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EFFECTS OF BERBERINE SUPPLEMENTATION ON THE POST-THAW QUALITY OF BOAR SEMEN

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Abstract

BACKGROUND: Berberine (BBR) is known for its strong free radical-scavenging and antioxidant activities and may alleviate oxidative stress-induced damage during semen cryopreservation. **OBJECTIVE:** This study aims to evaluate the impact of BBR supplementation in the freezing extender on the cryopreservation quality of boar semen. **MATERIALS AND METHODS:** BBR was incorporated into the freezing extender at concentrations of 0 (control), 5, 10, and 20 μmol/L. Frozen semen straws were prepared following a standard protocol. Post-thaw, assessments were conducted on sperm viability, motility, motility parameters, acrosome integrity, plasma membrane integrity, antioxidant capacity, reactive oxygen species (ROS) levels, and mitochondrial membrane potential (MMP). **RESULTS:** The 10 μmol/L BBR-treated group exhibited significantly higher sperm viability, motility, and velocity parameters, including straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP), compared to the control group ($P < 0.05$). Additionally, acrosome integrity and plasma membrane integrity were significantly enhanced in the 10 μmol/L BBR-treated group relative to the control group ($P < 0.05$). Furthermore, the group treated with 10 μmol/L BBR exhibited a significant reduction in malondialdehyde (MDA) content and ROS levels, while demonstrating the highest superoxide dismutase (SOD) activity, total antioxidant capacity (T-AOC), and MMP ($P < 0.05$). **CONCLUSION:** The addition of BBR to the freezing extender enhanced the post-thaw quality of boar sperm, with the 10 μmol/L concentration providing the most effective protective benefits among the concentrations evaluated.

Keywords: berberine; boar semen cryopreservation; frozen–thawed sperm; oxidative stress.

INTRODUCTION

Semen cryopreservation, which allows semen collection and artificial insemination (AI) to be temporally separated, is an important approach for the long-term preservation, genetic improvement, and interregional transportation of superior germplasm resources (1) . At present, this technology is relatively well established for bovine semen preservation, whereas liquid storage remains the most commonly used method for porcine semen preservation (2). Compared

with bovine sperm, porcine sperm are more sensitive to cold stress and osmotic changes during the freeze–thaw process, resulting in lower post-thaw viability and fertilizing capacity, which severely limits the widespread application of frozen boar semen technology (3). Studies have shown that the excessive production of reactive oxygen species (ROS) during freeze–thawing is a key factor contributing to porcine sperm damage (4). The plasma membrane of porcine sperm is rich in polyunsaturated fatty acids (PUFAs), making it highly susceptible to lipid peroxidation during cryopreservation. Lipid peroxidation can disrupt plasma membrane structure, compromise acrosome integrity, and impair mitochondrial function, ultimately leading to decreased sperm motility and even apoptosis (5). Therefore, supplementation with exogenous antioxidants to alleviate oxidative stress-induced damage and maintain the structural and functional stability of sperm has become an important research direction for improving the cryopreservation quality of boar semen.

Berberine (BBR) is a natural isoquinoline alkaloid that exhibits multiple biological activities, including antioxidant (6), anti-inflammatory, and energy metabolism-regulating effects (7, 8). Previous studies have demonstrated that BBR can scavenge free radicals, enhance antioxidant enzyme activity, and maintain mitochondrial function, thereby alleviating lipid peroxidation and oxidative DNA damage, improving cellular antioxidant defense capacity, and exerting protective effects in various cellular injury models (9). However, studies on the application of BBR in boar semen cryopreservation remain limited, and its optimal supplementation concentration and effects on the physiological functions of post-thaw boar sperm remain unclear. Therefore, this study aimed to investigate the effects of supplementing boar semen freezing extender with different concentrations of BBR on the cryopreservation quality of boar semen.

MATERIALS AND METHODS

Reagents and instrumentation

In this study, unless specified otherwise, all reagents were procured from Sigma-Aldrich and were of analytical grade. Coomassie Brilliant Blue G-250 was purchased from Shanghai Sangon Biotech Co., Ltd. The ROS assay kit was purchased from Jiangsu KeyGEN BioTECH Co., Ltd. Superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. The mitochondrial membrane potential assay kit with JC-1 was purchased from Beyotime Biotechnology Co., Ltd. The sperm viability assay kit was purchased from Beijing Leagene Biotechnology Co., Ltd.

The main instruments used in this study included a phase-contrast microscope (AE2000, Motic, China), an automatic sperm analyzer (ML-608JZ, Nanning Songjing Tianlun Biotechnology Co., Ltd., China), a Multiskan GO microplate reader (Thermo Fisher Scientific, USA), and an upright fluorescence microscope (BX63, Olympus, Japan).

