Animal Reproduction Science 210 (2019) 106178

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effects of *Isatis* root polysaccharide on boar sperm quality during liquid storage and *in vitro* fertilization

Zhiqiang Ren, Weike Shaoyong, Qian Li, Lu Ma, Junying Xiao, Junheng Jiao, Gongshe Yang, Weijun Pang*

Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Province, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China

ARTICLE INFO

Keywords:
Isatis root polysaccharide
Boar sperm
Liquid storage
IVF

ABSTRACT

Liquid preservation of boar semen is a preferred method in pig husbandry, and antioxidants to protect against sperm oxidative stress during periods of storage have become the focus of recent research. Through its antioxidant activity, *Isatis* root polysaccharide (IRPS), a plant extract, can effectively reduce the cellular lipid peroxidation caused by the accumulation of reactive oxygen species inside mitochondria. In the present study, there was examination of the effects of no supplementation (Control) of a semen extender with or supplementation in different concentrations of IRPS (0.2, 0.4, 0.6, 0.8, and 1.2 mg/mL) on sperm quality variables and antioxidant capacity during liquid storage. The results indicate that after prolonged storage (≥ 3 days), the sperm motility was greater in the group supplemented with 0.6 mg/mL IRPS than in the other groups ($P < 0.05$). The use of this IRPS concentration also resulted in maintanence of acrosome integrity, plasma membrane integrity, mitochondrial membrane potential, and antioxidant capacity of the sperm ($P < 0.05$). Furthermore, the results of an *in vitro* fertilization study indicate IRPS at 0.6 mg/mL markedly increased the sperm fertilization capacity ($P < 0.01$) and embryonic development to the blastocyst stage ($P < 0.05$). The addition of 0.6 mg/mL IRPS enhanced the antioxidant capacity of boar sperm, resulting in greater preservation of sperm motility and fertilization capacity during liquid storage. These findings indicate that IRPS has the potential to be used as a component of a semen-preserving diluent to maintain sperm quality during storage.

1. Introduction

Artificial insemination (AI), which is commonly used as an assisted reproductive technology in swine production to maximize the use of genetically superior sires, has effectively improved the genetics for pork production worldwide (Roca et al., 2016). There are two primary storage strategies for boar semen, cryopreservation and liquid preservation. Boar sperm, however, have been reported to be more vulnerable to oxidative stress during the freezing-thawing process when compared with those of other species. This problem is related to its unique physiological structure of boar sperm, and results in damage and death of these cells (Johnson et al., 2000; Knox, 2015). In parallel, compared with cryopreservation, liquid semen preservation is more convenient and economical, thus this approach for boar semen storage is generally used by companies processing this semen (Fang et al., 2017).

Reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) are

* Corresponding author at: No. 22 Xinong Road, Yangling, Shaanxi, Province 712100, China.
E-mail address: pwj1226@nwsuaf.edu.cn (W. Pang).

https://doi.org/10.1016/j.anireprosci.2019.106178
Received 12 April 2019; Received in revised form 12 August 2019; Accepted 4 September 2019
Available online 05 September 2019
0378-4320/

byproducts of oxygen metabolism inside sperm mitochondria (Gomez-Fernandez et al., 2013; Menegat et al., 2017). Although there is little basal ROS production in fresh boar spermatozoa, the ROS content increases in liquid storage as duration of storage advances and there are two primary effects on sperm function (Guthrie and Welch, 2006; Kumaresan et al., 2009). Accumulating evidence indicates ROS formation is a requisite for normal spermatozoan function (Aitken, 2017; Moraes and Meyers, 2018). At physiological concentrations, ROS induce sperm capacitation and an acrosome reaction that will contribute to the capacity of sperm to penetrate oocytes during the process of fertilization (Awda et al., 2009). In contrast, sperm oxidative stress can be induced when the homeostasis between ROS biosynthesis and degradation is disturbed, leading to lipid peroxidation of membranes, DNA fragmentation, and an overall decrease in semen quality (Hu et al., 2014; Zhang et al., 2016). A wide range of antioxidants, therefore, have been studied for use in the preservation of boar semen, including superoxide dismutase, astaxanthin, and astragalus polysaccharide, to ameliorate oxidative damage of sperm cells (Zhang et al., 2017; Basioura et al., 2018; Fu et al., 2018).

