ATTENUATIVE ROLE OF IDEBENONE ON DELTAMETHRIN MEDIATED PEROXIDATIVE TOXICITY OF RAM SEMEN STORED AT 4°C*

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Abstract

The current study was conducted to evaluate the different concentrations of deltamethrin (DEL; Exp. 1) and the protective role of idebenone (IDB) supplemented with toxic dose of deltamethrin (Exp. 2) during chilled storage of ram semen. Collected samples were pooled and diluted at 500×10^6 spermatozoa per mL. In Exp. 1, effect of DEL at 1, 2.5, 5, 10, 20, 40, 80 μM levels was evaluated on different variables of spermatozoa motion characteristics. In Exp. 2, different amounts of IDB (2, 4, and 8 μM) concurrent with constant doses of DEL (10 μM) were examined on semen quality upon chilled preservation up to 72 h. Indices of spermatozoa kinematics, functionality of plasma membrane and viability were recorded. Biochemical metabolites were measured in spermatozoa and its medium (extender) at different time points. In Exp. 1, different parameters of spermatozoa kinematics were affected by exposure to DEL in a dose dependent manner. In Exp. 2, combination of IDB with DEL resulted in a significant ($P < 0.05$) increase in total motility, forward progressive motility and curvilinear path velocity compared to DEL group at 24, 48 and 72 h. Simultaneous administration of IDB with DEL increased the percentage of spermatozoa with functional membrane and viability compared to DEL group at 72 h ($P < 0.05$). The amounts of lipid peroxidation index were lower in medium of combination groups compared to DEL group at 48 h and 72 h ($P < 0.05$). Antioxidant capacity was higher in spermatozoa and medium of IDB treated groups compared to DEL group at 72 h ($P < 0.05$). Amounts of total nitrate-nitrite and superoxide dismutase activity of spermatozoa and medium were not affected by treatment ($P > 0.05$). In conclusion, IDB could ameliorate oxidative and peroxidative damages induced by DEL mild toxicity upon cold preservation of ram semen.

Key words: deltamethrin, idebenone, ram spermatozoa, lipid peroxidation

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Ram spermatozoa is very susceptible to produced redox-active oxygen species during cooling, freezing and thawing process. Different molecules of reactive oxygen species (ROS) such as hydroxyl radical, superoxide and hydroperoxides showed high potential redox-active oxygen (Buhr et al., 1994). An excess formation or production of ROS molecules in spermatozoa (during liquid or freeze preservation), alter the structure of macromolecules, especially lipids, by oxidative toxicity process (Yeste, 2016). Maintenance of the normal spermatozoa function is a mandatory option in short or long term semen storage. Then, control of the redox balance is very important and necessary for maintaining the spermatozoa function during semen preservation (Holt, 2000).

The structure of all quinones consists of a cyclic dione structure. Idebenone (IDB) is a synthetic molecule and is very similar to CoQ10 in chemical structure. IDB is more hydrophilic than CoQ10. It was documented that IDB reacts with different reactive oxygen and nitrogen species and reduced the harmful effects of them (Cardoso et al., 1998; Mordente et al., 1998; Erb et al., 2012). In addition, protective role of IDB against oxidative stress during liquid-chilled and cryopreserved storage of ram spermatozoa was reported (Eslami et al., 2019; Lone et al., 2019).

Deltamethrin (DEL) belongs to the type II pyrethroid family and has been used in veterinary and agriculture fields (Forshaw et al., 2000). Detrimental effects of DEL on reproductive organs of male rats were reported (Andrade et al., 2002). Moreover, reduced spermatozoa quantity and quality, as well as libido and testosterone levels of male mice were documented following oral administration of DEL (Ben Slima et al., 2017). Previous study revealed that bioactive substances such as piperine and curcumin could ameliorate cytotoxic effect of DEL by inhibiting the apoptogenic signaling pathway in mice model (Kumar et al., 2018). Moreover, protective role of quercetin against induced toxicity by deltamethrin on reproductive system of male rats was reported (Sharma et al., 2018). The first aim of the present study was to investigate the effect of different levels of DEL on ram semen kinematics during storage at 4°C. After choosing the mild toxic dose of DEL, the second objective was to assess the probable protective role of IDB against DEL-induced toxicity on different parameters of chilled stored semen.

Material and methods

The procedure of semen collection via the artificial vagina, performed in the present study, was approved by the Animal Care Committee of the Urmia University (IR-UU-AEC-3/TDT/1818).

Chemicals

Tris hydroxymethyl amino methane, citric acid, fructose, ammonium iron (II) sulfate hexahydrate, trichloroacetic acid, thiobarbituric acid, hydrochloric acid, acetic acid, hydrogen peroxide, sodium benzoate, n-naphtyl, ethylene-diamine-tetra-acetic acid were purchased from Merck Company (Darmstadt, Germany). Eosin,

nigrosine, pyrogallol, sulfonyl amide, deltamethrin (D9315, Sigma-Aldrich) and idebenone (67805, Sigma-Aldrich) were procured from Sigma-Aldrich Company, and penicillin and streptomycin were bought from Jaber Ibn Hayyann Pharmaceutical Company (Tehran, Iran).

