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The Effect of L-Carnitine and Melatonin on Sperm Quality Parameters, DNA Fragmentation and Membrane Lipid Peroxidation during Sperm Freezing Process

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ABSTRACT

Background and Objective: During sperm freezing, its functional indices, including survival and motility, are damaged and lead to a decrease in sperm quality and fertility. Studies have been performed to lower these damages, including adding antioxidants to the freezing environment. This study was conducted to investigate the effects of antioxidants melatonin and L-carnitine on the quality of sperm parameters after freezing and thawing.

Methods: In this interventional study, 40 sperm samples were divided into 4 groups including: control group (no antioxidant), melatonin group (0.01 mmol melatonin), L-carnitine group (100 µmol L-carnitine) and combined group (0.01 mmol melatonin and 100 µmol L-carnitine). The samples were thawed two weeks after freezing, then the motility, survival and morphology parameters were evaluated by Eosin-nigrosin staining and were counted. Lipid peroxidation was evaluated by measuring MDA with thiobarbituric acid reaction and DNA fragmentation through Tunnel kit.

Findings: The results showed that the combined use of melatonin and L-carnitine in the freezing medium had a significant effect on the rate of motility and survival (increase from 15% to 25%), DNA fragmentation (decrease from 32% to 23%) and MDA level (decrease from 10 to 6.5 µm/dl). However, the examination of sperm morphology did not show any significant difference between the studied groups.

Conclusion: The results of the study showed that melatonin and L-carnitine can reduce the damage caused by sperm manipulation during the freezing and thawing process.

Keywords: *Melatonin, L-Carnitine, Sperm Freezing, Apoptosis.*

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Introduction

Infertility currently affects about 15% of couples worldwide regardless of race and ethnicity (1). Sperm freezing (sperm cryopreservation) is widely used as a routine technique at the clinic for fertility preservation and infertility treatment and to establish donor banks (2). In the 1960s, cryopreservation was introduced as a valuable method to preserve fertility in men (3). But this process causes damage to sperm parameters. In the study of Karimfar et al. on human sperm, who evaluated sperm parameters before and after freezing, the survival rate and motility in frozen sperm were significantly reduced compared to fresh sperm. Also, the amount of lipid peroxidation in frozen sperm was increased compared to fresh sperm, which shows that sperm freezing can have destructive effects on sperm parameters (4).

Oxidative stress is a powerful mechanism that degrades nucleic acid in spermatozoa. This condition is caused by the high concentration of reactive oxygen species (ROS) and its imbalance with antioxidant defense (5). During freezing, the amount of ROS in human sperm is overproduced. ROS causes lipid peroxidation in the plasma membrane of sperm, disrupts membrane fluidity and the activity of membrane enzymes and ion channels, and as a result causes poor sperm motility and fertility (6). Adding antioxidant compounds in the freezing medium is a specified method to prevent oxidative stress (7). Melatonin is mainly produced from the pineal gland (8). In addition to several known physiological functions, melatonin has a significant antioxidant role through direct scavenging of free radicals (9).

In previous studies, the use of antioxidants melatonin and L-carnitine has been able to improve the quality of sperm parameters. In the study of Najafi et al., Karimfar et al., Pariz et al., and Deng et al. on human sperm, the antioxidant melatonin was able to have positive effects on sperm quality after thawing (3, 4, 10, 11). L-carnitine is an essential and natural amino acid that is made in the body from lysine and methionine. L-carnitine facilitates and increases the entry of long chain fatty acids into mitochondria (12). In addition, its antioxidant activity has also been reported (13). Also, in the study of Ghorbani et al. and Chavoshi Nezhad et al. on human sperm, L-carnitine was able to have positive effects on sperm quality after thawing (13, 14). Despite the investigation of these antioxidants alone, the combined effect of melatonin and L-carnitine antioxidants has not been evaluated so far. Therefore, the aim of this study is to investigate the combined effect of these two antioxidants on sperm parameters after thawing.

