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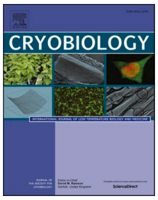

Antioxidant astaxanthin enhances cryosurvival and post-thaw functional parameters of rabbit spermatozoa

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ABSTRACT

This study evaluated the effect of astaxanthin (AX), a potent antioxidant carotenoid, on the post-thaw quality of cryopreserved rabbit sperm. Semen samples were frozen with AX supplementation at concentrations of 0 (CONTROL), 0.5, 1, or 2 μM. Post-thaw sperm quality was assessed by computer-assisted sperm analysis (CASA) for motility, and by flow cytometry to evaluate viability, early apoptosis, mitochondrial activity, reactive oxygen species (ROS) levels, and acrosomal membrane integrity. Supplementation with 0.5 and 1 μM AX significantly improved total motility, viability, and mitochondrial activity compared to the control group ($p < 0.05$). These concentrations also led to significantly reduced levels of apoptotic cells and ROS. Acrosomal damage was not significantly affected by AX supplementation. These findings demonstrate that low-dose AX addition during cryopreservation attenuates oxidative and apoptotic damage in rabbit spermatozoa and enhances several key post-thaw quality parameters. Astaxanthin may thus represent a promising additive for improving cryosurvival in rabbit sperm used for assisted reproduction.

1. Introduction

Rabbits (*Oryctolagus cuniculus*) are of significant importance among livestock species, due to their rapid growth and early sexual maturity, as well as their high reproductive rate and great potential for genetic selection. Rabbits are also widely used as valuable biomedical models in areas of research such as reproduction, toxicology, immunology, and biotechnology. For these reasons, the development of an optimal technique for cryopreserving rabbit sperm is of great importance. Advances in this field would offer numerous benefits, including the conservation of genetic diversity, the preservation of transgenic or selected lines, and the facilitation of reproductive technologies such as artificial insemination. However, a common challenge associated with sperm cryopreservation is the poor quality of spermatozoa obtained after thawing. This issue is due to critical structural and functional changes in the spermatozoa during the cryopreservation process: acrosome damage, viability reduction due to apoptosis, sperm motility diminution, and membrane integrity and permeability alterations. All these modifications can finally lead molecular changes, such as DNA damage and lipid peroxidation [18].

Lipid peroxidation, in particular, involves peroxidation of polyunsaturated fatty acids, leading to plasma membrane damage. This phenomenon occurs due to the formation of reactive oxygen species (ROS) within the cell, which is triggered by the significant temperature fluctuations that occur during the process of cryopreservation [20]. Several strategies have been developed to mitigate the adverse effects of oxidative stress. For example, it is important to maintain an optimal concentration of rabbit spermatozoa during cryopreservation, which should be between 25 and 35 million cells per straw [6]. Furthermore, researchers have identified BotuCrio as an effective sperm extender for rabbits [7]. The role of antioxidants in reducing cell damage during cryopreservation has also been studied. These molecules help reduce oxidative stress and minimize structural and functional alterations in the spermatozoa. Antioxidant effectiveness can be evaluated using parameters such as motility, viability and apoptosis, intracellular ROS, acrosome integrity and mitochondrial activity [14]. Astaxanthin (AX) is a carotenoid with the potential to act as an antioxidant. This molecule induces the expression of antioxidant genes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST). As shown in Fig. 1, this induction is achieved by activating erythroid 2-related factor 2 (Nrf2) protein, which is also inhibited within the Nrf2-Keap1 complex. When the cell detects ROS, Nrf2-Keap1 separates, allowing Nrf2 to translocate to the nucleus,

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and activate antioxidant gene expression [4]. It has been shown that AX improves the quality of spermatozoa after thawing in some species such as pigs [12], dogs [19], boars [2,11], mouse [9] and humans [5,10,13].

However, the effects of adding AX to semen extenders have not yet been systematically studied in rabbits. Although AX has demonstrated potential as an antioxidant in other species, its application in cryopreserving rabbit sperm remains unexplored. Therefore, it is essential to assess whether semen supplemented with AX retains its fertilising ability. Further *in vitro* studies are needed to identify the optimal AX concentration for cryopreservation.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise indicated.

2.2. Animals and semen sample collection

Entire ejaculates from nine sexually mature of the New Zealand White males, belonging to the M91 and P91 lines, were collected twice a week during two months, using a pre-heated artificial vagina. At the time of collection, the rabbits were 12–18 months old. The rabbits showing no clinical signs of genital tract infections were used in the experiments. They were housed individually at the breeding facility of the NPPC, RIAP Nitra, Lužianky, Slovak Republic. They were fed a commercial diet (KV; TEKRO Nitra, s.r.o., Slovakia) and had ad libitum access to water. The photoperiod was maintained at 14 h of light and 10 h of darkness. The environmental conditions in the facility were controlled, with temperatures ranging from 17 to 20 °C and relative humidity levels of 60–65 %. All experiments were performed with the approval of the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK U 18021, in accordance with the ethical guidelines presented in the Slovak Animal Protection Regulations (RD 377/12), which conforms to the Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

The semen samples were transported to the laboratory in a water bath at 37 °C in 10 min, and then evaluated using a CASA system (SpermVision™ Software, MiniTube, Tiefenbach, Germany) to record sperm concentration and motility. Only semen samples meeting the following criteria were selected for the study: progressive motility of at least 70 %, a concentration of at least 1.0×10^9 spermatozoa/mL, and less than 10 % abnormal sperm forms (see Supplementary Material S1 for details and representative images). Morphological abnormalities are studied using phase contrast microscopy. For each collected sample, four photographs were taken, spermatozoa with malformations were counted, and their proportion was calculated in relation to the total number of spermatozoa. Samples of lower quality were excluded from further analysis.

