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Sperm Quality, Antioxidant Capacity, Transcriptional Profile and Antifreeze-Related Genes of Frozen Rabbit Semen in Relation to Alpha Lipoic Acid Implication

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Abstract

THE aim of this study was to estimate the impact of alpha-lipoic acid (ALA) as an antioxidant at different levels (0.25, 0.5, and 1 mmol) on the post-thawed rabbit sperm parameters, in comparison to 200 µmol Trolox (vitamin E analogue). Key parameters included sperm quality, antioxidant defense status, mitochondrial activity, ROS levels, and expression level of selected antioxidant-associated genes and antifreeze related gene assessed using RT-qPCR. Semen samples from fifteen mature bucks were pooled based on a minimum progressive motility of 70%. Assessments were conducted post-thaw using TCG extender, focusing on motility, quality, and antioxidant-related expression. Results showed that the group of using (ALA 0.25 mmol) had a positive influence on post-thawed sperm progressive motility with significant difference ($p \leq 0.05$) between other groups. Moreover, (ALA 0.25 mmol) group significantly ($p \leq 0.05$) improved antioxidants enzymes activity in compared to all groups while malondialdehyde MDA value reduced in (ALA 0.25 mmol) group in comparison with other groups. The (ALA 0.25 mmol) group exhibited significantly ($p \leq 0.05$) higher mitochondrial membrane potential activity, lower ROS accumulation and DNA fragmentation levels than other groups, Antioxidant-related genes and HSP90 were up-regulated in sperm cryopreserved with (ALA 0.25 mmol) group in compared to other groups ($p \leq 0.05$). This study concluded that supplementation of alpha lipoic acid with low concentration (0.25 mmol) in buck semen promotes sperm quality characteristics, reduces ROS, upregulates antioxidants and antifreeze related genes post freezing.

Keywords: Cryopreservation, Rabbits semen, Alpha lipoic acid, Mitochondrial activity, Sperm motility.

Introduction

Sperm cryopreservation effectively preserves male reproductive capabilities in humans and livestock [1], allowing for long-term storage and transport of semen, which has significantly contributed to breeding practices through artificial insemination over the past seventy years [2-3] .

Recent advancements enhance genetic strategies in livestock breeding while addressing freezing-related damages, including osmotic, oxidative, and epigenetic issues [4].

Enhancements in the assessment of freeze damage in sperm concentrate on osmotic, oxidative, and epigenetic influences. Elevated amounts of polyunsaturated fatty acids (PUFA) in sperm heighten the risk of lipid oxidation during freezing, which is exacerbated by reactive oxygen species (ROS) [1]. ROS disrupt antioxidant defense, resulting in lipid oxidation, decreased membrane flexibility, lower mobility, increased cell mortality, issues with sperm-egg fusion, early pregnancy failures, and changes in embryonic gene expression [5-6]. Rabbit sperm display larger heads, which leads to a diminished survival rate [4], and while sperm typically contain high levels of polyunsaturated fatty acids, they have insufficient antioxidants [7]. Semen is sensitive to hypertonic solutions, which complicates preservation and increases oxidative damage, affecting motility and capacitation due to

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elevated reactive oxygen species and lactate dehydrogenase (LDH) levels [8-9].

In rabbits, artificial insemination is limited by poor semen quality, low fertility, and small litter sizes. Freezing and thawing semen increase oxidative stress, leading to reduced sperm quality and viability [4].Cold shock damages sperm acrosomes and membranes, resulting in increased DNA fragmentation [10], reduced motility, and decreased fertility [11]. Sperm's antioxidant systems are insufficient and impaired by chilling, limiting effective defense against oxidative stress [12-13]. Antioxidants, including vitamins E and C, along with selenium and zinc, contribute to addressing male infertility by lowering reactive oxygen species (ROS) and enhancing sperm movement and performance [14-15]. New research has examined the potential benefits of polyphenols, carotenoids, and substances such as resveratrol, quercetin, curcumin, and Alpha-lipoic acid for the preservation of chilled semen [16-17]. Animal sperm and seminal fluid contain natural antioxidant mechanisms, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and glutathione reductase, and nonenzymatic one [15] but these are often inadequate due to dilution or cooling [13].

Adding antioxidants like vitamin E can improve sperm quality by reinforcing membranes and shielding against oxidative damage [15]. A Trolox concentration of 200 µm maintains the quality and movement of sperm in rabbits during the freezing process, acting as a standard comparison [18]. Alpha-lipoic acid, recognized for its ability to combat oxidative stress, boosts the levels of antioxidant enzymes and plays a vital role in the function of mitochondria and the metabolism of energy via the Keap/Nrf2 signaling pathway [19-20]. Adding ALA to cryopreservation solutions enhances sperm quality in multiple species [21-23]. There for this research intends to evaluate the impact of ALA on various aspects of rabbit semen quality, such as movement, viability, membrane integrity, antioxidant capacity, mitochondrial function, accumulation of reactive oxygen species, integrity of DNA, and the expression of related genes after thawing.

Material and Methods

Experimental animals and semen collection

Fifteen healthy and mature male rabbit of the New Zealand breed were utilized in the present study. Their ages varied between 6 to 12 months. The average body weight was 3.5 ± 0.2 kg. All subjects were maintained under uniform management practices and stable environmental conditions. Each buck was placed individually in cages with measuring 60 × 40 × 35 cm. the cages were maintained within a relatively controlled farm system. The provided diet was a basal formulation designed to meet the essential nutritional requirement of the animals [24]

Semen samples were collected from each buck twice a week for a duration of 12 weeks. The collected samples were submerged immediately in the water bath that adjusted at 37 °C. Sperm motility in the freshly obtained semen was evaluated through computer-assisted sperm analysis (CASA; Sperm Vision™ software, version 3.9, Mini tube, Tiefenbach, Germany) following the removal of the gel clot with forceps. Progressive motility percentages were assessed, and only ejaculates with at least 70% progressive motility were pooled to control the individual variations and included in the present study.

Semen processing

Semen assessment was conducted utilizing TCG based extender (Tris and citric acid), which was reported by Viudes-De-Castro [25]. The pooled samples were subsequently diluted 1:1 (v:v) with the studied extender. The pH level of the freezing extender was finally adjusted to 7.00. After that, the extender was kept at -20°C until it was needed. Moreover, Trolox, a form of Vitamin E, was added at the concentration of 200µM to act as the positive control.

The experimental procedure employed Alpha Lipoic Acid (ALA); $C_8H_{14}O_2S_2$, (CAS#: 1077-28-7) sourced from USA. Finally, the current concentrations of (ALA) within TCG extender were adjusted to be (0.025 mmol, 0.50 mmol and 1 mmol).The diluted semen was cooled at 5°C for a duration of 2 hours as an equilibration period and packed into 0.25 mL French straws immediately following the equilibration process. The straws were placed in horizontal manner at a height of 5 cm above the liquid nitrogen (LN_2) surface for the period of 15 minutes, after which they were submerged into the LN_2. To thaw the frozen straws, they were placed in the water bath at 37°C for 30 seconds.

The Sperm kinetics

Sperm motion characteristics was performed by using Computer-assisted sperm analysis (CASA) post-thawing by monitoring approximately 2500 sperm cells from each group as described by Ashrafi [26].

The Sperm Viability

The sperm viability within each studied group was evaluated using the approach described by Murugesan and Mahapatra, with slight modifications. More precisely, the percentage of live sperm was determined by counting approximately 200 sperm in various microscopic fields on each slide[27].

The Acrosomal Integrity

In this present study, Acrosomal status was assessed by using Giemsa stain, following the guidelines set forth by Rakha et al [28] with minor modifications to the initial procedure. The evaluation of sperm cells with intact acrosomes was conducted using phase contrast microscopy, involving a count of 200 blue-stained cells from the Giemsa staining at a magnification of 1000.

Plasma Membrane Status

The methodology presented by Rakha et al [28] was included using the Hypoosmotic Swelling Test (HOST) to assess the sperm membrane functionality with a slight adjustment made. A total of 200 sperm cells were examined using a phase contrast microscope at a magnification of 1000× with oil immersion. The percentage of sperm cells displaying intact plasma membranes, shown through either swollen or curled tails, acts as an essential factor for determining the best performance of the plasma membrane [28].

