

## Research Article

# Protective Effects of Astaxanthin on Post-Thaw Sperm Quality in Normozoospermic Men

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Fighting against free radical accumulation during the cryopreservation process with the help of exogenous antioxidants has become an effective strategy to augment post-thaw sperm quality. The present study explored the effects of various concentrations of astaxanthin, as a potent antioxidant, on human sperm parameters during the freeze–thaw process. Twenty-five normozoospermic specimens were included in this prospective study. Each sample was divided into five equal parts: fresh group, and frozen–thawed groups including 0, 1, 10, and 50  $\mu\text{M}$  of astaxanthin. The prepared spermatozoa were cryopreserved by rapid freezing technique. Precryopreservation and post-thaw sperm motility parameters, sperm morphology, vitality, DNA fragmentation, reactive oxygen species (ROS) levels, and mitochondrial membrane potential (MMP) were investigated. All sperm parameters after thawing significantly decreased compared to before freezing. Treatment of spermatozoa with 50  $\mu\text{M}$  of astaxanthin significantly increased their total and progressive motility, viability, DNA integrity, and MMP and decreased their intracellular ROS levels compared with the control group. In total, 10  $\mu\text{M}$  of astaxanthin significantly improved total and progressive motility, DNA integrity, MMP, and decreased ROS levels, whereas, in the 1  $\mu\text{M}$  group, there were significant differences only in ROS levels. As a result, we found that astaxanthin can improve sperm quality after freezing/thawing and decrease the detrimental effects of this process on sperm parameters.

## 1. Introduction

Cryopreservation of spermatozoa is a widely used procedure in assisted reproductive technology (ART) centers for a variety of reasons, such as fertility preservation before cancer treatment or certain surgery that may result in testicular

dysfunction or ejaculatory dysfunction [1, 2], sperm storage in donor banks, and the minimization of infectious disease transmission [3]. Despite its tremendous importance, sperm cryopreservation has undesirable effects on sperm structure and function, decreasing sperm motility and viability and

ultimately compromising fertility potential [4]. During cryopreservation, sperm cells are exposed to cold shock, osmotic stress, and mainly oxidative stress that results in acrosomal damage, mitochondrial dysfunction, plasma membrane permeability changes, and DNA fragmentation [5, 6]. These sperm cryo-damages are mediated by reactive oxygen species (ROS) overproduction. Though ROS, in physiological amounts, is necessary for important sperm events such as hyperactivation, acrosomal reactions, capacitation, mitochondrial stabilization, and fertilization, elevated ROS causes polyunsaturated fatty acid peroxidation, DNA damage, membrane fluidity reduction, membrane enzyme and ion channel inactivity, and cellular apoptosis [6–8]. Various studies have shown that the addition of antioxidants to extender media before cryopreservation can be an effective strategy in the mitigation of cryopreservation-related oxidative stress [1, 3, 9, 10]. Generally, there are two types of antioxidants: enzymatic and nonenzymatic. Enzymatic antioxidants, such as SOD and catalase, function at the extracellular level [11, 12], while nonenzymatic antioxidants counteract ROS generation at the cell membrane, as seen with vitamin E [13], or at extra- and intra-cellular levels, as exemplified by tempol [14]. The antioxidants predominantly used for supplementation in cryo-protective media are nonenzymatic. They preserve sperm functions during cryopreservation by scavenging free radicals and mitigating oxidative stress through the mimicry of enzymatic activity [14].

Astaxanthin is a lipid soluble, red–orange pigment that extends across the entire plasma membrane and falls within the group of oxygen-containing carotenoids known as xanthophylls. It is naturally produced by algae, fungi, bacteria, yeast, and marine animals such as shrimps, salmon, trout, and lobsters [15]. Astaxanthin has a broad range of biological activities and effects, such as antioxidant, anti-inflammatory, and antiapoptotic properties [16], as well as inhibition of cell membrane peroxidation. In general, astaxanthin can exert an inhibitory effect on the development of oxidative stress and mitochondrial dysfunction [17].

