The Effect of L-Carnitine and Coenzyme Q10 on the Sperm Motility, DNA Fragmentation, Chromatin Structure and Oxygen Free Radicals During, before and after Freezing in Oligospermia Men

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Purpose: The aim of the present study is to assess the effect of L-carnitine and Coenzyme Q10 (CoQ10) on human sperm motility, DNA fragmentation, chromatin structure, and reactive oxygen species (ROS) during, before and after freezing in oligospermia men.

Materials and Methods: Semen was collected from 30 oligospermic men, who referred to infertility clinic of Beasat Hospital in Sanandaj, Iran. The samples of each individual were divided into 8 equal parts: 1. control group before freezing; 2. incubated with L-carnitine; 3. incubated with coenzyme Q10; 4. incubated with the combination of L-carnitine + CoQ10; 5. control freezing group; 6. the experimental freezing group with L-carnitine; 7. the experimental freezing group with coenzyme Q10 and 8. the experimental freezing with the combination of L-c + CoQ10. Sperm motility was assessed by WET MOUNT method. DNA fragmentation was evaluated by SCD (Sperm Chromatin Desperation), ROS, was evaluated by quantitative fluorescence reaction, and chromatin deficiency was determined by chromatin staining (CMA3).

Results: Antioxidant treatments, significantly reduced the number of ROS + in the pre and post freezing groups. Significant improvement was seen in the sperm motility of class B in the pre freezing groups with L-carnitine. Antioxidants also reduced the percentage of DNA fragmentation and protamine deficiency in pre-and post-freezing.

Conclusion: Addition of Coq10 and L-carnitine to human sperm medium significantly reduced the number of ROS. This reduction in ROS reduced sperm damage during cryopreservation.

Keywords: coenzyme Q10; l-carnitine; oligospermia; reactive oxygen species; sperm

INTRODUCTION

Infertility is an important medical and social problem in the world since 15% of couples are infertile; 40% of them are infertile because of male factor infertility, 40% because of female factor infertility, and in the remainder, both factors are associated[(1)]. Male infertility can be due to oligospermia, which has a sperm count of less than 15 to 20 million per ml in 16% of the 41% infertile couples[(2)]. With the advent of ART (Assisted Reproductive Technology), it has become possible to treat infertile men. The quality of semen in some diseases, such as oligospermia, is crucial for the success of ART[(3)]. Recently, much attention has been paid to the influence of ROS on sperm quality. Despite the advancement of ART techniques, gametes and embryos when handled, prepared and manipulated for ART procedures, are exposed to various potential ROS-inducing factors[(3)]. Another method used in ART to maintain the ability of men reproduction is cryopreservation techniques (freezing and thawing process). Most damage occurs during freezing and thawing. Major causes of damage during freezing are ROS formation and cell dehydration, which disrupt the cell wall and intracellular organelles. Many in vitro and in vivo studies have

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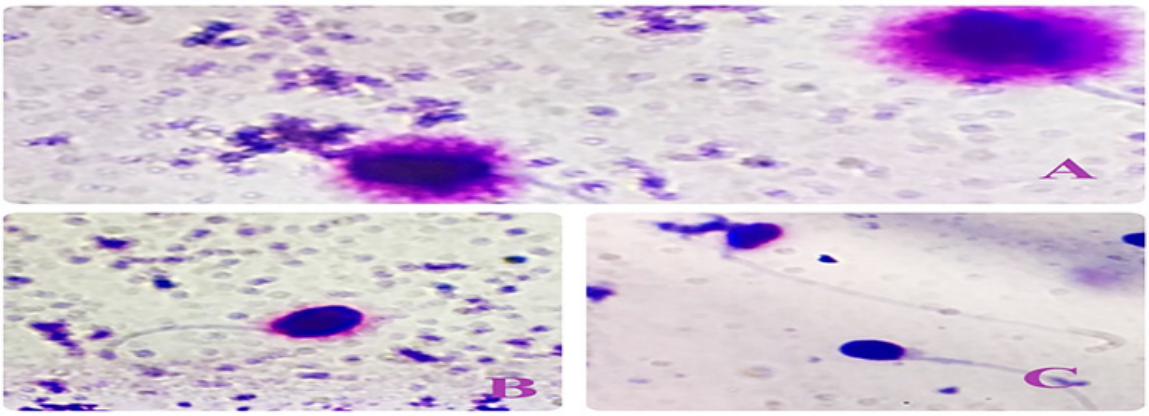