2.3. Experimental design, semen dilution and cryopreservation

All high-quality semen samples were centrifuged (5 min, 480×*g*) and the supernatant was discarded. The sperm pellet was resuspended in BotuCrio freezing medium (Nidacon, Sweden). After determining the sperm concentration, the ejaculate was evaluated for the following sperm parameters: total motility and progressive motility, viability, apoptosis, plasma membrane integrity, mitochondrial activity, and intracellular ROS level. The sample was then divided into four equal aliquots, one of which was assigned to the control group (without antioxidants), while the remaining aliquots were allocated to the experimental groups containing 0.5 μM, 1 μM and 2 μM of AX. Four straws (Minitüb, Tiefenbach, Germany) of 0.25 mL were filled for each group,

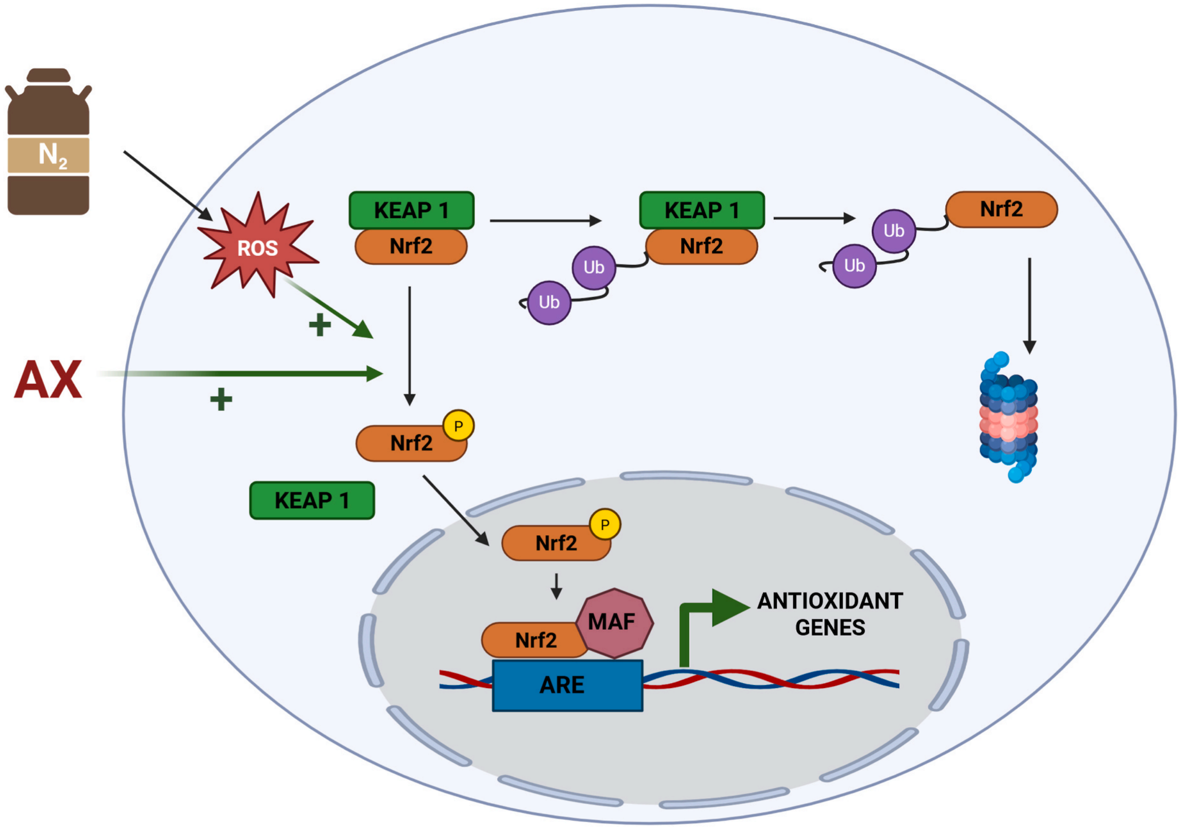

Fig. 1. Modulation of astaxanthin (AX) in the activation of Nrf2 to induce the expression of antioxidant genes. (Created in BioRender. Luque, P (2025) https://BioRender.com/g80xjbp).

achieving a final sperm concentration of approximately 50 × 10^6 cells/ml. Semen straws were equilibrated at 4 °C for 30 min, then placed 4 cm above liquid nitrogen vapor for 15 min, and finally submerged in liquid nitrogen for one week. After this period, cryopreserved samples were thawed in a water bath at 37 °C for 30 s, transferred to Eppendorf tubes and then evaluated for the same parameters as assessed prior to freezing (Fig. 2).

2.4. Sperm analyses

2.4.1. Sperm motility

Computer-Assisted Sperm Analysis was used to determine total motility (%) and progressive motility (%) of the spermatozoa. Ten μL of pre-diluted semen (1:10, vol/vol) in saline (0.9 % NaCl; Braun, Nuaille, Germany) were transferred to a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and analysed using SpermVision™ software under an AxioScope A1 light microscope (Carl Zeiss Slovakia, Bratislava, Slovakia).

