

The effect of astaxanthin on human sperm parameters after cryopreservation

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Abstract

Introduction: The aim of the study is to examine the protective efficacy of astaxanthin (ASTA) against the damage that occurs during sperm cryopreservation.

Methods: This experimental study was carried out on waste semen samples of 30 normozoospermic individuals who applied for semen analysis. Semen samples were divided into four equal volumes and 0 μ M (control group), 50 μ M, 100 μ M, and 500 μ M ASTA were added to each group, respectively. All groups were stored frozen in a liquid nitrogen tank. Semen samples were removed from liquid nitrogen after 72 hours and were thawed. Motility evaluation of sperm was performed. In addition, sperms were stained with acidic aniline blue to detect DNA chromatin condensation.

Results: The highest motility loss was found in the control group and the least motility loss was in the 100 μ M ASTA group. When examined in terms of sperm chromatin condensation, condensed sperm count was higher in the 100 μ M ASTA group than in the other groups.

Conclusions: It has been observed that ASTA added to the cryoprotectant substance during sperm cryopreservation positively affects sperm motility and reduces the number of decondensed sperm.

Key Messages

The primary goal in cryopreservation is to minimize the cellular damage. Astaxanthin, which is considered as the strongest and safest antioxidant in nature. Astaxanthin added to cryoprotectant during cryopreservation were found to be effective

Introduction

Approximately 8% of men in reproductive age have infertility problems ^{1,2}. Sperm-embryo cryopreservation is one of the commonly used treatment methods among assisted reproductive techniques. In cryopreservation, which is applied for individuals with infertility problems or those who have had a disease affecting reproductive functionality, sperms can be kept for many years and they can be thawed and used if necessary ^{3,4}. Cryopreservation is the cessation of biological activity of cells or tissues at very low temperatures and their long-term storage for future use with minimum damage and no loss of function ⁵. The primary goal in cryopreservation is to minimize the cellular damage that ice crystals may cause during the process of freezing and thawing with cryoprotectants ^{6,7}. However, cryopreservation process leads to damage by exposing the sperm to physical and chemical stress. After cryopreservation, sperms may have damages such as morphological changes, decrease in motility, disruption of acrosome structure and increase in DNA fragmentation ^{4,8–10}. In order to reduce these damages, it is recommended to add antioxidants such as ascorbic acid, catalase, Vitamin E in addition to cryoprotectant agents (CPAs) ¹¹. Astaxanthin (ASTA), which belongs to the ketone family of carotenoids, is a red pigment naturally found in sea creatures such as salmon, lobster, shrimp and crabs ^{12,13}. ASTA, which is considered as the strongest and safest antioxidant in nature, protects the cell membrane and tissue against lipid peroxidation and oxidative stress ^{14,15}. Antioxidant activity of ASTA is 100 times higher than Vitamin E ¹⁶, 6000 times higher than Vitamin C, 800 times higher than Coenzyme Q10, 4 times higher than lutein and 10 times higher than β -carotene ^{17–19}. Studies have shown that ASTA has an effective mechanism in protecting cell membrane structure ^{20,21} and increases human sperm motility ^{22,23}.

In this study, our aim was to show the protective efficacy of ASTA against damage in the cryopreservation process.

Methods

This experimental study was conducted on waste semen samples of male individuals between the ages of 25 and 40 who applied to Bolu Abant İzzet Baysal University Faculty of Medicine for semen analysis. 30 normozoospermic individuals according to WHO 2010 criteria who signed the “Informed Consent Form” were included in the study with the 2019/103 numbered Bolu Abant İzzet Baysal University Faculty of Medicine Clinical Researches Ethics Committee approval.

Preparation of semen samples

Semen samples were obtained from 30 male individuals after 3 days of sexual abstinence. Semen samples taken with masturbation method were kept for 20-30 minutes on a 37°C heating surface for liquefaction. Following this, macroscopic (color, odor, viscosity, volume) and microscopic (concentration, motility, morphology) examination was completed (Table 1). The pellet obtained after semen samples were centrifuged was mixed with DMEM (Dulbecco's Modified Eagle's Medium) washing solution (Gibco, Cat No.2007871, UK) at a ratio of 1:1.

