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Research Article

Astaxanthin Shields Sperm DNA against Freeze Damage: A Preliminary Study

Li F[1]; Arantes R[2,3]; Moutinho O[3]; Pinto-Leite R[2,3*]; Gaivão I[4]

[1]Faculty of Medicine, University of Coimbra, Portugal
[2]Genetics and Andrology Laboratory, Hospital Centre of Trás-os-Montes and Alto Douro, Portugal
[3]Department of Woman and Child, Hospital Centre of Trás-os-Montes and Alto Douro, Portugal
[4]Department of Genetics and Biotechnology and Veterinary and Animal Research Centre, University of Trás-os-Montes and Alto Douro, Portugal

***Corresponding author:** **Pinto-Leite R**
Laboratório de Genética, Centro Hospitalar de Trás-os-Montes e Alto Douro, Av. da Noruega, 5000-508 Vila Real, Portugal
Tel: 351 915 263 932
Email: mlleite@chtmad.min-saude.pt

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Abstract

Sperm cryopreservation is a valuable technique for preserving and managing male fertility. However, the freeze-thaw cycle often induces oxidative stress and damage to sperm cells. Astaxanthin (ASTX), known for its potent antioxidant properties, holds promise in mitigating the harmful effects of oxidative stress.

This study investigated the effects of ASTX and DMSO on post-thaw sperm parameters. Rigorous exclusion criteria were applied in the samples selection to ensure a precise and reliable analysis, eliminating potential confounding factors such as detrimental lifestyle habits, vitamin intake, history of diseases, and abnormal semen parameters. Subsequently, from an initial pool of 150 participants, six carefully selected semen samples were used, and seven experimental groups were established. The control group was comprised solely of Sperm Freezing Medium (SFM), and the remaining groups received SFM supplemented with three different concentrations of ASTX (0, 15, 25 μM), each dissolved in DMSO at concentrations of 2.5% and 5.0%. Sperm motility, vitality, morphology, and DNA damage both before and after freezing were assessed for each group.

5% DMSO was generally more beneficial, with significant improvement in sperm vitality (40.8%) and DNA damage (22.2%), over the 2.5% DMSO and control groups. Moreover, ASTX at 5.0% DMSO displayed the most marked impact on DNA damage. The concentrations of 15 μM and 25 μM resulted in minor increases in DNA damage (7.5% and 12%, respectively). In comparison, the control group exhibited significantly higher values (33.4%).

These results highlight the potential of ASTX in mitigating DNA damage during sperm cryopreservation.

Keywords: Sperm; Cryopreservation; Antioxidants; Astaxanthin; Cryoinjury

Abbreviations: ASTX: Astaxanthin; DMSO: Dimethylsulfoxide; DNA: Deoxyribonucleic Acid; ROS: Reactive Oxygen Species; SFM: Sperm Freezing Medium

Introduction

Sperm cryopreservation is a valuable service that allows men to preserve their fertility before undergoing procedures or exposures that could harm their reproductive potential. Additionally, sperm cryostorage is pivotal in cases of severe oligozoospermia (low sperm count), intermittent presence of motile sperm cells in semen, or partially successful infertility treatment. This technique also offers substantial advantages to assisted reproductive technologies [1].

Successful cell cryopreservation depends on the ability to induce and exit the low-temperature state without cell damage. However, the freezing and thawing cycle frequently changes the sperm membrane lipid composition, and acrosome status, severely hinders sperm motility and viability and has been reported to increase sperm Deoxyribonucleic Acid (DNA) damage [2]. Cell cooling not only causes physical injury but also results in the excessive production of free radicals that disrupt vital chemical structures in the sperm cell. In addition, sperm DNA

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is especially vulnerable to oxidative damage because the highly condensed nuclear structure of sperm prohibits the enzymatic repair of damaged DNA [3]. Therefore, to maintain functionality, sperm cells undergoing a freeze-thaw cycle need to balance their redox potential [4].

Currently, researchers are exploring various methods to safeguard sperm cells from freeze damage. One strategy relies on neutralising Reactive Oxygen Species (ROS) through freezing medium supplementation with antioxidants. In principle, implementing such action can enhance the antioxidant defence mechanisms of sperm and preserve, to a satisfactory degree, the biological potential of sperm cells post-thaw [5].

Astaxanthin (ASTX) is a marine xanthophyll (oxygenated carotenoid) produced by algal species (*Haematococcus pluvialis*, *Chlorella zofingiensis* and *Chlorococcum*), bacteria, and fungi [6]. This antioxidant has received increasing attention as an effective molecule to counteract and dilute the effects of conditions related to oxidative stress [7]. Numerous studies have been conducted on its anticancer, antidiabetic, anti-inflammatory, immune-stimulating, and antioxidant properties, suggesting a potential therapeutic effect [8–10].

