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RESEARCH ARTICLE

Influence of Lycopene on Sperm Quality, Antioxidant Capacity, Mitochondrial Potential, and Oxidation Status during 4°C Preservation of Ram Semen

Tariq Sohail[1], Yuxuan Sun[1], Fuhao Chen[1], Xiaomei Sun[1], Zhenwu Wei[2] and Yongjun Li[1]*

[1]Key Laboratory for Animal Genetics & Molecular Breeding of Jiangsu Province, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China; [2]Institute of Grassland Science, Yangzhou University, Yangzhou 225009, China.
*Corresponding author: yzliyongjun@163.com; liyj@yzu.edu.cn

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ABSTRACT

Ram sperm are very susceptible to oxidative stress damage during low-temperature preservation. Seminal oxidative stress results in membrane lipid peroxidation, changes in the normal physiological and enzymatic pathways, finally associated with spermatozoa motility loss, reduced fertility and compromised embryogenesis. Lycopene is one of the richest natural sources of antioxidants. The effects of lycopene (LYC) supplementation of the semen diluent upon the quality of Hu ram semen preserved for five days at 4°C were investigated in this study. Semen ejaculates collected from 5 rams twice weekly for 12 weeks were pooled on each collection day, diluted with an extender supplemented with 0.5, 1.5, 2.5, and 3.5µM of LYC, while the control group received no supplementation. Sperm viability, motility variables, and membrane integrity were evaluated daily up to five days. Semen biochemical indexes, including oxidation status, mitochondrial potential, and antioxidant activity, were assessed on the fifth day of storage. The results showed that supplementation of LYC at 2.5µM significantly ($P \leq 0.05$) increased the sperm viability, sperm total and progressive motility percentages, sperm plasma membrane integrity, and acrosome integrity during preservation at 4°C. It also significantly ($P \leq 0.05$) enhanced the total antioxidants content and antioxidant enzymes activities like catalase and superoxide dismutase. The mitochondrial membrane potential of sperm was correspondingly increased during preservation. However, supplementation of LYC at 2.5µM to the extender significantly ($P \leq 0.05$) reduced highly detrimental free reactive oxygen species production and malondialdehyde contents. In conclusion, the present study showed that supplementation of the extender with 2.5µM LYC significantly recovers the quality of sperm by improving the antioxidant enzyme activity and mitochondrial function, decreasing the damage caused due to free radicals, lipid peroxidation (MDA), and oxidative stress (ROS) to Hu rams' semen.

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INTRODUCTION

The Hu sheep is an average sized breed (overall height 117cm, withers height 91cm, body length 127cm), with white skin color, kept for milk and meat purpose, and is extensively distributed in Zhejiang, Jiangsu province, and Shanghai region of China (Eer *et al.*, 2020). It is a four-season-estrus breed, well-known for its tremendous growth rate, early sexual maturity, and higher prolificacy (Wan *et al.,* 2022; Wu *et al.*, 2021).

In small ruminant production, artificial insemination is a vital technique for breed improvement, which is accomplished using either fresh or cooled diluted semen to increase the lambing rate in sheep breeding (Gibbons *et al.*, 2019). Modern studies focus on the use of ram spermatozoa preserved in semen extender supplemented with some exogenous antioxidants (enzymes, vitamins, proteins, and amino acids) or related compounds for artificial insemination to improve their quality and fertilizing ability (Kameni *et al.*, 2021). These suggest that using chilled and diluted semen would be a useful technique because it slows down the metabolism and suppresses the growth of microorganisms and improves sperm quality and survival time (Hameed *et al.*, 2024).

Sperm are highly susceptible to oxidative damage as their plasma membrane contains higher polyunsaturated fatty acids (PUFAS), the major mark for oxidation. The reactive oxygen species (ROS) are produced and required during the physiological procedures related to sperm capacitation, acrosome reaction, and structural and functional maturation (Du Plessis *et al.*, 2015). Under normal conditions, both enzymatic (SOD, CAT, and glutathione reductase) and non-enzymatic (vitamins C, B and E, carotenoids and carnitines) antioxidants are present in the seminal plasma to make sure that ROS concentrations remain low (Durairajanayagam *et al.*, 2014). However, systemically excessive ROS concentration has been constantly correlated with reduced sperm quality during preservation (Silvestre *et al.*, 2021).

Lycopene ($C_{40}H_{56}$), with a pinkish or reddish color and a molecular weight of 536.85Da, is one of the richest natural sources of antioxidants. It is lipophilic, and all red colored fruit and vegetables are lycopene (LYC) sources (Naviglio *et al.*, 2008; Tvrda *et al.*, 2017). Some common dietary sources of LYC include watermelon, apricots, guavas, rosehips, papayas, and pink grapefruits, with processed tomato products having the highest amount of LYC (Khan *et al.*, 2021; Nunes *et al.*, 2023). Lycopene belongs to the beta-carotenoid family, synthesized by various plants and microorganisms, and holds distinctive biological properties. Its biochemical structure encloses highly unsaturated hydrocarbons comprising 11 conjugated and 2 unconjugated double bonds, which is responsible for its free radical scavenging and oxygen quenching activity, making it a powerful antioxidant that can offer protection against oxidative stress-facilitated damages (Chauhan *et al.*, 2011; Kim *et al.*, 2015).

Earlier studies have discovered the beneficial antioxidative effects of LYC on various mammalian (bull, buck, ram, canine) and avian species (turkey, broiler breeder) sperm during preservation. These studies found the protective role of LYC on sperm structural, functional, biochemical, and fertility results during semen preservation (Rostao *et al.*, 2012; Akalin *et al.*, 2016; Najafi *et al.*, 2018; Sheikholeslami *et al.*, 2020). The main aim of this study was to investigate the effects of lycopene on Hu rams spermatozoa motion characteristics (Total motility-TM and Progressve motility-PM), viability/vitality, plasma membrane integrity (PMI), acrosomes integrity (ACI), mitochondrial membrane potential (MMP), antioxidant enzymes activity (Catalase-CAT, superoxide dismutase-SOD), total antioxidant content (TAOC), lipid peroxidation (Malondialdehyde-MDA) level, and oxidation (Reactive oxygen species-ROS) status during storage at 4°C for five days.

