

Article

Optimizing Semen Cryopreservation in Inner Mongolia Cashmere Goats: Combined Effects of Centrifugation Parameters and L-Proline Supplementation

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Abstract

This study optimized the cryopreservation protocol for cashmere goat semen by testing centrifugation speeds (750, 1000, 1250, 1500 rpm) for seminal plasma removal and L-proline concentrations (10, 30, 50 mmol/L) in a freezing extender. Semen from six 3-year-old breeding bucks of Inner Mongolia cashmere goats was evaluated post-thaw in terms of motility, membrane integrity, antioxidant capacity, and artificial insemination (AI) outcomes ($n = 130$ does). The results demonstrated that the group that underwent centrifugation at 1250 rpm saw significantly improved sperm motility ($p < 0.05$), curvilinear velocity (VCL, $p < 0.05$), and straight-line velocity (VSL, $p < 0.05$) compared to the other groups. The addition of 30 mmol/L L-proline further enhanced post-thaw sperm motility ($p < 0.05$), plasma membrane integrity ($p < 0.05$), and acrosome integrity ($p < 0.05$), while significantly reducing reactive oxygen species (ROS, $p < 0.05$) and malondialdehyde (MDA, $p < 0.05$) levels. This group also exhibited the highest antioxidant capacity, as indicated by elevated levels of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) ($p < 0.05$). AI trials revealed that semen treated with 1250 rpm centrifugation and 30 mmol/L L-proline achieved the highest kidding rate (56.82%), significantly outperforming the control group (37.21%, $p < 0.05$). Meanwhile, no significant differences were observed in prolificacy or offspring sex ratio ($p > 0.05$). In conclusion, this study demonstrates that combining 1250 rpm centrifugation for seminal plasma removal with the addition of 30 mmol/L L-proline to the freezing extender significantly improves the quality of cryopreserved cashmere goat semen and enhances AI outcomes.

Keywords: cashmere goat; semen cryopreservation; centrifugation; antioxidant; artificial insemination; reproductive efficiency



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1. Introduction

The cashmere goat industry, which holds a unique germplasm resource and large-scale industrial advantages, positions China as the world's largest producer and exporter of cashmere. China's average annual cashmere production over the past decade has reached approximately 16,000 tons, accounting for over 70% of the world's total cashmere output [1]. With the implementation of ecological conservation policies and advancements

in large-scale breeding technologies, improving the reproductive performance of high-quality cashmere goats and enhancing the fiber-producing traits of the population have become critical tasks for the development of China's cashmere goat industry. Artificial insemination (AI) technology, as a key breeding technique in modern livestock production, has been widely used in cashmere goat production. This technology enables the control of offspring traits, enhances genetic improvement advantages, prevents the spread of infectious diseases, and reduces breeding costs. Spermatozoa cryopreservation refers to the preservation of semen in an ultra-low-temperature environment, which significantly reduces spermatozoa metabolism and enables the long-term storage of high-quality semen from superior male animals [2]. The quality of frozen semen is crucial to the success of AI.

However, spermatozoa inevitably suffer cryodamage through two primary mechanisms during cryopreservation. On the one hand, ice crystal formation can induce mechanical damage to the sperm plasma membrane. On the other hand, the excessive reactive oxygen species (ROS) generated during post-thaw metabolic recovery trigger lipid peroxidation, leading to altered membrane fluidity and DNA damage, which subsequently activate regulated cell death (RCD) pathways such as apoptosis and ferroptosis, ultimately compromising sperm integrity [3]. In addition, during the sperm cryopreservation process, sperm are also subjected to cryodamage caused by factors such as osmotic pressure changes, cold shock, and mechanical stress [3]. Particularly in ruminants like sheep and goats, the high abundance of polyunsaturated fatty acids in sperm membranes renders them more susceptible to lipid peroxidation, thereby diminishing frozen-thawed sperm quality [3,4]. Furthermore, current goat semen cryopreservation techniques face limitations due to interspecies variations. For instance, goat seminal plasma contains unique components such as egg-yolk-coagulating enzyme (EYCE), glycoprotein lipase, and anti-fertility factors, which further impair sperm motility, disrupt plasma membrane integrity, and reduce sperm survival capacity [5,6].

Studies have shown that removing seminal plasma before cryopreservation can mitigate the negative effects on spermatozoa [6,7]. Centrifugation is a commonly used method for seminal plasma removal. Typically, goat spermatozoa are washed by centrifugation at 550–950 g for 10–15 min [8]. However, the centrifugation process itself may induce damage to spermatozoa [7]. The extent of spermatozoa damage caused by centrifugation is closely related to the centrifugation intensity [7]. Therefore, determining the optimal centrifugation speed for seminal plasma removal is crucial for semen cryopreservation. Furthermore, the addition of antioxidants to the semen cryopreservation extender can protect spermatozoa from oxidative stress, improve post-thaw spermatozoa viability and motility, and enhance the success rate of AI [9,10]. L-proline is a naturally occurring amino acid with osmoprotective and antioxidant properties. It can scavenge free radicals and reduce oxidative stress damage, and has shown positive effects in animal semen cryopreservation. It effectively protects spermatozoa from oxidative damage, maintains spermatozoa motility and fertilizing ability, and improves conception rates [11–13].