Figure 1. SCD test under light microscope; normal sperm have large halo **(A)** around the head. Abnormal sperm have a small halo **(B)** or no halo **(C)**.

recommended the use of antioxidants as an adjunct to infertility treatment to improve sperm quality[4]. CoQ10 is an essential component for electron transport in oxidative phosphorylation of mitochondria. CoQ10 function as a potent antioxidant in testicular, and high levels of its reduced form ubiquinol are present in sperm[5,6]. In the mammalian epididymis, the free L-carnitine is taken up from the blood plasma, transported into the epididymal fluid and into the spermatozoa, and accumulated as both free and acetylated L-carnitine[7]. In humans and experimental models, carnitines play an important role in sperm energy metabolism and provide the primary fuel for sperm motility[8]. Previous studies have shown that seminal-free L-carnitine content correlates with the number of sperms and sperm motility. Carnitine, as a water-soluble antioxidant, protects the plasma membrane of sperm from damage by free radicals and prevents the oxidation of proteins, pyruvate and lactate and also, protect sperm DNA against the damage induced by ROS[7,8]. In this study, it is tried to improve the quality of sperm parameters of oligospermia men by using L-carnitine and COQ10.

PATIENTS AND METHODS

Inclusion criteria and exclusion criteria

Inclusion criteria, men with oligospermia and 25-40 years of age with abnormal spermogram should be done at least two examinations in two to three months, according to the WHO. Exclusion criteria included: patients over 40 years of age with underlying factors such as varicocele, testicular atrophy, ejaculatory disorders, patients with azoospermia, Sertoli cell syndrome and endocrine and anatomical disorders, seminal specimens which contained abundant bacteria and suspected of infection and the presence of leukocytes more than one million / mL[2].

Semen Collection

The samples were collected from patients after 3 to 4 days of abstinence and they completely agreed to participate in experimental tests by completing the consent form before collecting the sample. The supernatants sample were kept in a 37 ° C incubator without CO_2 for liquefaction for 20 to 30 minutes. Toxicity test was performed to obtain a responsive dose of L-carnitine and

Figure 2. ROS $^{+}$spermatozoa under fluorescence microscopy with a wavelength of 535-485 nm.

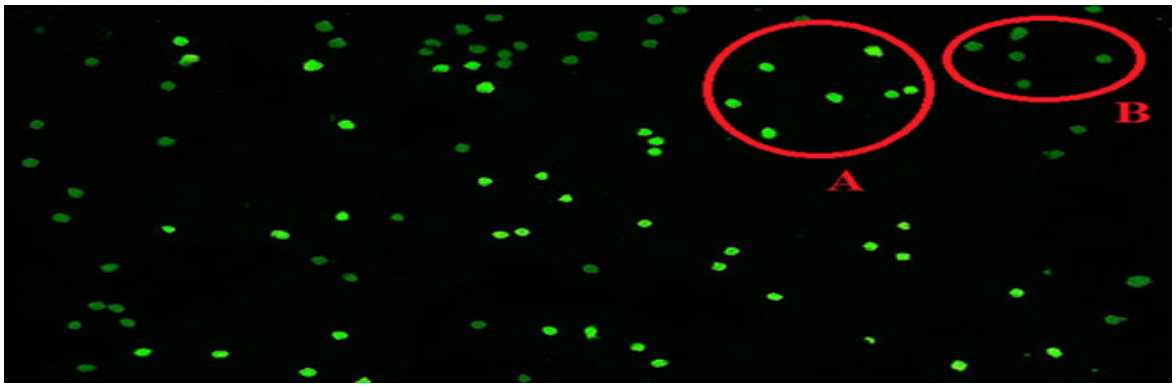

Figure 3. A: Luminous yellow sperm has protamine deficiency (CMA3 +). **B:** Sperm has normal protamine levels (CMA3-)

CoQ10 to prevent sperm infection. We obtained this dose at 100 µM[9].