MATERIALS AND METHODS

Rams selection and semen collection: Five sexually mature Hu rams aged 5±0.5 year with average body weight of 64±3.4kg and body condition score of 4.0±0.3, and having clinically normal reproductive organs, were used in the present study. These rams were kept on the experimental unit of Yangzhou University (32°23'40.0"N 119°24'46.2"E) under a free stall system and fed concentrate at the rate of 200-250g per animal twice daily, along with an adequate supply of clean water and hay.

This experimental work was executed in the breeding season of sheep from October 2023 to January 2024. Semen samples were collected twice/week for 12 weeks, taken to the lab within 30 minutes, and a preliminary assessment (volume, color, and consistency) was accomplished to check the suitability of the samples for the subsequent use. Merely ejaculates with volume of 0.8–1.7mL, total motility ≥80%, and concentration $\geq 2\times10^{9}$/mL were selected for the experiment. Each time, five ejaculates from five Hu rams were collected and pooled. Hence, 10 pooled semen samples were collected every week, and a total of 120 samples (24 repetitions) were used for measuring different sperm variables during the experiment. All indices were reiterated five times to ratify the results and evade significant differences in the dataset.

Source of reagents: Lycopene ($C_{40}H_{56}$) was acquired from Solarbio Science and Technology Corporation, Beijing, China, with Register No. R530576 and Slice No. R163517. Except for those listed, all other reagents and substances used in this experimental work were purchased from Sangon Biotechnology Corporation, Shanghai city, China.

Semen sample handling, supplementation groups with preservation method: The semen diluent was prepared via mixing tris (3.08g), citric acid (1.64g), fructose (2.00g), penicillin (0.03g), and streptomycin (0.07g) in 0.1L of distilled water. To protect against chilling storage (4°C) damage, soy lecithin (0.1g) was mixed with a basic extender by warming at 100°C. As the molecular weight of lycopene is 536.87g/mol, hence, a 10mM stock solution of lycopene (LYC) in dimethylsulfoxide (DMSO) was prepared by mixing 5.4mg of LYC powder into 1mL of DMSO in a 2mL centrifuge tube. A routine stock solution was allocated in a small ratio of 50µM by dividing 5µL into small tubes for subsequent use (Sidi *et al.*, 2022).

Afterward, a standard solution of LYC with DMSO was diluted by 1 mL of basic semen diluent. Subsequently, different applications of LYC were prepared by adding 1×10^{-5L} (0.5µM), 3×10^{-5L} (1.5µM), 5×10^{-5L} (2.5µM), 7×10^{-5L} (3.5µM) to pooled samples diluted using semen to extender ratio of 1:9 at 37°C, and the control group lacking supplementation.

- Therefore, control group contained a 1.3×10^{-4L} pooled semen sample diluted by 117×10^{-5L} basic semen extender.
- The 1×10^{-5L} (0.5µM) group contained a 1.3×10^{-4L} pooled ejaculate samples diluted through 116×10^{-5L} basic semen extender + 1×10^{-5L} (0.5µM) LYCO solution.
- The 3×10^{-5L} (1.5µM) group contained 1.3×10^{-4L} pooled ejaculate samples diluted with 114×10^{-5L} basic semen extender + 3×10^{-5L} (1.5µM) LYC solution.
- The 5×10^{-5L} (2.5µM) group contained 1.3×10^{-4L} pooled ejaculate samples diluted with 112×10^{-5L} basic semen extender + 5×10^{-5L} (2.5µM) LYC solution.
- The 7×10^{-5L} (3.5µM) group contained 1.3×10^{-4L} pooled ejaculate samples diluted with 110×10^{-5L} basic semen extender + 7×10^{-5L} (3.5µM) LYC solution.

After dilution, these samples were first wrapped with cotton and kept at room temperature (20-25°C) for 30 minutes. Then, these samples were cooled slowly to a

storage temperature (4°C) in a refrigerator to prevent cold shock damage, slow down metabolic processes, and preserve sperm quality characteristics.

Sperm viability analysis: The eosin-nigrosin staining technique was used to estimate sperm viability/vitality. For this purpose, 10µL diluted semen was mixed with 10µL of each eosin and nigrosin stain solution. After 30 sec, 6µL of the stained sample was placed on the slide, and examined under light microscope (400×). At least 300 spermatozoa were examined to estimate sperm viability percentage. Viable sperm excluded the stain and appeared white, while dead sperm took up the eosin stain and appeared red under the microscope, as shown in Fig. 1 and described by Sohail *et al.* (2024).

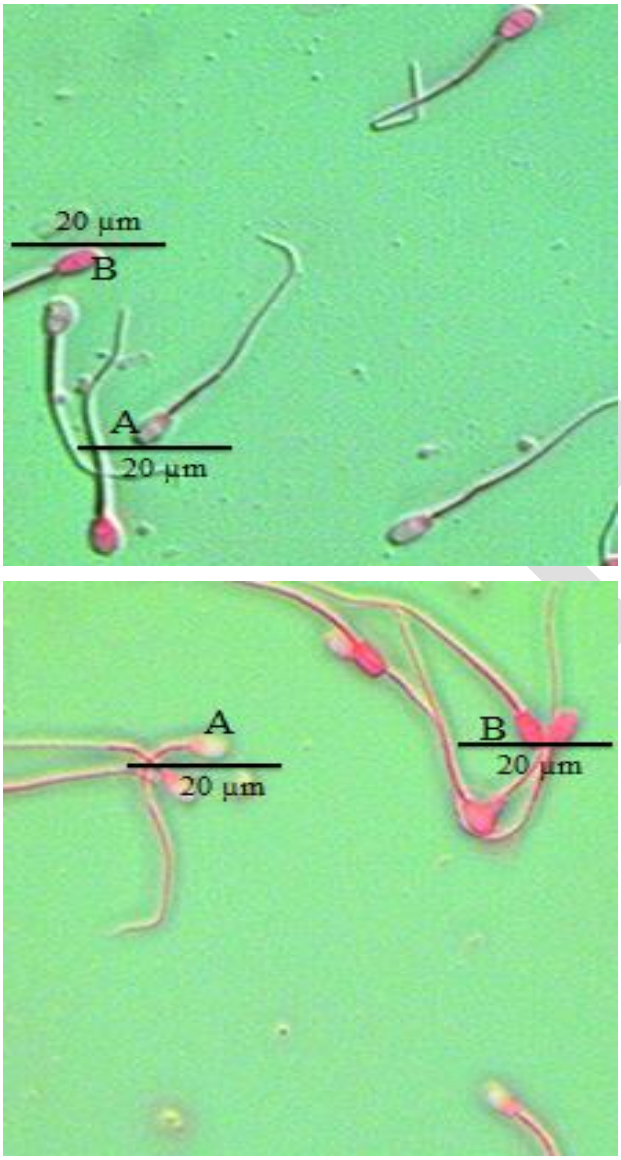

Fig. 1: The sperm viability was checked through the eosin-nigrosin staining test method. Viable sperm head type (A) remained unstained, while dead sperm type (B) stained pink or red after staining.

