A Study of Effects of L-Carnitine on Morphology and Apoptosis in Cryopreserved Sperm

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ABSTRACT

Introduction: Absence of proper access to the sperm during the fertilization is the cause of male infertility problems. Although the freezing of sperm is reduced the critical parameters, sperm cryopreservation technique is effective for this class of problems. Because of the essentiality of the optimality of the environment and freezing procedures, the effect of the using of the L-carnitine in the environment of sperm cryopreservation and its influence on some sperm vital parameters are perused in this study. Methods: 36 Semen samples normally of application of spermogram in the clinical laboratory in Jahrom are collected and after the assiance of being normal, they divided into 3 groups of 12 samples. We added sperm cryopreservation solution, L-carnitine with concentration 3.6 mM to experimental group 1 and L-carnitine 100 mM to experimental group 2 are added. Sperms are freezed with vitrification technique for 3 months. Then the sperm vital parameters such as morphology, DNA integrity and apoptosis are perused. And data is compared with each other with analysis statistical software and SPSS(17) duncan's test. Result: In this research, 100mM L-carnitine is added to the environment to DNA of sperm freezing and then it caused reducing in apoptosis rates and demolitions (p<0.5) and sperm motility significantly and increasing in sperm motility and it didn't have on morphology. Conclusion: According to the research, because of the potential of oxidative stress, the environment of sperm freezing endamages the sperm and proffered the using of L-carnitine for optimality the condition of sperm freezing with increasing the advocacy of cell anti-oxidant and with influence on sperm parameters with a function of the doze.

Key words: infertility, vitrification, antioxidants, L-carnitine

Introduction

Infertility and reduced fertility are one of the major problems in medical science. Infertility is the failure of a couple to conceive a pregnancy after at least 1 year of unprotected sex (intercourse). World Health Organization (WHO) considers infertility as an important issue of reproductive health. Approximately 10 to 15 percent of couples are infertile. In about half of these cases, male infertility plays a role [20]. Reduced male fertility is one of the major problems in the science of infertility and impaired sperm production is a major cause of male infertility. Male infertility results from varied factors such as infertility due to germ cell loss in men undergoing anti-cancer treatments such as chemotherapy and radiation therapy, or in patients suffering from ejaculation disorder.[30]

Semen cryopreservation is one of the most effective and acceptable methods to maintain male fertility potential. Long-term sperm storage, sperm banking and the possibility of storing the sperm of people with impaired germ cell production are the main objectives of using cryopreservation method. Unfortunately, despite many advantages of sperm Cryopreservation, this method reduces sperm fertilizing capacity through morphological damage, DNA damage and generally impaired sperm function [5]. Regardless of cryopreservation methods, each type of cryopreservation has negative effects on sperm motility and decreases the normal ratio of sperm vital parameters after thawing compared with the pre-cryopreservation step. Many researchers found that cryopreservation decreases sperm motility parameters [6,9]. In general, it can be said that cryopreservation decreases normal morphology and increases programmed sperm death in infertile men [25]. Cryopreservation decreases sperm fertility potential due to its physiological sensitivity to low temperatures. It maybe because of reduced glycosal (cell-surface carbohydrates) due to the presence and
increase of hydroxyl (OH) and superoxide (O2-) radicals, their negative effects on cells biochemical structure and reduced metabolism as a result of cryopreservation process [28, 18].

Researches show that the protection for sperm cryopreservation is significantly decreased due to sperm membrane dysfunction (Arabi & Anand, 2002; Nazm Bojnourdi et al. 2008).

In addition, cryopreservation protection leads to changes in its DNA [21].

It is extremely necessary to optimize cryopreservation method since it is an effective step in improving male infertility. Therefore, adding substances – that prevent damage to sperm - to cryopreservation environment can improve sperm cryopreservation condition. These substances include antioxidants that are capable of preventing sperm damage and destruction through removing oxygen free radicals. L-carnitine is one of these antioxidants, which plays an important role in increasing vital capabilities of the sperm.