Experimental groups

Each individual sample was divided into 8 equal parts. Control group was prepared without any intervention and freezing (control before freezing); Group 2 was incubated with L-carnitine (100 µM) for one-hour; Group 3 was incubated with CoQ10 (100 µM) for one hour; Group 4 was incubated with L-carnitine and CoQ10 (100 µM) for one hour; Group 5 (freezing control group) was mixed and frozen only with human sperm preservation medium (HSPM); Group 6 was frozen with CoQ10 (100 µM) and HSPM; Group 7 was frozen with L-carnitine (100 µM) and HSPM and group 8 was frozen with the combination of 100 µM (L-carnitine+ CoQ10) and HSPM for two weeks. Samples were then analyzed to evaluate parameters such as ROS, protamine, DNA fragmentation and motility according to WHO standards[9].

Freezing and thawing method

To reduce the damage caused by freezing and thawing, one-step freezing method was used. The sample was held in the vapor phase for ten minutes before being plunged into liquid nitrogen. Sperms were mixed with HSPM cryopreservation medium in 1: 1 ratio and they were transferred to cryovials. After 7 minutes of freezing treatment, the cryovials were placed on nitrogen vapor for 10 minutes and finally stored in a nitrogen tank. After 2 weeks, the sperms were thawed. They were placed under running water for 1 to 2 minutes to reach normal temperature[10].

Motility determination

Sperm motility was assessed by WET MOUNT method which included 3 types of progressive motility. It refers to sperms that swim in a mostly straight line or large circles in class (A). Non-progressive motility refers to sperms that do not travel in straight lines or swim in very tight circles in group (B) and sperms with no motion in group (C). At first, 10 µL of sperm sample was placed on the slide and on a 22 × 22 lamellae and it was examined with 40 lenses and the percentage of class A-B-C sperm motility was counted[11].

DNA fragmentation determination

SCD testing is one of the methods used to assess sperm DNA damage. Sperm DNA damage is detected by the presence of extracellular chromatin haloes around the sperm nucleus. Normal sperms have a large halo and abnormal sperms have a small halo or no halo around the head. DFI (DNA Fragmentation Index) was calculated with halo larger than or equal to sperm head and abnor-

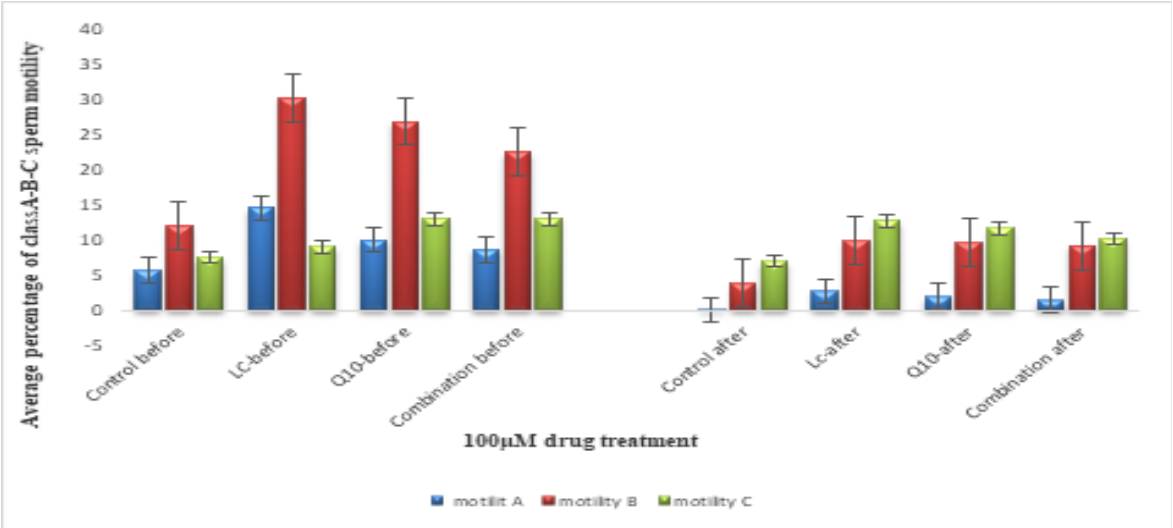

Figure 4. Comparison of sperm motility in different experimental and control groups

