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RESEARCH ARTICLE

Puerarin Supplementation into a Tris-based Extender Improves Sperm Quality, Antioxidant Capacity, and *in vitro* Fertility During Cryopreservation of Hu Ram Semen

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ABSTRACT

Semen cryopreservation is associated with cryo-damages that compromises sperm quality parameters post-thawing. Hence, the present study was planned to evaluate the influence of supplementation of puerarin (PUE) to Tris-based extender on post-thaw semen quality parameters of ram semen, with a view to determine the optimal puerarin concentration for the cryopreservation of Hu ram semen. For this purpose, 100 ejaculates collected twice weekly from five Hu rams were used. Ejaculates collected from 5 rams on each collection day were pooled to yield 20 pooled samples for further processing. These samples were diluted with a Tris-based extender supplemented with 0, 25, 50, 75 and 100µmol/L of PUE solution in DMSO, cryopreserved and stored in liquid nitrogen. Post-thaw results showed that the addition of 75µmol/L PUE significantly improved total sperm motility, progressive sperm motility, as well as some bio-kinetic characteristics, compared with the control and other PUE supplementation groups ($P<0.05$). Sperm bio-kinetic characteristics including amplitude of lateral head displacement, angular displacement and wobble in PUE 75 and 100µmol/L supplementations were also higher than those of the control group ($P<0.05$). Moreover, sperm acrosome integrity, plasma membrane integrity, along with semen total antioxidant content, activities of catalase and superoxide dismutase enzymes, and mitochondrial membrane potential were correspondingly higher in the PUE 75µmol/L group post-thaw ($P<0.05$). In addition, PUE 75µmol/L treatment significantly decreased semen ROS and malondialdehyde level ($P<0.05$). *In-vitro* fertilization rate of semen in the PUE 75µmol/L group (57.14%) was higher than 38.00% for the control group ($P<0.05$). In conclusion, supplementation of 75µmol/L PUE to a Tris-based extender was considered optimal, as it improved post-thaw sperm motility indexes, bio-kinetic characteristics, mitochondrial function, antioxidant enzymes activity, and fertilization potential of Hu ram semen.

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INTRODUCTION

Hu sheep are well-known medium-sized (body length 74-82cm, overall height 65-75cm, withers height 82-92cm), milk and meat purpose native sheep with white lamb-skin under first-class protection in China and widely distributed in Shanghai, Zhejiang and Jiangsu provinces (Liu *et al.*, 2025; Xiang *et al.*, 2025). It is popular for its distinctive behavioral and reproductive characteristics, such as year-around estrus, multiple fetuses per birth, early sexual maturity, excellent growth rate with high adaptability to intensive housing and feeding systems (Li *et al.*, 2024; Zhao *et al.*, 2024). Therefore, the application of reproductive biotechnology like artificial insemination (AI) is essential to rapidly expand the utilization of superior-quality genetic material of Hu sheep for breed improvement, control of sexually transmissible diseases, enhance reproductive efficiency, and reduce the cost of animal transportation while mitigating biosecurity risks (Souza-Fabjan *et al.*, 2023).

Semen cryopreservation involves the storage of superior quality male germplasm at sub-zero temperatures

in a liquid nitrogen (-196°C) tank after dilution with diluents containing various cryoprotective agents, an energy source, antibiotics, and osmotic pressure maintenance substances (Galarza *et al.*, 2023). Sperm cryopreservation suppresses sperm metabolism and biological activities without loss of functionality to overcome the constraint of time and location of use (Saha *et al.*, 2022). However, during the freeze-thawing process of sperm, their anatomical, physiological, and biochemical properties change due to ice crystal formation, osmotic imbalance, abrupt temperature fluctuations, and the reactive oxygen species (ROS) production (Ezzati *et al.*, 2020).

Abundant ROS interact with unsaturated fatty acids present in plasma membrane of the sperm, leading to membrane lipid peroxidation (LPO). This ROS imbalance and LPO lead to changes in sperm viability, membrane structure, mitochondrial matrix, DNA integrity, and proteomic, molecular, and epigenetic alterations, ultimately leading to reduced fertility rate after post-thaw insemination (Peris-Frau *et al.*, 2020). Therefore, various exogenous enzymatic and non-enzymatic antioxidants supplementation into basic semen extender is an effective strategy for mitigating oxidative stress (OS) damages caused by highly reactive (O_2^-, H_2O_2, and OH^-) free radicals to maintain higher post-thaw sperm quality (Berean *et al.,* 2024).

Puerarin ($C_{21}H_{20}O_9$) is a naturally occurring flavonoid compound isolated from the root of a leguminous herbal plant (*Pueraria lobata*), and has unique physicochemical properties (Liga and Paul, 2024). Its chemical structure (7,4-bihydroxy-8c-glycosyl-isoflavone) contains phenolic hydroxyl groups, which further enhance the intermolecular hydrogen bonding potency, making it poorly soluble in water and other lipid compounds (Wu *et al.*, 2025). Pueraria plant extracts contain phytoestrogens, known as triterpenoids, daidzein, genistein, and coumarins, commonly used in traditional Chinese medicine to enhance the immunity, antioxidant capacity, intestinal health, endocrine hormonal production, reproductive function, and growth rate (Guo *et al.*, 2023; Wang *et al.*, 2024; Du *et al.*, 2024). Puerarin exhibited a variety of pharmacological effects, such as preventing atherosclerosis, reducing cardiovascular diseases, inhibiting oxidation and inflammation, anti-diabetic, anti-viral, and anti- apoptotic properties (Wang *et al.*, 2022a; Scarpa *et al.*, 2024; Kou *et al.*, 2025).

An earlier study has shown that the puerarin supplementation through intragastric route to young and adult male mice improves busulfan-induced degeneration of seminiferous tubules, Sertoli cells, and spermatogenesis, and improves sperm quality. Further, it significantly reduces malondialdehyde (MDA) content, caspase-3 expression, suppresses phosphorylation, and inactivates the Mitogen-Activated Protein Kinase (MAPK) pathways to restore the abnormal gene expressions (Li *et al.*, 2023). Cong *et al.* (2017) reported a protective effect of puerarin for heat-stress-induced (42°C for 1.0h) changes in primary cultured bovine Sertoli cells. It significantly reduces oxidative stress damage and apoptosis rate (Bax to Bcl-2 ratio) in bovine Sertoli cells by inhibiting ROS production and upregulating heat shock protein (Hsp72) expression. However, there is relatively little information regarding the effects of supplementing puerarin into the Tris base extender on the post-thaw quality parameters of Hu ram semen. Thus, aim of the current study was to evaluate the efficacy of incorporating puerarin into the basic diluent on motility parameters, biokinetic characteristics, mitochondrial function, oxidative stress markers of sperm, and *in vitro* fertility rate, with a view to find the optimal puerarin supplementation level for cryopreservation of Hu ram semen.