Methods

After approval by the Ethics Committee of Qazvin University of Medical Sciences with the code IR.QUMS.REC.1400.322, this interventional study was conducted on 40 volunteers aged 20-40 who referred to a private histopathology laboratory in Qazvin. People were given a checklist to complete the information including history of smoking, drug addiction, alcohol and drug use, no history of surgery such as varicocele, diseases such as diabetes and oligospermia. People who did not have a history of any of the above cases were included in the study, and it was also recommended to avoid intercourse for 3-5 days. After obtaining informed consent, the samples were collected in plastic containers with lids and incubated for 15 to 30 minutes in an incubator with a temperature of 37 °C and 5% CO2 and turned from gel to liquid and sent to the embryology laboratory of Qazvin University of Medical Sciences.

During the freezing stage, each sample was poured into the falcon tube in the required amount and under sterile conditions of washing medium (origio) and 20% albumin was added to it in a ratio of 10 to 1 to dilute the sperm and the tube was centrifuged at 2500 rpm for 5 minutes. After centrifugation, the sperms were deposited in the form of a plate at the bottom of the tube, and the deposited layer contains healthy sperms. At this stage, some of the liquid on the top of the tube was discarded, and the remaining liquid inside the

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tube was mixed with the deposited plate again by shaking the tube and the washing process was done again, and finally, this obtained suspension was divided into four groups under the hood and transferred to the cryotube, and the freezing medium (origio) was added to them drop by drop and one drop every 30 seconds. These four groups include the control group (C) (without antioxidants and only with freezing medium), melatonin group (M) (with freezing medium and 0.01 mmol or 10 µmol of melatonin (4)), L-carnitine group (L) (with freezing medium and 100 µmol L-carnitine (14)) and the combined group (ML) (with freezing medium and both antioxidants with previous doses). Antioxidants were obtained from Behnogen company. Antioxidants were dissolved in 96% ethanol. Then the cryotubes were shaken for 3 minutes in the laboratory environment and placed at -5°C for 3 minutes, then induced for 1 minute with pre-cooled forceps and mixed again for 7 minutes at room temperature and was placed at -5°C and the cryovial containing the mixture was placed in nitrogen vapor for 30 minutes and finally the cryovial was immersed in the nitrogen tank along with the cryocane.

After two weeks, the cryotubes were removed from the nitrogen tank and placed in a water container at 37°C for 10 minutes at room temperature and then 5 minutes and the samples were melted. Again, the samples were washed with the sperm washing medium and placed in a centrifuge at 1500 rpm for 5 minutes, the liquid on the plate consisting of healthy sperms was discarded, and again the sperm washing and centrifuge at 1500 rpm was done for 5 minutes according to the above protocol and finally the sperm settled at the bottom of the falcon tube ready for analysis were analyzed in terms of movement, viability, shape, lipid peroxidation rate and its final product malondialdehyde and DNA fragmentation.

In order to evaluate the sperm motility, 10 samples from each group were placed on slides and a lamel was placed on it, and the slides were marked according to the group. Sperm motility was examined under the microscope with x40 magnification using the CASA (Computerized Sperm Analysis) device based on World Health Organization (WHO) criteria as progressive, non-progressive and non-motile. In this study, progressive and non-progressive sperm were considered as overall mobility.

Viability was assessed using eosin-nigrosin staining (Hancock Model Cytology and Histology). 25 microliters of 1% eosin Y were mixed with 50 microliters of the melted suspension, and after 30 seconds, 25 microliters of 10% negrosin was added to this mixture, and after 30 seconds from this mixture, 4 smears were obtained on marked slides. After drying the smears at room temperature, 200 sperms were counted under a light microscope with x100 magnification. Sperm morphology was evaluated by hematoxylin staining. After preparing a smear from the sample and drying it, it was washed with ethanol. After drying, it was stained with hematoxylin and sperm was counted under a light microscope with x100 magnification.

