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Effect of different doses of hydrated C_{60} fullerene nanoparticles on ram semen during cool storage

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Abstract: The aim of this study was to determine the effect of different doses of hydrated C_{60} fullerene (C_{60}HyFn) nanoparticles on ram semen during cool storage at 5 °C. Semen samples were collected from 7 healthy Akkaraman rams by artificial vagina during nonbreeding season (April) 3 times for two weeks (3 replications), and samples having good initial sperm motility (> 70 %) and count (> 2 billion / mL) were pooled and diluted with tris-egg yolk based extender at 37 °C. Then, the diluted semen samples were divided into 15 aliquots control and 14 different C_{60}HyFn groups (25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, 1 µM, 5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM), and stored at 5 °C up to 144th h. The motility (total, progressive, rapid, medium, slow, and static), kinematic (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF), and some other spermatological parameters (dead, abnormal, damaged acrosome, and membrane intactness) were examined at 0, 12, 24, 48, 72, 96, 120, and 144th h. All C_{60}HyFn doses except 25 nM, 50 nM, 60 µM, 80 µM, and 100 µM had numerically higher total and progressive motility, membrane intactness and numerically lower static, dead and total abnormal spermatozoon ratios at the end of 144th h than control. The differences between 400 nM dose and control in membrane intactness, and also between 400, 800 nM doses and control in dead spermatozoon ratio were statistically significant ($P < 0.0125$). In conclusion, it is suggested that supplementation of any doses of C_{60}HyFn between 100 nM and 40 µM may be beneficial to keep some spermatological parameters at high level during the short-term storage of ram semen at 5 °C.

Key words: Hydrated C_{60} fullerene, cool storage, spermatozoon, ram

1. Introduction

Under field conditions, the low success rate obtained from intracervical insemination with frozen-thawed ram semen in ewes is a reason why short-term storage of semen at 5 °C is still available to rams compared to the bulls. Nevertheless, marked reductions have been observed in the pregnancy rates obtained from intracervical insemination performed by ram semen stored for more than 24 h at 5 °C [1]. Short-term storage process commonly causes some biochemical reactions in semen due to the cold environment. The most important of these reactions is oxidative stress. Increases in reactive oxygen species (ROS) levels during cool-storage have been reported to cause peroxidation of lipids in the spermatozoon membrane [2, 3]. Thus, increased lipid peroxidation (LPO) has been associated with decreased motility, increased morphological disorder, increased acrosomal damage, decreased membrane integrity, increased dead spermatozoon ratio, decreased mitochondrial activity, and increased DNA damage [2, 4]. The above-mentioned damages related to LPO induced by cool storage are much more common in rams due to the high unsaturated fatty acid content of the plasma membrane of spermatozoa [5, 6]. On the other hand, it is suggested that these damages in ram spermatozoa are also related to the diluents used [7, 8], and that tris + egg yolk and tris + soy lecithin diluents are more effective in short-term storage than milk powder diluent [8].

There are numerous studies involving many antioxidant substances supplemented to ram semen during short-term storage in order to prevent damage to the spermatozoa and to ensure the long-term viability of spermatozoa. Because antioxidants have the ability to protect spermatozoa against ROS attacks and LPO damages, increased ROS levels during short-term storage may strongly be scavenged by

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enzymatic and nonenzymatic antioxidant additives [2, 4]. Fullerenes have been extensively investigated for their unique physicchemical properties and biological activities since they were discovered in 1985 [9–11]. Carbon 60 (C_{60}) fullerene is practically insoluble in water and this situation limits its biomedical application fields [12]. However, it is emphasized that the C_{60} fullerene nanoparticles prepared by different chemical dissolution and modification methods exhibit very different biological effects. Some functional fullerene derivatives have been reported to show significant toxic effects in vivo and in vitro, and toxic responses are largely related to the chemical structure of the functional solvents bound to C_{60}, but not related to the basic structure of it [13]. Currently, a water-soluble nanotechnological method has been developed without using any solvent or stabilizer to produce C60 fullerene. The product was named as hydrated C_{60} fullerene, (C_{60}HyFn). Chemically, C_{60}HyFn water molecules are highly hydrophilic [14]. The positive effects of C_{60}HyFn have been proven to be mainly determined by the unique bio-antioxidant properties that occur unexpectedly, even at super small concentrations and doses [15, 16]. To our knowledge, to date, there are two scientific studies about the in vitro effect of C_{60}HyFn on the quality of semen exposed to low temperature. Although, the positive effects of different doses of C_{60}HyFn on human [17] and boar [18] semen were observed in those studies, there is no evidence related to its effect on ram semen incubated in low temperature. That's why this study was conducted to determine the effect of different doses of C_{60}HyFn on ram semen during cool-storage at 5 °C by examining the changes in motility and kinematic parameters, membrane integrity, vitality, and morphology of spermatozoa.

2. Materials and methods

2.1. Chemicals

C_{60}HyFn was purchased from IPAC Co. (FWS 144 mg / L = 200 µM, 10 mL, Kharkiv, Ukraine). Penicillin (penicillin G potassium, 1.000.000 IU) and streptomycin (crystallized streptomycin sulphate, 1 g) were supplied from I.E. Ulagay Co. (İstanbul, Turkey). Diff-Quick rapid staining set was purchased from Gündüz-Kimya Co. (İstanbul, Turkey). Tris (hydroxymetyl) aminomethane, fructose, glucose, citric acid, eosin, nigrosine, and sodium citrate were supplied from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals, semen collection and dilution

The study protocol was approved by Fırat University Animal Experimentations Local Ethics Committee (Protocol No: 2016 / 149). In the study, 7 Akkaraman breed rams that are free of systemic and genital organ diseases and at the age of 1.5 years were used as animal material. The animals were housed in Fırat University Animal Hospital Hospitalisation Unit during the study. Before and during the study, the rams were fed with concentrated food (350 g / animal / day), hay (1 kg / animal / day), crushed barley (350 g / animal / day), and dried grape (100 g / animal / day), and drinking water was given ad libitum.