Assays of antioxidants biomarkers

The concentrations of total antioxidant capacity (TAC), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and Malondialdehyde (MDA) were measured. The total protein (TP) concentration in the samples was first measured with the biuret method, according to the instructions provided by the manufacturer of the colorimetric assay kit (TP-2020, Bio Diagnostic, Inc., Egypt). Three replicates were assembled for every treatment group by thawing two straws of cryopreserved sperm per replicate, following the methodologies previously stated [29]. Following the guidelines from the manufacturer, with all calculations for each assay normalized to milligrams of protein. Data for all tests were gathered using an automated scanning spectrophotometer (CE1010, Cecil Instruments Limited, Cambridge, United Kingdom).

Mitochondrial Membrane Potentiality

Ramalho-Santos et al. [30] indicated, with slight adjustments, the use of Mitotracker Red FM (Molecular Probes, Eugene, Oregon, USA), a crucial mitochondrial-specific dye that detects active mitochondria by their membrane potential, to evaluate mitochondrial function in sperm cells. The staining method followed the instructions from the manufacturer (M22425 for Mitotracker Red, Thermo Fisher Scientific, Waltham, MA, USA), which included incubating live sperm (1×10^6 sperm/ml) in a dark environment at 37 °C for 30 minutes with 1 µL of Mitotracker Red. Afterward, the samples underwent three washes with 1× phosphate-buffered saline (PBS, Sigma-Aldrich).

The samples were subsequently placed on a microscope slide and assessed through confocal imaging using a laser scanning confocal microscope, specifically the Zeiss LSM 880, which was enhanced with the super-resolution (SR) Airyscan system from Zeiss (Jena, Germany). The filter sets utilized consisted of a 488 nm filter with a bandpass covering 491-632 nm (laser HeNe1), and the imaging utilized the Plane-Apochromat 63×1.4 Oli DIC M27 objective.

Measurement of Intracellular Reactive Oxygen Species

According to McCloy et al [31] Intracellular levels of reactive oxygen species (ROS) in sperm were assessed utilizing a the DCF assay with the probe 2,7-dichlorodihydrofluorescein diacetate (DCFH2-DA). Concentration of 13 µM DCFH2-DA (obtained from Sigma-Aldrich) was introduced to a 150-µL sample of semen and subsequently incubated in the dark at 37 °C for a the duration of 30 minutes. The specificity of the fluorescent labeling for ROS detection was verified through confocal imaging performed with a Zeiss LSM 880 laser scanning confocal microscope, along with the super-resolution Airyscan system from Zeiss (Jena, Germany), following three washing steps using phosphate-buffered saline (PBS, Sigma-Aldrich).

DNA fragmentation

Following the methodologies by Henkel et al. [32], twenty microliters of thawed semen were spread on glass slides and allowed to dry. After being exposed to a fixative buffer for fifteen minutes, the slides were immersed in a staining solution of aniline blue for three minutes. Once the staining was completed, the slides were rinsed with distilled water and left to dry in the air. The evaluation of stained sperm heads involved analyzing 200 sperm cells, which were sorted according to the intensity of the staining: those that had strong or very strong aniline blue staining were determined to have fragmented DNA, while those with faint or no staining were considered to have non-fragmented DNA.

RNA extraction, complementary DNA (cDNA) synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR).

Three biological replicates from the negative and positive control groups, as well as from each Alpha Lipolic Acid treatment group, were removed from the liquid nitrogen tank and allowed to thaw at room temperature. The resultant contents were subsequently transferred to 1.5 ml Eppendorf tubes and centrifuged for 12 minutes at 680 rpm and 4 °C. RNA extraction was carried out following the TRIZOL RNA Isolation Protocol (Yale University, USA), adhering to the manufacturer's guidelines. To eliminate genomic DNA contamination, DNase I, as recommended by the RNase-free kit (Thermo Scientific, California, USA), was added. The RNA's quality and quantity were measured using a NanoDrop 2000c (Thermo Fisher Scientific Inc.,

USA). For cDNA synthesis, a Reverse Transcription Kit from Life Technologies Corporation (California, USA), which includes MultiScribe™ Reverse Transcriptase and random primers, was utilized. Gene-specific primers were designed based on sequences from the GenBank database (www.ncbi.nlm.nih.gov) using the Primer3 software (http://primer3.wi.mit.edu//), as detailed in Table 1.

Real-time PCR was performed according to the Two-step Quantitative Real-Time PCR Protocol (Applied Biosystems, California, USA). The reaction mixture consisted of 2 µl of reverse and forward primers, 10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, California, USA), 6 µl of nuclease-free water, and 2 µl of cDNA. The PCR conditions were set for one initial cycle at 95°C for 10 minutes, followed by 40 cycles comprising of 15 seconds at 95 °C and 60 seconds at 60 °C. The comparative cycle threshold (CT) method was employed to determine fold changes ($2^{-\Delta\Delta CT}$) for gene expression analysis, normalizing the expression of target transcripts (CAT, GPX1, PRDX1, SOD1, NFE2L2, and HSP90) to the housekeeping gene (GAPDH). Prior to gene expression analysis, the stability of GAPDH as a housekeeping gene was confirmed to be adequate [33].

Statistical Analysis

The data was analyzed statistically following the approach described by Wieder and Lang [34] subjected to statistical analysis using SAS version 9.1[35] (SAS Institute Inc., Cary, NC, USA). The influence of various Alpha Lipolic Acid concentrations (negative control, ALA 0.25 mmol, ALA 0.5 mmol, ALA1 mmol, and positive control 200 µ Trolox, a vitamin E analogue) on sperm traits as described previously. This was followed by a one-way ANOVA and Tukey's post hoc test ($\alpha = 0.05$). For gene expression analysis, the SAS General Linear Model (GLM) was employed. Mean values among treatment groups were compared using ANOVA. A significance threshold was set at ($P \leq 0.05$).

Results

Sperm motility attribute

The results illustrated in Table (2) indicated that the total motility percentage of frozen/thawed sperm rose by approximately 7% ($p \leq 0.05$) in the ALA 0.25 mmol treatment group when compared with the control and ALA 1 mmol groups. Howeve, the differences among the ALA 0.25 mmol and ALA 0.5 mmol groups were not statistically significant. Importantly, the ALA 0.25 mmol treatment had a positive effect on the progressive motility of post-frozen-thawed semen, reflecting an increase of approximately 18-19% ($p \leq 0.05$) when compared to the control and ALA 1 mmol groups, while no significant differences ($p \leq 0.05$) were observed between the ALA 0.5 mmol and 200µm Trolox groups.

Regarding the distance average path (DAP), the control group showed a slight rise of about 1 to 2% when compared to the other ALA groups, although there was no significant ($p \leq 0.05$) difference between all groups. Regarding the distance curved line (DCL), the control group registered an increase of approximately 2 to 4% relative to other treatment groups, with no significant ($p \leq 0.05$) differences observed. Similarly, the distance straight line (DSL) metric for the control group indicated no significant ($p \leq 0.05$) disparity across all groups.

The velocity average path (VAP) displayed significant differences ($p \leq 0.05$) for the control group comparing to the all groups but with no significant ($p \leq 0.05$) difference between control group and ALA 0.5 mmol group. The velocity curved line (VCL) exhibited a significant increase ($p \leq 0.05$) of about 10 to 11% was noted in the control group when contrasted with ALA 1 mmol group, however there was no significant ($p \leq 0.05$) difference between control, 200µm Trolox, ALA 0.25 mmol and ALA 0.5 mmol groups. The velocity straight line (VSL) showed no significant differences ($p \leq 0.05$) among the 200 µm Trolox, ALA 0.25 mmol, ALA 0.5 mmol, and ALA 1 mmol groups, although significant differences ($p \leq 0.05$) were present between the control and all ALA groups. Additionally, the straightness of post-thaw sperm (STR) indicated a notable increase ($p \leq 0.05$), estimated at around 0.02 to 0.03%, in the ALA 1 mmol and control groups compared to other treatment groups. Regarding linearity (LIN), a significant rise ($p \leq 0.05$) was found in the ALA 1 mmol group compared to all other groups. No significant differences were noted among all groups for wobble (WOB).

Lastly, the amplitude of lateral head displacement (ALH) presented a significant improvement ($p < 0.05$) in the control group, when contrasted with the other groups. Additionally, the beat cross frequency (BCF) did not reveal a significant rise ($p \leq 0.05$) in the control, 200 µm Trolox, and all ALA groups.