Currently, there is limited research on the optimal centrifugation speed for seminal plasma removal and the optimal concentration of L-proline in cryopreservation extenders for cashmere goats. Previous studies have primarily focused on semen quality, with fewer studies focusing on kidding rate and prolificacy [2,5]. Therefore, this study first investigated the optimal centrifugation speed for seminal plasma removal in cashmere goats. Based on this speed, the semen was centrifuged to remove seminal plasma. Subsequently, the protective effects of different concentrations of L-proline on cryopreserved cashmere goat spermatozoa were explored to identify the most effective dosage. Finally, we systematically integrated physical optimization (centrifugation parameters) with biological protection (antioxidant supplementation), and further validated the results through artificial insemination.

nation trials. This study aimed to establish the optimal centrifugation protocol for seminal plasma removal and determine the most effective L-proline concentration for cryopreservation, thereby providing both theoretical guidance and practical protocols for optimizing cashmere goat semen cryopreservation techniques.

2. Materials and Methods

2.1. Animal Management

The experiment was conducted at the Second Branch of Inner Mongolia Yiwei Cashmere Goat Co., Ltd. (Hohhot, Inner Mongolia, China) and Erdos Eco Ranch of Inner Mongolia ERDOS Resources Co., Ltd. (Ordos, Inner Mongolia, China). Six healthy 3-year-old Inner Mongolia cashmere goat bucks in good physical condition and with excellent reproductive performance were selected. A total of 130 healthy, non-pregnant Inner Mongolia cashmere goat does aged 2 years with a parity of 1 were used as recipients. The animals were managed under uniform conditions, with a combination of grazing and supplemental feeding. Each buck was supplemented daily with 600 g of corn and 150 g of black beans, while each doe was supplemented with 400 g of corn. The centrifugation and additive concentration tests used semen collected and cryopreserved in October 2023, while the artificial insemination trial involved semen processed in September 2024 with insemination conducted in October 2024.

2.2. Experimental Design

2.2.1. Optimization of Centrifugation Speed Experiment

Five groups were established, with the control group being the conventional frozen-semen group, where seminal plasma was retained and no centrifugation was performed. The experimental groups were subjected to centrifugation at four different speeds (750, 1000, 1250, and 1500 rpm, corresponding to $64\times g$, $111\times g$, $173\times g$, and $249\times g$, respectively, calculated using a rotor radius of 9.8 cm) for 10 min to remove seminal plasma using a low-speed centrifuge (SCIOLOGEX, DM0412, SCIOLOGEX Corporation, Rocky Hill, CT, USA). The treatment was replicated in each group three times ($n = 3$ technical replicates per group) to evaluate the effects of different centrifugation speeds for seminal plasma removal on the efficacy of semen cryopreservation. A centrifugal force of $173\times g$ was selected as optimal and used throughout subsequent L-proline and AI trials.

2.2.2. Optimal L-Proline Concentration Experiment

Following optimization of the centrifugation protocol for seminal plasma removal, subsequent experimental procedures were performed using the processed, seminal plasma-depleted semen samples. Four groups were established, with the control group consisting of the basic diluent without any added antioxidants. The experimental groups were prepared by supplementing the basic diluent with 10, 30, and 50 mmol/L L-proline (Solarbio, P0011, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), designated as the P1, P2, and P3 groups, respectively. The treatment in each group was replicated three times to evaluate the effects of different L-proline concentrations on semen cryopreservation. An amount of 30 mmol/L was chosen as the optimal cryoprotectant concentration and applied in all AI validation tests.

2.2.3. AI Experiment

A total of 130 experimental does were randomly divided into 3 groups: the control group, which received the basic diluent, consisted of 43 does; experimental group 1, which was treated with non-centrifuged (seminal plasma retained) semen supplemented with 30 mmol/L L-proline, comprised 44 does; and experimental group 2, which was treated

with centrifuged (seminal plasma removed) semen supplemented with 30 mmol/L L-proline, also comprised 44 does. The kidding rate, prolificacy, and kid sex ratio were compared among the groups to evaluate the combined effects of seminal plasma removal and L-proline supplementation on the efficacy of artificial insemination.

2.3. Solution Preparation

The base extender and washing solution were prepared according to the method described by Chai et al. [14]. The base extender consisted of 0.5 g D-glucose, 0.66 g D-fructose, 3.07 g Tris, 1.64 g citric acid, 100,000 IU ampicillin sodium, 100,000 IU streptomycin sulfate, 5 mL ethylene glycol, 15 mL egg yolk, and 100 mL ultrapure water. The washing solution consisted of 0.5 g D-glucose, 0.66 g D-fructose, 3.07 g Tris, 1.64 g citric acid, and 100 mL ultrapure water. All reagents were Solarbio® products (Beijing Solarbio Science & Technology Co., Ltd., China). Egg yolks were obtained from fresh hen eggs (Charoen Pokphand Group, Xiangyang, China). The mixed solution was homogenized using a magnetic stirrer, filtered through a 40 µm filter, aliquoted into sterile 100 mL centrifuge tubes, and stored at 4 °C for future use. The base extender served as the control, while the experimental groups were prepared by supplementing the basic diluent with 10 mmol/L, 30 mmol/L, and 50 mmol/L L-proline, designated as the P1, P2, and P3 groups, respectively.