A total of 3 ejaculates were taken from each ram for 2 weeks by artificial vagina during nonbreeding season (April); so 3 repetitions were performed. Ejaculates having good initial motility (> 70 %) and concentration (> 2 billion / mL) were evaluated. On the day of semen collection, C_{60}HyFn stock solutions were prepared by diluting it with distilled water to obtain 14 different C_{60}HyFn dose groups. In addition, 100 mL tris + egg yolk based extender [297.58 mM tris (hydroxymetyl) aminomethane + 96.32 mM citric acid + 82.66 mM fructose + 100.000 IU penicillin + 100 mg streptomycin + 15 mL egg yolk + enough distilled water to complete to 100 mL] [19] was also prepared, and the eggs were purchased from Fırat University, Agriculture and Livestock Research Centre. The ejaculates collected from each ram were mixed at 37 °C. Pooled ejaculates were divided into 15 equal volumes and diluted with tris + egg yolk extender containing 400 million motile spermatozoa per mL extender at 37 °C being 14 different C_{60}HyFn groups (25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, 1 µM, 5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM) and control group (not containing C_{60}HyFn), they were then placed in a cooling cabinet at 5 °C. After the temperature of the diluted semen samples was cooled to 5 °C, the samples were stored at this temperature and spermatological examinations were performed at 0, 12, 24, 48, 72, 96, 120, and 144th h.

2.3. Spermatological examinations

2.3.1. Motility and kinematic parameters

Motility and kinematic parameters were analysed by computer assisted sperm analyser (CASA, ISASv1, Proiser, Spain). Cooled semen samples were further diluted with tris buffer solution [tris (hydroxymethyl) aminomethane 0.3 M + glucose 0.027 M + citric acid + 0.1 M + distilled water 100 mL] in an Eppendorf tube at 37 °C. For the analysis, 3.5 µL of extra diluted samples with tris buffer were dropped onto a special slide (Spermtrack 20 µm) placed on the heated stage of the phase-contrast microscope microscope kelimesinden sonra (Nikon, Tokyo, Japan) connected to the CASA system. Total, progressive, rapid, medium, and slow motility ratios (%), static spermatozoon ratio (%) and values belonging to kinematic parameters [VCL: curvilinear velocity (µm / s); VSL: straight line velocity (µm / s); VAP: average path velocity (µm / s); LIN: linearity index (%); STR: straightness index (%), WOB: wobble (oscillation) index (%); ALH: average amplitude of lateral head moving (µm); BCF: beat cross frequency (Hz)] were recorded. The speed range of the CASA, which was adjusted for rams by the manufacturer, was static < 10 µm

/ s < slow < 45 μm / s < medium < 75 μm / s < rapid. The particle size range of the CASA was 15–70 μm.

2.3.2. Membrane intactness (Hypo-osmotic swelling, HOS) ratio

One hundred μL of the cooled semen samples were further extended with 400 μL of tris buffer solution at 37 °C. Then, 50 μL were taken from the mixture of tris buffer-cooled semen samples and mixed with 500 μL of hypotonic solution (0.49 g citric acid + 0.9 g fructose + 100 mL distilled water) and incubated in an incubator at 37 °C for 60 min. After incubation, 30 μL were taken from the mixture and 200 spermatozoa were examined at 40× magnification of the phase-contrast microscope. The ratio of intact spermatozoa with swollen and curved tail was expressed as a percentage [20].

2.3.3. Morphological and acrosomal damage ratio

Smears were done using 75 microliter of tris buffer-cooled semen mixture prepared for HOS test, and they were dried in air for 5-10 min. Commercial Diff - Quick rapid staining set was used for staining. For this purpose, as recommended by the manufacturer, the smears were stained by immersing them to A, B, and C solutions of the staining set for 10 s, 6 s and 6 s, respectively. The stained smears were washed with distilled water for approximately one minute and air dried. The smears were then examined using 60 or 100× magnification of the phase-contrast microscope. A total of 200 spermatozoa were examined in each smear and the ratio of spermatozoa having both abnormal shape (head, tail, and total) and acrosomal damage was expressed as percentage.

2.3.4. Dead spermatozoon ratio

Fifty μL of the cooled semen samples were further extended with 50 μL of tris buffer solution at 37 °C. 50 μL of tris buffer-cooled semen mixture were taken and dropped onto the heated slide at 37 °C. Then, 3 drops of eosin-nigrosin dye (1.67 % eosin + 10 % nigrosine + 0.1 M sodium citrate + 100 mL distilled water) were placed on the mixture and mixed. Smears were prepared from tris buffer-cooled semen staining solution and dried for 5–10 s. The smears were examined using 40× magnification of the phase-contrast microscope. A total of 200 spermatozoa were examined in each smear and the spermatozoa of which heads with pink colour were considered dead. The dead spermatozoon ratio was expressed as percentage [21].

