Original Article

Effects of astragalus polysaccharides on the quality of frozen-thawed Tibetan boar spermatozoa and levels of genomic DNA methylation in the sperm

Yan-Ling Zhao[1] Shan-Zheng Li[1] Zi-Li Ren[1*]

Abstract

Astragalus polysaccharides (APS) have strong antioxidant effects and may thus be useful for the preservation of semen at low temperatures *in vitro*. However, the effects of APS on the quality of frozen-thawed Tibetan boar sperm have yet to be clarified. Here, the effects of APS on the quality and levels of genomic DNA methylation in Tibetan boar spermatozoa were assessed. Tibetan boar semen was cryopreserved in high-density tubules in freezing extender supplemented with 0, 0.2, 0.4, 0.6, or 0.8 g ·L^{-1} APS to determine the optimal concentration of APS. Sperm genomic DNA methylation levels of the three groups (Fresh group represents fresh spermatozoa, No add group represents unsupplemented frozen spermatozoa, and Optimum add group represents frozen spermatozoa supplemented with the optimal concentration of APS) were detected using a fluorescence method. The results showed that sperm quality was significantly higher in the group supplemented with 0.4 g ·L^{-1} APS (increased sperm motility, acrosome integrity, and plasma membrane integrity, simultaneously decreased abnormality and the ROS level) than in all other groups. $p<0.05$), and the DNA methylation levels of frozen sperm in this group were significantly lower than that of the No add group ($p<0.05$). The results indicated that semen freezing extender supplemented with 0.4 g ·L^{-1} APS was suitable for Tibetan boar semen.

Keywords: Astragalus polysaccharides, Tibetan boar spermatozoa, cryopreservation, DNA methylation

[1]*College of Animal Science, Tibet Agricultural and Animal Husbandry University, 100 Yucai West Road, Bayi District, Linzhi, Tibet, 860000, China*

****Correspondence**: zlren0925@163.com* (Z. Ren)

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Introduction

The Tibetan pig, known as "the treasure of the plateau", is an important pig breed that has adapted to the harsh plateau environment (Yang *et al.,* 2011). The long-term cryopreservation of mammalian spermatozoa supports genetic breeding and improves germplasm resources (Ma, 2013). Thus, freezing is a practical and effective protection method that fosters artificial fertilization in farm animals (Li *et al.,* 2018). However, cryopreservation may damage spermatozoa, leading to unfavorable changes in membrane lipid composition, acrosome status, sperm motility, and sperm viability (Gangwar *et al.,* 2018). Although cryopreserved boar semen has been available since 1975, there have been no major breakthroughs in commercial application (Grossfeld *et al.,* 2008). Semen extenders used for freezing should contain a cryoprotective agent and an energy source, and should also be well buffered to prevent changes in pH and tonicity throughout the preservation period (Namula *et al.,* 2019). In addition, the addition of antioxidants to boar semen refrigerant may significantly improve the quality of the thawed semen (Zhang *et al.,* 2012). Astragalus polysaccharides (APS), the main component of *Astragalus mongholicus* (AM), has been applied extensively to alleviate immune stress and viral infections (Jiang *et al.,* 2010; Liu *et al.,* 2011). APS have strong antioxidant effects and may thus be useful for the preservation of semen at low temperatures *in vitro* (Fu *et al.,* 2018). The extender supplimented with 0.4 g ·L^{-1} APS can demonstrate desirable parameters of boar frozen-thawed spermatozoa (Yang *et al.,* 2014). The addition of 0.5 g ·L^{-1} APS to thawed sperm showed decreased the concentration of reactive oxygen species (ROS) and increased the cleavage rate and blastocyst rate (Weng *et al.,* 2018). Overwhelming evidence has shown that APS can inhibit mitochondrial injury by scavenging reactive ROS (Huang *et al.,* 2016). Boar spermatozoa are very susceptible to ROS, which may stimulate the acrosome reaction in boar sperm through membrane lipid peroxidation and phospholipase A activation (Awda *et al.,* 2009). Oxidative stress-related damage to sperm DNA impedes methylation, whereas antioxidant supplementation may reduce DNA damage and normalize sperm DNA methylation (Tunc and Tremellen, 2009). The supplementation of semen extender with 5.0 g ·L^{-1} vitamin C improved the quality of Tibetan boar semen kept at 4 °C and mitigated the effects of low temperature on DNA methylation in Tibetan boar spermatozoa (Ren *et al.,* 2016). Similarly, the supplementation of semen extender with 6 mg ·L^{-1} rhodiola polysaccharide improved quality of thawed Tibetan boar semen (Ren *et al.,* 2017). To our knowledge, however, the effects of APS on the quality of frozen-thawed Tibetan boar spermatozoa, as well as on the levels of genomic DNA methylation in the spermatozoa, have not yet been clarified.