Evaluation of lipid peroxidation (MDA) was evaluated using sperm reaction with TBA (Thyobarbituric Acid). One milliliter of the sample was mixed with one milliliter BHT (Butylated Hydroxyltoulene), one milliliter EDTA (Ethylenediamintetraacetic Acid) and two milliliters TCA (Trichloro acetic acid), respectively. Then the samples were centrifuged at 1200 rpm for 15 minutes. One milliliter of supernatant was mixed with one milliliter of TBA in a microtube. Then they were closed in microtubes and kept in a hot water bath at a temperature of 95 degrees for 15 minutes and kept at room temperature for 5 to 10 minutes. Then one milliliter of the resulting sample was poured into a cuvette. The concentration of MDA in the samples was determined by a spectrophotometer at a wavelength of 532 nm.

TUNNEL method and cell kit (Roche, Mannheim, Germany) were used to examine DNA damage. For this purpose, 20 microliters of each sample washed with PBS buffer was prepared as a smear on a clean, fat-free slide. After drying, it was washed with 4% paraformaldehyde and for 45 minutes, this substance was floated on the fixed sperm. After that, each slide was washed three times with PBS1X buffer (Phosphate-Buffered Saline) every five minutes. After one hour, 0.1% sodium citrate and Triton X100 were

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poured on the slides. Then, each slide was washed three times with PBS1X buffer at five-minute intervals and dried. The enzyme containing deoxynucleotide was added to the buffer medium in a ratio of 1 to 10 inside a microtube. Then this mixture of enzyme and buffer was poured on each slide in a dark environment and left in the incubator for half an hour. After that, each slide was washed every five minutes with PBS1X buffer and counted under a fluorescent microscope. Sperms that emitted green light were considered as having DNA fragmentation (15). TUNNEL method and cell kit (Roche, Mannheim, Germany) were used to investigate DNA damage. The enzyme was added to the buffer medium at a ratio of 1 to 10 inside a microtube. Then this mixture was poured on each slide in a dark environment and left in an incubator for 30 minutes. After that, each slide was washed with PBS1X buffer every five minutes and counted under a fluorescent microscope. Statistical analysis of the results was done by ANOVA method in SPSS software and Tukey HSD post hoc test and $p<0.05$ was considered significant.

Results

The results of the present study showed that the addition of melatonin and L-carnitine with millimolar and micromolar doses, respectively, to the freezing medium, either individually or in combination, significantly ($p<0.05$) increased total motility and survival compared to the control group and also decreased DNA breaks and MDA resulting from lipid peroxidation of sperm membrane (Table 1). The results showed that the increase in sperm motility in groups M, L and ML had a significant increase compared to group C ($p<0.05$). Also, there were significant changes in comparing two groups ($p<0.05$). However, there were no significant changes in the comparison between M and ML groups (Table 1). The results of sperm survival showed that survival in groups M, L and ML increased significantly compared to group C ($p<0.05$). Also, there were significant changes in the comparison of two groups. However, there were no significant changes in the comparison between M and ML groups (Figure 1, Table 1).

The results of sperm morphology investigation showed that in groups M, L, and ML, there was a significant difference in increasing the quality of morphological parameters compared to group C. This was despite the fact that no significant changes were seen between the treated groups (Figure 2, Table 1). The results of examining the amount of MDA resulting from the lipid peroxidation of the sperm membrane showed that MDA production showed a significant difference between all groups with group C ($p<0.05$). Also, there was the greatest decrease in MDA production in ML, M and L groups respectively (Table 1). The results related to the amount of DNA breaks showed that the amount of DNA breaks in groups M, L, and ML was significantly reduced compared to group C, as well as a two-by-two comparison of the treated groups ($p<0.05$) (Figure 3, Table 1).