Sperm Quality Attributes

Viability

The data presented in Table 3 reveals that the ALA 0.25 mmol group exhibited superior sperm quality parameters when compared to the other groups ($p \leq 0.05$). The viability was over 21% higher in ALA 0.25 mmol group compared to control group, while there was no significant difference between ALA 0.25 mmol group and 200 µm Trolox group but ALA 1 mmol group showed decreased in the viability ($p \leq 0.05$).

Acrosomal integrity

According to Table 3, the percentage of intact acrosomes increased notably ($p \leq 0.05$) by over 14% in the ALA 0.25 mmol group in comparison to the control group. Additionally, no significant difference was observed between the ALA 0.5 mmol and 200µm Trolox groups. In the ALA 1 mmol group, acrosome integrity declined by roughly 11 percent ($p \leq 0.05$) when compared to ALA 0.25 mmol group, and there was no significant difference ($p \leq 0.05$) noted between the ALA 1 mmol group and the control group.

Plasma membrane condition

The intact plasma membrane percentage in ALA 0.25 mmol notably ($p \leq 0.05$) rose by over 19% relative to the control group, as presented in Table 3. The integrity of the plasma membrane was reduced during the freezing of semen with ALA 1 mmol, showing a decline of about 11 percent ($p \leq 0.05$) when contrasted with the ALA 0.25 mmol group. Additionally, there were no significant ($p \leq 0.05$) differences observed between the ALA 0.5 mmol and 200µm Trolox groups.

Mitochondrial Membrane Functionality

The evaluation of mitochondrial activity in rabbit sperm, as shown by the mitochondrial membrane potential, was carried out using MitoTracker1-Red. The group that received treatment with ALA at a concentration of 0.25 mmol exhibited a notably higher level of mitochondrial potential activity, indicated by a high level of fluorescence intensity ($p \leq 0.05$), when contrasted with other treated groups (Fig. 1, A). In contrast, the group treated with ALA at 1 mmol showed the lowest fluorescence intensity in relation to all other experimental groups ($p \leq 0.05$). No significant differences ($p \leq 0.05$) were observed between the ALA 0.5 mmol group and the 200µmol Trolox group.

Measurement of Intracellular Reactive Oxygen Species.

The accumulation levels of reactive oxygen species (ROS) were measured in one hundred sperm samples from each test group. The group that received ALA at a concentration of 1 mmol showed the highest accumulation of ROS, as reflected by a notably elevated fluorescence intensity ($p \leq 0.05$). Conversely, the ALA 0.25 mmol group exhibited the least ROS accumulation, which was shown by the lower intensity of the fluorescent dye ($p < 0.05$). Furthermore, there was no significant difference ($p \leq 0.05$) between the 200 µmol group and the ALA 0.5 mmol group. (Fig. 1, B).

DNA fragmentation

The assessment of DNA fragmentation was conducted on one hundred sperm samples from each experimental group. The ALA 1 mmol group showed the highest level of DNA fragmentation, surpassing 33% ($p \leq 0.05$). on the other hand , the group with 0.25 mmol ALA presented the lowest level of DNA fragmentation, around 10%, with the 200 µm Trolox and 0. 5 mmol ALA groups closely behind, both demonstrating noteworthy findings ($p \leq 0.05$), as depicted in Fig. 2.

Semen-Based Antioxidant Indicators

The ALA 0.25 mmol group demonstrated the highest levels of total antioxidant capacity (TAC), glutathione peroxidase (GPx), super oxide dismutase (SOD), and catalase (CAT), with values of 0.009 µM/mg, 0.009 mU/mg, 5.11 U/mg, and 0.008 u/mg, respectively. Following this, the 200 µM Trolox and ALA 0.5 mmol groups also showed noteworthy results ($p \leq 0.05$). Conversely, the ALA 1 mmol group had the lowest SOD level, registering at 1.340 U/mg. In addition, the 200 µM Trolox group exhibited enhanced CAT activity ($p \leq 0.05$), with no significant ($p \leq 0.05$) variance between the 200 µmol and ALA 0.25 mmol groups. The lowest CAT values were observed in the control and ALA 1 mmol groups, which had 0.003 u/mg and 0.004 u/mg, respectively ($p \leq 0.05$). Importantly, adding ALA at a concentration of 0.25 mmol to the extender resulted in the minimal malondialdehyde (MDA) levels recorded (0.156 nM/mg) ($p \leq 0.05$). There was no significant ($p \leq 0.05$) difference found between the ALA 0.5 mmol and 200 µmol groups, both of which lowered MDA levels compared to the ALA 1 mmol and control groups (Table 4).

Expression of Genes Associated with Antioxidants and Anti-Freeze Mechanisms

Fig. 3 illustrates the the results related to the antioxidant and antifreeze-related gene expressions in rabbit sperm that has been cryopreserved with ALA. It is noteworthy that the genes such as CAT, GPX1, PRDX1, SOD1, and NFE2L2 showed significant upregulation ($p \leq 0.05$) in the ALA 0.25 mmol treatment group when compared to the other groups. There was no significant difference in the CAT gene among the ALA 1mmol, ALA 0.5 mmol, and 200 µm Trolox groups ($p \leq 0.05$). For the GPX1, SOD1, and PRDX1 genes, the expression levels in the ALA 0.5 mmol and 200 µm Trolox groups were significantly ($p \leq 0.05$) higher than in the control group. However, the 200 µm Trolox, control, and ALA 1 mmol groups did not show significant ($p \leq 0.05$) variations. Additionally, the expression of HSP90 was significantly increased in the ALA 0.25 mmol group relative to the other treatments ($p \leq 0.05$). In contrast, the ALA 1 mmol group exhibited significant ($p \leq 0.05$) downregulation of all the genes assessed, with the exception of HSP90.

Discussion

Alpha-lipoic acid act as an antioxidant and serves as a co-factor for mitochondrial enzymes essential

for generating energy and facilitating metabolic functions. [36], Research has shown that alpha-lipoic acid (ALA) is vital for the citric acid cycle's efficiency in mitochondria. This enhancement results in higher levels of reduced glutathione, adenosine triphosphate (ATP), enzymes related to the tricarboxylic acid (TCA) cycle, and improved activity of the electron transport chain complexes[37]. The influence of ALA on metabolic activities, along with the greater availability of mitochondrial co-enzymes and increased protection against free radicals, is believed to significantly contribute to minimizing mitochondrial dysfunction. Such a reduction ensures a sufficient supply of ATP, which is crucial for sperm motility [38].

This study investigated the impact of alpha lipoic acid supplementation at various doses (0.25, 0.5, and 1 mmol) in cryopreservation media on frozen-thawed rabbit sperm cells. This assessment involved a comparison with a control group that used TCG freezing extender without any supplements, as well as a positive control that added 200 µm of Trolox, a vitamin E analog.

Our findings revealed that within the range of concentrations examined, the ALA 0.25 mmol group significantly ($p \leq 0.05$) improved motion characteristics , as shown in Table 2. Additionally, this group demonstrated improvements in the percentages of acrosome and plasma membrane integrity, alongside a reduction in the quantity of dead and abnormal sperm. The ALA 0.5 mmol and 200 µm Trolox groups also followed this trend; however, both the control and ALA 1 mmol groups exhibited a significant decline ($p \leq 0.05$) in viability, as well as in the percentages of intact acrosome and plasma membrane integrity, as depicted in Table 3. Nonetheless, it is important to highlight that no significant differences ($p \leq 0.05$) were noted in PROG motility (%) among the groups receiving ALA 0.5, ALA 1 mmol, and 200 µm Trolox, as illustrated in Table 3. These findings support earlier studies [23] that indicated semen ejaculates from Nili-Ravi buffalo bulls were diluted with an extender containing various concentrations of α-lipoic acid (ALA), specifically 0.5, 1.0, 2.0, 3.0, and 4.0 mM.

Integrity percentages, along with a decrease in both the quantity of non-viable and abnormal sperm, were noted. This was followed by groups treated with ALA at 0.5 mmol and those with 200 µm Trolox. However, both the control and the ALA at 1 mmol group showed a significant reduction ($p \leq 0.05$) in viability, intact acrosome, and plasma membrane integrity percentages, as shown in Table 3. It is also important to highlight that there were no significant variations ($p \leq 0.05$) in PROG motility (%) among the ALA 0.5, ALA 1 mmol, and 200 µm Trolox groups, as detailed in Table 3. These findings support earlier studies [23] that found that semen samples from Nili-Ravi buffalo bulls were diluted with an extender containing different concentrations of α-lipoic acid (ALA), specifically at levels of 0.5, 1.0, 2.0, 3.0, and 4.0 mM.

