

Article

Ginsenoside Rc Improves Chilled Storage Quality of Lower-Layer X-Bearing Dairy Goat Spermatozoa Through Enhanced Mitochondrial Function and Antioxidant Capacity

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Simple Summary

The TLR7/8 agonist R848 was utilized by researchers to achieve in vitro separation of X- and Y-bearing spermatozoa in mice, cattle, and goats. In this work, we applied an X/Y sperm-specific activation and sorting approach to isolate dairy goat sperm. Different concentrations of ginsenoside Rc were added to a chilled storage (4 °C) extender, and lower-layer X-bearing sperm of the sorting system were chilled storage. Supplementation with ginsenoside Rc in the chilled extender enhances mitochondrial function, strengthens antioxidant defenses and mitigates oxidative damage.

Abstract

Sex-control technologies that alter offspring sex ratios can markedly improve the economic efficiency of dairy production. Recent studies have demonstrated that treatment with the TLR7/8 agonist R848 enables successful in vitro separation of X- and Y-bearing spermatozoa in mice, cattle, and goats. This is because high-quality semen is essential for successful artificial insemination, especially during chilled storage. In this study, X/Y sperm-specific activation and sorting methods were used to isolate sperm from dairy goats, and the effectiveness of sperm isolation was evaluated using TLR7 immunofluorescence staining. Different concentrations of ginsenoside Rc were added to the semen diluent as a natural preservative, and the lower-layer X-bearing sperm recovered from the sorting system were subjected to chilled storage. The effects of ginsenoside Rc on chilled storage performance were then assessed. Among the tested concentrations, 150 µg/mL of ginsenoside Rc exhibited the most potent protective effect, significantly improving the quality, mitochondrial function, antioxidant capacity and the relative protein abundance of SIRT1, PGC-1α, and Nrf1 of the lower-layer X-bearing sperm after chilled storage for 7 days. In summary, supplementation with ginsenoside Rc in the chilled extender improved the quality of lower-layer X-bearing dairy goat spermatozoa during chilled storage by maintaining mitochondrial function, enhancing antioxidant defense, and reducing oxidative damage, facilitating the application of lower-layer X-bearing sperm-selected sperm in production.

Keywords: dairy goat sperm; toll-like receptor 7/8; chilled storage; reactive oxygen species; ginsenoside Rc



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1. Introduction

Sex-control technology plays an important role in modern livestock production. By altering the offspring sex ratio, this technology allows sex-associated production traits, such as lactation, meat yield, and egg production, to be more effectively utilized, thereby improving production efficiency and reducing costs [1]. Among the available approaches, sex-sorted semen is one of the most practical strategies for producing offspring of a desired sex. At present, flow cytometric sperm sorting remains the dominant method for producing sex-sorted semen; however, its commercial application is mainly limited to dairy cattle. This technique is constrained by high capital and operating costs, limited sorting efficiency, and potential sperm damage during processing [2–4]. Thus, the development of efficient, cost-effective, and less damaging sperm sex-sorting technologies remains an important goal in animal reproduction.

During spermatogenesis, X- and Y-bearing spermatozoa exchange cytoplasmic components through intercellular bridges; however, some gene products may not be completely shared between them [5,6]. Toll-like receptor 7/8 (TLR7/8) genes are located on the X chromosome and have been implicated in the functional distinction between X- and Y-bearing spermatozoa [7]. Treatment with the agonist R848 targeting TLR7/8 inhibits mitochondrial and hexokinase activity in X-chromosome carrying sperm, thereby reducing sperm motility [7]. When combined with an upstream separation procedure, this approach successfully separated X- and Y-bearing spermatozoa in mice [7]. Similarly, the use of R848 to activate the X-sperm-associated TLR7/8 pathway successfully separated X- and Y-sperm in goat semen [8]. Recent studies have shown that R848 modulates mitochondrial function via the PI3K/GSK3 α / β /hexokinase and PI3K/NF- κ B/hexokinase pathways to regulate mitochondrial function [9]. It selectively inhibits the motility of X-chromosome carrying sperm without affecting Y-chromosome carrying sperm, and this mechanism was utilized to separate X- and Y-chromosome carrying sperm in beef cattle [9]. However, due to the short survival period of the separated dairy goat semen, artificial insemination must be completed within a short time, which greatly limits its application in production.

Semen preservation is essential for the application of artificial insemination. In vitro preservation extends sperm survival, supports insemination across estrous cycles, and facilitates long-distance transport [10]. Common preservation strategies include storage at ambient temperature, chilled storage, and cryopreservation. Compared with ambient-temperature storage, chilled storage provides a longer preservation period and better meets production requirements. Compared with cryopreservation, chilled storage requires less specialized equipment and technical expertise, causes less cryo-injury, and is more cost-effective [10]. Therefore, chilled storage is widely used in dairy goat production [10,11]. In view of this, this study conducted cold storage of lower-layer X-bearing sperm to extend their effective survival time, making it convenient for application in production.

Chilled storage reduces sperm metabolism, decreases nutrient consumption, and prolongs sperm survival [12]. However, spermatozoa continue to consume energy substrates during chilled storage, which can promote the accumulation of reactive oxygen species (ROS). Excessive ROS disrupt redox homeostasis and induce lipid peroxidation, membrane permeability changes, and DNA damage, ultimately compromising sperm quality and preservation efficiency [13]. Supplementation of semen extenders with exogenous antioxidants can enhance sperm antioxidant capacity, reduce oxidative stress-induced damage to motility, plasma membrane integrity, and acrosome integrity, and thereby improve preservation outcomes [14].

Ginsenosides are major bioactive components of ginseng and have been reported to possess anti-inflammatory, antitumor, antioxidant, and protective effects on the nervous and cardiovascular systems. They have been widely used in food, pharmaceutical, cosmetic,

and health-related products [15]. Ginsenoside Rc enhanced mitochondrial function in cells while suppressing ROS generation [16]. Moreover, Ginsenoside Rc enhanced the motility and forward movement ability of sperm in patients with asthenospermia [17]. The R848 treatment will inhibit the mitochondrial activity of sperm and that sperm will suffer oxidative damage during the refrigeration process. Therefore, in this study, different concentrations of ginsenoside Rc were added to the semen extender to evaluate their effects on the chilled storage of lower-layer X-bearing sperm obtained from the dairy goat sperm sorting system with the aim of extending the viable survival time of lower-layer X-bearing sperm, thereby facilitating their application in production.

2. Materials and Methods

2.1. Animals and Reagents

Ten healthy adult male dairy goats (2 to 3 years old) with excellent semen quality were selected from Shaanxi Aonike Dairy Goat Breeding Co., Ltd. (Weinan, China). The animals were managed by the company's technical staff. Experimental animal procedures were approved by the Experimental Animal Ethics Committee of the College of Animal Medicine, Northwest A&F University, China (Approval No. IACUC2025-0528 for the dairy goat experiments, approval date 16 May 2025). Unless otherwise stated, all chemicals and reagents were purchased from Solarbio (Beijing, China).

2.2. Semen Collection

Semen was collected from dairy goats using an artificial vagina and immediately diluted at a 1:3 ratio with a semen extender (Base diluent: glucose 3.5 g, lactose 4.2 g, sodium citrate 1.75 g, dissolved in 100 mL double-distilled water. All the reagents used were purchased from Sorelbo (Beijing, China). The diluted semen samples were rapidly transported to the laboratory, where initial evaluations were performed using a computer-assisted sperm analysis (CASA) system. Five qualified samples exhibiting a sperm progressive motility of >70% were selected and pooled for subsequent experiments. All evaluations of the pooled semen were performed in triplicate.

2.3. Sorting of X and Y Sperm in Dairy Goats

This experiment employed the method reported by Ren et al. [8] for sorting dairy goat sperm. First, the sperm sample was resuspended in 3 mL of semen sorting solution containing 0.6 µmol/L R848 (Semen sorting solution: Glucose 28.5 g/L, citric acid 0.49 g/L, lycopene 0.2 g/L, sodium citrate 2.0 g/L, EDTA 0.45 g/L, sodium bicarbonate 1.2 g/L, potassium chloride 0.7 g/L, Tris 5.7 g/L, etc., and Ultra-pure water 1 L. Mix well and store in a 4 °C refrigerator for later use. All the reagents used were purchased from Sorelbo). After incubation at 37 °C in a water bath for 30 min, the upper sperm fraction (1 mL) and lower sperm fraction (1 mL) were collected and transferred to new tubes. In the new tubes, add 2 mL of sorting solution to dilute to 3 mL, then add 0.4 µmol/L R848. Incubate at 37 °C in a water bath for 15 min. Collect the upper-layer sperm (1 mL) and lower-layer sperm (1 mL) separately to obtain upper-layer Y-bearing sperm and lower-layer X-bearing sperm. Centrifuge the lower-layer X-bearing sperm at 500 × g for 5 min, discard the supernatant, and resuspend the sperm in an appropriate amount of sorting solution. Repeat this procedure three times to wash the sperm. After discarding the supernatant following the third wash, add an appropriate amount of diluent to dilute and resuspend the sperm. The experiment comprised four groups: control (Con), upper-layer Y-bearing sperm (Up), lower-layer X-bearing sperm without elution (Low R848+), and lower-layer X-bearing sperm elution (Low R848-) (Figure 1).