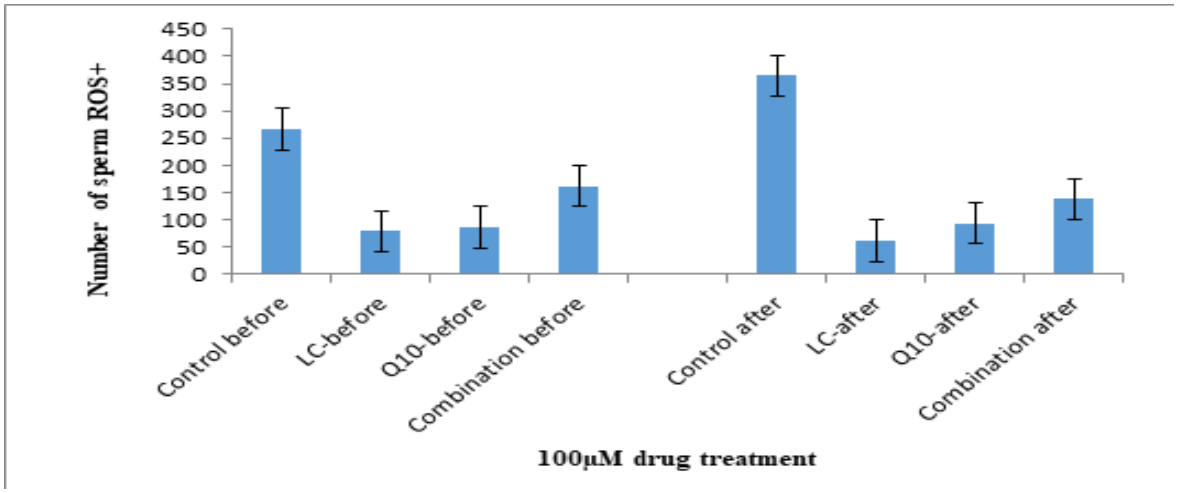

Figure 5. Comparison of mean ROS in different experimental and control groups

mal sperm (non-halo or smaller sperm head) around the sperm head during staining. 30 µL of spermatozoa was mixed with 70 µL of low melting agarose at 37 °C. The mixed sample was placed on a slide pre-coated with 65% agarose for 4 minutes at 4°C. Then, the lamellae were separated from the surface of the slide, and each slide was horizontally immersed in .08 normal hydrochloric acid solution for 7 minutes at room temperature and in the darkness. It was then placed in a lubricant solution for 25 minutes. Each slurry was washed with distilled water for 5 minutes and dehydrated in 70, 90, 100% alcohol 2 minutes, and then stained with Wright's color solution and washed with ordinary water after 10 minutes. Then, it was examined by light microscopy at a magnification of 100 (**Figure 1**)[12].

ROS measurement

We used DCFDA Cellular ROS Detection Assay Kit to measure the ROS of the sperm samples for each group (**Figure 2**)[13].

Determination of protamine deficiency

To evaluate deficiency of protamine, smear was prepared from the samples and investigated by ChromycinA3 (CMA3) staining[22]. After staining, sperms were observed and counted under each fluorescence microscope at a magnification of × 60. The percentage of bright yellow spermatozoa was recorded as CMA3+ (sperms lacking protamine deficiency) (**Figure 3**)[14].

Data analysis

Data analysis was conducted in SPSS software version 22 using Mann-Whitney test.