Here, freezing medium supplementation with ASTX was performed at different Dimethylsulfoxide (DMSO) concentrations to ascertain the possible beneficial effect on sperm cryopreservation.

Materials and Methods

Between March and July 2022, 150 semen samples were collected from men attending fertility support consultations at the Trás-os-Montes and Alto Douro Hospital Centre. A rigorous exclusion criterion was applied in these samples to ensure accurate and reliable data and to eliminate factors that could affect ROS generation and compromise sperm quality. Participants with a history of drug addiction, smoking, alcohol consumption, prolonged diseases, drug use (including vitamins), as well as samples exhibiting abnormal semen parameters (below the threshold set by the World Health Organization [11]) or suspected of leukocytospermia were excluded from the study. Furthermore, samples that did not liquefy within 30-40 minutes of incubation at 37°C or had a volume below 1.5 ml or a sperm concentration less than $20x10^6$ sperm cells/ml were also removed from the study. Consequently, only six out of the initial pool of 150 samples met the strict criteria and were included in the study and could be considered a pool of normal sperm cells.

The six semen samples were distributed into seven groups (A-G) (Table 1). Each sample with an adjusted concentration of $20x10^6$ sperm cells/ml underwent rapid freezing cryopreservation (the cryovials were placed 10 min at room temperature, followed by a 30 min exposure to nitrogen vapours before submersion in liquid nitrogen) with Sperm Freezing Medium (SFM) (ORIGIO, Måløv, Denmark) supplemented with six different concentrations of DMSO and ASTX (Sigma-Aldrich Company, St. Louis, MO) (Table 1): group A (control – SFM only), group B (2.5% DMSO), group C (15 µM ASTX in 2.5% DMSO), group D (25 µM ASTX in 2.5% DMSO), group E (5.0% DMSO), group F (15 µM ASTX in 5.0% DMSO), and group G (25 µM ASTX in 5.0% DMSO). After two weeks, the cryovials were thawed by submersion in a 37°C water bath for 5 min before immediate freezing medium removal. Sperm motility, vitality (Eosin Y 0.5%, Merck) morphology, and DNA damage (Alkaline Comet Assay) were evaluated before freezing and after thawing. DNA damage was assessed through the classification of comets into five categories (visual score) (0-4) according to amount of DNA in tail (100 cells were scored per gel). The obtained visual score, following Azqueta et al. [12], was divided by four and converted to the percentage of sperm DNA in tail.

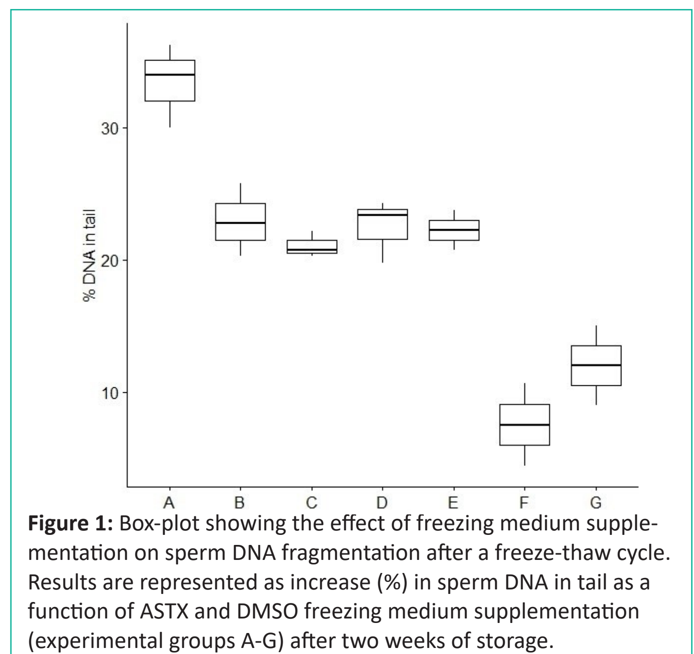

Figure 1: Box-plot showing the effect of freezing medium supplementation on sperm DNA fragmentation after a freeze-thaw cycle. Results are represented as increase (%) in sperm DNA in tail as a function of ASTX and DMSO freezing medium supplementation (experimental groups A-G) after two weeks of storage.

The normality of data was analysed by the Shapiro-Wilk test. Parameters were compared between groups using One Way-ANOVA and Kruskal-Wallis, followed by post hoc tests. The value of $p<0.05$ was regarded as statistically significant. The statistical analysis was performed using R Statistical Software (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

The semen samples enrolled in our study exhibited, before cryopreservation, an average volume of 3.0 ml, total motility of 64.5%, 71.6% vitality, sperm count of $45.9x10^6$ sperm cells/ml, 6.0% sperm cells with normal morphology and 25.5% of sperm DNA in tail.

