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The antioxidant roles of L-carnitine and N-acetyl cysteine against oxidative stress on human sperm functional parameters during vitrification

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Objective: Amino acids can protect sperm structure in cryopreservation due to their antioxidant properties. Therefore, the present study aimed to investigate the protective effect of L-carnitine (LC) and N-acetyl cysteine (NAC) on motility parameters, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), DNA damage, and human sperm intracellular reactive oxygen species (ROS) during vitrification.
Methods: Twenty normal human sperm samples were examined. Each sample was divided into six equal groups: LC (1 and 10 mM), NAC (5 and 10 mM), and cryopreserved and fresh control groups.
Results: The groups treated with LC and NAC showed favorable findings in terms of motility parameters, DNA damage, and MMP. Significantly higher levels of intracellular ROS were observed in all cryopreserved groups than in the fresh group ($p \leq 0.05$). The presence of LC and NAC at both concentrations caused an increase in PMI, MMP, and progressive motility parameters, as well as a significant reduction in intracellular ROS compared to the control group ($p \leq 0.05$). The concentrations of the amino acids did not show any significant effect.
Conclusion: LC and NAC are promising as potential additives in sperm cryopreservation.

Keywords: Acetylcysteine; Carnitine; Oxidative stress; Spermatozoa; Vitrification

Introduction

Sperm cryopreservation is an effective and useful solution for various conditions such as chemotherapy treatments, donor semen preservation, and infertility surgery that may affect sperm quality [1]. Successful sperm cryopreservation affects the results of infertility treatment [2]. Despite being effective, however, cryopreservation can have adverse effects on sperm functional parameters, as the process of semen cryopreservation can cause the formation of ice crystals, which affect sperm viability [3]. Some research has explored ways to prevent the formation of ice crystals during the process of cryopreservation, and the vitrification technique has been proposed [4]. In the vitrification technique, the processed samples are directly transferred into a liquid nitrogen container [5]. In addition to reducing the damage caused by cryopreservation, this method can be implemented in less time than with conventional methods of sperm cryopreservation, and it is also cheaper [6,7]. It has been found that reactive oxygen species (ROS) are produced during the freeze-thaw process. The unique characteristics of sperm cells, such as a large number of mitochondria and low levels of antioxidants, place sperm at an elevated susceptibility to damage caused by free radicals [8]. The sperm plasma membrane in fresh semen is partially protected against damage by the antioxidant system [8-10], but the antioxidant protection of the sperm plasma membrane decreases during cryopreservation; spermatozoa are largely deprived of protection,

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and finally, an increase in lipid peroxidation occurs [8]. Researchers have found that ultra-rapid cryopreservation (vitrification) reduces damage to the plasma membrane, motility, mitochondrial membrane integrity, and DNA damage [11]. Amino acids can be used in many ways to increase sperm resistance to cryopreservation cold shock. L-carnitine (LC) is a water-soluble amino acid with an IUPAC name of (3R)-3-hydroxy-4-(trimethylamine)butanoate and a structure derived from the amino acid lysine [8,12]. LC facilitates and increases the entry of long-chain fatty acids into the mitochondria [13]. LC is produced in mammalian epididymal tissue and transported to sperm. Increasing the concentration of LC at the epididymal level causes an increase in sperm motility [14]. Moreover, LC has also been reported to have antioxidant activity [15]. *In vivo* and *in vitro* studies have reported that through its antioxidant activity, LC reduces oxidative stress that leads to DNA damage [16,17].

N-acetyl cysteine (NAC) is a thiol-containing compound with strong antioxidant properties. NAC is a precursor to L-cysteine, which plays a role in eliminating free radicals by reacting with ROS. During oxidative stress, the decreased concentration of glutathione is compensated for by the use of NAC as an antioxidant. NAC prevents the sedimentation of membrane proteins in sperm cryopreservation and increases the amount of membrane proteins during cold shock [18].

This amino acid plays an antioxidant role against ROS activity. Numerous studies have documented the effect of NAC in cryopreservation media, leading to improved sperm functional parameters [19]. This study aimed to evaluate the antioxidant effect of NAC and LC on sperm parameters during cryopreservation and to determine the effect of different doses of these amino acids on motility, plasma membrane potential, mitochondrial membrane potential (MMP), intracellular ROS, and DNA damage.

Methods

1. Subject and semen collection

This study was carried out at the Research Center of Tehran University of Medical Sciences. Twenty normal sperm samples were prepared from the Aban Infertility Center from February 2020 to April 2020. This study was approved by the Ethics Committee of Tehran University of Medical Sciences. Consent was obtained from all participants orally. Samples were obtained from patients by masturbation after 4–6 days of sexual abstinence and kept in sterile cups. Routine sperm parameters were assessed according to the World Health Organization (2010). Sperm motility and concentration were assessed using the CASA system (Sperm Class Analyzer version 5.1; Barcelona, Spain). The inclusion criteria for this study were factors such as volume of 2–6 mL, a concentration of more than 1×10^9 sperm/mL, and progressive motility of 70%.

