Antioxidants Significantly Improve Qinling Giant Panda (*Ailuropoda Melanoleuca Qinlingensis*) Sperm Quality During Cryopreservation

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Abstract

Background: Semen cryopreservation has become an essential tool for the reproduction and long-term preservation of giant pandas (*Ailuropoda melanoleuca*). However, cryopreservation is severely detrimental to sperm quality, including motility, plasma membrane, acrosome integrity, and mitochondrial activity. It is necessary to screen a effective antioxidants for improvement of sperm quality. Eight groups of antioxidants were added to the freezing medium.

Results:The results showed that lycium barbarum polysaccharide (LBP), Laminaria japonica polysaccharides (LJP), or goujresveratrol (RSV) added to the freezing medium significantly improved sperm motility, acrosome integrity, and mitochondrial activity during the cryopreservation process. Furthermore, the activities of glutathione and superoxide dismutase were also improved. However, the levels of reactive oxygen species and malondialdehyde in semen were notably reduced. Regarding fertility, acrosin activity was significantly increased in LBP-treated sperm. Unfortunately, sperm viability, DNA fragmentation, and hyaluronidase activity were not significantly different between the above groups.

Conclusions: LBP (2.0 mg/mL) or RSV (50 μ M) are the best candidate antioxidants for inclusion in the freezing medium for improving the quality of panda spermatozoa, during semen cryopreservation.

Background

The giant panda (*Ailuropoda melanoleuca*), one of the most endangered animals in the world, is known as a “living fossil”, and has survived on earth for at least 80 million years [1, 2]. The Qinling panda (*A melanoleuca qinlingensis*), a subspecies of the giant panda, is highly endangered and a precious species because of its unique morphological characteristics and genetic features. From the fourth investigation report in 2015, about 345 pandas inhabited the Qinling Mountains, accounting for 18.51% of all wild pandas in China. Low mating opportunities are the main factor driving hypo-fertility of pandas, especially in captive-bred giant pandas that have fewer chances of finding a suitable mate compared to wild pandas. Therefore, only 10 percent of captive-bred giant pandas can mate naturally. Although captive breeding is an effective method to preserve endangered species, the low conception rate of giant pandas is a major challenge for artificial insemination. Currently, there is an increasingly demand for a reproductive method utilizing cryopreservation of giant panda semen to address infertility problems. This will be beneficial in maintaining the number of Qinling giant pandas, establishing a germplasm repository of the Qinling subspecies, and improving their conservation status.

There is increasing evidence shows that sperm cryopreservation induces the overproduction of reactive oxygen species (ROS), which are major deleterious factors affecting sperm quality [3]. Excessive ROS production leads to drastic changes in sperm membranes because natural antioxidants in the seminal plasma are diluted in the freezing medium before the cryopreservation process [4]. Recently, the addition of antioxidants to the sperm freezing medium for humans, horses, beers, dogs, goats, cattle, and boars has demonstrated that sperm ability is significantly enhanced [5–9]. Multiple studies have affirmed that

LBP, LJP and RSV contribute to the reduction of excessive ROS production from cryodamage [10–12]. LBP extracted from barbarums can improve sperm quality. Moreover, the addition of LBP to the freezing medium not only increases the activity of antioxidant enzymes to inhibit apoptosis, but also enhances spermatogenesis in male mice with type 1 diabetes [13]. Multiple studies have indicated that LJP, a polysaccharide extracted from seaweed, has anti-coagulant, anti-arteriosclerosis, anti-tumor, and antiviral properties. In addition, studies have demonstrated that LJP is another possible candidate to add to the freezing medium, acting as an ROS scavenger to improve sperm quality during the freeze-thawing process [14]. RSV is a polyphenol found in plants such as grapes [15]. Recent literature has shown that RSV modulates lipid metabolism and has anti-inflammatory, antioxidant, anti-allergic, and anti-cancer properties. Additionally, RSV displayed protective effects against free radicals, cardiovascular diseases, and allergies [16]. Evidence from animal model studies on male fertilization has confirmed that RSV plays a promoting role in spermatogenesis by stimulating the hypothalamic-pituitary gonadal axis [17]. In addition, RSV suppressed germ cell apoptosis, enhanced penile erection, elevated steroidogenesis, and facilitated sperm motility and spermatogenesis in rats and mice [18].