RESULTS

In all the groups, motility factors, ROS, DNA fragmentation and protamine were evaluated and compared and $P < .05$ was considered significant. The results of the statistical analysis of these data are as follows:

Figure 4 shows the mean percentages of different classes of motility and the level of significance between the experimental groups. By examining the results of the above diagram, in both pre and post freezing conditions, three drug treatments were able to increase sperm motility (A-B-C) compared to the pre and post freezing control group, (L-carnitine , COQ10 , L-carnitine + COQ10). Significant increase in class B ($p = .004$) with l-carnitine was seen in before freezing group. Overall freezing significantly decreased all three A-B-C mo-

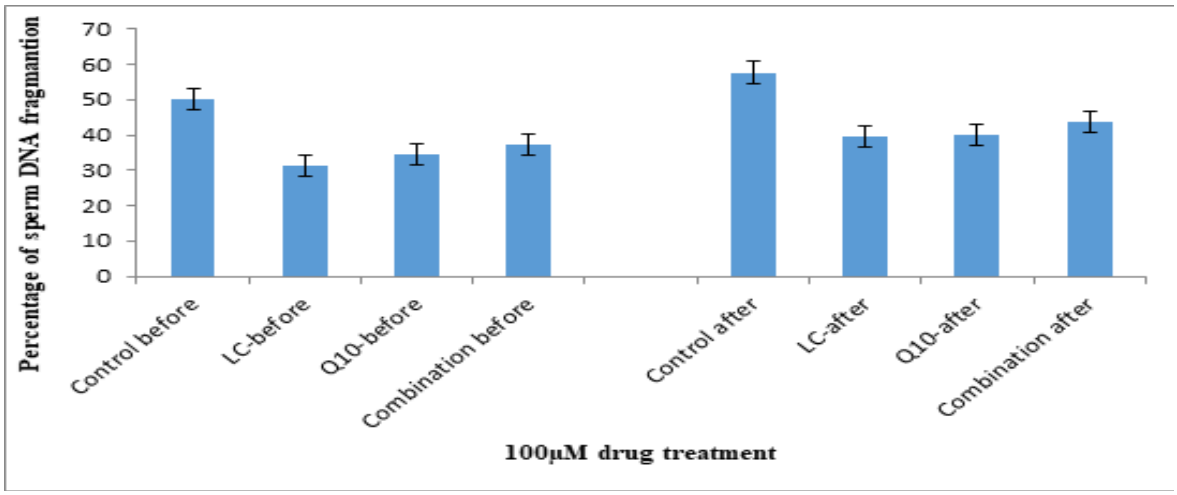

Figure 6. Comparison of mean percentage of DNA fragmentation in experimental and control groups