Table 1. Comparison of sperm motility, survival, morphology, lipid peroxidation and DNA damage in the control group and groups receiving antioxidants

Groups	Mobility Mean±SD	Survival Mean±SD	Morphology Mean±SD	Lipid peroxidation (MDA) Mean±SD	DNA breaks Mean±SD
Control (D)	15.70 ± 2.3555^{a}	20.2750 ± 2.6984^{a}	7.1512 ± 0.0297^{a}	9.6258 ± 0.0287^{a}	31 ± 2.4910^{a}
Melatonin (A)	23.90 ± 2.4889^{b}	25 ± 2.4390^{b}	7.1553 ± 0.0233^{a}	7.8370 ± 0.0226^{b}	26.6250 ± 2.4980^{b}
L-Carnitine (B)	20.8250 ± 2.9689^{c}	22.6250 ± 2.8795^{c}	7.1525 ± 0.0318^{a}	8.1073 ± 0.0236^{c}	28.60 ± 2.2735^{c}
Melatonin-L-Carnitine (AB)	24.50 ± 2.4910^{b}	26.425 ± 2.3412^{b}	7.1575 ± 0.0264^{a}	6.4647 ± 0.0279^{d}	22.10 ± 2.1697^{d}

The difference of letters in a column indicates a significant difference to each other.

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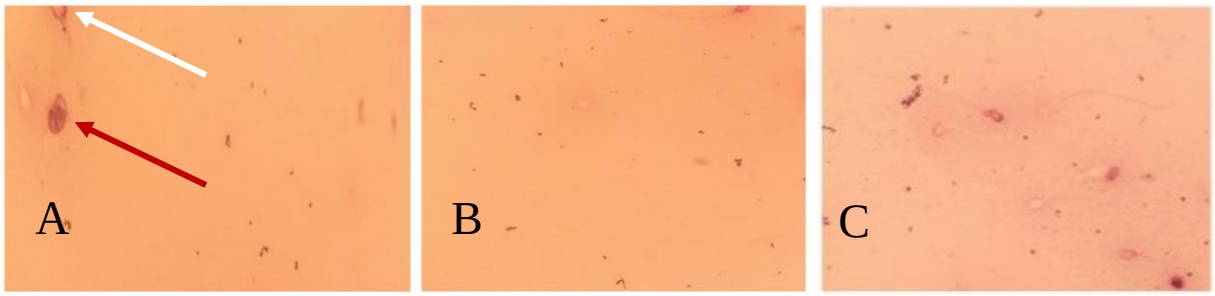

Figure 1. Sperm image after eosin-nigrosin staining

White arrow: live sperm with white head, red arrow: dead sperm with red head. A: melatonin group, B: L-carnitine group, C: combined group

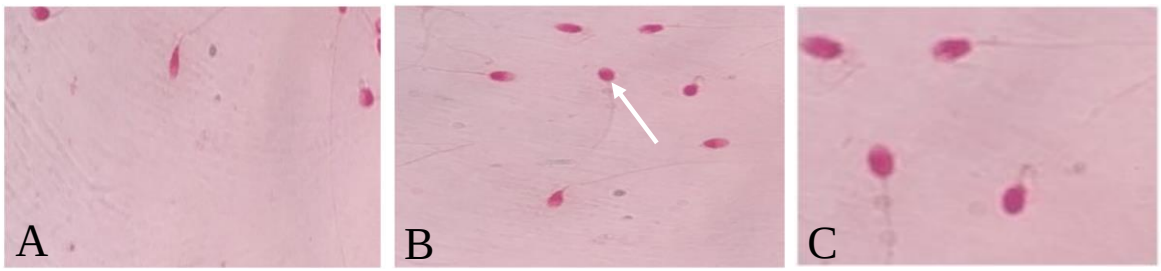

Figure 2. Image of sperm after hematoxylin staining

Sperm with a round head are considered abnormal. A: melatonin group, B: L-carnitine group, C: combined group

