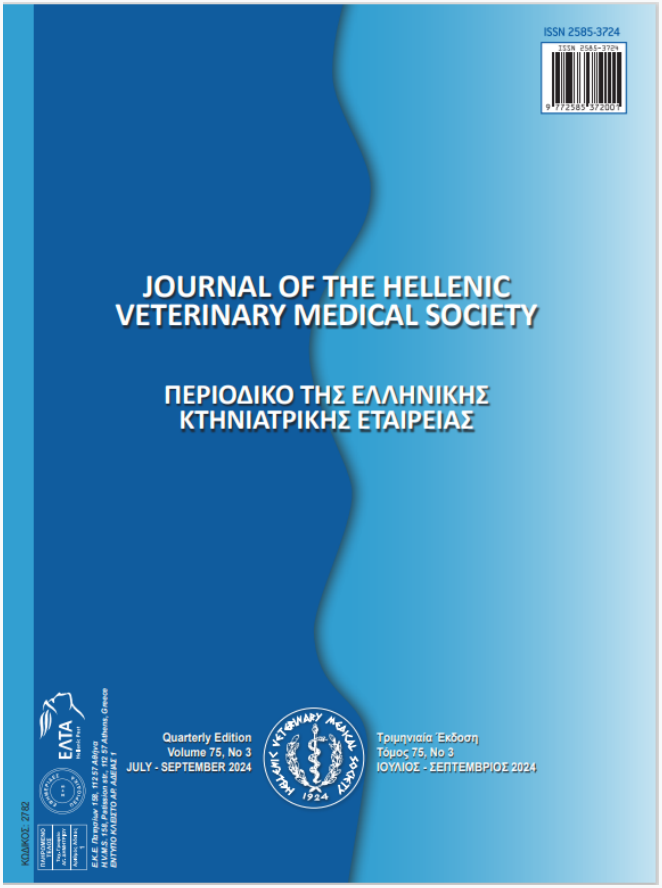
Journal of the Hellenic Veterinary Medical Society

Vol 75, No 3 (2024)



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doi: 10.12681/jhvms.36212

To cite this article:

Güngör, İH, Gür, S., Koca, R., Çakır Cihangiroğlu, A., Güler Ekmen, E., Acısu, T., Badıllı, N., Özer Kaya, Ş, Yüce, A., Sönmez, M., & Türk, G. (2024). Evaluation of the use of L-carnosine in short-term storage of ram semen with spermatological and flow-cytometric analyzes. *Journal of the Hellenic Veterinary Medical Society*, *75*(3), 7977–7992. https://doi.org/10.12681/jhvms.36212

J HELLENIC VET MED SOC 2024, 75 (3): 1-1
ΠΕΚΕ 2024, 75 (3): 1-1

Research article
Ερευνητικό άρθρο

Evaluation of the use of L-carnosine in short-term storage of ram semen with spermatological and flow-cytometric analyzes

İ.H. Güngör[1*], S. Gür[1], R.H. Koca[2], A. Çakır Cihangiroğlu[3], E. Güler Ekmen[4], T.C. Acısu[1], N. Badıllı[1], Ş. Özer Kaya[1], A. Yüce[4], M. Sönmez[1], G. Türk[1]

[1]*Fırat University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Elazığ, Türkiye*

[2]*Bingöl University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Bingöl, Türkiye*

[3]*Siirt University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Siirt, Türkiye*

[4]*Fırat Üniversitesi, Faculty of Veterinary Medicine, Department of Physiology, Elazığ, Türkiye*

ABSTRACT: This study was conducted to investigate the effect of adding different doses of L-carnosine to semen in vitro on the short-term storage of semen. Semen taken from 6 Akkaraman rams was used in the research. Semen taken from each animal was pooled. It was diluted with tris+egg yolk extender containing L-carnosine at different doses (1, 5, 10, 20, 50, 100 and 200 mM) and stored at +4°C for 96 hours. Motility and kinematic parameters, abnormal spermatozoa ratio, HOS (Hypo-osmotic swelling test), pH and flow-cytometric analyzes were performed in all experimental groups every 24 hours (0, 24, 48, 72 and 96 hours) during storage. Oxidative stress parameters were determined at 0 and 96 hours. It was determined that 1, 5, 10, 20 mM L-carnosine doses increased total, progressive and rapid motility, kinematic parameters VCL (Curvilinear velocity), VSL (Straight line velocity), VAP (Average path velocity) values and high mitochondrial membrane potential level during short-term storage. It was also determined that these doses protected membrane integrity, reduced the rate of abnormal spermatozoon, acrosomal damage and low mitochondrial membrane potential level. It was determined that a dose of 200 mM L-carnosine had a toxic effect and negatively affected the parameters [total and progressive motility, VCL, VSL, VAP, ALH (Amplitude of lateral head displacement), HOS Test, viability, acrosomal damage, HMMP (High Mitochondrial Membrane Potential), LMMP (Low Mitochondrial Membrane Potential)].
In conclusion, high doses of L-carnosine (200 mM) in semen extenders are harmful to ram semen characteristics after a short time storage. Moreover, low doses of L-carnosine (1, 5, 10 and 20 mM) supplementation may have beneficial effect on ram semen traits, depending on the time storage.

***Keywords*:** L-carnosine; Semen; Cooling; Flow-cytometric analysis; Ram

Corresponding Author:
İbrahim Halil Gungor, Fırat University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Elazığ, Türkiye
E-mail address: ihgungor@firat.edu.tr

Date of initial submission: 15-12-2023
Date of acceptance: 29-01-2024

INTRODUCTION

Crossbreeding studies are carried out to improve the genotypic structure in sheep. In hybridization studies, the artificial insemination method is used more widely than natural insemination (Akçapınar, 2001). When performing artificial insemination in sheep under field conditions, diluted fresh semen is generally preferred. Insemination of more than one ewe by taking the semen of a ram and diluting it with appropriate diluents not only saves animal owners from the burden of keeping a large number of breeding rams, but also is creates an opportunity for genetic improvement (Bearden and Faquay, 1992).

Low pregnancy rates obtained from intracervical inseminations performed with frozen-thawed ram semen make short-term storage at 5°C more important. However, pregnancy rates cannot reach the desired levels in intracervical inseminations performed with ram semen stored at 5°C for more than 24 hours (Maxwell and Salamon, 1993). For this reason, short-term storage of semen causes some biochemical reactions depending on the cold environment. One of the most important of these reactions is oxidative stress. Oxidative stress occurs as a result of oxidative imbalance due to an increase in ROS (Reactive oxygen species) or a decrease in antioxidant levels (Gulum et al., 2017). This process can cause damage to polyunsaturated fatty acids in the spermatozoon plasma membrane, as well as DNA, RNA and proteins (Atig et al., 2017; Kamkar et al., 2018). However, under physiological conditions, ROS are required for many conditions involving the reproductive process, such as spermatozoon capacitation, hypermotility, cellular activation, interaction with the zona pellucida, and the acrosome reaction (Banihani et al., 2018). Increased ROS levels during short-term storage can be strongly scavenged by enzymatic and non-enzymatic antioxidants (Budai, 2014). In this context, many antioxidant substances have been added to semen extenders used to extend the life of spermatozoa during short-term storage of semen and studies have been conducted. The aim of adding antioxidants is to protect spermatozoa against LPO (lipid peroxidation) damage. It has been reported that antioxidants such as cysteamine (Jumintono et al., 2021), ebselen (Bucak et al., 2019), taurine (Zhang et al., 2021) and idebenone (Eslami et al., 2019), which are added to semen extenders for short-term storage of semen in rams, contribute to the prolongation of the life of spermatozoa by reducing the amount of LPO.

Sperms have try to protect themselves against oxidative stress caused by free radicals, thanks to the antioxidants in seminal plasma (Kim and Parthasarathy, 1998). Protection of lipids in the cell membrane structure against LPO was the first identified effect of antioxidants. ROS cause lipid and protein peroxidation in spermatozoa. Increased ROS attacks cause breaks in spermatozoon DNA (Deoxyribo nucleic acid), even though it has a compact structure (Türk, 2013). The most critical defense mechanism to prevent oxidative stress caused by free radicals is provided by antioxidants. For this reason, research has been conducted by adding many antioxidant substances to ram semen extenders, and as a result of these studies, efforts have been made to contribute to the storability of ram semen (Özer Kaya et al., 2021; Bucak et al., 2021; Güngör et al., 2021). One of the antioxidant substances used for short-term storage of ram semen is C60HyFn. It has been reported that the addition of doses between 100 nM and 40 µM of this antioxidant substance to ram semen will contribute to short-term storage (Özer Kaya et al., 2021). In addition, it has been reported that antioxidants such as cysteamine (Peker Akalın et al., 2016), bovine serum albümin (Gökçe et al., 2017) and melatonin (Dai et al., 2019) added to ram semen reduce MDA levels, an indicator of LPO, and increase total antioxidant capacity and GSH levels during short-term storage.

L-carnosine is a dipeptide consisting of a complex of β-alanyl - L-histidine amino acids (*Fig. 1*) (Boldyrev, 2012; Wu, 2020). Carnosine is synthesized from histidine and β-alanyl amino acids by ATP-dependent carnosine synthase (Horinishi et al., 1978) and is broken down into its component amino acids by metal ion-dependent homodimeric peptidase (carnosinase) (Teufel et al., 2003). The most common analogues are carnosine variants such as anserine, ophidine (balenin), homocarnosine, acetylcarnosine.