Sperm motility evaluation: Sperm motility variables such as TM and PM were assessed using a computer-aided sperm analyzer (Mailang Enterprise, ML-608JZ 11, version 5, Nanning City, China). A 20µL of the sample stored at 4°C was diluted eight times with basic diluent and incubated for 4min at 37°C. A total of 1.5µL drop of incubated sperm sample was placed on the sperm calculation slide for estimation of sperm motility features (Sohail *et al.*, 2024).

Sperm plasma membrane integrity estimation: The hypo-osmotic swelling test (HOST) was used to assess the PMI of spermatozoa. First, the HOST solution (100mL) was prepared by blending fructose (0.90g) and sodium citrate (0.49g) in 100 mL sterilized water. Afterward, 10µL sperm samples were added to 100µL HOST solutions and incubated at 37°C for 30 min. A minimum of 300 sperm were examined under phase contrast microscope (400x) for bulge (HOST positive) and non-bulge (HOST negative) of sperm tails to estimate the proportion of spermatozoa with PMI or loss of integrity, as shown in Fig. 2, and described by Zhang *et al.* (2024b).

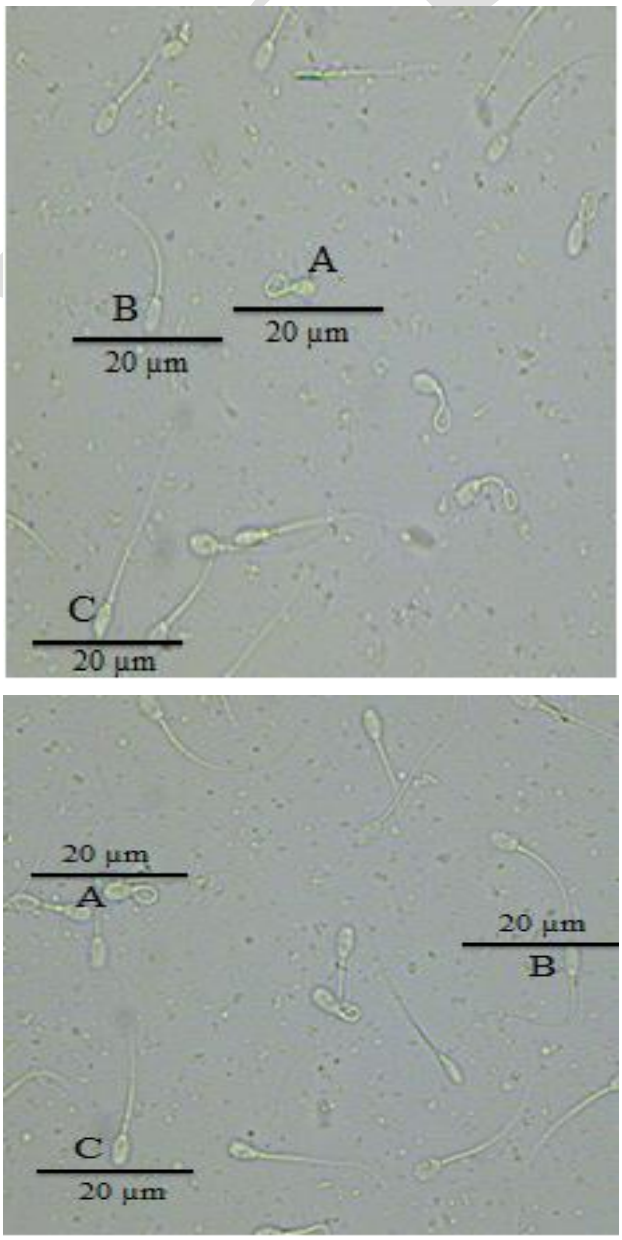

Fig. 2: Morphology of swelling (curly) tail of sperm during hypo-osmotic swelling test (HOST) method. The two types of curly sperm tails (A and B) show an intact plasma membrane. The tail non-curl type C was sperm with a damaged plasma membrane.

Sperm acrosome integrity estimation: Coomassie brilliant blue (G-250) staining was used to evaluate the ACI of spermatozoa. G-250 stain solution was prepared by mixing 0.10g of G-250 colorant in 50mL of 95% ethanol solution. Then 100mL of 85% phosphoric acid was added, and the ultimate volume of stain solution was fixed to 1.0L. Concisely, 50µL semen samples were taken in a 2ml Eppendorf tube. Later, 1mL of 4% paraformaldehyde was added to the semen sample and waited for 10 minutes for fixation of sperm. The resultant solution was centrifuged at 2500×g for 10 minutes; supernatant was discarded, and the remaining liquid semen sample was used. Afterward, 10µL of the sample was spread uniformly on a slide with the aid of a coverslip. Lastly, a G-250 colorant was used to color the smear for 35 minutes. A minimum of 300 spermatozoa heads were assessed using a 1000× oil immersion lens to define the percentage of sperm with ACI (blue sperm head) or loss of integrity (unstained sperm head), as shown in Fig. 3 and described by (Wang *et al.,* 2022).