In this study, we evaluated the potential effect on sperm viability, sperm plasma membrane, acrosome integrity, mitochondrial activity, and oxidative stress in Qinling giant panda semen during cryopreservation with LBP, LJP, or RSV. A further goal of this study was to provide a effective antioxidant with freeze medium.

Results

Improvement of sperm motility in freezing medium with antioxidants

To explore the roles of antioxidants (RSV, LJP, and LBP) in giant panda sperm during cryopreservation, we first detected sperm viability and motility. There was no effect on the viability of sperm exposed to RSV, LBP, or LJP alone at the indicated concentrations. Furthermore, the combination of RSV (100 μ M), LBP (2 mg/mL), and LJP (1 mg/mL) also did not change the viability of sperm after freeze-thawing when compared with the control (Figure. 1A, $P > 0.05$). In addition, RSV alone had no effect on sperm motility. Similar to RSV, there was no significant difference between the LJP and control group ($P > 0.05$). However, sperm in the LBP (2 mg/mL) freezing medium showed an improved motility compared to the control ($P < 0.05$). Furthermore, the combination of RSV (100 μ M), LBP (2 mg/mL) and LJP (1 mg/mL) notably improved sperm motility after freeze-thawing compared to the control (Figure. 1B $P < 0.05$). These data indicated that antioxidants did not change sperm viability but improved sperm motility.

Improvement of plasma membrane and acrosome integrity in freezing medium supplemented with antioxidants

There is increasing evidence shows that plasma membrane and acrosome integrity are associated with sperm motility [4]. Thus, we detected plasma membrane integrity and acrosome integrity in sperm treated with antioxidants. We found that 50 μ M RSV and 100 μ M RSV markedly improved plasma membrane integrity ($P < 0.05$), but LBP and LJP had no significant protective effects ($P > 0.05$). The combination of

RSV (100 μ M), LBP (2 mg/mL)), and LJP (1 mg/mL)) evidently protected the plasma membrane integrity compared to the control ($P < 0.05$), yet showed no difference in the protection of plasma membrane integrity in comparison to RSV (100 μ M) alone ($P > 0.05$, Figure 2A), suggesting that RSV alone had the ability to protect the plasma membrane integrity during cryopreservation.

We found that sperm in the freezing medium with 50 μ M RSV and 100 μ M RSV improved acrosome integrity ($P < 0.05$). Furthermore, 1.0 mg/mL LJP alone improved the protection of acrosome integrity ($P < 0.05$). As expected, the combination of RSV (100 μ M), LBP (2 mg/mL), and LJP (1 mg/mL) increased protection of the acrosome integrity more than the control ($P < 0.05$) (Figure 2B). These results showed that RSV and LJP at certain concentrations could protect acrosome integrity during cryopreservation.

Improvement of mitochondrial activity in freezing medium with antioxidants

To further analyze the protective effect of antioxidants, mitochondrial activity and DNA integrity were detected using PI and Rh123 staining and AO staining, respectively. The results showed that 50 μ M RSV and 100 μ M RSV significantly protected mitochondrion from freeze-thaw injury ($P < 0.05$), and the ability of protecting mitochondrial activity was increased in the freezing medium by adding 2.0 mg/mL LBP or 1.0 mg/mL LJP alone ($P < 0.05$). Furthermore, the combination of RSV (100 μ M), LBP (2 mg/mL) with LJP (1 mg/mL) significantly improved the mitochondrial activity of sperm when compared to the control (Figure 3A, $P < 0.05$). From the above results, 50 μ M resveratrol and the combined addition were best at protecting mitochondrial activity ($P < 0.05$). In addition, as shown in Figure 3B, no significant effect on the DNA integrity of sperm was found after treatment with RSV, LJP, LBP, or the combined addition compared with the control (Figure 3B, $P < 0.05$). These data suggest that antioxidants could improve the mitochondrial activity of sperm in freezing mediums.