Several studies have shown that the antioxidant activity of astaxanthin is notably more powerful than that of other carotenoids [18, 19]. Astaxanthin contains a polyene chain, polar ionone rings, and multiple double bonds which directly quench singlet oxygen, deactivate peroxy radicals, and scavenge other free radicals both at the surface and in the interior of the phospholipid membrane. Moreover, astaxanthin exhibits indirect antioxidant activity by regulating transcription factors and antioxidant enzymes, such as nuclear factor erythroid 2-related factor 2 (Nrf2), paraoxonase (PON1), haem oxygenase-1 (HO-1), superoxide dismutase (SOD), and catalase [15, 20]. In sperm cells, astaxanthin improved capacitation, acrosome reaction, and fertilization *in vitro* and *in vivo* [21, 22]. This antioxidant could improve endotoxin lipopolysaccharide (LPS)-impaired spermatozoa motility, viability, morphology, and activity; decrease LPS-induced spermatozoa oxidative stress; and alleviate LPS-impaired fertilization and embryo development through activating Nrf2/HO-1 antioxidant signaling pathway [4].

The protective effects of astaxanthin supplementation on sperm freezing media have been reported in various animal models, such as ram, pig, boar, and rooster [23–27]. It has been reported that astaxanthin could improve sperm parameters in diabetic rats [28] and rooster testes subjected to induced oxidative stress by cadmium [25].

In pigs, astaxanthin showed protective effects on frozen-thawed sperm parameters at different doses; antioxidant supplementation resulted in a higher percentage of sperm motility and provided strong protection against free radicals and lipid peroxidation in comparison to the control [24]. Based on these findings, the present study was undertaken to evaluate the effect of astaxanthin on various physiological functions of human sperm, such as motility, viability membrane integrity, intracellular ROS levels, and mitochondrial membrane potential (MMP) status during cryopreservation.

## 2. Material and Methods

**2.1. Chemicals.** In this study, all chemicals were purchased from Sigma (Sigma–Aldrich) unless noted. Astaxanthin was resolved with DMSO. After aliquoting the mixture, it was kept at  $-20^{\circ}\text{C}$  until use.

**2.2. Sample Collection.** Semen samples were obtained from 25 normozoospermic men (20–40 years old) who were referred to the IVF clinic of Shahid Mohammadi Hospital. Subjects with any history of drug addiction, smoking or alcohol consumption, varicocele, accessory gland infection, and vitamin consumption were not included. Semen samples were obtained after 3–5 days of sexual abstinence.

**2.3. Sperm Cryopreservation and Thawing.** After liquefaction, each semen sample was divided into five equal parts: (1) the fresh group, (2) the frozen–thawed group without astaxanthin (the samples in this group were frozen using only a sperm-freezing medium (Origio, Denmark), (3) frozen–thawed group containing  $1\ \mu\text{M}$  astaxanthin, (4) frozen–thawed group containing  $10\ \mu\text{M}$  astaxanthin, and (5) frozen–thawed group containing  $50\ \mu\text{M}$  astaxanthin. For cryopreservation, each aliquot was diluted by the same volume (1:1) of sperm freezing medium drop by drop and gently shaken the sperm-containing cryovial. Subsequently, the diluted samples were loaded into straws and frozen by exposure to liquid nitrogen vapor at a distance of 15–20 cm for 15 min, then immersed in liquid nitrogen [2]. After 2 weeks of storage,  $37^{\circ}\text{C}$  water was used to thaw the frozen straw. Then, samples were diluted by prewarm Ham's F10 medium with 5% HSA and centrifuged for 15 min (300 g). Finally, the pellets were resuspended with a sufficient amount of medium and processed in order to measure motility, morphology, vitality, DNA integrity, MMP, and intracellular ROS levels.

**2.4. Assessment of Sperm Motility and Morphology.** After being thawed, sperm motility was assessed by using a 400x magnification phase-contrast microscope and was categorized into three grades according to the WHO criteria (WHO, 2010): progressive, nonprogressive, and immotile. All the measurements were performed by one operator who was blinded to the treatments [29].

TABLE 1: Effect of different concentrations of astaxanthin added to freezing medium on sperm parameters after thawing.