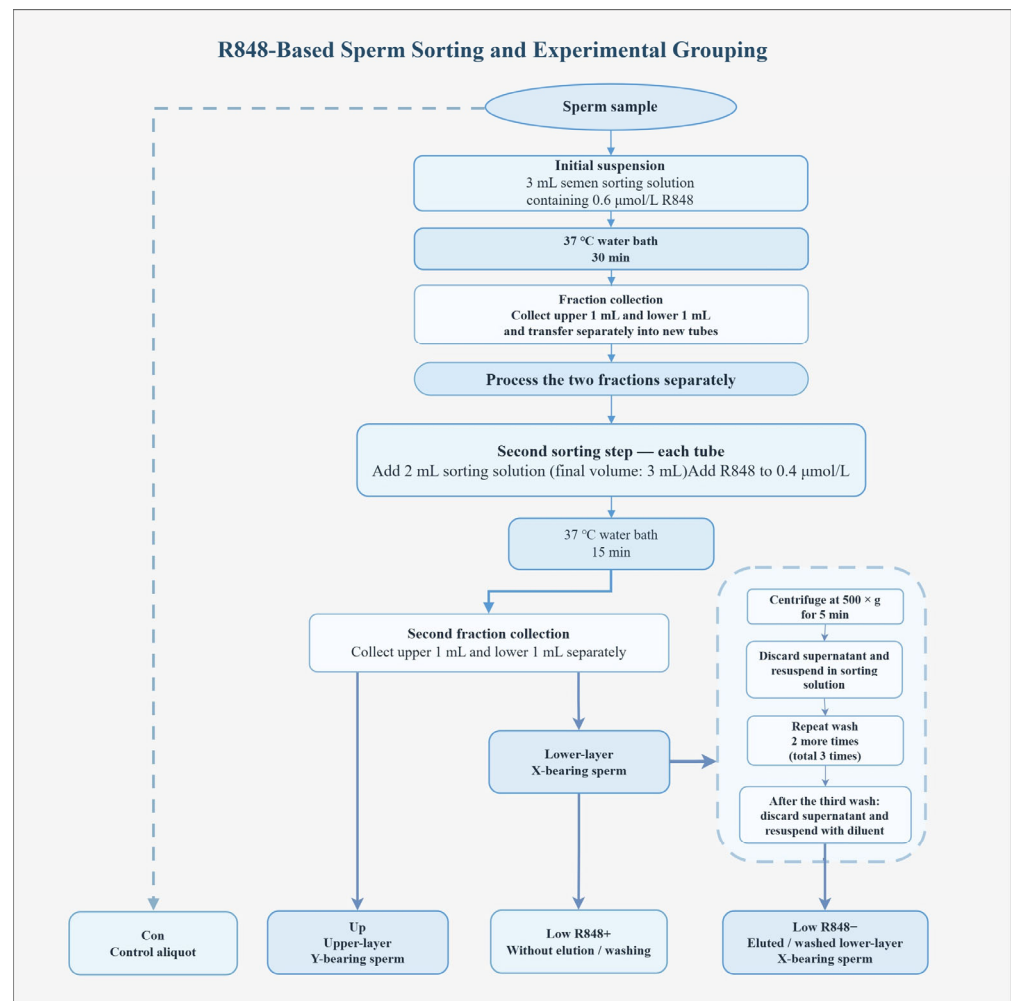


Figure 1. Sperm sorting process and grouping for X/Y sperm of dairy goats.

2.4. Chilled Storage of Lower-Layer X-Bearing Sperm

After the third wash of the lower-layer X-bearing sperm, the supernatant was discarded, and a semen refrigeration diluent containing different concentrations of ginsenoside Rc (Solarbio, Cat No: IG0210) was added (the semen refrigeration diluent was prepared by mixing 8.5 mL of the base diluent with 1.5 mL of fresh egg yolk). The mixture was gently shaken to mix and then allowed to equilibrate at room temperature for 1 h. Subsequently, the sample was wrapped tightly in 4–5 layers of towels and placed at 4 °C to cool slowly. Thereafter, it was gently shaken to mix once every 24 h to prevent excessive sedimentation until the experiment was completed. The ginsenoside Rc was first diluted to 1 mg/mL using double-distilled water. Then, the required volume of this stock solution was added to each semen dilution solution, and the volume difference was made up with double-distilled water. Based on the results of a preliminary experiment, the final addition concentrations of ginsenoside Rc were set to 0, 10, 50, 100, 150, and 200 μg/mL.

2.5. Analysis of Sperm Trajectories and Motility Using the CASA System

Sperm trajectories and motility parameters were evaluated using a computer-assisted sperm analysis (CASA) system (HVIEW-SSA V8.0, Nanning song jing tianlun biotechnology. co., ltd., Nanning, China) (Contrast: 200, Brightness: 0, Chroma: 0, Saturation: 0, Detection Diameter: 10–50 μm). Finally, 10 μL semen were added to a slide, and the movement trajectory of sperm at 60 Hz was captured by the CASA system (the standard parameter was 30 frames per second). Before analysis, semen samples from each group

were gently mixed, and an aliquot of each sample was placed on a pre-warmed glass slide for CASA examination. For each sample, at least five clear microscopic fields were selected for analysis. The CASA-derived parameters included sperm motility, average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), and mean amplitude of oscillation (MAD). Each biological sample was measured with multiple technical replicates, and the mean value was used for statistical analysis. Among them, sperm motility includes total motility and progressive motility. Total motility (%) was defined as the percentage of spermatozoa exhibiting any detectable movement, while progressive motility (%) was defined as the percentage of spermatozoa exhibiting predominantly forward, directional movement.

2.6. Assessment of Sperm Plasma Membrane and Acrosome Integrity

The integrity of the acrosome (FITC-PAN Cat: 14015.1.1, GENMED Shanghai, China) and plasma membrane (LIVE/DEAD™ Sperm Viability Assay Kit, Cat: L7011, Thermo Fisher, MA, USA) of the sperm was evaluated using the commercial reagent kit. First, place 30 µL of semen on a glass slide. Allow it to air-dry naturally, then fix with methanol for 10 min. Wash three times with PBS (3 min per wash). Incubate the sample with 30 µL of FITC-PNA (10 µg/mL) solution at 37 °C in the dark for 30 min. Subsequently, incubate with DAPI (1 µmol/L) at room temperature in the dark for 10 min. Acrosome integrity was assessed under a fluorescence microscope (LECIA DM6 B, Lecia, Wetzlar, DE). Additionally, sperm membrane integrity was analyzed using SYBR-14 (100 nM) and propidium iodide (PI: 12 µM). A 100 µL semen sample was placed in a 1.5 mL centrifuge tube. Add SYBR-14 staining solution and incubate for 10 min. Subsequently, add PI staining solution and incubate for 10 min. Finally, dispense 10 µL of the semen sample onto a microscope slide and immediately examine under fluorescence microscopy. The entire procedure was conducted in a light-protected environment. Five random fields of view were selected under fluorescence microscopy (each field containing at least 100 spermatozoa; five assessments per sample).

2.7. Analysis of ATP Levels and Mitochondrial Membrane Potential in Sperm

ATP was measured using a commercial kit (Nanjing Jiancheng, China) following the manufacturer's instructions. The sperm density was measured using a sperm density analyzer, and the sperm density of each treatment group was adjusted to 1×10^7 sperm/mL. Take 1 mL of the semen that has been adjusted to the desired density and transfer it to a new 1.5 mL centrifuge tube. Processed sperm were pelleted by centrifugation at $500 \times g$ and resuspended in an appropriate volume of ATP lysis buffer. The suspension was incubated on ice for 30 min, with gentle mixing every 5 min to ensure complete lysis. Lysates were clarified by centrifugation at $12,000 \times g$ for 10 min at 4 °C, and the supernatant was collected for analysis. For detection, 100 µL of ATP working solution was dispensed into each well/tube, incubated at room temperature for 3–5 min, then 20 µL of sample or ATP standard was added and mixed immediately. After at least 2 s, the signal was recorded on a microplate reader. Measurements were performed according to the kit protocol, with standards and blanks included for calibration.

The mitochondrial membrane potential assay kit was purchased from Solarbio. Then, 500 µL of semen was added to 0.5 µL of JC-1 working solution and mixed by inverting several times. The mixture was incubated at 37 °C in a cell culture incubator for 20 min. After incubation at 37 °C, the mixture was centrifuged at $600 \times g$ for 3–4 min at 4 °C, and the supernatant was discarded. The cells were washed twice with JC-1 staining buffer (1×), and then resuspended in an appropriate volume of JC-1 staining buffer (1×). Finally, the

samples were observed under a fluorescence microscope or analyzed using an enzyme-labeled instrument reader.

2.8. Sperm Antioxidant Marker Assay

Sperm antioxidant markers ROS (Cat: CA1410), MDA (Cat: BC0025), SOD (Cat: BC5130), and GSH (Cat: BC1175) were measured using the Solabio assay kits. Sperm samples were processed following the suspension cell protocol, strictly adhering to the kit instructions. Data analysis was performed after detection using an enzyme-labeled instrument reader.