Animals and their management for semen collection

Five males and two females of Qezel sheep, four years old, were used in the present study. The animals belonged to the Agriculture Faculty of Urmia University, West Azerbaijan, Iran, and kept under uniform nutritional and management conditions. The study was carried out during breeding season. Ejaculates collected by artificial vagina (twice a week) were immediately transferred to the laboratory and immersed in a water bath at 37°C. Only ejaculates with forward progressive motile spermatozoa greater than 70% and spermatozoa concentration higher than 2.5×10^9 mL were used in the study.

Semen processing and treatment

Ejaculates were diluted with a proposed medium (Evans and Maxwell, 1987) for ram (Tris 36.3 g/L, fructose 5 g/L, citric acid 19.9 g/L, egg yolk 14 mL/100 mL, 1000,000 IU penicillin and 1000 mg streptomycin/L) to achieve a concentration of 0.5×10^9 spermatozoa/mL for the purposes of the present study. In order to eliminate individual differences, equal volume of each diluted ejaculates was pooled. For each experiment, nine triplicate replications were carried out each time for every group.

Experiment 1

After collection and dilution, pooled semen samples were exposed to different concentrations of DEL (1, 2.5, 5, 10, 20, 40, 80 μ M). Kinematics of spermatozoa were assessed by computer assisted sperm analysis (CASA; Test Sperm 2.1; Videotest, St. Petersburg, Russia) system at 0, 24, 48 and 72 h after storage at refrigerator temperature (4°C).

Experiment 2

The pooled semen sample was divided into six aliquots and treated with dimethyl sulfoxide (DMSO; solvent of DEL and IDB, as control group), DEL alone (10 μ M) or combined with 2, 4, or 8 μ M of IDB. Moreover, an untreated group served as control (negative control group). All treated groups, except the negative control group, received the equal volume of DMSO alone such as control group (10 μ L in 1000 μ L semen sample), or DEL treated group (10 μ L in 1000 μ L semen sample: 5 μ L DEL dissolved in DMSO + 5 μ L DMSO alone) or in combination groups (10 μ L in 1000 μ L semen sample: 5 μ L DEL dissolved in DMSO + 5 μ L IDB dissolved in DMSO). The stock solution of 2 mM IDB was prepared by dissolving it in DMSO and stored at freeze temperature until usage. For 8 μ M group, 4 μ L of stock solution + 1 μ L DMSO alone was used in 1000 μ L sample (final volume 5 μ L), and for 4 μ L group, 2 μ L of stock solution + 3 μ L DMSO alone was used. Moreover, for 2 μ M group, 1 μ L of stock solution + 4 μ L DMSO alone was used. The volume of DMSO was 1% of diluted sample in treated groups. The final concentration of spermato-

zoa was set at 0.5×10^9 spermatozoa/mL. Treated samples were gradually cooled to 4°C and kept at this temperature for up to 72 h. Motility parameters (evaluated by CASA), membrane functionality and viability were evaluated at 0 h, 24 h, 48 h and 72 h intervals. Moreover, the extracellular (medium) and intracellular (spermatozoa) redox status was evaluated by measurement of superoxide dismutase activity, total antioxidant capacity (TAC), total nitrite/nitrate values and amounts of malondialdehyde (MDA; as secondary product of lipid peroxidation index). Protein level was measured in order to express the redox related indicators based on the protein.

Assessing the microscopic parameters of spermatozoa

To evaluate the spermatozoa kinematics, the CASA (Test Sperm 2.1; Videotest, St. Petersburg, Russia) system was used. The system contains a CCD-camera (SDC-313B, Samsung Techwin Co., Gyeong, Korea) attached to the phase contrast microscope (Olympus, BX41, Tokyo, Japan) with an electronic stage warmer. Spermatozoa kinematics was evaluated by putting the portion of semen (10 µL) on a slide, covered with a coverslip (22×22 mm) and examined under magnification of 200×. Spermatozoa were diluted with same diluent of each experimental group at 25×10^6 spermatozoa/mL before initiation of kinematics evaluation by CASA system. Videotaping of spermatozoa motility was conducted at a rate of 25 frames/s. The recorded parameters were total motility (TM; %), forward progressive motility (FPM; %), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), straightness (STR; %), and linearity (LIN, %). Spermatozoa with an average path velocity >20 µm/s were considered to be motile. Spermatozoa with an average path velocity of >65 µm/s with a straightness of >65% were considered to be progressively motile.

To evaluate the membrane functionality of ram spermatozoa the hypo-osmotic swelling (HOS) test was used (Correa and Zavos, 1994). In order for that, an aliquot of treated sample (20 µL) was mixed with HOS reagent (180 µL) and incubated for one hour at 37°C. After incubation, at least two hundred spermatozoa were assessed under phase contrast microscope (200×, Olympus, BX41, Tokyo, Japan). Finally, the number of spermatozoa with straight or swollen/coiled tail were recorded.

The eosin-nigrosine staining (Evans and Maxwell, 1987) was carried out by mixing one drop of treated sample with two drops of satin solution. At least two hundred spermatozoa were counted under light microscope (Olympus, Japan) to calculate the percentage of viability. Unstained spermatozoa were considered as live.

