in their studies, used nmol/mL for MDA level description and they found values of 9.2, 2.31, and 1.37, respectively. For unit measurement, µmol G^{-1} protein was used in this study for a description of MDA level and the value was 12.7. Mmol Trolox Eq G^{-1} protein was used for TAS level description , whereas Gavella et al. [21] used mmol/L .The value of 0.08 was found in TAS analyses in seminal plasma of this study. However, Gavella et al. [21] reported this value as 2.0. This can cause a contradiction when oxidative parameters are compared in different studies.

The level of ceruloplasmin impacts the seminal plasma antioxidant defense system and strongly correlates with semen quality [16]. As in this study, Verit et al. [22] did not observe any significant change in seminal plasma ceruloplasmin concentration in the experimental groups. It is known that seminal plasma is the main source of antioxidants [13]; however, there were no differences in CP levels between semen and pellets. This result shows that the ceruloplasmin level is not a relevant indicator of the semen antioxidant defense system. In a study conducted in rams, CP values were determined between 4.5 and 52.2 U/g protein [23]. In healthy people, ceruloplasmin levels were measurent between 537 and 2030 U/L in the difrent studies [24–26]. These results showed that ceruloplasmin levels also show a lot of variation in different studies. In addition, meaningful results could not be obtained in SH and OSI analyses.

TAS level (mmol Trolox Eq G^{-1} protein) was very low in all groups because of spermatozoa's limited TAS capacity [12]. Our expectation from homogenization methods is that the seminal plasma TAS level should be slightly lower than semen, but the sum of pellets and seminal plasma TAS level should be higher than semen. Only the ultrasonic homogenization method was in line with this theory. TAS levels were 0.09 in semen (C1) and 0.13 in the sum of pellet and seminal plasma (C2 + C3). TAS levels were 0.10 and 0.15 in U1, U2 + U3, respectively.

To conclude, the homogenization methods significantly affected the result. One homogenization method should be used in studies and ultrasonic homogenization method is more appropriate for analyses of oxidative and antioxidative status.

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