Sperm cryopreservation and thawing

Semen samples were divided in 4 equal volumes. Dimethyl sulfoxide (DMSO), which is used as a cryoprotectant, was added to each group in accordance with the amount of ASTA to be added. Afterwards, ASTA (Sigma-Aldrich, Cat No. 472-61-7, USA) dissolved in 1mg/ml DMSO (Sigma-Aldrich, Cat No.41639, USA) was added in amounts of 50 µM, 100 µM and 500 µM to each group, respectively. The group which was not added ASTA was evaluated as the control group. The tubes which were cooled in liquid nitrogen vapour for 10 minutes were stored frozen for 72 hours. The samples extracted from liquid nitrogen tank were thawed for 20 minutes by placing in 37 °C water bath. After the thawing process was completed, DMEM washing solution with a ratio of 1:1 was added on the samples to remove DMSO and ASTA. Next, they were centrifuged for 10 minutes at 1000 rpm. After this, the samples were analyzed in terms of sperm parameters.

Sperm motility assessment

Motility assessment was made after thawing process. Sperm motility was evaluated as progressive, non-progressive and immotile and proportioned to sperm concentration.

Aniline blue staining

Aniline blue, which is used for the detection of chromatin condensation in sperm DNA, is considered as a standard test ²⁴. Sperms with damaged DNA often show the presence of residual histones ²⁵. Based on this, the nuclei of decondensed sperms including histones with abundant lysine will be stained darker. Arginine and cysteine rich protamine including nucleus of condensed sperm will be stained lighter as it contains a small amount of lysine ²⁶⁻²⁸. The smears of groups were first fixed with 3% glutaraldehyde (Merck, Cat No.8206031000, Germany) for 30 minutes for acidic aniline blue staining. Later, staining was performed with acidic aniline blue (Carlo Erba, Cat No. 428582, France) (pH: 3,5) for 5-7 minutes. Preparates were washed twice in phosphate buffer solution (Phosphate Buffered Saline, PBS, Thermo Fisher Scientific, UK, pH 7.2). The preparates stained with acidic aniline blue were evaluated by counting 200 sperm cells at Nikon Eclipse 80i light photomicroscope. Then they were photographed with X100 lens.

Statistical analysis

Statistical analyses in our study were performed with SPSS version 21.0 analysis program. The data used were expressed as mean±standard deviation. The differences in data between groups which were normally distributed were evaluated with One-Way ANOVA. Tukey HSD test with post-hoc analysis was used to determine individual group differences. Kruskal-Wallis test and Tamhane's T2 test were used for non-parametric comparisons between groups which were not normally distributed. Statistical significance level of the results was taken as $p \leq 0,05$.

Results

Our study was conducted with waste semen samples of 30 normozoospermic male individuals. Sperm samples which were removed from liquid nitrogen 72 hours later were

thawed in 37 °C water bath. After thawing, sperm samples obtained were analyzed and evaluated statistically. When the pre-cryopreservation and post-cryopreservation motility values of sperms were compared, statistically significant overall decrease was observed in all groups in terms of motility when compared with pre-cryopreservation ($p<0,01$) (Figure 1).

The highest motility loss was observed in the control group (0 μ M ASTA). It was found that the motility of sperm cells including 100 μ M ASTA and 500 μ M ASTA was higher when compared with the sperm cells in the control group and the result was found to be statistically significant ($p<0,01$). When 100 μ M ASTA added sperm cells were compared with 50 μ M ASTA containing sperm cells, high number of motile sperms were found and they were found to be statistically significant ($p<0,01$). When 100 μ M ASTA added sperm cells were compared with 500 μ M ASTA containing sperm cells; although lower number of motile sperms were found in 500 μ M ASTA containing sperm cells (Table 2), no significance was observed (Figure 2).

The samples were stained with acidic aniline blue in order to evaluate sperm chromatin condensation. Sperms cells the nuclei of which were stained light were considered positive (intact DNA), while those stained dark were considered as negative (damaged DNA) (Figure 3).

The results were evaluated statistically. When the control group and the groups containing 100 μ M ASTA and 500 μ M ASTA were compared, decondensed sperms in the control group were statistically higher than those in the other groups ($p<0,01$) (Table 3). In addition, decondensed sperms in the group containing 50 μ M ASTA were significantly higher than the sperm cells which were added 100 μ M ASTA and 500 μ M ASTA ($p<0,01$). When the groups containing 100 μ M ASTA and 500 μ M ASTA were compared with each other, although decondensed sperm number was lower in the group containing 100 μ M ASTA (Table 4), no significant difference was found (Figure 4).

Discussion

Sperm cryopreservation is one of the most studied topics in recent years. The aim of this method is to preserve fertility by storing sperms before surgical operations and cytotoxic treatments such as chemotherapy/radiotherapy that may cause infertility^{4,29}. During the cryopreservation process, the most basic cell damages occur during freezing and especially thawing phase. Cryo-damage can be effectively reduced by methods such as adding CPA prior to cryopreservation and applying appropriate freezing-thawing techniques³⁰. However, CPAs are toxic for cells and they become gradually more toxic as concentration increases³¹. It is recommended to add antioxidant materials to CPAs in order to prevent cryopreservation damage^{11,32,33}.