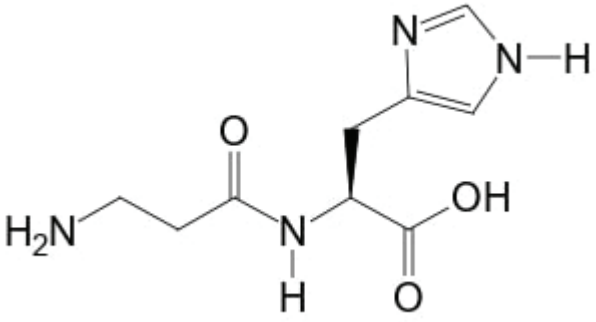

Figure 1: Chemical Structure of L-carnosine

It is known that in mammals, carnosine is present at millimolar levels in only two tissues (skeletal muscle and the olfactory center in the brain) (Boldyrev et al., 2013). It is also reported that carnosine is present in seminal plasma (Rocha et al., 2018; Sarkar et al., 2020). Carnosine is a well-soluble compound in water (1 g of carnosine in 3.1 ml of water at 25°C) and is characterized by three ionizable groups. These; carboxylic group (pKa 2.76), amino group of β-alanyl residue (pKa 9.32), and imidazole ring in histidine (pKa 6.72) (Vistoli et al., 2012). Carnosine has a functional imidazole ring that can easily donate hydrogen to free radicals so that they can be converted into non-radical substances (Kohen et al., 1988). Such ability of the imidazole ring is enhanced by the β-alanyl moiety in carnosine. The physiological functions of L-carnosine are mainly; It is known as pH buffering, ATP activation for energy conservation, metal ion (copper, zinc and iron) chelation and homeostasis. Additionally, L-carnosine exerts its antioxidant effect by inhibiting LPO, protein oxidation, advanced protein glycation, protein carbonylation and glycoxidation, directly scavenging reactive oxygen species and peroxyl radicals, and indirectly chelating metals (Barca et al., 2019). Among its antioxidant properties, it is very important that it scavenges advanced lipid oxidation end products such as nonenal and HNE (4-hydroxy-nonenal) (Zhou and Decker, 1999). It has been stated that carnosine inhibits lipoxidation and glycosidation end products, namely sugars (glucose, fructose, ribose, deoxyribose) and reactive carbonyls (malondialdehyde, glyoxal, methylglyoxal, acetaldehyde and formaldehyde) in vitro (Hipkiss et al., 1998) and reduces cellular glutathione consumption depending on the döşe (Xie et al., 2013). L-carnosine is a substance known for its antioxidant properties, is a very strong intracellular bufferer, protects cells against DNA damage, is an anti-aging molecule and has the ability to neutralize lactic acid.

The objective of the present study was to investigate the effect of different doses of L-carnosine on ram semen characteristics during a short-term storage

MATERIAL AND METHOD

Animal Materials and Ethical Approval

Approval of Fırat University Animal Experiments Local Ethics Committee (FÜHADYEK) dated 06.01.2021 and session number 2021/01 was received. In this study, 6 Akkaraman breed rams, aged 2 years old, with an average live weight of 60-65 kg, which were clinically healthy and did not show any pathological findings during genital organ examination, were used. Rams were housed in the Hospitalization Unit of Fırat University Faculty of Veterinary Medicine Animal Hospital. Before the experimental studies began and throughout the study, the rams were fed with high-quality forage and concentrate feeds. Drinking water was provided ad libitum.

Experimental Design

L-carnosine (99%, Crystalline, C9525- 5G, C9H-14N4O3, Sigma-Aldrich®) has a molecular weight of 226.23 g/mol. An amount of L-carnosine of this molecular weight (399.52 mg) was taken and dissolved in 2.2 ml of distilled water, and a stock solution of 800 mM was obtained. 1 ml of the prepared tris+egg yolk diluent was put into 8 different falcon tubes.

Prepared working solutions according to doses;

Working Solution 1: 2 mM (0.995 ml distilled water + 0.005 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 2: 10 mM (0.975 ml distilled water + 0.025 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 3: 20 mM (0.950 ml distilled water + 0.050 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 4: 40 mM (0.900 ml distilled water + 0.100 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 5: 100 mM (0.750 ml distilled water + 0.250 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 6: 200 mM (0.500 ml distilled water + 0.500 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 7: 400 mM (0.000 ml distilled water + 1.000 ml 800 mM stock solution + 1 ml tris + egg yolk diluent),

Working Solution 8: Control (1,000 ml distilled water + 0,000 ml 800 mM stock solution + 1 ml tris + egg yolk diluent). As a result, the working solutions were set as 8 tubes containing a total of 2 ml tris + egg yolk and different doses of L-carnosine.

Collecting Semen and Combining It with Working

Solutions

Throughout the research, rams and ewes were kept in separate paddocks. The study was conducted outside the breeding season. The sheep was brought into heat by applying 17-β-estradiol dissolved in sesame oil at a dose of 5 mg/2mL/sheep. In this way, the rams' libido was stimulated and they were enabled to release semen into the artificial vagina. Each semen collected from 6 rams using artificial vagina was brought to the laboratory in a beaker glass filled with 38°C water. Motility and concentration determinations of the semen brought to the laboratory were made using the CASA system. In order for the collected semen to be used in research, the total motility is required to be 70% and the concentration to be over 2 billion.

After the collected semen was brought to the laboratory, it was diluted with Tris+egg yolk diluent at a ratio of 1:1 in an oven at 38°C. After the necessary individual examinations (spermatological analyzes performed with CASA), with te collected ejaculates were pooled. After the motility and concentration of the resulting pooling were determined in the CASA system, it was rediluted to 800 million motile spermatozoon per ml. The final pooling volume was divided into 8 different tubes, 2 ml each. Experimental groups *(Group 1: Control, Group 2: 1 mM, Group 3: 5 mM, Group 4: 10 mM, Group 5: 20 mM, Group 6: 50 mM, Group 7: 100 mM, Group 8: 200 mM)* were formed by adding previously prepared working solutions containing 2 ml of tris+egg yolk and different doses of L-carnosine onto the semen. In the final, 4 ml of diluted semen containing different doses of L-carnosine and 400 million motile spermatozoa per ml was obtained. Diluted semen samples from all groups were placed in a cooling cabinet with a temperature down to 5°C. Examinations began after the temperature of the semen was gradually reduced to 5°C within 2 hours (Özer Kaya et al., 2021). This time period was recorded as hour 0, and motility and kinematic parameters, abnormal spermatozoon ratio, membrane integrity, pH, live spermatozoon ratio, acrosome integrity and mitochondrial membrane potential were determined every 24 hours (24, 48, 72, 96 hours). In addition, oxidative stress analyzes in semen were performed using biochemical methods at 0 and 96 hours.

Spermatological analysis

Motility and kinematic parameters were determined in the sperm of all groups using the CASA (ISASv1, Proiser, Spain) system. First of all, in order to reduce the number of spermatozoa per image area of CASA to 80-150, 15 µL of the semen was taken and 485 µL of tris buffer solution was added to dilute the semen. 3 µL of the diluted semen samples were placed on a special slide (Spermtrack 20 µm) designed to be compatible with the device for analysis. Then, this prepared slide was placed on the hot stage of the phase-contrast microscope integrated into the CASA system. Following this process, the motility module connected to the CASA system was used. Total, progressive motility (with rates of rapid, medium and slow moving sperm) rates (%) and kinematic parameter [VCL- curvilinear velocity (µm/s); VSL- straight line velocity (µm/s); VAP- average path velocity (µm/s); LIN- linearity (VSL/VCLx100, %); STR- straightness (VSL/VAPx100, %), WOB: (VAP/VCLx100, %), ALH- Amplitude of lateral head displacement (µm); BCF- beat cross frequency, Hz)] values were recorded via this module. For spermatozoa speed, the speed range set by the manufacturer for rams (still < 10 µm/s < slow < 45 µm/s < medium < 75 µm/s < fast) was used. The particle size of the device was set as 15-70 µm.

Determination of Abnormal Spermatozoon Ratio

Solutions A (Methanol based solution - Fixative), B (Eosin Y based solution - Staining) and C (Methylene blue - Thiazine) of the Diff-Quick Staining test were used respectively to determine the abnormal spermatozoon rate. First of all, 100 µL of the semen samples were taken and 400 µL of tris buffer solution was added to the dilution process. Then, 75 µL of this mixture was taken and dropped onto the slide to prepare a smear. After the smears were dried in air, they were immersed in solutions A, B and C respectively and kept for 30 seconds, 20 seconds and 30 seconds. Then, the smears were washed with distilled water and dried in an oven at 38°C for 15 minutes. After the drying process was completed, 200 spermatozoa were counted with the 400x magnification setting of the phase-contrast microscope. The proportion of sperm with head and tail abnormalities was stated as a percentage (Güngör et al., 2022).

Determination of Membrane Integrity - Hypoosmotic Swelling Test (HOST)

A hypotonic solution (0.49 g citric acid, 0.9 g fructose, 100 mL distilled water) was prepared to detect membrane integrity. After diluting the semen samples with tris buffer at a ratio of 1:5, 50 µL was taken and 500 µL hypotonic solution was added. This mixture was incubated for 60 minutes in a 38°C oven. After in-

cubation, 15 µL of the mixture was taken and dropped onto the slide and covered with a coverslip. 200 spermatozoa were counted using the 400x magnification of the phase-contrast microscope. The proportion of intact sperm with a curved tail was expressed as a percentage (Güngör et al., 2022).

Determination of pH

pH values of semen samples were measured using a digital pH meter (Hanna Instrument, Bucharest, Romania) device. For this process, semen samples were contacted with the electrode of the pH meter. Waited until the result was fixed on the screen. At the end of this process, the value on the digital screen of the pH meter was recorded as a result.