2.4. Semen Collection

Semen was collected using the artificial vagina method. Immediately after collection, 10 µL of semen was diluted at a 1:6 ratio with the basic diluent. Fresh semen quality was analyzed using a computer-assisted spermatozoa analysis (CASA) system (Hamilton Thorne,IVOS II, Hamilton Thorne Inc., Beverly, MA, USA) with standardized settings: a 38 °C stage temperature, a 30 frames/sec acquisition rate, and ≥ 5 fields per sample. The kinematic thresholds were set at $VAP \geq 25 \mu\text{m/s}$ and $STR \geq 75\%$, with daily calibration using 5 µm latex beads. Spermatozoa motility was expressed as the percentage of motile sperm (motile sperm count/total sperm count $\times 100\%$). The initial semen concentration of Inner Mongolia cashmere goats was approximately 6×10^9 sperm/mL [14]. Semen samples from six Inner Mongolia Cashmere bucks (with individual spermatozoa motility above 80%) were pooled to minimize inter-individual variability in subsequent experiments.

2.5. Semen Cryopreservation

Qualified fresh semen samples (motility > 80%) were processed through two protocols: (1) The first protocol was conventional freezing (control), where semen was diluted in a ratio of 1:6 with the extender, thoroughly mixed by repeated pipetting, and then loaded into 0.25 mL veterinary semen straws (IMV, 005838 0.25 mL, IMV Technologies, L'Aigle, France). When filling, the straws were immersed in the semen and approximately half-filled by mouth aspiration. The upper end was then sealed with the tongue tip to prevent backflow. After removing the straw, a small air bubble was aspirated before re-immersing to complete filling. Finally, the straws were sealed with polyvinyl alcohol-based powder. (2) For seminal plasma removal, qualified semen was mixed in a ratio of 1:1 with the washing solution under isothermal conditions and centrifuged at 750, 1000, 1250, or 1500 rpm for 10 min. After centrifugation, the supernatant was removed, and the process was repeated once. The resulting pellet was then subjected to the dilution procedure.

The packaged straws were wrapped in multiple layers of sterile towels and equilibrated at 4 °C for 3 h. Liquid nitrogen was poured into the homemade fumigation chamber (50 L capacity, $-80 \sim -130$ °C adjustable). After temperature stabilization at -100 °C (± 5 °C) was confirmed using an ultra-low-temperature thermometer (Huahanwei, HHW-50, Changzhou Huahanwei Electric Technology Co., Ltd., Changzhou, China),

the equilibrated frozen-semen straws were placed on a perforated fumigation plate for 3 min of vapor exposure. Then the straws were transferred to liquid nitrogen for freezing and storage. After 30 days of storage, the straws were thawed in a 38 °C water bath (Jinghong, DK-8B, Shanghai JingHong Experimental Equipment Co., Ltd., Shanghai, China) for 1 min for semen quality assessment.

2.6. Post-Thaw Spermatozoa Quality Assessment

2.6.1. Spermatozoa Motility and Kinematic Parameters

Post-thaw semen analysis was performed using the CASA system. A 10 µL aliquot of thawed semen was placed on a microscope slide pre-warmed to 37 °C and covered with a similarly preheated coverslip. The system assessed spermatozoa motility and three kinematic parameters: curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). For each sample, five randomly selected microscopic fields were analyzed.

2.6.2. Integrity of Spermatozoa Plasma Membrane

Membrane integrity was assessed via Annexin V-FITC/PI staining (Annexin V-FITC Apoptosis Detection Kit: BD Biosciences, 556547, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and analyzed by flow cytometry (BD, FACSCanto II, BD Biosciences, San Jose, CA, USA), and fluorescence signals were analyzed using FlowJo software (v10.8.1, FlowJo LLC, Ashland, OR, USA). Briefly, thawed semen samples were washed twice with PBS by centrifugation (1250 rpm, 5 min). The sperm pellet was resuspended in $1 \times$ Binding Buffer at a concentration of 1×10^6 – 1×10^7 sperm/mL. A 100 µL aliquot of the suspension was incubated with 5 µL FITC-Annexin V for 10 min in the dark, followed by 5 µL PI for 5 min. After staining, the sample was washed with 500 µL PBS (1350 rpm, 5 min) and resuspended in 400 µL $1 \times$ Binding Buffer. Then the sample was analyzed by flow cytometry.

Flow cytometry was performed using a BD FACSCanto II system equipped with 488 nm (FITC detection in FL1) and 633 nm (PI detection in FL3) lasers. Samples were acquired at a low flow rate (~30 µL/min) with 10,000 events recorded. Compensation was adjusted using single-stained controls. The data were analyzed with FlowJo software by gating with Forward Scatter/Side Scatter (FSC/SSC) to exclude debris. Cell populations were classified as Annexin V[−]/PI[−] (viable), Annexin V⁺/PI[−] (early apoptotic), and Annexin V⁺/PI⁺ (late apoptotic/necrotic). After the gating process, the proportions of different cell populations were calculated.