The assessment of sperm after thawing indicated that lower concentrations of ALA at 0.5 and 1.0 mM significantly enhanced the quality of semen, especially in terms of sperm motility and viability. Enhancements in semen quality associated with the incorporation of ALA into semen extenders have been noted in various species, including goats [39], Holstein Friesian bulls [40], Limousin bulls[41], and boars [42]. Adding of alpha-lipoic acid to freezing extenders could improve the survival, functionality, and structural integrity of sperm. This benefit is attributed to its capacity to boost energy production while reducing oxidative stress[43]. Alpha-lipoic acid is effectively distributed throughout various tissues and is swiftly transformed into its more powerful antioxidant form, dihydrolipoic acid [44]. The small size and high lipophilicity of this compound allow it to penetrate biological membranes effectively, enabling it to neutralize free radicals in both lipid and water-based settings [45]. Furthermore, alpha-lipoic acid has shown a protective role for boar semen during the freezing and thawing cycle[21], as well as during its storage in liquid form[42].

The results from our research showed a decrease in viable spermatozoa, with the highest occurrence of damaged acrosomes and plasma membranes observed at a concentration of 1 mmol of alpha lipoic acid, as shown in Table 3. This effect is associated with the harm caused to the plasma membrane during the freezing procedure. These findings align with Gohar et al.[23].

In this research, we investigated the antioxidant effects of ALA in a freezing medium for rabbits to reduce the build-up of reactive oxygen species (ROS) in sperm during the cryopreservation process, which could result in DNA damage. Preserving the sperm DNA's integrity is essential for effective fertilization and the development of healthy embryos. Various studies have highlighted a link between low temperature exposure and DNA oxidative damage in sperm[46]. Furthermore, maintaining the stability of the mitochondrial membrane potential is vital because research indicates it influences sperm fertility by regulating energy generation and movement[47]. Importantly, a negative relationship has been identified between mitochondrial membrane potential (MMP) and the levels of reactive oxygen species (ROS) in sperm subjected to freezing and thawing[47]. Mitochondria are recognized as the primary source of reactive oxygen species (ROS) within cells. The generation of ROS can impair the functional coupling of electron transport and oxidative phosphorylation in sperm cells, resulting in a decrease in the quantity of sperm exhibiting normal

mitochondrial activity and a reduction in their motility[48].

The findings from our study indicated that a concentration of 0.25 mmol of ALA was adequate to yield positive effects on the sperm cells of thawed rabbits. This treatment improved various sperm characteristics, minimized oxidative damage, and reduced the fragmentation of sperm DNA. These positive results were associated with a decline in the generation of reactive oxygen species (ROS) and an enhancement in the functionality of mitochondrial membrane potential, as shown in Fig. s 1 and 2. Alpha-lipoic acid has dual solubility, enabling it to dissolve in both water-based and fatty environments, which allows it to function effectively in both outside and inside the cells. This distinct feature boosts its capacity to cross cell membranes with ease. Research consistent with our findings has indicated that alpha-lipoic acid can help decrease DNA damage in buck spermatozoa following freezing and thawing processes [22], enhance mobility[43], and reduce (ROS) production in buck spermatozoa [39]. Alpha-lipoic acid, recognized for its antioxidant qualities, has been thoroughly examined to assess its potential impact on various conditions such as diabetes, ischemia, heavy metal toxicity, radiation damage, neurodegenerative diseases, and HIV infections, in addition to its role in improving sperm quality[23]. The current study highlights a significant ($p \leq 0.05$) enhancement in the enzymatic functions of total antioxidant capacity (TAC), catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in the group treated with ALA 0.25 mmol when compared to all other groups. At the same time, levels of malondialdehyde (MDA) were considerably ($p \leq 0.05$) lower in the ALA 0.25 mmol group when assessed against all groups, as shown in Table 4. This marked increase in antioxidant enzyme activities, such as CAT, SOD, and GPx, could affect the biological reaction to the modified MDA levels that lead to cellular damage and might hasten apoptosis. These cellular mechanisms frequently jeopardize cell membranes and DNA integrity, resulting in cell death [49] and impaired sperm motility is crucial for safeguarding sperm against reactive oxygen species (ROS) and lipid peroxidation. These results are consistent with studies indicating that incorporating alpha-lipoic acid into cryopreservation solutions, aimed at leveraging its antioxidant effects, yields numerous benefits for the reproductive characteristics in boars [21] and cattle [23].

This mechanism proficiently inhibits the formation of radical metabolites, removes free radicals, promotes the restoration of cellular damage, disrupts chain reactions, and strengthens the body's inherent antioxidant capabilities [36]. ALA has been recognized for its supplementary functions, notably its capability to regenerate vitamin C from its reduced form when glutathione is present. This mechanism aids in sustaining elevated antioxidant levels within the biological milieu without requiring an increase in the concentration of vitamin C in the sperm media, thus averting a transition to acidic conditions. The addition of ALA to the media does not significantly decrease the pH towards acidic values, as it is regarded as a weak acid. Moreover, recent studies have indicated that ALA can enter the Krebs cycle, thereby enhancing ATP production, which is crucial for spermatozoa viability [50].

In this investigation, we examined the gene expression profiles associated with antioxidant-related genes, specifically NFE2L2, CAT, SOD1, GPX1, and PRDX1, as well as the antifreeze-related gene HSP90. Our results indicated that treatment with 0.25 mmol of ALA (ALA) significantly upregulated ($p \leq 0.05$) the expression of all antioxidant-related genes CAT, SOD1, GPX1, and NFE2L2 when compared to all groups conditions. Additionally, the 0.25 mmol ALA treatment significantly enhanced ($p \leq 0.05$) the expression of the antifreeze-related gene HSP90 in comparison to all groups. Conversely, treatment with 1 mmol ALA resulted in a significant ($p \leq 0.05$) downregulation of all genes except HSP90 when compared to control, as illustrated in Fig. 3. Numerous studies have highlighted ALA's potent role as serves dual roles as both the direct and the indirect antioxidant, promoting the upregulation of antioxidant enzymes. Furthermore, it influences the expression of proteins essential for energy metabolism, mitochondrial biogenesis, and maintaining mitochondrial structural integrity. These actions are chiefly facilitated through the activation of the antioxidant response element (ARE), particularly through the Keap/Nrf2 signaling pathway [20]. Alpha lipoic acid (ALA), a type of short-chain fatty acid, is well-known for its potent antioxidant capabilities, which stem from its proficiency to operate efficiently in both oxidized and reduced forms [51]. Moreover, ALA is categorized as a weak acid, and its addition to semen extenders does not alter the seminal pH. The inclusion of ALA in the extender has been shown to improve the quality of low-grade semen derived from sub-fertile bulls [52].

Alpha-lipoic acid (ALA) serves as an antioxidant that is recognized for its ability to improve sperm quality during the cryopreservation process through the mitigation of oxidative stress. Nonetheless, the impact of ALA is contingent upon its concentration, with elevated levels potentially having adverse effects on sperm motility and gene expression, Research findings indicate that low levels of alpha-linolenic acid (ALA) can enhance sperm motility, whereas elevated concentrations may be detrimental. For example, a study involving ram spermatozoa revealed that a concentration of 0.1 mM ALA notably improved both total and progressive motility

percentages. In contrast, increased levels resulted in a reduction of motility metrics. This illustrates that an overabundance of ALA may disturb the redox equilibrium, subsequently causing oxidative stress and compromising sperm functionality [53].

Elevated levels of alpha-lipoic acid (ALA) have been linked to changes in gene expression that pertain to apoptosis within sperm cells. Research involving rooster spermatozoa indicated that ALA, at optimal concentrations, decreased apoptosis; however, when present in excessive amounts, it resulted in the upregulation of pro-apoptotic genes and heightened activation of caspase-3, an enzyme pivotal in the execution phase of apoptosis. This suggests that concentrations of ALA exceeding the optimal range could activate apoptotic pathways, thereby compromising sperm viability [54].

Although alpha-lipoic acid (ALA) functions as a protective antioxidant at suitable concentrations throughout the process of semen cryopreservation, elevated levels have the potential to trigger oxidative stress and initiate apoptotic pathways. This cascade can result in diminished sperm motility and modifications in gene expression.

Consequently, it is essential to meticulously optimize the concentration of ALA to enhance its positive impact on sperm quality.

