The effect of astaxanthin on human sperm parameters after cryopreservation

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Abstract

Introduction: The aim of the study was 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 µM (control group), 50 µM, 100 µM, and 500 µM ASTA were added to each group. 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, sperm was 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 µM ASTA group. When examined in terms of sperm chromatin condensation, condensed sperm count was higher in the 100 µM 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.

Introduction

Approximately 8% of men in reproductive age have infertility problems.[1,2] Sperm-embryo cryopreservation — where sperm can be kept for many years and thawed and used as needed[3,4] — is one of the most used methods among assisted reproductive techniques. 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 is to minimize the cellular damage that ice crystals may cause during the process of freezing and thawing with cryoprotectants;[6,7] however, the process can lead to damage, such as morphological changes, decrease in motility, disruption of acrosome structure, and increase in DNA fragmentation,[4,8-10] by exposing the sperm to physical and chemical stress.

KEY MESSAGES

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

To reduce this damage, it is recommended to add antioxidants, such as ascorbic acid, catalase, and 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] It is considered the strongest and safest antioxidant in nature, protecting the cell membrane and tissues 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, four times higher than lutein, and 10 times higher than β-carotene.[17-19] Studies have shown that ASTA has an effective mechanism to protect cell membrane structure[20,21] and increase 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 (Turkey) for semen analysis. Thirty normozoospermic individuals, based on World Health Organization 2010

criteria, who signed the informed consent form were included in the study, which received ethics committee approval.

Preparation of semen samples

Semen samples were obtained from 30 male individuals after three 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 Dulbecco's modified eagle's medium (DMEM) washing solution (Gibco, Cat No.2007871, U.K.) at a ratio of 1:1.

Sperm cryopreservation and thawing

Semen samples were divided in four 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, U.S.) dissolved in 1 mg/ml DMSO (Sigma-Aldrich, Cat No.41639, U.S.) was added in amounts of 50 µM, 100 µM, and 500 µM to each group. The group for which ASTA was not added was evaluated as the control group. The tubes that were cooled in liquid nitrogen vapor for 10 minutes were stored frozen for 72 hours. The samples extracted from the liquid nitrogen tank were thawed for 20 minutes by placing them in a 37 °C water bath.

After the thawing process was completed, DMEM washing solution in a ratio of 1:1 was added to 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.

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.

Sperm motility assessment

Motility assessment was made after thawing. 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 a standard test.[24] Sperm with damaged DNA often show the presence of residual histones.[25] Based on this, the nuclei of decondensed sperm, including histones with abundant lysine, will be stained darker. Arginine- and cysteine-rich protamine, including the nuclei 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, U.K.; 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 that 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 that were not normally distributed. Statistical significance was taken as $p \leq 0.05$.

Results

When the motility values of sperms were compared, a statistically significant overall decrease was observed in all groups in terms of motility in post-cryopreservation compared with pre-cryopreservation sperm ($p<0.01$) (Figure 1).

The highest motility loss was observed in the control group (0 µM ASTA). It was found that the motility of sperm cells with 100 µM ASTA and 500 µM ASTA was significantly higher when compared with the sperm cells in the control group ($p<0.01$). When 100 µM ASTA-added sperm cells were compared with 50 µM ASTA-containing sperm cells, a higher number of motile sperm were found ($p<0.01$). Although a