MATERIALS AND METHODS

Experimental animals and semen collection: In this study, five clinically healthy and fertile Hu rams (age, 3-4 years; body weight, 65-70kg; BCS, 3.6±0.4) were used. They were kept at the Yangzhou University (Jiangsu province, Yangzhou city, China) small ruminant experimental station (32.3972°N, 119.436°E) from July to October 2025. These rams were raised under intensive conditions, provided with 0.6kg of concentrate (80% TDN, 19% CP on DM basis) per ram each day, along with alfalfa grass, mineral salts, and free access to clean water.

One hundred and twenty (120) semen ejaculates (24 ejaculates per ram) were collected with the help of an artificial vagina (42°C) two-times a week over a period of 12 weeks. These ejaculates were taken to the laboratory within 25 minutes in a water bath at 37°C for preliminary assessment (Marco-Jimenez *et al.,* 2005). Ejaculates showing the volume of 0.80±0.14mL, pH 7.2±0.4, 87.5±1.5% live sperm, >80% total motility, >90% normal sperm, and >2 billion/mL sperm concentration were selected for further processing. In this way, 100 ejaculates were selected for further processing. Ejaculates collected from five rams on each collection day were pooled to remove the ram effect. Thus, 20 pooled semen samples were available for further processing.

Extender preparation: A common Tris-based extender for semen cryopreservation was used for this experiment. Fructose (0.252g), Tris (1.817g), and citric acid (0.912g) were dissolved in 50mL of sterile water. To protect against bacterial infection, both sodium penicillin and streptomycin sulfate 10000IU/50mL of diluent were mixed. Diluent 1 was obtained by adding 20% pure egg yolk to the Tris-based diluent, followed by stirring at room temperature (20°C). Diluent 2 was prepared by mixing 94% (v/v) diluent 1 with 6% (v/v) glycerol and storing it at 4°C (Zhang *et al.*, 2024a).

Experimental design with puerarin supplementations: Puerarin (Beijing Solarbio Biotechnology Co., Ltd., Catalogue No. 3881990), with molecular weight of 416.38g/mole, was used during the experiment. A 5mM stock solution of puerarin (PUE) in dimethylsulfoxide (DMSO) was prepared through dissolving 2.08mg of PUE in 1mL of DMSO (Cong *et al.*, 2017) and preserved in a refrigerator at 4°C. Pooled semen ejaculates were diluted with a tris-based extender and allocated into five different groups. The control group was without any PUE supplementation. Treatment groups contained 25, 50, 75, and 100µmol/L of puerarin solution. The whole study was divided into three experiments.

Experiment 1: During the first trial, thawed semen samples with control and puerarin supplementations were assessed for sperm motility indexes like total sperm motility (TM), progressive sperm motility PM), bio-kinetic parameters including average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), wobble (WOB), amplitude of lateral head displacement (ALH), linearity (LIN), beat cross frequency (BCF), mean angular displacement (MAD), and morphology parameters like sperm plasma membrane integrity (PMI) and sperm acrosome integrity (ACI). For this purpose, a computer-assisted sperm analyzer (CASA; Mailang Company ML608-JZ11, version 6, Nanning, China) was used.

Experiment 2: During the second trial, diluted samples with control and different PUE supplementations were assessed for oxidative stress parameters like ROS, malondialdehyde MDA), total antioxidant content (T-AOC), and antioxidant enzymes (catalase-CAT and superoxide dismutase-SOD) activities, and mitochondrial membrane potential (MMP).

Experiment 3: During the third trial, diluted samples with control and PUE (75µmol/L) supplementation were assessed for *in-vitro* fertilization (IVF) rates.

Dilution, freezing and thawing protocol for semen: Pooled semen samples were allocated into five equal fractions in 2ml test tubes with control and 25, 50, 75, and 100µmol/L of puerarin supplementation and diluted 1:3 (v/v) with diluent I at 37°C. After equilibration for 4h at 5°C, samples were diluted 1:2 (v/v) with diluent II and kept at 5°C for 4h. Then samples were loaded into 0.25mL mini straws, and sealed with polyvinyl alcohol (PVA) powder. For freezing, samples were placed horizontally in liquid nitrogen (LN_2) vapors, 5cm above the gas for 15min, plunged into the LN_2 tank and stored for two weeks before thawing. After thawing each straw at 37°C for 40sec, sperm concentration of approximately 3.22 billion/mL was achieved through dilution (1:5) with the basic extender and used for the evaluation of post-thaw sperm quality parameters (Zhang *et al.*, 2024b).

Sperm motility and bio-kinetics parameter evaluation: After thawing and dilution with the basic extender, a 1.7µL droplet of semen sample was placed on the sperm cell counting chamber for estimation of motility indexes and bio-kinetic evaluation. Five random fields of view were captured for each analysis, and the procedure was repeated three times. At least 400 sperm were examined using CASA fixed with a CCD camera at 100× magnification, as described earlier (Zhang *et al.*, 2025).

Sperm morphology assessment: The hypo-osmotic swelling test (HOST) was used to estimate the sperm PMI. Hypo-osmotic swelling (HOS) solution was prepared by mixing 0.49g sodium citrate and 0.90g fructose in 100mL distilled water. After thawing and dilution, 10µL sample was added to 100µL of HOS solution and incubated for 45min at 37°C. A 1.7µL drop of the mixed aliquot was spread on a glass slide and at least 300 sperm were examined under a phase contrast microscope (400x) for bending and non-bending of sperm tails (Fig. 1A) to estimate the proportion of sperm with intact or damaged plasma membrane (Sun *et al.*, 2024).

