Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Effect of Exogenous Curcumin on Post-thaw Sperm Parameters, Antioxidant Status, The Expression of Antioxidants and Antifreeze-Related Genes in Rabbits

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Abstract

THIS STUDY aims to investigate the impact of curcumin (CU) supplementation in semen extender on post/thawed rabbit sperm quality, and how curcumin prevents spermatozoa from harm during the preservation process. Semen was diluted with (TCG) extender supplemented with curcumin (CU) at levels of (CU 0, CU 0.25, CU 0.5 , and CU 1 mmol, respectively) compared to (TCG) extender supplemented with 200 µm of Trolox as a positive control, and the following parameters were analyzed: sperm motility, membrane integrity, acrosomal intact, viability, biochemical evaluation of antioxidants indices, mitochondrial activity, (ROS) level, DNA fragmentation, and expression level of selected antioxidant-related genes and antifreeze related gene using RT-qPCR. Our results revealed that groups of (CU 0.5 mmol, CU 0.25 mmol and 200 µm Trolox) have a positive influence on post-thawed sperm progressive motility with no significant difference. Moreover, CU 0.5 mmol group significantly ($p \leq 0.05$) improved antioxidants enzymes values compared to all groups while malondialdehyde MDA reduced in (CU 0.5 mmol, CU 0.25 mmol and 200 µm Trolox groups) in comparison with (control and CU 1mmol) groups. The CU 0.5 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 significantly ($p \leq 0.05$) up-regulated in sperm cryopreserved in CU 0.5 mmol group compared to other groups. This study concluded that supplementation of curcumin with concentration (0.5 mmol) promotes Sperm quality characteristics, reduces ROS, up regulates antioxidants and antifreeze related genes.

Keywords: Curcumin, Cryopreservation, HSP 90, mitochondrial activity, NFE2L2, Rabbit Spermatozoa.

Introduction

Over the past 70 years, animal breeding for artificial insemination has benefited significantly from the application of cryopreserved farm animal semen [1]. Innovations in sperm cryopreservation have made it easier to implement the present genetic breeding procedures. However, they have also led the way in identifying and minimizing the negative effects of cryopreservation, such as oxidative, osmotic, and epigenetic damage [2]. Mammalian spermatozoa have a small, spatulate head. However, the head of rabbit sperm is relatively big, and the dimensions of the ovoid head are about 7 × 4 × 0.5 µm [3], where there is a negative correlation between sperm head

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(Received 15 October 2024, accepted 27 November 2024)
DOI: 10.21608/EJVS.2024.328602.2428

size and the success of vitrification, which subsequently diminishes sperm survival, similar to the effects observed in human sperm [3]. Limited application of artificial insemination in rabbits, due to the low quality and fertility of rabbit semen and small litter sizes. In addition to the increase of oxidative stress of cryopreserved semen, the thawing process affects negatively affects rabbit sperm quality and viability [4].

Previous studies of rabbit semen demonstrate that the average survival rate after post-thawing varied between 10% and 60% for total motility and viability and 5% to 35% for progressive motility [4]. Rabbit sperm's high polyunsaturated fatty acid (PUFA) content and low antioxidant levels inside its cytoplasm make it susceptible to lipid peroxidation during cryopreservation due to the increased formation of reactive oxygen species (ROS) [2]. Due to semen's susceptibility to hypertonic solutions, the processes involved in diluting, freezing, or incubating it present various obstacles, and its vulnerability to oxidative damage, which causes sperm motility and sperm capacitation defects as a result of sperm metabolism's excessive generation of ROS [5-6]. Reactive oxygen species levels grow with rising seminal plasma lactate dehydrogenase (LDH) enzyme levels after cooling [5]. Cold shock also damages acrosomes and plasma membranes, and increases DNA fragmentation [7], this decreases sperm motility and fertility [6].

In general animal sperm and seminal plasma contain intrinsic enzymatic antioxidant systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as non-enzymatic [8]. Unfortunately, the capacity is insufficient and dilution or chilling can impair the effectiveness of antioxidants, reducing the benefits of endogenous antioxidant defense [9]. Antioxidants suppress oxidative processes by scavenging free radicals [8], so the addition of exogenous antioxidants including vitamins E and C, selenium (Se), and zinc (Zn) may aid in male infertility [8]. Vitamin E is commonly used as an effective antioxidant for removing oxidative stress in cryopreserved semen. Supplementation of vitamin E with cryopreservation media prevented oxidative damage to spermatozoa and improved semen quality in different animals [10]. It protects cell membranes from oxidative damage by trapping and scavenging free radicals and appears to have a dose-dependent effect [11].

From the previous studies we have concluded that the best concentration of Trolox (Vitamin E analogue) in rabbit freezing extender is 200 µm, which leads to increased rates of plasma, acrosome, and mitochondrial membrane potential integrity, as well as higher motility in frozen-thawed rabbit sperm [12], so we used it as appositive control to our research. Recently, polyphenols and carotenoids such as resveratrol, quercetin and curcumin have been used as antioxidants in semen cooled storage [13].

Curcumin is a powerful epigenetic regulator acting as a scavenger of reactive oxygen species. In vitro studies indicate that supplemented with curcumin enhances antioxidant enzymes. Additionally, it regulates enzyme levels and restores reactive oxygen species inhibits DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), breakdown of nuclear factor erythroid 2-related factor (Nrf2) and deacetylases (HDACs), as well as regulates micro ribonucleic acids (miRNA) [14]. Curcumin supplementation has been shown to improve the techniques used for the cryopreservation of sperm in bovine species [15].

Many studies concluded that curcumin at various doses protected frozen-thawed sperm characteristics in bulls, rats and rams where it improved sperm acrosome integrity and mitochondrial activity compared to the control group [16]. Therefore, the aim of this study were to examine the effect of curcumin supplementation to rabbit freezing extender media on semen quality (motility, viability), physiological response (acrosome and plasma membrane integrity), antioxidant capacity, mitochondrial membrane activity, ROS accumulation, DNA integrity and the expression of antioxidant and antifreeze related genes post-thawing and compare our results with the best concentration of Trolox (Vitamin A analogue) , which has been proven to be effective in preserving rabbit semen, which was 200 µm.

Material and Methods

Animals and semen collection

In the current investigation, a total of fifteen healthy male rabbits of the New Zealand breed were utilized. The ages of the subjects varied between 6 and 12 months, with an average body weight of 3.5 ± 0.2 kg. The bucks were maintained under uniform management practices and environmental conditions. Each animal was housed individually in standard cages measuring 60 × 40 × 35 cm, within a closed-system farm designed for controlled conditions. The bucks were provided with a basal diet specifically formulated to meet their nutritional needs [17].