In this study, Tibetan boar semen was cryopreserved in high-density tubules in a freezing extender supplemented with 0, 0.2, 0.4, 0.6, or 0.8 g ·L^{-1} APS. Sperm motility, abnormality, acrosome integrity, plasma membrane integrity, and ROS level were used as the indexes to assess sperm quality after thawing in order to determine the optimal concentration of APS. Then, the global methylation levels of the three experimental groups (Fresh group represents fresh spermatozoa, No add group represents unsupplemented frozen spermatozoa, and Optimum add group represents frozen spermatozoa supplemented with the optimal concentration of APS) were detected with a fluorescence method. Our results will provide a reference for further investigations aiming to mitigate the negative effects of cryopreservation on Tibetan boar semen, and will increase our understanding of the epigenetic mechanisms of sperm freezing injuries in Tibetan boars.

Materials and Methods

Animal: All of the experiments were performed using 10 Tibetan boars, which remained in their native high-altitude plateau habitat (Linzhi city, 3 000 m) for the duration of the experiment. The boars were 1.5 years old, approximately 35 kg, and with normal reproductive capacity. Throughout the experiment, nutrition levels were maintained as close to normal as possible; boars grazed and foraged freely, with artificial feed provided at fixed points. The procedures of the experiments have been approved by the Institutional Animal Care and Use Committee of Tibet Agricultural and Animal Husbandry University (Linzhi, Tibet; institutional certification number 12540000MB0P013721), and care was taken to minimize the number of animals used.

Main reagents: APS (B20562, Shanghai Yuanye Biological Technology Co. Ltd., Shanghai, China); coomassie brilliant blue R250, glucose, citric acid, glycerol (6104-59-2, 50-99-7, 77-92-9, 56-81-5, Shanghai Beyotime Ltd., Shanghai, China); perchloric acid (7601-90-3,Tianjin Damao Chemical Reagent Factory, Tianjin, China); fructose, Tris, (57-48-7 and 77-86-1, China National Medicine Shanghai Chemical Reagents Co. Ltd., Shanghai, China); N-acetyl-L-cysteine (NAC), ampicillin and streptomycin (YZ-140671, IA0340, and IS0360, Beijing solarbio science & technology co.,Ltd., Beijing, China); MethylFlash Methylated DNA Quantification Kit (P-1035-96; Epigentek Group Inc., New York, USA). Egg yolk was obtained from fresh eggs of Tibet Linzhi Gama Breeding Co. Ltd (Linzhi, China).

Coomassie brilliant blue staining solution formula: 50 mg was dissolved in 100 mL 3.5% solution, boiled to dissolve, which was filtered using the filter paper, and stored the filtrate in a brown bottle at 4 °C. Hypotonic solution formula: 1.351 2 g fructose and 0.735 2 g sodium citrate dihydrate were dissolved in 100 mL deionized water. Freezing extender formula: 1.65% glucose, 2.22% citric acid, 3.63% Tris, 0.03% N-acetyl-L-cysteine (NAC), 9.00% glycerol, 0.03% ampicillin, 0.09% streptomycin, 30.00% egg yolk (Hu, 2009).