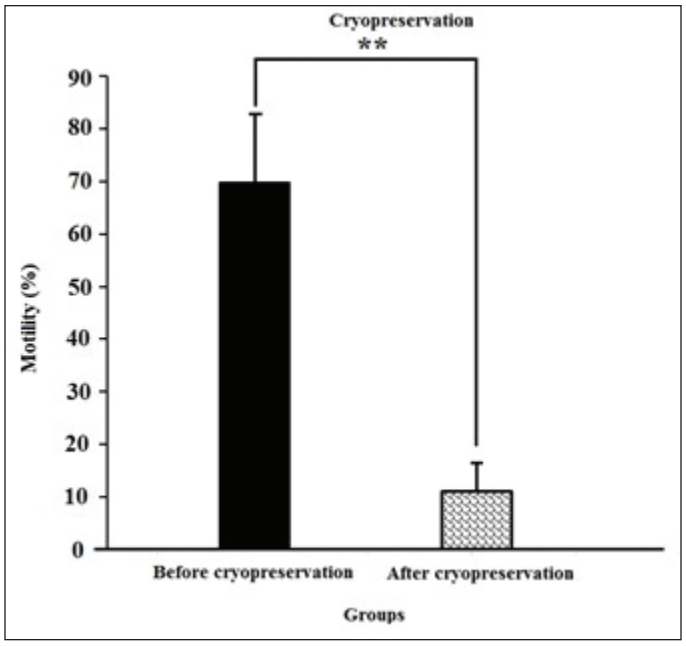

Figure 1. Pre-cryopreservation and post-cryopreservation sperm motility values.

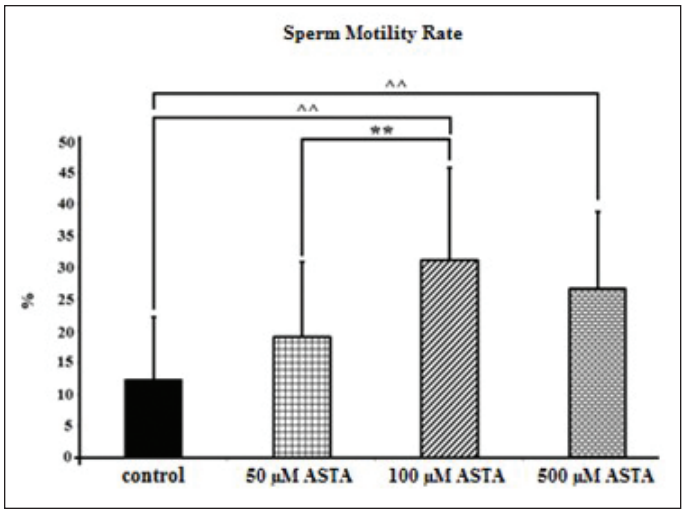

Figure 2. Post-cryopreservation motility values of the related groups.

lower number of motile sperm were found in 500 µM ASTA-containing sperm cells than 100 µM 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. Sperm cells the nuclei of which were stained light were considered positive (intact DNA), while those stained dark were considered negative (damaged DNA) (Figure 3).

The results were evaluated statistically. When the control group and the groups containing 100 µM ASTA and 500 µM 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 sperm in the group containing 50 µM ASTA were significantly higher than the sperm cells for which 100 µM ASTA and 500 µM ASTA were added ($p<0.01$). When the groups containing 100 µM ASTA and 500 µM ASTA were compared to each other, although decondensed sperm number was lower in the group containing 100 µM ASTA (Table 4), no significant difference was found (Figure 4).

Discussion

The aim of sperm cryopreservation is to preserve male fertility by storing sperm before a surgical procedure or cytotoxic treatments, such as chemotherapy and radiotherapy, that may cause infertility.[4,29] During the cryopreservation process, the most basic cell damage can occur during the freezing and especially the thawing phase. Cryo-damage can be effectively reduced by adding CPAs prior to cryopreservation and applying appropriate freezing-thawing techniques;[30]

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.

however, CPAs are toxic for cells and they become gradually more toxic as concentration increases.[31] It is recommended to add antioxidant materials to CPAs in order to prevent cryopreservation damage without toxicity.[11,32,33]

ASTA, a naturally occurring antioxidant, can be used to improve sperm quality and significantly decrease reactive oxygen species (ROS) production in seminal fluid. It has been reported that ASTA increases fertilization rate by increasing sperm concentration and linear progressive motility.[34,35]

In a study they conducted on cattle, Jang et a 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 µM ASTA for 24 hours and reported that ASTA had an antioxidant effect on the development of cattle embryo.[36] Gao et al observed that 100 mg/kg ASTA increased semen quality and antioxidant activity in a study they conducted in rooster sperm.[37] Comhaire et al gave 16 mg ASTA orally to 30 infertile male individuals for three months. In this study, authors found that ROS level decreased significantly and the progressive movement of sperm increased.[38]