2.4. Statistical analysis

All statistical analyses were performed using SPSS (Version 22.0, Inc, Chicago, IL, USA). Values are presented as mean ± standard error of means (SEM). Two-way analyses of variance with Bonferroni correction for repeated measurements was used to determine whether there is an interaction between time periods (8 time periods) and groups (15 groups) in terms of spermatological examinations. After the determination of an interaction, nonparametric Wilcoxon test with Bonferroni correction to compare time points in each group and nonparametric Mann–Whitney U test with Bonferroni correction to compare groups in each time point were then used. In order not to decrease the power in the repeated measurements with Bonferroni correction, α value (significance level) was taken to be 0.10. The number of comparisons (k) for the difference between the groups in the same time point was determined as 8 because the groups were compared for each of the 8 different time points. Therefore, the significance level for group comparisons in the same time point was set at $P < 0.0125$ (0.10 / 8 = 0.0125). Similarly, the number of dual comparisons (k) for the difference between time points in each group was calculated to be 28 (0 vs 12, 0 vs 24, 0 vs 48, 0 vs 72, 0 vs 96, 0 vs 120, 0 vs 144, 12 vs 24…….. and 120 vs 144). That's why the significance level for time point comparisons in the same group was set at $P < 0.00357$ (0.10 / 28 = 0.00357). However, since the significance level less than 0.01 is not desired in such tests, $P < 0.01$ was considered to be significant for time point comparisons in the same group [22].

3. Results

The data in different time points for each motility parameter (total, progressive, rapid, medium, slow, and static), each kinematic parameter (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) and each of other spermatological parameters (dead, head anomaly, tail anomaly, total anomaly, acrosomal damage, and HOS) were not presented to reduce table numbers, but the findings associated with them were presented only as text. Since the effectiveness of any substance in in vitro incubation is generally evaluated by considering the data to be obtained at the end of the storage period, only the data at 144th h for each parameter was presented as tables in this study.

3.1. Motility parameters

With respect to total, progressive, rapid, medium, slow motility, and static spermatozoon ratios at different time points (data not shown), the time-dependent regular decreases in total, progressive, and rapid motility ratios and also regular increases in static spermatozoon ratio were detected between time points in each group ($P < 0.01$). However, in medium and slow motility ratios, the time-dependent regular increases and/or decreases were not determined.

The data only at 144th h belonging to motility parameters are presented in Table 1. Compared to the control, although there were no statistically significant differences between the groups, 100 nM, 200 nM, 400 nM, 800 nM, 1 μM, 5 μM, 10 μM, 20 μM, and 40 μM C_{60}HyFn doses had numerically higher total and progressive motility ratios along with numerically lower static

Table 1. The spermatozoon motility parameters belonging to control and hydrated C_{60} fullerene (C_{60}HyFn)-supplemented ram semen at the end of short-storage period (144th h) at 5 °C.

Group	Motility parameters					
	Total (%)	Progressive (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
Control	52.61 ± 3.34	15.37 ± 0.92	25.61 ± 4.88^{B}	14.76 ± 4.04	12.24 ± 1.51^{AB}	47.39 ± 3.34
25 nM	47.51 ± 3.31	14.24 ± 2.74	17.48 ± 3.32^{AB}	18.33 ± 3.68	11.72 ± 1.62^{AB}	52.49 ± 3.31
50 nM	50.18 ± 2.88	14.89 ± 2.76	21.09 ± 4.40^{AB}	16.01 ± 2.76	13.08 ± 2.45^{AB}	49.82 ± 2.88
100 nM	54.72 ± 4.48	19.42 ± 2.67	18.30 ± 3.39^{AB}	25.17 ± 4.14	11.25 ± 1.26^{AB}	45.28 ± 4.47
200 nM	55.51 ± 4.68	18.86 ± 3.05	22.03 ± 6.79^{B}	17.87 ± 2.36	15.61 ± 1.93^{ABC}	44.49 ± 4.68
400 nM	56.18 ± 4.46	18.34 ± 2.09	30.77 ± 3.77^{B}	15.00 ± 1.97	10.41 ± 1.70^{A}	43.82 ± 4.46
800 nM	61.34 ± 3.69	20.63 ± 1.70	21.49 ± 4.67^{B}	25.42 ± 4.95	14.43 ± 1.57^{ABC}	38.66 ± 3.69
1 µM	52.72 ± 5.47	17.16 ± 3.12	21.96 ± 4.56^{B}	20.99 ± 2.55	9.77 ± 1.36^{A}	47.28 ± 5.47
5 µM	56.22 ± 3.25	18.40 ± 2.47	26.89 ± 3.33^{B}	17.69 ± 1.42	11.63 ± 1.74^{AB}	43.78 ± 3.25
10 µM	59.50 ± 2.46	18.71 ± 1.36	25.83 ± 5.42^{B}	21.80 ± 3.14	11.87 ± 1.79^{AB}	40.50 ± 2.46
20 µM	53.16 ± 3.04	17.76 ± 1.38	21.83 ± 4.90^{B}	18.41 ± 3.79	12.91 ± 3.16^{AB}	46.84 ± 3.04
40 µM	59.23 ± 2.43	20.38 ± 2.28	26.08 ± 6.88^{B}	19.80 ± 5.93	13.35 ± 3.01^{AB}	40.77 ± 2.43
60 µM	45.25 ± 5.07	14.23 ± 4.45	14.17 ± 5.70^{AB}	12.35 ± 3.02	18.73 ± 3.56^{BCD}	54.75 ± 5.07
80 µM	41.03 ± 5.70	8.40 ± 2.71	3.90 ± 1.39^{A}	13.55 ± 4.60	23.58 ± 2.27^{D}	58.97 ± 5.70
100 µM	51.07 ± 4.55	13.61 ± 4.50	17.70 ± 6.84^{AB}	12.58 ± 3.23	20.80 ± 4.40^{CD}	48.93 ± 4.55