Downregulation of ROS and upregulation of MDA, SOD, and GSH-PX inclusion of antioxidants in the freezing medium

To detect the protective capacity of antioxidants in thawed semen, antioxidant indices were determined using ELISA kits. It was found that the level of ROS was decreased in freezing mediums supplemented with 100 μ M RSV, but no change was found in the level of ROS after sperm was treated with LBP, LJP, or the combined addition (Figure 4A, $P < 0.05$). As shown in Figure 4B, all freezing media with RSV, LBP, LJP, or the combined addition had a significant protective effect against high levels of malondialdehyde (MDA) during freeze-thawing. The effective protection of RSV and the combined addition was highest (Figure 4B, $P < 0.05$). Meanwhile, 100 μ M RSV increased the level of superoxide dismutase (SOD) in giant panda frozen-thawed sperm ($P < 0.05$). In addition, LBP and LJP alone contributed to increased SOD levels ($P < 0.05$), and the combined addition showed a significant antioxidant effect compared to the control (Figure 4C, $P < 0.05$). Additionally, a facilitatory effect of 50 μ M or 100 μ M RSV alone on the level of glutathione (GSH-PX) was observed in sperm cryopreservation ($P < 0.05$). LBP and LJP alone also improved the level of GSH-PX in comparison with the control ($P < 0.05$). Similarly, the combination of RSV (100 μ M), LBP (2 mg/mL), and LJP (1 mg/mL) clearly increased the level of glutathione (GSH-PX)

compared to the control (Figure 4D, $P < 0.05$). These data support that antioxidants in the freezing medium could decrease ROS and MDA levels and increase SOD and GSH-PX levels.

Improvement of fertilization capacity in the freezing medium supplemented with antioxidants

To analyze the fertilization ability of giant panda sperm after cryopreservation, proteomics studies were performed using ELISA. As shown in Figure 5A, no significant difference in the hyaluronidase (HAase) in sperm was found after treatment with RSV, LJP, LBP, and the combined addition compared with the control (Figure 5 A, $P < 0.05$). However, supplementation of LBP with doses of 2.0 mg/mL and 4.0 mg/mL exerts a dramatic cryoprotective effect on sperm acrosomal protease (ACE) activity ($P < 0.05$). Furthermore, the difference in sperm acrosin activity between 2.0 mg/mL and 4.0 mg/mL was not significant (Figure 5 B, $P > 0.05$). These data indicate that the addition of LBP to the freezing medium improves the fertilization capacity.