Antioxidants act differently, but briefly we can mention the decrease in oxygen concentration, removal of metal catalysts and removal of free radicals such as superoxide, hydroxyl and hydrogen peroxide, etc [4].

During spermatogenesis, sperm cells lose a large amount of their associated cytoplasm along with antioxidant substances contained in it, and subsequently become sensitive to oxidative stress (OS). However, these cells are suitably protected against OS process because of immersion in sperm fluid containing many antioxidants such as ascorbic acid, urate, Turin, sulphhydryl (thiol) groups, catalases, superoxide dismutase and L-carnitine. In recent years, it has been demonstrated that sperm plasma of infertile men has lower antioxidant levels than that of fertile men [12,13].

Since the role of this substance on the maintenance and enhancement of sperm capabilities is observed in new environments, it seems that considering the importance of cryopreservation in infertility treatment, this substance may effectively reduce damages caused by cryopreservation process, because this substance has an antioxidant effect.

Free L-carnitine is a high polarized and water-soluble substance being first isolated from bovine muscle in 1905, and its chemical structure was identified in 1948. L-carnitine is the only carnitine isomer, which is biologically active [33].

L-carnitine is derived from lysine and methionine (amino acids) and is a foodstuff being found in meat and dairy products [17].

Free L-carnitine is essential for beta-oxidation (B-Oxidation) of long-chain fatty acids in mitochondrion. Fatty acids must be activated before entering the mitochondrion (making a connection with coenzyme A to form acetyl-CoA). Long-chain acetyl-CoA molecules cannot cross the inner mitochondrial membrane in the absence of L-carnitine. When fatty acids are entered the mitochondrion, beta-oxidation and adenosine triphosphate formation (ATP) will begin [16,23].

The epididymis has the highest carnitine concentration found in body. Carnitine concentration in epididymis is 2000 times higher than that in blood. Different studies have shown the reduced level of carnitine in infertile men’s semen [19,33].

Therefore, we decided to investigate the effect of this substance on morphology and the rate of apoptosis and sperm nuclear integrity after leaving cryopreservation state in order to provide suitable strategies for improving the quality of cryopreservation, storing sperm and preventing the risks of cryopreservation.

Materials and Methods

The present experiments were conducted on human semen samples from men referred to the pathology laboratory of Dr. Farrokhnia in Jahrom (Fars, Iran) to do spermogram test. After ensuring samples’ normality, some of it (2 ml) was considered for testing.

1-3-3: Classification method:

Study groups include:

1 - Control group: Including 12 samples. In this group, only sperm cryopreservation solution (0/7 ml) is added to the sample.

2 - Experimental group 1: Including 12 samples. In this group, in addition to 0/7 ml of sperm cryopreservation solution, 3/6 mM of L-carnitine drug was added.

3 - Experimental group 2: Including 12 samples. In this group, in addition to 0/7 ml of cryopreserved sperm solution, 100 mM of L-carnitine drug was added.

2-3-3: Sampling:

Sampling conditions and methods and a 3-4 day interval of no intercourse were explained to all men referred to the laboratory by urologists for spermogram test, and they were given a wide-mouth plastic jar.

Papanicolaou staining technique was used for the study of sperm morphology.

Prepared slides were observed by light microscope (optical microscope), and all sperms that were different from normal sperms - i.e. sperms that were short tail, two-headed, double tailed and without tails, or sperms with cytoplasmic components - were counted as abnormal sperms. To perform the calculation, at least 10 fields and 200 sperms were counted, and then the percentage of abnormal sperms was evaluated.

4-3: Sperm Cryopreservation:
The following steps were taken for semen cryopreservation:

1 - Adding S F10, HAM solution with a volume 2 times higher than that of semen.
2 - Adding 20% human serum albumin with a ratio of 1:10.
3 - Transferring tubes containing the sample to centrifuge device and performing centrifuge with 3000 round per minute and for 5 minutes.
4 - Removing the seminal plasma by pipette to obtain the sample volume of about 3 ml.
5 - Re-transferring the above-mentioned tubes to centrifuge with 3000 round per minute and for 5 minutes.
6 - Removing the seminal plasma by pipette to obtain the sample volume of 1 ml.