The animals underwent training for semen collection utilizing an artificial vagina two weeks prior to the commencement of the study. Ejaculates were obtained from each buck biweekly over a period of ten consecutive weeks. The samples were gathered in sterilized glass tubes and promptly placed in a water bath maintained at 37 ◦C following collection. Sperm motility of the fresh semen was assessed using computer-assisted sperm analysis (CASA; Sperm Vision™ software, version 3.9, Mini tube, Tiefenbach, Germany). The percentage of

progressive motility was assessed, and only those ejaculates exhibiting a minimum of 70% progressive motility were combined to mitigate individual variations for inclusion in the present study. The pooled samples were subsequently diluted at a ratio of 1:1 (v:v) with each type of extender.

Semen Preparation and Cryopreservation/Thawing Procedures

Semen analysis was performed utilizing a TCG (Tris-citric acid-based extender) developed by Viudes-De-Castro [18]. The pH of the freezing extender was meticulously adjusted to 7.00 and subsequently stored at $-20^{\circ C}$ until required. Additionally, Trolox, a Vitamin E analogue, was incorporated at a concentration of 200µM as a positive control. The experimental setup included Curcumin (CU) Brilliant Yellow S; 1,7-Bis (4-hydroxy-3-methoxyphenyl) 1,6-heptadiene-3,5-dione; Tumeric Yellow, Natural Yellow 3, with the chemical name Curcumin Crystalline and CAS number 458-37-7, sourced from India. The final concentrations of CU in the TCG extender were established at 0, 0.25 mmol, 0.50 mmol, and 1 mmol.

Following the preparation, the extended semen was equilibrated for 2 hours at $5^{\circ C}$. Immediately after equilibration, the diluted semen was transferred into 0.25 mL straws. These straws were positioned horizontally 5 cm above the liquid nitrogen (LN_2) surface for a duration of 15 minutes before being immersed in LN_2. Thawing of the frozen straws was conducted at $37^{\circ C}$ for 30 seconds.

Characteristics of sperm motility

Computer-assisted sperm analysis (CASA) was employed to evaluate sperm motility following thawing by applying a drop of semen onto a glass slide that had been pre-warmed to 37 $^{\circ C}$. Approximately 2500 cells from each sample were randomly selected and analyzed using the CASA system, as reported by Ashrafi [19].

Sperm Quality Attributes

Viability

The viability of sperm in each treatment group was assessed following the methodology outlined by Murugesan and Mahapatra, with minor adjustments. Specifically, the proportion of viable spermatozoa was assessed by enumerating around 200 spermatozoa in multiple microscopic fields on each slide [20].

Integrity of the Acrosome

Giemsa staining was employed in this research to evaluate the condition of the acrosome, adhering to the protocol established by Rakha *et al*. [21] with slight adjustments made to the original methodology.

Condition of the Plasma Membrane

The approach outlined by Rakha *et al.* [21] involved the utilization of the Hypoosmotic Swelling Test (HOST) to evaluate the integrity of the membrane surrounding the sperm, with a minor modification applied. The proportion of spermatozoa exhibiting intact plasma membranes, characterized by either swollen or coiled tails, serves as a critical parameter for identifying optimal plasma membrane functionality [21].

Semen Antioxidant Indicators

Three replicates were obtained for each treatment group by thawing two straws of frozen sperm per replicate, as outlined in the previously mentioned methodologies [22]. The total protein (TP) content in the samples was initially assessed using the biuret reaction method, adhering to the instructions provided by the manufacturer of the colorimetric assay kit (TP-2020, BioDiagnostic, Inc., Egypt). Subsequently, the levels of total antioxidant capacity (TAC), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and Malondialdehyde (MDA) were quantified. Standard curves were employed to evaluate the sensitivity and accuracy of the assays, in accordance with the manufacturer's guidelines, and all calculations for each assay were normalized to milligram of protein. Data for all analyses were collected using an automated scanning spectrophotometer (CE1010, Cecil Instruments Limited, Cambridge, United Kingdom).

Mitochondrial Membrane Functionality

Ramalho-Santos *et al.* [23] reported, with minor modifications, the utilization of Mitotracker Red FM (Molecular Probes, Eugene, Oregon, USA), a vital dye specific to mitochondria that identifies active mitochondria based on their membrane potential, to assess mitochondrial activity in sperm. The staining procedure adhered to the manufacturer's guidelines (M22425 for Mitotracker Red, Thermo Fisher Scientific, Waltham, MA, USA), involving the incubation of live sperm ($1\times10^{\wedge 6}$ sperm/ml) in the dark for 30 minutes at 37 °C with 1 µL of Mitotracker Red. Following this, samples were washed three times with 1× phosphate-buffered saline (PBS, Sigma-Aldrich). The prepared samples were then placed on a microscope slide and analyzed using confocal imaging with a laser scanning confocal microscope, specifically the Zeiss LSM 880, equipped with the super-resolution (SR) Airyscan system from Zeiss (Jena, Germany). The filter sets employed included a 488 nm filter with a bandpass of 491-632 nm (laser HeNe1), and imaging was conducted using the Plane-Apochromat 63×1.4 Oli DIC M27 objective.

Intracellular ROS Measurement

According to McCloy *et al*. [24] conducted measurements of intracellular reactive oxygen

species (ROS) levels in sperm using a dichlorofluorescein (DCF) assay, employing the 2,7-dichlorodihydrofluorescein diacetate (DCFH2-DA) probe. A concentration of 13 µ2-DA (Sigma-Aldrich) was introduced to a 150-µL semen sample, the sample was then placed in a dark environment for a 30 minutes at a temperature of 37 $^{\circ C}$. The specificity of the fluorescent labeling for ROS levels was confirmed through confocal imaging, utilizing a Zeiss LSM 880 laser scanning confocal microscope and the super-resolution (SR) Airyscan system from Zeiss (Jena, Germany), following three washes with phosphate-buffered saline (PBS, Sigma-Aldrich).

Quantification of fluorescence levels

To assess fluorescence levels indicative of mitochondrial activity and reactive oxygen species (ROS) accumulation in oocytes, Image J software (version 1.50i, NIH, USA, http://imagej.nih.gov/ij) was employed. For each sperm sample, mean fluorescence and background measurements were obtained. Fluorescence signals were quantified from a minimum of 200 individual sperm within each group. The total corrected fluorescence (TCF) was determined following established methodologies. [25].

DNA Fragmentation

According to Henkel *et al.* [25], a volume of twenty microliters of thawed semen was placed on glass slides and permitted to dry. Following a 15-minute exposure to a fixative buffer, the slides were then submerged in an aniline blue staining solution for three minutes. After the staining process, the slides underwent a rinsing process using distilled water and allowed to air dry. The assessment of stained sperm heads was conducted by analyzing 200 spermatozoa, which were categorized based on staining intensity: spermatozoa that displayed strong to very strong aniline blue staining were identified as having fragmented DNA, whereas those with weak or absent staining were classified as possessing non-fragmented DNA.