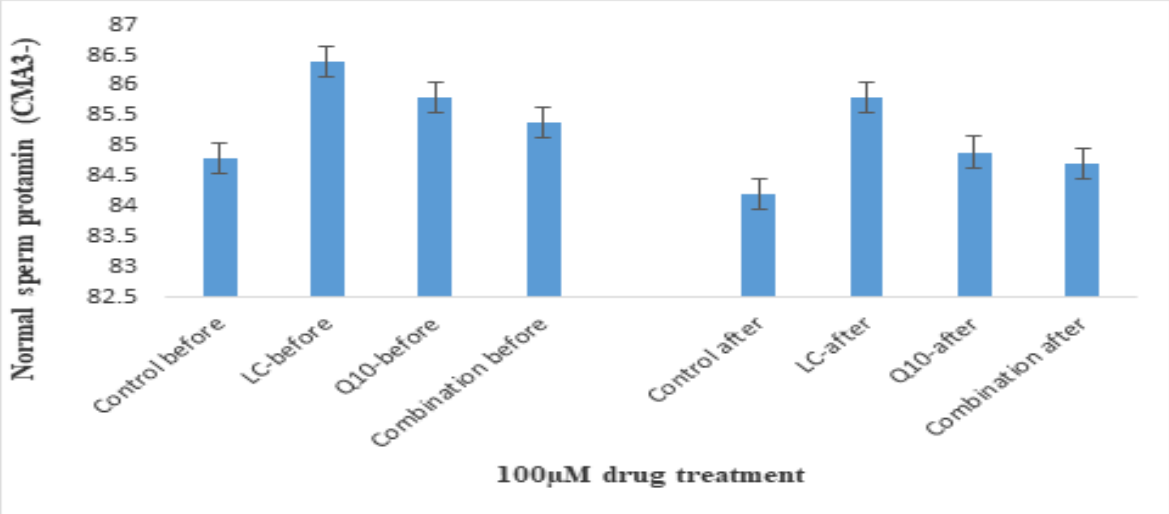

Figure 7. Comparison of the mean of protamine in experimental and control groups

tions in the control group. Freezing had the greatest effect on decreasing A motility (progressive motility), and increasing C
motility (no progressive motility). By the addition of these antioxidants, this decrease in progressive motility was partially prevented. L-carnitine had the most effect on the improvement of three classes of motility in after freezing group.

According to Figure 5, with the addition of COQ10 and L-carnitine to the sperms of the control group, there was a significant decrease in ROS mean compared to the control group (respectively $P = .04$, $P = .03$ and L Carnitine + COQ10 group $P = .2$). There is an overall increase in the average ROS. Adding antioxidants significantly reduced the ROS mean compared to the freezing control group. (L-carnitine ($P = .01$) , COQ10 ($P = .01$) , L-carnitine +COQ10 ($P = .03$)).

According to Figure 6, no significant change in the reduction of DNA fragmentation was observed with the addition of Q10 and L-carnitine separately and in combination before cryopreservation. After freezing, DNA fragmentation increased, but the addition of these treatments decreased in DNA fragmentation compared to the freezing control group. The increasing effect of cryopreservation on DNA fragmentation in the cryopreservation control group was significant ($P < .001$). Also, addition of CoQ10 and L-carnitine to the sperm of the control group increased the number of sperms with normal protamine but this difference was not significant (L-carnitine , Q10 , L-carnitine + Q10) ($p \leq .05$) (**Figure 7**).

Freezing reduces protamine in spermatozoa. With the addition of antioxidants, the average number of spermatozoa with normal protamine increased compared to the freezing control group, but it was not significant (L-carnitine , Q10, Carnitine + Q10) ($p \geq .05$) (**Figure 7**).

DISCUSSION

The present study demonstrated L-Carnitine and Coenzyme Q10 effects on the Sperm Motility, DNA Fragmentation, Chromatin Structure and Oxygen Free Radicals During, Before and After Freezing in Oligospermia Men. In oligozoospermic patients, the spermatozoa are the predominant source of ROS and generate extremely high levels of ROS compared to those produced by spermatozoa from normal fertile men[15]. The most important strategy to reduce oxidative stress is to use antioxidant-supplemented. Our results showed L-carnitine and CoQ10 significantly improved sperm motility before and after freezing. Freezing shows a decrease in all three types of sperm motility. But antioxidants partially prevented this reduction. The most effective treatment was L-carnitine treatment. L-carnitine had the greatest improvement in sperm motility in Class B before freezing and Class C after freezing. Decreased motility has been shown to be due to ROS-induce, primarily H2O2-mediated, peroxidation of lipids in the sperm membrane decreasing flexibility and by inhibition of motility mechanisms. The reduction in sperm motility is proportional to the amount of lipid peroxidation[15]. ROS-induced damage of mitochondrial DNA leads to decreased ATP and energy availability, impeding sperm motility[16]. Previous studies showed that quaternary antioxidant increased sperm motility by reducing sperm lipid peroxidation. These results are in line with the results of our study[5,17]. Melissa Rossi et al. showed that the addition of Q10 antioxidant to horse sperm freezing medium did not increase the sperm motility. This result was inconsistent with our study, which may be due to the different types of samples as well as differences in the method of freezing[18]. According to other studies, antioxidants appear to reduce ATPase K+ / Na pump activity, reduce phosphorylation of axonal proteins and alter membrane permeability by reducing membrane peroxidation (resulting from oxidative stress). Finally, sperm motility was maintained[15,16].