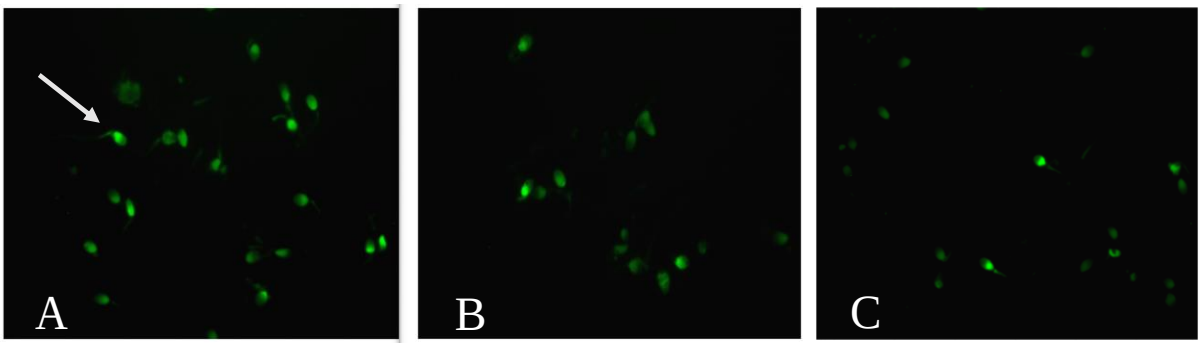

Figure 3. The image of sperm after staining with the tunnel kit

(White arrow indicates sperm with DNA breaks). A: L-carnitine group, B: Melatonin group, C: Combined group

Discussion

The results of this study showed that adding melatonin and L-carnitine individually and in combination to the freezing medium in men with normal sperm can improve the motility of the whole sperm, increase survival and decrease death, decrease the amount of DNA breaks and decrease the peroxidation of membrane lipids. These effects may be due to the antioxidant activity of melatonin and L-carnitine. Sperm freezing and thawing, mechanical damage, cold shock and exposure to oxygen atmosphere, in turn, increase the sensitivity to lipid peroxidation and cause more ROS production. Although ROS are important for various physiological functions, excessive amounts contribute to oxidative stress. The mechanism of action of ROS includes the lipid peroxidation of the sperm plasma membrane, which is very prone to oxidative damage due to the presence of a large amount of unsaturated fatty acids in its membrane, and this issue can

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have a negative effect on sperm motility, membrane fluidity, and its fertilization ability. In addition, ROS can damage sperm axoneme proteins by reducing the phosphorylation of proteins and accelerating the consumption of ATP, thus causing disruptions in the function of mitochondria and DNA. According to the results obtained from this study, adding these antioxidants to the freezing environment will not affect sperm morphology, and melatonin has a somewhat better antioxidant effect than L-carnitine. Therefore, the use of the combination of these two antioxidants may be able to reduce the harmful effects of the freezing and thawing process to some extent and help improve sperm performance. Melatonin has been noted for its anti-inflammatory, anti-apoptotic (programmed cell death), anti-cancer activities, and also removal and neutralization of free radicals such as hydroxyl, peroxyl and peroxynitrate anions. Melatonin itself can indirectly stimulate several antioxidant enzymes such as glutathione peroxidase, reductase, catalase and superoxidase dismutase. The study of Chavoshi Nejad et al. on human sperm L-carnitine was able to improve sperm survival and motility, which is in line with our study (14). The same concentration in this study has resulted in the same results as our study, but the results of some past studies are inconsistent with our results.

In the study of Alcay et al. on honey bee sperm, the amount of DNA damage in all groups was not significantly different, and the non-uniformity of the concentration in this study compared to our study caused inconsistent results (16). Also, in another study conducted by ChaithraShree et al. on cow sperm, adding melatonin to the freezing medium was able to improve sperm motility and maintain the integrity of the plasma membrane and acrosome at a concentration of 0.1 mmol, which is close to the concentration in this study, and has led to the same results with our study (17). In the study of Karimfar et al., the amount of sperm parameters changed significantly, which was consistent with our study. One of the reasons for being consistent with our study is that their concentration in this study was the same as our concentration (4). Also, in this study, L-carnitine was able to significantly improve sperm parameters, except morphology after thawing. In the studies conducted in the past, such as the results of the present study and similar to the data of the study by Inyawilert et al., who froze buffalo sperm and thawed the samples after 1 day, 7 days, 15 days, and 30 days, it was shown that in groups of treatment with melatonin with 4 different concentrations, positive effects were found on sperm motility compared to the control (18).