2.9. In Vitro Cervical Mucus Penetration Test

A fresh egg was taken, and an appropriate amount of egg white was separated. For the Slide Method, one drop each of the processed semen and egg white was placed onto a microscope slide, with the two droplets spaced 2–3 mm apart. A coverslip was gently placed over the slide to bring the two droplets into contact, and the slide was incubated at 37 °C for 10–15 min before being removed for analysis. CASA was used to capture a field of view at the same distance from the interface on the egg white side, and the number of motile sperm was analyzed and counted. For the Capillary Method, one end of a capillary tube was inserted into egg white, and the siphon effect was used to fill it with egg white (ensuring no air bubbles were present), after which one end was sealed. Then, 100 µL of semen was placed into a 1.5 mL centrifuge tube, 0.6 µL of Hoechst33342 was added, and the mixture was thoroughly mixed. The mixture was incubated at 37 °C for 30 min. Subsequently, the unsealed end of the capillary was inserted at a 45° angle into the treated semen, and the capillary was incubated at 37 °C for another 30 min. Thereafter, a fluorescence microscope was used to capture images at 10 mm and 15 mm from the unsealed end (focusing on the center of the capillary), and finally, the number of sperm was counted.

2.10. Sperm Immunofluorescence Staining

Processed semen (30 µL) was evenly spread onto microscope slides and air-dried. Slides were fixed in anhydrous methanol for 10 min, air-dried again, and washed three times with PBS (5 min each). Permeabilization was performed with 0.5% Triton X-100 (Solarbio, Beijing, China) for 30 min at room temperature, followed by three PBS washes. Non-specific binding was blocked with 5% BSA for 30 min at 37 °C. Diluted primary antibody was applied, and slides were placed flat in a humidified chamber and incubated overnight at 4 °C. After three PBS washes, diluted secondary antibody was added and slides were incubated for 1 h at 37 °C in the dark. Slides were then washed three times with PBS (5 min each), counterstained with DAPI for 10 min at room temperature in the dark, washed three times in PBS, and mounted with an anti-fade medium. Fluorescence images were acquired using a fluorescence microscope.

2.11. Western Blot

The processed semen was centrifuged at 500× g to remove the supernatant. The pellet was resuspended in an appropriate volume of cell lysis buffer (RIPA:PMSF = 100:1) and incubated on ice for 30 min, with vortexing every 5 min to ensure thorough mixing. The supernatant was obtained by centrifugation at 4 °C and 12,000× g for 10 min. Total protein concentration was determined using the BCA assay kit (Biyun Tian Biotechnology Co., Ltd., Shanghai, China). Protein samples were normalized using RIPA. Subsequently, sample buffer (Tris-HCl (pH 6.8): 125 mM; SDS: 4% (w/v); DMSO: 20% (v/v); Bromophenol Blue: 0.02% (w/v)) was added to the supernatant and boiled for 10 min to denature the mixture. Electrophoresis was performed using 12% FuturePAGE™ Protein Preformed

(ACE) Binder (BOYI-ACE Biotechnology, Changzhou, China) at 120 V for 90 min with 30 µg protein per well. Membrane transfer was conducted at 100 V for 60 min using a PVDF membrane. The membrane was then blocked with 5% BSA at room temperature for 2 h, followed by incubation with the primary antibody overnight at 4 °C. After the membrane was washed three times with TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (*v/v*) Tween 20), the secondary antibody specific for the target species was applied and incubated at 37 °C for 2 h. All antibodies used in this study are listed in Table 1. The membrane was washed six times with TBST (5 min per wash). Protein bands were detected using a chemiluminescent detection system (Solarbio ECL Kit), followed by quantitative analysis via density assessment using ImageJ (ImageJ-1.53e) software.

Table 1. Information on primary antibodies used in this study.

Target	Source	Supplier	Catalog Number	Use	Dilution Ratio (WB)	Dilution Ratio (IF)
NRF1	Rabbit	Proteintech	12482-1-AP	Primary antibody	1:2000	--
SIRT1	Rabbit	Proteintech	13161-1-AP	Primary antibody	1:3000	--
Beta Actin	Rabbit	Proteintech	20536-1-AP	Primary antibody	1:5000	--
PGC1a	Mouse	Proteintech	66369-1-Ig	Primary antibody	1:10,000	--
AMPK	Mouse	Proteintech	66536-1-Ig	Primary antibody	1:3000	--
GLUT1	Rabbit	Proteintech	81463-1-RR	Primary antibody	1:10,000	--
phosphorylated AMPK	Rabbit	Proteintech	86794-1-RR	Primary antibody	1:2000	--
TLR7	Rabbit	Proteintech	17232-1-AP	Primary antibody	--	1:400
Multi-rAb [®] HRP-Goat Anti-Rabbit	Rabbit	Proteintech	RGAR001	Secondary antibody	1:10,000	--
Multi-rAb [®] HRP-Goat Anti-Mouse	Mouse	Proteintech	RGAM001	Secondary antibody	1:10,000	--
Multi-rAb [®] CoraLite [®] Plus 594-Goat Anti-Rabbit	Rabbit	Proteintech	RGAR004	Secondary antibody	--	1:200

2.12. Data Analysis

Statistical analyses were performed using SPSS 23.0, and data organization and graphical presentation were conducted using GraphPad Prism 8.0.2. In this study, each pooled semen sample was composed of semen from five goats, which were considered as five biological replicates for the purpose of sample composition; however, the entire experimental procedure was independently repeated three times ($n = 3$ technical replicates) for each parameter measurement. The mean value of technical replicates was used for statistical analysis. Before statistical analysis, data were first tested for normality using the Shapiro–Wilk test. Normally distributed data were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test, whereas non-normally distributed data were analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparison test. Results are presented as mean $\pm$ standard deviation (SD). Statistical significance was defined as $p < 0.05$.

2.13. GenAI Tool

ChatGPT 5.5 was used for optimizing the graphical abstract. Firstly, we downloaded the sample of the graphical abstract from the MDPI website. Then, using the example as a template, ChatGPT 5.5 was employed to optimize the layout, color scheme, and shape of the graphic abstract. Finally, we conducted a rigorous inspection of the optimized graphic abstract to ensure its correctness and accuracy.

3. Results

3.1. Effect of R848 on Sperm Motility Performance in Dairy Goats

To investigate the effects of R848 on sperm motility performance, we analyzed sperm trajectories and motility-related parameters using CASA after co-incubation with R848. The results are shown in Figure 2. After co-incubation with R848, the progressive motility of most sperm in the lower layer of sperm was inhibited, exhibiting shorter trajectories and circular movements, whereas only a few sperm in the upper layer showed impaired progressive motility (Figure 2A). Additionally, CASA revealed that compared to the control group and upper-layer sperm, lower-layer sperm exhibited significantly reduced VAP, VSL, STR, LIN, and progressive motility ($p < 0.05$) (Figure 2D,E), while exhibiting significantly increased WOB and MAD ($p < 0.05$) (Figure 2E). After elution, the progressive motility, VAP, VSL, STR, LIN, WOB, and MAD parameters of the lower-layer sperm recovered to levels comparable to those of the control group or upper-layer sperm ($p > 0.05$). However, no significant differences in total motility were observed among the groups ($p > 0.05$) (Figure 2B,C). It indicates that the R848 treatment does not cause sperm death, but only affects the forward motility of the sperm, and it will recover after elution.

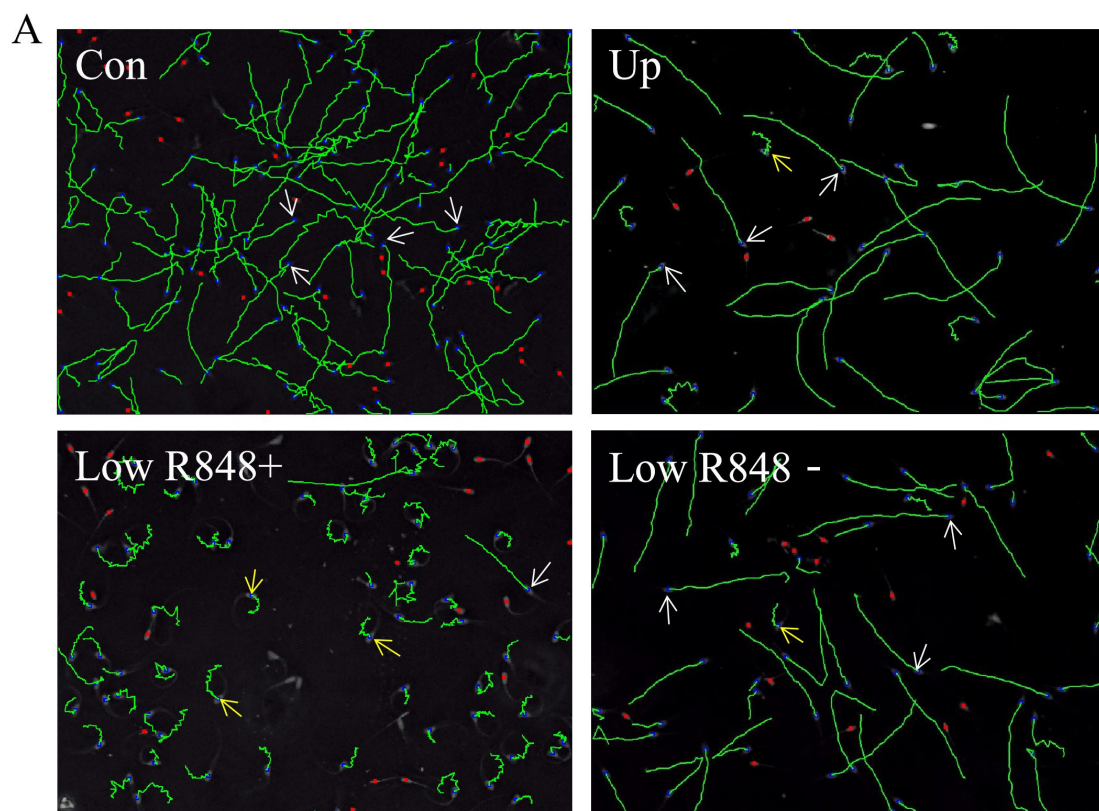


Figure 2. Cont.