Determination of Viability Rate by Flow-cytometry

Flow-cytometric evaluations of sperm samples were made with laser beam at 488 nm (50 mW laser output). For this purpose, 525 ± 40, 585 ± 42 and 610 ± 20 nm emission filters were used. The determination of the viability rate was carried out by modifying the method designed by Garner and Johnson (Garner and Johnson, 1995). LIVE/DEAD Sperm Viability Kit (L7011, ThermoFisher Scientific, USA) was used to perform viability examination on sperm samples. Dilution of the sperm was done using PBS and the density of spermatozoa in the eppendorf was ensured to be one million. 5 µL SYBR-14 and 5 µL PI were added to the diluted semen. This mixture was vortexed for one minute and incubated in a dark environment at 38°C for 30 minutes. After incubation, these tubes were placed in the tube scanning section of the flow-cytometry device (Cytoflex, Beckman-Coulter, USA). The number of spermatozoon counted by the device was set to 10,000. The total spermatozoon ratio was determined as a result of the FSC (Forward Scatter) - SSC (Side Scatter) comparison. Singlet cells were separated as a result of comparing FSC-Area (Forward Scatter-Area) and FSC-Height (Forward Scatter-Height). As a result of PE (Phycoerythrin) - FITCH (Fluorescein Isothiocyonate) comparison, dead and live spermatozoa among singlet cells were displayed as two separate compartments on the graph and expressed as a percentage (*Fig. 2*).

Determination of Acrosomal Damage Rate by Flow-cytometry

Detection of acrosomal damage was performed by modifying the method used by İnanç et al. (İnanç et al., 2019). Lectin PNA from Arachis hypogaea (peanut agglutinin), Alexa Fluor™ 488 Conjugate (L21409, ThermoFisher Scientific, USA) was used to measure the degree of acrosomal damage in sperm samples. The same dilution procedures were performed to determine the viability rate. 5 µL Lectin PNA and 3 µL PI dye were added to the diluted semen. This mixture was vortexed for one minute and incubated in a dark environment at 38°C for 30 minutes. Again, the same flow-cytometric comparison cytograms that were applied when determining the viability rate were applied. As a result, dead and live cells with acrosomal damage were displayed in two separate panes on the

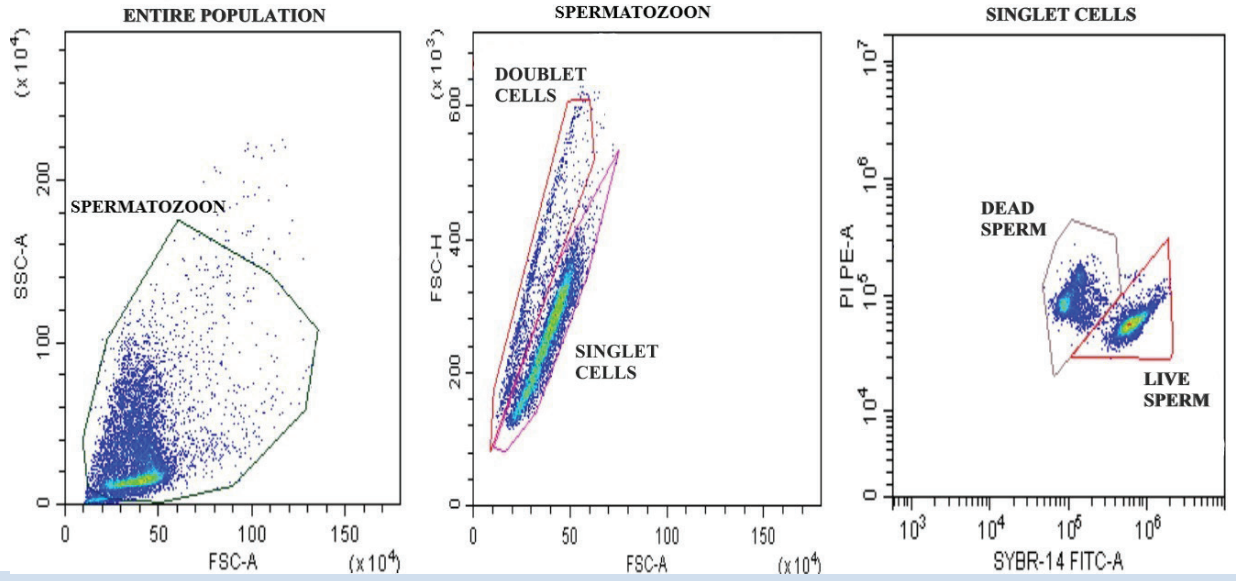

Figure 2: Sequential comparison cytograms used in determining viability

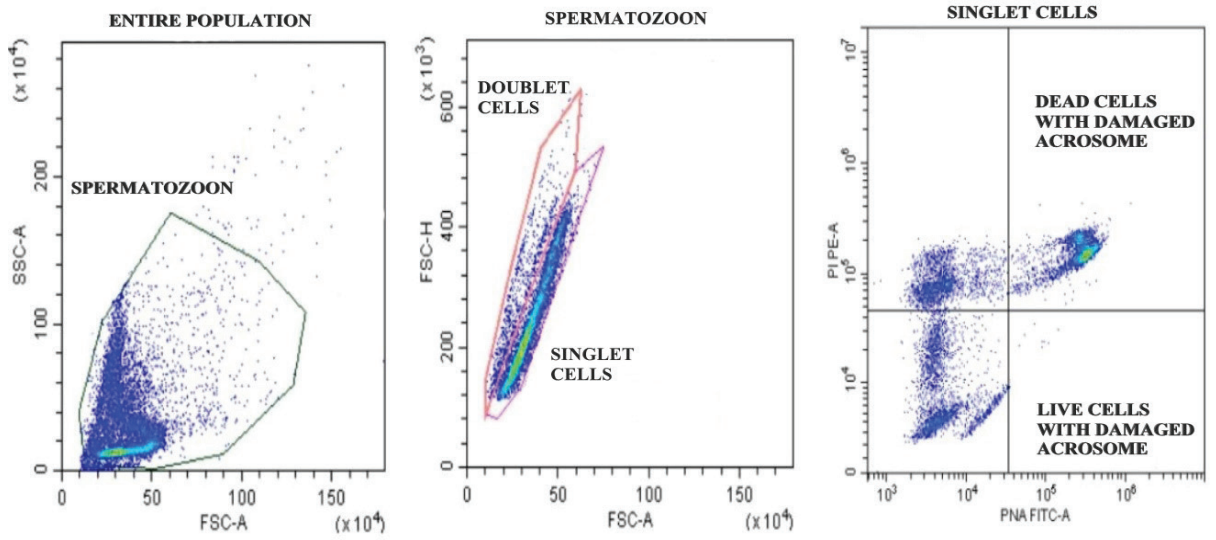

Figure 3: Sequential comparison cytograms used during detection of acrosomal damage

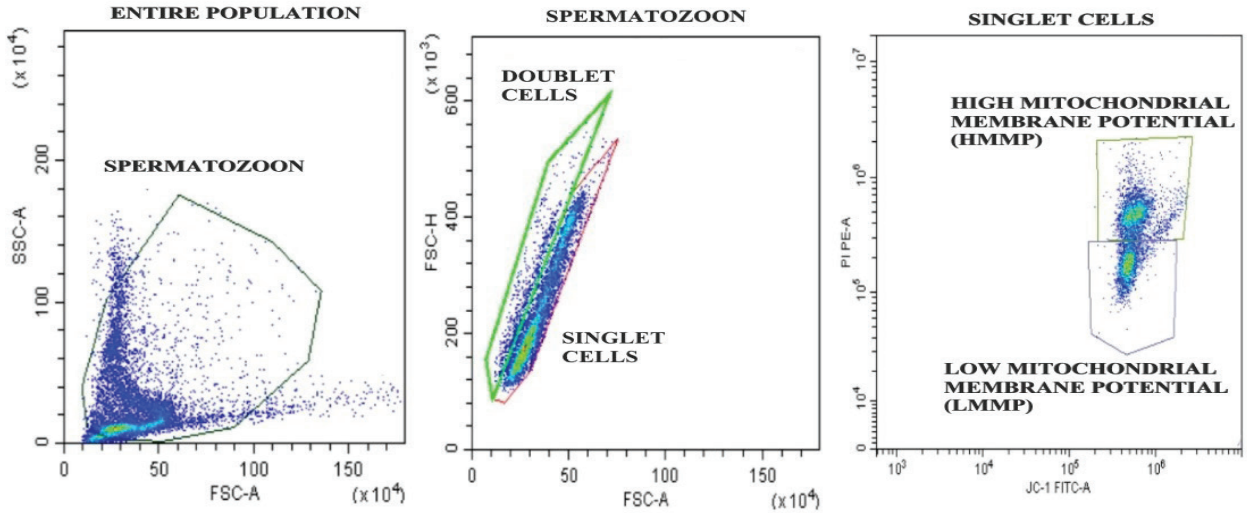

Figure 4: Sequential comparison cytograms used during determination of mitochondrial membrane potential

graphic screen. The two cell groups were collected and the total acrosomal damage rate was expressed as a percentage (*Fig. 3*).

Determination of Mitochondrial Membrane Potential by Flow-cytometry

The analysis was carried out by modifying the method used by İnanç et al. (İnanç et al., 2019) for the determination of mitochondrial membrane potential. 1,1',3,3'- Tetraethyl-5,5',6,6'-tetrachloroimidacarbocyanine iodide (JC-1 Dye, Mitochondrial Membrane Potential Probe, T3168, ThermoFisher Scientific, USA) was used to determine the mitochondrial membrane potential in sperm samples. The same dilution procedures were performed to determine the viability rate. 2.5 µL JC-1 and 2.5 µL PI dye were added to the diluted semen. This mixture was vortexed for one minute and incubated in a dark environment at 38°C for 30 minutes. Again, the same flow-cytometric comparison cytograms that were applied when determining the viability rate were applied. As a result, spermatozoa with high and low mitochondrial membrane potential were displayed in separate compartments on the flow-cytometry device and expressed as a percentage (*Fig. 4*).

Oxidative Stress Analysis

The lipid peroxidation level of semen samples

was determined according to the spectrophotometric method described by Placer et al. (1966). As a result of incubation of semen at 100°C for 1 hour under aerobic conditions and pH 3.5, the pink complex formed by malondialdehyde (MDA), the secondary product of LPO, and thiobarbituric acid (TBA) was measured at 532 nm with a spectrophotometer (UV-Vis Spectrophotometer UV-1700, Kyoto, Japan) device. MDA level was determined as nmol/ml.