2.4.2. Examination of sperm viability using SYBR-14

Sperm viability was assessed using the SYBR-14 green fluorescent dye (LIVE/DEAD® Sperm Viability Kit) and DRAQ7 far-red fluorescent nucleic acid dye (BioStatus Limited, Shepshed, UK). A semen sample containing 1 × 10^6 spermatozoa was incubated with SYBR-14 (100 nM final concentration) for 10 min at 37 °C in the dark. Without washing, DRAQ7 was added (3 μM final concentration), and the sample was incubated for a further 10 min at room temperature in the dark. Flow cytometry was then performed without additional washing. Viable spermatozoa were defined as SYBR-14+/DRAQ7−, while dead or membrane-compromised cells were categorized as SYBR-14+/DRAQ7+ or SYBR-14−/DRAQ7+, respectively.

2.4.3. Examination of apoptosis-like features using Yo-Pro-1

Apoptotic-like alterations in spermatozoa were detected using the Yo-Pro-1 nuclear green stain. Semen samples (1 × 10^6 spermatozoa) were diluted in 500 μL of Ca- and Mg-free PBS and incubated with Yo-Pro-1 (100 nM final concentration) for 15 min at room temperature in the dark. Following incubation, the samples were centrifuged (480×*g* for 10 min at room temperature), resuspended in PBS, and co-stained with DRAQ7 prior to flow cytometric analysis. The proportion (%) of spermatozoa positive for Yo-Pro-1 was considered to be the proportion of apoptotic-like spermatozoa.

2.4.4. Assessment of acrosome integrity using peanut agglutinin (PNA)

Acrosome integrity was evaluated using PNA conjugated with Alexa Fluor 488. A semen sample containing 1 × 10^6 spermatozoa in 200 μL of PBS was incubated with PNA (0.5 mg/mL final concentration) for 15 min at room temperature in the dark. After incubation, the samples were centrifuged (480×*g* for 10 min at room temperature), washed in PBS, and stained with DRAQ7 prior to flow cytometric analysis. Sperm positive for PNA were classified as acrosome-damaged.

2.4.5. Measurement of mitochondrial activity using MitoTracker® green FM

A semen sample containing 1 × 10^6 spermatozoa was diluted in 500 μL of PBS, after which it was incubated with MT Green (300 nM final concentration) for 10 min at 37 °C in the dark. The sample was washed (centrifugation at 480×*g* for 10 min at room temperature), stained with DRAQ7, and analysed by flow cytometry. Sperm positive for MT Green (MT Green+/DRAQ7−) were considered to have high mitochondrial membrane activity.

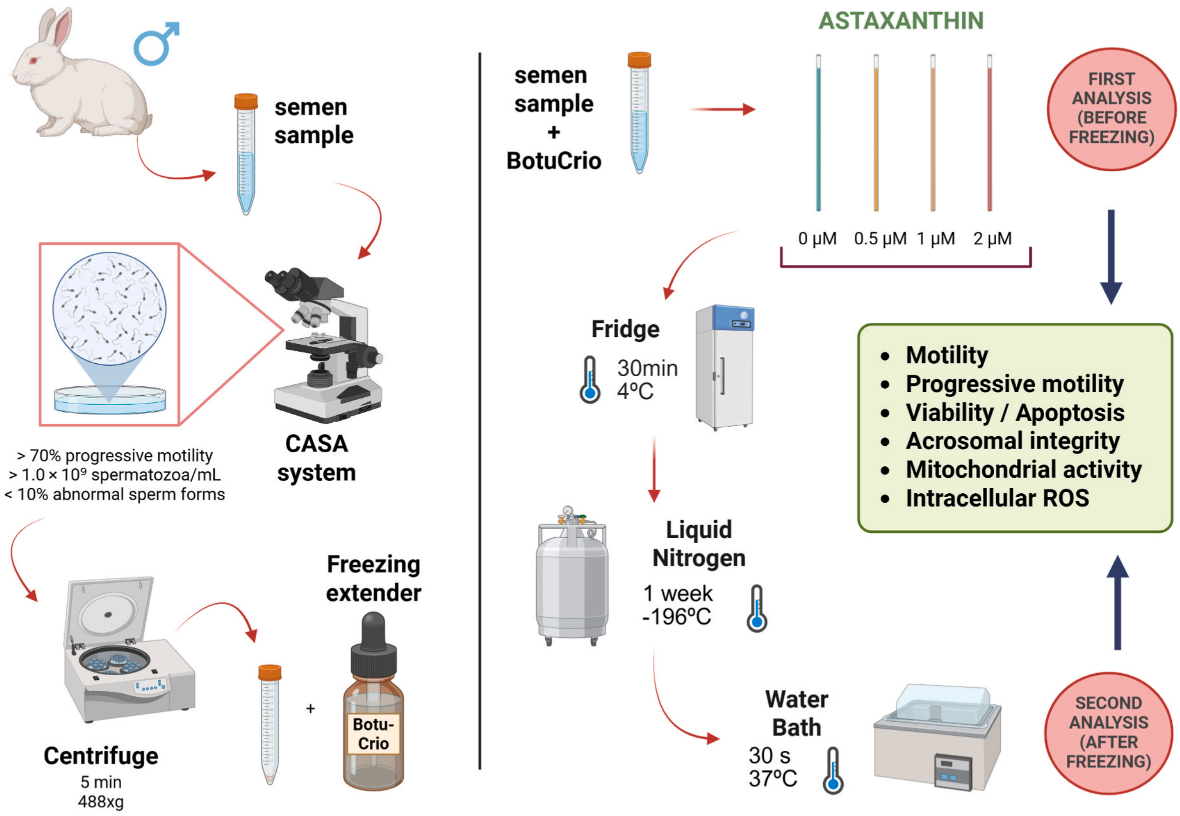

Fig. 2. Experimental design. Semen collection and selection using the CASA system. Centrifugation and resuspension in BotuCrio extender. Preparation of four straws with different astaxanthin concentrations (0, 0.5, 1, and 2 μM). Freezing in liquid nitrogen and thawing in a water bath. Analysis of parameters (motility, viability, apoptosis, acrosomal integrity, mitochondrial activity, intracellular ROS) before and after cryopreservation. (Created in BioRender. Luque, P (2025) https://BioRender.com/g2nm4xg).