RNA isolation, cDNA synthesis and (qRT-PCR) for antioxidant and antifreeze related genes

Three biological replicates from both the negative and positive control groups, as well as each CU treatment group, were retrieved from the liquid nitrogen storage and allowed to thaw at room temperature. The samples were subsequently transferred to 1.5 ml Eppendorf tubes and centrifuged for 12 minutes at 680 rpm and 4 ˚C. RNA extraction was conducted following the TRIZOL RNA Isolation Protocol (Yale University, U.S.A) in accordance with the manufacturer's guidelines. Genomic DNA digestion was performed by incorporating DNase I, as recommended by the RNase-free kit (Thermo Scientific, California, USA). The quality and quantity of the RNA were evaluated using the NanoDrop 2000c (Thermo Fisher Scientific Inc., USA). cDNA synthesis was carried out using a reverse transcription Kit (Life Technologies Corporation, California, USA), which included MultiScribe™ reverse transcriptase and random primers. Gene-specific primers were designed based on sequences available in the GenBank database (www.ncbi.nlm.nih.gov) utilizing Primer3 software (http://primer3.wi.mit.edu//), as detailed in Table 1. The real-time PCR was performed according to the Two-step Quantitative Real-Time PCR Protocol (Applied Biosystems, California, USA). The reaction mixture comprised 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 as follows: an initial cycle of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. The comparative cycle threshold (CT) method was employed to quantify fold changes ($2^{-\Delta\Delta CT}$) in gene expression data analysis, normalizing the target transcripts (CAT, GPX1, PRDX1, SOD1, NFE2L2, and HSP90) against the housekeeping gene (GAPDH). Prior to the gene expression analysis, the stability of the housekeeping gene was confirmed to be consistent [26].

Statistical Analysis

Statistical analysis of the data was conducted in accordance with the methodology outlined by Wieder and Lang [27], utilizing SAS software [28]. The impact of varying CU concentrations (negative control, CU 0.25 mmol, CU 0.5 mmol, CU 1 mmol, and positive control 200 µM Trolox, on CASA parameters, sperm viability, acrosome integrity, plasma membrane integrity, DNA fragmentation, mitochondrial activity, (ROS) levels, and antioxidant enzyme biomarkers were assessed. Data were log-transformed and subjected to one-way ANOVA, followed by Tukey's post-hoc test, with a significance threshold set at $\alpha = 0.05$. Additionally, gene expression data were evaluated using the SAS General Linear Model (GLM). Mean differences among treatment groups were analyzed using ANOVA, followed by the Student t-test, with significance determined at ($p \leq 0.05$).

Results

Characteristics of sperm motility

The data presented in Table 2 indicate that the total motility percentage of frozen/thawed sperm exhibited an increase of approximately 4% ($p \leq 0.05$) in the CU 0.25 mmol and CU 0.5 mmol groups compared to the control and CU 1 mmol groups. However, no significant differences were observed among the CU 0.25 mmol, CU 0.5 mmol, and 200 µm Trolox groups. Notably, the CU 0.25 mmol, CU 0.5 mmol, and 200 µm Trolox groups demonstrated a beneficial effect on progressive motility in post

frozen-thawed semen, which rose by roughly 10 to 11% ($p \leq 0.05$) relative to the control and CU 1 mmol groups. In terms of distance average path (DAP), the control group showed an increase of about 1 to 2% compared to the other CU groups, although this difference was not statistically significant when compared to the 200 µm Trolox group. The distance curved line (DCL) in the control group increased significantly ($p \leq 0.05$) by approximately 4 to 5% ($p \leq 0.05$) in comparison to the other groups. Similarly, the distance straight line (DSL) in the control group increased significantly ($p \leq 0.05$) by about 1 to 2% compared to the other CU groups, yet no significant difference was found with the 200 µm Trolox group. The velocity average path (VAP) showed significant differences ($p \leq 0.05$) between CU 0.25 group compared to control, while no significant differences among 200 µm Trolox, CU 1 mmol, and CU 0.5 mmol groups compared to control group. The velocity curved line (VCL) increased sigmificantly ($p \leq 0.05$) by approximately 10 to 11% in the control group relative to the other groups, Velocity straight line (VSL) exhibited no significant differences ($p \leq 0.05$) between the control, 200 µm Trolox, and CU 1 mmol groups, while there is a significant ($p \leq 0.05$) differences between CU 0.5 mmol and CU 0.25 mmol groups compared to control. Lastly, the post-thaw sperm straightness (STR) increased significantly ($p \leq 0.05$) by approximately 0.02 to 0.03% in the CU 1 mmol group compared to the other treatment groups. In comparison, the linearity of sperm (LIN) exhibited an increase significantly ($p \leq 0.05$) of approximately 0.03 to 0.04% in CU 1 mmol group relative to all other groups. No significant differences were observed among the CU 0.25 , CU 0.5 and CU 1 mmol groups concerning the wobble (WOB), but it increased significantly ($p \leq 0.05$) of approximately 0.03% in 200 µm Trolox compared to control group. Additionally, the amplitude of lateral head displacement (ALH) showed a significant increase ($P < 0.05$) in both the control and CU 1 mmol groups, with an enhancement of about 0.30 to 0.45% compared to CU o.25 and CU 0.5 groups. Furthermore, the beat cross frequency (BCF) also demonstrated a significant ($p \leq 0.05$) rise in control group and 200 µm Trolox group, with increases ranging from approximately 0.5 to 1% when compared to all other groups.

Sperm Quality Attributes

Viability, Integrity of the Acrosome and the state of the Plasma Membrane

As indicated in Table 3, the group treated with CU at a concentration of 0.5 mmol exhibited superior ($p \leq 0.05$) sperm quality parameters relative to the other experimental groups. Specifically, sperm viability improved by over 16%, while the integrity of the acrosome and plasma membrane both increased by more than 13% when compared to the control group. Furthermore, a decline in sperm viability, acrosome integrity, and plasma membrane integrity was observed upon freezing the semen in the CU 1 mmol group, with reductions of approximately 9%, 13%, and 15% respectively ($p \leq 0.05$) in comparison to the CU 0.5 mmol group.

Mitochondrial Membrane Functionality

The mitochondrial membrane potential of rabbit sperm, serving as a marker for mitochondrial activity, was assessed utilizing MitoTracker1-Red. The group treated with CU at a concentration of 0.5 mmol demonstrated a significantly elevated mitochondrial potential activity, indicated by higher fluorescence intensity ($p \leq 0.05$), in comparison to the other treatment groups (Figure 1, A). Conversely, the CU 1 mmol group displayed the lowest fluorescence intensity among all groups analyzed.