To ensure the existence of sperm in the sample after centrifuge, a drop of semen was removed and observed under a light microscope.

Sperm freeze solution kept at 4 °C in the refrigerator was brought out the refrigerator and was placed at 37 °C incubator for about 5 minutes without CO₂. Then transferring 1 ml of samples to a Cryo tube with 2 ml capacity and adding 0/7 ml of pure solution of sperm freeze (drop by drop) to 12 control tubes.

Before adding sperm freeze solution in experimental groups 1 and 2, the following actions took taken:

3/6 mM and 100 mM of L-carnitine were added to experimental group 1 and group 2, respectively. Sperm cryopreservation solution as the solvent of L-carnitine was considered.

It is noteworthy to say that concentrations applied in this study were used considering the research conducted by Tanphaichitr (1979), Shi et al. (2010), and Ali Abadi et al. (2012).

The following steps were taken for cryopreservation:
1 - Putting the samples in aluminum files of nitrogen tank
2 - Putting the samples on nitrogen vapor for 2 minutes
3 - Dipping files containing samples into 196_ °C nitrogen

5-3: Process of Thawing:

After 3 months, cryopreserved samples were removed from liquid nitrogen tank to be thawed. The following steps were taken to perform thawing process:

1 – Putting the removed samples in vitro for 10 min to reach equilibrium
2 – Transferring the samples to a 37 °C incubator without CO₂ after 10 min for complete thawing
3 – Adding HAM, S F10 medium to samples
4 – Performing a 3000 rpm centrifuge for 5 min

Samples were re-evaluated in terms of vital parameters such as the number of sperm per ml, motility, percentage of live sperms and morphology according to the method described previously. And apoptosis and DNA integrity evaluated by staining the help of the acridine orange.

In the present study, phosphoric, red and yellow sperms indicated healthy sperms with natural DNA, single-stranded and damaged DNA and apoptosis, and intermediate and damaging mode of DNA, respectively. To perform sperms’ calculation and investigation, at least 10 fields and 200 sperms were counted, and then the percentage of healthy, apoptotic, and intermediate sperms as well as sperms with damaged DNA was evaluated.

6-3: Statistical Analysis:

Obtained data were analyzed using the statistical package of SPSS (version 17), and the significant difference between groups was analyzed using analysis of variance (ANOVA) and Duncan's test. Where p was equal or less that 0.05, the difference was considered significant (p ≤0.05).

In the present study, results are shown as Mean ± SEM.

Results:

the present study aimed to investigate the effects of l-carnitine in sperm freeze environment as well as its effect on some vital parameters of sperm. Therefore, l-carnitine with a different concentration of 2 was added to cryopreservation environment, and parameters such as morphology, DNA integrity, nuclear integrity and apoptosis were evaluated and compared with the control group.

Obtained results were divided into two general parts, and then were analyzed:
- Results of sperm morphology assessment in control and experimental groups 1 and 2
- Results of assessing DNA integrity, nuclear integrity and the rate of apoptosis in control group and experimental groups 1 and 2.

3-4: Results of Sperm Morphology Assessment:

- According to analysis of variance Table (3-4) and Diagram (3-4), adding l-carnitine to sperm freeze environment for preserving its morphology investigated through papanicolaou staining was not statically significant at any investigated concentration compared to control group.
Table 1: Comparison of sperm morphology in study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration of 100 mM</th>
<th>Concentration of 3/6 mM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before cryopreservation</td>
<td>46/083±0/712a</td>
<td>45/666±0/710±0/00±0/685</td>
<td>Before cryopreservation</td>
</tr>
<tr>
<td>After cryopreservation</td>
<td>27/50±1/011a</td>
<td>29/916±0/811±30/083±0/732</td>
<td>After cryopreservation</td>
</tr>
</tbody>
</table>

Means in each row having at least one common letter are not significantly different at 5% level of Duncan's test.

Diagram 3-4: Comparison of study groups in terms of morphology:

Rows having at least one common letter are not significantly different at 5% level of Duncan's test.