Semen collection and experimental design

Semen was collected from six healthy adult Large White boars aged 18 to 24 months, housed at a commercial breeding facility in Chizhou, Anhui Province, China. Each boar was individually housed under standard management conditions, which included adequate ventilation, natural or artificial lighting, and a commercial boar diet with unrestricted access to clean drinking water. Semen collection occurred weekly from each boar. The use of these animals was sanctioned under ethical guidelines for research. The sperm-rich fraction of the ejaculate was collected utilizing the gloved-hand technique. Post-collection, the semen was filtered through sterile gauze to eliminate gel fractions and impurities, subsequently transported to the laboratory in an insulated container, and processed within 30 min. A routine assessment of semen quality was conducted at 37°C. Ejaculates exhibiting sperm motility of ≥ 80%, abnormal morphology of ≤ 20%, and sperm concentration within the normal range ($1–5 \times 10^8$ sperm/mL) were selected for further analysis. The qualified semen samples were thoroughly pooled for subsequent experimental use. Based on the concentration of BBR added to the cryopreservation extender, four treatment groups were established: 0 μmol/L, 5 μmol/L, 10 μmol/L, and 20 μmol/L BBR, with the 0 μmol/L group serving as the blank control. The pooled semen was diluted with the respective freezing extender to achieve a final concentration of 1.0×10^8 sperm/mL prior to cryopreservation. Each treatment was conducted in triplicate.

Preparation of extenders and reagents

The preparation of the fundamental extender for boar semen cryopreservation involved dissolving 3.3 g of glucose, 4.44 g of citric acid, 7.26 g of tris(hydroxymethyl)aminomethane (Tris), and 0.18 g of penicillin-streptomycin, followed by adjusting the solution to a final volume of 300 mL.

Subsequently, the cryopreservation extender was formulated by incorporating 60 mL of egg yolk and 9 mL of glycerol into the basic extender, with the final volume again adjusted to 300 mL. The resultant mixture was thoroughly stirred and subjected to centrifugation to eliminate any precipitates. This extender was freshly prepared immediately prior to use.

Semen freezing and thawing

Qualified boar semen samples underwent thorough mixing and centrifugation at 1,500 rpm for 3 min to facilitate the removal of seminal plasma. Then, the sperm pellet was resuspended in four distinct cryopreservation extenders, each containing varying concentrations of BBR, using a volume equivalent to that of the extracted seminal plasma (1:1, v/v). The resuspended semen was then loaded into 0.5-mL freezing straws, which were subsequently sealed at both ends with sealing powder. These straws were equilibrated at 4 ℃ for a duration of 2 h. Post-equilibration, the straws were positioned approximately 3 cm above the surface of liquid nitrogen and subjected to liquid nitrogen vapor exposure for 15 min. Thereafter, the straws were immersed in liquid nitrogen for storage within a liquid nitrogen tank. For the thawing process, the frozen semen straws were retrieved from the liquid nitrogen and thawed in a 37 ℃ water bath for 30 s. Upon thawing, both ends of the straws were cut open, and the semen was transferred into EP tubes, followed by incubation at 37 ℃ for 5 min prior to subsequent analyses.

Evaluation of sperm quality parameters

Assessment of sperm motility and kinematic parameters. A 10 μL aliquot of thawed semen was positioned centrally on a standard microscope slide, which had been prewarmed to 37°C, and was immediately covered with a coverslip. Following a 5-min equilibration period, parameters such as sperm motility, viability, straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP) were quantified utilizing an automated sperm analysis system. For each specimen, five microscopic fields were selected at random, with a minimum of 200 spermatozoa counted per field.

Assessment of acrosome integrity. The integrity of the sperm acrosome was evaluated using Coomassie Brilliant Blue staining, as per the methodology of Larson and Miller (10), with minor modifications. In brief, thawed spermatozoa were washed two to three times with PBS. Subsequently, 50 μL of semen was transferred into a centrifuge tube, combined with 1 mL of 4% paraformaldehyde, and fixed for 10 min. The sample was then centrifuged at 1,500 rpm for 3 min, after which the supernatant was discarded, and the pellet was resuspended in PBS.

A 10 μL aliquot of the sperm suspension was dispensed onto a glass slide to create a smear, which was subsequently allowed to air-dry naturally. The smear was then stained with Coomassie Brilliant Blue for a duration of 30 min, rinsed with water, air-dried, and subsequently examined under a microscope. Spermatozoa exhibiting uniform staining in the acrosomal region, characterized by a smooth, regular, and continuous acrosomal outline, were classified as having intact acrosomes; conversely, those not meeting these criteria were classified as acrosome-damaged. Observations were conducted across a minimum of five microscopic fields, with at least 200 spermatozoa counted per field to determine the acrosome integrity rate.

Assessment of plasma membrane integrity. The hypo-osmotic swelling test was employed to evaluate the plasma membrane integrity of frozen-thawed sperm. Specifically, 10 μL of thawed semen was thoroughly mixed with 100 μL of hypo-osmotic solution and incubated at 37°C for 30 min. The samples were then observed and counted under a microscope. Plasma membrane integrity was calculated using the formula: plasma membrane integrity (%) = (number of spermatozoa with coiled tails / total number of spermatozoa counted) × 100. The assay was repeated five times, with at least 200 spermatozoa counted in each field to assess plasma membrane integrity rate.

Assessment of antioxidant capacity. After sperm cells were disrupted using an ultrasonic cell disruptor, the levels of SOD, MDA, and T-AOC were determined strictly according to the instructions of the corresponding assay kits. Each group included three replicates. Absorbance values were measured using a microplate reader, and the results were statistically analyzed.