Glutathione (GSH) level of sperm samples was determined according to the method specified by Sedlak and Lindsay (1968). For example, the color intensity of the yellow complex formed with 5,5'-dithio-bis (2-nitrobenzoic acid: DTNB) is directly proportional to the GSH concentration in the medium. Therefore, samples were measured spectrophotometrically at a wavelength of 412 nm and the GSH level was determined in nmol/ml.

Glutathione peroxidase (GSH-Px) activity of semen samples was determined as described by Lawrence and Burk (1976). After the samples were mixed with 5,5-dithio-bis (2-nitrobenzoic acid: DTNB) solution as a coloring agent, the yellow complex formed was measured in a spectrophotometer at a wavelength of 412 nm. GSH-Px enzyme activity was determined as IU/g protein.

Catalase (CAT) activity of sperm samples was determined according to the method described by Goth (1991). Semen was incubated with a hydrogen peroxide (H2O2)-containing substrate, and then the H2O2 was broken down into H2O and O2 by CAT activity. The reaction was terminated by combining the added ammonium molybdate with H2O2. The resulting color change was measured spectrophotometrically at a wavelength of 405 nm and catalase (CAT) enzyme activity was determined as kU/g protein.

Statistical Analysis

SPSS (version 22) statistical program was used for statistical analysis. It was considered statistically significant when the p value was less than 0.05. Differences between time periods within the same group were determined with the non-parametric Friedman test. Wilcoxon test was used to compare two time periods in the same group. The non-parametric Kruskal-Wallis test was used to compare groups in the same time period. To compare the two groups, the non-parametric Mann-Whitney-U test was performed.

RESULTS

Motility and Kinematic Values

The effect of different doses of L-carnosine on sperm motility and kinematics after different time periods of storage is described in Tables 1 and 2. It was observed that sperm total motility was statistically significantly increased in 5 mM L-carnosine group compared to the control group after 72 hours of storage **(p <0.001)**. However, sperm total motility was significantly decreased in 100 mM L-carnosine group compared to the control group, after 96 hours of storage **(p <0.001)**.

It was detected that 10 mM L-carnosine increased progressive motility statistically significantly **(p<0.001)** compared to the control group at the 24th hour. Compared to the control group, it was determined that 100 mM L-carnosine dose reduced progressive motility at the 72nd hour.

It was noticed that 100 mM L-carnosine reduced the rate of rapid spermatozoon compared to the control group in all time periods except the 24th hour.

It was observed that 100 mM L-carnosine statistically significantly increased the rate of spermatozoon moving at medium speed compared to the control group at 0, 24 and 48 hours. It was observed that 50 mM L-carnosine increased the rate of medium spermatozoon compared to the control group in all time periods except the 96th hour. It was determined that 200 mM L-carnosine significantly reduced the rate of medium spermatozoon at the 96th hour compared to the control group **(p<0.001)**.

It was determined that 50 mM L-carnosine had a statistically significantly **(p<0.001)** lower VCL level than the control group at 0 and 72 hours. Compared to the control group, it was determined that 100 and 200 mM L-carnosine decreased the VCL value to a statistically significant degree **(p<0.001)** in all time periods except the 96th hour.

While 50 mM L-carnosine significantly **(p<0.001)** decreased the VSL value at the 0th hour, it was found that 100 mM carnosine significantly **(p<0.001)** decreased the VSL value in all other time periods except the 24th hour. On the other hand, 200 mM L-carnosine significantly **(p<0.001)** decreased the VSL value in all time periods compared to the control. It was determined that 50 mM L-carnosine caused a statistically significant decrease in STR values (except for the 0th hour) compared to the control group **(p<0.001).** It was

determined that 100 mM L-carnosine caused a statistical decrease compared to the control group only at the 24th hour **(p<0.001).**

It was detected that in all time periods, 50 (except the 96th hour), 100 and 200 mM L-carnosine significantly reduced the ALH value compared to the control group, and this decrease was statistically significant **(p < 0.001).**

It was noticed that 200 mM L-carnosine decreased the BCF value statistically significantly **(p<0.001)** compared to the control group at the 72nd and 96th hours.

It was observed that 200 mM L-carnosine decreased the values of motility (total, progressive and rapid) and kinematic parameters (VCL and VAP) statistically significantly **(p<0.001)** compared to the control group in all time periods.

Table 1. Effect of different doses of L-carnosine on sperm total motility (TM), progressive motility (PM), rapid motility (RM) and medium motility (MM), after different time periods of storage.

	Storage Periods				
TM (%)	0h	24h	48h	72h	96h
Control	76.56 ± 2.51^{bcA}	57.53 ± 3.85^{bB}	50.41 ± 2.41^{bBC}	38.33 ± 2.99^{bcCD}	34.30 ± 2.24^{cD}
1 mM	79.70 ± 2.62^{bcA}	57.53 ± 4.54^{bB}	55.80 ± 3.45^{bBC}	46.70 ± 3.80^{cdCD}	42.80 ± 3.03^{cD}
5 mM	81.01 ± 2.49^{cA}	63.95 ± 3.85^{bB}	55.53 ± 2.11^{bCD}	54.23 ± 3.37^{dD}	47.30 ± 2.33^{cE}
10 mM	76.88 ± 1.82^{bcA}	66.01 ± 2.91^{bB}	56.35 ± 2.23^{bC}	48.03 ± 3.39^{cdC}	45.70 ± 2.86^{cC}
20 mM	80.38 ± 1.20^{bcA}	68.10 ± 3.14^{bB}	50.20 ± 3.91^{bC}	42.75 ± 3.60^{cdD}	42.33 ± 3.15^{cD}
50 mM	79.05 ± 1.63^{bcA}	65.98 ± 3.75^{bB}	50.35 ± 2.51^{bC}	38.88 ± 2.56^{cD}	32.56 ± 2.96^{bcD}
100 mM	67.50 ± 2.23^{bA}	65.25 ± 3.42^{bB}	39.43 ± 2.10^{bC}	24.41 ± 3.13^{bD}	18.60 ± 2.60^{abE}
200 mM	31.23 ± 2.10^{aA}	18.50 ± 3.62^{aB}	12.40 ± 2.71^{aB}	5.71 ± 1.13^{aC}	3.56 ± 0.55^{aD}
PM (%)	0h	24h	48h	72h	96h
Control	37.41 ± 3.86^{bA}	20.46 ± 2.48^{bcB}	18.83 ± 2.29^{bB}	14.96 ± 1.45^{cdB}	14.43 ± 3.14^{bcB}
1 mM	35.10 ± 2.97^{bA}	25.33 ± 2.11^{bcdB}	21.86 ± 2.70^{bBC}	16.75 ± 0.93^{cdBC}	13.43 ± 2.08^{bcC}
5 mM	36.83 ± 1.88^{bA}	27.75 ± 1.13^{cdB}	22.48 ± 2.75^{bBC}	19.80 ± 1.71^{dC}	17.03 ± 2.96^{cC}
10 mM	33.57 ± 1.84^{bA}	30.23 ± 2.74^{dA}	20.31 ± 2.92^{bB}	18.96 ± 1.77^{dB}	18.26 ± 3.03^{cB}
20 mM	36.16 ± 1.81^{bA}	26.98 ± 1.74^{cdB}	19.98 ± 3.17^{bBD}	14.26 ± 1.79^{cdCD}	14.23 ± 3.21^{bcD}
50 mM	31.06 ± 3.46^{bA}	22.40 ± 2.43^{bcdB}	13.25 ± 1.80^{abC}	9.86 ± 1.02^{bcC}	7.41 ± 1.58^{abcD}
100 mM	28.56 ± 2.48^{abA}	17.83 ± 1.37^{bB}	14.90 ± 1.93^{abB}	6.35 ± 1.18^{abC}	4.66 ± 1.30^{abD}
200 mM	10.60 ± 2.15^{aA}	4.80 ± 1.24^{aB}	4.76 ± 2.53^{aB}	1.25 ± 0.35^{aBC}	0.81 ± 0.17^{aC}
RM (%)	0h	24h	48h	72h	96h
Control	64.81 ± 3.53^{cA}	40.25 ± 3.65^{bcB}	34.43 ± 4.31^{cBC}	25.70 ± 2.79^{bcC}	26.40 ± 4.64^{cC}
1 mM	64.65 ± 3.71^{cA}	46.01 ± 4.82^{bcB}	39.33 ± 3.43^{cB}	31.88 ± 3.36^{bcC}	28.41 ± 4.19^{cBC}
5 mM	68.03 ± 3.67^{cA}	51.10 ± 3.67^{cB}	39.78 ± 3.94^{cC}	36.63 ± 2.37^{cC}	31.76 ± 4.49^{cC}
10 mM	62.53 ± 1.86^{cA}	47.13 ± 3.13^{cB}	38.33 ± 4.62^{cC}	32.88 ± 2.90^{bcC}	30.78 ± 2.94^{cC}
20 mM	65.81 ± 2.34^{cA}	46.43 ± 2.36^{bcB}	33.76 ± 3.45^{cC}	27.36 ± 2.90^{bcD}	26.15 ± 3.96^{cCD}
50 mM	57.96 ± 4.84^{bcA}	44.75 ± 4.38^{bcB}	29.06 ± 3.75^{bcC}	22.15 ± 1.77^{bC}	17.01 ± 2.55^{bcD}
100 mM	44.08 ± 3.76^{bA}	31.51 ± 2.39^{bB}	16.48 ± 2.71^{abC}	9.96 ± 1.57^{aD}	7.75 ± 1.72^{abD}
200 mM	12.45 ± 2.26^{aA}	4.50 ± 1.23^{aB}	2.70 ± 0.65^{aBD}	0.91 ± 0.29^{aC}	1.13 ± 0.41^{aCD}
MM (%)	0h	24h	48h	72h	96h
Control	6.83 ± 0.84^{aAB}	7.93 ± 0.91^{aAB}	7.01 ± 0.63^{abAB}	5.46 ± 1.08^{abA}	7.40 ± 0.56^{bB}
1 mM	9.16 ± 1.03^{abAB}	8.61 ± 0.59^{abA}	8.83 ± 0.67^{bcdA}	7.66 ± 0.82^{bcAB}	6.33 ± 0.78^{bB}
5 mM	7.51 ± 1.60^{abA}	6.90 ± 0.94^{aA}	7.56 ± 0.21^{abA}	8.18 ± 0.52^{bcA}	7.93 ± 0.64^{bA}
10 mM	8.01 ± 1.38^{abA}	7.93 ± 0.58^{aA}	8.61 ± 0.57^{bcdA}	7.55 ± 0.77^{bcA}	7.60 ± 0.73^{bA}
20 mM	8.63 ± 1.33^{abA}	9.75 ± 0.60^{abcA}	8.30 ± 0.34^{bcA}	8.08 ± 0.80^{bcA}	8.20 ± 0.81^{bA}
50 mM	14.61 ± 3.08^{bAB}	13.58 ± 1.56^{cA}	11.78 ± 1.19^{dAB}	9.06 ± 0.56^{dB}	7.48 ± 1.22^{bB}
100 mM	14.83 ± 1.43^{bA}	13.01 ± 1.36^{bcAB}	11.16 ± 0.99^{cdB}	6.23 ± 1.02^{bcCD}	4.83 ± 0.97^{bD}
200 mM	11.48 ± 1.76^{abA}	6.35 ± 1.17^{aA}	4.30 ± 0.85^{aB}	2.36 ± 0.54^{aC}	0.80 ± 0.36^{aD}