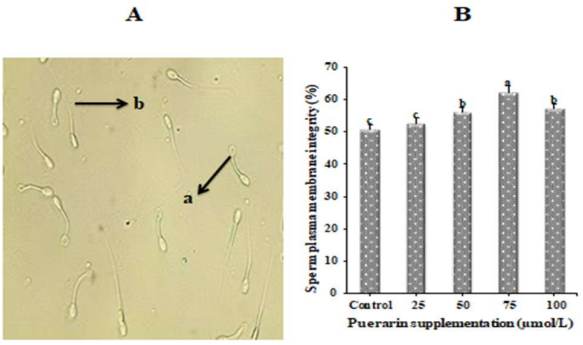

Fig. 1: Influence of puerarin supplementation on plasma membrane integrity (PMI) of sperm after cryopreservation. A: Photographic presentation of sperm with curled tail (a) and non-curled tail (b). B: Quantitative presentation of sperm PMI. Bars with different letters show significant differences among groups (P<0.05).

Sperm acrosome integrity of freeze-thaw samples was assessed using coomassie brilliant blue staining test (Sohail *et al.*, 2025). Briefly, staining solution was prepared by mixing 100mg of G-250 dye in 50mL of 95% ethanol solution, and then 100mL of 85% phosphoric acid was added, with the ultimate volume was fixed to 1.0L. After thawing and dilution with the basic extender, a 50µL semen sample was mixed with 1000µL of a 4% paraformaldehyde solution. After 15min, the solution was centrifuged at 3500×g for 5min, the supernatant was discarded, 10µL of the sample was spread on a slide, and the smear was colored with the staining solution. At least 300 sperm heads were evaluated under oil immersion lens (1000x) using the Smart digital imaging system (BX30; Olympus, Tokyo city, Japan), and 10 images under different fields were collected to define the fraction of sperm with intact or damaged acrosome. The intact acrosome sperm heads appeared blue, while damaged acrosome sperm heads appeared colorless (Fig. 2A).

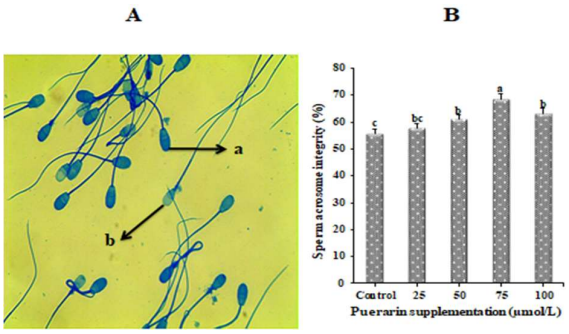

Fig. 2: Influence of puerarin supplementation on acrosome integrity (ACI %) of sperm after cryopreservation. A: Photographic presentation of spermatozoa head stained with a staining solution was observed under a microscope. Blue spermatozoa head indicated intact acrosome (a), while a stainless and colorless spermatozoa head means loss of acrosome integrity (b). B: Quantitative presentation of sperm ACI. Bars with different letters show significant differences among groups (P<0.05).

Estimation of lipid peroxidation and oxidative stress damage: A lipid peroxidation test kit (S0132S; Beyotime, Shanghai, China) was used to evaluate MDA content in the frozen-thawed samples. An antioxidant (S0131M-4) 18µL,

thiobarbituric acid (TBA) diluent (S0131M-3) 900µL, and TBA formulations (S0131M-2) 300µL were mixed to prepare 1218µL MDA working solution. The thawed semen sample was diluted with the basic extender, and semen samples and kit reagents were processed as per the instructions given with the kit. The standard curve was obtained by diluting the substance (1mM) in the kit to various concentration gradients against the MDA working solution. Results were attained by comparing MDA values with the standard curve following the kit directions, and expressed as nmol/mg of protein concentration (Sohail *et al.*, 2024).

The ROS assay kit (R0254; Solarbio Enterprise, Beijing city, China) was used to estimate oxidative stress damage to the sperm cells. Thawed semen samples were diluted with the basic extender, and semen samples and kit reagents were processed as per the instructions given with the kit. A microplate reader analysis system (Perkin Elmer, Waltham Corporation, Massachusetts, USA) was used to assess the fluorescence strength, an indicator of ROS. The excitation and emission wavelengths of the probe were set at 488/525nm for the magnitudes of fluorescence intensity.

Estimation of sperm mitochondria function and semen total antioxidant content: An MMP (Mitochondrial Membrane Potential) analysis kit with JC-1 probe (AS20082; Solarbio, Beijing, China) was used to evaluate the mitochondrial function of the sperm samples. The thawed samples were diluted with basic extender, washed two times with phosphate buffer saline (500µL), and centrifuged at 3500rpm for 8min. After discarding supernatant, 600mL of JC-1 working solution was added, mixed well, stored at 37℃ for 30min in the dark and washed twice with JC-1 dye. The fluorescence intensity fraction of red fluorescence with an excitation wavelength of 525nm and an emission wavelength of 590nm was used. The fluorescence intensity fraction of green fluorescence with an excitation wavelength of 488nm and an emission wavelength of 525nm was used. This red/green fluorescence intensity was calculated through a multifunctional enzyme marker system for MMP assessment.

A T-AOC (Total antioxidant capacity) assay kit (AOX-21; Nanjing Institute of Technology, China) was used to assess the total antioxidant content of semen samples, with a standard curve plotted through dilution of standard (10mM) Trolox solution with distilled water to various concentration gradients (0.1, 0.2, 0.4, 0.8, and 1.0mM). Thawed semen samples were diluted with basic extender, and supernatants were obtained through centrifugation at 1500rpm for 15min of 100µL of the thawed semen sample. Next, 15µL of supernatant was mixed into each hole of 96 well plates. Afterward, 170µL of ABTS working solution as substrate and 20µL of enzyme solution were added, mixed well, incubated at 37℃ for 20min under dark and examined at 405nm wavelength under a multifunctional fluorescence enzyme marker system. Results were attained by comparing the value with the standard curve and expressed as mmol/L of protein (Sohail *et al.*, 2025).

Determination of semen antioxidant enzymes activity: The catalase (CAT) and superoxide dismutase SOD) test kits (AB138897 and A0014, respectively) from Nanjing Jiancheng Bioengineering Institute, China, were used to assess the CAT and SOD enzyme activities of semen samples. Thawed semen samples were diluted with basic extender, and 200µL of semen sample was washed two times with phosphate buffer saline (600µL) and seminal plasma was discarded by centrifugation at 3500rpm for 8 min. After adding 20µL of sperm lysate to each well of the 96-well plate, 50µL each of CAT assay buffer, H_2O_2 substrate, and working solution were added and mixed well. Resultant solution was observed on a 405nm wavelength using a fluorescence detection system, with CAT activity assessed through a standard curve and stated as units/mL of semen sample.