In this study, an increase in sperm motility, plasma membrane integrity, and HSP90 expression was noted after the implementation of the freezing/thawing protocol at the concentration of ALA 0.25 mmol, as depicted in Table 2 and Fig. 3. These findings suggest that HSP90 expression may play a crucial role in enhancing plasma membrane integrity, reducing the accumulation of reactive oxygen species (ROS), improving mitochondrial membrane function, and boosting the activity of antioxidant enzymes in seminal plasma, all contributing to enhanced sperm motility post-thawing. These results are consistent with previous research highlighting the synergistic function of HSP90 and HSP70 in alleviating oxidative stress [55] and preventing programmed cell death [53].

In the present study, we sought to evaluate the antioxidant capabilities of the cryopreservation medium enhanced with low levels of alpha lipoic acid to reduce oxidative damage to sperm during the freezing process. Our results demonstrated that the only concentration that exhibited a positive effect on rabbit sperm cells after thawing was 0.25 mmol. Incorporating 0.25 mmol of ALA into the cryopreservation medium may improve sperm quality, reduce oxidative damage, and preserve sperm DNA integrity by decreasing the generation of reactive oxygen species (ROS) and influencing the expression of specific genes associated with antioxidant defense. Our study presents important insights into improving the effectiveness of sperm cryopreservation, an essential procedure for aiding those experiencing infertility or subfertility. The rabbit is an advantageous model for investigating human reproductive processes. In addition, these results may enhance artificial insemination techniques, particularly in livestock species that encounter reproductive issues, such as buffaloes.

Conclusion

The addition of Alpha lipoic acid (ALA) at 0.25 mmol enhances post-thaw quality of rabbit sperm in freezing extender media. It improves progressive motility, viability, plasma membrane integrity, acrosome integrity, and reduces sperm DNA fragmentation by preserving antioxidant-related transcripts and HSP90, thus lowering reactive oxygen species (ROS) and improving mitochondrial function.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Benha University, Egypt (ethics approval number; 49/11/2023).

TABLE. 1 Primer sequences utilized in qRT-PCR analysis of designated antioxidants and anti- freeze-related genes

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)
GAPDH	NG_027769.2	F: 5′- TGTTTGTGATGGGCGTGAA-3′	129
		R: 5′- CCTCCACAATGCCGAAGT-3′	
CAT	XM_002709045.4	F: 5′- GACAAAATGCTTCAGGGCCG-3′	173
		R: 5′- TAGTAATTGGGAGCACCGCC-3′	
SOD1	NM_001082627.2	F: 5′- GCCGCTGCGGAGTCAT-3′	195
		R: 5′- CTGCACTCGTACAGCCTTGT-3	
NFE2L2	XM_008258785.3	F: 5′- ACTACCACGGTTCCAAGTGC-3′	192
		R: 5′- TGTGGCATTTGAGTTCACGC-3′	
PRDX1	XM_002715138.4	F: 5′- TCACCTTTGTGTGCCCTACG-3′	100
		R: 5′- TGAGAATCCACGGAAGCACC-3′	
GPX1	NM_001085444.1	F: 5′- AGTTTGGGCATCAGGAGAACG-3′	70
		R: 5′- CCTCCAGGCCGGACATACTT-3′	
HSP90	XM_051834811.1	F: 5′- AGACCCAGACCCAAGACCAG-3′	166
		R: 5′- ATCTTGTCCAGAGCATCCGAC-3	

TABLE 2. The sperm parameters of post thawed rabbit semen influenced by three different concentrations of Alpha Lipoic Acid alongside 200 µm of Trolox.

Traits	Control	200 µm Trolox (vitamin E analogue)	ALA 0.25 mmol	ALA 0.5 mmol	ALA 1mmol
TMOT (%)	67.62^{d}±1.05	69.23^{cd}±1.01	74.58^{a}±1.01	73.22^{ab}±1.04	67.25^{d}±1.09
PROG (%)	30.44^{b}±1.58	36.51^{b}±1.51	48.51^{a}±1.52	39.22^{b}±1.56	29.32^{b}±1.64
DAP (um)	17.50^{a}±0.75	15.57^{a}±0.72	15.68^{a}±0.72	16.17^{a}±0.74	15.39^{a}±0.77
DCL (um)	26.73^{a}±1.52	23.40^{a}±1.45	24.93^{a}±1.46	24.54^{a}±1.50	22.75^{a}±1.57
DSL (um)	13.87^{a}±0.65	12.31^{a}±0.62	11.96^{a}±0.62	12.51^{a}±0.64	12.28^{a}±0.67
VAP (um/s)	44.46^{a}±1.76	38.58^{b}±1.68	38.18^{b}±1.69	39.98^{ab}±1.73	38.33^{b}±1.82
VCL (um/s)	66.83^{a}±3.45	57.40^{ab}±3.30	60.14^{ab}±3.32	59.92^{ab}±3.40	55.85^{b}±3.56
VSL (um/s)	35.49^{a}±1.52	30.74^{b}±1.45	29.26^{b}±1.46	31.12^{b}±1.50	30.80^{b}±1.57
STR (%)	0.79^{a}±0.0077	0.78^{ab}±0.0073	0.76^{cd}±0.0074	0.77^{abc}±0.0075	0.79^{a}±0.0079
LIN (%)	0.531^{ab}±0.008	0.531^{ab}±0.008	0.488^{c}±0.008	0.514^{b}±0.008	0.547^{a}±0.009
WOB (VAP/VCL)	0.665^{a}±0.007	0.669^{a}±0.007	0.635^{b}±0.007	0.662^{a}±0.007	0.682^{a}±0.007
ALH (um)	5.66^{a}±0.17	4.84^{bc}±0.16	4.56^{cd}±0.16	5.01^{bc}±0.17	4.87^{cd}±0.17
BCF	10.80^{a}±0.52	10.02^{a}±0.49	10.74^{a}±0.50	10.43^{a}±0.51	9.28^{a}±0.53

Data are illustrated as means ± standard error (SE). Significantly ($p \leq 0.05$) different means within the same row are denoted by different superscripts. TMOT: total motility; PROG: progressive motility; distance average path (DAP, µm), distance curved line (DCL, µm) distance straight line (DSL, µm)VAP: average velocity line (µm/s);, VCL: curved velocity line (µm/s); VSL: velocity straight line (µm/s); STR: straightness (VSL/VAP, %); LIN: linearity (VSL/VCL, %); WOB: wobble (VAP/VCL, %); ALH: amplitude of lateral head displacement (µm); BCF: beat cross frequency (Hz).

TABLE 3. Effects of Alpha Lipoic Acid concentrations as well as Trolox in freezing extender on the sperm parameters of post-thawed rabbit semen

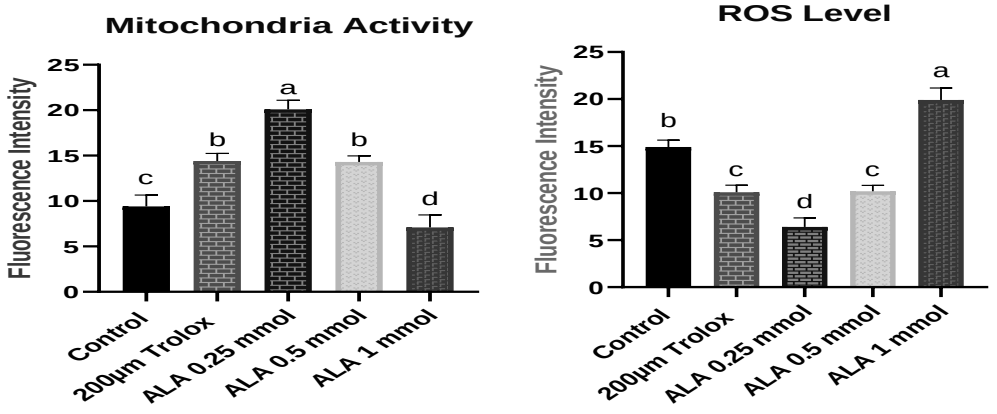
Parameters	Control	200 µm Trolox	ALA 0.25 mmol	ALA 0.5 mmol	ALA 1 mmol
Viability (%)	65.4^{c}±0.27	83.2^{b}±0.27	86.8^{a}±0.27	80.6^{b}±0.27	57.6^{d}±0.27
Intact acrosome (%)	74.31^{cd}±0.386	81.71^{b}±0.413	88.06^{a}±0.38	80.93^{b}±0.38	77.62^{c}±0.38
Intact plasma membrane (%)	69.56^{d}±0.489	80.35^{b}±0.522	88.06^{a}±0.38	80.93^{b}±0.38	77.62^{c}±0.38

Data are illustrated as means ± standard error (SE). Significantly ($p \leq 0.05$) different means within the same row are denoted by different superscripts.