Detection of intracellular ROS levels. Intracellular ROS levels were detected using a ROS assay kit from KeyGEN BioTECH according to the manufacturer's instructions. Briefly, the sperm concentration was adjusted to 1×10^6 cells/mL. The samples were centrifuged, and the supernatant was discarded. The pellet was resuspended in an appropriate volume of PBS and centrifuged at 1,000 × g for 10 min at room temperature. After removing the supernatant, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted 1:1,000 in PBS to a final concentration of 10 μmol/L. A positive control group was established by treating sperm samples with H_2O_2 before DCFH-DA staining. The sperm pellet was resuspended in the DCFH-DA working solution and incubated at 37 ℃ for 20 min, with gentle mixing every 3–5 min to ensure sufficient contact between the probe and sperm. The samples were then centrifuged at 1,500 rpm for 3 min, the supernatant was discarded, and the sperm were washed three times with PBS.

Finally, the sperm pellet was resuspended in PBS. Fluorescence images were captured using a fluorescence microscope, and the mean fluorescence intensity of sperm in each group was measured using ImageJ software. The fluorescence intensity was used to reflect the intracellular ROS level.

Assessment of mitochondrial membrane potential. The JC-1 staining working solution was prepared according to the instructions of the Beyotime MMP assay kit C2006 and kept on ice before use. The sperm concentration was adjusted to 1×10^6 cells/mL. Equal volumes of semen sample and JC-1 staining solution were mixed and incubated at 37 ℃ in the dark for 20 min.

After incubation, the samples were centrifuged at 600 × g for 3 min at 4 ℃, and the supernatant was discarded. The sperm were washed twice with precooled JC-1 staining buffer (1×) and then resuspended in an appropriate volume of staining buffer.

Red and green fluorescence images were captured separately using a fluorescence microscope. The mean red and green fluorescence intensities of sperm were measured using ImageJ software. The red/green fluorescence intensity ratio was calculated to evaluate the mitochondrial membrane potential.

Statistical analysis

Experimental data were organized using Excel 2021 and analyzed using SPSS 26.0.Before performing statistical analysis, the homogeneity of variance was assessed for all data. Group differences were analyzed by one-way analysis of variance (one-way ANOVA), and multiple comparisons were conducted using the least significant difference (LSD) test and Duncan's multiple range test. Graphs were prepared using GraphPad Prism 9.0, and fluorescence images were analyzed using ImageJ 1.8.0.

All quantitative data are presented as the mean ± standard deviation. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of BBR on motility and kinematic parameters of frozen–thawed boar sperm

Table 1. Effects of berberine (BBR) concentration on the viability, motility, and motility parameters of frozen–thawed boar spermatozoa (n＝5). Values within the same row with different lowercase superscript letters indicate significant differences ($P < 0.05$), whereas values sharing the same lowercase letter indicate no significant difference ($P > 0.05$).

Parameter	BBR concentration			
	Control	5 μmol/L	10 μmol/L	20 μmol/L
Sperm viability (%)	30.99±0.54^{b}	32.32±0.69^{b}	36.48±1.53^{a}	29.19±1.28^{c}
Sperm motility (%)	24.10±1.09^{b}	26.82±1.07^{ab}	29.38±3.99^{a}	23.97±1.51^{b}
VSL(μm/s)	24.53±3.06^{b}	26.91±2.72^{ab}	29.61±1.30^{a}	25.28±1.94^{b}
VCL(μm/s)	55.29±3.23^{b}	63.27±2.84^{a}	64.95±2.62^{a}	54.16±2.21^{b}
VAP(μm/s)	38.29±1.83^{c}	45.01±1.03^{b}	50.33±3.41^{a}	36.22±3.89^{c}

As demonstrated in Table 1, the administration of BBR resulted in an overall enhancement of sperm viability, motility, and kinematic parameters at lower concentrations, with a subsequent decline observed at the highest concentration. Among the treatments, supplementation with 10 μmol/L BBR produced the most favorable overall effect. Sperm viability, motility, VSL, VCL, and VAP in the 10 μmol/L BBR-treated group were significantly higher than those in the control group ($P < 0.05$), and viability and VAP were also significantly higher than those in the 5 and 20 μmol/L BBR-treated groups ($P < 0.05$). In the 5 μmol/L BBR-treated group, VCL and VAP were significantly higher than those in the control and 20 μmol/L BBR-treated groups ($P < 0.05$), whereas viability, motility, and VSL were higher than those in the control group but did not differ significantly ($P > 0.05$). In the 20 μmol/L BBR-treated group, sperm viability was significantly lower than that in the control group ($P < 0.05$), while the remaining motility parameters showed no significant differences compared with the control group ($P > 0.05$).

Effects of BBR on acrosome integrity and plasma membrane integrity of frozen–thawed boar sperm

As shown in Figures 1A and 1B, compared with the control group, sperm acrosome integrity showed an increasing trend in all BBR-treated groups. The 10 μmol/L BBR-treated group exhibited the highest acrosome integrity, which was significantly higher than that of the control group ($P < 0.05$). Although no significant differences were observed between the 10 μmol/L BBR-treated group and the 5 or 20 μmol/L BBR-treated groups ($P > 0.05$), the value in the 10 μmol/L BBR-treated group was numerically higher than those in the 5 and 20 μmol/L BBR-