2.4.6. Detection of intracellular reactive oxygen species (ROS) using CellROX green

A semen sample containing 1 × 10^6 spermatozoa was diluted in 500 μL of PBS and incubated with CellROX (2.5 μM final concentration) for 30 min at 37 °C. The sample was then co-stained with DRAQ7, as previously described, and analysed by flow cytometry. ROS-positive spermatozoa were classified as CellROX+/DRAQ7− or CellROX+/DRAQ7+.

2.4.7. Flow cytometric analysis

Processed samples were analysed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with a 488 nm argon ion and a 635 nm red diode lasers. Green fluorescence was recorded using the FL1 channel (530/30 nm bandpass filter) and red fluorescence using the FL3 channel (670 nm long-pass filter). The fluorescence data were collected and analysed using Cell Quest Pro™ software (BD Biosciences). A minimum of 10,000 spermatozoa were counted per sample.

2.4.8. Statistical analysis

All experiments were independently replicated six times ($n = 6$). Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. Differences between the experimental groups for each parameter were analysed using one-way ANOVA followed by a Tukey's post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$. All results are presented as mean ± standard deviation (SD). Letters above the columns in the figures indicate statistically homogeneous groups ($p < 0.05$). Statistical analysis was performed in R. Figures were prepared using Excel (Microsoft 365).

3. Results

As expected, all evaluated parameters—including motility, viability, mitochondrial activity, and membrane integrity—were significantly better in the fresh (non-frozen) semen group (FRESH) compared to any of the cryopreserved groups ($p < 0.05$). Therefore, statistical comparisons of experimental effects were primarily focused among cryopreserved groups (AX0.5, AX1, AX2, and CONTROL).

3.1. Sperm motility

Total motility (TM) was significantly higher in AX1 (59.28 ± 2.29 %) and AX0.5 (56.94 ± 3.07 %) compared to the CONTROL group (48.41 ± 1.37 %). AX2 (50.53 ± 1.88 %) did not differ significantly from CONTROL. (Fig. 3). Progressive motility (PM, Fig. 4) followed a similar trend. Although all cryopreserved groups showed reduced PM compared to FRESH (68.06 ± 2.90 %), no statistically significant differences were found among AX0.5 (37.31 ± 3.16 %), AX1 (39.01 ± 2.89 %), AX2 (34.62 ± 2.69 %), and CONTROL (34.74 ± 3.16 %).

3.2. Sperm viability

Sperm viability, assessed using SYBR-14 staining, was significantly higher in AX0.5 (53.20 ± 2.33 %) and AX1 (55.10 ± 2.22 %) compared to CONTROL (41.63 ± 4.40 %). AX2 (49.73 ± 2.55 %) showed intermediate values, significantly lower than AX1 but not differing from AX0.5 (Fig. 5). The proportion of membrane-damaged spermatozoa (DRAQ-7 positive) was significantly highest in CONTROL (57.78 ± 4.21 %). All AX-supplemented groups (AX0.5, AX1, AX2) showed significantly reduced levels of membrane damage (Fig. 6).

3.3. Sperm apoptosis

Early apoptotic spermatozoa, detected by Yo-Pro-1 staining, were most abundant in CONTROL (22.10 ± 1.06 %), followed by AX2 (19.25 ± 2.51 %). AX0.5 (10.53 ± 0.85 %) and AX1 (10.35 ± 1.26 %) showed significantly reduced apoptosis (Fig. 7).

3.4. Acrosomal membrane integrity

The highest levels of acrosomal damage, assessed by PNA staining, were found in AX2 (11.16 ± 1.19 %), followed by CONTROL (10.28 ± 1.05 %), AX1 (10.16 ± 0.61 %) and AX0.5 (9.67 ± 0.54 %), with no significant differences among cryopreserved groups. FRESH semen showed a significantly lower percentage of acrosomal damage (1.46 ± 0.68 %) (Fig. 8).**3.5. Mitochondrial activity**.

Mitochondrial activity, evaluated using MitoTracker Green, was significantly higher in AX0.5 (52.13 ± 1.90 %) and AX1 (51.53 ± 2.17 %) compared to CONTROL (37.57 ± 3.17 %) and AX2 (42.81 ± 3.13 %) (Fig. 9).