2. Vitrification and thawing procedure

Cryopreservation of sperm samples was performed using the micro-droplet technique. Sperm samples were suspended in human tubal fluid (HTF; Sigma, St. Louis, MO, USA) solution and diluted in a solution containing 0.5 mol/L of sucrose and 5% human serum albumin (HSA, Sigma). Next, 1 and 10 mM LC and 5 and 10 mM NAC (Sigma) were added separately to the previous solution. Finally, a 30-μL drop of the suspension was transferred to a liquid nitrogen container and stored for 1 week. For the warming process, the HTF medium was heated at 37℃ for 2 hours. In this phase, the samples were immersed in 5 mL of HTF with 1% HSA. Sperm suspensions were then incubated at 37℃ and exposed to 5% CO_2 for 5 minutes. Finally, samples were centrifuged (400 $\times g$, 5 minutes) and the pellets were suspended in 50 μL of HTF [19].

3. Determination of sperm motion characteristics

To investigate sperm motility, 10 μL of a sample was placed on a Makler slide at 37℃ and examined using CASA. The evaluated parameters included motility (%), progressive motility (%), average path velocity (VAP; μm/sec), curvilinear velocity (VCL; μm/s), linearity (LIN; %), and straight-line velocity (VSL; μm/s). Finally, five microscopic fields for 500 spermatozoa were selected to be evaluated.

4. Determination of sperm plasma membrane integrity

To assess the integrity of the sperm plasma membrane, the hypo-osmotic solution (HOS) test was used. This solution contains 1.35 g of fructose (Merck, Branchburg, NJ, USA) and 0.73 g of sodium citrate (Merck). The HOS solution was diluted with 100 mL of water (osmolality ~190 mOsm/kg). Next, 500 μL of this solution was mixed with 50 μL of the sample at 37℃ for 45 minutes, and then 10 μL of the suspension was transferred to a slide. Finally, the samples were evaluated using phase-contrast microscopy (Olympus BX20) [20].

5. Determination of sperm MMP

A lipophilic cationic dye, JC-1 (T4069; Sigma-Aldrich, USA), was used to investigate MMP. Samples were first centrifuged at 500 $\times g$ for 5 minutes, mixed with 1×10^6 sperm/mL in phosphate-buffered saline (PBS), and 1 mL of this suspension was stained with 1 μg of JC-1 dye. Finally, samples were evaluated using flow cytometry with FL1 (530 nm) and FL2 (585 nm) detectors [21].

6. Determination of sperm DNA damage

To investigate DNA damage, chromatin was stained using acridine orange. For this purpose, samples were first centrifuged at 5 $\times g$ for 5 minutes and were then mixed with Tris-Null-EDTA buffer, which contained 0.15 mM NaCl and 10 mM Tris. Next, 1.2 mL of acridine orange solution and 400 μL of detergent acid solution were added. Finally,

flow cytometry with the FL1 (500–530 nm) and FL2 (620 nm) detectors was used for normal DNA and abnormal DNA, respectively [20].

7. Determination of intracellular ROS

Intracellular ROS levels were evaluated using dihydroethidium. DNA intercalates and emits red fluorescence due to reaction between ethidium bromide and the superoxide anion. Samples were mixed with PBS and a sperm concentration of 1×10^6 sperm/mL was obtained. Next, 1 mL of suspension was added to 10 μL of dihydroethidium solution (Sigma-Aldrich) and incubated at 25°C for 20 minutes. Finally, flow cytometry with the FL2 (525–625 nm) detector was used for the final investigation [19].

8. Flowcytometric analysis

Flowcytometric analysis was performed using the Calibur FACS, with 488-nm excitation of an argon laser. After removing debris, 100,000 sperm cells were assessed through flowcytometry using Cyflogic software version 2.5.1 (Cell Imaging Core, Turku Center).

9. Statistical analysis

The Kolmogorov-Smirnov test was used the confirm the normal distribution of data. One-way analysis of variance and the Tukey test were also used. The statistical analysis was carried out using IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard error of the mean. A p-value ≤ 0.05 was considered to indicate statistical significance.

Results

As shown in Table 1, no significant difference was observed in total motility in the LC (1 and 10 mM) and NAC (5 and 10 mM) groups compared to the control group ($p \geq 0.05$), but NAC (5 mM) led to a significant increase ($p \leq 0.05$). Significantly higher progressive motility and motility characteristics (VSL, VCL, LIN, and VAP) were found in all groups receiving LC and NAC than in the control group.