In the present study, L-carnitine and CoQ10 reduced DNA damage before and after cryopreservation. Free radicals have the ability to directly damage sperm DNA by attacking the purine and pyrimidine bases[19]. ROS cause damage via single and double strand DNA breaks, cross links, and chromosomal rearrangements[20]. Infertile men often have deficient protamination which may make their sperm DNA more vulnerable to ROS damage[21]. A study by Talevi et al. using an antioxidant compound (Zinc, D –aspartate, CoQ10) in in-vitro environment showed increased sperm DNA

integrity and prevented fragmentation in oligospermic patients[5]. The researchers also showed that addition of CoQ10 antioxidant to sperm freezing medium had a significant effect on reducing sperm DNA fragmentation after cryopreservation[22]. According to the results of other research, it seems that antioxidants prevent the oxidation of purine and pyrimidine bases by eliminating free radicals which leads to preventing the breakage of one or two strands of DNA. They also prevent the formation of transverse cells between DNA and protein and, ultimately, maintain chromatin structure and DNA integrity[19-21]. The study showed that addition of coenzyme antioxidants CoQ10 and L-carnitine decreased the number of ROS-positive spermatozoa before and after cryopreservation. The freezing process produces ROS. But these antioxidants significantly reduced the number of ROS-positive sperm in the pre and post vitrification treatment groups. In vitro incubation of sperm in the absence of seminal plasma shows a significant increase in markers for oxidative stress[23]. According to research, it seems that antioxidants prevent sperm membrane lipid peroxidation and ultimately protect sperm by removing oxygen free radicals and oxidative stress (24). In the present study, the addition of CoQ10 and L-carnitine reduced the number of protamine deficient sperms compared to the pre-cryopreserved control group, but had no significant effect. Oxidative stress may affect the levels of protamine through influencing the spermatogenesis process. Proteins are one of the main targets for oxidative damage[25] and cysteine residues are particularly sensitive to oxidation because the thiol group (-SH) in cysteine can be oxidized[26]. A recent study showed that L-carnitine and coenzyme in 40 µg dose improve protamine deficiency[27]. Also, Aliabad et al., explained that L-carnitine and acetyl L-carnitine improved protamine by acetyltransferase (Ache) transfer[28].

CONCLUSIONS

The use of antioxidants in-vitro in the clinical laboratory setting during ART procedures should also be considered, alongside improvement of ART techniques and optimization of the laboratory environment. Addition of CoQ10 and L-carnitine antioxidants to human sperm medium by reducing the number of ROS, improves motility, protamine deficiency and reduces DNA percentage of sperm fragmentation before and after freezing. Undeniably, excessive ROS leading to oxidative stress conditions has a serious impact on the outcome of assisted reproduction which leads to lower fertilization, implantation and pregnancy rates. In conclusion, prophylactic oral antioxidant therapy and supplementation of medium for culture, incubation/handling and cryopreservation can possibly improve gamete quality and fortify the developing embryo. However, the appropriate antioxidants and dosages (whether as a sole compound or as a combination) for different forms of infertility issues still remain an ongoing area of research.

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CONFLICTING INTEREST

The authors declared no potential conflicts of interest.