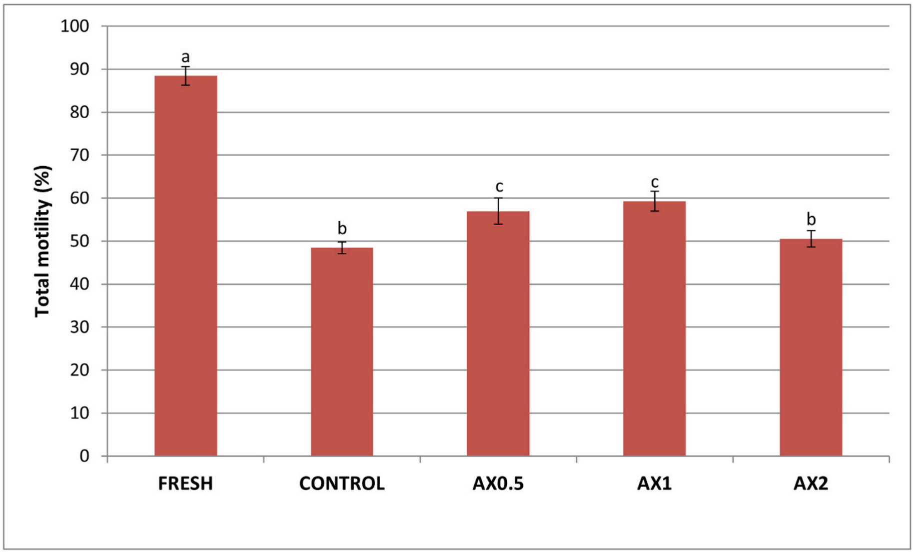

Fig. 3. Bar graph with total motility assessment of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

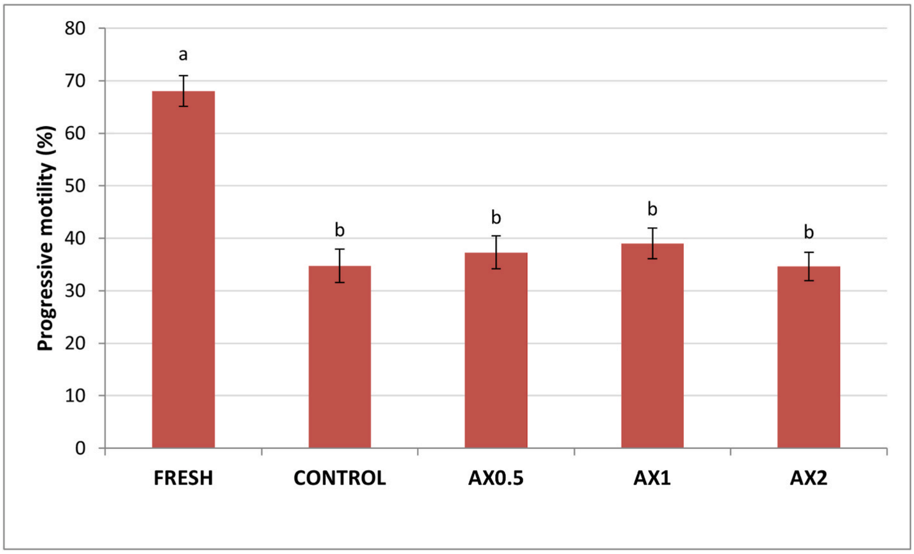

Fig. 4. Bar graph with progressive motility assessment of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

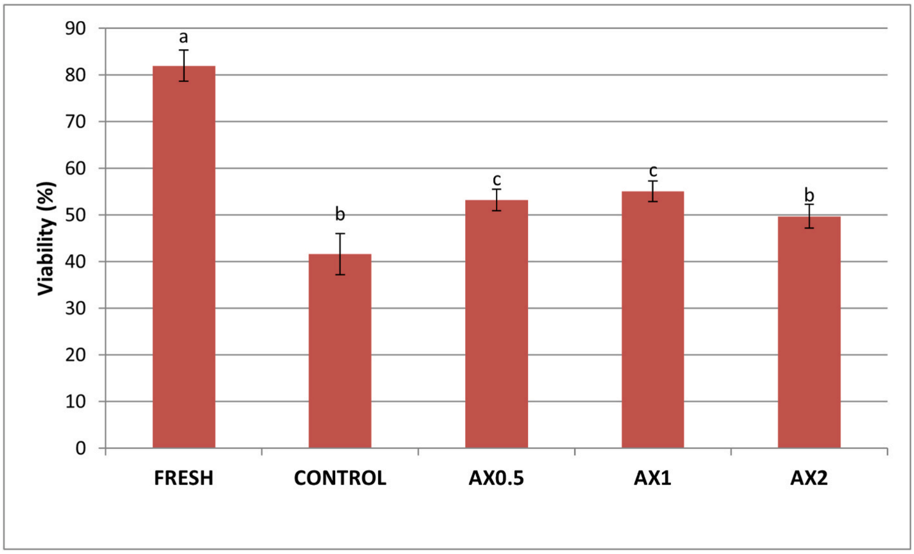

Fig. 5. Bar graph with viability assessment (SYBR-14) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

3.5. Oxidative stress

Intracellular reactive oxygen species (ROS) levels, determined via CellROX staining, were significantly reduced in AX0.5 (34.77 ± 3.00 %) and AX1 (33.98 ± 3.35 %) compared to CONTROL (41.13 ± 1.58 %) and AX2 (39.57 ± 2.58 %) (Fig. 10).

4. Discussion

This study demonstrates that supplementation with astaxanthin (AX) during the cryopreservation process, especially at concentrations of 0.5 and 1 μM, significantly improves several sperm quality parameters after thawing in rabbits. Specifically, these concentrations increased total motility, viability, and mitochondrial activity, while reducing intracellular ROS levels and the rate of apoptosis. These results are consistent with the known antioxidant properties of AX, a xanthophyll-type carotenoid characterised by its high capacity to eliminate reactive oxygen species and stabilise cell membranes [1].