Discussion

Giant pandas are one of the most endangered mammal species on earth, and protection of the Qinling subspecies is the most pressing challenge. Apart from the establishment of nature reserves, semen cryopreservation is an effective approach for protecting of giant pandas. However, the negative effect of cryopreservation on the viability and motility of giant panda sperm is inevitable. Substantial evidence suggests that cryopreservation severely compromises plasma membrane fluidity, antioxidant ability, acrosome enzyme activity, mitochondrial potential, and motility, thereby affecting the quality of sperm. Studies have demonstrated that the addition of cryoprotective agents is important for reducing damage to sperm during cryopreservation. In the area of cryoprotectant type, silicane-coated silica colloid particles were added to the freezing medium to improve the ability of spermatozoa to fertilize prior to giant panda semen freezing [19]. INRA96 freezing dilutions increased the quality of giant panda semen after thawing, compared with TEST extender [20]. In addition, cryoprotectants, such as glycerol, sucrose, vitamin B12, vitamin E, vitamin C, dimethyl sulfoxide (DMSO), trehalose, and others, were added to the freezing medium of giant pandas. At the transcript level, differentially expressed lncRNAs, microRNAs and mRNAs were analyzed in frozen and fresh sperm of giant pandas using high-throughput sequencing technology [21, 22]. The above evidence shows that the negative effect of cryopreservation on giant panda sperm ability and motility is inevitable [23]. A previous report showed that RSV, LBP, and LJP were added to semen to improve the quantity of sperm [5]. While the protective effect of antioxidants on ruminant sperm has been widely investigated, few studies have explored the required doses of LBP, LJP, and RSV for panda sperm cryopreservation. To develop new techniques or cryopreservation methods, novel findings regarding giant panda sperm freezing supplemented with antioxidants are in high demand. Because the cytotoxic effects generated beyond high concentrations, the optimal dose and concentration of LBP, LJP, and RSV were referenced from their use in cattle, sheep, pigs, and other animals. In our study, eight groups of antioxidants were added to the freezing medium to obtain more results. This study provides an important reference for future dose preparation.

It was reported that buffalo semen freezing medium supplemented with RSV (50 μ M) could markedly improve the post-thaw quality of sperm [24]. Moreover, the addition of RSV (10 μ M and 50 μ M) to the freezing medium prior to cryopreservation also attenuated cryodamage and enhanced the motility of goat sperm [5]. Research has shown that 2.0 mg/mL LBP + 1.0 mg/mL LJP could significantly improve the quality of cashmere goat sperm. In our study, we detected the protective effects of LBP, LJP, and RSV on giant panda semen after cryopreservation. Similarly, we confirmed that supplementation of the freezing medium with 2.0 mg/mL LBP and the combined addition was effective in protecting giant panda sperm motility. Unfortunately, RSV and LJP alone had no protective effect on giant panda sperm motility (Fig. 1B). During the freeze-thawing process, the plasma membrane fluidity and permeability is increased. The redistribution of antioxidant enzymes, phospholipids, and cholesterol is observed in the sperm plasma membrane and is responsible for structural and functional changes. In addition, plasma membrane integrity, which is involved in metabolism and osmotic pressure, is a critical factor for fertility [25]. Recently, evidence has confirmed that cryopreservation damaged plasma membrane integrity and acrosome integrity in bulls, boars, rams and sheep. To improve plasma membrane integrity and acrosome integrity, 50 μ M RSV was an effective antioxidant for boar semen cryopreservation. Supplementation of tris citric acid extender with 50 μ M RSV and 100 μ M RSV significantly enhanced the plasma membrane integrity of buffalo [26]. Based on the antioxidant function of RSV, we further investigated whether RSV exerts a cryoprotective effect on giant panda sperm. Our study showed that RSV 50 μ M, 100 μ M, and the combined addition had a cryoprotective effect on giant panda sperm plasma membrane integrity and acrosome integrity, which was consistent with the above-mentioned report. In addition, we found that LJP-1.0 mg/mL and the combination were beneficial for acrosome integrity (Fig. 2). Recently, evidence from cryopreservation has confirmed that sperm with damaged DNA have been detected in cats, humans and bovines [27–29]. Furthermore, antioxidants, extenders, and new approaches for humans have been used to improve sperm chromatin quality during freezing [27–29]. However, there were no protective effects of LBP, LJP, and RSV on giant panda semen after cryopreservation in our study (Fig. 3).