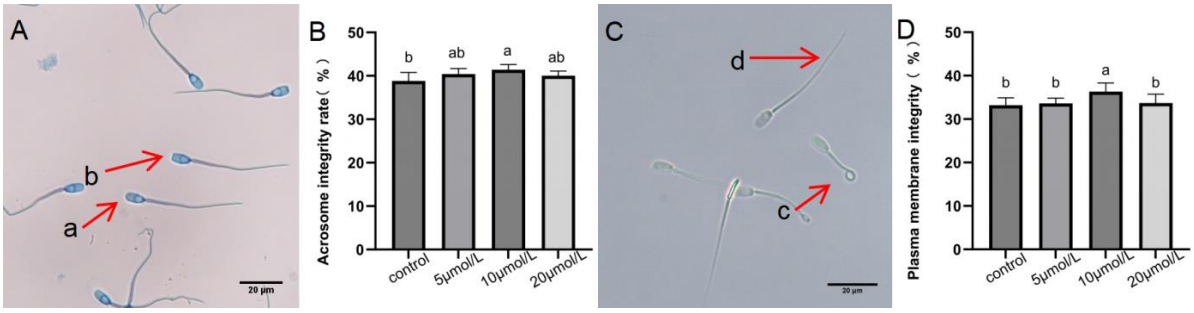

Figure 1. Effects of BBR concentration on acrosome and plasma membrane integrity of boar spermatozoa. (A) Assessment of sperm acrosome integrity, where a indicates an intact acrosome and b indicates a damaged acrosome. (B) Acrosome integrity (%) with treatment. (C) Assessment of sperm plasma membrane integrity, where c indicates an intact plasma membrane and d indicates a damaged plasma membrane. (D) Plasma membrane integrity (%) with treatment. Different letters above the bars indicate significant differences (P < 0.05; n＝5).

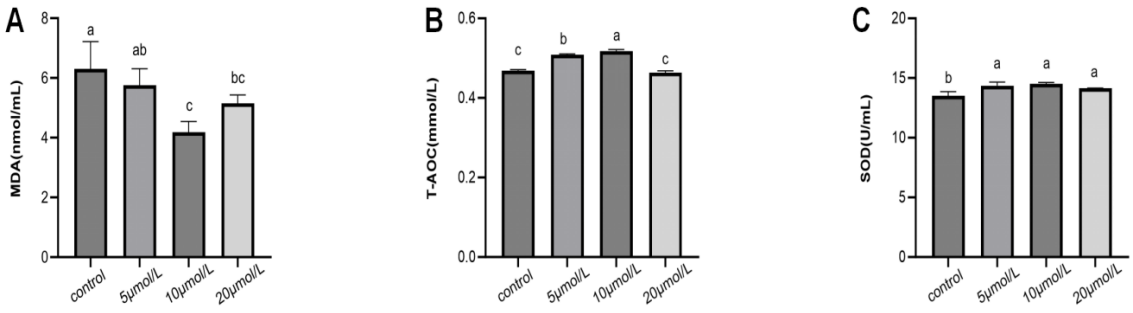

Figure 2. Effects of BBR concentration on the antioxidant capacity (MDA, T-AOC and SOD) of boar spermatozoa. Different letters above the bars indicate significant differences between treatments (P < 0.05; n = 3).

treated groups. Sperm plasma membrane integrity was increased in all BBR-treated groups compared with the control group (Figs 1C, 1D). Plasma membrane integrity in the 10 μmol/L BBR treatment group was significantly higher than that in the other groups. ($P< 0.05$).

Effects of BBR on antioxidant capacity in frozen–thawed boar sperm

As shown in Figure 2A, compared with the control group, MDA content was significantly decreased in the 10 and 20 μmol/L BBR-treated group ($P < 0.05$), with the lowest MDA content observed in the 10 μmol/L BBR-treated group. The 5 μmol/L BBR-treated group also showed a slight decrease in MDA content compared with the control group, but the difference was not significant. As shown in Figure 2B, T-AOC levels were significantly increased in the 5 and 10 μmol/L BBR-treated groups compared with the control group ($P < 0.05$). Among all groups, the 10 μmol/L BBR-treated group exhibited the highest T-AOC level, which was significantly higher than those of the other groups ($P < 0.05$), whereas no significant difference was observed between the 20 μmol/L BBR-treated group and the control group. As shown in Figure 2C, SOD activity was significantly higher in all BBR-treated groups, including the 5, 10, and 20 μmol/L BBR-treated groups, than in the control group ($P < 0.05$).

Effects of BBR on ROS levels in frozen–thawed boar sperm

As shown in Figure 3A–E, the fluorescence images correspond to the PC (positive control), control, 5, 10 and 20 μmol/L BBR-treated groups, respectively. Compared with the control group, ROS-associated fluorescence intensity was significantly decreased in the 10 μmol/L BBR-treated group ($P < 0.05$), and the lowest ROS level was observed at this concentration. The ROS-associated fluorescence intensity in the 5 and 20 μmol/L BBR-treated groups was slightly lower than that in the control group, but the differences were not significant ($P > 0.05$). In addition, although ROS fluorescence intensity was numerically lower in the 10 μmol/L BBR-