Semen collection: Ten fresh semen samples were obtained from each of the 10 Tibetan boars using the gloved-hand technique. After semen collection, no adverse effects on the health and growth of the Tibetan pigs were observed. Semen samples were filtered through three layers of gauze. Sperm with a motility

greater than 80% were isothermally diluted with sperm washing solution at a 1:1 ratio, placed in a thermos flask, and immediately transported to the laboratory. Semen diluent was prepared as described previously (Guo et al., 2010).

Preparation and thawing of frozen semen from high density thin tubes: Each semen sample was centrifuged, the supernatant was discarded, and the semen density was adjusted to 1×10^9 cells·mL^{-1}. Samples were incubated for 3-4 h in a freezer at 4 °C. The freezing extender was supplemented with 0, 0.2, 0.4, 0.6, or 0.8 g·L^{-1} astragalus polysaccharide. The balanced semen was inhaled with a 1 mL syringe after the needle was removed. The 0.5 mL thin tube was injected and sealed. Then, these tubes were frozen in liquid nitrogen. The sealed end of the thin tube was clamped with tweezers, and then the tube was defrosted in a water bath at 52 °C for 9 s. Next, the ends of the tube were removed, and the tube was placed in thawing solution preheated to 37 °C. The solution was mixed gently and evenly (Han et al., 2013).

Determination of sperm quality

Sperm motility: Sperm motility was assessed using a Computer-Assisted Semen Analysis (CASA) system (Hamilton Thorne Biosciences, MA) following the manufacturer's instructions. In short, put the semen to incubation at 37 °C for 10 min, then 3 µL semen was dropped into the preheated Makler sperm count board at 37 °C, sperm motility was assessed by using CASA system. This experiment was repeated three times, to observe 5 visual fields each time, 200 spermatozoa were counted at least each visual field.

Abnormality and acrosome integrity: Following the method of Zou et al (Zou and Yang 2000), coomassie brilliant blue staining solution was preheated in a 37 °C water bath for 10 min, the sperm smear was soaked in the staining solution for 5-7 min, then was rinsed with tap water, dried and sealed. Sperm abnormalities and acrosome integrity were examined under a 1 000× microscope to identify. The experiments were repeated three times, 200 spermatozoa were counted at least each time.

***Plasma membrane integrity*:** The low permeability swelling test (HOST) was used to determine plasma membrane integrity. In breifly, Mix 5 µL semen into 1 mL hypotonic solution to incubate for 30 min, and take 20 µL the mixture to drip into a blood count board under a 400 × phase contrast microscope, the sperm tail was observed and the sperm plasma membrane was analyzed. The details of this test were given in Guo et al (Guo *et al.*, 2010). This experiment was repeated three times, 200 spermatozoa were counted at least each time.

Sperm ROS level assay: According to the manufacturer's protocol of ROS assay kit (S0033M, Shanghai Beyotime Biotechnology Co. Ltd, Shanghai, China), sperm ROS level was evaluated by using the probe 2', 7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA). Breifly, Sperm suspensions (1×10^7 cells·mL^{-1}) were incubated with 10 µmol·L^{-1} DCFH-DA at 37 °C in the dark for 25 min. For intracellular DCFH-DA was oxidized by ROS to produce dichlorofluorescein with strong fluorescence, the fluorescence intensity could be monitored using a fluorescent microplate reader (Biotek Synergy, SynergyH4, USA) at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm. The treatments were replicated 3 times.

***DNA methylation levels in the sperm genome*:** Sperm genomic DNA was extracted from three groups (Fresh group represents fresh spermatozoa, No add group represents unsupplemented frozen spermatozoa, and Optimum add group represents frozen spermatozoa supplemented with the optimal concentration of APS) using the phenol-chloroform method. DNA concentration and purity were measured using a NanoDrop nd-1000 ultraviolet spectrophotometer. DNA samples were then diluted to 100 ng·mL^{-1}. The genomic DNA methylation levels in the three groups of spermatozoa were measured using MethylFlash Methylated DNA Quantification Kit following the manufacturer's instructions. In brief, standard curves were derived, DNA binding was performed, and signals were detected using a NanoQuant infinite M200 ELISA (TECAN Inc., Mannedorf, Switzerland). Finally, the amount of DNA methylation (5 mC) was calculated. This experiment was repeated three times, 1×10^7 spermatozoa were used to extract genomic DNA in each group each time.