5-4: Results of Assessing DNA Integrity, Nuclear Integrity and Apoptosis:

Adding l-carnitine to sperm freeze environment for preserving DNA integrity, nuclear integrity as well as apoptosis reduction was significant in the experimental group 2 having received 100 mM compared to control group. In addition, these changes were significant in comparison between two experimental groups. Table (6-4) and Diagram (5-4) show the analysis of variance and the comparison of the effect of l-carnitine addition in different concentrations in all three study groups, respectively.

Table 2: Comparison of different groups in terms of DNA integrity and study sperm apoptosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Green</td>
<td>a76/083±0/972</td>
</tr>
<tr>
<td>DNA Yellow</td>
<td>b13/833±0/534</td>
</tr>
<tr>
<td>DNA Red</td>
<td>b11/833±1/266</td>
</tr>
</tbody>
</table>

Means in each row having at least one common letter are not significantly different at 5% level of Duncan's test. This table is depicted to compare different groups in terms of DNA integrity, nuclear integrity and apoptosis parameters.
Table 2: Comparison of the examined groups and apoptosis in DNA sperm

Diagram 2: Comparison of study groups in terms of DNA and sperm apoptosis assessment:

Rows having at least one common letter are not significantly different at 5% level of Duncan's test.

Fig. 1: Determination of sperm morphology. The head of the arrow indicates a normal sperm after cryopreservation in the group receiving 100 mM of l-carnitine (pap staining, light microscopy, magnification of 40 ×).

Fig. 2: Determination of sperm morphology. The head of white arrow indicates a normal sperm and the head of black arrow indicates an abnormal sperm after cryopreservation in control group (pap staining, light microscopy, magnification of 100 ×).

Fig. 3: Determination of sperm morphology. The head of the arrow indicates an abnormal twisted-tail sperm in control group after cryopreservation (pap staining, light microscopy, magnification of 100 ×).
Fig. 4: A green and brilliant sperm with healthy DNA and without apoptosis after cryopreservation in a sample of the experimental group 2 receiving 100 mM of l-carnitine (Acridine Orange staining, fluorescence microscopy, magnification of 40 ×).

Fig. 5: A red sperm with damaged DNA and cell apoptosis of control group after cryopreservation (Acridine Orange staining, fluorescence microscopy, magnification of 100 ×).

Fig. (7-4): A red sperm with damaged DNA and cell apoptosis of control group after cryopreservation (Acridine Orange staining, fluorescence microscopy, magnification of 100 ×).

Discussion and Conclusion:

Researches show that the fertilization rate is significantly decreased after cryopreservative storage due to sperm membrane dysfunction. In addition, cryopreservative protection leads to changes in DNA, sperm cytoskeleton, inhibition of sperm-ovule binding, and destruction of sperm axoneme, and consequently reduction of its mobility. Results of studies indicate that antioxidants protect the sperm plasma membrane against oxidative reactions during cryopreservation storage. In fact, reducing the formation of oxygen free radicals, antioxidants change cellular conditions so that the sperm vital parameters are preserved [21].

3-1-5: Effect on sperm morphology:

According to Diagram and Table (3-4), in the present study, adding l-carnitine for preserving normal sperm morphology in the receiver group made no significant difference statistically. Seminal plasma is an important source of antioxidants, which is separated during processes performed on sperm for preparation for cryopreservation. Therefore, sperms’ sensitivity to various antioxidants is increased. In addition, lipid distribution in the plasma membrane
of frozen-thawed sperm is significantly higher than that of fresh human sperm and leads to the displacement of phosphatidylserine from the inner half to the outer half. This is one of the first biochemical events of apoptosis or cell death disrupting the membrane asymmetry [1]. Superoxide anion is the first substance produced by sperm, but these short-lifetime radicals are quickly transformed into $\text{H}_2\text{O}_2$ [11]. This causes membrane lipid peroxidation and is the greatest factor contributing to sperm damage [27]. Since human sperm contains large amounts of unsaturated fatty acids, it is extremely sensitive to lipid peroxidation [3].