In another study by Comhaire and Decleer, it was found that ASTA given as a food supplement for three months to infertile couples improved sperm morphology. They also found that ASTA decreased ROS level of semen and significantly increased sperm motility and amount.[39] In a similar study, Tunç et al researched whether antioxidant supplements could improve DNA integrity in male individuals who were found to show oxidative stress and applied ASTA treatment orally to 50 male individuals for three months. They found that the ASTA treatment provided significant improvements in sperm DNA integrity. As a result, they recorded a decrease in seminal ROS production and apoptosis.[40] Basioura et al showed higher plasma membrane integrity, viability, motility, and progressive motility after thawing in pigs.[41] Nejafi et al reported a positive effect of ASTA on male semen quality after thawing.[42]

In a study conducted on miniature pig sperm, Lee and Kim added increasing concentrations of 0, 10, 50, 100, and 500 µM ASTA to a CPA. In parallel with our study, they found that in all experimental groups for which ASTA was added sperm motility increased significantly when compared with the control group, and the number of sperm with advanced mobile sperm also increased. In addition, it was found that ROS levels were lower in all groups with ASTA supplementation.[43] In another study, Neamah and Houbi observed that the percentage of viable sperm improved 24 hours after the

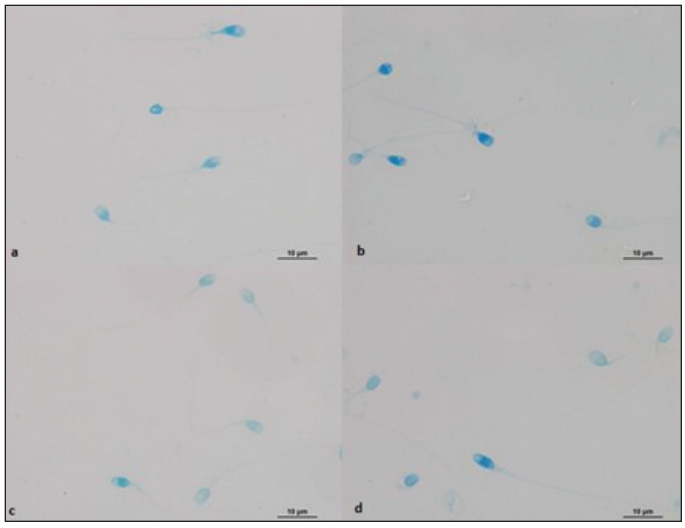

Figure 3. Control group (0 µM astaxanthin [ASTA]) **(a)** 50 µM ASTA group, **(b)** 100 µM ASTA group, **(c)** 500 µM ASTA group, **(d)** 1000X, Scale bar: 10 µm. Condensed sperms are seen to be lightly stained with aniline blue in pictures a, b, c, and d; decondensed sperms are seen to be darkly stained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

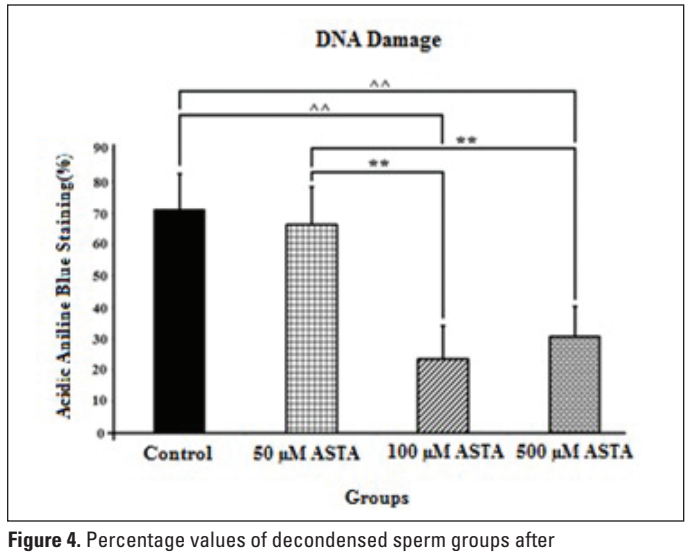

Figure 4. Percentage values of decondensed sperm groups after cryopreservation. ASTA: astaxanthin.