Intracellular ROS Measurement

The level of (ROS) accumulation was assessed in one hundred sperm samples from each experimental group. The group treated with CU 1 mmol exhibited the highest ROS accumulation, indicated by a significant ($p \leq 0.05$) increase in fluorescence intensity. Conversely, the group treated with CU 0.5 mmol demonstrated significantly ($p \leq 0.05$) the lowest ROS accumulation, as evidenced by a reduced intensity of the fluorescent dye (Figure 1, B).

DNA Fragmentation

DNA fragmentation was assessed in one hundred sperm samples from each experimental group. The CU 1 mmol group exhibited the highest ($p \leq 0.05$) degree of DNA fragmentation, exceeding 20%. In contrast, the CU 0.5 mmol group demonstrated the minimal degrees of DNA fragmentation approximately 10%, followed by the 200 µm Trolox and CU 0.25 mmol groups, both of which also showed significant ($p \leq 0.05$) differences (Figure 2).

Semen Antioxidant Indicators

The highest values for (TAC) and (GPx) were observed in the CU 0.5 mmol group, and reached 0.117 µM/mg and 0.007 mU/mg, respectively. This was followed by the 200 µM Trolox group, as well as the CU 0.25 and CU 1 mmol groups, with statistically significant differences ($p \leq 0.05$). In contrast, the control group exhibited the lowest values, recorded at 0.007 µM/mg for TAC and 0.002 mU/mg for GPx. Furthermore, the CU 0.5 mmol treatment significantly enhanced the activities of (SOD) and (CAT), yielding values of 7.565 u/mg and 0.009 u/mg, respectively, when compared to the other groups. The 200 µM Trolox and CU 0.25 mmol groups also showed improved activity ($p \leq 0.05$), while the control and CU 1 mmol groups demonstrated significantly ($p \leq 0.05$) the lowest CAT activity at 0.003 u/mg. The control group also recorded the minimum SOD value at 5.125 u/mg.

Notably, the inclusion of CU at concentrations of 0.5 and 0.25 mmol in the extender resulted in the lowest (MDA) levels ($p \leq 0.05$), measuring 0.170 nM/mg and 0.205 nM/mg, respectively, similar to the 200 µM Trolox group, which had an MDA level of 0.231 nM/mg, in comparison to the other groups ($p \leq 0.05$) (Table 4).

Antioxidants and Anti-Freeze Related Genes Expression

Figure 3 presents the results concerning the expression of antioxidant and anti-freeze-related genes in rabbit sperm subjected to cryopreservation with CU. The data indicate that the genes CAT, SOD1, GPX1, and NFE2L2 exhibited significant ($p \leq 0.05$) up regulation in the CU 0.5 mmol treatment group when compared to the other experimental groups. Notably, the PRDX1 gene also showed significant ($p \leq 0.05$) upregulation in the CU 0.5 mmol and 200 µm Trolox treatments relative to the other conditions. Additionally, the CU 0.5 mmol group demonstrated a significant ($p \leq 0.05$) increase in HSP90 expression compared to the other groups. Conversely, the CU 1 mmol treatment resulted in a significant ($p \leq 0.05$) downregulation of all genes analyzed, with the exception of SOD1.

Discussion

Sperm cryopreservation is a technique that allows for the preservation of male gametes, thereby facilitating the maintenance of fertility. It is widely recognized that these preservation methods can lead to a decline in sperm quality, ultimately reducing the potential for fertilization. A significant biological challenge associated with sperm cryopreservation is the disruption of normal cellular functions. Exposure to low temperatures can inflict irreversible damage on sperm cells, which in turn diminishes both motility and reproductive capability [29].

The effectiveness of sperm freezing is influenced by the intrinsic characteristics of the sperm as well as extrinsic factors, including the formulation of the diluent, the type and concentration of cryoprotectants, dilution rates, and the methodologies employed during cooling, equilibration, freezing, and thawing [30].Cryoprotectants, along with suitable freezing and thawing protocols, serve to shield sperm from dehydration, elevated salt concentrations, and thermal shock, thereby preserving the integrity of the cell membrane and optimizing the osmolarity of the extracellular fluid. Nevertheless, the mere presence of cryoprotectants does not fully mitigate the stress induced by the freezing process, which can lead to a loss of membrane integrity, mitochondrial impairment, alterations in metabolic and functional states, lipid peroxidation, increased levels of reactive oxygen species (ROS) in the cytoplasm, and DNA damage. It is important to note, however, that low levels of ROS are essential for sperm maturation, as they play a critical role in processes such as capacitation, the acrosomal reaction, mitochondrial stability, and motility [31].

The imbalance between (ROS) and antioxidant defenses can lead to oxidative stress. Human sperm exhibit a high susceptibility to the toxicity of free oxygen radicals, primarily resulting in lipid peroxidation. Among the most detrimental products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which can cause significant protein dysfunction and DNA damage. Additionally, these compounds can attract leukocytes through their chemotactic properties and inhibit cellular proliferation. Due to the limited effectiveness of intracellular gene repair mechanisms in sperm, the primary protective measures involve the compact organization of the genome and the presence of antioxidants in the male reproductive tract and seminal plasma. [32].

Nrf2 plays a crucial role in combating oxidative stress by promoting the expression of antioxidant proteins and phase II detoxification enzymes, as well as genes responsible for encoding catalase (Cat), superoxide dismutase, glutathione S-transferase (GST), and haem oxygenase-1 [33]. Natural and nonenzymatic antioxidants are vital for the protection of male gametes, as they can mitigate DNA damage both in vivo and in vitro. The therapeutic potential of antioxidants is significant, as they reduce reactive oxygen species (ROS) and oxidative stress, thereby enhancing reproductive capabilities in both natural and assisted reproductive technology (ART) contexts; they also provide protective effects during embryonic development. [34]. In light of curcumin's antioxidant properties and its ability to enhance semen quality by reducing oxidative stress[35] , we examined the effects of curcumin supplementation at doses of 0.25, 0.5, and 1 mmol in freezing media for frozen-thawed rabbit sperm cells. This was compared to a control group using TCG freezing extender media without any supplements and a positive control group with 200 µm of Trolox.

Our findings indicate that among the concentrations evaluated, the CU 0.5 mmol group significantly ($p \leq 0.05$) improved sperm motility criteria, as detailed in Table 2. This group also demonstrated enhanced acrosome integrity and plasma membrane integrity, alongside a reduction in both dead sperm and abnormalities. The CU 0.25 mmol group exhibited similar effects, comparable to the 200 µm Trolox group when contrasted with the control and CU 1 mmol groups, as illustrated in Table 3. However, it is noteworthy that no significant differences were observed in TMOT and PROG motility among the CU 0.5, CU 0.25 mmol, and 200 µm Trolox groups, as evidenced in Table 2. These findings align with previous research indicating that high doses of curcumin and curcumin nano-emulsion significantly enhance sperm quality, including living sperm count, individual, and progressive motility,

while simultaneously reducing the presence of dead and malformed sperm in rats subjected to a protein-deficient diet [36]. The authors of that study proposed that curcumin exerts an antioxidative effect by mitigating lipid and protein oxidation within the sperm cell membrane. Furthermore, Alizadeh et al. [37] reported that curcumin's antioxidant properties can enhance sperm quality in asthenozoospermic males by decreasing oxidative stress levels. Consistent with our results, earlier studies have shown that the incorporation of curcumin into Tris-yolk fructose extender improves sperm viability and reduces necrotic and apoptotic sperm in frozen/thawed semen [37].