ASTA, which is known with its antioxidant activity, can be used to improve sperm quality and it decreases Reactive oxygen species (ROS) production in seminal fluid significantly. It has been reported that astaxanthin increases fertilization rate by increasing sperm concentration and linear progressive motility. This shows that it creates positive effects on male infertility^{34,35}.

In a study they conducted on cattle, Jang et al. researched the antioxidant effects of ASTA against oxidative stress created in epithelium cell culture. They observed an increase in the viability of oviduct cells exposed to 500 μ M ASTA for 24 hours. They reported that ASTA had an antioxidant effect on the development of cattle embryo³⁶. Gao et al. observed that 100 mg/kg ASTA increased semen quality and antioxidant activity in a study they conducted in rooster sperm³⁷. Comhaire et al. gave 16 mg ASTA orally to 30 infertile male individuals for 3 months. In this study they conducted on the effects of ASTA on infertility, they found that ROS level decreased significantly and the progressive movement of sperm increased³⁸. In another study conducted by Comhaire and Decleer, it was found that ASTA given as a food supplement for 3 months to infertile couples improved sperm morphology. They also found that astaxanthin decreased ROS level of semen and also significantly increased sperm motility and amount³⁹. In a similar study, Tunç et al. researched whether antioxidant supplement could improve DNA integrity in male individuals who were found to show oxidative stress and applied ASTA treatment orally to 50 male individuals for 3 months. They found that the ASTA treatment applied provided significant improvements in sperm DNA integrity. As a result, they recorded a decrease in seminal ROS production and apoptosis⁴⁰. Basioura et al.⁴¹ showed higher plasma membrane integrity, viability, motility and progressive motility after thawing in pigs. Nejafi et al.⁴² reported a positive effect of astaxanthin on male semen quality after thawing.

In a study Lee and Kim conducted on miniature pig sperm, they added increasing concentrations of 0, 10, 50, 100, and 500 μ M ASTA to cryoprotectant. In parallel with our study, they found that in all experimental groups which were added ASTA, sperm motility increased significantly when compared with the control group and the number of sperms with advanced mobile sperm increased. In addition, it was found that ROS levels were lower in all groups with ASTA supplementation⁴³. In a study of Neamah and Houbi observed that the percentage of viable sperm improved 24 hours after the addition of ASTA to the semen sample, and sperm motility increased 72 hours later²³. Hao, who researched the effects of ASTA on semen quality of diabetes patient mice, applied 0, 10, 50 or 100 mg/kg ASTA treatment to 60 mice. In terms of sperm motility and morphology, it was found that diabetes patient mice which were applied ASTA improved significantly when compared with mice in the control group. Thus, it was reported that ASTA affected semen quality positively⁴⁴. In a similar study, the effects of ASTA on the sperms of 30 adult diabetic rats were researched. In the sperms of the group that was treated with ASTA for 56 days, increase was found in normal morphology and viable sperms when compared with other groups. Based on this, they found that ASTA improve sperm viability, morphology and DNA integrity⁴⁵. Kumalic et al. in a study of their effect on semen parameters; observed that oral astaxanthin intake did not change semen parameters in patients in a study¹⁶. On the contrary, Dona et al. conducted on human sperm, they added 0 μ M, 0,5 μ M, 1 μ M and 2 μ M concentrations of ASTA to semen samples taken from 24 male individuals with normozoospermia and incubated for 180 minutes. When all groups were compared, the number of viable sperms was higher in the group that was added 2 μ M ASTA. This way, they showed that ASTA could improve human sperm parameters³⁴. In a similar study conducted with semen samples taken from 51 healthy

male individuals, the effects of ASTA on human sperm were researched. As a result of the study, Andrisani et al. proved that ASTA could be used to decrease male infertility by improving human sperm quality ²².

Conclusions

In summary, Different concentrations of ASTA doses added to cryoprotectant during cryopreservation were found to be effective. It was realized that 100 μ M ASTA dose affected the motility of sperms positively. It was also found that ASTA was effective in decreasing chromatin condensation. These findings are promising. Considering the positive results of this new methodology, molecular studies are needed to prevent possible chromatin damage and increase motility in human sperm before starting clinical application.