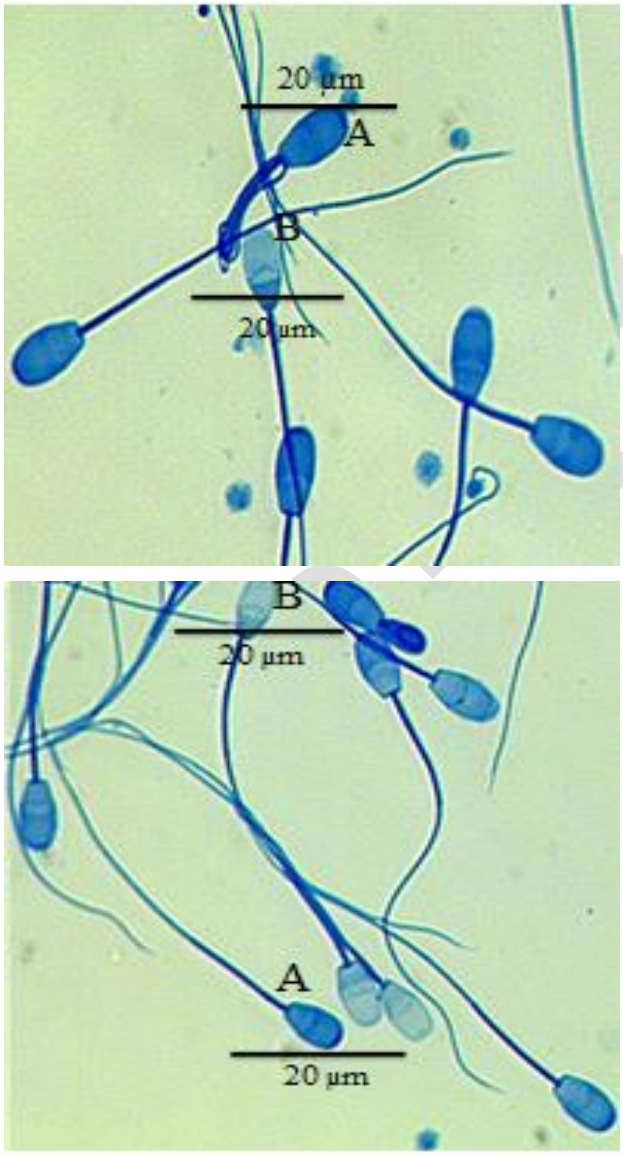

Fig. 3: Acrosome morphology of the sperm stained with Coomassie Brilliant Blue. There were two types of sperm heads after staining: Type A and Type B. If the sperm head is blue, that means the acrosome is intact (A). If the sperm head is unstained and colorless, that means loss of acrosome integrity (B).

Estimation of reactive oxygen species concentration: The ROS test kits (Solarbio Enterprises, Beijing, China) were used for the estimation of oxidation stress damage to sperm cells. Phosphate buffer saline, 600µL, was used to rinse 50µL of semen samples. These samples were treated with 350µL of dichlorodihydrofluorescein diacetate (DCFH-DA) functioning solution and kept warm at 37°C for 30 min. The resulting solution was washed thrice with phosphate buffer saline to eliminate the DCFH-DA from sperms, and supernatant was attained utilizing centrifugation at 2000×g for 10 min. A microplate reader analysis system was used to assess the fluorescence intensity of oxidative stress. The excitation and Emission wavelengths of dichlorofluorescein (DCF) were 488/525 nm for measurements of fluorescence magnitude of oxidation (Sun *et al.*, 2024).

Evaluation of malondialdehyde concentration: A lipid peroxidation test kit from Solarbio Enterprises, Beijing, China, was used to evaluate Malondialdehyde content in semen samples, and results were acquired by equating with a standard curve according to the kit directions. The MDA working solution was prepared by mixing Thiobarbituric acid (TBA) diluent, antioxidant, and TBA formulations. Concisely, 200µL semen samples were used from the control group and different LYC solutions test tubes and centrifuged at 2500 rpm for 10 min to attain the supernatant. Malondialdehyde working solution (200µL) was mixed with 100µL of supernatant, and the resultant solution was placed under dark in a hot water bath at 70°C for 20 min. After 20 min, it was taken out from the hot water bath, allowed 5min to cool down, then centrifuged at 2000 rmp for 10min. Then 100µL of each of the three replicates from the control group and different LYC treatments were poured inside 96-well plates and samples were examined at 532nm wavelength via a multifunctional fluorescence detection system (Perkin Elmer Corporate, NYSE: PKI, Waltham city, Massachusetts, USA). Malondialdehyde concentration was calculated as micromoles per liter of protein (Zhang *et al.*, 2024a).

Evaluation of sperm MMP: A mitochondrial function (MMP) analysis kit purchased from Beyotime Enterprises, Shanghai, China) was utilized to grade the MMP of the semen samples. This procedure works on the principle that higher MMP instigates the accretion of JC-1 inside mitochondrial surroundings, develop polymer, and produce red tint fluorescence. Lower mitochondrial potential leads to failure of JC-1 to assemble in mitochondrial surroundings, remains as a monomer, and generates green color fluorescence. Semen samples were washed two times with PBS; the supernatant was removed by centrifugation at 2500 rpm for 10 minutes. JC-1 working solution (500µL) was mixed and kept in the dark at 37°C for 30min according to kit recommendations. After incubation, it was washed away thrice with JC-1 staining liquid. The fluorescence intensity ratio of red fluorescence (Excitation/Emission= 525/590) and green fluorescence (Excitation/Emission= 488/525) was recorded via multifunctional plate reader (Perkin Elmer Corporate, NYSE: PKI, Waltham city, Massachusetts, USA), commonly used to assess the extent of mitochondrial potential (MMP) of samples (Zhang *et al.*, 2020).

Estimation of CAT enzyme activity: A catalase test kit (Beyotime Enterprises, Shanghai, China) was used to estimate the CAT activity of semen samples according to the kit instructions. The estimation of catalase activity is based on the fact that the catalase breakdown reaction of H_2O_2 can be swiftly blocked by adding ammonium molybdate. The residual H_2O_2 reacts with ammonium molybdate to create a light-yellow complex, which can be evaluated at 405nm wavelength. Before the required reagents, 200µL of the semen samples were centrifuged at 2500×g for 15 minutes, and the supernatant was removed. Then, the required reagents were mixed up according to the kit's directions. Lastly, results were recorded according to the guidelines described previously (Sun *et al.*, 2024).