Our research indicated a reduction in viable spermatozoa, with the highest proportion of compromised acrosome and plasma membrane observed at a concentration of 1 mmol CU, as illustrated in Table 3. This decline is attributed to plasma membrane damage resulting from the adverse effects of the freezing process. This finding consistent with previous studies, such as that by NAZ [38], which demonstrated that high doses of curcumin adversely affected motility, capacitation, and acrosome reaction in both human and mouse sperm in a concentration-dependent manner. Additional research has confirmed that curcumin can exhibit detrimental effects at elevated concentrations (1 mM) [39], which is consistent with our observations at the 1 mmol CU concentration.

In this investigation, we assessed the antioxidant properties of curcumin within a rabbit freezing medium to mitigate the accumulation of reactive oxygen species (ROS) in sperm due to cryopreservation, which is associated with DNA fragmentation. The integrity of sperm DNA is crucial for successful fertilization and subsequent embryo development. Numerous studies [40] have linked exposure to low temperatures in sperm to oxidative DNA damage, as well as to mitochondrial membrane potential activity. The status of mitochondria is critical for sperm fertility, as it influences energy levels and motility, an inverse relationship exists between mitochondrial membrane potential (MMP) and ROS levels in frozen-thawed spermatozoa [41]. Mitochondria serve as the primary source of intracellular ROS production, and excessive ROS can disrupt the coupling of electron transport and oxidative phosphorylation in sperm, leading to a reduction in the number of sperm with normal mitochondrial function and diminished motility [41]. Our findings demonstrated that a concentration of 0.5 mmol CU was adequate to elicit beneficial effects on rabbit sperm cells following thawing, enhancing sperm parameters, reducing oxidative damage, and preserving sperm DNA integrity by decreasing ROS production and increasing mitochondrial membrane potential activity, as depicted in Fig. 1 and 2.

The findings align with those of Soleimanzadeh and Saberivand [42], who determined that curcumin therapy could safeguard the antioxidant equilibrium and significantly diminish DNA fragmentation. Additionally, it has been shown to offer protection against DNA damage, facilitate appropriate development of testicular tissue [36], and avert the death of spermatogenic cells [39]. In contrast, extender solutions diluted with 1 mmol of curcumin exhibited elevated levels of fragmented DNA. This phenomenon may be attributed to the high concentrations of curcumin, which could elevate reactive oxygen species (ROS) levels, thereby impairing sperm motility through membrane damage, inducing apoptosis, and reducing mitochondrial membrane potential [42].

The present investigation demonstrates a notable significant ($p \leq 0.05$) increase in the activities of TAC, CAT, GPx, and SOD in the CU 0.5 mmol group when compared to all other groups. Conversely, the levels of MDA were significantly ($p < 0.05$) reduced in the CU 0.5 mmol, CU 0.25 mmol, and 200 µm Trolox groups relative to the control and CU 1 mmol groups, as illustrated in Table 4. This heightened activity of antioxidant enzymes such as CAT, SOD, and GPx may influence the response to the altered MDA concentrations, which are implicated in cellular damage and the acceleration of apoptosis. Such processes typically compromise cell membranes and DNA integrity, ultimately leading to cell death and adversely affecting sperm motility by protecting sperm from the detrimental effects of (ROS) and lipid peroxidation. This finding agrees with the work of Ogbuewu *et al*. [43], who proposed that curcumin enhances the seminal plasma's capacity to mitigate oxidative stress (OS) and boosts the activity of protective enzymes like SOD and GSH in seminal plasma by preserving mitochondrial redox signaling and respiratory functions. Furthermore, curcumin may alleviate OS damage by down-regulating H_2O_2 levels, thereby safeguarding spermatozoa from inflammatory agents and contributing to the preservation of sperm quality [4].

In this investigation, we examined the expression of genes associated with antioxidant activity, specifically NFE2L2, CAT, SOD1, GPX1, and PRDX1, alongside the antifreeze-related gene HSP90. The results indicated that treatment with 0.5 mmol of CU significantly ($p \leq 0.05$) upregulated all antioxidant-related genes—CAT, SOD1, GPX1, and NFE2L2—when compared to the control, 200 µm Trolox, 0.25 mmol CU, and 1 mmol CU. Notably, the PRDX1 gene exhibited significant ($p \leq 0.05$) upregulation in response to both the 0.5 mmol CU and 200 µm Trolox treatments relative to the control, 0.25 mmol CU, and 1 mmol CU. Additionally, the 0.5 mmol CU treatment significantly enhanced ($p < 0.05$) the expression of the antifreeze-related gene HSP90 compared to the control, 200 µm Trolox, 0.25 mmol

CU, and 1 mmol CU. Conversely, the 1 mmol CU treatment resulted in significant downregulation of all genes except for SOD1 when compared to the control, as illustrated in Figure 3. Numerous studies have highlighted curcumin's role as a potent epigenetic regulator, inhibiting deoxyribonucleic acid (DNA), DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), and deacetylases (HDACs), while also modulating micro ribonucleic acids (miRNA) [44]. This evidence implies that curcumin not only shields sperm from reactive oxygen species (ROS) but also functions at the molecular level to enhance the innate defenses of the cells.

The findings of our study support the theories suggesting that the antioxidant properties of curcumin, which include phenolic, β-diketone, and methoxy groups, can positively influence sperm parameters when administered in specific amounts. Curcumin appears to disrupt the keap1-Nrf2/NFE2L2 complex, thereby stabilizing Nrf2/NFE2L2 and facilitating its translocation to the cell nuclei. This process subsequently promotes the transcription of various antioxidant genes associated with the antioxidant response, including (CAT), (SOD), (GST), (PRDX1), (GPx1), and haem oxygenase-1 [45]. In contrast, Zhou *et al*. [39] demonstrated that curcumin may improve asthenozoospermia by inhibiting the production of (ROS) and modulating Nrf2 levels. They also noted that curcumin can enhance sperm motility in a manner that is not dependent on dosage, although excessive concentrations may lead to toxicity affecting sperm motility. Specifically, a concentration of 100 nM curcumin was found to significantly enhance sperm motility by reducing ROS levels and apoptosis, whereas higher concentrations of 1 mM and 1 M resulted in decreased total and progressive motility compared to the control group [39].