a,b,c,d: *The difference between average values with different letters in the same column is important* **(p<0.001).**
A,B,C,D,E: *The difference between average values with different letters within the same row is important* **(p<0.001).**
Values are given as Mean ± SEM.

Table 2. Effect of different doses of L-carnosine on sperm kinematic parameters (VCL, VSL, STR, ALH and BCF) after different time periods of storage.

	Storage Periods				
VCL (μm/s)	0h	24h	48h	72h	96h
Control	137.36 ± 4.73^{dA}	118.31 ± 5.37^{cdB}	110.88 ± 6.32^{cdB}	109.70 ± 6.03^{dB}	88.11 ± 16.64^{abB}
1 mM	129.33 ± 6.06^{cdA}	121.46 ± 6.65^{cdA}	116.73 ± 5.16^{dA}	115.76 ± 2.65^{dA}	109.33 ± 8.32^{bA}
5 mM	138.01 ± 6.58^{dA}	132.43 ± 2.64^{dA}	117.46 ± 3.64^{dB}	113.66 ± 1.80^{dB}	111.61 ± 7.12^{bB}
10 mM	127.86 ± 5.97^{cdAB}	124.66 ± 5.69^{dA}	110.80 ± 4.14^{cdBC}	114.53 ± 4.72^{dABC}	108.41 ± 4.44^{bC}
20 mM	127.18 ± 5.56^{cdA}	115.68 ± 3.03^{cdB}	108.80 ± 4.79^{cdBC}	100.55 ± 3.55^{cdC}	102.48 ± 5.12^{bC}
50 mM	105.86 ± 6.61^{bcA}	103.76 ± 5.63^{bcAB}	91.81 ± 4.73^{bcABC}	92.13 ± 3.97^{bcB}	86.58 ± 1.58^{abC}
100 mM	94.78 ± 1.27^{bA}	93.51 ± 2.33^{bA}	75.33 ± 3.00^{abB}	76.31 ± 2.66^{bB}	75.73 ± 5.97^{abB}
200 mM	71.20 ± 3.65^{aA}	60.08 ± 3.03^{aB}	59.05 ± 3.39^{aBC}	52.15 ± 3.02^{aC}	51.56 ± 7.57^{aCD}
VSL (μm/s)	0h	24h	48h	72h	96h
Control	59.67 ± 2.28^{cA}	39.58 ± 1.87^{bcB}	38.12 ± 1.75^{bcB}	43.72 ± 1.90^{cdC}	34.20 ± 1.90^{bcC}
1 mM	54.17 ± 0.95^{bcA}	46.40 ± 1.43^{cdB}	43.40 ± 1.79^{cB}	42.25 ± 2.94^{dB}	34.25 ± 2.59^{bcC}
5 mM	58.23 ± 3.38^{cA}	51.97 ± 1.74^{dAB}	45.85 ± 3.34^{cBC}	41.40 ± 1.28^{cdC}	38.23 ± 2.37^{cC}
10 mM	57.73 ± 3.31^{cA}	52.37 ± 1.47^{dA}	39.20 ± 2.57^{cB}	46.68 ± 1.56^{cdB}	40.15 ± 2.51^{cB}
20 mM	53.30 ± 1.34^{bcA}	48.83 ± 2.65^{dA}	43.18 ± 2.37^{cB}	34.67 ± 1.59^{bcC}	36.80 ± 1.53^{bcC}
50 mM	45.63 ± 2.69^{bA}	35.87 ± 3.71^{bcB}	28.65 ± 1.79^{abBC}	27.55 ± 1.53^{bcC}	22.57 ± 1.75^{abC}
100 mM	41.03 ± 2.04^{bA}	31.47 ± 2.41^{bB}	26.83 ± 1.96^{aB}	23.92 ± 1.17^{bC}	20.78 ± 1.43^{aC}
200 mM	20.30 ± 4.77^{aA}	20.67 ± 1.32^{aB}	20.90 ± 0.74^{aB}	16.62 ± 1.68^{aB}	15.37 ± 1.52^{aB}
STR (%)	0h	24h	48h	72h	96h
Control	77.26 ± 2.60	74.55 ± 1.95^{b}	75.55 ± 0.71^{b}	74.18 ± 2.71^{b}	72.68 ± 2.80^{b}
1 mM	74.56 ± 2.57	76.58 ± 1.97^{b}	74.68 ± 2.81^{b}	74.51 ± 2.96^{b}	71.03 ± 2.98^{b}
5 mM	76.38 ± 1.93	78.45 ± 2.38^{b}	75.73 ± 2.38^{b}	74.68 ± 2.89^{b}	72.91 ± 2.51^{b}
10 mM	77.61 ± 3.93	79.17 ± 0.99^{b}	73.13 ± 2.52^{b}	75.90 ± 3.92^{b}	73.26 ± 4.13^{b}
20 mM	74.98 ± 2.00^{A}	76.26 ± 1.34^{bA}	73.80 ± 3.31^{bA}	66.75 ± 2.09^{abB}	69.21 ± 3.84^{abB}
50 mM	69.13 ± 2.56^{A}	62.83 ± 1.09^{aAB}	58.10 ± 2.01^{aBC}	57.16 ± 2.13^{aC}	54.31 ± 3.20^{aC}
100 mM	72.43 ± 1.95^{A}	63.31 ± 2.39^{aB}	65.03 ± 1.31^{abB}	59.63 ± 2.05^{abB}	58.36 ± 2.22^{abB}
200 mM	71.26 ± 3.01	74.75 ± 1.81^{b}	73.60 ± 4.50^{b}	69.01 ± 6.55^{ab}	69.55 ± 4.53^{b}
ALH (μm)	0h	24h	48h	72h	96h
Control	4.98 ± 0.14^{bAB}	5.35 ± 0.17^{cA}	5.05 ± 0.16^{cAB}	4.71 ± 0.10^{deB}	4.58 ± 0.34^{cdAB}
1 mM	4.83 ± 0.10^{b}	5.13 ± 0.14^{c}	4.76 ± 0.07^{c}	5.06 ± 0.19^{e}	4.90 ± 0.23^{d}
5 mM	5.05 ± 0.23^{b}	5.30 ± 0.20^{c}	5.00 ± 0.19^{c}	4.88 ± 0.12^{e}	4.88 ± 0.18^{d}
10 mM	4.68 ± 0.17^{bAB}	5.05 ± 0.08^{cA}	4.83 ± 0.17^{cA}	4.68 ± 0.14^{deAB}	4.45 ± 0.18^{cdB}
20 mM	4.60 ± 0.25^{bAB}	4.68 ± 0.13^{cA}	4.46 ± 0.16^{cAB}	3.98 ± 0.28^{cdB}	4.28 ± 0.20^{cdAB}
50 mM	3.66 ± 0.13^{aAB}	3.98 ± 0.15^{bA}	3.48 ± 0.09^{bB}	3.51 ± 0.16^{bcAB}	3.55 ± 0.14^{bcB}
100 mM	3.76 ± 0.17^{aA}	3.46 ± 0.14^{bAB}	3.16 ± 0.09^{abAB}	2.88 ± 0.20^{abB}	3.01 ± 0.29^{bB}
200 mM	3.15 ± 0.06^{aA}	4.45 ± 0.15^{aB}	2.76 ± 0.22^{aAB}	1.51 ± 0.24^{aC}	1.46 ± 0.37^{aC}
BCF (Hz)	0h	24h	48h	72h	96h
Control	9.51 ± 0.57	8.23 ± 0.51	8.21 ± 0.42	8.30 ± 0.24^{bc}	8.46 ± 0.41^{b}
1 mM	9.53 ± 0.51	8.83 ± 0.33	8.25 ± 0.34	8.30 ± 0.34^{bc}	8.36 ± 0.43^{b}
5 mM	9.52 ± 0.55^{A}	8.58 ± 0.41^{AB}	8.53 ± 0.34^{AB}	8.80 ± 0.11^{abB}	8.31 ± 0.30^{bAB}
10 mM	10.11 ± 0.48^{A}	8.76 ± 0.27^{AB}	8.40 ± 0.29^{B}	8.76 ± 0.26^{bcB}	9.21 ± 0.33^{bAB}
20 mM	10.05 ± 0.64	9.08 ± 0.41	9.40 ± 0.31	8.60 ± 0.16^{bc}	9.60 ± 0.44^{b}
50 mM	10.43 ± 0.44	9.70 ± 0.21	9.60 ± 0.61	9.38 ± 0.43^{bc}	9.11 ± 1.00^{b}
100 mM	10.18 ± 0.52	9.78 ± 0.31	9.53 ± 0.40	10.25 ± 0.44^{c}	9.91 ± 0.75^{b}
200 mM	10.15 ± 0.12^{A}	9.03 ± 0.82^{A}	9.98 ± 0.68^{A}	5.86 ± 1.15^{aB}	4.76 ± 1.01^{aB}

a,b,c,d,e: *The difference between average values with different letters in the same column is important* **(p<0.001).**
A,B,C,D: *The difference between average values with different letters within the same row is important* **(p<0.001).** Statistical significance in BCF values is at **p<0.05.**
Values are given as Mean ± SEM.