Estimation of superoxide dismutase enzyme activity: A superoxide dismutase analysis kit with water-soluble tetrazolium salt-8 (WST-8) purchased from Beyotime Enterprises, Shanghai, China, was used to quantify the SOD activity of semen samples. Semen samples (100µL) were fully lysed using 300µL of phosphate buffer saline (PBS), centrifuged at 10000×g for 10min to remove the supernatant. Working solution of WST-8, SOD, standard and xanthine oxidase were prepared according to kit instructions. Then, 20µL of sperm samples from control and treatment groups were added to hole of 96-well plate in triplicate. Then, 20µL of SOD standard solution, 160µL of WST-8, and 20µL of xanthine oxidase working solution were added to each well as specified in the kit protocol, and incubated at 37°C for 30min to initiate the reaction. After incubation, the absorbance of each well was measured at 450nm via a multifunctional microplate reader (Perkin Elmer Corporate, NYSE: PKI, Waltham, Massachusetts, USA). Finally, the results were determined by the use of a formula: SOD activity=absorbance of blank-absorbance of sample/absorbance of blank×100, given in the kit instructions. Superoxide dismutase activity was expressed as units/mg of protein and determined from a standard curve, as described earlier (Zhang *et al.*, 2021).

Estimation of total antioxidant content: Antioxidant contents measurements of semen samples were performed using the total antioxidant content (TAOC) analysis kit (Nanjing Institute of Technology, China). The standard Trolox solution (10 mM) was diluted using refined water to 0.1, 0.2, 0.4, 0.8, and 1.0mM to get a standard curve. Supernatants were obtained by centrifugation of 100µL of the semen sample at 2500 rpm for 10min. After centrifugation, 10µL of supernatant was poured into every hole of the 96-well plates. Later, 20µL of enzyme solutions and 170µL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate were added and mixed well, conforming to kit instructions. The subsequent mixture was permitted to react at room temperature for 6 minutes and detected using a 405nm wavelength fluorescence multifunctional detection system. Results were gained via a standard curve and recorded as milimoles per liter of antioxidant content (Wang *et al.*, 2022).

Statistical analysis: Experimental data collected during research were statistically analyzed using SPSS, IBM version 24. All results of the dataset analyzed were presented as mean±SEM. The data on sperm viability, TM, PM, PMI and ACI were analyzed by two-way ANOVA. Tukey's honestly significant difference (HSD) multiple range test was used to compare the mean values. Data on antioxidant enzymes activity (CAT, SOD), TAOC, MMP, MDA, and ROS contents were analyzed by use of one-way ANOVA and mean values were compared by Tukey HSD multiple range test. Statistically significant difference was set at ($P \leq 0.05$).

RESULTS

Effects of Lycopene treatment on spermatozoa viability: Result exhibited that spermatozoa vitality of lycopene (LYC) 1.5µM and 2.5µM groups was significantly higher ($P<0.05$) compared to control and other LYC treatments on the 1st and 3rd day. Spermatozoa vitality of the 1.5µM group was non-significantly higher than that of the 0.5µM and 3.5µM groups on the 2nd, 4th, and 5th days. The sperm viability of control group was significantly ($P<0.05$) lower compared to all lycopene (LYC) treatments throughout the storage process. Sperm vitality of the control and all LYC treatments decreased ($P<0.05$) as the preservation time increased up to five days, as presented in Table 1.

Effects of Lycopene treatment on total sperm motility: Spermatozoa total motility of 1.5µM and 2.5µM groups was statistically ($P<0.05$) higher as compared to control group and 3.5µM group on the 5th day of conservation. The spermatozoa total motility of the 2.5µM group was significantly ($P<0.05$) higher relative to the control and 3.5µM group throughout the preservation process. Total motility of the 1.5µM lycopene treatment was non-significantly higher relative to 0.5µM, and 3.5µM groups on the 1st, 2nd, and 3rd day. The total motility of the control group was significantly ($P<0.05$) lower compared to all LYC treatments throughout the preservation process, except 3rd day. Total motility of the control and all LYC treatment groups was significantly ($P<0.05$) decreased as the storage time increased up to five days, as displayed in Table 1.

Effects of Lycopene treatment on progressive motility: Spermatozoa progressive motility of 1.5µM and 2.5µM LYC treatments was higher compared to control and other groups on the 5th day of conservation ($P<0.05$). However, it was non-significantly higher than 0.5µM and 3.5µM groups on the 2nd day. Similarly, progressive motility of 0.5µM and 3.5µM treatments was non-significantly higher than that of the control group on the 1st, 2nd, and 4th day. The progressive motility of the control group was lower ($P<0.05$) relative to all LYC treatments on the 3rd and 5th day. Progressive motility of the control and all LYC treatments decreased ($P<0.05$) as the preservation time increased up to five days (Table 1).

Effects of Lycopene treatment on plasma membrane integrity: Sperm plasma membrane integrity (PMI) of the 1.5µM and 2.5µM LYC treatments was higher ($P<0.05$) compared to the control group throughout the conservation process. Plasma membrane integrity of 2.5µM treatment was higher ($P<0.05$) relative to the 3.5µM treatment on the 3rd and 5th day. The PMI of 0.5µM and 3.5µM LYC treatments was non-significantly

higher relative to the control group on the 1st, 2nd, and 3rd day. The PMI of the control group was significantly (P<0.05) lower relative to all LYC treatments on the 4th and 5th days. Sperm PMI of the control and all LYC treatments statistically (P<0.05) decreased as the preservation time increased up to five days (Table 2).

Effects of Lycopene treatment on sperm acrosome integrity: The sperm acrosome integrity of 2.5µM treatments was higher (P<0.05) than the control group during all conservation days. The sperm acrosome integrity of 1.5µM treatments was higher (P<0.05) than the control group during all conservation days, except the 2nd day. The acrosome integrity of 0.5µM and 3.5µM LYC treatments was non-significantly higher relative to the control group during the conservation process, except on the 5th. The ACI of the control group was significantly (P<0.05) lower compared to all LYC treatments on the 5th day. Sperm ACI of the control and all LYC treatments significantly (P<0.05) decreased as the preservation time increased up to five days (Table 2).

Effects of Lycopene treatment on mitochondrial membrane potential: The effect of LYC treatments on the MMP during semen preservation on the 5th day is shown in Table 3. Results showed that the mitochondrial potential of 2.5µM and 1.5µM treatment was higher (P<0.05) than the control, 0.5µM and 3.5µM groups. The differences in MMP among control, 0.5µM and 3.5µM groups were non-significant. The same was true for difference between 1.5µM and 2.5µM groups.