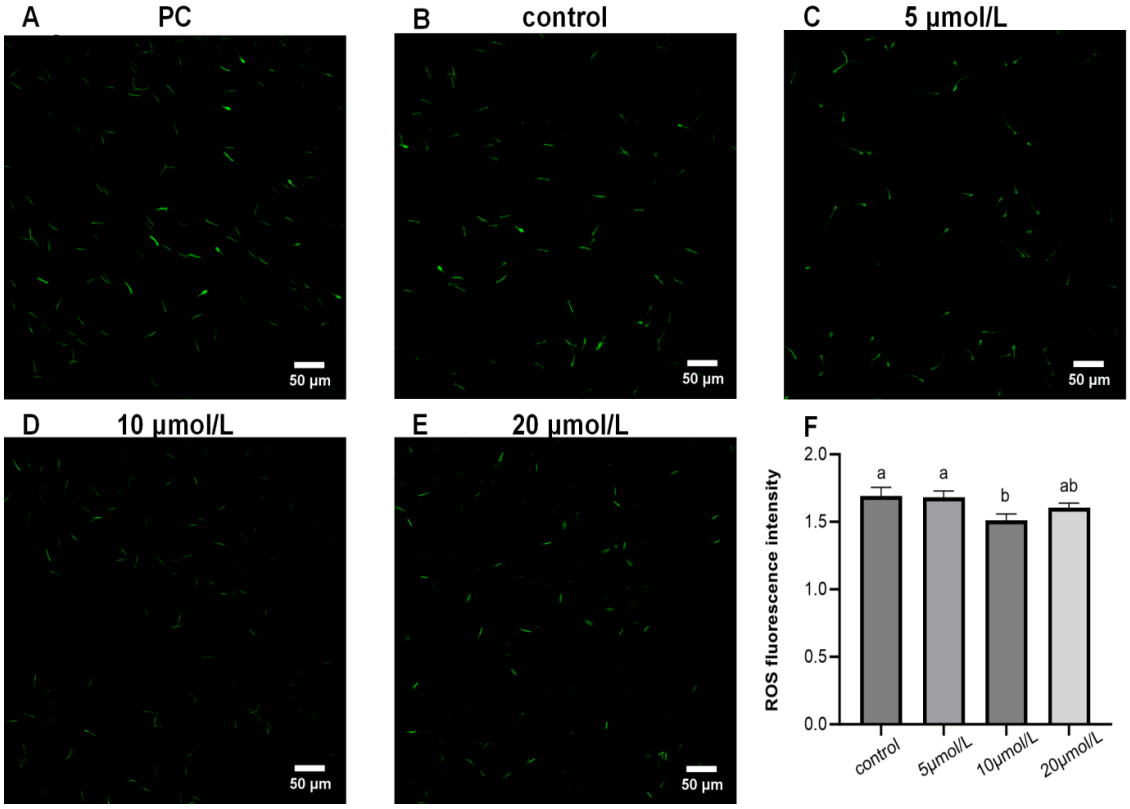

Figure 3. Effects of BBR concentration on intracellular ROS levels in boar spermatozoa (A-E). Note: The positive control (PC) was used only for ROS detection and was excluded from statistical comparisons (F). Different letters above the bars indicate significant differences ($P < 0.05$; n＝3).

treated group than in the 20 μmol/L BBR-treated group, the difference between these two groups was not statistically significant (P > 0.05).

Effects of BBR on MMP in frozen–thawed boar sperm

As shown in Figure 4A, spermatozoa exhibiting red fluorescence indicate high mitochondrial membrane potential, whereas those exhibiting green fluorescence indicate low mitochondrial membrane potential. The quantitative analysis shown in Figure 4B