2.6.3. Integrity of Spermatozoa Acrosome

Acrosome integrity was determined using PNA-FITC/PI staining (PNA-FITC: Sigma, L7381, Sigma-Aldrich Corporation, Germany; PI: Solarbio, P8080, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and analyzed by flow cytometry and FlowJo software. Thawed semen was washed twice with PBS (1250 rpm, 5 min) and resuspended in PBS to a final concentration of 1×10^6 – 1×10^7 sperm/mL. A 1 mL aliquot was stained with 15 µL PNA-FITC (1 mg/mL, 20 min, dark), followed by centrifugation (1250 rpm, 5 min). The pellet was resuspended in 1 mL PBS, stained with 10 µL PI (1 mg/mL, 10 min, dark), and washed twice with PBS. The sample was resuspended in 1 mL PBS, then analyzed by flow cytometry.

The same instrument settings were applied (BD FACSCanto II, FL1/FL3 channels, 10,000 events). Populations were defined as PNA[−]/PI[−] (acrosome-intact), PNA⁺/PI[−] (acrosome-reacted), and PI⁺ (dead). After the gating process, the proportions of different cell populations were calculated.

2.6.4. Antioxidant Properties

Antioxidant capacity was evaluated through measurements of reactive oxygen species (ROS), malondialdehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The specific catalog numbers and assay principles were as follows: ROS (DCFH-DA method, E004-1-1), MDA (TBA method, A003-1-2), T-AOC (ABTS method, A015-2-1), SOD (WST-1 method, A001-3-2), CAT (ammonium molybdate method, A007-1-1), GSH-Px (DTNB colorimetric method, A005-1-2). All assays were performed strictly according to the manufacturer's protocols.

2.7. Estrus Detection and Artificial Insemination

Estrus was detected each morning using a harness-fitted teaser buck (1:15 buck-to-doe ratio). Does exhibiting a standing reflex (remaining immobile for ≥ 3 s when mounted) were identified as in estrus. For AI, selected does were restrained in dorsal recumbency. After vulval disinfection with 75% ethanol, 0.5 mL thawed semen was deposited 0.5–2.0 cm into the cervical os using a caprine AI gun (Shuangxin, SX-AI-50, Inner Mongolia Saikexing Breeding Biotechnology Group Co., Ltd., Hohhot, China) and a caprine vaginal speculum (Muduobang, MDB-SP10, Xinxiang Muduobang Biotechnology Co., Ltd., Xinxiang, China). All instruments were sterilized with ethanol and rinsed with saline between procedures. Inseminated does were kept undisturbed for ≥ 2 h, with a second insemination performed 8–10 h later. The complete AI protocol, from initial estrus detection to final insemination, was constrained within a 24 h period to coincide with the optimal fertility window. All groups were inseminated in parallel during a single breeding season (October 2024) to eliminate potential confounding effects of seasonal variation on reproductive outcomes.

All reproductive parameters were determined by tracking kidding outcomes five months after artificial insemination (AI). The kidding rate was the percentage of does that gave birth of the inseminated females. Prolificacy rate refers to the percentage of live kids per doe kidded. The kid sex ratio was recorded as the male-to-female proportion among all live births.

2.8. Statistical Analysis

The data were organized using Microsoft Excel 2020 (Microsoft Corp., Redmond, WA, USA) and analyzed via one-way analysis of variance (ANOVA) in SPSS Stat 28 software (IBM Corp., Armonk, NY, USA). The results are expressed as the mean $\pm$ standard deviation. The LSD method was used for significance analysis, with $p < 0.05$ indicating a statistically significant difference and $p > 0.05$ indicating no significant difference.

Intergroup differences in artificial insemination prolificacy were analyzed using Pearson's chi-square test in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The test assumptions were satisfied (all expected frequencies ≥ 5 , no cells with an expected count < 1). Effect sizes were evaluated with the Phi coefficient and Cramer's V.

3. Results

3.1. Effects of Centrifugation Speed on Post-Thaw Spermatozoa Motility and Kinematic Parameters

The effects of different centrifugation speeds on the post-thaw spermatozoa motility and kinematic parameters are shown in Table 1. Spermatozoa motility and VCL were significantly higher in the 750 and 1000 rpm groups compared to the control group ($p < 0.05$), while VSL and VAP showed no significant differences ($p > 0.05$). The 1250 rpm group had significantly higher spermatozoa motility, VCL, and VSL compared to the control and other

experimental groups ($p < 0.05$). The 1500 rpm group showed no significant differences in any parameters compared to the control group ($p > 0.05$).

Table 1. Effects of centrifugation speeds on the motility and kinematic parameters of frozen–thawed cashmere goat spermatozoa ($n = 3$ replicates/group).