Effects of Lycopene treatment on reactive oxygen species: The effect of LYC treatments on ROS content on the 5th day of preservation is shown in Table 3. Results exhibited that ROS levels of 2.5µM and 1.5µM treatment were significantly (P<0.05) lower compared with the control and 0.5µM treatment. The ROS content of the 3.5µM group was non-significantly lower compared than the 0.5µM treatment. ROS content of the 0.5µM treatment was also statistically (P<0.05) lower than control group. ROS content of the control group was statistically (P<0.05) higher relative to all LYC treatments.

Effects of Lycopene treatment on catalase enzyme activity: The effect of LYC treatments on catalase enzyme activity on the 5th day of semen storage indicated that the activity of 2.5µM and 1.5µM treatments was statistically (P<0.05) higher than the control and 3.5µM treatment. Catalase activity of the 1.5µM group was non-significantly higher than the 0.5µM treatment. However, catalase activity of the control group was statistically (P<0.05) lower than 2.5µM and 1.5µM treated groups (Table 3).

Effects of Lycopene treatment on superoxide dismutase enzyme activity: As shown in Table 3, SOD activity of 2.5µM treatment was non-significantly higher than 1.5µM and 3.5µM, but significantly (P<0.05) higher than the control and 0.5µM treatment. SOD activity of the control group was non-significantly lower than the 0.5µM treatment and significantly (P<0.05) lower than all other LYC treatments.

Table 1: Effect of lycopene treatments on sperm viability, total motility and progressive motility in semen preserved for different days at 4°C.

Parameters	Time (d)	Control	LYC (0.5 µM)	LYC (1.5 µM)	LYC (2.5 µM)	LYC (3.5 µM)
Viability (%)	0	82.23 ± 1.67^{A}	83.45 ± 0.78^{A}	83.44 ± 0.98^{A}	83.67 ± 1.23^{A}	82.55 ± 0.86^{A}
	1	74.83 ± 1.39^{Bc}	78.79 ± 0.62^{Bb}	80.56 ± 1.22^{Aa}	81.69 ± 0.57^{Aa}	78.48 ± 1.26^{Bb}
	2	67.10 ± 0.41^{Cc}	71.19 ± 1.02^{Cb}	74.63 ± 0.36^{Bab}	76.19 ± 1.33^{Ba}	71.32 ± 1.77^{Cb}
	3	59.87 ± 0.76^{Dc}	63.75 ± 1.26^{Db}	68.27 ± 0.49^{Ca}	69.71 ± 1.34^{Ca}	64.71 ± 1.13^{Db}
	4	52.34 ± 1.10^{Ec}	55.22 ± 1.24^{Ebc}	58.23 ± 0.58^{Db}	63.82 ± 0.89^{Da}	57.94 ± 1.40^{Eb}
	5	40.84 ± 1.54^{Fc}	47.55 ± 1.25^{Fb}	49.69 ± 0.35^{Eab}	51.38 ± 1.41^{Ea}	46.57 ± 0.15^{Fb}
Total motility (%)	0	80.77 ± 1.47^{A}	81.89 ± 0.98^{A}	81.49 ± 1.28^{A}	81.99 ± 1.32^{A}	80.68 ± 0.66^{A}
	1	72.92 ± 0.49^{Bc}	76.89 ± 0.72^{Bb}	78.65 ± 1.55^{Aab}	80.59 ± 0.37^{Aa}	76.28 ± 1.46^{Ab}
	2	64.11 ± 0.51^{Cc}	69.09 ± 1.02^{Cab}	71.73 ± 0.16^{Bab}	73.09 ± 1.23^{Ba}	68.22 ± 2.17^{Bb}
	3	57.17 ± 0.66^{Dc}	61.85 ± 1.06^{Dbc}	64.17 ± 0.29^{Cab}	66.81 ± 2.44^{Ca}	62.70 ± 1.03^{Cbc}
	4	48.44 ± 1.20^{Ec}	53.12 ± 1.54^{Eb}	55.03 ± 0.68^{Db}	61.72 ± 0.99^{Da}	55.74 ± 1.50^{Db}
	5	36.94 ± 1.64^{Fc}	44.25 ± 1.35^{Fab}	45.19 ± 0.45^{Ea}	46.58 ± 1.01^{Ea}	41.27 ± 1.35^{Eb}
Progressive motility (%)	0	75.66 ± 0.97^{A}	76.78 ± 1.68^{A}	75.31 ± 0.95^{A}	76.62 ± 1.30^{A}	76.98 ± 0.76^{A}
	1	66.65 ± 2.24^{Bc}	69.24 ± 2.03^{Bbc}	72.86 ± 1.09^{Aab}	75.48 ± 0.67^{Aa}	71.05 ± 1.07^{Babc}
	2	57.79 ± 1.63^{Cb}	61.06 ± 1.32^{Cab}	63.89 ± 0.93^{Ba}	64.99 ± 1.36^{Ba}	62.22 ± 2.45^{Cab}
	3	50.39 ± 1.58^{Dc}	53.61 ± 0.99^{Db}	54.87 ± 0.55^{Cb}	59.38 ± 1.67^{Ca}	54.32 ± 1.65^{Db}
	4	40.31 ± 2.56^{Eb}	43.70 ± 1.81^{Eb}	45.12 ± 1.18^{Dab}	50.87 ± 1.86^{Da}	46.89 ± 2.75^{Eab}
	5	30.68 ± 0.92^{Fc}	34.11 ± 0.37^{Fb}	36.21 ± 0.74^{Ea}	37.40 ± 0.63^{Ea}	33.70 ± 1.32^{Fb}

Values with different lowercase letters within a row differ significantly from one another (P<0.05); values with different uppercase letters within a row differ significantly from one another (P<0.05).

Table 2: Effects of lycopene treatments on the plasma membrane integrity and acrosome integrity of Hu ram sperms in semen stored for different days at 4°C.