For SOD activity, thawed semen samples were diluted with basic extender, 100µL of the sample was washed two times with phosphate buffer saline (600µl), and supernatant was removed by centrifugation at 8500rpm for 15min. Sperm lysate was obtained by using the lysis buffer supplied in the kit, and 10µL was added to a 96-well plate following the addition of 20µL xanthine oxidase, 20µL SOD standard, and 160µL WST-8. The mixture was incubated at 37°C in the dark for 30min. Finally, samples were examined at 450nm wavelength using a fluorescence enzyme marker system, with results gained through comparing values with the standard curve, and expressed as units/mg of protein in semen samples (Wang *et al.*, 2022b).

Determination of In-vitro fertilization (IVF) rates: Ovaries collected from female sheep after slaughter were used for aspiration of cumulus oocyte complexes (COCs) with follicular fluid from 2-6mm diameter ovarian follicles. Follicular fluid with COCs was washed with washing solution (10mM HEPES buffer solution, 10% bovine serum albumin-BSA, 50µg/mL Gentamycin, pH 7.4), allowed to settle for 15min and searched for COCs under the microscope. Four Petri dishes containing tissue culture medium (TCM-199), added with 10% fetal bovine serum (FBS), 50µg/mL gentamycin, 0.5µg/mL FSH, 0.5µg/mL LH, 1µg/mL estradiol 17-β, with pH 7.2 and osmolarity 280 mOsm/kg, were pre-incubated for 3h before maturation. The COCs were then transferred into the petri dishes and incubated for one day at 38.8℃ under 5% CO_2+ 95% humidity. After incubation, COCs enclosed by a minimum of five granulosa cell layers with clear homogeneous cytoplasm, a thin perivitelline space, an intact polar body, and surrounded by expanded, fluffy cumulus cells were used for in-vitro fertilization (Lorenzo-Torres *et al.,* 2022).

In vitro fertilization of 120 mature oocytes was carried out using 10µL of frozen-thawed semen each for control (50 mature oocytes) and 75µmol/L puerarin (70 mature oocytes) supplementation groups, as described previously (Anzalone *et al.,* 2021). Zygotes were then incubated at 38.8˚C for 2 days in a humidified incubator (5% O_2+5% CO_2 and 95% humidity). Finally, fertilized oocytes were observed under an inverted microscope (Nikon Eclipse TS2; Nikon, Tokyo city, Japan) for pronucleus formation or cleavage rate, as shown in Fig 6A. Fertility rate was determined by the number of fertilized oocytes developed into multicell embryos divided by the total number of mature oocytes used (Lorenzo-Torres *et al.*, 2025).

Statistical analysis: The data set was statistically analyzed using Statistical Product and Service Solutions (SPSS 28.0 Inc., Chicago, IL, USA). The Shapiro–Wilk normality test was used to see whether the data followed the normal distribution. Since the data set exhibited normal distribution, One-way ANOVA was used to assess the statistical differences in sperm quality parameters post-thaw among control and puerarin supplemented groups. Tukey's HSD (honestly significant difference) method was used to compare the mean value among groups. Chi-square test was used to analyze the data on *in vitro* fertility rates to confirm the statistical significances between the control and 75µmol/L puerarin supplementation groups. Results were expressed as the mean±SEM, and a P-value ≤0.05 was considered statistically significant. The data set was repeated five times to confirm the final results to avoid substantial variation.

RESULTS

Sperm motility post-thaw: Sperm total motility (TM) of the PUE 75µmol/L supplementation was higher compared with control and other PUE supplementations (P<0.05). Sperm TM of the PUE 100µmol/L group was higher than the PUE 25 and 50µmol/L supplementations (P<0.05); however, there was no difference in TM between the latter two groups. Sperm TM of the control group was the lowest (P<0.05) relative to TM of all PUE supplementations (Table 1).

Table 1: The effect of different levels of puerarin supplementation on total and progressive motility and some bio-kinetic parameters of sperm after cryopreservation

Treatment (µmol/L)	TM (%)	PM (%)	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)
Control	48.56±1.81^{d}	40.67±1.58^{d}	36.17±0.35^{c}	55.28±0.74^{c}	41.03±0.31^{c}
Pue 25	56.90±4.16^{c}	47.70±4.51^{c}	38.13±0.26^{bc}	56.06±1.06^{c}	44.74±0.50^{b}
Pue 50	58.17±1.30^{c}	50.11±0.75^{b}	40.50±0.56^{b}	58.93±1.15^{b}	45.79±0.23^{b}
Pue 75	66.51±0.65^{a}	56.59±1.46^{a}	45.29±0.18^{a}	64.61±0.96^{a}	48.58±0.83^{a}
Pue 100	61.36±0.82^{b}	51.00±1.96^{b}	41.69±1.55^{b}	59.90±0.54^{b}	45.95±0.46^{b}

Note: $^{a-d}$Values with different superscripts in a column differ significantly from one another (P<0.05).

Sperm progressive motility (PM) of the PUE 75µmol/L group was significantly higher compared to that of the control and other PUE supplementations (P<0.05). Sperm PM for the PUE 25µmol/L treatment was lower compared to the PUE 50 and 100µmol/L treatments (P<0.05), the difference in PM between the latter two groups was non-significant. The PM of the control group was the lowest relative to that for all PUE supplementations (P<0.05; Table 1).

Sperm bio-kinetic parameters post-thaw: The sperm VSL of the PUE 75µmol/L group was higher (P<0.05) than that of the control and other PUE supplementations. There was non-significant difference in the VSL among the PUE 25, 50, and 100µmol/L supplementations. The VSL of the control group was significantly lower (P<0.05) relative to that of all PUE supplementation groups, except PUE 25µmol/L (Table 1).

The sperm VCL of the PUE 75µmol/L group was statistically (P<0.05) higher relative to that of the control and other PUE supplementations. There was non-significant difference in the VCL among the PUE 50 and 100µmol/L supplementations. The VCL of the control and PUE 25µmol/L treatment was significantly lower (P<0.05) compared to other PUE supplementations (Table 1).

The sperm VAP of the PUE 75µmol/L supplementation group was significantly (P<0.05) higher relative to the control and other PUE supplementations. There were non-significant differences in the VAP among the PUE 25, 50, and 100µmol/L supplementations. The VAP of the control group was lower (P<0.05) than that of all PUE supplementations (Table 1).