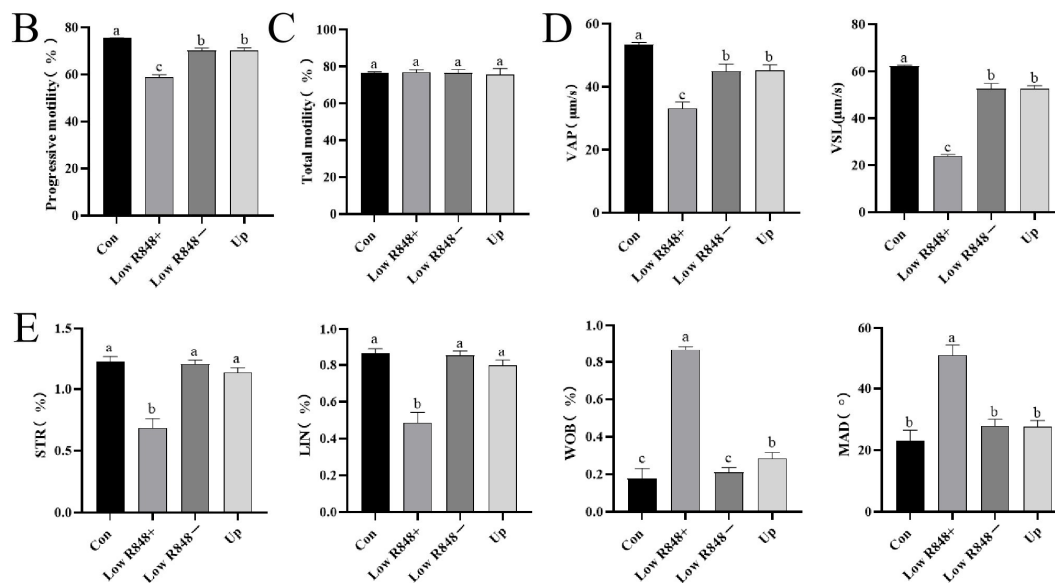


Figure 2. Effects of R848 on dairy goat sperm kinematic parameters. (A) Plot of sperm movement trajectories captured by CASA; trajectories indicated by white arrows represent linear movements, while those indicated by yellow arrows represent nonlinear movements. (B,C) Effects of R848 on dairy goat sperm progressive motility (B) and total motility (C). (D) Effects of R848 on the mean path velocity and straight-line path velocity of dairy goat sperm. (E) Effects of R848 on movement parameters of dairy goat sperm. Different lowercase letters indicate significant differences among groups ($p < 0.05$), whereas identical letters indicate no significant difference ($p \geq 0.05$).

3.2. The Effect of R848 on Sperm Quality and the Proportion of TLR7-Positive Sperm

To investigate whether R848 adversely affects dairy goat sperm, the acrosome integrity rate and plasma membrane integrity rate were detected using fluorescence staining in the control group (Con), the upper layer sperm (Up), and lower-layer sperm eluted after treatment (Figure 3A,B). Results showed that the membrane integrity rate and acrosome integrity rate of spermatozoa in the lower and upper layers after elution did not differ significantly from the control group ($p > 0.05$) (Figure 3C,D). After co-incubation with R848, both ATP levels and JC-1 levels in lower-layer sperm were significantly lower than those in the control group and upper-layer sperm ($p < 0.05$) (Figure 3E,F). Following elution, ATP levels in the lower-layer sperm recovered to levels comparable to those in the upper-layer sperm ($p > 0.05$) (Figure 3E). Although JC-1 levels were significantly elevated, they remained markedly lower than those in the control group and upper-layer sperm ($p < 0.05$) (Figure 3F). We further examined the mitochondrial structure and the '9 + 2' microtubule arrangement in the principal piece of the sperm tail using transmission electron microscopy. The results revealed no significant differences between the eluted lower-layer sperm and the control group (Figure 3G).

Additionally, we performed TLR7 immunofluorescence staining on lower-layer sperm, upper-layer sperm, and the control group (Figure 3H). Results showed that in the control group, the proportions of TLR7-positive (TLR7⁺) sperm and TLR7-negative (TLR7⁻) sperm were 48.02% and 51.98%, respectively. In the lower layer, the proportions of TLR7⁺ and TLR7⁻ spermatozoa were 84.67% and 15.33%, respectively, while in the upper layer, the proportions were 9.35% and 90.65% (Figure 3I).

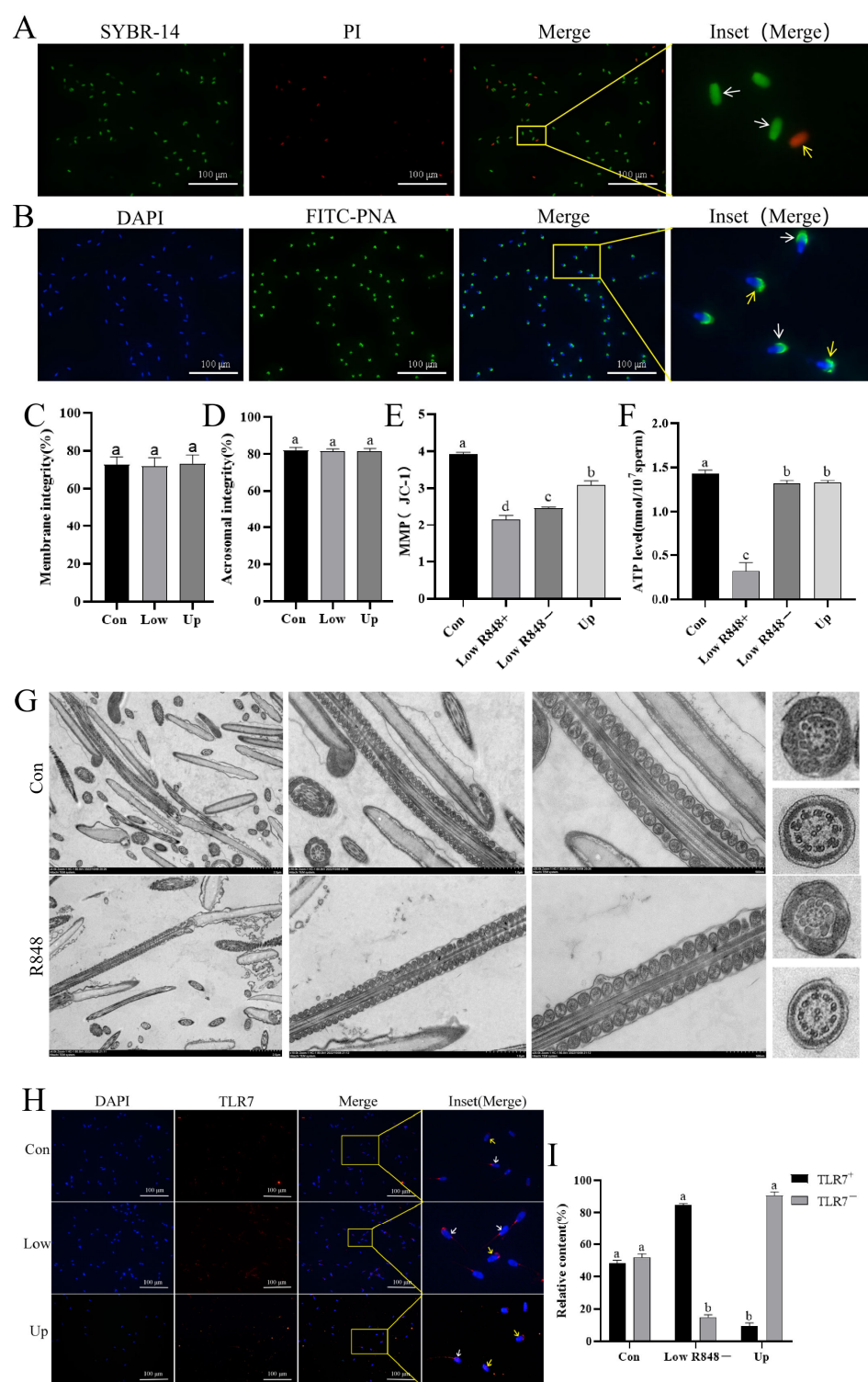


Figure 3. Effects of R848 on dairy goat sperm quality and TLR7 immunofluorescence staining. (A,C) Effects of R848 on dairy goat sperm plasma membrane integrity. Yellow arrows indicate sperm with damaged membranes; white arrows indicate sperm with intact membranes. (B,D) Effects of R848 on dairy goat sperm acrosome integrity. Yellow arrows indicate sperm with damaged acrosomes; white arrows indicate sperm with intact acrosomes. (E) Effects of R848 on ATP levels in dairy goat sperm. (F) Effect of R848 on JC-1 levels in dairy goat sperm. (G) Effect of R848 on Mitochondrial sheath and “9 + 2” microtubule structure in dairy goat sperm. (H,I) TLR7 immunofluorescence staining of dairy goat sperm. White arrows indicate TLR7-positive sperm; yellow arrows indicate TLR7-negative sperm. Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$), whereas the same letters indicate no significant differences ($p > 0.05$).