Parameters	Time (d)	Control	LYC (0.5 µM)	LYC (1.5 µM)	LYC (2.5 µM)	LYC (3.5 µM)
Plasma membrane integrity (%)	0	66.24 ± 1.37^{A}	67.48 ± 0.86^{A}	67.28 ± 1.26^{A}	68.62 ± 1.42^{A}	67.58 ± 1.47^{A}
	1	55.53 ± 1.58^{Bb}	56.14 ± 1.76^{Bb}	62.60 ± 1.63^{Ba}	64.63 ± 1.48^{Ba}	61.34 ± 1.65^{Bab}
	2	45.79 ± 1.34^{Cb}	48.35 ± 1.27^{Cab}	51.75 ± 1.53^{Ca}	52.27 ± 1.31^{Ca}	49.88 ± 1.26^{Cab}
	3	37.44 ± 1.36^{Dc}	44.78 ± 0.66^{Cbc}	47.38 ± 1.65^{Cab}	49.72 ± 1.54^{Ca}	45.38 ± 1.45^{Cbc}
	4	34.87 ± 0.53^{Dc}	37.32 ± 0.92^{Db}	41.59 ± 1.97^{Da}	42.63 ± 1.82^{Da}	39.63 ± 1.25^{Dab}
	5	28.46 ± 0.79^{Ed}	31.40 ± 1.28^{Ec}	36.46 ± 1.51^{Eab}	38.65 ± 1.49^{Da}	34.32 ± 1.33^{Ebc}
Acrosome integrity (%)	0	87.12 ± 0.66^{A}	86.47 ± 1.35^{A}	$87.26\pm0.77A$	87.46 ± 0.37^{A}	86.79 ± 0.15^{A}
	1	80.42 ± 1.44^{Bb}	81.11 ± 1.63^{Bab}	83.14 ± 1.29^{Ba}	84.41 ± 0.63^{Aa}	82.34 ± 0.93^{Bab}
	2	79.51 ± 1.25^{Bb}	80.67 ± 1.37^{Bb}	$81.13\pm0.^{89Bab}$	82.83 ± 0.92^{Ba}	81.26 ± 1.22^{Bab}
	3	77.41 ± 1.43^{Cb}	79.02 ± 0.75^{Bab}	80.22 ± 1.02^{Ca}	81.67 ± 0.87^{Ba}	78.54 ± 1.31^{Cb}
	4	75.39 ± 1.18^{Cb}	77.86 ± 1.30^{Cab}	78.48 ± 0.36^{Ca}	78.98 ± 1.11^{Ca}	76.85 ± 2.34^{Cab}
	5	69.49 ± 0.92^{Dc}	71.73 ± 1.38^{Db}	74.33 ± 1.14^{Da}	75.82 ± 1.47^{Ca}	72.71 ± 1.59^{Dab}

Values with different lowercase letters within a row differ significantly from one another (P<0.05); values with different uppercase letters within a row differ significantly from one another (P<0.05).

Table 3: Effects of lycopene treatments on the ROS, MDA, MMP, TAOC, CAT, and SOD activity of the semen samples stored at 4°C on the fifth day.

Parameters	Control	LYC (0.5 µM)	LYC (1.5 µM)	LYC (2.5 µM)	LYC (3.5 µM)
ROS	568.66±5.93^{a}	546.68±4.63^{b}	515.76±6.63^{cd}	505.35±5.64^{d}	536.33±6.37^{bc}
MDA	6.37±0.35^{a}	5.57±0.31^{ab}	5.09±0.15^{b}	4.47±0.28^{c}	5.30±0.13^{ab}
MMP	3.36±0.12^{b}	3.52±0.14^{b}	4.14±0.15^{a}	4.27±0.21^{a}	3.64±0.09^{b}
TAOC	6.36±0.23^{c}	6.89±0.16^{bc}	7.41±0.13^{ab}	7.95±0.31^{a}	6.94±0.21^{bc}
CAT	3.32±0.18^{c}	3.76±0.22^{bc}	4.04±0.16^{ab}	4.34±0.29^{a}	3.44±0.13^{c}
SOD	86.07±6.19^{c}	104.92±8.73^{bc}	127.32±12.13^{ab}	150.47±8.47^{a}	122.17±6.23^{ab}

Values with different superscripts within a row are statistically different (P<0.05).

Effects of Lycopene treatment on malondialdehyde concentration: Results indicated that the MDA concentration of the control group was higher (P<0.05) relative to 2.5µM and 1.5µM groups, and non-significantly higher than 0.5µM and 3.5µM groups. MDA concentration of the 2.5µM LYC treatment was significantly lower (P<0.05) compared to all other LYC treatments. However, MDA concentration of the 1.5µM treatment was non-significantly lower compared to 0.5µM and 3.5µM groups (Table 3).

Effects of Lycopene treatment on total antioxidant content: The **total antioxidant content** (TAOC) of the 2.5µM group was non-significantly higher than the 1.5µM group, but significantly (P<0.05) higher than the control, 0.5µM, and 3.5µM treatments. TAOC of the 1.5µM group was non-significantly higher relative to 0.5µM and 3.5µM groups. TAOC of the control group was non-significantly lower than 0.5µM and 3.5µM treatments, but significantly (P<0.05) lower relative to 1.5µM and 2.5µM treatments, as shown in Table 3.

DISCUSSION

The result of this study revealed that the addition of LYC (2.5µM) to basic diluent significantly improved the sperm viability, total motility, progressive motility, PMI and ACI during preservation at 4°C. LYC (2.5µM) addition also significantly enhanced sperm cells biochemical profile like mitochondrial activity (MMP), antioxidant enzymes activity (CAT, SOD), and antioxidant capacity (AOC). Contrary to this, LYC (2.5 µM) reduced oxidative stress (ROS) and MDA-mediated damages during liquid preservation of Hu ram spermatozoa for five days. Similar to our results, LYC addition to Akkaraman ram semen diluent reduced the cryopreservation-induced damages in spermatozoa morphology, motility, viability, and acrosome integrity. Lycopene modifies pro- and anti-apoptotic (Bcl-2/Bax) gene expression which reduces sperm apoptosis rate to improve their morphology and viability (Qu *et al.*, 2024). LYC also mitigates ROS generation within the mitochondrial matrix to raise energy production and sperm motility indexes. It reduces lipid peroxidation; adjusts calcium influx into cells, and delays premature acrosome reaction to protect their integrity (Uysal and Bucak, 2007).