DRAFT

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[Consider removing Table 1 as per reviewers’ comments and author recommendations]

Figures and Tables

Figure 1. Pre-cryopreservation and post-cryopreservation sperm motility values.

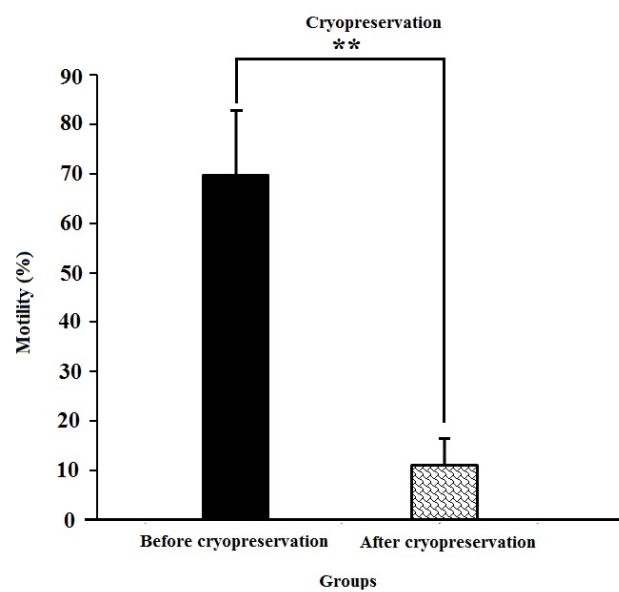


Figure 2. Post-cryopreservation motility values of the related groups.

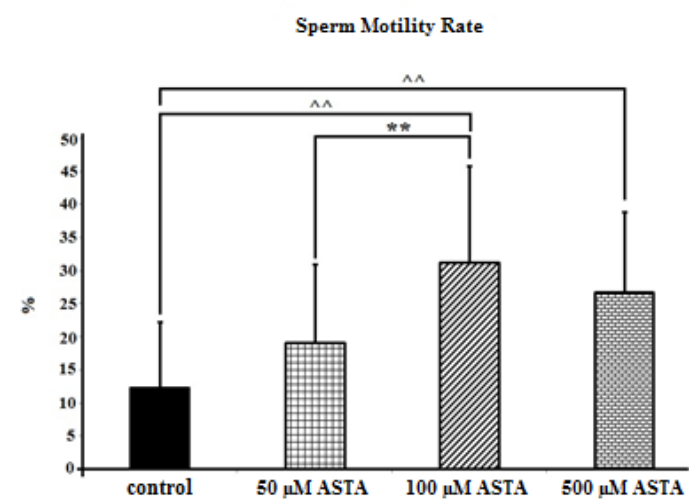


Figure 3. Control group (0 μ M astaxanthin [ASTA]) (a) 50 μ M ASTA group, (b) 100 μ M ASTA group, (c) 500 μ M ASTA group, (d) 1000X, Scale bar: 10 μ m. Condensed sperms are seen to be lightly stained with aniline blue in pictures a, b, c, and d; decondensed sperms are seen to be darkly stained with aniline blue in pictures a, b, c, and d.

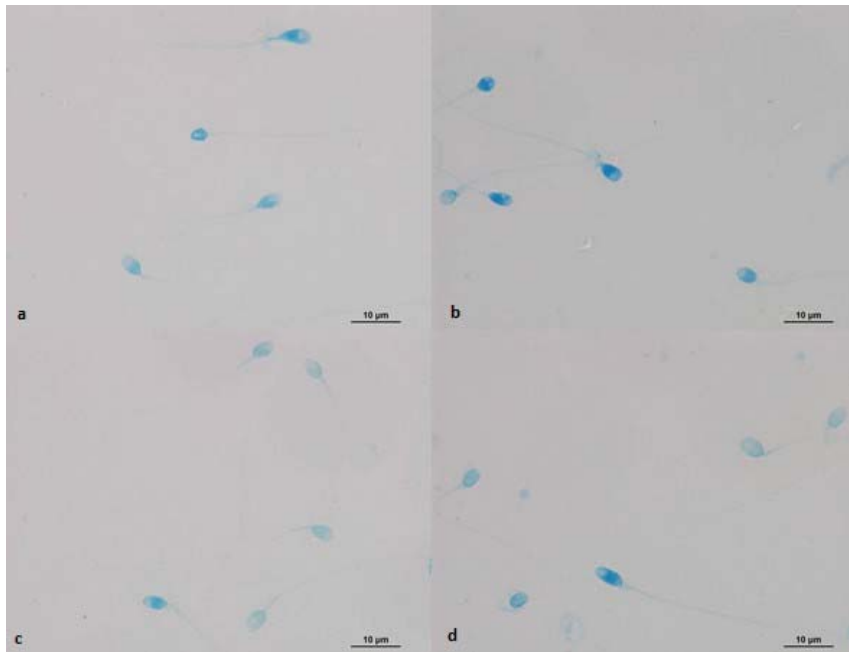


Figure 4. Percentage values of decondensed sperm groups after cryopreservation. ASTA: astaxanthin.