3.3. Effects of Ginsenoside Rc on Chilled Storage of Lower-Layer X-Bearing Dairy Goat Sperm

To facilitate application in production, dairy goat chilled storage diluent was supplemented with ginsenoside Rc at concentrations of 0, 10, 50, 100, 150, and 200 µg/mL. The lower-layer X-bearing sperm isolated from dairy goat sperm using X/Y sperm-specific protein activation and sorting technology was stored at 4 °C. The effects of ginsenoside Rc on chilled storage viability of dairy goat sperm of the lower layer are shown in Table 2. On day 7 of chilled storage, the group supplemented with 150 µg/mL ginsenoside Rc in the dilution solution exhibited significantly higher progressive motility than the control group and other concentration groups ($p < 0.05$) (Table 1).

Table 2. Effect of ginsenoside Rc on chilled storage viability (%) of lower-layer X-bearing dairy goat spermatozoa.

Concentration of Ginsenoside Rc	Time				
	2 h	1 d	3 d	5 d	7 d
0 µg/mL	70.63 ± 2.75 ^b	69.25 ± 0.80 ^b	66.50 ± 1.71 ^c	63.41 ± 0.87 ^d	58.08 ± 0.52 ^d
10 µg/mL	72.37 ± 0.35 ^{ab}	70.54 ± 2.47 ^b	68.63 ± 1.00 ^{bc}	64.08 ± 1.00 ^d	60.67 ± 0.70 ^c
50 µg/mL	72.57 ± 0.71 ^{ab}	71.72 ± 2.24 ^{ab}	69.79 ± 1.42 ^{ab}	65.96 ± 1.09 ^c	61.48 ± 0.58 ^c
100 µg/mL	73.09 ± 1.82 ^{ab}	72.31 ± 1.39 ^{ab}	70.88 ± 1.94 ^{ab}	67.71 ± 1.17 ^{bc}	63.48 ± 0.60 ^b
150 µg/mL	75.39 ± 1.19 ^a	74.75 ± 1.01 ^a	72.61 ± 2.04 ^a	70.82 ± 0.87 ^a	66.07 ± 0.58 ^a
200 µg/mL	73.65 ± 1.55 ^{ab}	72.36 ± 1.65 ^{ab}	70.64 ± 2.19 ^{ab}	67.57 ± 0.41 ^b	63.65 ± 1.28 ^b

Data are presented as mean ± SD ($n = 3$ technical replicates). Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$), whereas the same letters indicate no significant differences ($p > 0.05$).

To investigate the effect of ginsenoside Rc on chilled storage efficacy of lower-layer X-bearing dairy goat sperm, further assessments were conducted on the plasma membrane integrity and acrosome integrity of chilled storage sperm (Figure 4A,C). Results showed that at ginsenoside Rc concentrations of 50, 100, 150, and 200 µg/mL in the diluent, sperm plasma membrane integrity and acrosome integrity were significantly higher than in the control group ($p < 0.05$). The 150 µg/mL ginsenoside Rc group exhibited the highest rates of plasma membrane integrity (65.38%) and acrosome integrity (73.15%), both significantly higher than other groups ($p < 0.05$) (Figure 4B,D).

Studies have shown that treatment with ginsenoside Rc can increase cellular ATP production and glucose uptake while enhancing mitochondrial function. To further evaluate mitochondrial function, we measured JC-1 and ATP levels in lower-layer X-bearing dairy goat sperm after chilled storage. Results showed that when the concentration of ginsenoside Rc in the dilution solution reached 150 µg/mL, the JC-1 level and ATP level in sperm reached their maximum values of 5.82 $\Delta\Psi_m$ and 13.52 nmol/ 10^7 sperm, respectively (Figure 4E–G). The relative protein abundance of proteins associated with sperm energy metabolism (including GLUT1, AMPK, and p-AMPK) were significantly higher in the 150 µg/mL group than in the control group (Figure 4H,I and Figure S1).

3.4. Ginsenoside Rc Enhances the Antioxidant Capacity Chilled Storage Lower-Layer X-Bearing Sperm

Ginsenoside Rc possesses antioxidant properties and can effectively alleviate oxidative damage to cells caused by excessive accumulation of ROS. To assess oxidative stress and lipid peroxidation, we further investigated the effects of ginsenoside Rc on ROS levels and MDA content in lower-layer X-bearing dairy goat sperm after 7 days of chilled storage. The results showed that at ginsenoside Rc concentrations of 50, 100, 150, and 200 µg/mL in the dilution solution, sperm ROS levels and MDA content were significantly lower than in the control group ($p < 0.05$, Figure 5B,C), with the lowest levels observed in the 150 µg/mL ginsenoside Rc group. Similarly, adding ginsenoside Rc to the dilution solution significantly

increased glutathione (GSH) and superoxide dismutase (SOD) content in chilled storage dairy goat sperm. with the 150 µg/mL ginsenoside Rc group exhibiting the highest GSH and SOD content, significantly higher than the control group ($p < 0.05$, Figure 5D,E).

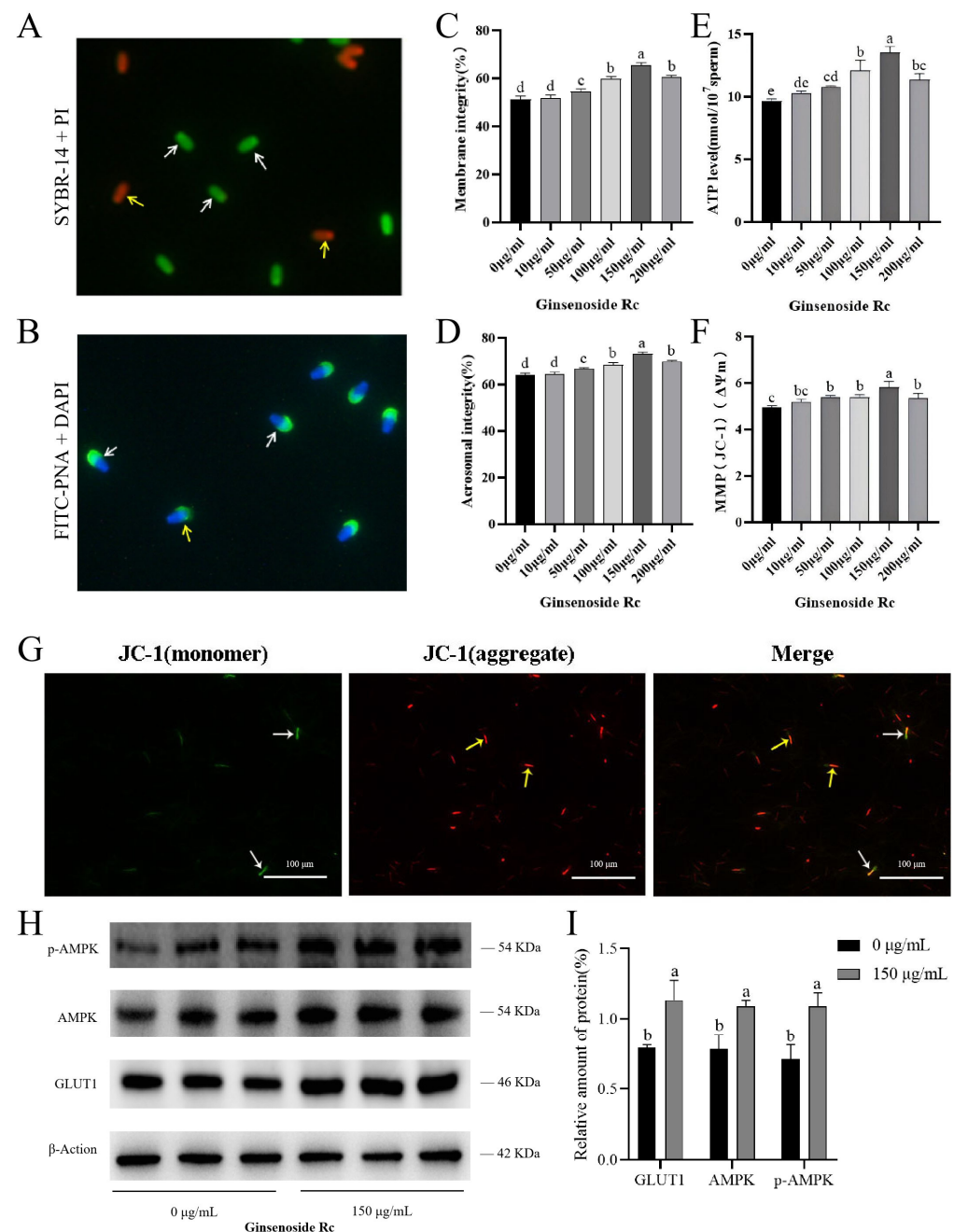


Figure 4. Effects of Ginsenoside Rc on chilled storage of lower-layer X-bearing dairy goat sperm. (A,B) Effects of Ginsenoside Rc on plasma membrane integrity of lower-layer X-bearing dairy goat sperm. Yellow arrows indicate intact plasma membranes; white arrows indicate sperm with damaged plasma membranes. (C,D) Effects of ginsenoside Rc on acrosome integrity in lower-layer X-bearing dairy goat sperm. Yellow arrows indicate sperm with damaged acrosomes; white arrows indicate sperm with intact acrosomes. (E) Effects of ginsenoside Rc on ATP levels in lower-layer X-bearing dairy goat sperm. (F,G) Effects of ginsenoside Rc on JC-1 levels in lower-layer X-bearing dairy goat sperm. White arrows indicate sperm with low mitochondrial membrane potential, yellow arrows indicate sperm with high mitochondrial membrane potential. (H,I) Effects of ginsenoside Rc on the relative protein abundance of GLUT1, AMPK, and p-AMPK in lower-layer X-bearing dairy goat sperm. Different lowercase letters indicate significant differences among groups ($p < 0.05$), whereas identical letters indicate no significant difference ($p \geq 0.05$).