Parameters	Groups				
	Fresh	Control	Astaxanthin 1 $\mu$ M	Astaxanthin 10 $\mu$ M	Astaxanthin 50 $\mu$ M
Progressive motility (PR) (%)	52.55 $\pm$ 7.23	17.13 $\pm$ 9.72***	18.1 $\pm$ 8.1***	23.63 $\pm$ 8.18***,+	27.54 $\pm$ 9.2***,++
Total motility (PR + NP) (%)	85.1 $\pm$ 6.3	46.72 $\pm$ 4.02***	48.8 $\pm$ 5.24***	65.53 $\pm$ 6.18***,+++	67.83 $\pm$ 8.34***,+++
Normal morphology	5.1 $\pm$ 2.6	4.5 $\pm$ 2.3	4.7 $\pm$ 1.9	4.9 $\pm$ 2.33	4.9 $\pm$ 2.78

Note: Data are presented as mean  $\pm$  SEM. \*\*\* $p$  < 0.001 compared with the fresh group. + $p$  < 0.05, ++ $p$  < 0.01, and +++ $p$  < 0.001 compared with the control group.

Morphology was evaluated by Diff-quick rapid sperm staining (Ravan Sazeh Co., Iran). At least 200 cells per slide were counted under a light microscope ( $\times$ 100 objectives). The measurement of sperm morphology was carried out according to the WHO 2010 guideline [29, 30].

**2.5. Assessment of Metabolic Activity.** The metabolic activity of sperm was determined using the MTT (Sigma–Aldrich, USA) method. Briefly, 100  $\mu$ l of sperm sample, 100  $\mu$ l of sperm buffer, and 100  $\mu$ l of MTT solution were added to a microtube and slowly mixed. Then, the mixture was incubated for 3 hr at 37°C and in complete darkness. After incubation, the samples were centrifuged for 2 min at a speed of 10,000 rpm and a temperature of 4°C. Then, 1  $\mu$ l of dimethyl sulfoxide (DMSO) was added to the sperms and thoroughly stirred using a vortex. Then, 200  $\mu$ l of each sample was transferred to a 96-well plate, and its absorbance was measured at 570 nm [31].

**2.6. Measurement of DNA Integrity.** DNA fragmentation was determined using a sperm DNA fragmentation assay kit (SDFA; Ravan Sazeh, Iran). For this assessment, 30  $\mu$ l of the sperm samples were first mixed with 70  $\mu$ l of 1% agarose. Then, 50  $\mu$ l of this mixture was placed on a slide that was already covered with 0.65% agarose dried at 80°C, covered with a coverslip, and left to solidify at 4°C for 4 min. Afterward, the coverslips are carefully removed, and the slides are immersed in 0.08 N HCl for 7 min at 22°C in a dark place. The slides were left in the lysing solution for 25 min, then they were washed in distilled water for 5 min and dehydrated in 70%, 90%, and 100% ethanol, respectively (2 min each), and air-dried. Cells are stained with Wright's staining solution and PBS. Nucleoids were examined under a bright-field microscope with an immersion oil 100 $\times$  objective. At least 200 spermatozoa were scored per semen sample. Sperm cells with no halos or small halos contain fragmented DNA [2].

**2.7. Measurement of Intracellular ROS.** One hundred microliters of sperm sample were mixed with 1,000  $\mu$ l of potassium chloride solution (at a weight/volume ratio of 1.15%) and 100  $\mu$ l of dichlorofluorescein reagent (26  $\mu$ M, Sigma–Aldrich, USA). It was stirred slowly and incubated for 30 min at 37°C in complete darkness. After incubation, the mixture was centrifuged for 2 min at a speed of 10,000 rpm and a temperature of 4°C. Then, the fluorescence absorption of 100  $\mu$ l of the supernatant solution was measured at wavelengths between 485 and 520 nm. Next, 100  $\mu$ l of the same supernatant solution was taken and slowly mixed for 15 min with 100  $\mu$ l of Bradford's

solution, then its absorbance was measured at 596 nm wavelength [31].

**2.8. Measurement of MMP.** One hundred microliters of the sperm sample with 1,000  $\mu$ l of sperm buffer and 100  $\mu$ l of Rhodamine 123 reagent (26  $\mu$ M, Sigma–Aldrich, USA) were mixed and incubated for 30 min at 37°C in the dark. After incubation, the mixture was centrifuged for 2 min at 10,000 rpm and 4°C. The fluorescence absorption of 100  $\mu$ l of the supernatant solution was measured at a wavelength of 485–520 nm. Again, 100  $\mu$ l of the same supernatant solution was mixed with 100  $\mu$ l of Bradford solution for 15 min and its absorbance was measured at 596 nm [31].