Firstly, it was observed that supplementation with astaxanthin (AX) during the cryopreservation process, especially at concentrations of 0.5 and 1 μM, significantly improved the total motility of rabbit sperm after thawing, while 2 μM did not produce additional benefits. Similar results have been reported in other species: Ghantabpour et al. (2022) described an increase in human sperm motility with 1 μM AX [10], Qamar et al. (2021) reported positive effects in dogs with the same concentration [19], and Guo et al. (2017) in boars with 2 μM [11]. In contrast, Dede and Saylan (2020) observed that higher doses (100 μM) were necessary in humans to prevent post-cryopreservation motility loss [5].

Sperm viability increased significantly with 0.5 and 1 μM AX, which is consistent with the results obtained in dogs (Qamar et al., 2021) [19] and humans (Ghantabpour et al., 2022) [10], where these concentrations also reduced intracellular ROS levels. In boars, Guo et al. (2017) described a similar effect with 2 μM AX [11], while in miniature pigs,

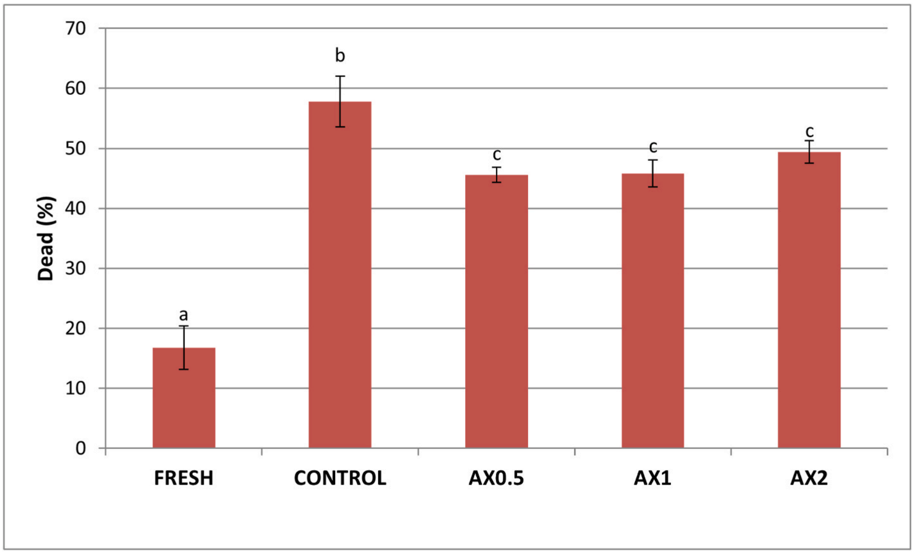

Fig. 6. Bar graph with membrane damage assessment (DRAQ-7) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

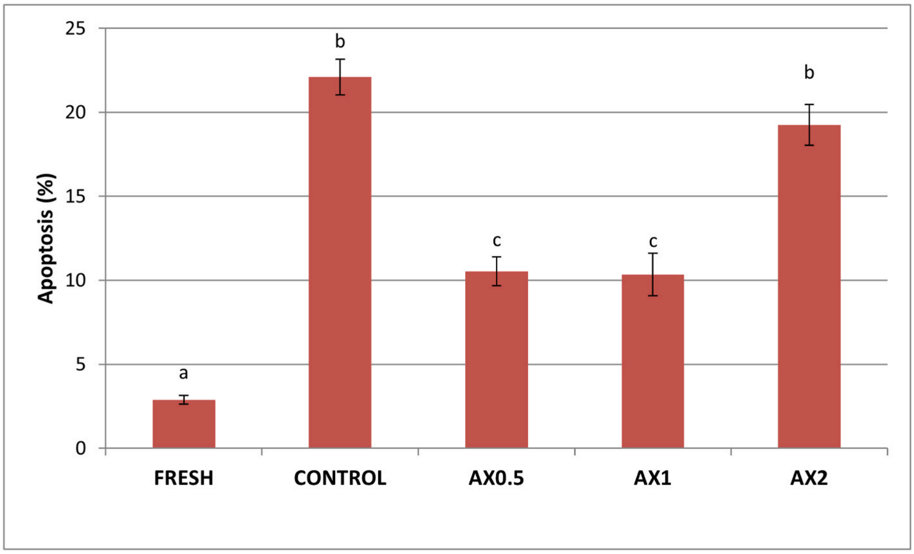

Fig. 7. Bar graph with apoptosis assessment (Yo-Pro-1) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

Lee and Kim (2018) found improvements with higher doses, up to 500 μM [12]. Optimal concentrations of 0.5–1 μM AX also increased mitochondrial activity in rabbit sperm after thawing, consistent with the findings of Qamar et al. (2021) in dogs, who reported an increase in mitochondrial function with 1 μM [19], and by Lee and Kim (2018) in miniature pigs with 500 μM [12].

Regarding apoptosis, the addition of 0.5 and 1 μM AX significantly reduced the percentage of apoptotic sperm cells, a result consistent with those obtained by Basioura et al. (2018) in boars [2], where 15 μM AX decreased apoptotic lipid changes, and by Dede and Saylan (2020) in humans [5], with a significant reduction in apoptosis after the use of 100 μM AX. These coincidences indicate that AX exerts a generalised anti-apoptotic effect, although the effective concentration varies between species.