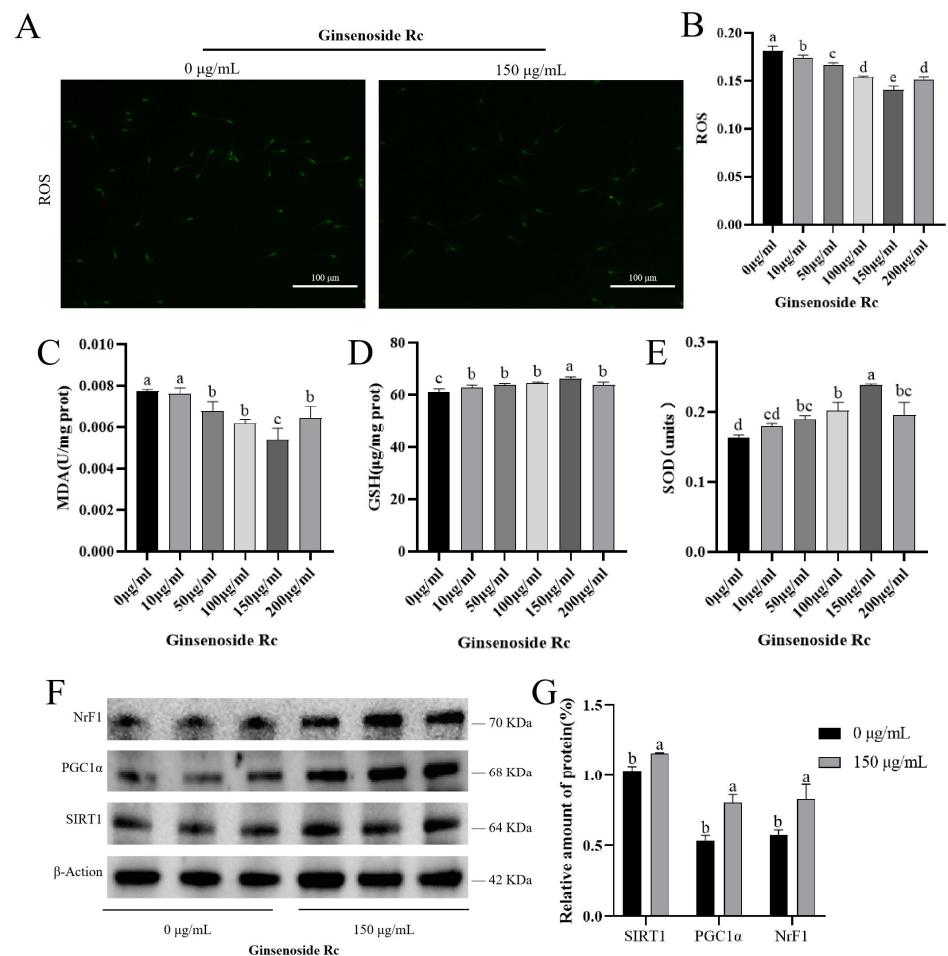


Figure 5. Effects of ginsenoside Rc on antioxidant capacity of lower-layer X-bearing dairy goat sperm. (A,B) Effects of ginsenoside Rc on ROS levels in lower-layer dairy goat sperm. (C) Effects of ginsenoside Rc on MDA levels in lower-layer X-bearing dairy goat sperm. (D,E) Effects of ginsenoside Rc on GSH and SOD content in sperm from lower-layer X-bearing dairy goat sperm. (F,G) Effects of ginsenoside Rc on relative protein abundance of SIRT1, PGC-1 α , and Nrf1 in sperm from lower-layer X-bearing dairy goats. Different lowercase letters indicate significant differences among groups ($p < 0.05$), whereas identical letters indicate no significant difference ($p \geq 0.05$).

Furthermore, compared with the control group, 150 $\mu\text{g/mL}$ ginsenoside Rc can significantly increase the relative protein abundance of SIRT1 and PGC-1 α in chilled storage lower-layer X-bearing dairy goat sperm ($p < 0.05$, Figure 5F,G). Meanwhile, adding 150 $\mu\text{g/mL}$ ginsenoside Rc to the dilution solution significantly increased the relative protein abundance of Nrf1, a downstream protein of the SIRT1/PGC-1 α pathway ($p < 0.05$, Figure 5F,G and Figure S2). This enhanced sperm antioxidant capacity and maintained sperm redox balance.

3.5. Ginsenoside Rc Enhances the Ability of Lower-Layer X-Bearing Sperm to Penetrate Cervical Mucus *In Vitro*

We further investigated the fertilization potential of lower-layer X-bearing sperm of the sorted system after chilled storage through the cervical mucus penetration test. The results show that the number of motile sperm in egg white and the number of sperm reaching the 10 mm and 15 mm marks in the capillary were significantly higher in the ginsenoside Rc-treated group than in the control group (Figure 6A,B).

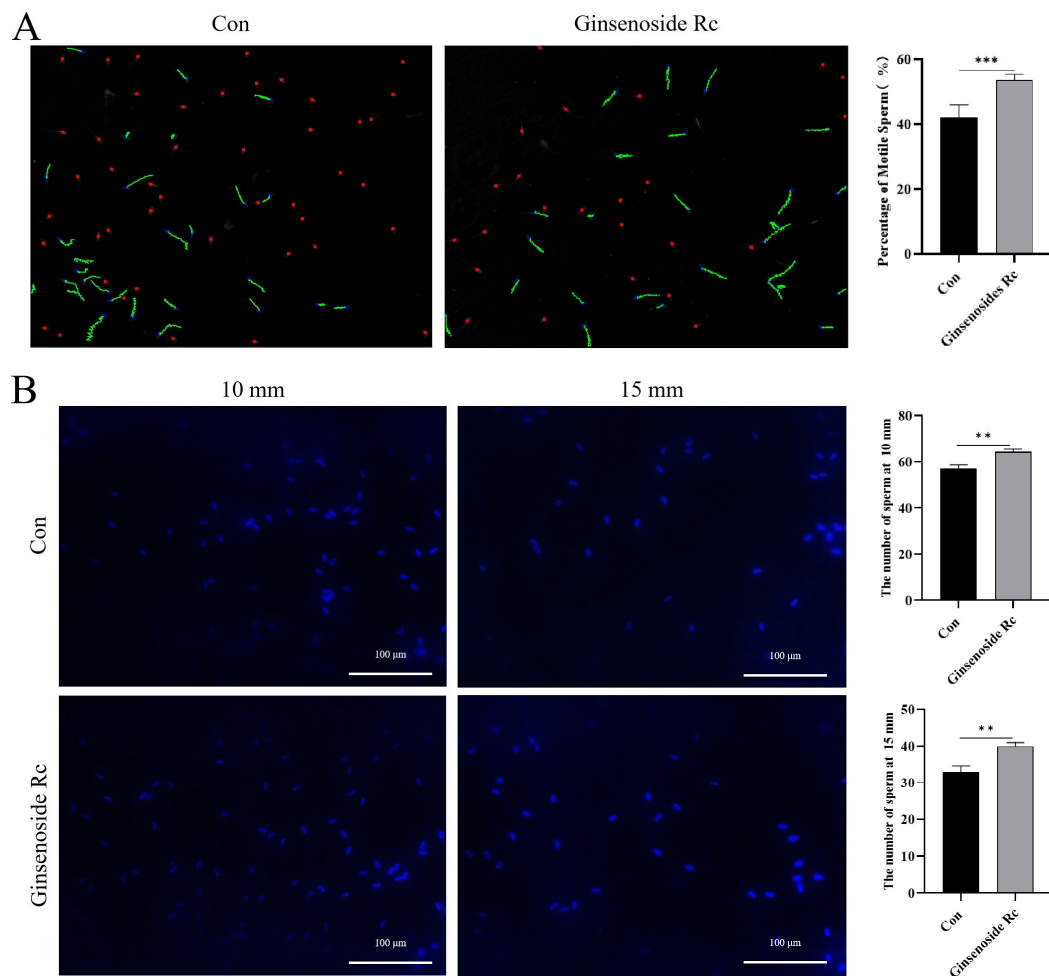


Figure 6. Effects of ginsenoside Rc on the ability of chilled storage lower-layer X-bearing dairy goat sperm to penetrate cervical mucus in vitro. **(A)** The effect of Ginsenoside Rc on sperm penetration of cervical mucus as detected by the glass slide method. **(B)** The effect of Ginsenoside Rc on sperm penetration of cervical mucus as detected by the capillary method. Data are presented as mean $\pm$ SEM. ns, not significant; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

In animal husbandry, producing offspring of a desired sex is important for improving production efficiency. The separation of X- and Y-bearing spermatozoa is one of the most effective strategies for producing sex-controlled offspring [18]. Recent studies have shown that co-incubation of spermatozoa with TLR7/8-specific ligands significantly reduces the motility of X-bearing spermatozoa without markedly affecting Y-bearing spermatozoa, thereby enabling the separation of X- and Y-bearing spermatozoa [7–9].