The root of *Isatis* (*Radix Isatidis*), in which polysaccharides are the primary active ingredients, is a traditional Chinese medicine that has been extensively used clinically because it cost very little and there are few side effects when it is consumed (Li et al., 2016). *Isatis* root polysaccharide (IRPS), isolated from a Radix Isatidis aqueous extract, has various biological activities, particularly antioxidant, bacteriostatic, and antiviral (Wei et al., 2011). Most of the previous research on IRPS focused on its antibacterial and antiviral activities for the treatment of associated diseases; however, evidence indicates IRPS also has a marked scavenging capacity for ROS free radicals (Han et al., 2011). For example, IRPS-treated mouse alveolar macrophages have relatively greater glutathione contents and superoxide dismutase activity, as well as lesser ROS concentrations (Du et al., 2013). Other plant extracts, such as those of rooibos and *Rosmarinus officinalis*, have proven to be useful as natural sources of antioxidants for boar semen preservation, but these compounds do not protect the sperm plasma membrane or acrosome (Malo et al., 2010; Ros-Santaella and Pintus, 2017). Considering the ready availability and economical cost of IRPS, the compound may be a viable antioxidant additive to a boar semen extender.

The aim of the present study, threfore, was to investigate the antioxidant effects of IRPS on boar sperm quality during liquid storage and *in vitro* fertilization (IVF). During the 7 days of preservation, sperm motility, plasma membrane integrity, acrosomal integrity, mitochondrial membrane potential, total antioxidant capacity (T-AOC), malondialdehyde (MDA) content, and ROS concentrations were evaluated. Furthermore, there was an IVF assessment of fertilizing capacity and subsequent embryonic development after 5 days of liquid storage.

2. Materials and methods

All experiments involving animals were conducted in compliance with animal research guidelines of the Animal Welfare and Ethics Code of China, and experimental procedures were approved by the Animal Care and Use Committee of Northwest A&F University.

2.1. Reagents and chemicals

The IRPS (purity 98%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Unless otherwise indicated, other reagents and chemicals used in the present study were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Experimental design

To evaluate the effects of no treatment (Control) with or with IRPS treatments of 0.2, 0.4, 0.6, 0.8, and 1.2 mg/mL on sperm quality variables and mitochondrial activity, the sperm motility, acrosome integrity, plasma membrane integrity, and mitochondrial membrane potential were evaluated after 1, 3, 5, and 7 days of liquid storage.

The T-AOC of the sperm and the MDA content of the semen were qunatified during the entire period of liquid storage in samples where there was no treatment (Control) with or treatments with 0.2, 0.4, 0.6, 0.8, and 1.2 mg/mL of IRPS. In addition, there was selection of the IRPS concentration of 0.6 mg/mL for ROS assessment to determine the antioxidant capacity of IRPS.

Based on the experimental data and the most commonly used AI procedure at swine farms, there was liquid storage of semen for 3–5 days. Semen supplemented with 0.6 mg/mL IRPS was preserved for 5 days for an assessment of IVF and subsequent embryo development to the blastocyst stage.

All experiments were performed with at least five biological and three technical replicates.

2.3. Semen collection

Sperm-rich fraction of ejaculates were collected using the gloved-hand technique from six healthy and sexually mature Guanzhong black boars, aged between 1.5 and 2 years. Immediately after collection, the semen samples were stored in a 37 °C vacuum flask and transported to the laboratory within 30 min of collection. The interval between successive semen collections was 3 days, and a total of 60 ejaculates (ten ejaculations per boar) were collected for analysis in this study.

2.4. Semen processing

The basic Beltsville thawing solution (BTS) diluent used in this experiment consisted of 37.15 g glucose, 1.25 g EDTA, 0.75 g KCl, 6.00 g disodium citrate, 1.25 g sodium bicarbonate, and 0.25 g gentamicin, which were dissolved in 1 L deionized water. With a 0.22 μm bacterial filter, the diluent was filtered and placed in a thermostat water bath at 37 °C.

After collecting fresh semen, the sperm concentration was determined using a sperm densitometer (Iberspectro v11, Portugal). Sperm quality variables were evaluated using a phase-contrast microscope (Nikon Eclipse E600, Tokyo, Japan) in combination with a computer-assisted sperm analysis (CASA) system (Hamilton Thorne Research, Beverly, MA, USA). Only samples that met the following criteria were used in this study: sperm concentration $\geq 2.0 \times 10^8$ spermatozoa/mL, motility $\geq$ 80%, and normal sperm morphology $\geq$ 85% (Fang et al., 2017; Li et al., 2018). To reduce the effect of individual boar differences, equal volumes (10 mL) of qualified semen doses from six boars were pooled and homogenized. Subsequently, six aliquots were prepared with a BTS extender, which did not contain (Control) or did contain 0.2, 0.4, 0.6, 0.8, and 1.2 mg/mL IRPS. The diluted semen (2.0×10^7 spermatozoa /mL) was stored in a 17 °C incubator throughout the study.