Similarly, these concentrations (0.5–1 μM) of astaxanthin significantly reduced intracellular levels of reactive oxygen species (ROS) after thawing, confirming its potent antioxidant effect. Equivalent results have been reported in humans with 1 μM (Ghantabpour et al., 2022) [10] and 100 μM (Dede and Saylan, 2020) [5], in miniature pigs up to 500 μM (Lee and Kim, 2018) [12] and in dogs with 1 μM (Qamar et al., 2021) [19], with a dose-dependent relationship between AX concentration and reduction in oxidative stress observed in all cases.

Acrosomal integrity showed no significant differences between the treated groups and the control. However, Guo et al. (2017) reported better preservation of acrosome integrity in boar sperm treated with 2 μM AX [11], while Lee and Kim (2018) observed a protective effect in miniature pigs at concentrations of up to 500 μM [12]. The absence of significant effects in rabbits could be due to structural differences in the acrosome between species or because the doses used were not sufficient to exert detectable protection at that level. Overall, the results of this study confirm that astaxanthin, at low concentrations, improves motility, viability and mitochondrial activity, and reduces apoptosis and

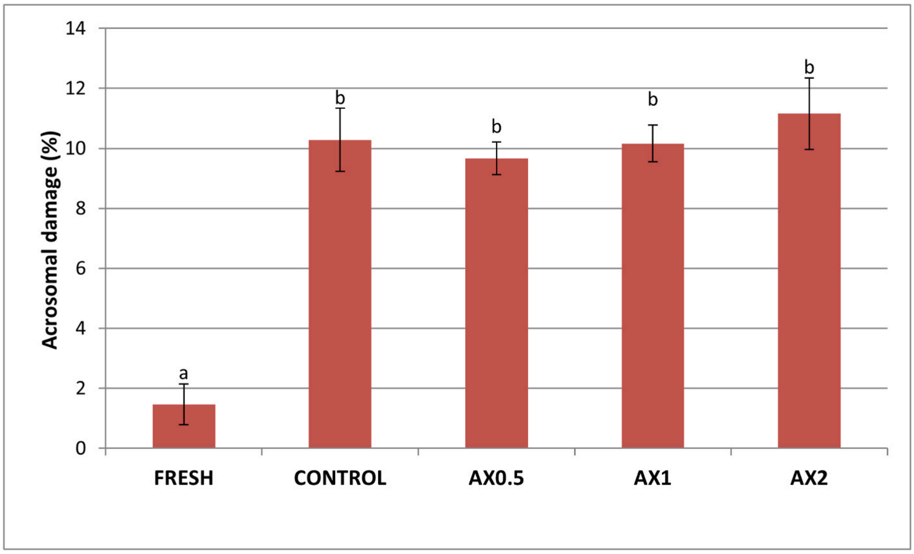

Fig. 8. Bar graph with acrosome integrity assessment (PNA) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at P < 0.05 are marked with letters a, b, and c.

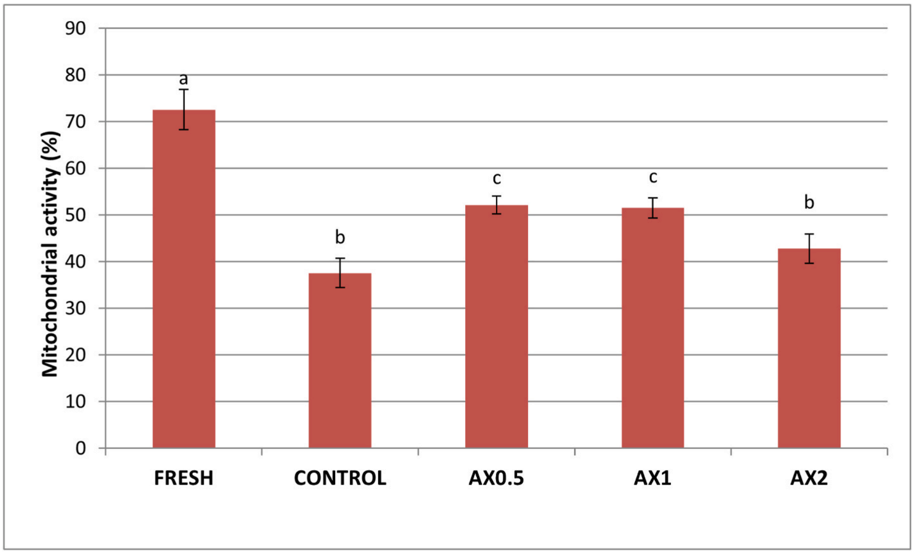

Fig. 9. Bar graph with mitochondrial activity assessment (MT Green) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at P < 0.05 are marked with letters a, b, and c.

intracellular ROS levels in rabbit sperm after cryopreservation. The effective concentrations (0.5–1 μM) are comparable to those described in humans and dogs, while species with lower oxidative susceptibility, such as boars or miniature pigs, require higher doses.

Both S. Fati et al. (2025) [9] and A. Mohammed et al. (2024) [15] also studied the addition of astaxanthin in mice and humans, respectively, both concluding that the optimal concentration is 10 μM. This variability among species can be largely attributed to differences in the lipid composition of sperm plasma membranes. Rabbit spermatozoa are particularly rich in polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA) and arachidonic acid, which confer high membrane fluidity but also render the cells highly susceptible to lipid peroxidation during freezing and thawing [3].