The sperm ALH of the PUE 75µmol/L supplementation group was significantly higher (P<0.05) relative to the control and PUE 25 and 50µmol/L supplementation groups. There was non-significant difference in the sperm ALH between PUE 50 and 100µmol/L treatment groups. Similarly, there was non-significant difference in the ALH between the control and PUE 25µmol/L supplementation, and was lower (P<0.05) compared to other PUE supplementations (Table 2).

Table 2: The effect of different levels of puerarin supplementation on some bio-kinetic parameters of sperm after cryopreservation

Treatment (µmol/L)	ALH (µm/s)	WOB (%)	BCF(Hz)	LIN (%)	MAD°/s
Control	15.58±0.45^{c}	0.48±0.02^{c}	0.54±0.02^{c}	0.63±0.02^{c}	32.46±1.74^{b}
Pue 25	15.99±0.68^{c}	0.53±0.01^{b}	0.56±0.03^{bc}	0.66±0.02^{b}	35.31±0.76^{a}
Pue 50	17.15±0.67^{b}	0.54±0.02^{ab}	0.59±0.02^{b}	0.67±0.03^{b}	35.59±0.46^{a}
Pue 75	19.69±0.21^{a}	0.56±0.03^{a}	0.63±0.02^{a}	0.70±0.03^{a}	36.70±0.68^{a}
Pue 100	18.53±0.38^{ab}	0.55±0.03^{a}	0.58±0.03^{b}	0.66±0.02^{b}	35.53±0.42^{a}

Note: $^{a-c}$Values with different superscripts in a column show significant differences (P<0.05).

There was non-significant difference in the sperm wobble (WOB) movement between the PUE 75 and 100µmol/L treatment groups, while it was significantly higher in both these groups compared to the control and PUE 25µmol/L group. The difference in WOB between PUE 25 and 50µmol/L groups was also non-significant. The WOB motion of the control group was statistically lower than that of all PUE supplementations (Table 2).

The sperm BCF of the PUE 75µmol/L group was higher (P<0.05) compared to that of the control and other PUE supplementations. There was no difference in the BCF among the PUE 25, 50, and 100µmol/L groups. The BCF of the control group was lower (P<0.05) than that of all PUE supplementations, except PUE 25µmol/L group (Table 2).

The sperm linearity (LIN) of the PUE 75µmol/L group was higher (P<0.05) compared to that of the control and other PUE supplementations. There was non-significant difference in the LIN among the PUE 25, 50, and 100µmol/L groups. The LIN of the control group was lower than that of all PUE supplementations post-thaw (Table 2). There was non-significant difference in the sperm MAD among all PUE supplementation groups. However, all four PUE supplementation groups showed significantly higher MAD values (P<0.05) than the control group (Table 2).

Sperm morphology and membrane integrity: Sperm plasma membrane integrity (PMI) of the PUE 75µmol/L supplementation group was higher than that of the control and other PUE supplementations (P<0.05). However, there was no difference in PMI between the PUE 50 and 100µmol/L supplementations and also between control and PUE 25µmol/L supplementation groups. The PMI of the

control and PUE 25µmol/L supplementation was lower (P<0.05) than that for other PUE supplementations (Fig. 1B).

Sperm acrosome integrity (SAI) of the PUE 75µmol/L group was higher than the control and other PUE supplementations (P<0.05). There was no difference in the SAI among the PUE 25, 50, and 100µmol/L supplementations and also between control and PUE 25µmol/L groups. The SAI of the control group was lower (P<0.05) compared to PUE 50, 75, and 100µmol/L supplementations (Fig. 2B).

Sperm mitochondria function and semen total antioxidant content: The sperm mitochondrial membrane potential (MMP) of the PUE 75µmol/L group was significantly higher compared to that of the control and other PUE supplementations (P<0.05). There was non-significant difference in the sperm MMP between PUE 50 and 100µmol/L supplementations, but both groups showed higher MMP than 25µmol/L group (P<0.05). The MMP of the control group was lower than that of all PUE supplementations (P<0.05; Fig. 3A).

Semen total antioxidant content (T-AOC) of the PUE 75µmol/L group was higher compared to that of the control and other PUE supplementations (P<0.05). T-AOC of the PUE 100µmol/L group was also higher than that for the control, PUE 25, 50µmol/L supplementations. However, there was no difference in the T-AOC between the control and PUE 25µmol/L supplementations (Fig. 3B).

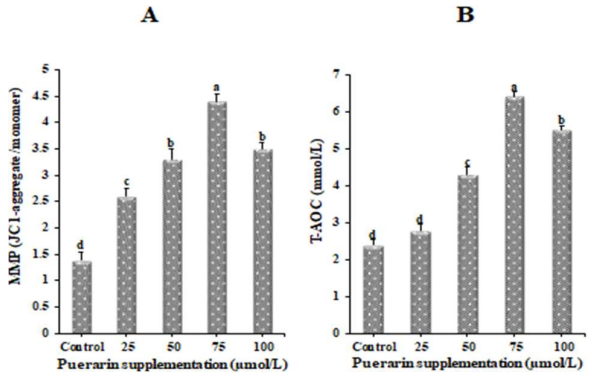

Fig. 3: Influence of different levels of puerarin supplementation on sperm MMP (A), and semen T-AOC after cryopreservation (B). Bars with different letters show significant differences among groups for the same parameter (P<0.05).

Sperm reactive oxygen species and semen malondialdehyde content: The sperm reactive oxygen species (ROS) level of the PUE 75µmol/L group was lower compared to that of the control and other PUE supplementations (P<0.05). There were no differences in the ROS levels between PUE 50 and 100µmol/L supplementations, but both these groups had significantly lower ROS levels compared with the control group. The ROS content of the control group was the highest (P<0.05) when compared to that of PUE supplementations (Fig. 4A).

Semen MDA content in the PUE 75µmol/L group was lower compared with those of the control and other PUE supplementations (P<0.05). There were non-significant differences in the MDA contents between PUE 50 and 100µmol/L treatments, but these groups had lower MDA contents than the control and PUE 25µmol/L groups (P<0.05). The MDA contents of the control and PUE 25µmol/L groups were statistically higher compared to that of other PUE supplementation groups (Fig. 4B).