**2.9. Statistical Analysis.** All sperm parameters are expressed as mean  $\pm$  standard error (mean  $\pm$  SEM). Graphs created with the Prism GraphPad version 8 software. The one-way ANOVA, following the Tukey post hoc test, was used to compare differences between groups. The statistically significant level was set at  $p$  < 0.05.

### 3. Results

**3.1. Sperm Motility and Morphology.** The effects of the various concentrations of astaxanthin on sperm motility and morphology are presented in Table 1. Our results indicate that sperm progressive motility and sperm total motility significantly decreased after freezing in the control group compared to the fresh group (\*\* $p$  < 0.001). The addition of 10 and 50  $\mu$ M of astaxanthin to the freezing medium significantly improved progressive and total motility compared with those in the control (0 mM) group (+ $p$  < 0.05, ++ $p$  < 0.01, respectively). No significant difference was observed between 1  $\mu$ M of astaxanthin and the control group when motility was compared.

Normal morphology decreased, but not significantly, in the control group due to the cryopreservation compared to the fresh group. No significant difference was seen between the various astaxanthin groups (1, 10, and 50  $\mu$ M) and the control group (0  $\mu$ M astaxanthin) when morphology was compared.

**3.2. Sperm Metabolic Activity.** The sperm metabolic activity after freezing, especially in the control group, decreased significantly (\*\* $p$  < 0.001). Supplementation with 50  $\mu$ M of astaxanthin significantly (+ $p$  < 0.01) increased the metabolic activity of spermatozoa compared to the control group after the freezing process (Figure 1).

**3.3. DNA Fragmentation.** Cryopreservation induced a meaningful increase in the percentage of sperms with fragmented

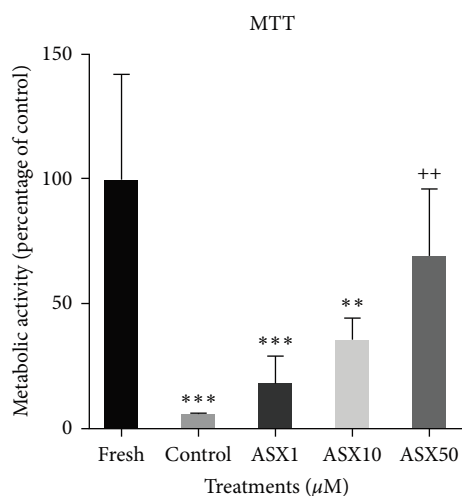


FIGURE 1: Effect of different concentrations of astaxanthin added to freezing medium on sperm metabolic activity after thawing. Fresh group (Fresh); freezing group without antioxidant (Control); freezing group containing  $1\ \mu\text{M}$  of astaxanthin (ASX1); freezing group containing  $10\ \mu\text{M}$  of astaxanthin (ASX10); and freezing group containing  $50\ \mu\text{M}$  of astaxanthin (ASX50). Data are presented as mean  $\pm$  SEM. \*\*\* $p < 0.001$  versus the fresh group, \*\* $p < 0.01$  versus the fresh group, and ++ $p < 0.01$  versus the control group. The one-way ANOVA test followed by Tukey's post hoc test was used to compare the groups.