Mitochondrial ATP for energy homeostasis is generated by oxidative phosphorylation and ATP synthases, which play a vital role in sperm motility [30]. Mitochondria are the primary organelles responsible for ROS production due to disturbances in the electron transport chain. In addition, the capacity of antioxidant enzymes against oxidative stress was significantly reduced because the abundance was diluted and had a negative effect on the cryopreservation process [31]. Therefore, excessive ROS levels impair the mitochondrial proteins, resulting in severely damaged mitochondrial [26]activity. Recently, evidence has shown that the addition of RSV, LBP, and LJP to freeze-thawed semen could significantly improve the mitochondrial activity of sperm [11] [5, 32, 33]. In our study, we verified that the addition of RSV-50 μ M, RSV-100 μ M, LBP-2.0 mg/mL, LJP-1.0 mg/mL or the combined addition into the freezing medium, mitochondrial activity of sperm was improved (Fig. 3A), which was consistent with a previous study. Previous reports have shown that cryopreservation damaged mitochondrial function and increased the production of ROS, and excessive production of ROS attacked polyunsaturated phospholipids and triggered the MDA level. In addition, the antioxidant activity of SOD and the level of GSH were reduced

during cryopreservation. Finally, the semipermeable properties of the membrane are affected by peroxidative attack. However, the addition of RSV, LJP, and LBP to semen reduces the negative effects of excessive ROS levels [32–35]. Our study verified that supplementation of the freezing medium with RSV (50 μ M), LBP (2.0 mg/mL), LJP (1.0 mg/mL) and combined addition significantly enhanced activity of SOD and GSH for oxygen radical (O_2^-) scavenging. Our results showed that the addition of antioxidants prior to the freezing medium protected sperm from cryodamage induced by MDA, whereas RSV-50 μ M was the best potential candidate as a cryoprotective agent (Fig. 4).

In mammals, spermatozoa penetrate the zona not only by mechanical force, but also by acrosomal enzymes. Among acrosomal enzymes, acrosin was identified as the most important factor for strong hydrolyzing activity. Evidence has shown that sperm mitochondrial activity affects human sperm acrosin activity [36]. In addition, hyaluronidase is important for digesting hyaluronic acid during the acrosome reaction process [37]. Therefore, hyaluronidase activity in sperm is responsible for fertilization. In our study, acrosin was improved in the LBP (2.0 mg) and RSV (50 μ M) treated group, but had no protective effect on the acrosomal enzymes (Fig. 5).

Conclusion

To summarize, our study revealed that LJP, LBP, RSV, and the combined treatment enhanced sperm plasma membrane, acrosome integrity and mitochondrial activity by increasing antioxidant enzyme levels and suppressing ROS production from oxidative damage during the freezing process. RSV (50 μ M) and LBP (2.0 mg/mL) were potential candidates for cryopreservation supplementation of the freezing medium against cryodamage. The study provides a reference on the improvement of Qinling giant panda semen cryopreservation.

Material And Methods

Semen collection

Semen from sexually mature male giant pandas housed in Shaanxi Wild Animal Research Center (SWARC; Louguantai, Zhouzhi County, Xi'an city, 34°06' N, 108°32' E) was collected via electrostimulation during the breeding season. The giant pandas were anesthetized by injection with ketamine hydrochloride (8 mg/kg, IM) and halothane after 12 h of fasting. The pandas were then given 10–90 mA electroejaculation of semen collection for 5 s with an interval of 2 s. Fresh semen was collected into sterile cups after a 37 °C pre-warmed process and with a diluted 1:1 with a diluent (Irvine Scientific 90129). This work involved in giant pandas semen collection were in compliance with the Wildlife Protection Law of the Peoples Republic of China and Regulations of Shaanxi Province on the Protection of Wild Animals and Plants. Furthermore, all the protocols were approved by Committee for the Ethics on Animal Care and Experiments in Northwest A&F University.

Experimental design

The semen was aliquoted into eight pre-heated tubes before freezing. The original cryoprotectant was added to one tube prior to the freezing process and was used as the control. Prior to cryopreservation, three tubes were diluted in a freezing medium with 50 μ M, 100 μ M, or 150 μ M RSV. Two tubes received LBP (2 mg/mL and 4 mg/mL); LJP (1 mg/mL) was added to the freezing medium in one tube, and the remaining tube of semen received a combination of LJP (1 mg/mL), RSV (100 μ M), and LBP (2 mg/mL).