addition of ASTA to the semen sample, and sperm motility increased 72 hours later.[23]

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 whose sperm was applied ASTA improved significantly when compared with mice in the control group. Thus, it was reported that ASTA affected semen quality positively.[44] In a similar study, the effects of ASTA on the sperm of 30 adult diabetic rats were researched. In the sperm of the group that was treated with ASTA for 56 days, an increase in normal morphology and viable sperms was found when compared to other groups. Based on this, authors found that ASTA improve sperm viability, morphology, and DNA integrity.[45]

Kumalic et al, in a study of its effect on semen parameters, observed that oral ASTA intake did not change semen parameters in adult patients.[16] On the contrary, Dona et al added 0 µM, 0,5 µM, 1 µM, and 2 µM 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 sperm was higher in the group that was added 2 µM ASTA, showing that ASTA could indeed improve human sperm parameters.[34] In a similar study conducted with semen samples taken from 51 healthy male individuals, Andrisani et al showed that ASTA could be used to decrease male infertility by improving human sperm quality.[22]

Conclusions

Different concentrations of ASTA doses added to CPAs during sperm cryopreservation were found to be effective. An ASTA dose of 100 µM ASTA positively affected the motility of sperm. It was also found that ASTA was effective in decreasing chromatin condensation. Considering the positive results of this new sperm preservation method, molecular studies are needed to prevent possible chromatin damage and increase motility in human sperm before clinical application.

Competing interests: The authors do not report any competing personal or financial interests related to this work.

This paper has been peer-reviewed.