Values Related to HOS test, pH and Abnormal Spermatozoon Rates

HOS test, pH and abnormal sperm rates in the control and L-carnosine containing groups at different time periods are presented in Table 3. It was observed that 100 mM L-carnosine decreased HOST values at a statistically significant level **(p<0.001)** compared to the control group at 0 hours, and 200 mM L-carnosine decreased HOST values at all time periods.

Compared to the control group, it was found that 20 mM L-carnosine significantly increased pH at 24, 48 and 96 hours, and 50, 100 and 200 mM L-carnosine significantly increased pH in all time periods **(p<0.001)**.

It was determined that in all time periods, all groups except 200 mM L-carnosine (100 mM, except the 96th hour) reduced the rate of abnormal spermatozoon at a statistically significant rate **(p < 0.001)** compared to the control group.

Findings Determined as a Result of Flow-cytometric Analyzes

Flow-cytometric analysis findings of the control and L-carnosine containing groups at different time periods are presented in Table 4. 100 mM L-carnosine significantly (p<0.001) reduced the rate of live spermatozoon at 24 and 48 hours compared to the control group. The increase in the rate of acrosomal damage in the 200 mM L-carnosine group compared to the control at all time periods except the 96th hour was found to be statistically significant **(p<0.001)**. It was determined that 100 mM L-carnosine significantly **(p<0.001)** reduced the high mitochondrial membrane potential at the 48th hour compared to the control group. 100 mM L-carnosine significantly **(p<0.001)**

Table 3. HOST, pH and Abnormal Spermatozoon (AS) Rates of Control and L-carnosine Containing Semens in Different Time Periods

	Storage Periods				
HOST (%)	0h	24h	48h	72h	96h
Control	56.17 ± 4.65^{bA}	38.33 ± 2.56^{bcB}	33.33 ± 3.16^{bcBC}	31.83 ± 2.62^{bcBC}	28.50 ± 2.43^{bcC}
1 mM	62.17 ± 3.20^{bA}	47.67 ± 2.51^{cB}	43.17 ± 2.02^{cBC}	35.83 ± 3.63^{cCD}	34.83 ± 2.33^{cD}
5 mM	67.17 ± 2.96^{bA}	48.17 ± 3.27^{cBC}	45.83 ± 3.26^{cB}	37.00 ± 3.30^{cCD}	33.83 ± 2.86^{cD}
10 mM	59.17 ± 2.19^{bA}	46.00 ± 1.98^{cB}	40.00 ± 3.08^{cBC}	38.17 ± 1.77^{cC}	35.50 ± 2.94^{cC}
20 mM	58.00 ± 3.65^{bA}	49.83 ± 3.56^{cAB}	43.00 ± 3.99^{cB}	42.33 ± 2.91^{cB}	36.00 ± 2.98^{cB}
50 mM	56.83 ± 4.99^{bA}	40.67 ± 2.80^{bcB}	32.67 ± 3.70^{bcBC}	35.00 ± 3.23^{cBC}	28.17 ± 2.10^{bcC}
100 mM	37.67 ± 4.40^{aA}	28.67 ± 4.65^{bAB}	22.83 ± 3.21^{abB}	21.17 ± 2.54^{abB}	17.50 ± 1.92^{abB}
200 mM	20.83 ± 3.92^{aA}	11.17 ± 1.77^{aB}	9.33 ± 1.11^{aB}	10.50 ± 1.78^{aB}	9.00 ± 1.52^{aB}
pH	0h	24h	48h	72h	96h
Control	6.76 ± 0.16^{a}	6.79 ± 0.06^{a}	6.88 ± 0.03^{a}	6.91 ± 0.04^{a}	6.84 ± 0.03^{a}
1 mM	6.84 ± 0.10^{aAB}	6.81 ± 0.07^{aAB}	6.92 ± 0.03^{aA}	6.90 ± 0.03^{aAB}	6.85 ± 0.03^{aB}
5 mM	6.95 ± 0.05^{a}	6.96 ± 0.04^{a}	7.00 ± 0.05^{ab}	6.97 ± 0.04^{a}	6.91 ± 0.04^{a}
10 mM	6.96 ± 0.17^{aAB}	7.09 ± 0.05^{abAB}	7.12 ± 0.04^{bA}	7.09 ± 0.06^{aAB}	6.97 ± 0.06^{abB}
20 mM	7.25 ± 0.07^{abAB}	7.32 ± 0.05^{bcAB}	7.32 ± 0.03^{cA}	7.20 ± 0.05^{aAB}	7.19 ± 0.05^{bB}
50 mM	7.66 ± 0.12^{bc}	7.62 ± 0.08^{c}	7.77 ± 0.02^{d}	7.69 ± 0.11^{b}	7.67 ± 0.04^{c}
100 mM	7.98 ± 0.11^{c}	8.02 ± 0.07^{d}	8.10 ± 0.03^{e}	8.05 ± 0.09^{c}	7.99 ± 0.04^{d}
200 mM	8.18 ± 0.16^{cAB}	8.29 ± 0.08^{dAB}	8.28 ± 0.01^{fA}	8.22 ± 0.09^{cAB}	8.10 ± 0.06^{dB}
AS (%)	0h	24h	48h	72h	96h
Control	14.25 ± 2.28^{aA}	14.75 ± 2.09^{aAB}	16.00 ± 2.12^{aB}	17.00 ± 2.41^{aB}	19 ± 2.58^{abB}
1 mM	5.25 ± 0.94^{cAB}	5.50 ± 0.64^{bA}	6.75 ± 0.25^{bAB}	7.25 ± 0.47^{bB}	8.50 ± 0.28^{cC}
5 mM	5.00 ± 0.91^{cA}	6.25 ± 0.47^{bAB}	7.75 ± 0.25^{bB}	8.50 ± 0.28^{bC}	9.00 ± 0.10^{cC}
10 mM	6.40 ± 0.92^{bcAB}	6.40 ± 0.67^{bA}	7.20 ± 0.58^{bB}	7.80 ± 0.58^{bAB}	8.60 ± 0.98^{cAB}
20 mM	6.33 ± 0.88^{bc}	7.00 ± 1.15^{b}	7.00 ± 2.00^{b}	7.33 ± 0.33^{b}	8.67 ± 0.33^{c}
50 mM	5.80 ± 0.37^{cA}	6.60 ± 0.24^{bAC}	7.80 ± 0.20^{bB}	8.40 ± 0.40^{bB}	8.20 ± 0.80^{cBC}
100 mM	7.83 ± 1.19^{bcAB}	8.17 ± 1.04^{bA}	9.83 ± 1.42^{bA}	10.67 ± 1.30^{bB}	12.50 ± 1.87^{bcB}
200 mM	11.50 ± 0.99^{abA}	14.50 ± 0.61^{aB}	15.50 ± 0.56^{aB}	17.50 ± 0.99^{aC}	19.50 ± 1.17^{aD}

a,b,c,d,e,f: *The difference between average values with different letters in the same column is important* **(p<0.001).**
A,B,C,D: *The difference between average values with different letters within the same row is important* **(p<0.001).**
Values are given as Mean ± SEM.

increased the rate of low mitochondrial membrane potential at 48 and 72 hours compared to the control group.

It was detected that 200 mM L-carnosine significantly **(p<0.001)** reduced the rate of live spermatozoon and high mitochondrial membrane potential compared to the control group in all time periods. Additionally, it was observed that 200 mM L-carnosine caused a statistically significant **(p<0.001)** increase in all time periods.