Also, in the study of Karimfar et al., melatonin had an effective antioxidant effect on human sperm in reducing the amount of MDA, and the amount of MDA in the treatment groups with melatonin was dependent on the concentration, which was significantly reduced at the desired concentration in our study; similar concentration in this study compared to ours has caused the results to be the same as our results (4). However, in some studies, melatonin as a supplement in the freezing environment has not been able to have a significant effect on the parameters of the sperm after thawing. In the study of Chen et al., which was conducted on mouse sperm, the samples were divided into fresh, control, and melatonin groups containing 0.12, 0.25, and 0.5 mg/ml. The results of the studies showed that the addition of melatonin could not have a significant effect on the survival rate after thawing, and the difference in the concentration in this study and our study caused the results to be different (19). Another study was conducted on the effect of melatonin on frozen and thawed goat sperm. The samples were divided into several control groups, 0.5, 1, 2 and 4 mmol. According to the results obtained from the study, melatonin could not have a positive effect on semen, even in some cases (4 mmol) it could be harmful for semen (20). Oral intake of L-carnitine has been able to improve sperm quality in idiopathic asthenozoospermia (causes unknown) (21). Researchers have found that L-carnitine has a therapeutic role in azoospermia patients because it can increase sperm count both in these patients and in patients who exhibit abnormal sperm maturation (22). The effect of L-carnitine on improving human sperm motility in oligozoospermia, asthenozoospermia and teratozoospermia individuals has been reported in both humans and mice. Researchers have shown that the consumption of L-carnitine

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in oligospermic adult mice reduces the toxic effects of busulfan and preserves cell energy (16, 23, 24). In our study, L-carnitine had a positive effect on motility, survival, and reduction of MDA and reduction of DNA breaks, which has been proven in many studies as an antioxidant. However, in some studies that have been done in the past, the results obtained are contrary to the results of the present study. In the study of Alcay et al. conducted on bee sperm, the amount of DNA damage in all groups did not differ significantly after melting, and the difference in concentration in this study with our study has caused non-identical results (16). The result obtained in this study showed that the combination of these two antioxidants can play a more effective role in improving most sperm parameters compared to using them individually.

In fact, many antioxidants have been used to treat infertile people and improve sperm parameters, DNA health, and fertility, so that satisfactory results can be achieved by reducing the level of oxidative stress (25, 26). But the important point is that the type of infertility, the severity of infertility, the type of antioxidant chosen, the dose of antioxidant and the duration of its use are very important and the difference in the results of the articles depends on these factors. There is still no meta-analysis that determines which antioxidants are useful for each group of infertile people, but this point should be kept in mind that the best duration of antioxidant use is between 3-6 months. It is possible that excessive consumption of antioxidants leads to destructive effects on the physiological level of ROS, and therefore reverse effects occur, and natural processes such as capacitation, acrosome reaction, which require physiological ROS, cannot achieve successful conception and fertility due to the reduction of ROS level (27).

The results of this study show that although the body needs oxygen to carry out chemical reactions, in some conditions, such as manipulation of sperm in the melting and freezing process, oxygen oxidation occurs and excessive production of ROS leads to the damage of vital macromolecules, including protein, lipids, carbohydrates and nucleic acids. These damages in sperm, in addition to DNA damage, can cause the oxidation of membrane unsaturated fatty acids and membrane damage, which itself affects the connection of sperm with the egg and all subsequent processes related to fertilization, and in addition, it can reduce the life and motility of sperm, and ultimately lead to a decrease in natural fertility. Knowing the cellular and molecular pathways of these processes leads to the use of therapeutic solutions, including the use of antioxidants (melatonin and L-carnitine) to minimize the amount of damage to sperm during the freezing and thawing process, which can partially reduce the harmful effects of this process.

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