REFERENCES

1. Khadim A.H.A., Al-Wayelli D.A.O.J., Al-Rekabe BKK., Evaluation of serum FSH, LH and Testosterone levels in infertile patients affected with different male infertility factors after IUI technique. Thi-Qar Med J. 2010; 4: p. 112-122.
2. Dohle, G., Weidner W., Jung A., guidelines on male infertility. Europ Urol J. 2005; 48: p. 703-711.
3. Palermo, G., Joris H, Devroey P., Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet J. 1992; 340: p. 17-18.
4. Balercia, G., Buldreghini E, Vignini A, et al., Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double-blind randomized trial. Fertil Steril J. 2009; 91: p. 1785-1792.
5. Talevi R., Barbato V., Fiorentino I., et al., Protective effects of in vitro treatment with zinc, d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. Reprod Biol and Endo J. 2013; 11: p. 81.
6. Safarinejad, M.R., Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men. Urol J. 2009; 182: p. 237-248.
7. Mehni, N.M., Ketabchi A.A., Hosseini E., Combination effect of Pentoxifylline and L-carnitine on idiopathic oligoasthenoteratozoospermia. Iranian reprod Med J. 2014; 12: p. 817.
8. Jeulin, C. and L.M. Lewin, Role of free L-carnitine and Acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Hum Reprod Update J. 1996; 2: p. 87-102.
9. Beydola, T., Sharma RK, Lee W., Sperm preparation and selection techniques. Male Infertility Practice. Jaypee Brothers Med Publishers. 2013; p. 244-51.
10. Di Santo, M., et al., Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Advances in Urol. 2011; 2012.
11. Taylor, C.T., Antioxidants and reactive oxygen species in human fertility. Environmental tox and pharma J. 2001; 10: p. 189-198.
12. DelamiIr E., and Gagnon C., Reactive oxygen species and human spermatozoa. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. Andro J. 1992; 13: p. 379-386.
13. Hammadeh M., Radwan M., Al-Hasani S. Comparison of reactive oxygen species concentration in seminal plasma and semen parameters in partners of pregnant and non-pregnant patients after IVF/ICSI. Reprod biomed J. 2006;13: p. 696-706.
14. Nasr E., Abasi H, Razavi S., Varicocelectomy: semen parameters and protamine deficiency.

International Andro J. 2009; 32: p. 115-122.
15. Morielli T, O'Flaherty C. Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. Reprod J . 2015; 149: p.113–23.
16. Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. J Androl .1992; 13: p.368–78.
17. Wail Wafa. Saeed AM, El-Nagar HA, Effect of Coenzyme Q10 as an Antioxidant Added to Semen Extender During Cryopreservation of Buffalo and Cattle. Animal J and Poultry Prod., Mansoura Univ . 2016; 7: p. 403 -408.
18. Melissa R., Maria E., Roberto., M Role of coenzyme Q and vitamin E on semen motility evaluated both in frozen and cooled- stored semen, Italian Animal J. 2016; 4. p. 595-603
19. Geva E., Lessing JB., Lerner-Geva L., Free radicals, antioxidants and human spermatozoa: clinical implications. Hum Reprod.1998; 13: p.1422–4.
20. Bisht S., Faiq M., Tolahunase M, Oxidative stress and male infertility. Nat Rev Urol .2017; 14: p.470–85.
21. Oliva R. Protamines and male infertility. Hum Reprod Update. 2006; 12: p.417–35.
22. Kadenisa N., Charoenchai CH., Pachara V., Effects of coenzyme Q10 added in freezing medium on sperm quality and DNA fragmentation after vitrification in normozoospermia. Thammasat Uni. 2015.
23. Kobayashi T., Miyazaki T., Natori M., Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. Hum Reprod. 1991; 6: p.987–91.
24. Alkan I, Simsek F, Haklar G, et al. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. Urol J. 1997; 157: p.140–3.
25. Jung T, Bader N, Grune T. Oxidized proteins: intracellular distribution and recognition by the proteasome. Arch Biochem Biophys. 2007; 462: p.231–237.
26. Erenpreiss J, Spano M, Erenpreisa J, Sperm chromatin structure and male fertility: biological and clinical aspects. Asian Androl J . 2006; 8: p.11–29.
27. Fakhridlin M., Jabbar Y., Majida M.H., Effect of L- Carnitine and COQ10 Addition to SMART Pro Medium on Human Sperm Concentration, Sperm Morphology. IOSR J of Pharma and Biol Sciences 2017;12: p.51-55.
28. Aliabadi E., Soleimani M., h.D., Borzoei Z., Effects of L-carnitine and L-acetyl-carnitine on testicular sperm motility and chromatin quality. Iran Reprod Med J. 2012; 10: p. 77–82.