Table 4. The impact of varying concentrations of ALA and 200 µm Trolox in freezing extender on the antioxidant biomarkers in rabbit sperm following thawing.

Parameters	Control	200 µm Trolox	ALA 0.25 mmol	ALA 0.5 mmol	ALA 1mmol
TAC(µM/mg)	0.0001^{c}±0.00	0.008^{b}±0.00	0.009^{a}±0.01	0.0077^{b}±0.01	0.001^{c}±0.00
GPx(mU/mg)	0.006^{c}±0.00	0.008^{ab}±0.003	0.009^{a}±0.003	0.007^{b}±0.0006	0.006^{c}±0.002
SOD (U/mg)	2.832^{c}±0.217	4.180^{b}±0.121	5.11^{a}±0.00	4.494^{b}±0.312	1.340^{d}±0.195
CAT(u/mg)	0.003^{c}±0.001	0.008^{a}±0.001	0.009^{a}±0.000	0.006^{b}±0.0067	0.004^{c}±0.001
MDA(nM/mg)	0.486^{a}±0.03	0.272^{b}±0.03	0.156^{c}±0.007	0.307^{b}±0.06	0.498^{a}±0.09

Data are illustrated as means ± standard error (SE). Significantly ($p \leq 0.05$) different means within the same row are denoted by different superscripts.



(A) **(B)**

Fig. 1. illustrates several key parameters following the thawing of rabbit sperm subjected to different treatments. Panel (A) presents the fluorescence intensity corresponding to mitochondrial membrane potential activity, as measured in sperm from the various treatment groups. Statistical analysis reveals significant differences among groups, indicated by distinct letters ($P \leq .05$) for values a, b, c, and d. Panel (B) outlines the fluorescence intensity of ROS, utilizing the H2DCFDA stain, for sperm across different treatment groups, with significant differences also identified among the groups ($P \leq .05$) through the use of distinct letters for values a, b, c, and d

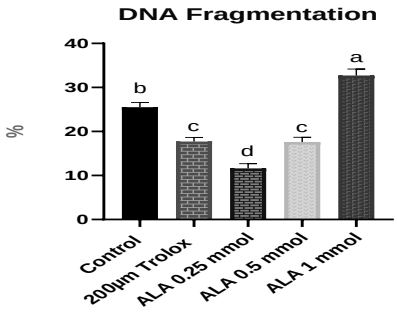


Fig. 2. Illustrate the DNA fragmentation observed in rabbit sperm following freezing and thawing, utilizing an extender that was supplemented with varying concentrations: 0 (negative control), 200 µm Trolox (positive control), as well as 0.25 mmol, 0.5 mmol, and 1 mmol of ALA. The values labeled a, b, c, and d are statistically significant, indicating differences at the level of P ≤0.05

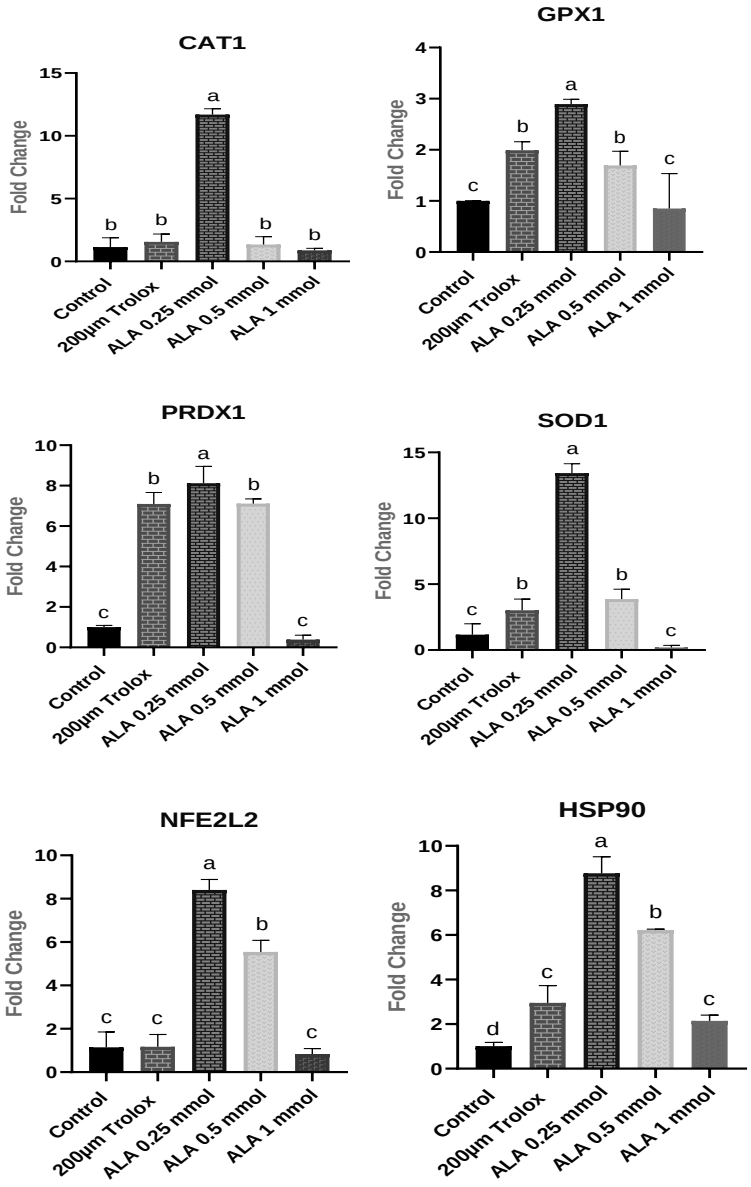


Fig. 3. The impact of different concentrations of ALA compared to 200 µm Trolox E in freezing extender on the antioxidants and antifreeze associated gene expression of rabbit sperm post-thawing, (FC: fold change) ± standard error (SE). a,b,c,d,e Means with different superscripts, within the same gene, are significantly different (p ≤ 0.05). antioxidants related genes: (CAT: catalase, GPX1: glutathione peroxidase 1, PRDX1: peroxiredoxin 1, SOD1: superoxide dismutase 1 and NFE2L2: nuclear factor erythroid

References

1. Hezavehei, M Sharafi, M., Kouchesfahani, H. M., Henkel, R. Agarwal, A., Esmaeili, V. and Shahverdi, A, Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reprod. Biomed. Online*, **37**(3),327–339(2018). doi: 10.1016/j.rbmo.2018.05.012.

2. Hammerstedt, R.H., Graham J.K., J. N. Cryopreservation of mammalian species. *Journal of Andrology*, **11**(3), 73–88(1990).

3. Sharafi, M., Borghei-Rad, S. M., Hezavehei, M., Shahverdi, A. and Benson, J. D. Cryopreservation of Semen in Domestic Animals: A Review of Current Challenges, Applications, and Prospective Strategies. *Animals*, **12**(23),1–24(2022). doi: 10.3390/ani12233271

   Kumar, A., Prasad, J. K. Srivastava, N. and Ghosh, S. K. Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa, Biopreserv. *Biobank.*, **17**(6), 603–612(2019). https://doi.org/10.1089/bio.2019.0037.

5. Pons-Rejraji, H., Sion, B., Saez, F., Brugnon, F., Janny, L. and Grizard, G. Role of reactive oxygen species (ROS) on human spermatozoa and male infertility. *Gynecologie, Obstetrique & Fertilite*, **37**(6), 529–535(2009). https://doi.org/10.1016/j.gyobfe.2009.04.015

6. Yánez-Ortiz, I.; Catalán, J.; Rodríguez-Gil, J. E.; Miró, J. and Yeste, M. Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Animal Reproduction Science*, **246**,1–18 (2022). https://doi.org/10.1016/j.anireprosci.2021.106904

7. Ismail, A. A., Abdel-Khalek, A. E., Khalil, W. A., Yousif, A. I., Saadeldin, I. M., Abomughaid, M. M. and El-Harairy, M. A. Effects of mint, thyme, and curcumin extract nanoformulations on the sperm quality, apoptosis, chromatin decondensation, enzyme activity, and oxidative status of cryopreserved goat semen. *Cryobiology*, **97**, 144–152(2020). https://doi.org/10.1016/j.cryobiol.2020.09.002

8. De Lamirande, E., Jiang, H., Zini, A., Kodama, H. and Gagnon, C. Reactive oxygen species and sperm physiology. *Reviews of Reproduction*, **2**(1), 48–54 (1997). https://doi.org/10.1530/ror.0.0020048

9. Bansal, A. K. and Bilaspuri, G. S. Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med. Int.*, **2011**, 686137 (2011). doi: 10.4061/2011/686137.