Items	Centrifugation Speeds				
	Control Group	750 rpm	1000 rpm	1250 rpm	1500 rpm
spermatozoa motility (%)	47.97 ± 0.19 ^d	51.96 ± 1.07 ^c	55.66 ± 1.04 ^b	60.13 ± 2.67 ^a	49.15 ± 0.54 ^d
VCL (µm/s)	60.42 ± 0.16 ^d	62.53 ± 0.36 ^c	68.31 ± 0.32 ^b	73.03 ± 1.54 ^a	61.64 ± 0.54 ^{cd}
VSL (µm/s)	39.67 ± 0.23 ^b	40.10 ± 0.74 ^b	40.63 ± 1.20 ^b	43.68 ± 1.87 ^a	39.54 ± 0.83 ^b
VAP (µm/s)	45.03 ± 0.39 ^b	45.71 ± 2.80 ^{ab}	46.42 ± 2.29 ^{ab}	49.41 ± 1.86 ^a	46.80 ± 1.88 ^{ab}

Note: Data represent mean ± SD of triplicate measurements ($n = 3$) from pooled semen of 6 bucks. Different superscripts within row indicate significant differences between centrifugation treatments ($p < 0.05$), while same letter indicates no significant difference ($p > 0.05$).

3.2. Effects of L-Proline on Post-Thaw Spermatozoa Motility and Kinematic Parameters

The effects of different L-proline concentrations on the post-thaw spermatozoa motility and kinematic parameters are shown in Table 2. Compared to the control group, the spermatozoa motility in the 10 mmol/L L-proline group (P1 group) showed no significant difference ($p > 0.05$). In contrast, spermatozoa motility was significantly higher in the 30 mmol/L L-proline group (P2 group) and 50 mmol/L L-proline group (P3 group) compared to the control group ($p < 0.05$), with the P2 group showing the highest motility ($p < 0.05$). The VCL was significantly higher in the P1 group compared to the control group ($p < 0.05$), while VSL and VAP showed no significant differences ($p > 0.05$). In the P2 group, VCL and VAP were significantly higher than in the control group ($p < 0.05$), while VSL showed no significant difference ($p > 0.05$). In the P3 group, VCL was significantly higher than in the control group ($p < 0.05$), while VSL and VAP showed no significant differences ($p > 0.05$).

Table 2. Effects of L-proline on motility and kinematic parameters of frozen–thawed cashmere goat spermatozoa ($n = 3$ replicates/group).

Items	Groups			
	Control Group	P1 (10 mmol/L)	P2 (30 mmol/L)	P3 (50 mmol/L)
spermatozoa motility (%)	60.13 ± 2.67 ^c	64.04 ± 1.50 ^{bc}	79.52 ± 2.17 ^a	68.21 ± 1.55 ^b
VCL(µm/s)	73.03 ± 1.54 ^c	77.70 ± 1.27 ^b	90.81 ± 3.14 ^a	78.33 ± 1.74 ^b
VSL(µm/s)	43.68 ± 1.87 ^a	44.23 ± 1.49 ^a	46.68 ± 0.77 ^a	44.54 ± 0.39 ^a
VAP(µm/s)	49.41 ± 1.86 ^b	50.61 ± 0.74 ^b	53.66 ± 0.88 ^a	51.89 ± 0.29 ^{ab}

Note: Data represent mean ± SD of triplicate measurements ($n = 3$) from pooled semen of 6 bucks. Different superscripts within row indicate significant differences between centrifugation treatments ($p < 0.05$), while same letter indicates no significant difference ($p > 0.05$).

3.3. Effects of L-Proline on Spermatozoa Membrane and Acrosome Integrity

The effects of different L-proline concentrations on the spermatozoa membrane and acrosome integrity are shown in Table 3. The spermatozoa membrane and acrosome integrity were significantly higher in the P1, P2, and P3 groups compared to the control group ($p < 0.05$), with the P2 group showing the highest integrity ($p < 0.05$).

Table 3. Effects of L-proline on integrity of plasma membrane and acrosome of frozen–thawed cashmere goat spermatozoa ($n = 3$ replicates/group).

Items	Groups			
	Control Group	P1 (10 mmol/L)	P2 (30 mmol/L)	P3 (50 mmol/L)
Membrane integrity (%)	48.66 ± 1.12 ^d	52.55 ± 0.59 ^c	61.27 ± 0.52 ^a	56.30 ± 0.38 ^b
Acrosome integrity (%)	60.60 ± 0.95 ^d	63.98 ± 1.41 ^c	79.36 ± 1.04 ^a	68.38 ± 0.94 ^b

Note: Data represent mean ± SD of triplicate measurements ($n = 3$) from pooled semen of 6 bucks. Different superscripts within row indicate significant differences between centrifugation treatments ($p < 0.05$), while same letter indicates no significant difference ($p > 0.05$).

3.4. Effects of L-Proline on Spermatozoa Antioxidant Capacity

The effects of different L-proline concentrations on spermatozoa antioxidant capacity are shown in Table 4. ROS and MDA levels were significantly lower in the P2 group compared to the other groups ($p < 0.05$), while T-AOC, SOD, CAT, and GSH-Px activities were significantly higher ($p < 0.05$). Compared to the control group, the P1, P2, and P3 groups all showed significantly lower ROS and MDA levels ($p < 0.05$) and significantly higher T-AOC, SOD, CAT, and GSH-Px activities ($p < 0.05$).