After evaluation of microscopic parameters of spermatozoa, the medium and spermatozoa were separated by two times centrifugation (600 g for 15 min) and saved at freeze temperature (−20°C) until biochemical assay. To evaluate the biochemical parameters within spermatozoa, the sediment of spermatozoa (after centrifugation) was homogenized with a cell homogenizer (T10 Basic, IKA®, Werke GmbH & Co. KG, Staufen, Germany) for 2 min after addition of 500 µL PBS.

Measurement of biochemical indices

Malondialdehyde, the second product of lipid peroxidation, was measured according to the method published by Stern et al. (2010). In brief, the prepared reagent

was mixed with sample (medium or homogenized spermatozoa) in a glass tube and incubated for 15 minutes at boiling water. After centrifugation (2500 g, for 15 min), the optical density of upper layer was read at 535 nm with spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II). Results were shown as $\mu\text{mol/g}$ protein. The data were normalized according to the amounts of protein.

The protocol of Koracevic et al. (2001) was used to evaluate the amounts of total antioxidant capacity (TAC). In a glass tube, sample (10 μL) was mixed with 490 μL PBS, 500 μL sodium benzoate, 1000 μL acetic acid and 200 μL complex of Fe-EDTA and hydrogen peroxide, respectively. Then, tubes were incubated in a water bath (at 37°C) for 60 min. Thiobarbituric solution and acetic acid were added to the tubes and second stage of incubation was carried out at 100°C for 10 min. Finally, absorbance of the generated solution was read at the wavelength of 532 nm with spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II). Amounts of TAC were expressed as mmol/g protein.

Measurement of total nitrate-nitrite amounts was carried out by preparation of Griess reagent (Green et al., 1982). A 50 μL sample was mixed with 50 μL reagent in 96-well plates and the plates were incubated in a dark place at laboratory temperature for 15 minutes. The optical density of generated solution was read by ELISA reader (DANA, Iran) at 540 nm. The standard plot was drawn by preparation of different concentrations of sodium nitrate. Amounts of total nitrate-nitrite were normalized by measurement of total protein and finally reported as $\mu\text{mol/g}$ protein in the samples.

Superoxide dismutase (SOD) activity was measured based on the level of pyrogallol oxidation (Marklund and Marklund, 1974). The optical density of the enzymatic reaction was read at 1.5 and 3.5 min (420 nm) after addition of pyrogallol solution to the sample with spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II). Activity of SOD was normalized according to the amounts of protein, and reported as unit/mg protein in the samples.

Different concentrations of bovine serum albumin (BSA) were prepared by dissolving an appropriate amount of BSA in sodium chloride 0.9% and used as standard in the protocol of protein measurement (Bradford, 1976). The reagent (Bradford reagent) was prepared by dissolving 100 mg Coomassie blue (G250) in 50 mL ethanol 95% and adding of 100 mL phosphoric acid 85% to the solution. Final volume of prepared solution was adjusted to 1000 mL with distilled water. In the next step, appropriate amounts of reagent were mixed with sample and incubated at 20–25°C for 15 min. Then, the optical density of generated solution was read at 595 nm by visible spectrophotometry (Shimadzu, Japan).

Statistical analysis

Arcsine square root transformation was carried out on percentage data. Effect of treatment was analyzed by one-way analysis, followed by a Holm-sidak *post hoc* test at each time point. Changes in variables among different time points in a group (within group analysis) were assessed using repeated measure ANOVA to reveal time effects. SigmaStat software (Version 3.5; Chicago, IL) was used for analysis. Results are reported as the mean $\pm$ SEM. Probability value lesser than 0.05 was not considered.

Results

Experiment 1

Percent of TM and FPM of spermatozoa was shown in Table 1. According to the result, the dose of 10 μM was chosen for the next experiment.

Table 1. Percentage of total and forward progressive motility of ram spermatozoa following exposure to different concentrations of deltamethrin (DEL) and stored for various time points at 4°C