Oxidative Stress Findings

Oxidative stress findings of the control and L-carnosine containing groups in different time periods are

Table 4. Flow-cytometric Parameter [Viability (Via. %), Acrosomal Damage (AR %), High Mitochondrial Membrane Potential (HMMP %) and Low Mitochondrial Membrane Potential (LMMP %)] Values of Control and L-carnosine Containing Semens in Different Time Periods

	Storage Periods				
Via. %	0h	24h	48h	72h	96h
Control	74.72 ± 1.92^{bAB}	73.79 ± 3.14^{cAB}	72.51 ± 2.66^{cA}	60.95 ± 2.39^{bAB}	59.76 ± 2.41^{bB}
1 mM	78.73 ± 4.26^{bAB}	74.56 ± 2.79^{cAB}	73.53 ± 2.61^{cA}	63.06 ± 2.73^{bAB}	62.12 ± 2.00^{bB}
5 mM	73.08 ± 2.45^{bA}	73.06 ± 2.98^{bcAB}	71.91 ± 2.87^{cA}	64.47 ± 2.43^{bAB}	59.62 ± 2.99^{bB}
10 mM	72.61 ± 3.39^{bAB}	72.31 ± 3.05^{bcAB}	71.06 ± 2.65^{bcA}	66.74 ± 2.54^{bAB}	60.63 ± 2.12^{bB}
20 mM	71.21 ± 2.96^{b}	70.91 ± 3.22^{bc}	69.76 ± 2.19^{bc}	62.12 ± 2.14^{b}	61.83 ± 2.44^{b}
50 mM	68.98 ± 3.17^{7b}	66.41 ± 2.66^{bc}	65.43 ± 2.14^{bc}	63.49 ± 2.99^{b}	59.49 ± 2.68^{b}
100 mM	63.18 ± 3.11^{bA}	60.86 ± 3.63^{bAB}	60.00 ± 2.41^{bA}	51.90 ± 2.38^{abB}	48.34 ± 2.20^{abB}
200 mM	49.44 ± 1.97^{aA}	42.22 ± 2.21^{aA}	40.73 ± 3.02^{aA}	32.36 ± 2.94^{aAB}	25.25 ± 2.75^{aB}
AR %	0h	24h	48h	72h	96h
Control	7.41 ± 0.82^{abA}	14.31 ± 2.03^{abB}	15.50 ± 2.38^{aB}	16.19 ± 1.51^{aB}	19.54 ± 3.51^{abB}
1 mM	6.81 ± 0.31^{aA}	13.33 ± 2.13^{aB}	14.64 ± 1.72^{aB}	14.66 ± 2.66^{aB}	17.65 ± 3.03^{aB}
5 mM	7.10 ± 0.54^{aA}	14.11 ± 2.24^{aB}	14.99 ± 1.98^{aB}	15.36 ± 1.98^{aB}	17.29 ± 2.90^{aB}
10 mM	6.99 ± 0.25^{aA}	13.33 ± 2.12^{aB}	15.20 ± 1.90^{aB}	15.53 ± 1.43^{aB}	17.39 ± 2.53^{aB}
20 mM	6.53 ± 0.42^{aA}	13.63 ± 1.88^{aB}	15.05 ± 1.45^{aB}	15.83 ± 2.21^{aB}	17.12 ± 2.28^{aB}
50 mM	9.51 ± 0.99^{abA}	17.58 ± 2.17^{abB}	17.83 ± 1.53^{aB}	18.58 ± 1.14^{aB}	19.33 ± 2.00^{abB}
100 mM	11.83 ± 1.66^{bcA}	24.54 ± 2.13^{bcB}	25.96 ± 2.98^{abB}	26.49 ± 3.56^{abB}	28.68 ± 2.60^{abB}
200 mM	15.43 ± 1.68^{cA}	33.03 ± 3.16^{cB}	34.03 ± 3.27^{bB}	36.38 ± 5.02^{bB}	37.04 ± 3.54^{bB}
HMMP %	0h	24h	48h	72h	96h
Control	76.12 ± 2.27^{bcA}	80.98 ± 1.43^{bcB}	80.69 ± 2.54^{cB}	$79,05 \pm 3,01^{bAB}$	74.67 ± 3.00^{bA}
1 mM	79.03 ± 1.66^{bcA}	81.68 ± 1.17^{cB}	80.70 ± 2.61^{cAB}	76.91 ± 4.31^{bAB}	77.50 ± 3.05^{bAB}
5 mM	80.01 ± 2.19^{c}	81.95 ± 1.30^{c}	80.74 ± 3.08^{c}	80.16 ± 3.01^{b}	74.85 ± 4.70^{b}
10 mM	78.75 ± 1.19^{bc}	80.86 ± 1.31^{bc}	80.21 ± 1.70^{bc}	79.46 ± 1.81^{b}	77.56 ± 3.24^{b}
20 mM	74.71 ± 2.26^{bcA}	79.44 ± 2.19^{bcB}	80.45 ± 1.89^{cB}	76.84 ± 2.01^{bAB}	76.96 ± 3.16^{bAB}
50 mM	70.87 ± 2.66^{bcA}	75.76 ± 2.37^{bcB}	75.61 ± 2.00^{bcB}	75.44 ± 3.37^{bAB}	73.61 ± 3.02^{bAB}
100 mM	67.92 ± 3.61^{abcAB}	69.34 ± 3.68^{bA}	68.15 ± 3.09^{bAB}	65.27 ± 3.43^{bB}	63.61 ± 4.23^{bAB}
200 mM	61.11 ± 2.87^{aA}	51.96 ± 2.97^{aAB}	45.29 ± 3.79^{aBC}	39.17 ± 2.63^{aBC}	34.12 ± 2.40^{aC}
LMMP %	0h	24h	48h	72h	96h
Control	16.60 ± 1.83^{abAB}	14.28 ± 0.96^{abAB}	14.14 ± 1.63^{aA}	$14.14 \pm 0,96^{aAB}$	19.15 ± 2.77^{aB}
1 mM	15.90 ± 1.56^{a}	13.34 ± 0.97^{ab}	12.90 ± 1.02^{a}	16.70 ± 3.18^{a}	16.00 ± 1.70^{a}
5 mM	15.29 ± 1.71^{aA}	13.02 ± 1.65^{aB}	12.96 ± 1.22^{aAB}	14.94 ± 1.15^{aAB}	15.34 ± 1.95^{aAB}
10 mM	16.31 ± 1.77^{abA}	13.11 ± 1.41^{aB}	13.89 ± 1.61^{aAB}	15.43 ± 1.01^{aAB}	15.00 ± 1.23^{aAB}
20 mM	17.49 ± 0.93^{abAB}	14.54 ± 1.56^{abA}	14.89 ± 1.40^{aA}	17.93 ± 1.71^{abB}	16.58 ± 1.71^{aAB}
50 mM	22.78 ± 1.59^{ab}	19.52 ± 1.83^{ab}	19.76 ± 2.10^{ab}	19.12 ± 2.08^{ab}	20.70 ± 2.05^{a}
100 mM	24.36 ± 1.82^{b}	24.57 ± 3.33^{b}	27.26 ± 2.35^{b}	27.31 ± 2.47^{b}	27.05 ± 1.76^{a}
200 mM	33.17 ± 2.94^{cA}	43.23 ± 5.26^{cA}	50.96 ± 3.39^{cB}	59.27 ± 3.91^{cB}	59.85 ± 6.06^{bB}

a,b,c: *The difference between average values with different letters in the same column is important* **(p<0.001).**
A,B,C: *The difference between average values with different letters within the same row is important* **(p<0.001).**
Values are given as Mean ± SEM.

Table 5. Oxidative Stress Levels of Control and L-carnosine Containing Semens in Different Time Periods.

	Storage Periods							
	MDA (nmol/ml)		GSH (nmol/ml)		GSH-Px (IU/ml)		CAT (IU/ml)	
	0h	**96h**	**0h**	**96h**	**0h**	**96h**	**0h**	**96h**
Control	4.23 ± 0.19	4.04 ± 0.47	2.28 ± 0.18	2.71 ± 0.13	31.17 ± 0.49^{a}	30.88 ± 0.48^{a}	19.26 ± 2.21	24.12 ± 6.56
1 mM	4.21 ± 0.20	4.35 ± 0.23	2.59 ± 0.23	2.64 ± 0.18	33.01±0.66^{Aa}	28.31 ±1.31^{Ba}	18.23 ± 2.35	20.27 ± 2.28
5 mM	4.11 ± 0.17	4.61 ± 0.38	2.42 ± 0.80	2.45 ± 0.16	30.88 ± 1.49^{a}	30.77 ± 0.98^{a}	18.34 ± 2.00	19.94 ± 3.63
10 mM	4.45 ± 0.21	4.75 ± 0.30	2.63 ± 0.19	2.65 ± 0.24	30.65 ± 1.03^{a}	32.85 ± 2.00^{a}	17.77 ± 3.25	15.43 ± 3.16
20 mM	4.16 ± 0.21	4.48 ± 0.34	2.52 ± 0.15	2.85 ± 0.10	30.54 ± 1.61^{a}	28.55 ± 0.72^{a}	19.84 ± 3.29	25.22 ± 2.63
50 mM	4.17 ± 0.23	4.60 ± 0.24	2.69 ± 0.32	2.85 ± 0.08	31.08 ± 1.52^{a}	31.28 ± 1.93^{a}	18.17 ± 2.43	24.02 ± 3.18
100 mM	3.75 ± 0.18^{A}	4.83 ± 0.30^{B}	2.59 ± 0.13	2.72 ± 0.31	35.16±0.61^{Ab}	26.19±1.58^{Bb}	18.65 ± 4.63	25.38 ± 3.86
200 mM	3.82 ± 0.17^{A}	5.08 ± 0.42^{B}	2.62 ± 0.21	2.57 ± 0.26	31.34 ± 1.66^{a}	30.53 ± 1.57^{a}	14.59 ± 1.53	24.30 ± 4.70

A,B: *The difference between average values with different letters in the same column is important* **(p<0.05)**.
a,b: *The difference between average values with different letters within the same row is important* **(p<0.05)**.
Values are given as Mean ± SEM.

presented in Table 5. While no statistical difference could be determined between the groups in MDA values, it was determined that there was a numerical increase in all groups except the control at the 96th hour compared to the 0th hour, and this increase observed in the 100 and 200 mM L-carnosine groups was statistically significant **(p<0.05)**.

When GSH-Px values were examined, it was seen that 100 mM L-carnosine provided a statistically significant **(p<0.05)** increase compared to the control group at the 0th hour and a significant **(p<0.05)** decrease at the 96th hour. Additionally, it was observed that GSH-Px levels of 1 mM and 100 mM L-carnosine decreased statistically significantly **(p<0.05)** at the 96th hour compared to the 0th hour.