The letters (A, B, C, D) in the same column show significant ($P < 0.0125$) differences between the groups.

spermatozoon ratio. On the other hand, 25 nM, 50 nM, 60 µM, 80 µM, and 100 µM doses had numerically lower total and progressive motility ratios along with numerically higher static spermatozoon ratio. However, these doses couldn't show the same effects, which were observed in other motility parameters, on rapid, medium, and slow motility ratios. In other words, some of the doses between 100 nM and 40 µM increased the rapid, medium, or slow motility ratios though some of them decreased. While only 80 µM C_{60}HyFn caused a statistically significant ($P < 0.0125$) decrease in rapid motility ratio, 80 µM and 100 µM doses led to statistically significant ($P < 0.0125$) increase in slow motility ratio.

3.2. Kinematic parameters

With regard to VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF values at different time points (data not shown), VCL, VSL, VAP values at each time points up to 72nd h and after 72nd h in almost all groups were determined to be ~ 100–120 and ~ 80 - 100 µm / s, ~ 50–60 and ~ 35–50 µm / s, ~ 80–100 and ~ 50–65 µm / s, respectively and statistical differences ($P < 0.01$) were found between some time points in the same group with regard to these parameters. A tendency to decrease was observed in VCL, VSL, and VAP values after 72nd h. Besides, the changes regardless of time were observed in the same group in terms of LIN, STR, WOB, ALH, and BCF values. While these changes observed in STR and WOB values between some time points in each group reached statistical significance ($P < 0.01$) but not in LIN, ALH, and BCF values.

The data only at 144th h belonging to kinematic parameters are shown in Table 2. Although there were significant ($P < 0.0125$) differences in VCL values between the groups, any dose of C_{60}HyFn didn't affect VCL values significantly compared to the control. Only 80 µM C_{60}HyFn addition to semen caused significant ($P < 0.0125$) diminution in VAP values in comparison with the control. The changes observed in other kinematic parameters between the groups were statistically insignificant. However, as in total, progressive motility and static spermatozoon parameters, regular dose-dependent increments/decrements were not detected.

3.3. Other spermatological parameters

In terms of dead, head abnormality, tail abnormality, total abnormality, acrosomal damage, and HOS ratios at different time points (data not shown), the time-dependent significant ($P < 0.01$) enhancements in dead spermatozoon ratio and time-dependent significant ($P < 0.01$) reductions in HOS ratio were determined in each group. With respect to other parameters, the changes regardless of time in the same group were not statistically significant.

Table 2. The kinematic parameters belonging to control and hydrated C_{60} fullerene (C_{60}HyFn)-supplemented ram semen at the end of short-storage period (144th h) at 5 °C.

Group	Kinematic parameters							
	VCL (μm / s)	VSL (μm / s)	VAP (μm / s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Control	91.11 ± 5.16^{ABC}	41.20 ± 1.95	66.29 ± 4.72^{B}	45.49 ± 1.62	62.89 ± 2.32	72.53 ± 1.89	3.00 ± 0.10	9.43 ± 0.48
25 nM	94.69 ± 7.47^{ABC}	40.52 ± 2.16	60.20 ± 3.26^{B}	43.98 ± 2.78	68.08 ± 3.65	65.07 ± 3.30	3.27 ± 0.23	10.22 ± 0.55
50 nM	100.58 ± 5.56^{AC}	46.12 ± 4.46	73.47 ± 5.80^{B}	45.22 ± 2.45	62.44 ± 2.85	72.48 ± 2.29	3.18 ± 0.21	11.07 ± 0.58
100 nM	76.17 ± 4.75^{B}	37.52 ± 3.72	55.04 ± 4.63^{AB}	48.62 ± 2.45	67.70 ± 1.87	71.54 ± 1.95	2.70 ± 0.16	10.17 ± 0.53
200 nM	90.40 ± 6.59^{ABC}	44.01 ± 4.90	63.06 ± 6.27^{B}	48.00 ± 2.53	69.52 ± 2.31	69.09 ± 2.79	3.59 ± 0.27	10.44 ± 0.47
400 nM	107.97 ± 6.98^{AC}	46.06 ± 2.25	72.32 ± 3.22^{B}	43.82 ± 2.89	63.84 ± 1.77	68.42 ± 3.74	3.51 ± 0.24	9.88 ± 0.41
800 nM	89.79 ± 3.85^{ABC}	42.80 ± 2.46	62.67 ± 3.90^{B}	49.21 ± 3.01	69.12 ± 2.95	69.37 ± 2.55	2.92 ± 0.08	9.92 ± 0.46
1 μM	88.18 ± 4.24^{ABC}	42.12 ± 3.46	65.47 ± 4.15^{B}	47.38 ± 1.95	64.18 ± 2.11	73.87 ± 1.93	2.89 ± 0.09	10.70 ± 0.64
5 μM	98.24 ± 3.06^{C}	41.78 ± 2.80	66.37 ± 3.49^{B}	42.53 ± 2.48	62.94 ± 2.53	67.86 ± 3.58	3.17 ± 0.17	9.89 ± 0.53
10 μM	95.36 ± 7.96^{ABC}	41.83 ± 6.43	64.86 ± 8.93^{B}	41.99 ± 3.51	63.89 ± 2.36	65.54 ± 4.62	2.98 ± 0.20	11.49 ± 0.83
20 μM	100.88 ± 8.33^{ABC}	40.31 ± 5.73	65.34 ± 8.39^{B}	39.07 ± 3.17	61.48 ± 2.68	62.12 ± 3.70	3.42 ± 0.24	11.013 ± 0.60
40 μM	104.27 ± 11.06^{ABC}	50.48 ± 6.52	76.05 ± 10.19^{B}	47.95 ± 1.71	66.97 ± 2.55	71.83 ± 2.45	3.55 ± 0.38	10.53 ± 0.82
60 μM	81.75 ± 5.35^{AB}	33.58 ± 5.38	51.42 ± 7.64^{B}	40.92 ± 5.68	65.92 ± 4.70	62.20 ± 7.67	3.00 ± 0.28	10.08 ± 1.20
80 μM	74.12 ± 4.82^{B}	27.12 ± 3.77	39.35 ± 4.91^{A}	35.85 ± 2.85	68.75 ± 2.37	52.05 ± 3.33	2.68 ± 0.40	9.63 ± 1.91
100 μM	87.42 ± 8.12^{ABC}	34.79 ± 6.79	52.26 ± 9.13^{AB}	38.89 ± 5.20	66.52 ± 3.89	57.43 ± 6.21	3.17 ± 0.21	11.11 ± 1.01