Table 2 shows the results of DNA damage, intracellular RSO, plasma membrane integrity (PMI), and MMP of human frozen-thawed sperm. According to the obtained results, the presence of LC and NAC at both concentrations led to a significant increase in MMP and PMI in comparison to the control group ($p \leq 0.05$). LC and NAC significantly reduced DNA damage and intracellular ROS compared to the control group. However, the specific concentrations of LC and

Table 1. Motility parameters of post-thawed human spermatozoa supplemented with different concentrations of LC and NAC

Variable	Fresh-C	Frozen-C	LC		NAC	
			1 mM	10 mM	5 mM	10 mM
Motility (%)	55.21 ± 15.42	21.33 ± 10.34[a]	25.26 ± 7.51	25.47 ± 7.23	43.17 ± 8.21[b]	29.15 ± 8.33
Progressive motility (%)	40.52 ± 4.77	18.64 ± 4.22[a]	31.82 ± 4.6[b]	28.60 ± 4.89[b]	30.27 ± 4.86[b]	27.42 ± 4.91[b]
VCL (μm/sec)	50.16 ± 5.21	31.39 ± 5.65[a]	52.47 ± 5.41[b]	52.18 ± 5.47[b]	52.43 ± 5.77[b]	50.71 ± 6.11[b]
VSL (μm/sec)	25.12 ± 8.52	12.11 ± 7.73[a]	23.31 ± 8.15[b]	23.91 ± 7.75[b]	25.59 ± 7.23[b]	26.33 ± 7.44[b]
VAP (μm/sec)	36.14 ± 8.23	21.19 ± 7.51[a]	35.29 ± 8.14[b]	38.35 ± 7.82[b]	38.62 ± 8.11[b]	38.79 ± 8.91[b]
LIN (%)	56.32 ± 4.99	35.42 ± 4.74[a]	53.22 ± 5.66[b]	53.45 ± 6.12[b]	57.72 ± 4.83[b]	57.15 ± 5.17[b]
BCF	13.47 ± 5.49	10.14 ± 5.19[a]	13.50 ± 5.61	13.67 ± 5.12	14.60 ± 5.89[b]	14.88 ± 4.32

Values are presented as mean±standard error of the mean.
LC, L-carnitine; NAC, N-acetyl cysteine; C, control; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; BCF, beat cross frequency.
[a]$p<0.05$ significant differences vs. the fresh group; [b]$p<0.05$ significant differences vs. the frozen control group.

Table 2. PMI, DNA damage, intracellular ROS and MMP of post-thawed human spermatozoa supplemented with different concentrations of LC and NAC

Variable	Fresh-C	Frozen-C	LC		NAC	
			1 mM	10 mM	5 mM	10 mM
PMI	61.5 ± 3.1	29.7 ± 3.5[a]	63.1 ± 3.4[b]	61.3 ± 5.4[b]	66.5 ± 3.1[b]	66.7 ± 3.1[b]
MMP	60.2 ± 6.4	31.3 ± 6.2[a]	38.6 ± 6.5[b]	39.1 ± 7.0[b]	43.2 ± 7.5[b]	43.2 ± 7.1[b]
DNA damage	6.2 ± 5.1	9.6 ± 5.5[a]	2.7 ± 1.9[b]	2.9 ± 1.9[b]	3.2 ± 1.5[b]	4.5 ± 1.2[b]
ROS intracellular	41.4 ± 8.2	62.0 ± 8.1[a]	52.1 ± 8.5[b]	52.6 ± 8.9[b]	51.4 ± 8.3[b]	51.7 ± 8.0[b]

Values are presented as mean±standard error of the mean.
PMI, plasma membrane integrity; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; LC, L-carnitine; NAC, N-acetyl cysteine; C, control.
[a]$p<0.05$ significant differences vs. the fresh group; [b]$p<0.05$ significant differences vs. the frozen control group.

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NAC did not show significant relationships with most sperm motility parameters, PMI, DNA damage, intracellular ROS, and MMP. It should be emphasized that there were no differences in the results depending on the concentration of LC, which has been found to be a potent antioxidant capable of improving spermatogenesis.