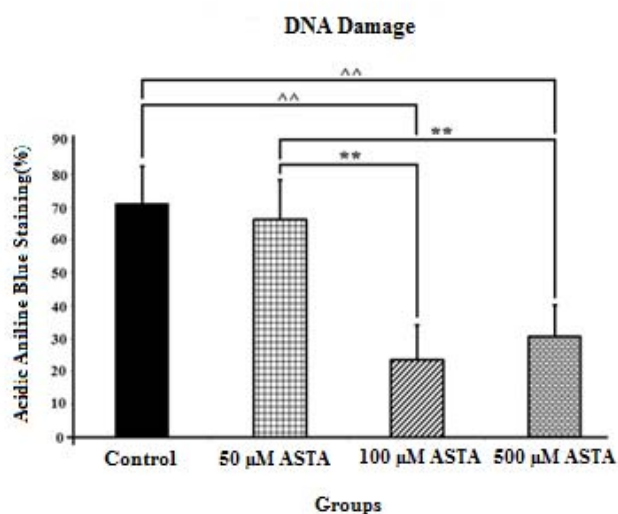


Table 1. Cutoff reference values for normal semen characteristics as published in consecutive WHO manuals and values in this study		
Parameters	WHO 2010	Values in this study
Semen volume (ml)	≥1.5	3.74
Total sperm concentration (10 ⁶)	≥39 (33–46)	279
Sperm concentration (10 ⁶ /ml)	≥15 (12–16)	74.6
Total motility (PR+NP,%)	≥40 (38–42)	69.7
Progressive motility, (%)	≥32 (31–34)	58.3
Sperm morphology (normal form, %)	≥4.0	9.7
pH	≥7.2	≥7.2
Peroxidase-positive leukocyte (10 ⁶ /ml)	<1.0	<1.0

NP: non-progressive; PR: progressive; WHO: World Health Organization.

Table 2. ASTA associated evaluation of sperm motility grades			
Groups	Groups	Mean difference	Significance
Control	50 µM ASTA	-6900	0.101
	100 µM ASTA	-18 967*	0.000
	500 µM ASTA	-14 467*	0.000
50 µM ASTA	Control	6900	0.101
	100 µM ASTA	-12 067*	0.005
	500 µM ASTA	-7567	0.099
100 µM ASTA	Control	18 967*	0.000
	50 µM ASTA	12 067*	0.005
	500 µM ASTA	4500	0.737
500 µM ASTA	Control	14 467*	0.000
	50 µM ASTA	7567	0.099
	100 µM ASTA	-4500	0.737

*The mean difference is significant at the 0.05 level. ASTA: astaxanthin.

Table 3. ASTA associated evaluation of sperm chromatin decondensation			
Groups	Groups	Mean difference	Significance
Control	50 µM ASTA	4700	0.347
	100 µM ASTA	47 400*	0.000
	500 µM ASTA	40 367*	0.000
50 µM ASTA	Control	-4700	0.347
	100 µM ASTA	42 700*	0.000
	500 µM ASTA	35 667*	0.000
100 µM ASTA	Control	-47 400*	0.000
	50 µM ASTA	-42 700*	0.000
	500 µM ASTA	-7033	0.067
500 µM ASTA	Control	-40 367*	0.000
	50 µM ASTA	-35 667*	0.000
	100 µM ASTA	7033	0.067

*The mean difference is significant at the 0.05 level. ASTA: astaxanthin.

Table 4. Mean ± SD values belonging to sperm motility and chromatin decondensation										
Groups	Before cryo-preservation (n=30)		After cryo-preservation Control (n=30)		After cryo-preservation 50 µM ASTA (n=30)		After cryo-preservation 100 µM ASTA (n=30)		After cryopreservation 500 µM ASTA (n=30)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total motility (PR+NP,%)	69.7	12.969	12.3	10 018	19.2	11 773	31.3	14 598	26.8	12 135
Chromatin decondensation (%)	18.2	6.002	71.16	11 543	66.4	11 936	23.7	10 474	30.8	9625

NP: non-progressive; PR: progressive; SD: stanard deviation.