Table 4. Effects of L-proline on antioxidant capacity of frozen–thawed cashmere goat spermatozoa ($n = 3$ replicates/group).

Items	Groups			
	Control Group	P1 (10 mmol/L)	P2 (30 mmol/L)	P3 (50 mmol/L)
ROS (DCFH-DA, RFU/10 ⁶ sperm)	9931.67 ± 591.24 ^a	5876.00 ± 71.14 ^b	3490.67 ± 146.33 ^d	4692.00 ± 128.73 ^c
MDA (nmol/mg prot.)	31.99 ± 1.24 ^a	18.76 ± 1.17 ^b	10.15 ± 0.67 ^d	15.32 ± 0.51 ^c
T-AOC (mmol/g prot.)	0.38 ± 0.01 ^c	0.65 ± 0.02 ^b	1.05 ± 0.05 ^a	0.71 ± 0.02 ^b
SOD (U/mg prot.)	12.2 ± 0.17 ^d	20.62 ± 1.43 ^c	36.81 ± 0.62 ^a	26.33 ± 1.57 ^b
CAT (U/mg prot.)	19.29 ± 0.19 ^d	31.56 ± 1.08 ^c	50.52 ± 1.53 ^a	40.71 ± 1.35 ^b
GSH-Px (U/mg prot.)	483.01 ± 9.85 ^d	633.41 ± 11.72 ^c	784.19 ± 8.35 ^a	692.06 ± 16.76 ^b

Note: Data represent mean ± SD of triplicate measurements ($n = 3$) from pooled semen of 6 bucks. Different superscripts within row indicate significant differences between centrifugation treatments ($p < 0.05$), while same letter indicates no significant difference ($p > 0.05$).

3.5. Effects of L-Proline on Kidding Rate and Prolificacy in AI

The effects of L-proline supplementation on AI kidding rate are shown in Table 5. The kidding rate was highest in experimental group 2 (56.82%), followed by experimental group 1 (51.16%) and the control group (37.21%). The prolificacy was highest in experimental group 1, followed by the control group and experimental group 2. The results of the chi-square test indicated no significant differences in kidding rates among the three groups ($\chi^2(2) = 3.5357$, $p = 0.1707$). The effect size analysis revealed a Phi coefficient of 0.1649 and Cramer's V of 0.1649, suggesting a weak association between groups.

Table 5. The effects of adding L-proline to the cryopreservation diluent on the artificial insemination kidding rate and prolificacy of cashmere goats ($n = 130$).

Items	Groups		
	Control Group	Experimental Group 1	Experimental Group 2
Number of breeding does	43	43	44
Number of conceiving does	16	22	25
Kidding rate (%)	37.21	51.16	56.82
Number of kids produced	20	28	31
Prolificacy	1.25	1.27	1.24

Note: The control group received the basic diluent; experimental group 1 was treated with L proline only; experimental group 2 was treated with L proline and centrifuged. All inseminations were conducted concurrently within one breeding season.

3.6. Effects of L-Proline on Kid Sex Ratio

The effects of L-proline supplementation on kid sex ratio are shown in Table 6. The number of male kids was slightly higher than that of female kids in all groups, with the male-to-female ratio being lower in experimental groups 1 and 2 compared to the control group.

Table 6. Effects of L-proline for frozen storage on gender of kids born from artificial insemination in cashmere goats ($n = 130$).

Items	Groups		
	Control Group	Experimental Group 1	Experimental Group 2
Number of male kids	11	15	16
Number of female kids	9	13	15
Kid sex ratio	1.22:1	1.15:1	1.07:1

Note: The control group received the basic diluent; experimental group 1 was treated with L proline only; experimental group 2 was treated with L proline and centrifuged. The kid sex ratio is expressed as male to female. All inseminations were conducted concurrently within one breeding season.

4. Discussion

4.1. Effect of Centrifugation Speed on Quality of Cashmere Goat Frozen Semen

Composed primarily of water, carbohydrates, amino acids, and lipids, seminal plasma critically supports spermatozoa motility, preservation, and metabolic function [15]. However, studies have shown that seminal plasma contains various anti-fertility factors that may impair spermatozoa vitality by inhibiting spermatozoa capacitation or inducing a premature acrosome reaction [6–8]. Particularly in goat semen, the presence of high levels of yolk-coagulating enzyme in the seminal plasma, coupled with the heightened sensitivity of goat spermatozoa to temperature fluctuations during freezing, makes spermatozoa susceptible to physical, chemical, and oxidative damage during the freeze–thaw process. This leads to a significant decline in post-thaw semen quality, severely limiting the widespread application of frozen-semen technology in goats.