In the present study, R848 treatment altered the motility pattern of spermatozoa in the lower fraction of the suspension system. Most spermatozoa in the lower layer exhibited shorter trajectories and circular movement, whereas only a small proportion of spermatozoa in the upper layer showed impaired motility. R848 co-incubation significantly reduced ATP levels and mitochondrial membrane potential in lower-layer spermatozoa. After elution, sperm progressive motility, ATP levels, and mitochondrial membrane potential were significantly restored. Moreover, the plasma membrane and acrosome integrity of eluted lower-layer spermatozoa did not differ significantly from those of the control and upper-layer spermatozoa. These results suggest that R848-induced motility inhibition in lower-layer spermatozoa is reversible and does not cause obvious structural damage to the plasma membrane or acrosome. Studies have reported that TLR7 and TLR8 are expressed

only in X-chromosome-carrying sperm [9,19]. We used TLR7 immunofluorescence staining as an alternative method to evaluate sperm separation in dairy goats. The results showed that 84.67% of the sperm in the lower layer were TLR7⁺, whereas only 9.35% of the sperm in the upper layer were TLR7⁺. A limitation of this study is that we used only one method for evaluation.

These findings are consistent with previous reports. Zhu et al. [20] reported that incubation with TLR agonists in the extender reduced sperm ATP levels by increasing the phosphorylation of GSK3 α and PI3K, thereby decreasing sperm motility. Umehara et al. [7] demonstrated that TLR7/8 agonists reduce the motility of X-bearing spermatozoa without affecting Y-bearing spermatozoa, and that the inhibitory effect can be reversed by centrifugation and washing. Ren et al. [8] found that activation of TLR7/8 increased the phosphorylation levels of glycogen synthase kinase α/β (GSK3 α/β) and nuclear factor κ B (NF- κ B) in X-bearing spermatozoa, resulting in reduced mitochondrial activity, ATP production, and sperm motility. Wen et al. [9] further showed that the TLR7/8 agonist R848 regulates mitochondrial function in X-bearing spermatozoa through the PI3K/GSK3 α/β /hexokinase and PI3K/NF- κ B/hexokinase pathways, thereby reducing ATP content and motility while leaving Y-bearing sperm motility largely unchanged. Together, these studies support the feasibility of R848-mediated separation of X- and Y-bearing spermatozoa.

During chilled storage, spermatozoa are susceptible to cold stress and oxidative damage. Cold stress can accelerate the production and accumulation of ROS, which promote lipid peroxidation of polyunsaturated fatty acids in the sperm plasma membrane. This process impairs sperm motility, plasma membrane integrity, acrosome integrity, and mitochondrial function, ultimately reducing semen preservation quality [21,22]. Supplementation of semen extenders with exogenous antioxidants can enhance sperm antioxidant capacity, reduce oxidative damage during storage, and improve semen preservation outcomes [23].

In this study, supplementation with 150 μ g/mL ginsenoside Rc significantly improved the chilled storage quality of lower-layer X-bearing dairy goat spermatozoa. Specifically, ginsenoside Rc improved sperm motility, plasma membrane integrity, and acrosome integrity, and enhanced mitochondrial function, as reflected by increased mitochondrial membrane potential assessed by JC-1 staining and elevated ATP levels. In addition, ginsenoside Rc increased the relative protein abundance of GLUT1, AMPK, and phosphorylated AMPK, suggesting that it may help maintain energy metabolism during chilled storage. These findings indicate that ginsenoside Rc can improve the preservation efficiency of lower-layer X-bearing dairy goat spermatozoa during chilled storage.

Similar protective effects of ginsenosides and other antioxidant additives have been reported in previous studies. Chen et al. [18,24] found that supplementation with Panax notoginseng extract or ginsenoside Rc improved sperm motility in patients with asthenozoospermia. Dai et al. [25] demonstrated that melatonin supplementation improved the motility and mitochondrial activity of goat spermatozoa stored at 5 days of 4 °C storage. Similarly, Zhang et al. [26] found that carboxylated ϵ -polylysine mitigated cryo-injury and improved post-thaw motility, mitochondrial membrane potential, and ATP levels in goat spermatozoa. Adding 60 μ g/mL geniposide (GNP) to the dilution solution can enhance the motility of chilled storage goat sperm after five days of refrigeration [27].

ROS accumulation during chilled semen storage increases with prolonged storage time. Excessive ROS can induce lipid peroxidation of sperm membrane lipids and increase the production of aldehyde compounds such as MDA, thereby disrupting sperm structure and function and reducing preservation efficiency [22,28]. In the present study, 150 μ g/mL ginsenoside Rc effectively reduced ROS accumulation and MDA content in lower-layer dairy goat spermatozoa after chilled storage. Meanwhile, ginsenoside Rc increased SOD content and GSH content, indicating an enhanced antioxidant defense capacity. These

results suggest that ginsenoside Rc protects lower-layer X-bearing spermatozoa from oxidative damage during chilled storage.

These observations are consistent with previous studies on antioxidant supplementation in semen preservation. Jumintono et al. [29] reported that cysteamine supplementation increased antioxidant potential and total glutathione levels while reducing lipid peroxide content in cryopreserved sheep spermatozoa. Xie et al. [30] showed that schisandrin B enhanced total antioxidant capacity, catalase activity, and SOD expression in low-temperature-stored boar spermatozoa, while reducing ROS and MDA levels. Therefore, the reduction in ROS and MDA levels, together with the increase in SOD activity and GSH content observed in this study, suggests that ginsenoside Rc improves chilled semen preservation partly by strengthening the antioxidant defense system.

Furthermore, ginsenoside Rc treatment significantly increased the relative protein abundance of SIRT1, PGC-1 α , and Nrf1 in lower-layer X-bearing dairy goat spermatozoa. The SIRT1/PGC-1 α pathway is closely associated with mitochondrial biogenesis, cellular energy metabolism, and oxidative stress regulation. Huang et al. [31] reported that ginsenoside Rc increased Nrf1 and Nrf2 levels in H9c2 and PC12 cells by activating the SIRT1/PGC-1 α pathway, thereby promoting biogenesis and improving energy metabolism under both normal and ischemia–reperfusion injury conditions. The research indicates that adding resveratrol to the dilution solution enhances the quality and antioxidant capacity of cryopreserved goat sperm by activating the AMPK/SIRT1/FoxO1 pathway [32]. MitoQ can reduce oxidative stress by regulating mitochondrial function during the cryopreservation process of buck sperm [33]. Based on this, ginsenoside Rc may protect the stability of the lower-layer X-bearing goat sperm during chilled storage by regulating the relative abundance of proteins related to the SIRT1/PGC-1 α /Nrf1 pathway, enhancing mitochondrial function and reducing oxidative stress. Excessive addition may disrupt the redox balance of sperm, thereby causing damage to sperm function. Unfortunately, we only examined the relative protein abundance changes in a few pathway-related proteins. Therefore, this pathway can only be regarded as a potential mechanism of action. Another drawback is that this study only measured the sperm quality indicators and did not conduct in vitro fertilization or artificial insemination to verify the effect of ginsenoside Rc on sperm fertilization ability.

Overall, this study demonstrates that R848 can separate X- and Y-chromosome-carrying dairy goat sperm by selectively inhibiting the motility and mitochondrial activity of the underlying sperm, and that this inhibitory effect can be reversed after elution. Furthermore, the addition of 150 $\mu\text{g}/\text{mL}$ ginsenoside Rc improved the chilled storage quality of lower-layer X-bearing sperm. The potential mechanism of its action may involve regulating the relative abundance of proteins in the SIRT1/PGC-1 α /Nrf1 pathway, enhancing antioxidant defense, and alleviating mitochondrial oxidative damage, thereby maintaining mitochondrial function. These findings provide a theoretical basis for the application of ginsenoside Rc in the chilled storage of sex-elected dairy goat semen.

5. Conclusions

In conclusion, our method of activating treatment with TLR7/8 agonist R848 enabled us to obtain lower-layer X-bearing dairy goat sperm. We then added different concentrations of ginsenoside RC to the dilution solution and chilled storage it. Ginsenoside RC can enhance the activity of lower-layer X-positive dairy goat sperm during chilled storage. Its mechanism of action may involve regulating the relative protein abundance of SIRT1/PGC-1 α /N2RF1 and modulating mitochondrial function to reduce oxidative damage.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15151298/s1>. Figure S1: Original, uncropped Western

blot images corresponding to Figure 4H in the main text. Figure S2: Original, uncropped Western blot images corresponding to Figure 5F in the main text.

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Institutional Review Board Statement: The research performed in this study complies with all relevant ethical regulations. All animal procedures were approved by the Experimental Animal Ethics Committee of the College of Animal Medicine, Northwest A&F University, China (Approval No. IACUC2025-0528 for the dairy goat experiments, approval date 16 May 2025), and all methods were performed in accordance with the institutional guidelines and national regulations for the care and use of laboratory animals.