The letters (A, B, C) in the same column show significant ($P < 0.0125$) differences between the groups.

The data only at 144th h belonging to other spermatological parameters are given in Table 3. When compared with the control values, statistically significant diminutions in dead (only at 400 and 800 nM groups) and tail abnormality (only at 200 nM, 800 nM and 40 μM groups) values but statistically significant increment (only at 400 nM group) and decrement (only at 80 μM group) in HOS value were detected in C_{60}HyFn groups ($P < 0.0125$). Moreover, like in total, progressive motility and static spermatozoon parameters, while all doses between 100 nM and 40 μM provided numerical (some of them is statistical) decreases in dead spermatozoon ratio and in tail and total abnormal spermatozoon ratios as well as increases in HOS ratio; however, 25 nM, 50 nM, 60 μM, 80 μM, and 100 μM doses had opposite effects in comparison to the control group.

4. Discussion

Spermatozoon plasma membrane is the primary target for cryopreservation damage [23]. Temperature change occurring through cool-storage and freeze-thawing processes entails physical and chemical stress in mammalian spermatozoa, causing changes in the lipid composition of plasma membrane [24, 25]. Ram spermatozoa are more sensitive to cold-shock damage than those of bull, rabbit and even human [6]. The low cholesterol/ phospholipid ratio and high unsaturated fatty acid level in the spermatozoon plasma membrane of rams are shown to be possible reason for this sensitivity [5]. The most important biochemical reaction occurring through cool-storage of semen is peroxidation that is formed in lipids due to an increase in ROS levels [2,3]. Unsaturated fatty acids are the most exposed molecules to ROS attacks, and ram spermatozoa are extremely sensitive to increased ROS-induced LPO damage during cool-storage, as they are rich in unsaturated fatty acids [2,4]. On the other hand, not only the ROS but also the diluent used in storing the semen in liquid and frozen forms was effective on the damages and it was reported that the diluents that gave the least damage to the ram spermatozoa are tris + egg yolk and tris + soy lecithin [8]. To minimize the diluent effect, tris + egg yolk extender was used in this study. Besides, since the effect of any substance on the short-term storage of semen is mostly evaluated considering the data at the end of the storage period, the data obtained in the 144th h in this study were taken into consideration in the discussion section.

Since antioxidants have effective roles in preventing damages caused by increased ROS levels [2,4], it has been reported that different antioxidant substances [3, 26-28]

Table 3. Some spermatological parameters belonging to control and hydrated C_{60} fullerene (C_{60}HyFn)-supplemented ram semen at the end of short-storage period (144th h) at 5 °C.

Group	Spermatological parameters					
	Dead spermatozoon ratio (%)	Abnormal spermatozoon ratio (%)			Damaged acrosome ratio (%)	HOS (%)
		Head	Tail	Total		
Control	22.29 ± 1.02^{A}	4.58 ± 0.26	3.33 ± 0.50^{B}	7.91 ± 0.44^{AB}	2.13 ± 0.32	31.00 ± 2.48^{BC}
25 nM	23.00 ± 2.66^{A}	4.75 ± 0.54	3.50 ± 0.43^{AB}	8.25 ± 0.85^{AB}	1.00 ± 0.29	30.33 ± 4.50^{BC}
50 nM	23.00 ± 1.76^{A}	4.67 ± 0.48	3.50 ± 0.81^{AB}	8.17 ± 0.90^{AB}	1.00 ± 0.22	28.50 ± 0.91^{CD}
100 nM	18.50 ± 1.65^{AB}	3.17 ± 0.94	2.25 ± 0.54^{AB}	5.42 ± 1.25^{A}	1.67 ± 0.25	35.50 ± 0.18^{B}
200 nM	18.25 ± 2.04^{AB}	3.92 ± 0.49	1.58 ± 0.20^{A}	5.50 ± 0.67^{A}	1.17 ± 0.31	37.33 ± 2.16^{AB}
400 nM	16.33 ± 1.22^{B}	3.67 ± 0.42	2.75 ± 0.70^{AB}	6.42 ± 0.89^{AB}	1.50 ± 0.72	41.17 ± 3.27^{A}
800 nM	16.25 ± 0.98^{B}	4.33 ± 0.84	2.08 ± 0.20^{A}	6.41 ± 1.02^{AB}	1.17 ± 0.28	39.50 ± 4.29^{AB}
1 µM	19.00 ± 0.90^{AB}	4.40 ± 0.81	2.80 ± 0.41^{AB}	7.20 ± 1.08^{AB}	0.80 ± 0.37	36.17 ± 4.93^{AB}
5 µM	18.92 ± 1.70^{AB}	4.67 ± 0.76	2.08 ± 0.49^{AB}	6.75 ± 0.87^{AB}	1.33 ± 0.44	35.50 ± 3.93^{B}
10 µM	19.58 ± 0.95^{AB}	4.00 ± 0.86	2.00 ± 0.63^{AB}	6.00 ± 0.93^{AB}	1.75 ± 0.17	32.83 ± 3.01^{BC}
20 µM	19.58 ± 1.72^{AB}	4.08 ± 0.78	2.33 ± 0.21^{A}	6.41 ± 0.93^{AB}	1.33 ± 0.36	35.17 ± 4.66^{BC}
40 µM	19.75 ± 2.61^{AB}	4.42 ± 0.82	3.13 ± 0.52^{AB}	7.25 ± 1.11^{AB}	1.25 ± 0.52	32.25 ± 3.56^{BC}
60 µM	24.00 ± 1.84^{A}	4.00 ± 0.50	5.00 ± 3.00^{AB}	9.50 ± 1.12^{B}	1.75 ± 0.11	28.67 ± 1.52^{C}
80 µM	23.50 ± 2.01^{A}	6.25 ± 1.31	3.50 ± 0.95^{AB}	9.75 ± 1.55^{B}	1.38 ± 0.62	24.83 ± 1.70^{D}
100 µM	25.67 ± 2.18^{A}	4.33 ± 0.33	4.42 ± 0.42^{B}	8.75 ± 0.54^{B}	1.50 ± 0.45	28.00 ± 4.00^{CD}