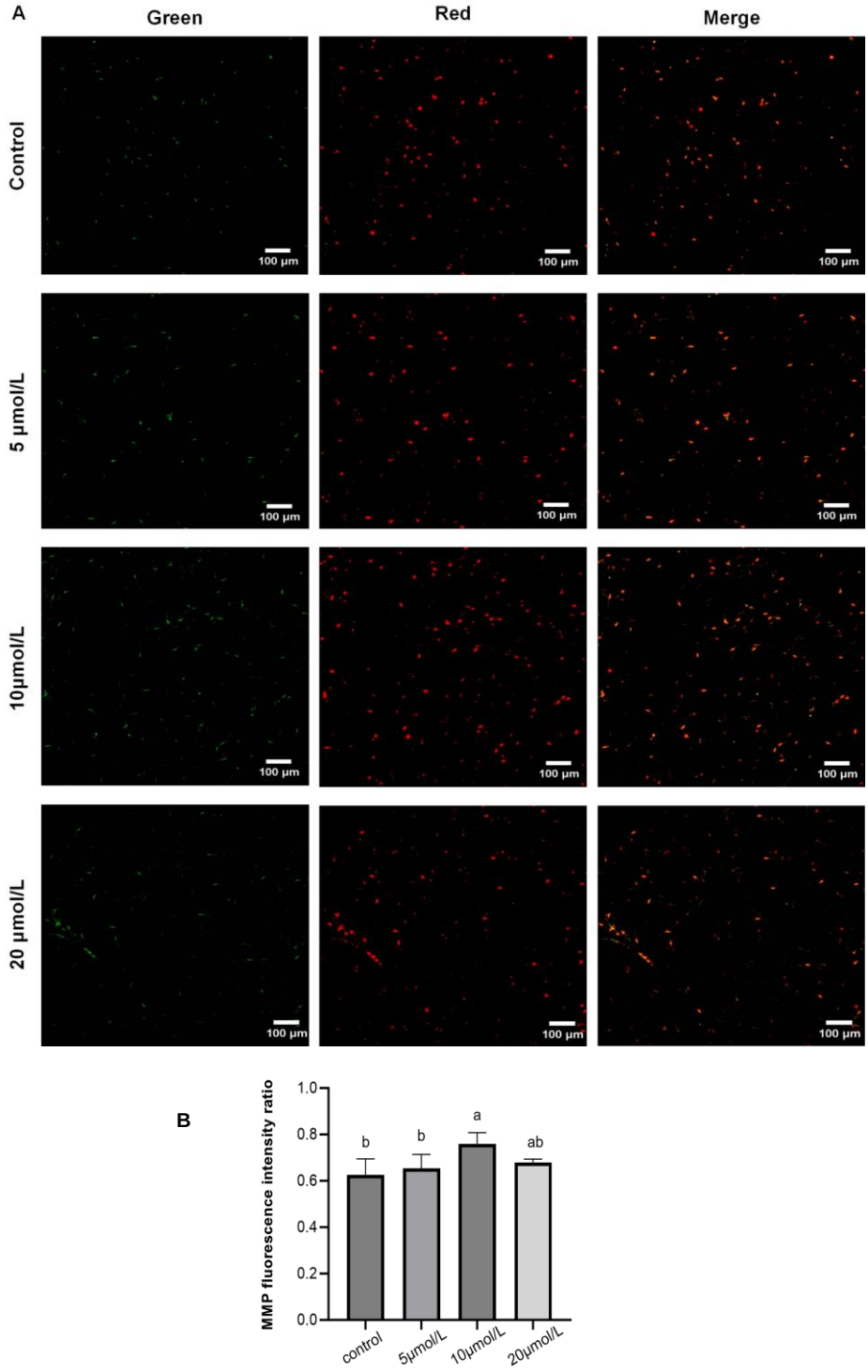

Figure 4. Effects of BBR concentration on the MMP of frozen-thawed boar spermatozoa. (A) JC-1 Fluorescence staining. (B) MMP level. Different letters above the bars indicate significant differences (P < 0.05; n＝3).

indicated that, compared with the control group, the red/green fluorescence intensity ratio of JC-1 staining was significantly increased in the 10 μmol/L BBR-treated group ($P < 0.05$), with the highest MMP level observed at this concentration. No significant difference in the red/green fluorescence intensity ratio of JC-1 staining was observed between the 5 μmol/L BBR-treated group and the control group ($P > 0.05$). The red/green fluorescence intensity ratio of JC-1 staining in the 20 μmol/L BBR-treated group was slightly higher than that in the control group, but the difference was not significant ($P > 0.05$). In addition, no significant difference was observed between the 20 and 10 μmol/L BBR-treated groups.

DISCUSSION

Effects of BBR on motility parameters and membrane integrity of boar spermatozoa

Sperm viability, motility, and motility parameters are important indicators for evaluating the efficiency of semen cryopreservation and are also key factors affecting the fertilizing capacity of spermatozoa (11). The results of this study showed that supplementation of the cryopreservation extender with 10 μmol/L BBR significantly improved the viability and motility of frozen-thawed boar spermatozoa, as well as motility parameters, including VSL, VCL, and VAP. Piri et al. found that the addition of 10 μmol/L BBR to the cryopreservation extender for ram spermatozoa significantly improved sperm motility and viability (12) and our findings are consistent with that study. Maintenance of sperm motility depends on the structural integrity of the flagellum and a continuous ATP supply generated mainly through mitochondrial oxidative phosphorylation (13). However, cryoinjury can disrupt the flagellar structure and impair mitochondrial energy metabolism. In addition, excessive production of ROS during the freeze-thaw process can further damage the inner mitochondrial membrane, interfere with the normal function of the electron transport chain, reduce ATP synthesis, and ultimately impair sperm motility.

The integrity of the sperm plasma membrane and acrosomal structure is an essential basis for maintaining normal sperm fertilizing capacity. During the freeze–thaw process, porcine sperm are susceptible to membrane damage caused by cold shock (14) and osmotic stress (15). In addition, the sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore particularly vulnerable to ROS-induced lipid peroxidation, which reduces membrane stability and subsequently affects sperm capacitation, the acrosome reaction, and sperm–oocyte binding. The results of this study showed that the addition of 10 μmol/L BBR significantly increased plasma membrane integrity and acrosome integrity in frozen–thawed sperm. Lee et al. reported that BBR inhibited lipid peroxidation in a liposome model and exerted a similar effect in a mouse model of TNBS-induced colitis (16). These findings suggest that BBR may reduce lipid peroxidation-related membrane damage during cryopreservation.

Effects of BBR on antioxidant capacity and ROS levels in boar spermatozoa

Oxidative stress is one of the major mechanisms underlying sperm cryoinjury (17). During the freezing–thawing process, low-temperature stress and osmotic pressure changes can induce mitochondrial dysfunction, leading to excessive production of ROS. When ROS accumulation exceeds the scavenging capacity of the endogenous antioxidant defense system in spermatozoa, oxidative stress is triggered, thereby inducing lipid peroxidation (18). This process further damages the sperm membrane structure, mitochondrial function, and sperm physiological functions (19). MDA, an important end product of lipid peroxidation, is commonly used as an indicator of the degree of cellular oxidative damage (20).