The removal of seminal plasma prior to semen cryopreservation has been demonstrated to mitigate its negative effects on spermatozoa. Studies by Zhang Jianjun et al. [7] and Yang Junxiang et al. [16] have shown that centrifuging Boer goat semen at 1000 rpm for 8 min to remove seminal plasma significantly improves post-thaw spermatozoa motility and acrosome integrity while substantially reducing spermatozoa damage. Similarly, Zhao Wenjun et al. [17] reported that centrifuging Shaanbei cashmere goat semen at 1250 rpm for 6 min significantly enhanced post-thaw spermatozoa motility, markedly improved acrosome integrity, and increased conception rates. In this study, the 1250 rpm group exhibited significantly higher post-thaw spermatozoa motility, curvilinear velocity, straight-line velocity, and average path velocity compared to the control and other experimental groups, indicating that 1250 rpm is the optimal centrifugation speed for seminal plasma removal in cashmere goats. This result is consistent with the findings reported by Zhao Wenjun et al. [17].

The improvement in frozen-semen quality following seminal plasma removal may be attributed to several mechanisms. First, the centrifugation process eliminates anti-fertility factors in the seminal plasma, particularly the yolk-coagulating enzyme, which hydrolyzes lecithin into lysolecithin, causing damage to spermatozoa [5]. Second, the removal of leukocytes, bacteria, and various enzymes from the seminal plasma reduces the likelihood of heterogeneous nucleation during freeze–thaw cycles, thereby minimizing ice-crystal-induced damage to spermatozoa [6]. Additionally, the centrifugation process removes dead spermatozoa, further enhancing post-thaw spermatozoa motility and motion parameters.

Notably, this study observed that semen quality initially improved with increasing centrifugation speed, peaking at 1250 rpm, but declined at higher speeds (e.g., 1500 rpm), consistent with the findings of Cheng Ming et al. [18]. This phenomenon may be related to the balance between separation efficiency and spermatozoa damage. At lower centrifugation speeds (e.g., 750 rpm), effective separation of spermatozoa from seminal plasma cannot be achieved, whereas excessively high speeds (e.g., 1500 rpm) may cause physical damage to spermatozoa due to excessive compression. The comparable outcomes between the 1500 rpm and control groups suggest a transitional zone where centrifugal force is neither sufficient to remove detrimental factors nor excessive enough to cause overt sperm damage. This non-linear response highlights the need for the breed-specific optimization of centrifugation protocols. Therefore, precise control of centrifugation speed is critical to achieving optimal separation while minimizing spermatozoa damage, which is essential for refining the seminal plasma removal process. These findings provide a theoretical foundation for improving cashmere goat semen cryopreservation techniques and have significant practical implications for enhancing artificial insemination outcomes.

4.2. Effect of L-Proline on Frozen-Semen Quality

To address the cryodamage caused by cold stress and other factors during cryopreservation, antioxidant supplementation has been widely investigated. Studies have shown that the addition of antioxidants to freezing and/or thawing media can effectively improve post-thaw spermatozoa motility [9,10]. Proline, a natural amino acid, exhibits ideal osmoprotective and antioxidant properties and is widely used as a cryoprotectant in fields such as biology, medicine, and agriculture [19]. A number of studies have confirmed the positive effects of L-proline in semen cryopreservation. For example, Li et al. [11] found that adding an appropriate concentration of L-proline to donkey semen extender significantly improved post-thaw spermatozoa motility and motion parameters, and protected spermatozoa DNA, plasma membrane integrity, acrosome integrity, and mitochondrial function, while reducing ROS levels. However, when the concentration of L-proline exceeded 80 mmol/L, it had a negative impact on donkey spermatozoa. Similarly, Liu et al. [12] demonstrated that adding 10 mmol/L L-proline to Duroc boar semen extender significantly enhanced post-thaw spermatozoa motility, acrosome integrity, and mitochondrial activity compared to the control, 50 mmol/L, and 90 mmol/L groups. Hosseini et al. [20] observed that adding 5 mmol/L proline to buck semen extender significantly improved spermatozoa parameters and reduced overall abnormalities post-thaw. Additionally, Moradi et al. [21] reported, in human spermatozoa studies, that 4 mmol/L L-proline significantly improved post-thaw progressive motility and viability, while significantly reducing MDA and ROS levels and mitigating chromatin damage.

The results of this study show that adding L-proline to cashmere goat semen extender significantly improved post-thaw spermatozoa motility, motion parameters, plasma membrane integrity, and acrosome integrity. Notably, the 30 mmol/L L-proline group exhibited significantly higher values for these parameters compared to the control, 10 mmol/L, and 50 mmol/L groups, which is consistent with the findings of Li [11] and Liu [12]. L-proline enhances the cryotolerance of sperm through the synergistic effects of physical protection (membrane stabilization), chemical protection (antioxidation), and metabolic support (energy supply) [13]. However, high concentrations of L-proline may significantly increase the osmotic pressure of the extender, which could be one of the reasons for acrosome damage [22].

During semen cryopreservation, the polyunsaturated fatty acids in spermatozoa plasma membranes are highly susceptible to oxidative stress and ROS attacks, leading to lipid peroxidation. This process not only compromises membrane integrity but also

impairs membrane fluidity, thereby affecting spermatozoa function [23]. Although spermatozoa chromatin is generally resistant to harsh environments, the accumulation of ROS during cryopreservation can cause harmful effects on chromatin structure, leading to DNA damage [24]. Furthermore, ROS can initiate lipid peroxidation chain reactions by attacking methyl groups in spermatozoa membrane phospholipids, causing cold shock and further exacerbating membrane damage [25]. Although spermatozoa possess inherent antioxidant defenses, these mechanisms are often insufficient to fully counteract the effects of ROS under extreme cryopreservation conditions, resulting in a decline in spermatozoa quality.