References

1. Lipovac M, Bodner F, Schütz A, et al. Increased hyaluronan acid binding ability of spermatozoa indicating a better maturity, morphology, and higher DNS integrity after micronutrient supplementation. *Eur Med J* 2014;1:60-5.
2. Hu H, Yan B, Zhang W, et al. A new method for evaluating the quality of single sperm by detecting reactive oxygen species. *Chin Chem Letters* 2022 [ahead of print]. https://doi.org/10.1016/j.cclet.2022.04.016
3. Özsait B, Gör A, Özcan T, et al. [Sperm kriyoprezervasyonu: kriyo-hasar ve DNA fragmantasyonu İlişkisi.] *Androloji Bülteni* 2014;16:264-8.
4. Abdi-Benemar H, Khalili B, Zamiri MJ, et al. Effects of astaxanthin supplementation on the freezability, lipid peroxidation, antioxidant enzyme activities and post-thawing fertility of ram semen. *Small Ruminant Research* 2020;192. https://doi.org/10.1016/j.smallrumres.2020.106213
5. Mahmoudi R. In vitro maturation and fertilization capacity of mouse GV- stage oocyte following stepwise vitrification. *J Clin Diagn Res* 2008;2:1234-39.
6. Aksoy E, Güven AM, Mermer MS. [Normozoospermik ve oligozoospermik erkeklerde dondurma çözdürme işlemlerin sperm DNA kondensasyonuna etkisi.] *Zeynep Kamil Tıp Bülteni* 2013;44:176-82.
7. Delilbaşı L. [A'dan Z'ye Tüp Bebek Laboratuvar.] *Veri MedikalYayıncılık* 2008:229-58.
8. Ozkavukcu S, Erdemli E, Isik A, et al. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. *J Assist Reprod Gen* 2008;25:403-11. https://doi.org/10.1007/s10815-008-9232-3
9. Wang S, Wang W, Xu Y, et al. Proteomic characteristics of human sperm cryopreservation. *Proteomics* 2014;14:298-310. https://doi.org/10.1002/pmic.201300225
10. Liu T, Gao J, Zhou N, et al. The effect of two cryopreservation methods on human sperm DNA damage. *Cryobiology* 2016;72:210-5. https://doi.org/10.1016/j.cryobiol.2016.04.004
11. Tunalı G. [Sperm kriyoprezervasyon teknikleri ve fertilizasyon başarısındaki rolü.] *Androloji Bülteni* 2012;16:123-8.
12. Medhi J, Kalita MC. Astaxanthin: An algae-based natural compound with a potential role in human health-promoting effect: An updated comprehensive review. *J Applied Bio Biotech* 2021;9:114-23. https://doi.org/10.7324/JABB.2021.9115
13. Lee E, Kim D. Effects of astaxanthin on miniature pig sperm cryopreservation. *BioMed Res Int* 2018;2018:6784591. https://doi.org/10.1155/2018/6784591
14. Pertiwi H, Nur Mahendra MY, Kamaludeen J. Astaxanthin as a potential antioxidant to improve health and production performance of broiler chicken. *Vet Med Int* 2022;2022:1-9. https://doi.org/10.1155/2022/4919442
15. Vasarri M, Degl'innocenti D. Antioxidant and anti-inflammatory agents from the sea: A molecular treasure for new potential drugs. *Marine Drugs* 2022;20:132. https://doi.org/10.3390/md20020132
16. Kumalic SI, Klun IV, Bokal EV, et al. Effect of the oral intake of astaxanthin on semen parameters in patients with oligo-astheno-teratozoospermia: A randomized double-blind placebo-controlled trial. *Radiol Oncol* 2020;55:97-105. https://doi.org/10.2478/raon-2020-0062
17. Nakajima Y, Inokuchi Y, Shimazawa M, et al. Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo. *Journal of Pharmacy and Pharmacology* 2008;60:1365-74. https://doi.org/10.1211/jpp/60.10.0013
18. Lee D hee, Lee YJ, Kwon KH. Neuroprotective of astaxanthin in oxygen-glucose deprivation in SH-SY5Y cells and global cerebral ischemia in rat. *J Clin Biochem Nutr* 2010;47:121-9. https://doi.org/10.3164/jcbn.10-29
19. Choi HD, Kim JH, Chang MJ, et al. Effects of astaxanthin on oxidative stress in overweight and obese adults. *Phytother Res* 2011;1818:1813-8. https://doi.org/10.1002/ptr.3494
20. Torrissen OJ, Christiansen R. Requirements for carotenoids in fish diets. *J Applied Ichthyology* 1995;11:225-30. https://doi.org/10.1111/j.1439-0426.1995.tb00022.x
21. Torrissen OJ, Hardy RW, Shearer KD. Pigmentation of salmonids carotenoid deposition and metabolism. *Crit Rev Aquat Sci* 1989;1:209-25.
22. Andrisani A, Donà G, Tibaldi E, et al. Astaxanthin improves human sperm capacitation by inducing lyn displacement and activation. *Marine Drugs* 2015;13:5533-51. https://doi.org/10.3390/md13095533
23. Neamah HJ, Houbi AA. Effect of adding astaxanthin and taurine to the Tris extender on the semen quality of Awassi rams during cooling storage. *Ann Tropical Med Public Health* 2020;23. https://doi.org/10.36295/ASRO.2020.23732
24. Nabi A, Khalili MA, Halvaei I, et al. Seminal bacterial contaminations: Probable factor in unexplained recurrent pregnancy loss. *Int J Reproduct BioMed* 2013;11:925-32.
25. Koyuncu H. Methods for the determination of sperm DNA damage. *Turk Urol Sem* 2011;2:18-23. https://doi.org/10.5152/tus.2011.04
26. Hammadeh ME, Al-Hasani S, Stieber M, et al. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. *Human Reprod* 1996;11:2468-71. https://doi.org/10.1093/oxfordjournals.humrep.a019139
27. Yüksel Ş. [Infertil çiftlerde sperm genom stabilitesini belirlenmesinde dna fragmantasyonu analiz yöntemleri.] *İnönü Üniversitesi Sağlık Hizmetleri Meslek Yüksek Okulu Dergisi* 2019;1:67-80. https://doi.org/10.33715/inonusaglik.527180