Reduced ROS level following LYC supplementation observed in this study is probably due to the presence of conjugated double bonds in lycopene, which acts as an effective scavenger of singlet oxygen and contains various free electrons that can be donated to reactive oxygen radicals, resulting in their neutralization (Choi and Seo, 2013). Likewise, the shielding effects of LYC (2.5µM) on mitochondrial function witnessed in our study were consistent with the previous findings, which witnessed a marked improvement in the sperm mitochondrial function after lycopene treatment (Qu *et al.*, 2024; Long *et al.*, 2025). Improved mitochondrial activity (MMP) after LYC supplementation is due to the reason that LYC sustains mitochondrial morphology, optimizes electron transport chain (ETC) efficiency, and upturn mitochondrial ATP synthesis (Aly *et al.*, 2012; Bucak *et al.*, 2015).

According to (Sheikholeslami *et al.* (2020), supplementation of 500µg/mL and 750µg/mL of LYC significantly increased sperm motility, viability, HOST test-positive sperms, DNA integrity, and total antioxidant content during chilled storage of canine semen. It also reduced the MDA level of canine sperm. Higher plasma membrane integrity in the 2.5 µg/mL LYC supplemented group compared to the control observed in the present study looks to be the result of incorporation of lycopene into sperm membrane to maintain optimal fluidity, which later prevents membrane damage. Similarly, the reduced MDA content in semen preserved in extender containing LYC is due to the binding and chelating ability of LYC with metal ions, like iron and copper, to prevent lipid peroxidation damage to spermatozoa. Improved total antioxidant content (TAOC) might be due to the regulation of nuclear factor erythroid-2 (Nrf2), and other antioxidant response elements (ARE) related gene expression that enhances overall antioxidant capacity during storage.

Mangiagalli *et al.* (2010) investigated the effect of drinking water supplementation with lycopene (0.5g/L) on the semen quality, fertility and immunity of broiler breeders. They reported significant improvements in the semen quality, fertility, and immunity of broiler breeders compared to controls. Similarly, the addition of LYC in turkey semen extender noticeably reduced the freezing damages and secured DNA integrity along with the improvement of viability and osmotic resistance of spermatozoa due to reduced peroxidation level. LYC has been shown to cause inhibition of oxidative stress damages to sperm DNA structure, maintains telomere length to develop their genetic stability during storage (Rosato *et al.*, 2012). In vitro shielding properties of LYC administration to various sperm quality parameters along with the maintenance of antioxidant enzyme activity (SOD, CAT, and $GP_{x,}$) recorded in the present study is due to the decrease in hydrogen peroxide ($H_2O_{2),}$ hydroxyl (-OH) and nitrogen dioxide ($-NO_2$) ion production in the male reproductive cells and tissues (Imran et al., 2020).

The positive effects of LYC on improving sperm quality variables recorded in this study might be due to two reasons. Firstly, LYC is a lipophilic substance that could simply and quickly infiltrate through the biological

membrane and enter the sperm cells. The second probable mechanism is that LYC plays an important role in the defense of sperm cell membranes and lipoproteins against oxidative harm. It might be due to the pro-vitamin A activity of β-carotene to play its direct operative role in shelter mechanisms (Paiva and Russell, 1999; Vardi *et al.*, 2009).

Earlier research in Merino rams has indicated that the 0.5mM LYC treatment was found to be the most effective addition of LYC to increase sperm motility and mitochondrial activity rate. According to Akalin *et al.* (2016), 2mM treatment of LYC was capable of improving antioxidant status, such as total glutathione level, compared with the control group. Consistent with our finding, LYC at a dose rate of 1-4mg/mL significantly improved the post-thaw quality of Sapudi ram semen (Bintara *et al.*, 2023). Another study with Cashmere goats (Ren *et al.*, 2018) indicated that the addition of 1.0mg/ml of LYC causes an elevation of natural antioxidants in the seminal plasma. (Tvrda *et al.*, 2016) reported that the addition of 1 or 2 mmol/liter of LYC neutralized the ferrous ascorbate (FeAA) associated oxidative damages and improved sperm quality and antioxidative profile of buffalo bull spermatozoa.

Previous studies have shown the effects of lycopene in ram sperm cryopreservation or liquid preservation at low temperature up to 72hr (Uysal and Bucak, 2007; Akalin *et al.*, 2016; Bintara *et al.*, 2023). But this is the first study to evaluate the effects of lycopene on ram semen preservation at 4℃ for five days. Ideal lycopene dose for a semen extender for sperm preservation involves several factors. These variable reasons include composition of basic extender (egg yolk, chemical, or milk base), source of lycopene (tomato extract, synthetic, or natural), and medium of dilution for lycopene (water, ethanol, acetone, chloroform or DMSO). According to our result, LYC (2.5μM) was established to be the ideal application for Hu rams' semen conservation for five days. Low levels of LYC (0.5μM and 1.5μM) were not adequate to completely trigger the enzyme protection systems and mitochondrial electron transport chain (ETC) to deliver energy in the form of adenosine triphosphate (ATP) for the best sperms cell functioning. Similarly, higher level of LYC (3.5μM or above) may have some damaging impact on Hu ram sperm cells functioning and antioxidant enzyme defense system.

Conclusions: This is a key research study regarding lycopene (LYC) treatment of Hu ram spermatozoa preserved at 4℃ for up to five days. The addition of 2.5μM LYC to Hu ram semen extender maintained the higher viability, TM, PM, PMI, ACI, and antioxidant enzymes activity, as well as the mitochondrial potential of sperm cells, compared with the control group. On the contrary, the addition of 2.5μM LYC significantly decreased the ROS and MDA contents in semen compared with the control group, thereby increasing stability and fertilization capacity of semen.

Ethical Statement: Experimental procedures conducted during the whole research work were permitted by the Ethical Committee for Animal Care of Yangzhou University, China (ID-202206132).

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Author's contributions: TS executed the research work. TS and YS statistically analyzed and envisioned the data. YS and FC backed to data acquirement in the laboratory. TS prepared the script and YL drafted and revised the manuscript. YL XS and ZW analytically reviewed the final the manuscript. All the authors have cautiously read, examined and approved the final version of the manuscript.

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