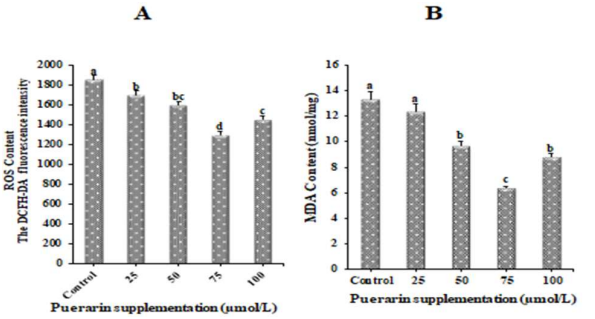

Fig. 4: Influence of different levels of puerarin supplementation on sperm ROS (A) and semen MDA (B) content after cryopreservation. Bars with different letters show significant differences among groups for the same parameter (P<0.05).

Semen antioxidant enzymes activity: Semen superoxide dismutase (SOD) activity of the PUE 75µmol/L group was higher compared to that of the control and other PUE supplemented groups (P<0.05). The SOD activity of PUE 50 and 100µmol/L treatment groups was significantly (P<0.05) higher compared to that of the control and PUE 25µmol/L supplementation. The SOD activity of the control group was the lowest when compared to the four PUE supplemented groups (P<0.05; Fig. 5A).

Semen catalase (CAT) activity of the PUE 75µmol/L supplemented group was the highest when compared to other groups, including control (P<0.05). However, there was no difference in the CAT activity between PUE 50 and 100µmol/L supplementations. The CAT activity of the control group was lower (P<0.05) than that of all PUE supplemented groups (Fig. 5B).

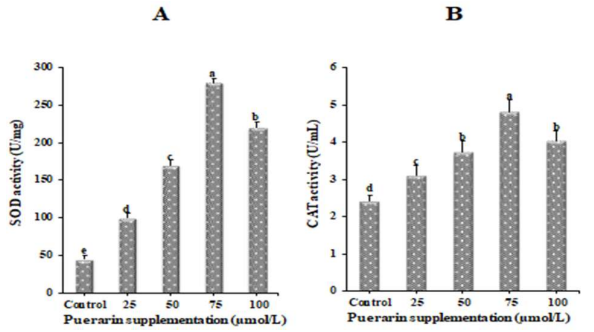

Fig. 5: Influence of different levels of puerarin supplementation on semen SOD (A) and CAT (B) enzyme activity after cryopreservation. Bars with different letters show significant differences among groups for the same parameter (P<0.05).

Sperm *in-vitro* fertilization rate: Sperm *in-vitro* fertilization (IVF) rates were compared between control and PUE 75µmol/L supplementation groups (Fig. 6B). Overall, 70 mature oocytes were used to determine IVF rate of the PUE (75µmol/L) group and 50 for the control group; out of these 40 and 19 were fertilized, respectively. These results showed that sperm IVF rate of the PUE 75µmol/L group (57.14%) was significantly higher than 38.00% (P<0.05) recorded for the control group.

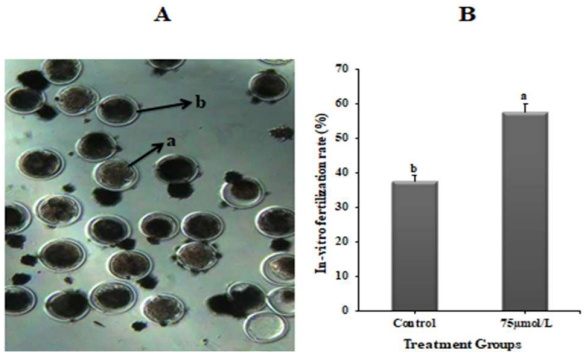

Fig. 6: Influence of 75µmol/L puerarin supplementation on sperm *in vitro* fertilization (IVF) rates after cryopreservation. A: Photographic presentation of fertilized (a) and non-fertilized (b) oocytes. B: Quantitative presentation of in-vitro fertilization rates. Bars with different letters show significant differences between groups (P<0.05).

DISCUSSION

During cryopreservation, sperm are highly vulnerable to damage due to oxidative stress. However, for ensuring successful fertilization after insemination, it is necessary that high-quality of spermatozoa is maintained throughout the collection and freeze-thawing process. Therefore, this study was planned to explore the effect of supplementation of Tris base extender with different concentrations of puerarin on the Hu ram sperm quality parameters, such as sperm motility, bio-kinetic characteristics, oxidative stress markers, mitochondrial function, antioxidant activity, and *in-vitro* fertilization potential after cryopreservation. Results of the current study indicated that the PUE (75µmol/L) supplementation of the Tris-based freezing extender significantly improved sperm morphological, functional, and biological parameters post-thawing than the control group. A highly protective effect of PUE (75µmol/L) on sperm motility parameters and other bio-kinetic parameters (VSL, VCL, VAP, LIN, WOB, MAD, BCF and ALH was observed in this study. This may be due to the strong antioxidant and anti-apoptotic capacity of puerarin, which scavenges oxidative stress markers (ROS, MDA) and upregulates heat shock protein (Hsp72) expression, thereby suppressing the mitochondrial matrix-based apoptotic pathway, such as changes in the Bax/Bcl-2 ratio, during sperm cryopreservation (Cong *et al.*, 2017; Liu *et al.,* 2023).

Significant increase in plasma membrane and acrosome integrity of post-thaw sperm observed in 75µmol/L PUE supplementation group in the current study is supported by findings of a previous study in mice (Li *et al.,* 2023), which showed that dietary puerarin supplementation restored the normal testicular morphology, spermatogenesis, and sperm quality by suppressing phosphorylation and inactivating testicular Mitogen-Activated Protein Kinase (MAPK) signaling pathways. Puerarin interacts with sperm membranes, reducing oxidative stress and lipid peroxidation, maintaining their integrity and fluidity to reduce ice crystal formation and osmotic pressure changes during cryopreservation. Zhang *et al.* (2024c) reported the neuro-protective effect of puerarin through activating chaperon-mediated autophagy, and increasing proteasome activity to prevent toxic intracellular protein accumulation.