Parameter	Treatment	Time of storage (h)			
		0	24	48	72
Total motility	Negative control	86.21 $\pm$ 3.35	79.54 $\pm$ 2.76	76.33 $\pm$ 3.01	55.47 $\pm$ 2.84
	Control	84.21 $\pm$ 2.80	78.32 $\pm$ 3.45	72.71 $\pm$ 2.93	53.51 $\pm$ 2.98
	DEL 1 μM	83.48 $\pm$ 3.18	80.09 $\pm$ 2.87	74.77 $\pm$ 3.21	52.91 $\pm$ 4.77
	DEL 2.5 μM	84.34 $\pm$ 4.38	77.91 $\pm$ 3.46	70.54 $\pm$ 2.56	50.21 $\pm$ 2.87
	DEL 5 μM	81.53 $\pm$ 3.21	75.63 $\pm$ 2.86	65.84 $\pm$ 2.88*	40.28 $\pm$ 3.09*
	DEL 10 μM	78.68 $\pm$ 2.59	66.86 $\pm$ 3.70*	55.55 $\pm$ 3.43*	18.25 $\pm$ 1.34*
	DEL 20 μM	70.55 $\pm$ 4.76*	35.28 $\pm$ 2.21*	20.11 $\pm$ 1.52*	5.21 $\pm$ 0.87*
	DEL 40 μM	50.25 $\pm$ 3.71*	15.22 $\pm$ 1.52*	0*	0*
	DEL 80 μM	31.35 $\pm$ 4.82*	2.57 $\pm$ 0.08*	0*	0*
Forward progressive motility	Negative control	79.66 $\pm$ 2.11	60.25 $\pm$ 1.88	55.28 $\pm$ 2.07	28.25 $\pm$ 2.46
	Control	78.11 $\pm$ 3.56	61.66 $\pm$ 2.83	52.32 $\pm$ 1.81	27.36 $\pm$ 1.71
	DEL 1 μM	80.52 $\pm$ 1.88	58.74 $\pm$ 2.34	53.48 $\pm$ 1.92	29.65 $\pm$ 1.55
	DEL 2.5 μM	78.39 $\pm$ 1.25	57.23 $\pm$ 1.74	50.24 $\pm$ 1.45	30.48 $\pm$ 0.92
	DEL 5 μM	77.51 $\pm$ 2.31	53.29 $\pm$ 2.11	42.21 $\pm$ 2.15*	18.25 $\pm$ 1.19*
	DEL 10 μM	75.46 $\pm$ 3.11	41.79 $\pm$ 2.86*	37.55 $\pm$ 2.10*	10.17 $\pm$ 0.97*
	DEL 20 μM	55.23 $\pm$ 2.88*	14.55 $\pm$ 2.12*	5.77 $\pm$ 1.01*	1.21 $\pm$ 0.08*
	DEL 40 μM	24.11 $\pm$ 2.55*	2.58 $\pm$ 0.21*	0*	0*
	DEL 80 μM	10.77 $\pm$ 2.10*	0*	0*	0*

*Indicate significant difference ($P < 0.05$) with control and negative control groups.

Experiment 2

Lesser percent of TM and FPM were recorded in DEL group compared to other groups at 24, 48 and 72 h ($P = 0.010$; Table 2). Within group analysis revealed that TM was lower at 72 h compared to 0 h in the treated groups ($P = 0.003$; Table 2). The highest FPM was recorded at 0 h, which is significantly different compared to other time points in all groups ($P < 0.001$; Table 2). Straight linear velocity and average path velocity were lower in DEL treated group compared to other groups at 72 h ($P = 0.04$; Table 3). Moreover, VCL index was lower in DEL group compared to controls and combination groups at 24, 48 and 72 h ($P < 0.001$; Table 3). The indices of straightness and linearity did not differ among groups at studied time points ($P > 0.05$; Table 3).

There were no significant differences among groups in viability rate at 0, 24 and 48 h ($P > 0.05$; Table 4), while lower viability rate was recorded in DEL group compared to other treated groups at 72 h ($P = 0.005$; Table 4).

Table 2. Percentage of total and forward progressive motility of ram spermatozoa following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C

Parameter	Treatment	Time of storage (h)			
		0	24	48	72
Total motility	Negative control	84.77 $\pm$ 1.54 Aa	80.79 $\pm$ 1.72 Aa	78.24 $\pm$ 1.79 Aa	53.92 $\pm$ 2.93 ACb
	Control	83.26 $\pm$ 3.49 Aa	82.34 $\pm$ 2.88 Aa	75.13 $\pm$ 2.05 Aa	46.61 $\pm$ 2.39 Ab
	DEL	80.95 $\pm$ 1.59 Aa	71.53 $\pm$ 3.26 Bb	59.53 $\pm$ 1.26 Bc	15.73 $\pm$ 0.43 Bd
	DEL + 2 μ M IDB	85.63 $\pm$ 2.76 Aa	86.75 $\pm$ 1.91 Aa	74.62 $\pm$ 3.65 Aa	50.80 $\pm$ 1.13 Ab
	DEL + 4 μ M IDB	89.20 $\pm$ 3.41 Aa	83.26 $\pm$ 6.25 Aa	77.75 $\pm$ 2.56 Aa	51.05 $\pm$ 2.12 Ab
	DEL + 8 μ M IDB	87.73 $\pm$ 2.51 Aa	85.62 $\pm$ 4.20 Aa	74.40 $\pm$ 0.51 Aab	62.65 $\pm$ 2.52 Cb
Forward progressive motility	Negative control	80.77 $\pm$ 1.58 Aa	62.69 $\pm$ 2.34 Ab	58.69 $\pm$ 1.60 Ab	30.81 $\pm$ 1.98 Ac
	Control	81.75 $\pm$ 2.25 Aa	61.81 $\pm$ 2.66 Ab	59.34 $\pm$ 1.64 Ab	27.96 $\pm$ 1.42 Ac
	DEL	78.11 $\pm$ 1.93 Aa	45.99 $\pm$ 2.35 Bb	40.89 $\pm$ 3.85 Bb	7.41 $\pm$ 0.28 Bc
	DEL + 2 μ M IDB	78.45 $\pm$ 2.89 Aa	56.16 $\pm$ 1.89 Ab	54.08 $\pm$ 2.21 Ab	31.57 $\pm$ 1.17 Ac
	DEL + 4 μ M IDB	86.25 $\pm$ 2.22 Aa	59.50 $\pm$ 1.06 Ab	55.89 $\pm$ 2.55 Ab	31.53 $\pm$ 2.36 Ac
	DEL + 8 μ M IDB	83.47 $\pm$ 1.12 Aa	59.54 $\pm$ 2.17 Ab	54.65 $\pm$ 2.38 Ab	35.51 $\pm$ 2.58 Ac

A, B, C – values with different letters indicate difference ($P < 0.05$) among groups at each time point.

a, b, c – values with different letters indicate difference ($P < 0.05$) between the data at the same row.