The letters (A, B, C, D) in the same column show significant ($P < 0.0125$) differences between the groups.

added to the diluent during the short-term storage of ram semen reduce the increased LPO and also increase the decreased total antioxidant capacity induced by cool-storage. It is known that C_{60} fullerene and its some derivatives provide effective protection against oxidative stress in vitro and in vivo without creating acute or subacute toxicity [29,30]. In the study conducted in goat epididymal spermatozoa, increments in glutathione-peroxidase, glutathione-reductase, and superoxide dismutase activities as well as decrement in LPO level in spermatozoa supplemented with 1, 10, or 100 µM fullerenol during incubation period at 32 °C for 3 h were found [31]. In addition, a current evidence has suggested that C_{60}HyFn is able to protect boar spermatozoa against ROS injuries and energy insufficiencies by preventing protein dephosphorylation via the cAMP-PKA signalling pathway during cool-storage [18]. Although oxidative stress markers were not measured in this study, our unpublished data showed that each dose of C_{60}HyFn between 100 nM and 40 µM provided significant reductions in LPO and significant increments in antioxidants such as glutathione, glutathione-peroxidase and catalase in frozen-thawed ram semen compared to the values in control. Findings of Murugan et al. [31], Li et al. [18] and our unpublished study clearly show that C_{60}HyFn has potent antioxidant effect against oxidative stress caused by liquid-storage at low temperature and freeze-thawing process in mammalian semen.

Considering the studies investigating the effects of antioxidants on spermatological parameters during the short-term-storage of ram semen at low temperature, it has been seen that the addition of argan oil to tris- and milk-based diluents provided significant increases in progressive motility, vitality, and membrane integrity for 48 h compared to control [26], and 1 mM cysteamine and 0.5 mM lycopene supplementations increased the motility for 48 and 72 h, respectively in comparison to control [3], and 5 mg bovine serum albumin addition to semen extended with tris protected motility, membrane integrity, and acrosomal structure until the end of the 72nd h versus control [27]. Likewise, at the end of 72 h storage period at 4–5 °C when compared with the their control values, it was demonstrated that trehalose (10 and 25 mM) and 4 mM cysteine are effective in the protection of motility, vitality, acrosomal structure, and mitochondrial activity [32], and curcumin (0.5 and 1 mM) and eugenol (1 mM) provided significant protection in motility, normal-shaped spermatozoa ratio, membrane intactness and