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References

1. Kurtz, S.; Petersen, B. Pre-determination of sex in pigs by application of CRISPR/Cas system for genome editing. *Theriogenology* **2019**, *137*, 67–74. [[CrossRef](#)] [[PubMed](#)]
2. Butler, S.T.; Hutchinson, I.A.; Cromie, A.R.; Shalloo, L. Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal* **2014**, *8*, 165–172. [[CrossRef](#)] [[PubMed](#)]
3. Qin, Y.; Yang, S.; Xu, J.; Xia, C.; Li, X.; An, L.; Tian, J. Deep insemination with sex-sorted Cashmere goat sperm processed in the presence of antioxidants. *Reprod. Domest. Anim.* **2018**, *53*, 11–19. [[CrossRef](#)] [[PubMed](#)]
4. Maicas, C.; Holden, S.A.; Drake, E.; Cromie, A.R.; Lonergan, P.; Butler, S.T. Fertility of frozen sex-sorted sperm at 4×10^6 sperm per dose in lactating dairy cows in seasonal-calving pasture-based herds. *J. Dairy Sci.* **2020**, *103*, 929–939. [[CrossRef](#)] [[PubMed](#)]
5. Hendriksen, P.J. Do X and Y spermatozoa differ in proteins? *Theriogenology* **1999**, *52*, 1295–1307. [[CrossRef](#)] [[PubMed](#)]
6. Rahman, M.S.; Pang, M.G. New Biological Insights on X and Y Chromosome-Bearing Spermatozoa. *Front. Cell Dev. Biol.* **2020**, *7*, 388. [[CrossRef](#)] [[PubMed](#)]
7. Umehara, T.; Tsujita, N.; Shimada, M. Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. *PLoS Biol.* **2019**, *17*, e3000398. [[CrossRef](#)] [[PubMed](#)]
8. Ren, F.; Xi, H.M.; Ren, Y.J.; Li, Y.; Wen, F.; Xian, M.; Zhao, M.J.; Zhu, D.W.; Wang, L.Q.; Lei, A.M.; et al. TLR7/8 signalling affects X-sperm motility via the GSK3 α/β -hexokinase pathway for the efficient production of sexed dairy goat embryos. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 89. [[CrossRef](#)] [[PubMed](#)]
9. Wen, F.; Liu, W.D.; Li, Y.; Zou, Q.L.; Xian, M.; Han, S.Q.; Zhang, H.Y.; Liu, S.Q.; Feng, X.Z.; Hu, J.H. TLR7/8 agonist (R848) inhibit bovine X sperm motility via PI3K/GSK3 α/β and PI3K/NF κ B pathways. *Int. J. Biol. Macromol.* **2023**, *232*, 123485. [[CrossRef](#)] [[PubMed](#)]

10. Yu, H.M. Effect of Different Factors on Cold Storage of Semen of Inner Mongolia Cashmere Goat. Master's Thesis, Inner Mongolia Agricultural University, Hohhot, China, 2020. [\[CrossRef\]](#)
11. Liu, Z.Z.; Li, X.L.; Gong, Y.F.; Zang, W.S. Effect of different centrifugal speed on preservation effect of boer goat semen in low temperature. *Grass-Feed. Livest.* **2002**, *37*, 37–39. [\[CrossRef\]](#)
12. Bailey, J.L.; Bilodeau, J.F.; Cormier, N. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *J. Androl.* **2000**, *21*, 1–7. [\[CrossRef\]](#)
13. Marchetti, C.; Obert, G.; Deffosez, A.; Formstecher, P.; Marchetti, P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum. Reprod.* **2002**, *17*, 1257–1265. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Asadi, N.; Bahmani, M.; Kheradmand, A.; Rafieian-Kopaei, M. The impact of oxidant stress on testicular function and the role of antioxidant in improving it: A Review. *J. Clin. Diagn. Res.* **2017**, *11*, IE01–IE05. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Zhang, X.Y.; Hong, X.Y.; Gao, X.Y.; Gao, L.C.; Ma, Y.H.; Han, L. Study on Preparation of six Ginseng Soaps and Their Antioxidant Activity in Vitro. *World Latest Med. Inf.* **2018**, *18*, 14–17. [\[CrossRef\]](#)
16. Han, W.; Wang, Z.; Guo, H.; Wang, M.; Cao, L.; Sun, G. Ginsenoside Rc inhibits diabetic cardiomyopathy by reducing lipotoxicity through the adiponectin and autophagy pathways. *Phytomedicine* **2026**, *150*, 157615. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Chen, J.C.; Chen, L.D.; Tsauer, W.; Tsai, C.C.; Chen, B.C.; Chen, Y.J. Effects of Ginsenoside Rb2 and Rc on inferior human sperm motility in vitro. *Am. J. Chin. Med.* **2001**, *29*, 155–160. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Soleymani, B.B.; Mansouri, K.; Rastegari-Pouyani, M.; Parvaneh, S.; Khademi, F.; Tabar Sharifi, M.; Mostafaie, A. Production of monoclonal anti-body against recombinant bovine sex-determining region Y (SRY) and their preferential binding to Y chromosome-bearing sperm. *Reprod. Domest. Anim.* **2021**, *56*, 270–777. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Umehara, T.; Yamanaka, T.; Shimada, M. Toll-like receptors in mammalian sperm. *Reprod. Med. Biol.* **2025**, *24*, e12651. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Zhu, X.X.; Shi, D.Y.; Li, X.Q.; Gong, W.J.; Wu, F.J.; Guo, X.J.; Xiao, H.; Liu, L.X.; Zhou, H. TLR signalling affects sperm mitochondrial function and motility via phosphatidylinositol 3-kinase and glycogen synthase kinase-3 α . *Cell Signal* **2016**, *28*, 148–156. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Eriksson, B.M.; Petersson, H.; Rodriguez-Martinez, H. Field fertility with exported boar semen frozen in the new FlatPack container. *Theriogenology* **2002**, *58*, 1065–1079. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Tuncer, P.B.; Bucak, M.N.; Sariözkan, S.; Sakin, F.; Yeni, D.; Çiğerci, I.H.; Ateşşahin, A.; Avdatek, F.; Gündoğan, M.; Büyükleblebici, O. The effect of raffinose and methionine on frozen/thawed Angora buck (*Capra hircus ancyrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology* **2010**, *61*, 89–93. [\[CrossRef\]](#) [\[PubMed\]](#)
23. He, J.J. Effects of Epicatechin on Boar Semen Cryopreservation. Master's Thesis, Northwest A & F University, Yangling, China, 2021. [\[CrossRef\]](#)
24. Chen, J.C.; Xu, M.X.; Chen, L.D.; Chen, Y.N.; Chiu, T.H. Effect of panax notoginseng extracts on inferior sperm motility in vitro. *Am. J. Chin. Med.* **1999**, *27*, 123–128. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Dai, G.C.; Meng, Y.; Zhang, L.K.; Du, Y.Q.; Wen, F.; Feng, T.Y.; Hu, J.H. Effect of addition of melatonin on liquid storage of ram semen at 4 °C. *Andrologia* **2019**, *51*, e13236. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Zhang, W.J.; Cui, H.X.; Ding, K.X.; Zhou, K.F.; Li, Y.J.; Hoque, S.A.M.; Min, L.J.; Zhu, Z.D. Carboxylated ϵ -Poly-l-lysine Improves Post-Thaw Quality, Mitochondrial Functions and Antioxidant Defense of Goat Cryopreserved Sperm. *Biology* **2023**, *12*, 231. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Wei, W. Effect of Geniposide on Semen Cryopreservation in Dairy Goats. Master's Thesis, Northwest A & F University, Yangling, China, 2025. [\[CrossRef\]](#)
28. Moazamian, R.; Polhemus, A.; Connaughton, H.; Fraser, B.; Whiting, S.; Gharagozloo, P.; Aitken, R.J. Oxidative stress and human spermatozoa: Diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol. Hum. Reprod.* **2015**, *21*, 502–515. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Jumintono, J.; Alkubaisy, S.; Yáñez Silva, D.; Singh, K.; Turki Jalil, A.; Mutia Syarifah, S.; Fakri Mustafa, Y.; Mikolaychik, I.; Morozova, L.; Derkho, M. Effect of Cystamine on Sperm and Antioxidant Parameters of Ram Semen Stored at 4 °C for 50 Hours. *Arch. Razi Inst.* **2021**, *76*, 1115–1123. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Xie, Y.F.; Chen, Z.Y.; Wang, Y.L.; Peng, X.Y.; Feng, N.; Wang, X.Y.; Tang, Y.S.; Li, X.; Xu, C.R.; Hu, C.H. Supplementation of Schisandrin B in Semen Extender Improves Quality and Oxidation Resistance of Boar Spermatozoa Stored at 4 °C. *Animals* **2023**, *13*, 848. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Huang, Q.X.; Su, H.; Qi, B.; Wang, Y.; Yan, K.I.; Wang, X.I.; Li, X.Y.; Zhao, D.Q. A SIRT1 Activator, Ginsenoside Rc, Promotes Energy Metabolism in Cardiomyocytes and Neurons. *J. Am. Chem. Soc.* **2021**, *143*, 1416–1427. [\[CrossRef\]](#) [\[PubMed\]](#)

32. Zheng, J.K.; Zhao, J.Y.; Li, M.H.; Cheng, Y.Q.; Fang, X.Q.; Zhao, L.; Zhao, Y.J. Resveratrol improves cryopreserved goat sperm quality and antioxidant capacity through AMPK/SIRT1/FoxO1 activation. *J. Anim. Sci.* **2026**, *104*, skag152. [[CrossRef](#)] [[PubMed](#)]
33. Hatami, M.; Masoudi, R.; Hatefi, A.; Alipour-Jenaghard, P.; Esmaeili, V. The effects of MitoQ as a mitochondrial-targeted antioxidant in a plant-based extender on buck sperm quality parameters during cryopreservation. *Anim. Reprod. Sci.* **2024**, *266*, 107517. [[CrossRef](#)] [[PubMed](#)]

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