The observed decrease in ROS and apoptosis is consistent with activation of the Nrf2/Keap1 pathway, as reported in somatic cells and potentially applicable to sperm physiology. This pathway promotes the phosphorylation of Nrf2, allowing its release from Keap1 and subsequent translocation to the nucleus [4,8,17]. Once there, Nrf2 binds to antioxidant response element (ARE) promoters, inducing the transcription of antioxidant genes such as *SOD* (superoxide dismutase), *CAT* (catalase), and *GPX* (glutathione peroxidase). These enzymes effectively reduce oxidative stress by eliminating reactive oxygen species (ROS) and protecting lipid membranes from peroxidation [8,16]. This mechanism explains the decrease in intracellular ROS observed in our study after thawing, as well as the preservation of mitochondrial integrity, since the reduction in oxidative stress helps maintain a stable mitochondrial membrane potential. Similar results have been reported in ram semen [22], supporting the involvement of this pathway in the protection of sperm cells during cryopreservation.

AX also inhibits the pro-inflammatory NF-κB pathway by preventing the phosphorylation and degradation of its inhibitor IκB [3]. This, in turn, blocks NF-κB translocation to the nucleus and the subsequent

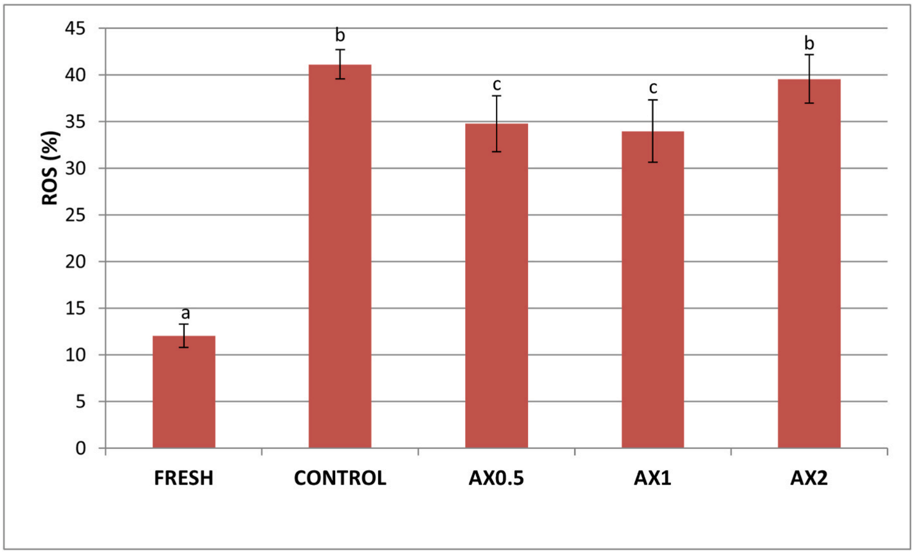

Fig. 10. Bar graph with intracellular reactive oxygen species (ROS) assessment (CellROX) of rabbit sperm samples analysed before cryopreservation (fresh) and after thawing: control (without antioxidant), and with different concentrations of astaxanthin (0.5, 1, and 2 μM). Significant differences between groups at $P < 0.05$ are marked with letters a, b, and c.

transcription of inflammatory cytokines (*IL-1β*, *TNF-α*) and pro-apoptotic genes [13]. Inhibition of this pathway directly contributes to enhanced sperm viability by reducing intracellular inflammation and preventing the activation of the intrinsic apoptotic cascade during cryopreservation and thawing [4,13,17].

Furthermore, AX modulates the CREB/Bcl-2 signaling pathway [17], increasing the expression of the *BCL-2* gene, which encodes an anti-apoptotic protein located in the outer mitochondrial membrane [23]. Elevated Bcl-2 levels prevent cytochrome-c release and thereby block the activation of Caspase-9 and the intrinsic apoptotic pathway [21]. This mechanism complements the antioxidant action of AX, enhancing post-thaw cell survival and overall sperm viability.

Collectively, the activation of the Nrf2/ARE and CREB/Bcl-2 pathways, together with the inhibition of NF-κB, explains the simultaneous improvements in viability, mitochondrial activity, and sperm motility, as well as the reduction in ROS generation and apoptosis observed after cryopreservation. Given the critical role of oxidative stress in cryoinjury and sperm dysfunction [20], future studies should focus on optimizing cryopreservation protocols by exploring potential synergistic effects between astaxanthin and other antioxidants or cryoprotective agents. The improvements observed in total motility, viability, mitochondrial function, and oxidative status indicate that astaxanthin supplementation enhances the functional integrity of rabbit sperm after cryopreservation. Although this study evaluated post-thaw quality through *in vitro* analysis, it remains essential to determine whether these improvements translate into increased fertilisation capacity and embryonic development. Therefore, well-designed *in vivo* trials with adequate statistical power are required to validate the applicability of astaxanthin in reproductive technologies involving rabbit semen.

5. Conclusion

This study provides the first evidence that astaxanthin supplementation during cryopreservation improves post-thaw quality of rabbit spermatozoa. The observed enhancements in viability, mitochondrial activity, motility, and reduction of apoptosis suggest that astaxanthin exerts a protective effect against cryodamage, likely through its antioxidant properties. The most effective concentrations identified were 0.5 and 1 μM, indicating a dose-dependent response. These findings support the potential of astaxanthin as a functional additive for optimizing rabbit sperm cryopreservation protocols.

CRediT authorship contribution statement

Paula Luque: Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Lenka Kuželová:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Jakub Vozaf:** Formal analysis. **Andrej Baláži:** Formal analysis. **Peter Chrenek:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2025.105564.

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