Experiment design

***The Effect of APS on the quality of frozen-thawed Tibetan boar spermatozoa*:** The experiment was performed using five groups. APS with different mass concentrations (0, 0.2, 0.4, 0.6, and 0.8 g·L^{-1}) were added to the cryogenic release solution to make 0.5 mL of high-density fine-tube cryosperm. Sperm motility, abnormality, acrosome integrity, plasma membrane integrity, and sperm ROS level after thawing were used as the indexes to determine the optimal concentration of astragalus polysaccharide.

***Effects of APS on the methylation levels on the genomic DNA of Tibetan boar spermatozoa*:** To investigate the effects of APS on the methylation levels of genomic DNA from thawed cryopreserved Tibetan boar semen, the genomic DNA methylation levels of the three groups (Fresh group, No add group, and Optimum add group) of spermatozoa were detected using MethylFlash.

Statistical analyses: Statistical analyses were performed using IBM SPSS Statistics for Windows v17.0 (SPSS Inc., Chicago, IL, USA). Graphs were prepared using SigmaPlot 10.0 (Systat Software, San Jose, CA, USA). One-way analyses of variance (ANOVAs) were used to calculate the significance of differences among means, and multiple comparisons were performed. All quantitative data are presented as means ± standard deviation (S.D.). We considered $p<0.05$ statistically significant.

Results

***The Effects of APS on the quality of frozen-thawed Tibetan boar spermatozoa*:** Sperm motility (reached to 0.39), was significantly higher in the group supplemented with 0.4 g·L^{-1} APS than in all other groups (Table 1; p<0.05). Abnormality and RFU（ROS fluorescence Unit，which represent sperm ROS level ）were 50.11% and 33.6 respectively, which were significantly lower in this group than in all other groups (p<0.05). Acrosome integrity and plasma membrane integrity were 60.62% and 55.42% respectively, which were significantly higher in this group than in all other groups (p<0.05). Obviously, semen freezing extender supplemented with 0.4 g·L^{-1} APS increased sperm motility, acrosome integrity, and plasma membrane integrity, simultaneously decreased abnormality and the ROS level. Thus, the addition of 0.4 g·L^{-1} APS to the semen freezing extender improved the quality of frozen-thawed Tibetan boar spermatozoa.

Genomic DNA methylation level: The six points in the standard curve corresponded to 0, 0.5, 1.0, 2.0, 5.0, and 10.0 ng of 50% methylated DNA in the positive control. The fitted linear regression equation for the first four points (Fig. 1a) was y=1 119.1x+ 187 (R^2=0.987 6). The DNA methylation levels in the three groups (Fresh group, No add group, and Optimum add group), were 0.646 5, 0.910 2, and 0.704 0, respectively. Although the DNA methylation level of frozen spermatozoa supplemented with the optimal concentration of APS was significantly greater than that in the fresh sperm (p<0.05), DNA methylation level in this group was significantly lower than in the unsupplemented frozen sperm (p<0.05; Fig. 1b). Thus, APS significantly ameliorated the effects of freezing on genomic DNA methylation level in Tibetan boar spermatozoa.

Table 1 Effects of APS on the quality of frozen-thawed Tibetan boar spermatozoa

Concentration (g·L^{-1})	Motility	Abnormality (%)	Acrosome integrity (%)	Plasma membrane integrity (%)	RFU
0	0.30 ± 0.03^{c}	55.82 ± 2.67^{a}	53.03 ± 1.83^{c}	50.03 ± 1.83^{d}	51.4 ± 6.04^{a}
0.2	0.34 ± 0.02^{b}	53.74 ± 2.75^{ab}	58.01 ± 1.83^{b}	53.61 ± 1.90^{b}	44.2 ± 5.31^{b}
0.4	0.39 ± 0.04^{a}	50.11 ± 2.60^{c}	60.62 ± 2.91^{a}	55.42 ± 2.01^{a}	33.6 ± 4.60^{c}
0.6	0.32 ± 0.02^{bc}	52.93 ± 1.79^{b}	57.63 ± 2.12^{b}	52.51 ± 2.07^{bc}	44.45 ±4.39^{b}
0.8	0.31 ± 0.04^{c}	53.62 ± 2.41^{ab}	54.81 ± 2.66^{c}	51.22 ± 1.99^{cd}	54.2 ± 4.92^{a}