DNA integrity [33], and 50 mM trehalose addition has beneficial effect to maintain normal-shaped spermatozoa ratio at high level [34]. Moradi et al. [35] have alleged that royal jelly supplementation to diluted ram semen prevents significantly the diminutions in vitality, VSL, and the damages in membrane integrity for 120 h storage-period at low temperature by reducing reactive nitric oxide levels compared to the control. In a study performed by Dai et al. [28], the subjective motility, membrane integrity, and mitochondrial activity values of ram spermatozoa in tris + egg yolk diluent containing 0.05 or 1 mM melatonin were found to be high than those of control group at the end of the 6th day of cool-storage period. Beyond the general beneficial effects of antioxidants added to the ram semen during cool-storage, it has also been reported that supplementation of some antioxidants like vitamin E and β-mercaptoethanol could decrease the progressive motility and vitality [36], L-arginine (0.1, 0.5, 1 and 5 mM) [37], 50 mM taurine, 5 mM glutathione [38], and 0.6 mM butylated hydroxytoluene [34] have no effects on parameters such as motility, vitality, and abnormality. In this study, at the end of 144 h storage-period, when compared with the control, although each of C_{60}HyFn doses used between 100 nM and 40 μM had slightly positive effects on total and progressive motility, the ratios of spermatozoa with static, dead, tail abnormality, and total abnormal spermatozoon ratio and membrane integrity; however, 25 and 50 nM doses had no effect and 60 μM and higher doses had slightly negative effects on these parameters. Moreover, the effects of some of these doses on some parameters were also found statistically significant. However, in terms of kinematic and other analysed parameters, these doses couldn't show the same effects as mentioned above. In other words, some of the doses between 100 nM and 40 μM had no positive effects though some of the doses between 60 and 100 μM had no negative effects. In accordance with our results, in a study, where 10, 20, 30, 40, 100, or 200 μg / mL C_{60}HyFn were added to native human semen and left incubation for 1–3 h at room temperature, it was determined that the doses of 10 and 20 μg / mL provided significant increases in spermatozoon motility but not spermatozoon DNA and membrane integrity, and 40 μg / mL and higher doses decreased motility and damaged spermatozoon DNA and membrane integrity compared to control [17]. Similarly, in a recent study [18], it was observed that the addition of 1, 2, 3, or 4 μg / mL C_{60}HyFn to boar semen stored at 4 °C resulted in higher motility, acrosome integrity, and mitochondrial activity at the end of the 10th day. The main underlying mechanism for the positive effect of C_{60}HyFn through short-term storage of ram semen is strongly related to the prevention of peroxidation of lipids in the spermatozoon plasma membrane thanks to reducing the increased ROS induced by cool-storage with its strong antioxidant feature. Moreover, keeping the ATP level at a high level by C_{60}HyFn may accompany the maintenance of motility during short-term storage. On the other hand, the possible hypothesis for the effect of C_{60}HyFn on reducing the ROS level may be that it shows affinity to the electrons on the surface of the spermatozoon plasma membrane [18]. Slight negative effects observed at some high doses can also be explained by an overdose.

Consequently, adding any C_{60}HyFn dose between 100 nM and 40 μM to ram semen may be beneficial to keep some spermatological parameters at high level during the short-term at 5 ˚C.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Maxwell WM, Salamon S. Liquid storage of ram semen: a review. Reproduction, Fertility and Development 1993; 5: 613-638. doi: 10.1071/rd9930613
2. Budai C, Egerszegi I, Olah J, Javor A, Kovacs A. The protective effect of antioxidants on liquid and frozen stored ram semen-review. Scientific Papers Animal Science and Biotechnology 2014; 47: 46-52.
3. Peker Akalın P, Bucak MN, Güngör Ş, Başpınar N, Çoyan K et al. Influence of lycopene and cysteamine on sperm and oxidative stress parameters during liquid storage of ram semen at 5 °C. Small Ruminant Research 2016; 137: 117-123. doi: 10.1016/j.smallrumres.2016.03.017
4. Arı UÇ, Öztürkler, Y. Oxidative stress during long and short term storage of sperm and usage of antioxidant. Turkiye Klinikleri Journal of Reproduction and Artificial Insemination-Special Topics 2015; 1: 16-21. (in Turkish with an abstract in English).
5. Scott T, Voglmayr JK, Setchell BP. Lipid composition and metabolism in testicular and ejaculated ram spermatozoa. Biochemical Journal 1967; 102: 456-461. doi: 10.1042/bj1020456
6. Watson PF. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science 2000; 60-61: 481-492. doi: 10.1016/s0378-4320(00)00099-3

7. Lopez Saez A, Ortiz N, Gallego L, Garde JJ. Liquid storage (5 °C) of ram semen in different diluents. Archives of Andrology 2000; 44: 155-164. doi: 10.1080/014850100262335

8. Maksimovic N, Milovanovic A, Barna T, Caro Petrovic V, Pantelic V et al. Short-term liquid storage of ram semen in various extenders. South African Journal of Animal Science 2018; 48: 717-723. doi: 10.4314/sajas.v48i4.13

9. Prato M. Fullerene chemistry for materials science applications. Journal of Material Chemistry 1997; 7: 1097-1109. doi: 10.1039/A700080D

10. Bosi S, Da Ros T, Spalluto G, Prato M. Fullerene derivatives: an attractive tool for biological applications. European Journal of Medicinal Chemistry 2003; 38: 913-923. doi: 10.1016/j.ejmech.2003.09.005

11. Satoh M, Takayanagi I. Pharmacological studies on fullerene C_{60}, a novel carbon allotrope, and its derivatives. Journal of Pharmacological Sciences 2006; 100: 513-518. doi: 10.1254/jphs.cpj06002x

12. Ruoff RS, Tse DS, Malhotra R, Lorents DC. Solubility of C_{60} in a variety of solvents. Journal of Physical Chemistry 1993; 97: 3379-3383. doi: 10.1021/j100115a049

13. Kolosnjaj J, Szwarc H, Moussa F. Toxicity studies of fullerenes and derivatives. In: Chan WCW (editor). Bio-applications of Nanoparticles. Advances in Experimental Medicine and Biology. New York, USA: Springer; 2007. pp. 168-80. doi: 10.1007/978-0-387-76713-0_13

14. Andrievsky GV, Kosevich MV, Vovk OM, Shelkovsky VS, Vashchenko LA. On the production of an aqueous colloidal solution of fullerenes. Journal of the Chemical Society, Chemical Communications 1995; 12: 1281-1282. doi: 10.1039/C39950001281

15. Andrievsky GV, Bruskov VI, Tykhomyrov AA, Gudkov SV. Peculiarities of the antioxidant and radioprotective effects of hydrated C_{60} fullerene nanostructures in vitro and in vivo. Free Radical Biology and Medicine 2009; 47: 786-793. doi: 10.1016/j.freeradbiomed.2009.06.016

16. Andrievsky G, Shakhnin D, Tronza A, Zhernosekov D, Tykhomyrov A. The acceleration of blood plasma clot lysis in the presence of hydrated C_{60} fullerene nanostructures in super-small concentration. Fullerenes Nanotubes and Carbon Nanostructures 2010; 18: 303-311. doi: 10.1080/15363831003785257

17. Pavlovich EV. Analysis of fullerene's action on human sperm before and after cryopreservation (in Russia). Bulletin of Problems Biology and Medicine 2014; 3: 178-182.