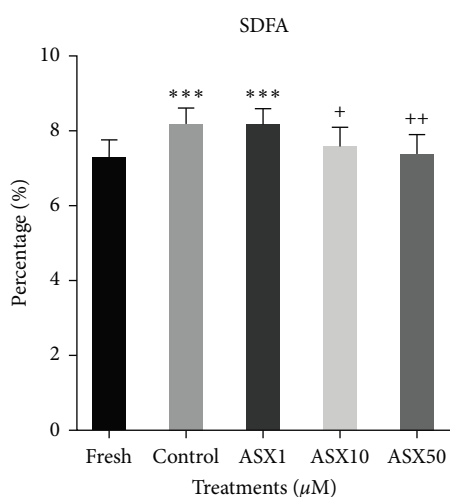


FIGURE 2: Effect of different concentrations of astaxanthin added to freezing medium on DNA fragmentation in human sperm cells after thawing. Fresh group (Fresh); freezing group without antioxidant (Control); freezing group containing  $1\ \mu\text{M}$  of astaxanthin (ASX1); freezing group containing  $10\ \mu\text{M}$  of astaxanthin (ASX10); and freezing group containing  $50\ \mu\text{M}$  of astaxanthin (ASX50). Data are presented as mean  $\pm$  SEM. \*\*\* $p < 0.001$  versus the fresh group, + $p < 0.05$  versus the control group, and ++ $p < 0.01$  versus the control group. The one-way ANOVA test followed by Tukey's post hoc test was used to compare the groups.

DNA (\*\*\* $p < 0.001$ ). In total,  $50\ \mu\text{M}$  of astaxanthin significantly decreased the level of DNA fragmentation after the freezing process (++ $p < 0.01$ ; Figure 2).

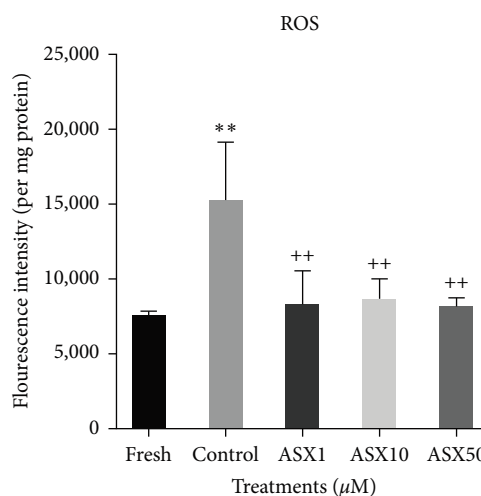


FIGURE 3: Effect of different concentrations of astaxanthin on added to freezing medium on intracellular ROS levels in semen samples after thawing. Fresh group (Fresh); freezing group without antioxidant (Control); freezing group containing  $1\ \mu\text{M}$  of astaxanthin (ASX1); freezing group containing  $10\ \mu\text{M}$  of astaxanthin (ASX10); and freezing group containing  $50\ \mu\text{M}$  of astaxanthin (ASX50). Data are presented as mean  $\pm$  SEM. \*\* $p < 0.01$  versus the fresh group. ++ $p < 0.01$  versus the control group. The one-way ANOVA test followed by Tukey's post hoc test was used to compare the groups.

3.4. *Intracellular ROS Levels.* The results revealed that the amount of intracellular ROS in the control group notably increased when compared to the fresh group (\*\* $p < 0.01$ ). The intracellular ROS levels in all groups of astaxanthin significantly decreased in comparison with the control group (++ $p < 0.01$ ; Figure 3).

3.5. *Mitochondrial Membrane Potential.* The results demonstrated that the amount of MMP in the control group significantly decreased compared to the fresh group (\*\*\* $p < 0.001$ ), indicating that cryopreservation enhanced mitochondrial damage in sperm. Supplementation with  $10$  and  $50\ \mu\text{M}$  of astaxanthin significantly improved MMP amount (++ $p < 0.01$ , +++ $p < 0.001$ , respectively). However, there was no significant difference between the  $1\ \mu\text{M}$  astaxanthin supplemented frozen-thawed group in relation to MMP compared to the control group (Figure 4).

## 4. Discussion

In this study, we evaluated the effects of various concentrations of astaxanthin on frozen-thawed human sperm quality. We found that all concentrations of astaxanthin had positive effects on most of the parameters studied; however, the  $50\ \mu\text{M}$  of astaxanthin showed the largest effects on sperm quality.