28. Sellami A, Chakroun N, Ben Zarrouk S, et al. Assessment of chromatin maturity ın human spermatozoa: Useful aniline blue assay for routine diagnosis of male ınfertility. *Adv Urol* 2013;2013:578631. https://doi.org/10.1155/2013/578631
29. Thomson LK, Fleming SD, Schulke L, et al. The DNA ıntegrity of cryopreserved spermatozoa separated for use ın assisted reproductive technology ıs unaffected by the type of cryoprotectant used but ıs related to the DNA ıntegrity of the fresh separated preparation. *Fertil Steril* 2009;92:991-1001. https://doi.org/10.1016/j.fertnstert.2008.07.1747
30. Chen Y, Liu R hi. Cryopreservation of spermatozoa. *Zhonghua Nan Ke Xue* 2007;13:734-8.
31. Best BP. Cryoprotectant toxicity: Facts, ıssues, and questions. *Rejuvenation Res* 2015;18:422-36. https://doi.org/10.1089/rej.2014.1656
32. Askari HA, Check JH, Peymer N, et al. Effect of natural antioxidants tocopherol and ascorbic acids ın maintenance of sperm activity during freeze-thaw process. *Syst Bio Reproduct Med* 1994;33:11-5. https://doi.org/10.3109/01485019408987797
33. Wetzels A, Bras M, Lens JW, et al. Laboratory aspects of in vitro fertilization. *Cryopreservation Theory* 1996:229-44.
34. Donà G, Kožuh I, Brunati AM, et al. Effect of astaxanthin on human sperm capacitation. *Marine Drugs* 2013;11:1909-19. https://doi.org/10.3390/md11061909
35. Ambati RR, Phang SM, Ravi S, et al. Astaxanthin: Sources, extraction, stability, biological activities and ıts commercial applications-a review. *Marine Drugs* 2014;12:128-52. https://doi.org/10.3390/md12010128
36. Jang HY, Ji SJ, Kim YH, et al. Antioxidative effects of astaxanthin against nitric oxide-induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. *Reproduct Domestic Animals* 2010;45:967-74. https://doi.org/10.1111/j.1439-0531.2009.01469.x
37. Gao S, Heng N, Liu F, et al. Natural astaxanthin enhanced antioxidant capacity and improved semen quality through the MAPK/Nrf2 pathway in aging layer breeder roosters. *J Animal Sci Biotech* 2021;12:112. https://doi.org/10.1186/s40104-021-00633-8
38. Comhaire FH, El Garem Y, Mahmoud A, et al. Combined conventional/antioxidant "astaxanthin" treatment for male ınfertility: A double-blind, randomized trial. *Asian J Androl* 2005;7:257-62. https://doi.org/10.1111/j.1745-7262.2005.00047.x
39. Comhaire FH, Decleer W. Beneficial effect of food supplementation with the nutriceutical ımprove for the treatment of ınfertile couple. *Asian Pacific J Reproduct* 2012;1:169-76. https://doi.org/10.1016/S2305-0500(13)60072-X
40. Tunc O, Thompson J, Tremellen K. Improvement in pperm DNA quality using an oral antioxidant therapy. *Reproduct BioMed Online* 2009;18:761-8. https://doi.org/10.1016/S1472-6483(10)60024-7
41. Basioura A, Tsakmakidis IA, Martinez EA, et al. Effect of astaxanthin in extenders on sperm quality and functional variables of frozen-thawed boar semen. *Animal Reproduct Sci* 2020;218:106478. https://doi.org/10.1016/j.anireprosci.2020.106478
42. Najafi D, Taheri RA, Najafi A, et al. Effect of astaxanthin nanoparticles in protecting the post-thawing quality of rooster sperm challenged by cadmium administration. *Poultry Sci* 2020;99:1678-86. https://doi.org/10.1016/j.psj.2019.12.006
43. Lee E, Kim D. Effects of astaxanthin on miniature pig sperm cryopreservation. *BioMed Res Int* 2018;2018:6784591. https://doi.org/10.1155/2018/6784591
44. Hao Z. Astaxanthin ımproves serum cytokine expression and semen quality of diabetes mellitus kkay mice. *Chemico-Bio Interact* 2020;332:109303. https://doi.org/10.1016/j.cbi.2020.109303
45. Bahmanzadeh M, Vahidinia A, Mehdinejadiani S, et al. Dietary supplementation with astaxanthin may ameliorate sperm parameters and DNA ıntegrity in streptozotocin ınduced diabetic rats. *Clin Exp Reprod Med* 2016;43:90-6 https://doi.org/10.5653/cerm.2016.43.2.90

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