After two weeks of storage, the findings in Table 2 reveal that all the studied parameters were negatively impacted, irrespective of the conditions tested. Regarding motility and morphology, DMSO alone or combined with ASTX yielded no statistically significant improvements over the control group A. Indeed, total motility (progressive and *in situ* movements) varied between 10.1%, group C (15 µM ASTX in 2.5% DMSO), and 20.8%, group E (5.0% DMSO). On average, the groups with 5.0% DMSO alone or in combination with ASTX (E-G) displayed higher values for total motility (20.8, 17.2, 18.7%, for groups E-G, respectively) when compared to groups with 2.5% DMSO (11.2, 10.1, 13.1% for groups B-D, respectively) and control group A (14.0%). When analysing morphology, group D (25 µM ASTX in 2.5% DMSO) exhibited the lowest percentage of normal sperm cells (only 2.5%), while Group F (15 µM ASTX in 5.0% DMSO) showed the highest (5.3%). Once again, better morphology was seen in the 5.0% DMSO groups (5.3, 5.3 and 4.7, for groups E-G, respectively, vs 3.4, 3.8, 4.3 and 2.5 for groups A-D, respectively).

Table 1: Study design.

Groups	A	B	C	D	E	F	G
	SFM						
DMSO (%)	-	2.5			5.0		
ASTX (µM)	-	0	15	25	0	15	25

ASTX: Astaxanthin; DMSO: Dimethylsulfoxide; SFM: Sperm Freezing Medium.

Table 2: Post-thaw comparison of sperm parameters.

	Control	2.5% DMSO			5.0% DMSO		
ASTX (μM)	-	0	15	25	0	15	25
Groups	A	B	C	D	E	F	G
Parameters (%)							
Total Motility	14.0 ± 2.9	11.2 ± 3.6	10.1 ± 0.9	13.1 ± 9.1	20.8 ± 6.1	17.2 ± 10.4	18.7 ± 5.5
Vitality	17.2 ± 4.3[b]	19 ± 0.5[ab]	18.3 ± 5.5[ab]	21.7 ± 10.9[ab]	40.8 ± 3.9[a]	38.7± 7.1[ab]	37.7 ± 3.7[ab]
Normal Forms	3.4 ± 1.9	3.8 ± 2.3	4.3 ± 2.4	2.5 ± 0.7	5.3 ± 1.5	5.3 ± 3.2	4.7 ± 0.6
DNA in tail*	33.4 ± 3.2[a]	22.9 ± 2.6[b]	21.0±1.0[bc]	22.5± 2.4[b]	22.2 ± 2.1[b]	7.5 ± 4.4[d]	12.0 ± 4.2[c]

Note: Data are presented as mean ± standard deviation. Different letters indicate statistically significant differences (p<0.05). * Increase in DNA in tail. ASTX: Astaxanthin; DMSO: Dimethylsulfoxide; SFM: Sperm Freezing Medium.

Table 3: Obtained p-value for multiple comparisons of means for post-thaw sperm parameters.

	Vitality	% DNA in tail
Groups	**p-value**	
B-A	1.0000	0.0116035*
C-A	1.0000	0.0034462*
D-A	1.0000	0.0085763*
E-A	0.0211*	0.0163227*
F-A	0.0915	0.0000123*
G-A	0.0741	0.0000775*
C-B	1.0000	0.9800033
D-B	1.0000	0.9999926
E-B	0.0667	0.9999649
F-B	0.2913	0.0014431*
G-B	0.2362	0.0189624*
D-C	1.0000	0.9952487
E-C	0.054	0.9989200
F-C	0.2362	0.0040701*
G-C	0.1915	0.0587698
E-D	0.0600	1.0000000
F-D	0.2623	0.0018494*
G-D	0.2127	0.0249874*
F-E	1.0000	0.0042519*
G-E	1.0000	0.0486060*
G-F	1.0000	0.7028412

*p-value<0.05

Concerning vitality, compared to the control group A, only group E, with an average of 40.8% vitality, demonstrated a statistically significant improvement in this parameter (p=0.021) (Table 3). Although no statistically significant difference could be detected for the remaining groups, the highest values for sperm vitality can be found in the 5.0% DMSO alone or in combination with ASTX groups (40.8, 38.7 and 37.7% for groups E-G, respectively, vs 17.2, 19, 18.3 and 21.7% for groups A-D, respectively).

Lastly, significant differences were found in sperm DNA damage (Figure 1 and Tables 2 & 3). Group A, the control, showed, in comparison with all the remaining groups, the highest DNA damage with the most significant gain in % of DNA in tail (33.4%), followed by Groups B-E (22.9, 21.0 and 22.5% DNA in tail, respectively), and lastly, by groups F and G (7.51 and 12%, respectively).