Among group analysis showed that the percent of spermatozoa with functional membrane was significantly lower in DEL group compared to other experimental groups in all studied time points ($P = 0.008$; Table 5).

The interaction of treatment $\times$ time was effective on MDA amounts of culture media, where amounts of MDA were significantly greater in medium of DEL group compared to other experimental groups at 0, 24, 48 and 72 h of experiment ($P = 0.001$; Figure 1). Within group analysis indicated greater amounts of MDA at 24, 48 and 72 h compared to 0 h in all experimental groups ($P = 0.005$; Figure 1).

Intra sperm amounts of MDA were less in negative control group compared to DEL group at 24 and 48 h of storage ($P = 0.033$; Figure 2). Moreover, higher intra-cellular amounts of MDA were observed at 72 h compared to 0 h in all experimental groups ($P = 0.012$; Figure 2).

The TAC amounts of culture media did not differ among groups at 0, 24, and 48 h of storage ($P > 0.05$; Figure 3), while its amounts were lesser in DEL groups compared to other experimental groups at 72h ($P = 0.001$; Figure 3). In negative control, control and DEL groups a significant increase in TAC values were observed at 48 h compared to 0 h, but their amounts were reduced at 72 h compared to 48 h ($P = 0.021$; Figure 3).

There were no significant differences in intra sperm TAC values among groups during the experiment intervals, except among DEL group compared to 4 and 8 μ L combination groups at 72 h ($P = 0.013$; Figure 4).

Table 3. Motion characteristics of ram spermatozoa analyzed by CASA following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C