10. Tavilani, H., Goodarzi, M. T., Doosti, M., VaisiRaygani, A., Hassanzadeh, T., Salimi, S., and Joshaghani, H. R Relationship between seminal antioxidant enzymes and the phospholipid and fatty acid composition of spermatozoa. *Reprod. Biomed. Online*, **16**, (5),649–656(2008). https://doi.org/10.1016/S1472-6483(10)60478-6 8

11. Mocé, E. and Vicente, J. S. Rabbit sperm cryopreservation: A review. *Anim. Reprod. Sci.*, **110** (1–2), 1–24(2009). doi: 10.1016/j.anireprosci.2008.08.015.

12. Aitken, R. J. Free radicals, lipid peroxidation and sperm function. *Reproduction, Fertility, and Development*, **7**(4), 659–668(1995). https://doi.org/10.1071/rd9950659

13. Al-Mutary, M. G. Use of antioxidants to augment semen efficiency during liquid storage and cryopreservation in livestock animals: A review. *J. King Saud Univ. Sci.*, **33**(1),101226(2021). doi: 10.1016/j.jksus.2020.10.023.

14. He, L.; He, T.; Farrar, S.; Ji, L.; Liu, T. and Ma, X. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cellular Physiology and Biochemistry*, **44**(2), 532–553(2017). https://doi.org/10.1159/000485089

15. Amidi, F., Pazhohan, A., Shabani Nashtaei, M., Khodarahmian, M. and Nekoonam, S. The role of antioxidants in sperm freezing: a review. *Cell and Tissue Banking*, **17**(4), 745–756(2016). https://doi.org/10.1007/s10561-016-9566-5

16. Ďuračka, M.; Halenár, M. and Tvrdá, E. In vitro efects of selected biologically active compounds on rabbit spermatozoa motility behaviour. *Journal of Microbiology, Biotechnology and Food Sciences*, **6**(6), 1290–1294(2017). https://doi.org/10.15414/jmbfs.2017.6.6.1290-1294

17. Ozturk, C. and Ataman, M. B. Effect of paclitaxel and resveratrol on New Zealand rabbit semen. *Asian Pacific Journal of Reproduction*, **7**(6), 266–269 (2018). https://doi.org/10.4103/2305-0500.246345

18. Zhu, Z.; Fan, X.; Lv, Y.; Zhang, N.; Fan, C.; Zhang, P. and Zeng, W. Vitamin E analogue improves rabbit sperm quality during the process of cryopreservation through its antioxidative action. *PLoS ONE*, **10**(12), 1–16 (2015). https://doi.org/10.1371/journal.pone.0145383

19. Mohamed, W. R.; Mehany, A. B. M. and Hussein, R. M. Alpha lipoic acid protects against chlorpyrifos-induced toxicity in Wistar rats via modulating the apoptotic pathway. *Environmental Toxicology and Pharmacology*, **59**, 17–23(2018). https://doi.org/10.1016/j.etap.2018.02.007

20. Phillipson, O. T. Management of the aging risk factor for Parkinson's disease. *Neurobiology of Aging*, **35**(4), 847–857(2014). https://doi.org/10.1016/j.neurobiolaging.2013.10.073

21. Shen, T., Jiang, Z. L., Li, C. J., Hu, X. C., and Li, Q. W. Effect of alpha-lipoic acid on boar spermatozoa quality during freezing-thawing. *Zygote (Cambridge, England)*, **24**(2), 259–265(2016). https://doi.org/10.1017/S0967199415000155

22. Ibrahim, S. F.; Osman, K.; Das, S.; Othman, A. M.; Majid, N. A. and Rahman, M. P. A.. A study of the antioxidant effect of alpha lipoic acids on sperm quality. *Clinics*, **63**(4), 545–550(2008a). https://doi.org/10.1590/S1807-59322008000400022

23. Gohar, A.; Khan, H.; Yousaf, M. S.; Ahmad, J.; Ali, Q.; Khan, M.; Khan, D.; Hayat, Y.; Ali, F.; Ahmad, I. and others. Assessment of alpha lipoic acid inclusion in semen extender on cryopreservation of Nili-Ravi buffalo bull spermatozoa. *Life Sci. J.*, **11**(9s), 45–50 (2014).

24. NRC-Nutrient Requirements of Rabbits.pdf. (1977).

25. Viudes-De-Castro, M. P.; Lavara, R.; Safaa, H. M.; Marco-Jiménez, F.; Mehaisen, G. M. K. and Vicente, J. S. Effect of freezing extender composition and male line on semen traits and reproductive performance in rabbits. *Animal*, **8**(5),765–770(2014). doi: 10.1017/S1751731114000135.

26. Ashrafi, I.; Kohram, H. and Ardabili, F. F. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Anim. Reprod. Sci.*, **139**(1–4), 25–30(2013). https://doi.org/10.1016/j.anireprosci.2013.03.016

27. Murugesan, S. and Mahapatra, R. Cryopreservation of Ghagus chicken semen: Effect of cryoprotectants, diluents and thawing temperature. *Reprod. Domest. Anim.*, **55**(8),951–957(2020). doi: 10.1111/rda.13734.

28. Rakha, B. A.; Ansari, M. S.; Hussain, I.; Anwar, M.; Akhter, S.; Raza, S. Q. and Blesbois, E Cryopreservation of Indian Red Jungle Fowl (Gallus gallus murghi) spermatozoa: A first approach. *Avian Biol. Res.*, **10** (1),6–11(2017). doi: 10.3184/175815617X14799886572940.

29. Hamad, S. K.; Elomda, A. M.; Sun, Y.; Li, Y.; Zong, Y.; Chen, J. and Mehaisen, G. M. The In Vitro Evaluation of Rooster Semen Pellets Frozen with Dimethylacetamide. *Animals*, **13**(10),1–14 (2023). doi: 10.3390/ani13101603.

30. Ramalho-Santos, J.; Amaral, A.; Sousa, A. P.; Rodrigues, A. S.; Martins, L.; Baptista, M. and Gamboa, S. Probing the Structure and Function of Mammalian Sperm using Optical and Fluorescence Microscopy. *Mod. Res. Educ. Top. Microsc.*, **351**, (239), 397–402(2007). [Online]. Available: http://www.formatex.org/microscopy3/pdf/pp394402.pdf

31. McCloy, R. A.; Rogers, S.; Caldon, C. E.; Lorca, V.; Castro, A. and Burgess, A. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell Cycle*, **13**(9),1400–1412(2014). doi: 10.4161/cc.28401.

32. Henkel, R.; Menkveld, R.; Kleinhappl, M. and Schill, W. B. Seasonal changes in human sperm chromatin condensation. *J. Assist. Reprod. Genet.*, **18**(7), 371–377 (2001). doi: 10.1023/A:1016618405570.

33. Livak, K. J. and Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method,‖ *Methods*, **25** (4),402–408 (2001). doi: 10.1006/meth.2001.1262.

34. Wieder, R. K. and Lang, G. E. A critique of the analytical methods used in examining decomposition data obtained from litter bags. *Ecology*, **63** (6), 1636–1642 (1982). doi: 10.2307/1940104.

35. SAS/STAT, ―The ANOVA procedure, SAS OnlineDoc, Version 8., p. Chapter 17, Pag. 338-393, (2013). [Online]. Available: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:The+ANOVA+Procedure#0.

36. Karaca, E. G. Lipoik asit: Evrensel antioksidan. Afyon Kocatepe Üniversitesi Fen Ve Mühendislik *Bilimleri Dergisi*, **8**(1), 231–245(2008).