The improved MMP and total antioxidant activity observed in this study following PUE supplementation compared to the control group is in line with results of an earlier study in rats (Chen *et al.*, 2018), which revealed that puerarin administration promoted mitochondrial function and prevented the oxidation of fatty acids in skeletal muscle cells, thereby inhibiting intracellular lipid accumulation and demonstrating anti-diabetic activity. This might have been due to the fact that puerarin reversed mitochondrial dysfunction through preventing the accumulation of intramuscular lipids and increasing the copy number of mitochondrial DNA and the expression of the mitochondria-encoded gene cytochrome oxidase-I (MTCO-1) to facilitate the expression levels of mitochondrial sirtuin, and uncoupling proteins (SIRT-1, SIRT-3, UCP-2, UCP-3), superoxide dismutase-2 (SOD-2), total antioxidant content (TAC), and oxidative phosphorylation (OXPHOS) gene in skeletal muscles (Brand and Esteves, 2005; Sparks *et al.*, 2005; Qiu *et al.*, 2010).

Consistent with findings of the present study, recent research has reported improvement in immune response, antioxidant enzyme activity (CAT, SOD), and total antioxidant content, along with the significant decline in oxidative stress and lipid peroxidation (MDA) following dietary puerarin supplementation in sows (Cao *et al.*, 2024) and domestic pigeons (Wang *et al.*, 2024). Puerarin probably reduced excessive generation of ROS, disrupting mitochondrial-dependent cytochrome-C release into cytosol, caspase-3 activation, and apoptosis-related gene (Bax/Bcl-2) expression, which directly protects antioxidant enzyme activity (Kasahara *et al.*, 2002; Gao *et al.*, 2003). Puerarine has also been shown to inhibit the generation of free radicals (H_2O_2, O_2^-, and OH^-), activate nuclear factor erythroid (Nrf2) to promote the synthesis of antioxidant enzymes (CAT, GSH, SOD, γ-GCS) to combat oxidative stress (ROS) through activation of Nrf2/ARE signaling pathway to exhibit its antioxidant activity (Li *et al.*, 2013; Cheng *et al.,* 2016).

No previous reports regarding the positive effect of puerarin on *in-vitro* fertilization rate of frozen-thawed sperm could be traced in the available literature. However, the antioxidant, anti-apoptotic, and membrane-stabilizing properties of puerarin may contribute to its ability to improve the IVF capacity of sperm after cryopreservation. It might retain higher sperm quality along with fertility through suppressing the MAPK pathways and oxidative phosphorylation (Cong *et al.*, 2017; Li *et al.*, 2023). Puerarin belongs to a phytoestrogen compound that shows concentration and dose-dependent effects. Supplementation of optimal concentration of puerarin to semen extender is associated with improvements in sperm quality parameters post-thaw, while higher concentrations might adversely affect the sperm quality. Our findings support an earlier study that confirmed improved sperm function with an optimal dose, but not for long-term supplementation of puerarin (Cederroth *et al.*, 2010; Gray *et al.,* 2015).

Conclusions: In conclusion, PUE (75µmol/L) maintained higher sperm quality parameters after cryopreservation compared with the control and other puerarin supplementation groups. Supplementation of PUE

(75µmol/L) extensively maintained higher physiological and bio-kinetic characteristics, membrane integrity, Total-AOC, antioxidant enzyme activity (SOD, CAT), mitochondrial function (MMP), and *in-vitro* fertility potential of sperm/semen post-thawing. Correspondingly, PUE (75µmol/L) supplementation of semen extender reduced the oxidative stress marker (ROS) along with malondialdehyde (MDA) content in the post-thaw Hu ram semen.

Competing interests: No potential conflict of interest relevant to this article was reported.

Availability of data and materials: Upon reasonable request, the datasets of this study will be available from the corresponding author.

Ethics approval and consent to participate: Experimental procedures conducted during the whole research work were duly approved by the Ethical Committee for Animal Care of Yangzhou University (ID 202206132), China.

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

REFERENCES

Anzalone DA, Palazzese L, Czernik M, *et al.*, 2021. Controlled spermatozoa–oocyte interaction improves embryo quality in sheep. Scientific Reports 11(1): 22629.

Berean DI, Bogdan LM and Cimpean R, 2024. Advancements in understanding and enhancing antioxidant-mediated sperm cryopreservation in small ruminants. Challenges and perspectives. Antioxidants 13(6): 624.

Brand MD and Esteves TC, 2005. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell Metabolism 2(2): 85-93.

Cao S, Li X, Yin H, *et al.*, 2024. Dietary puerarin supplementation improves immune response and antioxidant capacity of sows. Antioxidants 13(3): 290.

Cederroth CR, Zimmermann C, Beny JL, *et al.*, 2010. Potential detrimental effects of a phytoestrogen-rich diet on male fertility in mice. Molecular and Cellular Endocrinology 321(2): 152-160.

Chen Xf, Wang L, Wu YZ, *et al.*, 2018. Effect of puerarin in promoting fatty acid oxidation by increasing mitochondrial oxidative capacity and biogenesis in skeletal muscle in diabetic rats. Nutrition and Diabetes 8(1):1. Doi: 10.1038/s41387-017-0009-6.

Cheng Y, Leng W and Zhang J, 2016. Protective effect of puerarin against oxidative stress injury of neural cells and related mechanisms. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 22: 1244-49.

Cong X, Zhang Q, Li H, *et al.*, 2017. Puerarin ameliorates heat stress–induced oxidative damage and apoptosis in bovine Sertoli cells by suppressing ROS production and upregulating Hsp72 expression. Theriogenology 88: 215-227.

Du X, Zhao D, Pian H, *et al.*, 2024. Effects of puerarin as a feed additive on the laying performance, egg quality, endocrine hormones, antioxidant capacity, and intestinal morphology of aged laying hens. Poultry Scince 103(5): 103420.

Ezzati M, Shanehbandi D, Hamdi K, *et al.*, 2020. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. Cell and Tissue Banking 21(1): 1-15.

Galarza DA, Duma M, Samaniego JX, *et al.*, 2023. Cryopreservation of domestic and wild animal spermatozoa: Update of Knowledge. In: Silva AR (ed), Theriogenology-Recent Advances in the Field. Intechopen, pp:1-33. DOI: 10.5772/intechopen.111918.

Gao HB, Tong MH, Hu YQ, *et al.*, 2003. Mechanisms of glucocorticoid-induced Leydig cell apoptosis. Molecular and Cellular Endocrinology 199(1-2): 153-163.

Gray SL, Lackey BR and Boone WR, 2015. Impact of Kudzu and puerarin on sperm function. Reproductive Toxicology 53: 54-62.