Sperm cryopreservation, as a key process in ART laboratories, compromises sperm quality, through physical-chemical modifications and ROS overproduction. Polyunsaturated fatty acids (PUFAs) in spermatozoa membranes are highly susceptible to oxidative damage and lipid peroxidation (LPO), in which more than 50% of the membrane fatty acids are lost, diminishing their fluidity and membrane integrity. ROS are also responsible for reduced sperm motility by intracellular ATP depletion,

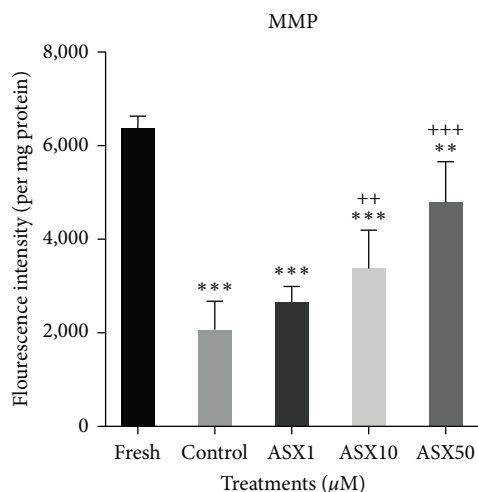


FIGURE 4: Effect of different concentrations of astaxanthin on added to freezing medium on MMP of sperm cells after thawing. Fresh group (Fresh); freezing group without antioxidant (Control); freezing group containing  $1 \mu\text{M}$  of astaxanthin (ASX1); freezing group containing  $10 \mu\text{M}$  of astaxanthin (ASX10); and freezing group containing  $50 \mu\text{M}$  of astaxanthin (ASX50). Data are presented as mean  $\pm$  SEM. \*\*\* $p < 0.001$  versus the fresh group, \*\* $p < 0.01$  versus the fresh group. ++ $p < 0.01$  versus the control group and +++ $p < 0.001$  versus the control group. The one-way ANOVA test followed by Tukey's post hoc test was used to compare the groups.

damage of electron transport chain (ETC) proteins, mitochondrial DNA mutations, axonemal damage, and increased mid-piece sperm morphological defects. Moreover, ROS are capable of invading the DNA, causing fragmentation and loss of the DNA base structure, and damage to DNA integrity. In the present study, total motility, progressive motility, viability, and MMP were significantly decreased in the control group compared with the fresh group. As well, cryopreservation increased DNA fragmentation and ROS levels in line with previous studies. Due to the low amount of cytoplasm, the antioxidant capacity of sperm cells is insufficient to scavenge excessive amounts of ROS generated during cryopreservation. Antioxidants are molecules that support sperm cells and can mitigate any form of oxidative/nitrosative stress or its consequences induced by the freezing process. Astaxanthin is a keto-carotenoid that its antioxidant activity is ten-fold higher than that of other carotenoids and a hundredfold more than that of tocopherol [27, 32]. The protective effects of astaxanthin against reactive species are mediated both by donating electrons to unpaired electrons and by bonding with the free radical to form a nonreactive adduct. In addition, the presence of a series of conjugated bonds in the nonpolar middle segment of the astaxanthin enables the molecule to remove free radicals from the cell interior and conduct them out of the membrane so that these are neutralized by other antioxidants located outside the cell membrane [33].

In the present study, the addition of  $10$  and  $50 \mu\text{M}$  astaxanthin to the cryopreservation medium significantly increased the percentages of total and progressive sperm motility and viability compared with that of the control group. In agreement with our findings, the addition of astaxanthin (from  $0.5$  to  $5 \text{ mM}$ ) to the boar sperm freezing medium improved the values

of total and progressive motility compared with the control group [23]. In another study supplementation of the cryopreservation medium with  $1 \mu\text{M}$  of astaxanthin significantly improved post-thaw percentages of motile and viable dog sperm [32]. Comhaire et al. [34] also indicated that the administration of  $60 \text{ mg}$  astaxanthin to infertile men for 3 months significantly decreased seminal ROS levels and increased the progressive motility of sperm. In a parallel investigation, Dede and Saylan [35] introduced varying concentrations of astaxanthin ( $0$ ,  $50$ ,  $100$ , and  $500 \mu\text{M}$ ) into semen samples collected from 30 healthy male participants. Their findings revealed noteworthy enhancements in sperm motility, particularly with a dosage of  $100 \mu\text{M}$  astaxanthin treatment [35]. It appears that astaxanthin has the potential to improve sperm motility in a dose-independent manner, indicating a potential saturation point beyond which further increases in concentration may not yield additional benefits and/or could instead induce toxicity to sperm motility. The mechanisms underlying astaxanthin's effects on sperm motility postcryopreservation are multifaceted. By scavenging free radicals and reducing lipid peroxidation, astaxanthin preserves sperm membrane integrity and mitochondrial function, thereby enhancing motility.