DISCUSSION

Low pregnancy rates obtained from intracervical inseminations performed with frozen-thawed ram semen make short-term storage at 5°C more important (Maxwell and Salamon, 1993). Adami et al. (2020) stated that the addition of 20 and 50 mM doses of L-carnosine to human semen increased total and progressive motility compared to the control group. Rocha (Rocha, 2017) measured the carnosine levels in the seminal plasma of semen taken from stallions and determined that the group containing low carnosine concentration had a better total motility than the group containing high carnosine concentration. In our study, positive effects of adding low dose (5 mM) L-carnosine to semen extenders were detected on motility. Our results are not similar to the findings of other authors. It is thought that the reason for this situation is due to species differences. Ram semen has a different lipid composition compared to other species. For this reason, the resulting LPO level is quite high. The antioxidative activity of the L-carnosine substance we used did not reach a sufficient level for a high level of protection. Another reason why our results are not similar to other studies is thought to be due to the difference in the L-carnosine doses used.

In addition, this analysis gives serious results about the important features of spermatozoa, such as reaching the fertilization site and ensuring fertilization, which is their main function. For this reason, this analysis is very important for all spermatological studies. The motility-enhancing effect of L-carnosine used in low doses is thought to be due to its ability to neutralize lactic acid (Davey, 1960), ATP activation for energy conservation (Rocha et al., 2018), and antioxidant properties (Hipkiss et al., 1998; Zhou and Decker, 1999; Barca et al., 2019). This antioxidant effect of L-carnosine comes from its suppression of LPO, the end product of lipooxidation, and its scavenging of ROS and peroxyl radicals (Barca et al., 2019). In addition, L-carnosine, which has the properties of many antioxidant substances, has an anti-aging effect by preventing the shortening of telomeres, a feature that is not found in other antioxidants. It is thought that this anti-aging effect enables motility parameters to be preserved for a longer period of time, and this effect may be due to the ability of L-carnosine to prevent protein-peptide carbonylation by reacting with electrophilic aldehydes and ketones (Hipkiss, 1998). It was determined that the 200 mM dose of L-carnosine decreased total motility, progressive motility and rapid motility values compared to the control group in all time periods. As proven in this study, high doses of L-carnosine change the pH of the environment in an alkaline direction, causing the loss

of conditions necessary for the survival of spermatozoa. Additionally, L-carnosine has the ability to react with substrates of the glycolytic pathway, which will reduce energy production through both glycolysis and oxidative phosphorylation. The main mechanism of ATP synthesis for cellular energy production is the oxidative phosphorylation pathway. During this process, there are some substrates (Citrate, Glucose phosphate, Fructose-2-6-bisphosphate) that will reduce energy production. L-carnosine reacts with these substrates, paving the way for energy production (Holliday and McFarland, 1996). Therefore, it is thought that the addition of high doses of carnosine may cause changes in sperm energy metabolism (Rocha et al., 2018) and, as a result, a decrease in motility. For these reasons, it has been determined that a dose of 200 mM L-carnosine has a toxic effect on spermatozoa.

In order to determine the effects of L-carnosine on human semen, Adami et al. (2020) reported that the addition of 20 and 50 mM doses of L-carnosine increased the level of kinematic parameters VCL, VSL, VAP and BCF compared to the control group. Rocha (Rocha, 2017) suggested in their study that the addition of low doses of carnosine increased spermatozoon kinetics. In this study, positive effects of low-dose L-carnosine use were observed in terms of VCL, VSL and VAP values, as well as motility parameters. This increase in kinematic parameters is thought to be due to the fact that low doses of L-carnosine maintain oxidative balance without disrupting energy metabolism (Rocha et al., 2018) and increase mitochondrial activity (Adami et al., 2020). It was determined that the 200 mM dose of L-carnosine reduced VCL, VSL and VAP values in all time periods compared to the control group and caused a toxic effect. This decrease may be associated with the increase in the pH value of the semen in the alkaline direction in parallel with the increase in the dose of L-carnosine. In the current study, it was determined that the 200 mM L-carnosine dose decreased the BCF value compared to the control group at the 72nd and 96th hours. However, it was determined that the other doses provided a numerical increase in the BCF value compared to the control group at all hours. The results of our study and the BCF levels of Adami et al. (2020) study are similar. It is thought that this increase in BCF level may be related to the increase in mitochondrial activity (Adami et al., 2020), as in other kinematic parameters. BCF value expresses the average crossing frequency of the spermatozoon head as it moves along the curvilinear path. This determined value is very important in terms of examining the direction changing rhythm of tail movements (Sönmez et al., 2022). This numerical increase observed in the BCF level shows that spermatozoa in groups containing L-carnosine can perform stronger whipping movements. It is thought that L-carnosine may achieve this effect thanks to its contribution to energy metabolism (Rocha et al., 2018). In this study, it was determined that 50, 100 and 200 mM L-carnosine doses decreased the ALH value compared to the control group in all time periods. ALH data obtained during short-term storage by adding L-carnosine to ram semen extenders are the first data in the literature. It is thought that this decrease may occur as a result of high doses of L-carnosine causing changes in the energy metabolism of spermatozoa (Rocha et al., 2018).

Membrane integrity is very important for spermatozoa. In particular, the unique lipidic structure of ram semen becomes even more important for this species, as the ROS level that occurs during the cooling of the semen causes damage to the membrane surface. Kawai (Kawai, 2017) added 1, 50 and 100 mM doses of carnosine to stallion semen and determined that the addition of 50 mM carnosine reduced sperm plasma membrane damage compared to the control group. Fattah et al. (Fattah et al., 2017) added different doses of L-carnitine (isolated from the same tissue as L-carnosine) to semen extenders during short-term storage of rooster semen. They determined that 1 and 2 mM L-carnitine protected membrane integrity better in semen stored for 48 hours after supplementation. In the presented study, it was determined that 1, 5, 10 and 20 mM L-carnosine doses provided a numerical increase by preserving spermatozoon membrane integrity in all time periods compared to the control group, while 200 mM L-carnosine dose caused decrease. It is thought to be effective in protecting membrane integrity due to the basic antioxidant properties of carnosine, which easily donates hydrogen to free radicals (Kohen et al., 1988), has a scavenging effect on hydroxyl, peroxyl, superoxide radicals and singlet oxygen (Xie et al., 2013), and has a membrane stabilizing effect (Cho et al., 1991).

The most suitable living environment pH for spermatozoa is neutral or near-neutral pH levels. Thanks to the metabolic activity of spermatozoa in the environment, the pH level shifts towards acidity over time. The most important reason for this situation is lactic acid. It is aimed to benefit from the lactic acid neutralizing feature of L-carnosine (Davey, 1960). In

the study, the pH levels showed statistically insignificant increases in the 1, 5, and 10 mM L-carnosine doses (excluding the 48th hour) when compared to the control group. However, there was a statistically significant increase observed in the 20 mM dose at 24, 48, and 96 hours, as well as in the 50, 100, and 200 mM L-carnosine doses. These increases are thought to be due to the reported ability of L-carnosine to buffer pH (Barca et al., 2019) and neutralize lactic acid (Davey, 1960).

In their study to improve semen quality in ducks, Al-Daraji and Tahir (Al-Daraji and Tahir, 2014) reported that adding 50, 100 and 150 mg/kg L-carnitine to the daily diet reduced the rate of abnormal spermatozoon compared to the control group. In their study to investigate the effect of L-carnitine on semen quality in pigs, Jacyno et al. (Jacyno et al., 2007) reported that the addition of 500 mg L-carnitine to the daily diet resulted in a decrease in the rate of abnormal spermatozoon in the semen collected for 5 weeks compared to the control group. In our study, it was determined that all doses except the 200 mM L-carnosine dose (100 mM, except the 96th hour) in all time periods reduced the rate of abnormal spermatozoon at a statistically significant rate ($p < 0.001$) compared to the control group. The results of our study are compatible with the literature information. This decrease in the proportion of abnormal spermatozoon may be associated with the membrane stabilizing effect (Cho et al., 1991) of L-carnosine.

Fattah et al. (Fattah et al., 2017) determined that the addition of 1 and 2 mM L-carnitine to rooster semen stored for 48 hours caused an increase in viability, membrane integrity and mitochondrial activity parameters and a decrease in the LPO rate compared to the control group. In our study, it was determined that 1, 5, 10 and 20 mM doses of L-carnosine numerically reduced the acrosomal damage rate (except for the 72nd hour) compared to the control group. Additionally, it was determined that 1 and 5 mM L-carnosine doses increased the high mitochondrial membrane potential (except for the 48th hour), while 1, 5 and 10 mM L-carnosine doses decreased the low mitochondrial membrane potential. The effects of 1, 5, 10 and 20 mM L-carnosine doses are similar to the study conducted by Fattah et al. (Fattah et al., 2017) and the current study. The positive effects determined on high mitochondrial membrane potential can be explained by the fact that L-carnosine increases mitochondrial functions and energy production as a result of its effect on Cytochrome C (Baek et al., 2014) and its membrane stabilizing effect (Cho et al., 1991). It was determined that 200 mM L-carnosine dose increased the dead spermatozoon rate, acrosomal damage rate (except the 96th hour) and low mitochondrial membrane potential in all time periods compared to the control group, and decreased the live spermatozoon rate and high mitochondrial membrane potential. It is thought that these negative effects caused by the 200 mM dose of L-carnosine occur as a result of the pH of the environment increasing in the alkaline direction and causing changes in sperm energy metabolism (Rocha et al., 2018). In this study, compared to the control group, 100 mM L-carnosine caused a significant increase in the GSH-Px level at the 0th hour and a significant decrease in the level at the 96th hour. The differences observed between the groups in both time periods in terms of MDA, GSH and CAT values did not reach statistical significance. The reason for the significant differences in GSH-Px levels is not fully known and more detailed molecular analyzes are needed.

CONCLUSIONS

In conclusion, high doses of L-carnosine (200 mM) in semen extenders are harmful to ram semen characteristics after a short time storage. Moreover, low doses of L-carnosine (1, 5, 10 and 20 mM) supplementation may have beneficial effect on ram semen traits, depending on the time storage.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This article was produced from the doctoral thesis. I would like to thank the Scientific and Technological Research Council of Turkey (TÜBİTAK) for the funding support that enabled us to complete this thesis.

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