2.5. Evaluation of sperm quality variables and mitochondrial membrane potential

After 0, 1, 3, 5, and 7 days of preservation, aliquots of each stored sample were removed to evaluate the sperm motility, acrosome integrity, plasma membrane integrity, and mitochondrial membrane potential.

2.5.1. Assessment of sperm motility

The total sperm motility was evaluated using the CASA system (Hamilton Thorne Research, Beverly, MA, USA) and a phase-contrast microscope (Nikon Eclipse E600, Tokyo, Japan) at 400x magnification (Fang et al., 2017; Weng et al., 2018), with standard parameter settings as previously described (Pintus et al., 2018). Briefly, after incubation at 37 °C for 15 min, 10 μL of each sample was loaded into a chamber (Cell-Vu Sperm Counting Chamber® DRM-600) and warmed at 37 °C for 30 s before microscopic examination. Subsequently, five visual fields, containing at least 200 spermatozoa, were randomly selected to record the motility data.

2.5.2. Assessment of sperm acrosome integrity

The acrosome integrity of spermatozoa was analyzed using staining with fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) and 4′,6-diamidino-2-phenylindole (DAPI), according to the procedure described previously (Yamashiro et al., 2013; Pei et al., 2018). In short, a 30 μL aliquot of semen was applied to a glass slide and fixed in anhydrous methanol for 15 min at room temperature. Thirty microliters each of 10 μg/mL DAPI working solution and 100 μg/mL FITC-PNA working solution were sequentially added to each slide with a 10 min interval between additions, and the slide was placed in a wet box at 37 °C for 30 min. Slides were then rinsed three times with phosphate buffered saline (PBS) and air-dried. A fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) was used at 400x magnification to analyze the percentages of sperm acrosomes that were intact, disrupted, or undergoing exocytosis. For each sample, the staining of at least 200 spermatozoa was evaluated.

2.5.3. Assessment of sperm plasma membrane integrity

The LIVE/DEAD® Sperm Viability Kit (Molecular Probes Inc., Eugene, OR, USA) was used to assess the sperm plasma membrane integrity as previously described, with some modifications (Garner and Johnson, 1995). Each 1 mL of semen was incubated at 37 °C with 1 μL of a 100 nM SYBR-14® stock solution in dimethyl sulfoxide for 10 min, followed by incubation with 5 μL of a 12 μM propidium iodide (PI) solution for 5 min. After the incubation, 10 μL of the stained semen was pipetted onto a glass slide and observed using a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) at 400x magnification. Sperm with an intact plasma membrane emitted green fluorescence, while spermatozoa with red-orange fluorescence had a disrupted plasma membrane and the cells were considered to be dead. The staining characteristics were evaluated for at least 200 spermatozoa/microscopic field to assess plasma membrane integrity.

2.5.4. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit (Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The BTS was used to adjust the concentration of each sample to 2.0×10^6 spermatozoa/mL. A tube containing 1 mL of semen with 0.5 mL of the JC-1 solution was shaken and then incubated at 37 °C for 20 min, followed by observation using the fluorescence microscope at 400x magnification to determine the membrane potential (at least 200 spermatozoa/field). A relatively greater mitochondrial membrane potential was indicated by the JC-1 concentration in the mitochondrial matrix that were observed as red fluorescent aggregates, while a relatively lesser mitochondrial membrane potential was indicated by green fluorescence.

2.6. Evaluation of sperm T-AOC, MDA content, and ROS concentratios

The sperm T-AOC, MDA content, and ROS concentrations were assessed in the semen samples after 1, 3, 5, and 7 days of preservation. For these assessments, 500 μL of each semen sample (2.0×10^7 spermatozoa /mL) was transferred to a 1.5 mL centrifuge tube and incubated in a water bath at 37 °C for 5 min.