Parameter	Treatment	Time of storage (h)			
		0	24	48	72
VAP (μ m/s)	Negative control	22.06 $\pm$ 1.45 Aa	19.78 $\pm$ 0.31 Aa	17.85 $\pm$ 1.68 Aa	16.44 $\pm$ 0.55 Aa
	Control	21.04 $\pm$ 1.33 Aa	18.99 $\pm$ 0.70 Aab	15.44 $\pm$ 0.39 Ab	15.87 $\pm$ 0.49 Ab
	DEL	21.17 $\pm$ 2.06 Aa	18.03 $\pm$ 0.83 Aab	12.66 $\pm$ 1.63 Abc	9.35 $\pm$ 0.61 Bc
	DEL + 2 μ m IDB	20.30 $\pm$ 1.84 Aa	17.60 $\pm$ 1.03 Aa	15.36 $\pm$ 1.68 Aa	16.86 $\pm$ 1.31 Aa
	DEL + 4 μ m IDB	20.43 $\pm$ 1.02 Aa	17.67 $\pm$ 0.39 Aab	15.40 $\pm$ 1.25 Aab	14.16 $\pm$ 1.12 Ab
	DEL + 8 μ m IDB	20.28 $\pm$ 0.48 Aa	18.45 $\pm$ 0.78 Aa	17.57 $\pm$ 1.54 Aa	16.54 $\pm$ 1.04 Aa
VSL (μ m/s)	Negative control	14.13 $\pm$ 0.95 Aa	14.33 $\pm$ 0.25 Aa	14.47 $\pm$ 0.94 Aa	12.47 $\pm$ 1.13 Aa
	Control	15.46 $\pm$ 0.50 Aa	13.80 $\pm$ 0.52 Aa	14.01 $\pm$ 0.66 Aa	12.57 $\pm$ 1.48 Aa
	DEL	13.55 $\pm$ 0.88 Aa	13.00 $\pm$ 0.86 Aa	12.56 $\pm$ 1.22 Aa	8.04 $\pm$ 0.69 Bb
	DEL + 2 μ m IDB	14.47 $\pm$ 0.58 Aa	12.43 $\pm$ 0.66 Aa	12.55 $\pm$ 1.16 Aa	13.38 $\pm$ 0.77 Aa
	DEL + 4 μ m IDB	12.72 $\pm$ 0.77 Aa	11.91 $\pm$ 1.14 Aa	12.46 $\pm$ 1.07 Aa	11.79 $\pm$ 1.19 Aa
	DEL + 8 μ m IDB	13.63 $\pm$ 0.16 Aa	12.96 $\pm$ 0.56 Aa	12.91 $\pm$ 1.05 Aa	12.46 $\pm$ 0.70 Aa
VCL (μ m/s)	Negative control	97.11 $\pm$ 4.84 Aab	87.92 $\pm$ 4.74 Aa	72.89 $\pm$ 2.45 Ab	50.55 $\pm$ 3.24 Ac
	Control	94.25 $\pm$ 6.50 Aa	86.45 $\pm$ 4.17 Aab	68.95 $\pm$ 1.70 Abc	52.38 $\pm$ 2.80 Ac
	DEL	89.59 $\pm$ 2.73 Aa	59.95 $\pm$ 0.83 Bb	41.35 $\pm$ 3.40 Bc	27.66 $\pm$ 2.61 Bc
	DEL + 2 μ m IDB	104.88 $\pm$ 8.15 Aa	84.29 $\pm$ 2.69 Aa	61.12 $\pm$ 3.37 Ab	57.48 $\pm$ 0.54 Ab
	DEL + 4 μ m IDB	102.35 $\pm$ 3.87 Aa	94.74 $\pm$ 3.18 Aa	67.07 $\pm$ 4.17 Ab	53.74 $\pm$ 2.88 Ab
	DEL + 8 μ m IDB	95.16 $\pm$ 7.35 Aa	82.24 $\pm$ 3.08 Aab	63.41 $\pm$ 3.51 Abc	54.25 $\pm$ 2.20 Ac
STR (%)	Negative control	70.55 $\pm$ 1.86 Aa	72.19 $\pm$ 1.32 Aa	70.89 $\pm$ 2.27 Aa	71.25 $\pm$ 1.75 Aa
	Control	67.52 $\pm$ 1.01 Aa	68.94 $\pm$ 2.05 Aa	69.29 $\pm$ 2.58 Aa	66.66 $\pm$ 1.22 Aa
	DEL	71.12 $\pm$ 1.70 Aa	71.63 $\pm$ 2.14 Aa	71.70 $\pm$ 0.86 Aa	70.80 $\pm$ 1.85 Aa
	DEL + 2 μ m IDB	70.57 $\pm$ 1.68 Aa	71.76 $\pm$ 1.45 Aa	72.89 $\pm$ 1.47 Aa	71.38 $\pm$ 1.83 Aa
	DEL + 4 μ m IDB	73.53 $\pm$ 1.10 Aa	72.73 $\pm$ 1.83 Aa	72.21 $\pm$ 1.15 Aa	70.67 $\pm$ 1.28 Aa
	DEL + 8 μ m IDB	71.31 $\pm$ 0.13 Aa	70.04 $\pm$ 1.29 Aa	69.94 $\pm$ 1.09 Aa	69.75 $\pm$ 1.45 Aa
LIN (%)	Negative control	26.01 $\pm$ 3.28 Aa	26.05 $\pm$ 0.59 Aa	28.33 $\pm$ 0.69 Aa	26.31 $\pm$ 1.06 Aa
	Control	24.52 $\pm$ 0.47 Aa	24.80 $\pm$ 0.75 Aa	24.60 $\pm$ 1.66 Aa	23.25 $\pm$ 1.19 Aa
	DEL	27.57 $\pm$ 1.82 Aa	26.43 $\pm$ 2.34 Aa	25.38 $\pm$ 1.14 Aa	27.79 $\pm$ 0.61 Aa
	DEL + 2 μ m IDB	25.50 $\pm$ 1.89 Aa	25.44 $\pm$ 1.28 Aa	26.71 $\pm$ 0.98 Aa	25.17 $\pm$ 1.67 Aa
	DEL + 4 μ m IDB	25.89 $\pm$ 1.38 Aa	24.82 $\pm$ 2.54 Aa	25.58 $\pm$ 0.32 Aa	25.48 $\pm$ 1.74 Aa
	DEL + 8 μ m IDB	26.27 $\pm$ 1.25 Aa	24.98 $\pm$ 1.09 Aa	24.44 $\pm$ 1.17 Aa	24.63 $\pm$ 1.20 Aa

A, B – values with different letters indicate difference ($P < 0.05$) among groups at each time point.

a, b, c – values with different letters indicate difference ($P < 0.05$) between the data at the same row.

Total nitrate-nitrite values of culture medium (Figure 5) and spermatozoa (Figure 6) did not show any significant changes among groups at studied time points ($P > 0.05$).

Table 4. Percentage of viable spermatozoa in ram semen following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C

Treatment	Time of storage (h)			
	0	24	48	72
Negative control	85.44 $\pm$ 0.76 Aa	85.06 $\pm$ 0.85 Aa	82.34 $\pm$ 1.03 Aa	75.46 $\pm$ 0.28 Ab
Control	83.67 $\pm$ 2.57 Aa	80.41 $\pm$ 1.92 Aa	80.44 $\pm$ 1.79 Aa	76.55 $\pm$ 2.08 Aa
DEL	83.12 $\pm$ 0.88 Aa	84.21 $\pm$ 0.99 Aa	75.77 $\pm$ 2.54 Aa	64.50 $\pm$ 2.84 Bb
DEL + 2 μ M IDB	84.58 $\pm$ 0.68 Aa	82.42 $\pm$ 0.96 Aa	82.20 $\pm$ 2.49 Aa	80.15 $\pm$ 0.36 Aa
DEL + 4 μ M IDB	87.60 $\pm$ 0.60 Aa	86.06 $\pm$ 1.41 Aab	79.93 $\pm$ 2.50 Ab	81.07 $\pm$ 0.72 Aab
DEL + 8 μ M IDB	85.16 $\pm$ 0.38 Aa	86.66 $\pm$ 1.69 Aa	80.07 $\pm$ 2.23 Aa	78.58 $\pm$ 4.98 Aa

A, B – values with different letters indicate difference ($P < 0.05$) among groups at each time point.

a, b – values with different letters indicate difference ($P < 0.05$) between the data at the same row.