In this study, compared to the group without L-proline, the addition of 10 mmol/L, 30 mmol/L, and 50 mmol/L L-proline to the extender significantly reduced spermatozoa ROS and MDA levels while increasing T-AOC and the activities of SOD, CAT, and GSH-Px. The 30 mmol/L group showed the most significant effects, indicating that L-proline enhances spermatozoa antioxidant capacity in a dose-dependent manner. It is noteworthy that the 30 mmol/L group exhibited superior cryopreservation outcomes compared to the 50 mmol/L group. This may be attributed to the fact that higher concentrations of L-proline disrupt the balance between ROS and the antioxidant system [26] while also increasing the osmotic pressure of the extender. These factors can lead to spermatozoa shrinkage and wrinkling during cryopreservation, ultimately compromising spermatozoa quality. Additionally, L-proline may play an important role in protecting mitochondrial and DNA integrity, mitigating damage caused by cold shock and oxidative stress [27]. These results demonstrate that 30 mmol/L L-proline optimally balances cryoprotection and osmotic stability, making it the recommended concentration for cashmere goat semen extenders.

4.3. Combined Effects of Plasma Removal and L-Proline on AI Outcomes

AI technology is widely used in the livestock industry, and the kidding rate is a critical indicator of its success, reflecting the quality of spermatozoa to some extent. Only spermatozoa with high motility and strong movement capabilities can rapidly navigate the female reproductive tract, successfully penetrate the egg's barriers, and achieve fertilization [28]. A common method for assessing spermatozoa fertilization potential is the spermatozoa penetration assay, which uses zona-free heterologous eggs (typically hamster eggs) for in vitro fertilization experiments [29,30]. Ma Yi [31] reported that the spermatozoa penetration rate in the seminal plasma removal group of Boer goats (82.00%) was significantly higher than that in the control group (69.00%). Similarly, Tang Daoling et al. [32] found that the spermatozoa penetration rate in the seminal plasma removal group of Yangtze River Delta white goats (40.00%) was significantly higher than that in the control group (27.00%). In this study, the kidding rate in experimental group 2 (seminal plasma removed, 56.82%) was higher than that in experimental group 1 (seminal plasma retained, 51.16%). This is consistent with the findings of Ma Yi and Tang Daoling, indicating that the removal of seminal plasma can enhance the spermatozoa penetration rate, thereby improving the kidding rate.

The results of this study showed that the kidding rates in experimental group 1 (51.16%) and experimental group 2 (56.82%) were significantly higher than that in the control group (37.21%), with experimental group 2 achieving the highest kidding rate. This aligns with the findings of our preliminary study, which demonstrated that adding 30 mmol/L L-proline to the semen cryopreservation extender significantly enhanced the antioxidant capacity of cashmere goat frozen semen, reducing oxidative stress during cryopreservation. This improvement in antioxidant capacity led to increased spermatozoa motility, plasma membrane integrity, and acrosome integrity, ultimately improving the outcomes of semen cryopreservation and increasing the kidding rate. Additionally, the

lower kidding rate compared to that typically observed under field conditions may be attributed to the genetic factors and feeding management of both bucks and does.

The addition of antioxidants to the semen cryopreservation extender did not significantly affect prolificacy. Ma Wenkui et al. [33] conducted a study in which frozen semen supplemented with different concentrations of melatonin was used for cervical insemination in Hu sheep. Their results showed no significant differences in prolificacy between the experimental and control groups. Similarly, in this study, the prolificacy levels in the control group (1.25), experimental group 1 (1.27), and experimental group 2 (1.24) were comparable. This may be attributed to the fact that prolificacy is primarily influenced by the genetic factors and management practices of rams and ewes, with frozen-semen quality having a relatively minor impact [34]. Additionally, in this study, the number of male kids slightly exceeded that of female kids in the control group, experimental group 1, and experimental group 2. Meanwhile, the male-to-female ratio in experimental groups 1 and 2 was lower than that in the control group. Future studies should increase the number of experimental animals to further explore the specific effects and mechanisms of frozen-semen quality on prolificacy.

5. Conclusions

The optimal centrifugation conditions for seminal plasma removal in cashmere goats are 1250 rpm for 10 min. The addition of L-proline to the semen extender significantly improves the quality of frozen semen by balancing antioxidant protection and osmotic stability, with an optimal concentration of 30 mmol/L. Furthermore, the inclusion of L-proline in the cryopreservation extender enhances the kidding rate of artificial insemination in cashmere goats, and the removal of seminal plasma further improves this effect. Optimizing the frozen-semen production process and identifying suitable antioxidants can improve the quality of cashmere goat frozen semen and enhance the efficiency of artificial insemination, which holds significant importance for the development of the cashmere goat industry.

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