The results of this study showed that ROS levels and MDA content in frozen-thawed spermatozoa treated with 10 μmol/L BBR were significantly lower than those in the control group, whereas SOD activity and T-AOC were significantly increased. Previous studies have demonstrated that BBR can alleviate cellular damage by scavenging ROS and ameliorating mitochondrial dysfunction (21). It can also enhance the activities of endogenous antioxidant enzymes, such as SOD, glutathione peroxidase (GPx), and CAT, which play crucial roles in neutralizing ROS and preventing oxidative cellular damage (22). Furthermore, BBR can act as a free radical scavenger, further supporting its role as a potent antioxidant (23). Taken together, these findings suggest that 10 μmol/L BBR-treatment may alleviate oxidative stress-induced cryodamage in spermatozoa during the freeze-

thaw process by reducing ROS accumulation and lipid peroxidation, as well as by enhancing the endogenous antioxidant defense capacity of spermatozoa.

Furthermore, this study found that the addition of 20 μmol/L BBR did not further enhance the antioxidant effect, and some spermatozoa parameters even showed a downward trend, suggesting that the protective effect of BBR does not increase continuously in a concentration-dependent manner. Kuželová et al. reported that the addition of 10 μmol/L BBR to the cryopreservation extender significantly improved the post-thaw quality of rabbit sperm, whereas the effect was attenuated at 50 μmol/L (24). Our finding is generally consistent with the attenuated effect observed at higher concentrations of BBR in other studies. However, the sensitivity to high concentrations of BBR and the optimal supplementation dose may vary among species. This attenuation may be associated with potential cytotoxic or metabolism-modulating effects of BBR at relatively high concentrations (25). Excessive antioxidant supplementation may also disturb the physiological redox balance required for sperm capacitation and normal cellular signaling.

Effects of BBR on mitochondrial function in boar spermatozoa

Mitochondrial membrane potential（MMP) is an important indicator of cellular health and functional status (26). A decline in MMP generally reflects compromised mitochondrial membrane integrity, dysfunction of the electron transport chain, and reduced ATP-generating capacity. In the present study, the MMP level of frozen–thawed spermatozoa treated with 10 μmol/L BBR was significantly higher than that of the control group. Similarly, Ming et al. reported that pretreatment with 1 μM BBR significantly increased MMP levels in bone marrow mesenchymal stem cells under a simulated type 2 diabetic microenvironment and reversed the abnormal decline in MMP (27). Mitochondria are not only a major source of ROS generation but also a primary target of ROS-mediated damage (28). Excessive mitochondrial ROS can induce mitochondrial damage, including mtDNA damage and mutations, as well as the release of pro-apoptotic proteins (29). Considering the reduced ROS levels, enhanced antioxidant capacity, and improved motility parameters observed in this study, BBR may preserve mitochondrial membrane integrity and maintain normal oxidative phosphorylation by reducing ROS accumulation and alleviating mitochondrial oxidative damage. This may, in turn, improve ATP production efficiency and sustain sperm motility. Moreover, stable mitochondrial function may contribute to reducing the activation of apoptosis-related signaling pathways, thereby decreasing the risk of apoptosis-like changes in spermatozoa during the freezing–thawing process and further improving post-thaw sperm quality.

In summary, supplementation of boar semen freezing extender with an appropriate concentration of BBR can alleviate oxidative damage during the freeze-thaw process by enhancing antioxidant capacity, reducing ROS levels, inhibiting lipid peroxidation, and maintaining mitochondrial homeostasis. These effects contribute to improved post-thaw sperm motility and membrane integrity, thereby enhancing the overall quality of boar spermatozoa. Among the tested concentrations, 10 μmol/L BBR-treated group exhibited a relatively favorable protective effect and may be considered an appropriate additive concentration for boar semen freezing extender. However, the effects of BBR on the fertilization potential and in vivo reproductive outcomes of frozen-thawed boar spermatozoa require further validation. In addition, the molecular pathways underlying the protective effects of BBR were not investigated. Future studies should incorporate both in vitro and in vivo fertilization assays to systematically evaluate its effects on fertilization capacity and reproductive performance. In addition, molecular biological techniques should be employed to further elucidate the key signaling pathways by which BBR regulates sperm antioxidant defense, mitochondrial homeostasis, and anti-apoptotic processes. These studies will provide theoretical and technical support for the optimization and industrial application of this approach and enhance its practical value in boar semen cryopreservation.

CONCLUSION

The results of this study demonstrated that supplementation of boar semen freezing extender with 10 μmol/L BBR significantly improved the post-thaw quality of boar spermatozoa. This improvement was characterized by enhanced sperm motility, kinematic parameters, acrosome integrity, plasma membrane integrity, antioxidant

capacity, and mitochondrial membrane potential, as well as reduced ROS accumulation and MDA content. These findings suggest that BBR protects boar spermatozoa during cryopreservation, at least in part, by alleviating oxidative stress and lipid peroxidation. Among the tested concentrations, 10 μmol/L BBR was the most effective and may be considered a promising additive for boar semen cryopreservation.