37. Palaniappan, A. R. and Dai, A. Mitochondrial ageing and the beneficial role of alpha-lipoic acid. *Neurochemical Research*, **32**(9), 1552–1558(2007). https://doi.org/10.1007/s11064-007-9355-4

38. Moreira, P. I.; Harris, P. L. R.; Zhu, X.; Santos, M. S.; Oliveira, C. R.; Smith, M. A. and Perry, G. Lipoic acid and N-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer disease patient fibroblasts. *Journal of Alzheimer's Disease*, **12**(2), 195–206(2007). https://doi.org/10.3233/JAD-2007-12210

39. Ma, H.; Quan, F.; Chen, D.; Zheng, Y.; Zhang, B.; Wang, Y. and Zhang, Y. Protective function of alpha-lipoic acid on sperm motility and mitochondrial function during goat sperm-mediated gene transfer. *Small Ruminant Research*, **99**(2–3), 191–198(2011). DOI:10.1016/j.cryobiol.2018.08.006

40. Büyükleblebici, O.; Büyükleblebici, S.; Tuncer, P. B.; Özgürta\cs, T. and Tacsdemir, U. Effect of αLipoic acid supplementation to extender on quality of frozen-thawed bull semen. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. **21**(3),307-311(2015). DOI: 10.9775/kvfd.2014.12366

41. Khairul Osman, K. O.; Siti Fatimah Ibrahim, S. F. I.; Nor Azlina Zakaria, N. A. Z.; Mohd Iswadi Ismail, M. I. I.; Amat Aswadi, A. K. and Zawawi Ismail, Z. I. Effects of alpha lipoic acids on cattle sperm kinetics using computer assisted semen analysis. *Research Journal of Biological Sciences*, **7**(2), 77(2012) .doi 10.3923/rjbsci.2012.73.77

42. Pindaru, L. P. and Groza, I. Ștefan.. Effects of alpha-lipoic acids on sperm membrane integrity during liquid storage of boar semen. Scientific Papers *Animal Science and Biotechnologies*, **48**(1), 162(2015).

43. Fayyaz, M. H.; Ahmad, M. and Ahmad, N. (2017). Survival of buffalo bull spermatozoa: effect on structure and function due to alpha-lipoic acid and cholesterol-loaded cyclodextrin. *Andrologia*, **49**(4), (2017). DOI:10.1111/and.12652

44. Packer, L.; Witt, E. H. and Tritschler, H. J. Alpha-lipoic acid as a biological antioxidant. *Free Radical Biology and Medicine*, **19**(2), 227–225(1995). DOI: 10.1016/0891-5849(95)00017-r

45. Suzuki, Y. J.; Tsuchiya, M. and Packer, L. Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. *Free Radical Research Communications*, **15**(5), 255–263 (1991). doi: 10.3109/10715769109105221

46. González-Marin, C.; Gosálvez, J. and Roy, R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *International Journal of Molecular Sciences*, **13**(11), 14026–14052 (2012). doi: 10.3390/ijms131114026

47. Kasai, T.; Ogawa, K.; Mizuno, K.; Nagai, S.; Uchida, Y.; Ohta, S.; Fujie, M.; Suzuki, K., Hirata, S. and Hoshi, K. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility

potential. *Asian Journal of Andrology*, **4**(2), 97–103 (2002). doi.org/10.1016/j.fertnstert.2011.03.059

48. Chatterjee, S. and Gagnon, C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Molecular Reproduction and Development*, **59**(4), 451–458(2001). https://doi.org/10.1002/mrd.1052
49. Meng, X.; Zhou, J.; Zhao, C. N.; Gan, R. Y. and Li, H. Bin. Health benefits and molecular mechanisms of resveratrol: A narrative review. *Foods*, **9**(3), 1–27 (2020). https://doi.org/10.3390/foods9030340
50. Ibrahim, S. F.; Osman, K.; Das, S.; Othman, A. M.; Majid, N. A. and Rahman, M. P. A. A study of the antioxidant effect of alpha lipoic acids on sperm quality. *Clinics*, **63**, 545–550(2008b). DOI:10.1590/S1807-59322008000400022
51. Rochette, L.; Ghibu, S.; Richard, C.; Zeller, M.; Cottin, Y. and Vergely, C. Direct and indirect antioxidant properties of $α$-lipoic acid and therapeutic potential. *Molecular Nutrition and Food Research*, **57**(1), 114–125(2013). https://doi.org/10.1002/mnfr.201200608
52. Ibrahim, S. F.; Jaffar, F. H. F.; Osman, K. and Syed, S. F. Bull spermatozoa motility: optimization of coenzyme q10 and alpha-lipoic acid concentration. *IIOAB. J.*, **2**, 8–13(2011)..
53. Sun, X.; Zhang, L.; Kang, Y.; Wang, X.; Jiang, C.; Wang, J.; Sohail, T. and Li, Y. Alpha-lipoic acid improves the quality of ram spermatozoa stored at 4°C by reducing oxidative stress and increasing mitochondrial potential. *Frontiers in Veterinary Science*, **10**(January), 1–10(2023). https://doi.org/10.3389/fvets.2023.1345016
54. Najafi, A.; Daghigh Kia, H. and Hamishehkar, H. Does alpha-lipoic acid–loaded nanostructured lipid carriers improve post-thawed sperm quality and ameliorate apoptosis-related genes of rooster sperm? *Poultry Science*, **100**(1), 357–365(2021).. https://doi.org/10.1016/j.psj.2020.10.007
55. Li, J. and Buchner, J. Structure, function and regulation of the Hsp90 machinery. *Biomedical Journal*, **36**(3), 106–117(2013).. https://doi.org/10.4103/2319-4170.113230

جودة الحيوانات المنوية، وقدرتها المضادة للأكسدة، ونمط النسخ، والجينات المرتبطة بمضادات التجمد في السائل المنوي المجمد للأرانب فيما يتعلق بتأثير حمض ألفا ليبويك

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الملخص

هدفت هذه الدراسة إلى تقدير تأثير حمض ألفا ليبويك (ALA) كمضاد للأكسدة بمستويات مختلفة (٠.٢٥، ٠.٥، و١ ملي مول) على الحيوانات المنوية بعد إذابتها، مقارنةً بـ ٢٠٠ ميكرومول من ترولوكس (نظير فيتامين هـ). وشملت المعايير الرئيسية جودة الحيوانات المنوية، وحالة دفاعها ضد مضادات الأكسدة، ونشاط الميتوكوندريا، ومستويات أنواع الأكسجين التفاعلية، وتقييم مستويات التعبير الجيني لبعض الجينات المرتبطة بمضادات الأكسدة والجينات المرتبطة بمضادات التجمد باستخدام تفاعل البوليميراز المتسلسل العكسي الكمي(RT-qPCR) . جُمعت عينات السائل المنوي من خمسة عشر ذكرًا بناءً على حد أدنى للحركة التقدمية بنسبة ٧٠%. أُجريت التقييمات بعد إذابة التجميد باستخدام مُوسِّع TCG، مع التركيز على الحركة والجودة والتعبير المرتبط بمضادات الأكسدة. أظهرت النتائج أن المجموعة التي استخدمت ALA 0.25) ملي مول (كان لها تأثير إيجابي على الحركة التقدمية للحيوانات المنوية بعد إذابتها مع وجود فرق كبير ($p \leq 0.05$) بين المجموعات الأخرى. علاوة على ذلك، فإن مجموعة (ALA 0.25 mmol) قد حسنت بشكل ملحوظ ($p \leq 0.05$) نشاط إنزيمات مضادات الأكسدة بالمقارنة مع جميع المجموعات بينما انخفضت قيمة المالونديالدهيد MDA في (ALA 0.25 mmol) بالمقارنة مع المجموعات الأخرى. أظهرت مجموعة (ALA 0.25 mmol)نشاطًا محتملًا أعلى للغشاء الميتوكوندريا بشكل ملحوظ ($p \leq 0.05$) وتراكمًا أقل لأنواع الأكسجين التفاعلية ومستويات تجزئة الحمض النووي من المجموعات الأخرى، وقد تم تنظيم الجينات المرتبطة بمضادات الأكسدة و HSP90بشكل تصاعدي في الحيوانات المنوية المحفوظة بالتبريد باستخدام مجموعة (ALA 0.25 mmol) بالمقارنة مع المجموعات الأخرى .($p \leq 0.05$) وخلصت هذه الدراسة إلى أن إضافة حمض ألفا ليبويك بتركيز منخفض(٠.٢٥ mmol) في السائل المنوي للذكور يعزز خصائص جودة الحيوانات المنوية ويقلل من أنواع الأكسجين التفاعلية وينظم الجينات المرتبطة بمضادات الأكسدة ومضادات التجمد بشكل تصاعدي بعد التجميد.

الكلمات الدالة: الحفظ بالتجميد، السائل المنوي للأرانب، حمض ألفا ليبويك، نشاط الميتوكوندريا، حركة الحيوانات المنوية.