Mitochondrial integrity and intact MMP are prerequisites for sperm motility [36]. Increased oxidative stress can harm mitochondria and subsequent mitochondrial dysfunction generates excesses of ROS that cause cellular damage [17]. Our data indicated that cryopreservation reduced MMP and increased ROS levels compared to the fresh group. Previous studies have shown that astaxanthin can reduce oxidative stress, maintain mitochondrial integrity, and prevent loss of MMP [37, 38]. Astaxanthin decreased ROS levels and increased MMP in an *in vitro* model of inflammatory pre-eclampsia [39]. All concentrations of used astaxanthin in the present study decreased ROS levels and improved MMP value, though  $50 \mu\text{M}$  of astaxanthin had better effects. Similarly, it was shown that astaxanthin has a great protective effect in preserving the mitochondrial function of dog sperm during freeze-thaw procedures. In a study conducted on miniature pig sperm, Lee and Kim [24] added increasing concentrations of  $0$ ,  $10$ ,  $50$ ,  $100$ , and  $500 \mu\text{M}$  astaxanthin to freezing extenders. In parallel with our study, they found that in all experimental groups for which astaxanthin was added, ROS levels decreased significantly when compared with the control group [24]. Further, Amidi et al. [10] conducted a human study that illustrated that administering  $1 \mu\text{M}$  astaxanthin before the freezing-thawing process displays protective properties against oxidative stress and reduces the detrimental effects of this process on sperm quality [40].

The results obtained by the SCD test also showed that the sperm DFI was significantly decreased in  $10$  and  $50 \mu\text{M}$  of astaxanthin compared to the frozen sperm group without astaxanthin added to the cryopreservation medium. In addition, we found that  $1 \mu\text{M}$  of astaxanthin had no beneficial effects on DNA fragmentation. Preserving sperm DNA integrity against oxidative stress produced by cryopreservation procedures is of great importance for further fertilization outcomes, such as normal development of the embryo, and the birth of healthy offspring. A previous study in dogs also

confirmed that astaxanthin-supplemented extender causes a significant increase in post-thaw sperm DNA integrity and a lower expression of the oxidative-induced DNA damage repair gene (OGG1), indicating reduced DNA damage [32]. Administration of astaxanthin for 4 weeks also resulted in a significant decrease in the levels of the DNA damage marker (plasma 8-OHdG) in humans [41].

## 5. Conclusion

Our findings showed that spermatozoa treatment with astaxanthin, especially at 50  $\mu$ M concentration before the cryopreservation process has protective effects against oxidative stress and could decrease the detrimental effects of this process on sperm quality. Further studies are needed to study the molecular mechanism of astaxanthin as a potent antioxidant on sperm parameters.

## Abbreviations

DNA:	Deoxyribonucleic acid
ROS:	Reactive oxygen species
MTT:	2,5-Diphenyl-2H-tetrazolium bromide
MMP:	Mitochondrial membrane potential
PUFAs:	Polyunsaturated fatty acids
LPO:	Lipid peroxidation
ETC:	Electron transport chain
SCD:	Sperm chromatin dispersion
DFI:	DNA fragmentation index
OGG1:	Oxidative-induced DNA damage repair gene
ART:	Assisted reproductive technology
Nrf2:	Nuclear factor erythroid 2-related factor 2
PON1:	Paraoxonase 1
SOD:	Superoxide dismutase
LPS:	Lipopolysaccharide
IVF:	<i>In vitro</i> fertilization
HAS:	Human serum albumin
DMSO:	Dimethyl sulfoxide
S DFA:	Sperm DNA fragmentation assay.

## Data Availability

Data will be available upon request.

## Ethical Approval

The study was ethically approved by the Ethics Committee of Hormozgan University of Medical Sciences (IR.HUMS.REC.1400.124).

## Consent

All participants signed written informed consent forms and the study was carried out according to the Helsinki Declaration.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Afshin Mohammadi-Bardbori and Amir Shadboorestan contributed equally to this manuscript.

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