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REFERENCES

1. Bolarin A, Berndtson J, Tejerina F, Cobos S, Pomarino C, D'Alessio F, Blackburn H & Kaeoket K (2024) *Anim Reprod Sci* **269**, 107496. https://doi.org/10.1016/j.anireprosci.2024.107496
2. Mili B, Chutia T, Buragohain L, Palanisammi A & Kumaresan A (2025) *Anim Reprod Sci* **278**, 107871. https://doi.org/10.1016/j.anireprosci.2025.107871
3. Yánez-Ortiz I, Catalán J, Rodríguez-Gil JE, Miró J & Yeste M (2022) *Anim Reprod Sci* **246**, 106904. https://doi.org/10.1016/j.anireprosci.2021.106904
4. Kim E, Yu IJ, Lee J & Jeon Y (2024) *Antioxidants* (Basel) **13**, 672. https://doi.org/10.3390/antiox13060672
5. Delgado-Bermúdez A (2024) *Anim Reprod Sci* **269**, 107547. https://doi.org/10.1016/j.anireprosci.2024.107547
6. Zhang P, Ma D, Wang Y, Zhang M, Qiang X, Liao M, Liu X, Wu H & Zhang Y (2014) *Food and Chemical Toxicology* **74**, 225-232. https://doi.org/10.1016/j.fct.2014.10.005
7. Huang Y, Zhang J, You H, Ye F, Yang Y, Zhu C, Jiang YC & Tang ZX (2024) *Eur J Pharmacol* **985**, 177109. https://doi.org/10.1016/j.ejphar.2024.177109
8. Alpaslan Ağaçdiken AH & Göktaş Z (2025) *PeerJ* **13**, e18924. https://doi.org/10.7717/peerj.18924
9. Wang Y, Liao J, Luo Y, Li M, Su X, Yu B, Teng J, Wang H & Lv X (2023) *International Journal of Molecular Sciences* **24**, 3257. https://doi.org/10.3390/ijms24043257
10. Larson JL & Miller DJ (1999) *Mol Reprod Dev* **52**, 445-449. https://doi.org/10.1002/(SICI)1098-2795(199904)52:4<445::AID-MRD14>3.0.CO;2-6
11. Tanga BM, Qamar AY, Raza S, Bang S, Fang X, Yoon K & Cho J (2021) *Anim Biosci* **34**, 1253-1270. https://doi.org/10.5713/ab.21.0072
12. Piri M, Mahdavi AH, Hajian M, Nasr-Esfahani MH, Soltani L & Vash NT (2024) *Sci Rep* **14**, 24171. https://doi.org/10.1038/s41598-024-75792-5
13. O'Connell M, McClure N & Lewis SEM (2002) *Hum Reprod* **17**, 704-709. https://doi.org/10.1093/humrep/17.3.704
14. Morris GJ, Acton E, Murray BJ & Fonseca F (2012) *Cryobiology* **64**, 71-80. https://doi.org/10.1016/j.cryobiol.2011.12.002
15. Yeste M (2016) *Theriogenology* **85**, 47-64. https://doi.org/10.1016/j.theriogenology.2015.09.047
16. Lee IA, Hyun YJ & Kim DH (2010) *Eur J Pharmacol* **648**, 162-170. https://doi.org/10.1016/j.ejphar.2010.08.046
17. Fang L, Bai C, Chen Y, Dai J, Xiang Y, Ji X, Huang C & Dong Q (2014) *Cryobiology* **69**, 386-393. https://doi.org/10.1016/j.cryobiol.2014.09.005
18. Ortiz-Rodriguez JM, Balao Da Silva C, Masot J, Redondo E, Gazquez A, Tapia JA, Gil C, Ortega-Ferrusola C & Peña FJ (2019) *PLoS One* **14**, e0211994. https://doi.org/10.1371/journal.pone.0211994
19. Guthrie HD & Welch GR (2012) *Theriogenology* **78**, 1700-1708. https://doi.org/10.1016/j.theriogenology.2012.05.002

20. Ayala A, Muñoz MF & Argüelles S (2014) *Oxid Med Cell Longev* **2014**, 360438. https://doi.org/10.1155/2014/360438
21. Gao Y, Wang F, Song Y & Liu H (2020) *Chin Med* **15**, 7. https://doi.org/10.1186/s13020-020-0288-z
22. Shakeri F, Kiani S, Rahimi G & Boskabady MH (2024) *Phytotherapy Research* **38**, 1882-1902. https://doi.org/10.1002/ptr.8077
23. Shams G, Allah SA, Ezzat R & Said MA (2024) *Open Vet J* **14**, 292-303. https://doi.org/10.5455/OVJ.2024.v14.i1.26
24. Kuželová L, Svoradová A, Baláži A, Vašíček J, Langraf V, Kolesárová A, Sláma P & Chrenek P (2024) *Antioxidants* (Basel) **13**, 1360. https://doi.org/10.3390/antiox13111360
25. Miłek M, Dżugan M, Pieńkowska N, Galiniak S, Mołoń M & Litwińczuk W (2024) *Curr Issues Mol Biol* **46**, 13193-13208. https://doi.org/10.3390/cimb46110787
26. Perelman A, Wachtel C, Cohen M, Haupt S, Shapiro H & Tzur A (2012) *Cell Death Dis* **3**, e430. https://doi.org/10.1038/cddis.2012.171
27. Ming Y, He X, Zhao Z, Meng X, Zhu Y, Tan H, Yang G, Hu Y & Zheng L (2024). *International Journal of Nanomedicine* **19**, 10263–10282. https://doi.org/10.2147/IJN.S475320.
28. Dunn JD, Alvarez LA, Zhang X & Soldati T (2015) *Redox Biol* **6**, 472-485. https://doi.org/10.1016/j.redox.2015.09.005
29. Youle, R. J. & A. M. van der Bliek (2012) Science (New York, N.Y.) **337**, 1062-1065. https://doi.org/10.1126/science.1219855