Note: All quantitative data are presented as means ± standard deviation (S.D.), n = 3 independent replicates. RFU = Reactive oxygen species (ROS) fluorescence Unit，which represent sperm ROS level; superscript letters in same column reflect significance: different letters indicate that the values are significantly different (p<0.05), whereas the same letter indicates that the values are not significantly different (p>0.05).

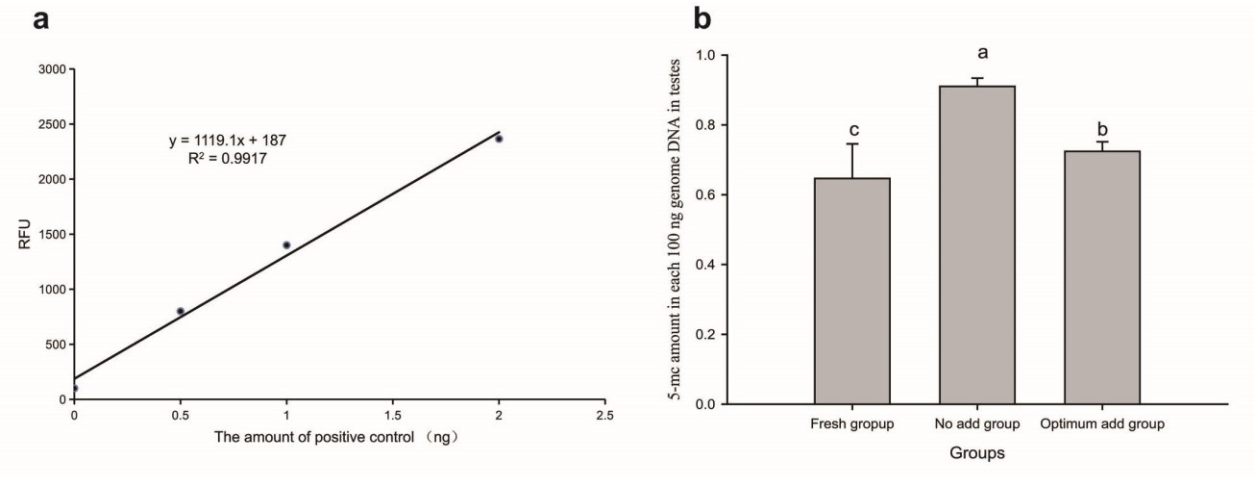

Figure 1 (a) The standard curve. (b) Genomic DNA methylation levels in Tibetan boar spermatozoa. Fresh group = fresh spermatozoa, No add group = unsupplemented frozen spermatozoa, and Optimum add group = frozen spermatozoa supplemented with the optimal concentration of APS. Each bar represents mean ± standard deviation (S.D.), n = 3 independent replicates. Different letters indicate significant differences (p<0.05), while identical letters indicate insignificant differences (p> 0.05).HS

Discussion

The Effect of APS on the quality of frozen-thawed Tibetan boar spermatozoa: Due to the high concentrations of unsaturated fatty acids in the plasma membranes of porcine spermatozoa, these cells are particularly sensitive to cold shock and peroxidation. However, antioxidants can protect the plasma membranes of frozen porcine sperm (Grossfeld *et al.*, 2008). The use of exogenous antioxidants may hold the key to alleviating the harmful effects of oxidative stress caused by inappropriate formation of ROS in boar sperm (Lee and Park, 2015). For example, the addition of high concentrations of fennel to diluted solutions of pig semen significantly improves the viability of pig spermatozoa after freezing, but has no effect on replacement integrity (Malo *et al.*, 2012). It has been proposed that an extender containing 0.5 g·L^{-1} Gynostemma pentaphyllum polysaccharide might be a more effective cryoprotective medium (Hu *et al.*, 2009).