Curcumin exhibits notable antioxidant properties, which can positively influence sperm parameters at certain concentrations. Additionally, curcumin has the ability to disrupt the keap1-Nrf2 complex, leading to the stabilization of Nrf2, which subsequently translocates to the cell nucleus [46]. This translocation initiates the transcription of various antioxidant genes that play a crucial role in the cellular antioxidant response. Furthermore, curcumin may impact sperm motility, capacitation, and overall functionality by inhibiting the tyrosine phosphorylation of surface proteins and calcium channels, lowering the intracellular pH of sperm, and inducing hyperpolarization of the sperm cell membrane [47].

HSP90, classified within the heat shock protein family, is implicated in the regulation of the development and functionality of the reproductive system. It has emerged as a significant predictive biomarker for male fertility and is involved in various cellular processes [48]. Additionally, research by Wang *et al*. [49] highlighted the presence of HSP90 in the sperm tail, indicating its role in sperm fertility.

In the present investigation, it was observed that sperm motility, plasma membrane integrity, and HSP90 expression were significantly enhanced following the freezing/thawing process at a concentration of CU 0.5 mmol, as illustrated in Table 2 and Figure 3. These findings imply that the upregulation of HSP90 expression may contribute to improved plasma membrane integrity, a reduction in reactive oxygen species (ROS) accumulation, enhanced mitochondrial membrane functionality, and increased activity of antioxidant enzymes in seminal plasma, ultimately leading to improved sperm motility after thawing. This agrees with previous studies that have indicated the protective role of HSP90, in conjunction with HSP70, against oxidative stress and its ability to inhibit apoptosis [50]. Conversely, research by Benko *et al.* [51] revealed that cryopreservation resulted in decreased sperm motility and lower HSP90 gene expression in bull spermatozoa when compared to fresh samples. Additionally, in chicken sperm, HSP90 expression was found to be greater in fresh samples than in those that had undergone freezing and thawing [52]. These observations underscore the potential of curcumin supplementation in rabbit freezing media to enhance HSP90 expression, thereby improving various sperm parameters in rabbits.

In this investigation, we aimed to assess the antioxidant properties of a freezing medium enriched with low concentrations of curcumin to mitigate oxidative damage to sperm resulting from cryopreservation. Our findings indicated that the only concentration that yielded a beneficial effect on rabbit sperm cells post-thawing was 0.5 mmol. The addition of 0.5 mmol CU to the sperm freezing medium can enhance sperm parameters, diminish oxidative damage, and preserve sperm DNA integrity by decreasing reactive oxygen species (ROS) production and modulating the expression of certain antioxidant-related genes.

These results hold significant implications for enhancing the efficacy of sperm cryopreservation, particularly for individuals facing infertility or subfertility, as rabbits serve as a suitable model for human reproductive studies. Furthermore, this research may contribute to advancements in artificial insemination techniques, especially in livestock species experiencing reproductive challenges, such as buffaloes. It suggests that antioxidants, particularly curcumin, may play a crucial role in restoring reproductive function in individuals affected by oxidative stress-related impairments.

Conclusion

The addition of curcumin at a lower concentration (0.5 mmol) to freezing extender media

has been demonstrated to improve the post-thaw quality of rabbit sperm. This enhancement positively affects various parameters, such as progressive motility, viability, plasma membrane integrity, acrosome integrity, and sperm DNA fragmentation. The protective mechanism at play involves the preservation of adequate levels of antioxidant-related transcripts and the antifreeze-related transcript HSP90, which together reduce the accumulation of reactive oxygen species (ROS) and improve mitochondrial function, This ultimately results in improved post-thaw viability and motility of cryopreserved spermatozoa, which is essential for the advancement of assisted reproductive technologies (ART) in rabbits and other livestock species, thereby supporting the conservation of genetic resources in the face of challenges such as climate change and declining fertility rates in farm animals.

Acknowledgments

Not applicable.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

The protocols adopted in this study have been approved and accomplished following the Institutional Animal Care and Use Committee (CU-IACUC) policies at Cairo University (Approval no. CU-II-F-41-23).

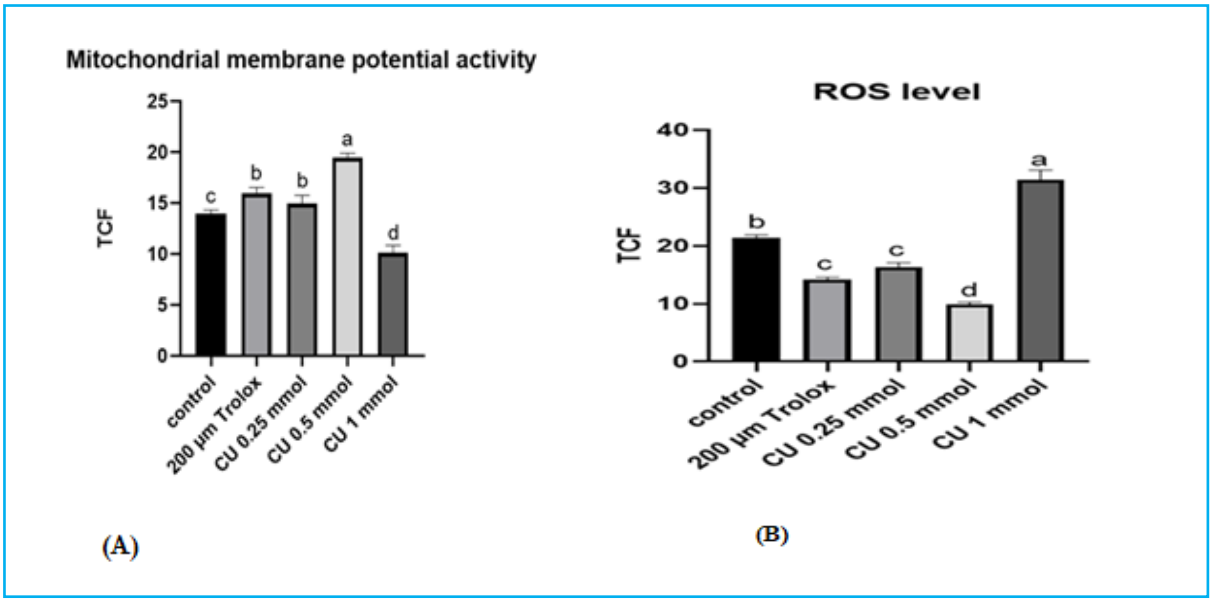

Fig. 1. Illustrate (A) Total corrected fluorescence (TCF) levels of Mitochondrial membrane potential activity (MitoTracker1-Red stain) and (B) Total corrected fluorescence (TCF) levels of ROS levels (H2DCFDA stain) of rabbit freezing sperm after thawing with extender supplemented of 0 (control),200 µm Trolox, 0.25 mmol, 0.5 mmol and 1 mmol of curcumin. $^{a, b, c, d}$ values with different letters are significantly different ($p \leq 0.05$).