18. Li X, Wang L, Liu H, Fu J, Zhen L et al. C_{60} fullerenes suppress reactive oxygen species toxicity damage in boar sperm. Nano - Micro Letters 2019; 11: 104, 1-17. doi: 10.1007/s40820-019-0334-5

19. Omur AD, Coyan K. Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. Veterinarni Medicina - Czech 2016; 61: 10-16. doi: 10.17221/8677-VETMED

20. Söderquist L, Madrid-Bury N, Rodriguez-Martinez H. Assessment of ram sperm membrane integrity following different thawing procedures. Theriogenology 1997; 48: 1115-1125. doi: 10.1016/s0093-691x(97)00344-0

21. Sönmez M, Demirci E. The effect of ascorbic acid on the freezability of ram semen diluted with extenders containing different proportions of glycerol. Turkish Journal of Veterinary and Animal Sciences 2004; 28: 893-899.

22. Akgül A. Tıbbi Araştırmalarda İstatistiksel Analiz Teknikleri: SPSS Uygulamaları (in Turkish). 3rd ed. Ankara, Turkey: Emek Ofset; 2005.

23. Bailey JL, Bilodeau J - F, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. Journal of Andrology 2000; 21: 1-7. doi: 10.1002/j.1939-4640.2000.tb03268.x

24. Schiller J, Arnhold J, Glander HJ, Arnold K. Lipid analysis of human spermatozoa and seminal plasma by MALDI-TOF mass spectrometry and NMR spectroscopy - effects of freezing and thawing. Chemistry and Physics of Lipids 2000; 106: 145-156. doi: 10.1016/s0009-3084(00)00148-1

25. Perez - Pe R, Grasa P, Fernandez-Juan M, Peleato ML, Cebrian-Perez JA et al. Seminal plasma proteins reduce protein tyrosine phosphorylation in the plasma membrane of cold-shocked ram spermatozoa. Molecular Reproduction and Development 2002; 61: 226-233. doi: 10.1002/mrd.1152

26. Allai L, Druart X, Contell J, Louanjli N, Moula AB et al. Effect of argan oil on liquid storage of ram semen in Tris or skim milk based extenders. Animal Reproduction Science 2015; 160: 57-67. doi: 10.1016/j.anireprosci.2015.07.003

27. Gökçe E, Alçay S, Gül Z. Positive effect of BSA supplemented soybean lecithin based extender on liquid storage of ram semen at 5 °C. Veterinary Journal of Ankara University 2017; 64: 313-320.

28. Dai GC, Meng Y, Zhang LK, Du YQ, Wen F et al. Effect of addition of melatonin on liquid storage of ram semen at 4 °C. Andrologia 2019; 51: e13236. doi: 10.1111/and.13236

29. Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR et al. Fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. Nano Letters 2005; 5: 2578-2585. doi: 10.1021/nl051866b

30. Beuerle F, Lebovitz R, Hirsch A. Antioxidant properties of water-soluble fullerene derivatives. In: Cataldo F, Da Ros T (editors). Medicinal Chemistry and Pharmacological Potential of Fullerene Carbon Nanotubes. Carbon Materials. Chemistry and Physics. Dordrecht, Netherlands: Springer; 2008. pp. 51-78. doi: 10.1007/978-1-4020-6845-4_3

31. Murugan MA, Gangadharan B, Mathur PP. Antioxidative effect of fullerenol on goat epididymal spermatozoa. Asian Journal of Andrology 2002; 4: 149-152.

32. Gungor S, Ozturk C, Omur AD. Positive effects of trehalose and cysteine on ram sperm parameters. Veterinarni Medicina-Czech 2017; 62: 245-252. doi: 10.17221/131/2016-VETMED

33. Avdatek F, Yeni D, Gündoğan M. The effects of antioxidants supplementation on spermatological parameters and DNA damage in liquid storage process in Merinos ram semen (in Turkish with an English abstract). Kocatepe Veterinary Journal 2018; 11: 126-133. doi: 10.30607/kvj.376568

34. Ceylan A, Aksoy M, Serin İ, Küçük N, Uçan U et al. In vitro and in vivo evaluation of Kıvırcık ram semen stored short-term with different antioxidants. Animal Health, Production and Hygiene 2013; 2: 216-220. (in Turkish with an English abstract).

35. Moradi AR, Malekinejad H, Farrokhi-Ardabili F, Bernousi I. Royal Jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small Ruminant Research 2013; 113: 346-352. doi: 10.1016/j.smallrumres.2013.03.003

36. Soltanpour F, Moghaddam G, Asadpour R, Rafat SA. Effect of antioxidant combinations on sperm quality of cross breed rams during liquid storage. International Journal of Advanced Biological and Biomedical Research 2014; 2: 732-740.

37. Özer Kaya Ş, Gür S. Effect of L-arginine addition on short term storability of ram semen. Fırat University Veterinary Journal of Health Sciences 2017; 31: 73-79. (in Turkish with an English abstract).

38. Bucak MN, Tekin N, Kulaksız R. Effect of antioxidants on the liquid storage of ram semen. Journal of Lalahan Livestock Research Institute 2007; 47: 15-21. (in Turkish with an English abstract).