Table 5. Percentage of ram spermatozoa with functional membrane (evaluated by hypo-osmotic swelling test) following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C

Treatment	Time of storage (h)			
	0	24	48	72
Negative control	85.88 $\pm$ 1.41 Aa	85.51 $\pm$ 2.53 Aab	75.78 $\pm$ 2.90 Aab	72.42 $\pm$ 2.49 Ab
Control	83.11 $\pm$ 2.66 Aa	80.45 $\pm$ 1.94 Aab	76.66 $\pm$ 2.46 Aab	70.75 $\pm$ 2.16 Ab
DEL	73.64 $\pm$ 1.32 Ba	68.11 $\pm$ 2.27 Bab	61.74 $\pm$ 2.66 Bbc	40.42 $\pm$ 3.93 Bd
DEL + 2 μ M IDB	82.88 $\pm$ 2.33 Aa	77.30 $\pm$ 1.39 Aa	75.37 $\pm$ 2.65 Aa	73.58 $\pm$ 1.99 Aa
DEL + 4 μ M IDB	85.37 $\pm$ 1.48 Aa	81.83 $\pm$ 1.31 Aa	76.37 $\pm$ 1.96 Aa	72.59 $\pm$ 6.08 Aa
DEL + 8 μ M IDB	83.76 $\pm$ 2.33 Aa	77.30 $\pm$ 1.17 Ab	72.10 $\pm$ 2.03 Ac	71.32 $\pm$ 1.98 Ac

A, B – values with different letters indicate difference ($P < 0.05$) among groups at each time point.

a, b, c, d – values with different letters indicate difference ($P < 0.05$) between the data at the same row.

Figure 1. Amounts of malondialdehyde (μ mol/g protein) in medium of ram spermatozoa following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C. A, B – values with different letters indicate difference ($P < 0.05$) among experimental groups at each time point. a, b, c, d – values with different letters indicate difference ($P < 0.05$) over time within the experimental groups

Figure 4. Intra sperm total antioxidant capacity (TAC; mmol/g protein) following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C. A, B – values with different letters indicate difference ($P<0.05$) among groups at each time point. a, b – values with different letters indicate difference ($P<0.05$) over time within experimental groups

Figure 5. Total amounts of nitric oxide ($\mu\text{mol/g}$ protein) in medium of ram spermatozoa following exposure to deltamethrin (DEL; $10 \mu\text{M}$) alone or concurrent with idebenone (IDB; $2, 4, 8 \mu\text{M}$) and stored for various time points at 4°C . No significant differences were observed among groups or within a group among different time points

Figure 6. Intra sperm amounts of nitric oxide ($\mu\text{mol/g}$ protein) following exposure to deltamethrin (DEL; $10 \mu\text{M}$) alone or concurrent with idebenone (IDB; $2, 4, 8 \mu\text{M}$) and stored for various time points at 4°C . No significant differences were observed among groups at each time point. a, b – values with different letters indicate a difference ($P < 0.05$) over time within experimental groups

Figure 7. Superoxide dismutase activities (unit/mg protein) in medium of ram spermatozoa following exposure to deltamethrin (DEL; $10 \mu\text{M}$) alone or concurrent with idebenone (IDB; $2, 4, 8 \mu\text{M}$) and stored for various time points at 4°C . No significant differences were observed among groups or within a group among different time points

Figure 8. Intra sperm activity of superoxide dismutase (unit/mg protein) following exposure to deltamethrin (DEL; 10 μ M) alone or concurrent with idebenone (IDB; 2, 4, 8 μ M) and stored for various time points at 4°C. No significant differences were observed among groups or within a group among different time points

No significant changes were observed in SOD activity of medium (Figure 7) and spermatozoa (Figure 8) among experimental groups at 0, 24, 48 and 72 h ($P>0.05$). Moreover, activity of SOD was not different among time points of experimental groups ($P>0.05$).

Discussion

The current study was designed to evaluate the different concentrations of DEL on kinematics of ram spermatozoa. Moreover, effect of idebenone on redox balance was evaluated concurrent with mild toxic doses of DEL. The present study indicated that: (1) DEL reduced the TM and FPM of chilled stored ram spermatozoa in a dose dependent manner; (2) idebenone attenuated the oxidative toxicity of stored ram semen caused by DEL.