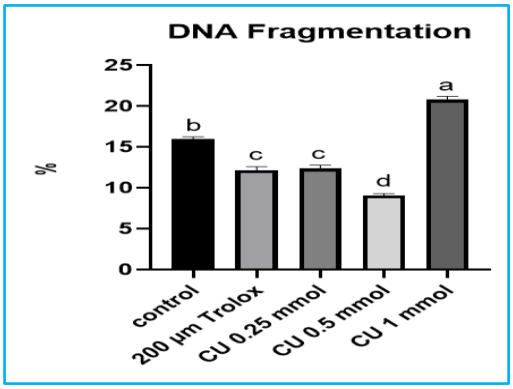

Fig. 2. Illustrate DNA fragmentation of rabbit freezing sperm post-thawing with extender supplemented of 0 (negative control), 200 µm Trolox (positive control), 0.25 mmol, 0.5 mmol and 1 mmol of curcumin, $^{a, b, c, d}$ values with different letters are significantly different ($p \leq 0.05$).

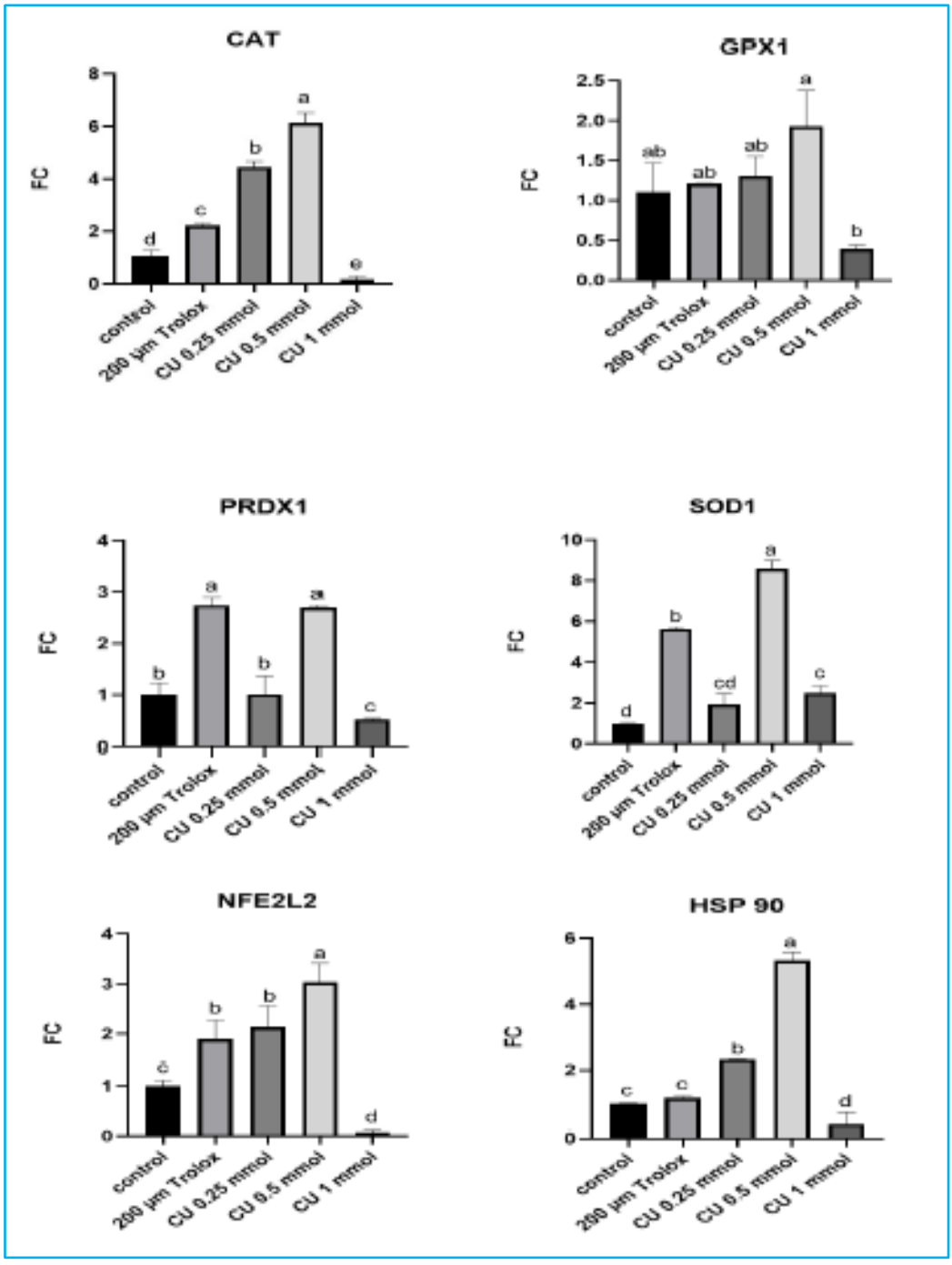

Fig. 3. The impact of different concentrations of curcumin compared to 200 µm Trolox E in freezing extender on the antioxidants and antifreeze associated gene expression of rabbit sperm post-thawing, (FC: fold change) ± standard error (SE). [a,b,c,d,e] Means with different superscripts, within the same gene, are significantly different ($p \leq 0.05$), antioxidants related genes: (CAT: catalase, GPX1: glutathione peroxidase 1, PRDX1: peroxiredoxin 1, SOD1: superoxide dismutase 1 and NFE2L2: nuclear factor erythroid 2-related factor 2(NRF2) and antifreeze related gene: (HSP 90: heat shock protein 90), respectively.

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′ R: 5′- CCTCCACAATGCCGAAGT-3′	129
CAT	XM_002709045.4	F: 5′- GACAAAATGCTTCAGGGCCG-3′ R: 5′- TAGTAATTGGGAGCACCGCC-3′	173
SOD1	NM_001082627.2	F: 5′- GCCGCTGCGGAGTCAT-3′ R: 5′- CTGCACTCGTACAGCCTTGT-3′	195
NFE2L2	XM_008258785.3	F: 5′- ACTACCACGGTTCCAAGTGC-3′ R: 5′- TGTGGCATTTGAGTTCACGC-3′	192
PRDX1	XM_002715138.4	F: 5′- TCACCTTTGTGTGCCCTACG-3′ R: 5′- TGAGAATCCACGGAAGCACC-3′	100
GPX1	NM_001085444.1	F: 5′- AGTTTGGGCATCAGGAGAACG-3′ R: 5′- CCTCCAGGCCGGACATACTT-3′	70
Hsp90	XM_051834811.1	F: 5′- AGACCCAGACCCAAGACCAG-3′ R: 5′- ATCTTGTCCAGAGCATCCGAC-3′	166

TABLE 2. Spermatic parameters of Rabbit semen as affected by three levels of curcumin and 200 µm of Trolox (vitamin E analogue) as positive control post-thaw.