Normally, antioxidants such as Superoxide dismutase, Glutathione peroxidase, Glucose-6-phosphate dehydrogenase enzymes prevent damage to sperm membrane through super oxide and hydrogen peroxide anions breakdown [7].

Although the results of the present study (Figures (1), (2) and (3)) are consistent with the results of researches conducted on the effect of vitamins E and C as antioxidant on the quality of frozen-thawed sperm using rapid cryopreservation method in the comparison of oligospermia patients and normal people, the lack of significant effect on improving normal morphology of the sperm [21] or the use of oral carnitine for 6 months, and the lack of effect on the increase of normal sperm forms [17], a reverse result was expected due to the antioxidant effect of l-carnitine. Studies conducted by Stradalli in 2004 showed that the rate l-carnitine antioxidant in seminal plasma is significantly associated with sperm morphology. In other words, increasing l-carnitine antioxidant, less sperms are morphologically damaged [29]. In addition, l-carnitine antioxidant plays a role in protecting the sperm membrane [31]. The difference in the result is possibly due to differences in methodology or above-mentioned factors (dose, consumption duration), etc.

5-1-5: Effect on Programmed Cell Death and DNA Integrity:

Adding l-carnitine antioxidant to sperm freeze environment significantly decreased the rate of apoptosis and DNA damage in the group having received 100 mM of l-carnitine (Diagram (2) and Table (2)).

Programmed cell death is a kind of cell death associated with biochemical and morphological changes [23]. In this case, the cell may be transformed and this transformation is associated with cell contents compression and chromatin condensation in the form of a uniform mass adjacent to the nuclear membrane. Apoptosis is controlled by a genetic system [1]. Sperm cryopreservation significantly increases the programmed cell death [23]. Increasing concentrations of ROS in the environment – called OS oxidative stress –lead to serious damages in sperms’ DNA [5]. In molecular structure of DNA in body cells such as sperm, free radicals can lead to the oxidation of purine and pyrimidine bases, breakage in one or two chromosome strands, formation of positions lacking base, formation of cross-bridges between DNA and protein, and change in deoxyribose sugar. Free radicals can oxidatively attack critical bio-molecules such as DNA and change DNA structure causing infertility [26].

Guanine (G) is the most common organic base attacked oxidatively by free radicals and is converted to 8-hydroxyguanin (8-OHG) [14]. Since DNA changes are increased in cryopreservative protection due to the increase of oxidative stress, it is necessary to add external antioxidants to the sperm to compensate the lack of shield [24].

Antioxidants act differently, but briefly we can mention the decrease in oxygen concentration, removal of free radicals such as superoxide ($\text{O}_2^-$), hydroxyl (OH) and hydrogen peroxide, etc [21]. According to recent explanations, adding some antioxidants such as l-carnitine to sperm freeze environment can lead to the protection of DNA and cell membrane against damages caused by oxygen free radicals [17].

Studies conducted by Samimi in 2010 showed that adding taurine as an antioxidant to sperm freeze environment prevents DNA fragmentation and cell apoptosis in experimental groups receiving antioxidants [27]. In addition, adding vitamin E as an antioxidant decreases the amount of oxidative stress, and consequently prevents damage to DNA and especially chromosome y [7,10]. In the study of l-carnitine effects on testicular tissue conducted by Zare, it was found that treatment with l-carnitine significantly increases the quality of sperm chromatin [33].

Conclusions:

According to the findings of this study, sperm freeze environment decreases sperm damages due to its oxidative stress. The use of l-carnitine as a substance with antioxidant properties can at least affect some sperm parameters such as dose-dependent reduction of DNA damage through preventing more production of free radicals and enhancing antioxidant defense of cell. Since positive results of the drug in the present study were only observed in the group receiving the highest dose, it seems that lower doses are not enough and higher doses (provided that know lethal dose) may provide better effects. Therefore, this substance may improve sperm cryopreservation quality, reduce damages caused by cryopreservation and improve sperm fertility potential.
Reference

1. Aggarwal, B.B., 2000, 


18. Long, J.A., 2006, 