Fresh semen samples were diluted with cryopreservation agents at a 1:1 ratio in a 15 mL tube placed in a beaker of water at 37°C. Subsequently, the tubes were incubated into 4 °C for 3 h. After that, glycerin was added to the cryopreservation semen at a final concentration of 400×10^6 spermatozoa/mL. Diluted semen was equilibrated at 4 °C for 1 h. Finally, diluted semen aliquots were packed into prelabeled 250 mL straws, frozen in liquid nitrogen vapor for 2 min, and stored in nitrogen for long-term preservation.

For thawing, frozen straws were removed from liquid nitrogen and immediately placed in a water bath at 37 °C. After shaking back and forth for 60 s, the thawed semen was transferred into a 2.5 mL tube for subsequent experiments.

Semen evaluation

Sperm viability and motility tests

Spermatozoa motility parameters of thawed semen were examined using an automatic analyzer (Naturegene Sperm Tracker).

Sperm plasma membrane integrity testing

As per previous evidence, the integrity of the sperm plasma membrane was assessed using HOST staining. Briefly, hypotonic solutions were mixed with the rapidly thawed giant panda semen, and the mixture was incubated at 37 °C for 30 min. A total of 200 spermatozoa were counted under an inverted microscope to identify the expansion percentage of sperm tails from a 10 μ L mixture.

Acrosome integrity assessment

Acrosome integrity was assessed by fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA) staining (Vector Laboratories, United States). Thawed semen with a final concentration of 105/mL was prepared for sperm smears and kept dry. The cells were then fixed with methanol for 10 min and incubated with 30 μ L FITC-PNA at 37 °C for 30 min under an environment of light avoidance. Then, sperm smears was sealed with a colorless nail polish after washing with PBS in triplicate. Finally, more than 200 sperms were evaluated using fluorescence microscopy.

Mitochondrial activity analysis

Tubes containing 1 μ L PI (0.02 mg/mL in PBS solution) (Solarbio, Beijing, China) and 1 μ L Rh123 (0.2 mg/mL in DMSO) (Solarbio, Beijing, China).were protected against light at room temperature for 10 min and carefully added to the 50 μ L suspension of sperm which was freeze-thawed for 30 min. After sperm

smears were prepared with 10 μ L of the incubated sample, estimation of sperm mitochondrial activity parameter was performed using an inverted microscope at 400 $\times$ magnification. Nonviable in head sperm were identified as live sperm with high mitochondrial membrane potential ($\Delta\Psi$ m). Therefore, the percentage of spermatozoa with intact mitochondria was also calculated.

Evaluation of DNA integrity

DNA integrity was assessed by acridine orange (AO) staining (Solarbio, Beijing, China). The sperm were washed three times with PBS and suspended in PBS at a concentration of 2×10^4 /mL. The suspension of sperm was cased on clean glass slides and air-dried for the preparation of smears. The smears were then rinsed in a fixative solution (3:1, absolute ethanol: glacial acetic acid) for 5 min. After fixation, the slides were stained with AO solution for 10 min and thoroughly washed with distilled water. Finally, the air-dried smears were sealed with paraffin oil and analyzed by fluorescence microscopy. At least 300 sperm were counted per smear.

ELISA estimation of ROS, MDA, SOD and GSH-PX levels

The reagent kits were equilibrated at room temperature for 30 min. ROS, MDA, SOD, and GSH were determined via ELISA (Jining, Shanghai, China) in triplicate following the manufacturer's instructions. The optical density of each sample was measured using a Spectra MR microplate reader (Dynex Technologies) at a wavelength of 450 nm. Subsequently, the levels of ROS, MDA, SOD, and GSH were calculated against each standard curve and expressed as pg concentration.