Guo S, Hu J, Ai S, *et al.*, 2023. Effects of pueraria extract and curcumin on growth performance, antioxidant status and intestinal integrity of broiler chickens. Animals 13(8): 1276.

Kasahara E, Sato EF, Miyoshi M, *et al.*, 2002. Role of oxidative stress in germ cell apoptosis induced by di (2-ethylhexyl) phthalate. Biochemical Journal 365(3): 849-856.

Kou B, Meng L, Zhao M, *et al.*, 2025. Unveiling the power of Pueraria lobata: a comprehensive exploration of its medicinal and edible potentials. Frontiers in Pharmacology 16: 1578472.

Li HT, Zhong K, Xia YF, *et al.*, 2023. Puerarin improves busulfan-induced disruption of spermatogenesis by inhibiting MAPK pathways. Biomedicine and Pharmacotherapy 165: 115231.

Li R, Liang T, Xu L, *et al.*, 2013. Puerarin attenuates neuronal degeneration in the substantia nigra of 6-OHDA-lesioned rats through regulating BDNF expression and activating the Nrf2/ARE signaling pathway. Brain Research 1523: 1-9.

Li T, Xing F, Zhang N, *et al.*, 2024. Genome-Wide association analysis of growth traits in Hu sheep. Genes 15(12): 1637.

Liga S and Paul C, 2024. Puerarin—a promising flavonoid: biosynthesis, extraction methods, analytical techniques, and biological effects. International Journal of Molecular Sciences 25(10): 5222.

Liu X, Huang R and Wan J, 2023. Puerarin: A potential natural neuroprotective agent for neurological disorders. Biomedicine and Pharmacotherapy 162: 114581.

Liu Z, Zhang N, Wen Z, *et al.*, 2025. Genomic insights into the origin, high fecundity and environmental adaptation of Hu sheep. Advanced Science 12(37): e06492.

Lorenzo-Torres A, Rangel-Santos R, Bautista-Pére YV, *et al.*, 2025. The presence and size of the corpus luteum influence the in vitro production of sheep embryos. Veterinary Sciences 12(8): 690.

Lorenzo-Torres A, Rangel-Santos R, Ruiz-Flores A, *et al.*, 2022. In vitro embryo production from ewes at different physiological stages. Journal of Veterinary Science 24(1): e10. doi: 10.4142/jvs.22168.

Marco-Jimenez F, Puchades S, Gadea J, *et al.*, 2005. Effect of semen collection method on pre-and post-thaw Guirra ram spermatozoa. Theriogenology 64(8): 1756-1765.

Peris-Frau P, Soler AJ, Iniesta-Cuerda M, *et al.*, 2020. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. International Journal of Molecular Sciences 21(8): 2781.

Qiu X, Brown K, Hirschey MD, *et al.*, 2010. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metabolism 12(6): 662-667.

Saha A, Asaduzzaman M and Bari FY, 2022. Cryopreservation techniques for ram sperm. Veterinary Medicine International 2022(1): 7378379.

Scarpa ES, Antonelli A, Balercia G, *et al.*, 2024. Antioxidant, anti-inflammatory, anti-diabetic, and pro-osteogenic activities of polyphenols for the treatment of two different chronic diseases: type 2 diabetes mellitus and osteoporosis. Biomolecules 14(7): 836.

Sohail T, Sun Y, Chen F, *et al.*, 2025. Influence of lycopene on sperm quality, antioxidant capacity, mitochondrial potential, and oxidation status during 4°C preservation of ram semen. Pakistan Veterinary Journal 45(3): 1235-1243.

Sohail T, Zhang L, Wang X, *et al.*, 2024. Astaxanthin improved the quality of Hu ram semen by increasing the antioxidant capacity and mitochondrial potential and mitigating free Radicals-Induced oxidative damage. Animals 14(2): 319.

Souza-Fabjan J, Oliveira M, Guimarães M, *et al.*, 2023. Non-surgical artificial insemination and embryo recovery as safe tools for genetic preservation in small ruminants. Animal 17(1): 100787.

Sparks LM, Xie H, Koza RA, *et al*., 2005. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes 54(7): 1926-1933.

Sun X, Zhang L, Kang Y, *et al*., 2024. Alpha-lipoic acid improves the quality of ram spermatozoa stored at 4 C by reducing oxidative stress and increasing mitochondrial potential. Frontiers in Veterinary Science 10: 1345016.

Wang D, Bu T, Li Y, *et al*., 2022a. Pharmacological activity, pharmacokinetics, and clinical research progress of puerarin. Antioxidants 11(11): 2121.

Wang R, Li T, Pan Z, *et al*., 2024. Effect of dietary puerarin supplementation on growth performance, immune response, antioxidant capacity, and intestinal morphology in domestic pigeons (Columba livia). The Journal of Poultry Science 61: 2024003.

Wang Y, Zhang L, Sohail T, *et al*., 2022b. Chlorogenic acid improves quality of chilled ram sperm by mitigating oxidative stress. Animals 12(2): 163.

Wu Q, Chen K, Ma L, *et al*., 2025. Pueraria: A comprehensive review of chemical composition, health benefits, and current applications. Comprehensive Review in Food Science and Food Safety 24(3): e70177.

Xiang X, Guo Y, Zhao L, *et al*., 2025. Whole genome sequencing and structural variations provide insights into the body size traits of Hu sheep. Scientific Data 12(1): 1373.

Zhang L, Jiang C, Sun Y, *et al*., 2025. β-Carotene addition into a Tris-based diluent improves Hu ram sperm parameters after cryopreservation. Cryobiology 119: 105254.

Zhang L, Jiang C, Wang X, *et al*., 2024a. Freezing Hu ram semen: influence of different penetrating cryoprotectants and egg yolk level on the post-thaw quality of sperm. Animal Bioscience 37(9): 1548.

Zhang L, Wang X, Jiang C, *et al*., 2024b. Effects of different diluents and freezing methods on cryopreservation of Hu ram semen. Veterinary Sciences 11(6): 251.

Zhang N, Guo P, Zhao Y, *et al*., 2024c. Pharmacological mechanisms of puerarin in the treatment of Parkinson's disease: An overview. Biomedicine and Pharmacotherapy 177: 117101.

Zhao L, Yuan L, Li F, *et al*., 2024. Whole-genome resequencing of Hu sheep identifies candidate genes associated with agronomic traits. Journal of Genetics and Genomics 51(8): 866-876.