Discussion

This study investigated the effects of different concentrations of LC and NAC on human sperm parameters during the vitrification process and post-thaw process. ROS products have toxic effects on sperm structure and function in the cryopreservation process. By being converted into hydrogen peroxide, the superoxide anion plays an important role in ROS production. Therefore, by determining the level of intracellular ROS, it is possible to elucidate its effects on sperm structure and function [22]. During the vitrification process, sperm viability and motility are the parameters associated with increased male fertility potential [23]. Antioxidants affect sperm quality and function during the cryopreservation process. Previous studies have emphasized that cryopreservation reduces and even destroys the antioxidant defenses [24,25]. The reduction of antioxidants or inhibition of antioxidant enzymes leads to oxidative stress and, eventually, disruption of membrane fluidity, membrane integrity, impaired sperm motility, and DNA damage during the vitrification process [26]. Oxidative stress, which occurs as a result of ROS accumulation, can have adverse effects on PMI and DNA damage. LC strengthens lipid metabolism, preserves the potential of the plasma membrane, and improves mitochondrial function. However, LC is an apoptosis inhibitor. The antioxidant property that protects the membrane against ROS depends on the oxidation process during which beta-oxidation products are transferred to the mitochondria and finally enter the Krebs cycle [27]. The results of our study confirm that NAC and LC exert a protective function against oxidative stress, but a significant reduction was observed in the level of intracellular ROS in all amino acid-receiving groups at both concentrations. The results of this part of the study are consistent with those of other studies conducted on human and animal sperm, which indicated that lipid peroxidation and intracellular ROS levels are reduced in the presence of NAC and LC [19,28-30]. Evidence indicates that NAC can increase the antioxidant activity of enzymes such as glutathione peroxidase and catalase [31,32]. The reduction of intracellular ROS can thus be explained by the increased activity of antioxidant enzymes. Oxidative phosphorylation in the mitochondria leads to the production of ROS. In the LC and NAC-receiving groups (at both concentrations), the sperm MMP was higher than in the control group. The MMP seems to increase through reductions in intracellular ROS levels. Normal motility is a main characteristic of sperm needed for their physiologi-

cal activity, and therefore motility can affect fertility outcomes. From the present study, it is inferred that the presence of NAC (10 mM) and LC (1 and 10 mM) in the cryopreservation groups did not improve sperm compared to the control group. One possibility is that the doses of LC and NAC should be adjusted to improve the results in terms of sperm parameters, or the method of administering these amino acids could be changed. These results are inconsistent with studies in the literature showing that LC at both concentrations (1 and 10 mM) can effectively improve sperm motility compared to the control group, but are in agreement with the results of previous studies indicating that cysteine (5 and 10 mM) did not improve outcomes in cryopreservation groups compared to the control group [19]. However, NAC (5 and 10 mM) and LC (1 and 10 mM) improved sperm motility compared to the control group. This result can probably be attributed to the lack of reduction of the superoxide anion in the presence of NAC and LC at the given concentrations. The motility characteristics (VSL, VCL, VAP and LIN) improved in the NAC and LC-receiving groups compared to the control group. In this study, the results of sperm PMI were similar in the NAC and LC-receiving groups, and NAC and LC caused an increase in PMI compared to the control and fresh groups. This effect was probably associated with the reduced level of the superoxide anion in the presence of these amino acids. The findings from the analysis of DNA damage in this study show that NAC and LC have the ability to prevent DNA damage, as significantly less DNA damage was observed in groups containing these two amino acids in cryopreservation media. This result is inconsistent with that obtained by Banihani et al. [12], who showed that LC did not prevent DNA damage. LC has important effects on sperm metabolism and spermatogenesis [23]. These results are also inconsistent with previous studies on sperm ROM, indicating that cysteine did not prevent DNA damage; this inconsistency can most likely be explained by differences in the consumed doses of amino acids, sperm species (human and animals), test conditions, and chromatin density. However, these results are similar to those reported by Shahverdi, Vatankhah, and Thawanut, who stated that the presence of cysteine in cryopreservation media could reduce DNA damage in buffalo and human sperm [19,33,34]. Despite these inconsistent findings, it can be stated that LC and NAC can probably inhibit DNA damage by affecting the structure and density of DNA. We suggest that more functional parameters, such as sperm acrosome integrity and malondialdehyde levels, should be assessed in the future to elucidate the antioxidant potential of these two amino acids in the vitrification process.

In cryopreservation media, NAC and LC can reduce the level of oxidative stress, their products, intracellular ROS, and DNA damage, and following this reduction, all sperm functional parameters affected and damaged by these products in the cryopreservation media are

recovered. The protective and beneficial effects of these two amino acids were observed for PMI, MMP parameters, and sperm motility characteristics. Thus, NAC and LC can be used to improve sperm function in cryopreservation media.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Author contributions

Conceptualization: KL, Data curation: FG, Formal analyziz: KL, Funding acquisition: KL, Metodology: YK, Project administration: ZN, Visualization: ZN, Writing orginal draft: KL, Writing review & editing: KL.

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