ELISA detection of HAase and ACE level

The levels of HAase and ACE in semen were assessed using ELISA kits (Jining, Shanghai, China). Each sample in triple well was performed at 450 nm according to the manufacturer's manufacturer's instructions. Finally, HAase and ACE contents were analyzed using each standard curve.

Statistical analysis

All experiments were performed in triplicates. Sperm quality metrics for antioxidant capacity, including CASA value, HOST, DNA integrity, acrosomal integrity, and ROS and MDA were analyzed using GraphPad Prism 7.00 software. Normality of the data was checked using the Kolmogorov–Smirnov normality test. All final data were shown as mean $\pm$ standard deviation. Statistical significance values was set at $p \leq 0.05$.

Abbreviations

LBP: lycium barbarum polysaccharide, LJP: laminaria japonica polysaccharides; RSV:resveratrol; ROS: reactive oxygen species; SOD: superoxide dismutase GSH-PX: glutathione; MDA : malondialdehyde; Haase: hyaluronidase; ACE : acrosomal protease

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by Wildlife Protection Law of the Peoples Republic of China and Regulations of Shaanxi Province on the Protection of Wild Animals and Plants

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this study will be available by the corresponding author.

Competing Interests

All authors declared no competing conflict of interest.

Founding

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Authors' Contributions

The experiment was designed by Pengfei Lin and Yaping Jin; the samples were collected and performed by Ruixue Zhang, Hemeng Dong, Pengpeng Zhao, Hang Qi, Chunmei Shang, Yongjie Ma, Chuxi Gao, Danhui Zhang, Jiena Shen; all the datas were analyzed by Pengfei Lin, Ruixue Zhang and Hemeng Dong; All the figures were prepared by Pengfei Lin. The manuscript was drafted by Ruixue Zhang; Pengfei Lin, Yinghu Lei and Yaping Jin revised the manuscript. Finally, all authors reviewed the final manuscript.

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Figures

A

B

Figure 1

Viability and motility of giant panda spermatozoa treated by antioxidants. (A) The viability of freeze-thawed sperm in the presence of antioxidants. (B) Sperm motility in freezing medium supplemented with antioxidants was evaluated by using Naturegene Sperm Tracker. Values are presented as mean $\pm$ SD (n = 5). Asterisks represent significant differences (*P < 0.05).

Figure 2

The effects of antioxidants on integrity of plasma membrane and acrosome integrity in freeze-thawed spermatozoa. (A) Plasma membrane integrity of spermatozoa was evaluated by HOST staining. (B) The acrosome integrity in post-thawed giant panda spermatozoa was stained by FITC-PNA. Data are shown as mean $\pm$ SD; Asterisks indicate statistically significant differences among treatments and control (* $P < 0.05$).

A

B

Figure 3

Evaluation the effect of antioxidants on mitochondrial activity and DNA integrity in giant panda spermatozoa. (A) Images unvisualize the percentage of fluorescence in the head of all spermatozoa stained with PI and Rh123. (B) DNA integrity was detected in the antioxidant groups using AO staining. Values are shown as mean $\pm$ SD (n = 5). Asterisks present significant differences.

Figure 4

Antioxidants enhance oxidation resistance of giant panda spermatozoa during the freeze-thaw process. The levels of ROS (A), MDA (B), SOD (C) and GSH-PX (D) in giant panda semen treated with antioxidants or control (Con) were detected by using ELISA. The values are shown as means $\pm$ SD of five independent repeats. Significant differences are shown with asterisks. (* $p < 0.05$; Student's t-test).

Figure 5

Improvement of fertilizing capability in giant panda spermatozoa. (A) Semen pretreated with antioxidants had no effect on HAase by using ELISA. (B) Acrosin activity of spermatozoa was measured using ELISA. All the experimental values are shown as means $\pm$ SD of five repeats. Significant differences are shown with asterisks. (* $p < 0.05$; Student's t-test).