The optimal concentration of the γ-oryzanol required for boar semen cryopreservation in lactose-egg yolk freezing extender is not only dependent on the individual boar but also on the breed of boar; that is, 0.16 mM was required for Duroc boars, whereas 0.24 mM was required for Large White and Landrace boars (Chanapiwat and Kaeoket, 2015). The addition of APS to thawed boar sperm improved the antioxidant abilities of the spermatozoa, the parameters *in vitro* fertilization, and the outcomes of embryonic development (Weng *et al.,* 2018). As ROS have been recognized as problems for sperm survival and fertility when its production exceeds formation and membrane lipid peroxidation, the reduction in motility may have been due to a ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum (Guthrie and Welch, 2012). APS treatment could help maintain the boar sperm antioxidant capacity and ameliorate the accumulation of ROS, which were mainly due to the elimination of excessive mitochondrial ROS, the improvement of antioxidant capacities and the enhancement of ATP levels (Fu *et al.,* 2018). The extender supplimented with 0.4 g·L^{-1} but not 0.8 g·L^{-1} APS can demonstrate desirable parameters of boar frozen-thawed spermatozoa (Yang *et al.,* 2014). These results were consistent with the results herein. That is, while 0.4 g·L^{-1} of APS was effective, 0.8 g·L^{-1} did not work in overcoming the detrimental effects of cryopreservation in spermatozoa, hinting that too high concentration of APS might increase osmotic pressure of the freezing extender, which might be ineffective to improve the quality of frozen-thawed spermatozoa. Overall, semen freezing extender supplemented with 0.4 g·L^{-1} APS increased sperm motility, acrosome integrity, and plasma membrane integrity, simultaneously decreased abnormality and the ROS level. Thus, the addition of 0.4 g·L^{-1} APS to the semen freezing extender improved the quality of frozen-thawed Tibetan boar spermatozoa.

Genomic DNA methylation level: Abnormal sperm DNA methylation patterns are associated with infertility (Capra *et al.,* 2019). The methylation levels of sperm cytosine-phosphoric acid-guanine (CpGs) differed significantly between cases and the control group (Alkhaled *et al.,* 2018). Semen processing for cryopreservation increased DNA methylation levels in stallion semen (Aurich *et al.,* 2016). Global DNA methylation is a robust parameter for biotechnologies such as hormonal spermiation induction and sperm cryopreservation, but this parameter can be altered when optimal sperm manipulation conditions are not met (Depince *et al.,* 2020). Supplementation with ethylene diamine tetraacetic acid (EDTA), EDTA-rosmarinic acid (EDTA-RA), and EDTA-melatonin (EDTA-MLT) during freeze-drying preserved the morphological integrity and methylation status of rabbit sperm (Mercati et al. 2020). Previously, we found that vitamin C significantly reduced the effects of preservation at 4 °C on the methylation levels of genomic DNA from Tibetan boar spermatozoa (Ren *et al.,* 2016). We also found that rhodiola polysaccharide significantly reduced the influence on DNA methylation of Tibetan boar spermatozoa after freezing (Ren *et al.,* 2017). Here, APS significantly reduced the influence on DNA methylation of boar spermatozoa after freezing. However, further study is required to determine how to normalize the DNA methylation levels of Tibetan pig sperm after freezing.

In summary, supplementation of semen freezing extender with 0.4 g·L^{-1} APS not only improved the quality of frozen-thawed Tibetan boar spermatozoa, but also significantly reduced the effects of freezing on the level of genomic DNA methylation in the spermatozoa (under the conditions of this experiment). Our results provide a reference for further investigations aiming to mitigate the negative effects of cryopreservation on Tibetan boar semen, and will increase our understanding of the epigenetic mechanisms of sperm freezing injuries in Tibetan boars.

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