Discussion

Sperm cryopreservation is a challenging process. Freezing injury is frequent and impairs sperm function. In agreement with several studies, the freeze-thaw cycle caused severe damage to the analysed sperm cells [13,14]. For human sperm cryopreservation, glycerol is the preferred cryoprotective agent, as, for sperm cells, it is three times more permeable than DMSO [15,16]. In the present study, all samples were cryopreserved in a glycerol-based (concentration not specified by the manufacturer) SFM, and DMSO was added at two different concentrations. Groups B (2.5% DMSO) and E (5.0 DMSO) were created to evaluate the potential impact of DMSO. Glycerol alone, especially at low concentrations (<20%), as often found in SFMs [17], is insufficient to prevent crystallisation entirely [16]. Indeed, compared to the control group, only 5.0% DMSO significantly improved sperm vitality. The failure of 2.5% DMSO to enhance sperm vitality may be attributed to its reduced capacity to delay ice crystallisation. This observation highlights the limited ability of cryoprotectants at lower concentrations to protect against irreversible structural damage [18]. Similar results, although at different concentrations/combinations of glycerol and DMSO, have been reported for sperm in various species [19,20]. In this study, as standard practice, DMSO was employed primarily as (ASTX) solvent. However, our preliminary findings indicate that the percentage of DMSO (v/v) is crucial, as it can significantly impact sperm parameters. The data obtained reveal a positive effect of 5.0% DMSO supplementation (groups E-G), over the control group, across all the studied parameters, with statistical significance reported only for vitality and DNA damage.

ASTX has been reported to improve sperm quality through oral supplementation by decreasing ROS production in seminal plasma and improving sperm motility and morphology [21]. In mice, ASTX treatment showed a protective effect on sperm DNA against cyclophosphamide-induced damage [22]. Regarding cryopreservation studies, Basioura et al. reported higher viability and motility in frozen-thawed boar semen using ASTX [23]. Similar positive effects of ASTX on post-thaw semen quality were also observed in other species, such as roosters [24], miniature pigs [25], and ram sperm [26]. More importantly, in a recent study, the effects of ASTX on cryopreserved semen samples from 30 males were evaluated [27]. Although not reporting the applied DMSO concentration, the authors found that 100 μM ASTX effectively decreased chromatin damage and improved sperm motility.

Regarding our preliminary results, both tested ASTX concentrations at 5.0% DMSO showed a protective effect against DNA damage. Group F (15 μM ASTX in 5.0% DMSO) and G (25 μM ASTX in 5.0% DMSO) exhibited the lowest increase in DNA in tail, with only 7.5% and 12%, respectively. A study conducted on the DNA binding property of ASTX, both *in silico* and *in vitro*, suggests that ASTX exerts this protective action through binding to the major and minor grooves [28]. However, at the lowest tested DMSO concentration (2.5%), ASTX's protective role against DNA damage was not evident. Three factors combine to damage sperm cells and increase the rate of ROS production during cell cryopreservation: osmotic stress, intracellular and extracellular ice formation and oxidative stress. Here, the

presence of 5.0% DMSO in a glycerol-based SFM reduced intracellular ROS levels by preventing intracellular ice formation. Such action, in turn, allowed ASTX at the low concentrations of 15 and 25 µM to exert a more noticeable positive effect on DNA integrity. Thus, the observed low percentage of DNA fragmentation in groups F and G reinforces the reported antioxidative activity of ASTX [22,27].

These initial findings point to a beneficial combined effect of DMSO and ASTX that warrants further exploration. Furthermore, increasing the concentration of ASTX may prove a beneficial strategy for motility, as Dede & Saylan [27] reported a positive effect of 100 µM ASTX for this parameter. Nonetheless, achieving the ideal formulation that maximises the protective properties of both DMSO and ASTX will require careful balance, as high concentrations of cryoprotectants are prone to exhibit cytotoxic effects [2].

Lastly, a limitation of this work concerns sample size. In order to ensure a precise evaluation of ASTX and DMSO supplementation effect, we conducted a meticulous sample selection process. This enabled us to treat the selected samples as a representative pool of normal spermatozoa.

Conclusion

Cryopreservation is a complex procedure that requires the precise regulation of many factors to ensure (minimal) success. The developed preliminary study, although severely hindered by the low number of semen samples, yielded promising results regarding ASTX's protective effect on human sperm genomic integrity. Furthermore, our findings suggest that 5.0% DMSO in combination with ASTX shows promise in improving sperm vitality and reducing DNA damage. The interplay between DMSO, ASTX, and their concentrations can be essential for effective sperm cryopreservation strategies and thus merits further investigation.

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