Traits	Control	200 µm Trolox (vitamin E analogue)	CU 0.25 mmol	CU 0.5 mmol	CU 1mmol
TMOT (%)	68.62±0.91 ^{b}	71.55± 0.89 ^{ab}	73.27±0.99 ^{a}	73.56±0.87 ^{a}	69.73±1.50 ^{b}
PROG (%)	34.20±1.38 ^{b}	44.87±1.34 ^{a}	48.05±1.40 ^{a}	49.56±1.33 ^{a}	35.36±1.34 ^{b}
DAP (um)	19.85±0.40 ^{a}	18.87±0.39 ^{ab}	17.78±0.41 ^{b}	18.30±0.39 ^{b}	18.01±0.66 ^{b}
DCL (um)	33.60±0.87 ^{a}	30.68±0.84 ^{b}	28.68±0.88 ^{b}	29.36±0.83 ^{b}	27.95±1.43 ^{b}
DSL (um)	15.08±0.32 ^{a}	14.19±0.31 ^{ab}	13.15±0.33 ^{b}	13.69±0.31 ^{b}	13.87±0.54 ^{b}
VAP (um/s)	47.82±0.95 ^{a}	45.88±0.91 ^{ab}	43.26±0.96 ^{b}	44.63±0.91 ^{ab}	44.76±1.5 ^{ab}
VCL (um/s)	79.77±1.92 ^{a}	73.59±1.86 ^{b}	68.86±1.95 ^{c}	70.76±1.84 ^{bc}	68.48±3.16 ^{c}
VSL (um/s)	36.51±0.76 ^{a}	34.75±0.73 ^{ab}	32.25±0.77 ^{b}	33.58±0.73 ^{b}	34.83±1.25 ^{ab}
STR (%)	0.75±0.005 ^{ab}	0.75±0.004 ^{b}	0.74±0.005 ^{b}	0.74±0.004 ^{b}	0.77±0.008 ^{a}
LIN (%)	0.47±0.006 ^{b}	0.47±0.006 ^{b}	0.46±0.006 ^{b}	0.47±0.006 ^{b}	0.50±0.011 ^{a}
WOB (VAP/VCL)	0.61±0.006 ^{b}	0.64±0.006 ^{a}	0.62±0.006 ^{ab}	0.62±0.006 ^{ab}	0.63±0.010 ^{ab}
ALH (um)	5.72±0.12 ^{a}	5.40±0.12 ^{ab}	5.29±0.12 ^{b}	5.26±0.11 ^{b}	5.73±0.20 ^{a}
BCF	13.71±0.36 ^{a}	12.99±0.35 ^{ab}	12.31±0.37 ^{b}	12.41±0.35 ^{b}	12.17±0.60 ^{b}

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. Impact of three different curcumin concentrations in semen freezing extender on the quality criteria of post-thawed Rabbit sperm

Parameters	Control	200 µm Trolox (vitamin E analogue)	CU 0.25 mmol	CU 0.5 mmol	CU 1 mmol
Viability (%)	60.4±0.27 ^{d}	83.2±0.27 ^{b}	80.8±0.27 ^{b}	86.6±0.27 ^{a}	77.6±0.27 ^{c}
Intact acrosome (%)	75.35±0.38 ^{d}	87.25±0.38 ^{b}	87.57±0.38 ^{b}	88.10±0.38 ^{ab}	74.50±0.50 ^{d}
Intact plasma membrane (%)	73.35±0.54 ^{c}	86.37±0.71 ^{ab}	86.42±0.54 ^{ab}	87.10±0.54 ^{a}	72.67±0.54 ^{c}

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

تأثير الكركمين على مقاييس الحيوانات المنوية بعد الإسالة، مضادات الأكسدة، التعبير الجيني للجينات المرتبطة بمضادات الاكسدة والتجميد في الارانب

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الملخص

تهدف هذه الدراسة إلى دراسة تأثير إضافة الكركمين (CU) في مخفف تجميد السائل المنوي للارانب على مدى جودة الحيوانات المنوية بعد الاساله من التجميد وبيان مدى قدرة الكركمين على تقليل أضرار عملية التجميد والاجهاد التأكسدى على الحيوانات المنوية للأرانب ، تم تخفيف السائل المنوي باستخدام مخفف (TCG) المضاف إليه الكركمين (CU) بمستويات (0 و0,25 و 0,5 و 1) مللى مول على التوالي) مقارنة بمخفف (TCG) المضاف إليه 200 ميكرومول من فيتامين أ (ترولوكس) كمعاملة ايجابية وتم دراسة الخصائص التالية :i) حركة الحيوانات المنوية، (ii) سلامة الغشاء البلازمى، (iii) سلامة الأكروسوم، (iv) الحيوية، (v) التقييم الكيميائي الحيوي لمؤشرات مضادات الأكسدة، (vi) نشاط الميتوكوندريا، ومستوى جزيئات الاكسجين الحرة، وسلامه الحمض النووي، و(vii) مستوى التعبير الجينى لبعض الجينات المرتبطة بمضادات الأكسدة و بمضاد التجمد باستخدام RT-qPCR. أظهرت النتائج أن مجموعات (0.5 CU ملى مول و 0.25 CU ملى مول و 200 ميكرومولر Trolox) لها تأثير إيجابي معنوي ($p \leq 0.05$) على الحركة التقدمية للحيوانات المنوية بعد التجميد وعلاوة على ذلك، فإن مجموعة 0.5 CU ملى مول حسنت بشكل كبير ($p \leq 0.05$) قيم إنزيمات مضادات الأكسدة مقارنة بجميع المجموعات بينما انخفض MDA في مجموعات (0,5 CU ملى مول و 0,25 CU ملى مول و 200 ميكرومولر Trolox) بالمقارنة مع مجموعات (التحكم و 1 CU ملى مول). وفقا لهذه الدراسة فأن اضافة الكركمين بتركيز (0,5 CU ملى مول) الى مخفف التجميد وجد انه يحسن من خصائص جودة الحيوانات المنوية، ويقلل تراكم جزيئات الاكسجين الحرة، وتعمل على زيادة التعبير الجينى للجينات المرتبطة بمضادات الأكسدة ومضادات التجمد فى السائل المنوى للأرانب.

الكلمات الدالة: الكركمين، الحفظ بالتبريد، HSP 90، نشاط الميتوكوندريا، NFE2L2، الحيوانات المنوية للأرانب.