Previous studies confirmed that exposure of PC 12 neuron cells to DEL led to the production of ROS and activated caspase-3 and -9, and finally induced apoptosis of cells (Li et al., 2007; Park et al., 2017). Moreover, oxidative toxicity of DEL on SH-SY5Y cell line was demonstrated via ROS generation (Romero et al., 2012). In addition, the ability of DEL to decrease the anti-apoptotic genes such as Bcl-2 and increase the pro-apoptotic genes such as p53 and Bax had been confirmed (Wu and Liu, 2000; Wu et al., 2000). Similar results and depletion of GSH, an enzymatic antioxidant, were demonstrated by *in vivo* and *in vitro* experiments on mice, when exposed to DEL (Kumar et al., 2014, 2018). Our results revealed that DEL reduced spermatozoa total motility and progressive motility in a dose dependent manner, and the findings are in accordance with the previous study (Park et al., 2017). It was shown that, as early as 1 hour after addition of DEL (at 25 μ M level) to primary culture of splenocytes, amounts of ROS and gene marker of apoptosis were increased (Kumar et al., 2016). Based on the results of first experiment, the dose of 10 μ M DEL was chosen for the next experiment, as mild toxic dose. Different *in vivo* and

in vitro experiments confirmed the attenuative role of IDB on cell viability during exposure to toxicants (Miyamoto et al., 1989; Miyamoto and Coyle, 1990; Bruno et al., 1994; Pereira and Oliveira, 2000). In the second experiment, deleterious effect of mild toxic doses of DEL was confirmed on different spermatozoa kinematics indices. Moreover, co-treatment of DEL with IDB, reduced the toxic effect of DEL on ram semen. Improvement in kinematic variables, viability and membrane functionality of spermatozoa could be attributed to protective effects of IDB upon semen cold storage.

Spermatozoa have some unique characters compared to other cells. First of all, amounts of polyunsaturated fatty acids in the membrane of spermatozoa are very high, especially in ram. Moreover, total antioxidant capacity of spermatozoa is nadir. Accordingly, spermatozoa are very sensitive to endogenous produced ROS species during cold storage or added exogenous toxic substances which mimic oxidative toxicity process. During cooling from 35°C to 5°C, the phase of plasma membrane lipids changes from fluid to a crystalline condition. Moreover, organization and composition of membrane lipids alter dramatically (Holt and North, 1986). Other reported changes are the decreased stabilization of spermatozoa plasma membrane, alteration of membrane ATPase activity and peroxidation of lipids due to generation of ROS (Buhr et al., 1994; Holt and North, 1986; Jones and Mann, 1977). It was reported that cooling of spermatozoa induces irreversible damages, and changes the spermatozoa function and fertilization potential (Evans and Maxwell, 1987). Then, addition of protective substances to semen extender have been examined by different studies, to ameliorate oxidative toxicity upon preservation (Câmara et al., 2011; Akalin et al., 2016; Eslami et al., 2019). The current study indicated greater amounts of MDA in DEL exposed group compared to combination groups. In this regard, it was confirmed that oxidative stress has an important role in the toxicity of DEL to exposed cells (Romero et al., 2012; Park et al., 2017). Another study demonstrated that oral administration of DEL to mice increased MDA levels and decreased activity of enzymatic antioxidants such as glutathione and catalase in kidney samples (Khalatbary et al., 2016). The negative correlation between levels of MDA and quality of semen was reported previously (Lewis et al., 1995; Bucak et al., 2010). Scavenging potential of IDB against toxicity of different reactive oxygen species was shown previously (Mordente et al., 1998). Studies conducted on ram revealed that IDB treated groups (at proper concentration) had lesser hydroperoxide and malondialdehyde values compared to control sample upon liquid storage or after freezing/thawing process of semen (Eslami et al., 2019; Lone et al., 2019). Probable protective role of IDB may be due to attenuation of oxidative and peroxidative toxicity induced by DEL during low temperature liquid semen storage.

Results of current study indicated that TAC values in 4 and 8 µM IDB plus DEL groups were greater compared to DEL group at final studied time point, but SOD activities were not affected by treatment. It was shown that oral administration of DEL to mice not only increased the amounts of MDA, but also decreased the activity of enzymatic antioxidants such as glutathione and catalase in kidney samples (Khalatbary et al., 2016). Previous study indicated that rooster semen enrichment with quercetin ameliorates peroxidative and nitrosative damages induced by H₂O₂ during

preservation at refrigerator temperature (Ghaniei et al., 2019). It was reported that IDB has about 7 times greater antioxidant potential compared to vitamin E (Zhai et al., 2008). Moreover, antioxidative role of IDB was confirmed by *in vitro* and *in vivo* studies (Rustin et al., 1999; Abdel Baky et al., 2010; Eslami et al., 2019; Lone et al., 2019). It was reported that pre-treatment of retinal epithelial cells with IDB, reduced the formation of ROS and sustained viability of cells when oxidative toxicity was induced by hydrogen peroxide (Arend et al., 2015). Potential of IDB in transferring electrons to complex III of mitochondria (Imada et al., 1989), might lead to prevent excessive ROS generation and ameliorate peroxidation process (Lone et al., 2019).

Conclusion

In conclusion, exposure of ram semen to mild toxic dose of DEL, reduced kinematics and viability of spermatozoa. Moreover, potential of IDB to ameliorate the DEL induced oxidative toxicity of ram semen was proven. Further studies are needed to reveal the role of IDB on semen protection of other animal species during normal and toxic situation upon semen preservation.

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