2.6.1. T-AOC determination

Changes in the sperm T-AOC were detected using a T-AOC Assay Kit (FRAP; Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) using the manufacturer's instructions. Because antioxidant compounds in sperm can reduce Fe^{3+} to Fe^{2+}, which forms stable complexes with phenanthrolines, the antioxidant capacity can be quantified using colorimetry. Prior to any evaluation, supernatants were collected using the method described by Fu et al. (2018). Supernatants were then mixed with the reaction buffer and incubated at room temperature for 10 min. Absorbance of each sample was quantified spectrophotometrically at 520 nm with repetition three times.

2.6.2. Determination of the MDA content

An MDA Assay Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to determine the concentrations of MDA, following the manufacturer's protocol. The MDA, produced as a result of lipid peroxide degradation, condenses with thiobarbituric acid (TBA) to form a red substance, which can be quantified colorimetrically. In brief, the semen samples (2.0×10^7 spermatozoa/mL) were mixed with the reaction buffer, and incubated in a water bath at 95 °C for 40 min. The sample was subsequently cooled with running water, and the supernatant was collected by centrifugation at 3000 × *g* for 10 min. The absorbance of each supernatant was quantified three times using a spectrophotometer at a wavelength of 532 nm.

2.6.3. Determination of ROS concentrations

Intracellular ROS concentrations were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; ROS Assay Kit; Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). In the presence of ROS, DCFH-DA is oxidized to dichlorofluorescein (DCF), with the fluorescence intensity being proportional to the amount of ROS. The sperm samples (2.0×10^7 spermatozoa/mL) were incubated with DCFH-DA (10 μM) at 37 °C in a darkened area for 30 min, then there was centrifugation at 1000 × *g* for 5 min, and the pellet was rinsed twice with PBS (1000 × *g* for 5 min). The pellet was subsequently re-suspended in PBS, and the emitted fluorescence was quantified at excitation/emission wavelengths of 500/525 nm.

2.7. In vitro maturation of pig oocytes

In vitro maturation of pig oocytes was performed as previously described (Spinaci et al., 2018; Weng et al., 2018; Shaoyong et al., 2019). After pig ovaries were collected at a local slaughterhouse (Yangling, China), cumulus-oocyte complexes (COCs) were extracted from ovarian follicles using an 18-gauge needle attached to a 10 mL disposable syringe. Using a stereomicroscope, intact COCs, with oocytes surrounded by at least three uniform layers of compact cumulus cells, were selected and transferred into maturation medium (TCM199 base medium supplemented with 2.012 g/L sodium bicarbonate, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 15 ng/mL epidermal growth factor, 2.5 IU/mL follicle-stimulating hormone, 10 IU/mL human chorionic gonadotropin, 10 IU/mL pregnant mare serum gonadotropin, 75 μg/mL penicillin G potassium, and 50 μg/mL streptomycin sulfate). The medium was incubated at 38.5 °C with 5% CO_2 and saturation humidity for 42 to 44 h. After maturation, oocytes were denuded by gentle repeated pipetting.

2.8. In vitro fertilization

An IVF procedure was conducted to assess the fertilization capacity of sperm using previously described methods (Abeydeera and Day, 1997; Shaoyong et al., 2019). Semen samples were divided into a control and treatment group, respectively, with there being no treatment with or treatment with 0.6 mg/mL IRPS. Two groups of semen, stored for 5 days, were subjected to a capacitation treatment by incubating sperm cells for 30 min in capacitation medium (95 mM NaCl, 4.8 mM KCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.56 mM glucose, 1 mM sodium pyruvate, and 0.6% [w/v] bovine serum albumin [BSA]). The semen samples were subsequently washed three times with BTS supplemented with 1% BSA, then centrifuged at 800 × *g* for 10 min, and re-suspended in IVF medium. Subsequently, capacitated sperm cells and mature oocytes were transferred to IVF medium at a ratio of 150:1 (oocytes = 50) and incubated at 38.5 °C in a humidified atmosphere with 5% CO_2. After 6 h, a subsample of oocytes was fixed in 4% paraformaldehyde for 30 min and washed in polyvinyl alcohol (PVA) -PBS three times, then stained with Hoechst 33342 for 10 min. Rates of sperm-oocyte binding were evaluated using fluorescence microscopy at 200x magnification. The remaining cultured oocytes were washed and transferred to PZM-3 medium and incubated for 2 or 7 days with the medium that was replaced every 2 days. After fixing in 1:3 acetic acid/ethanol for 24 h and staining with Hoechst 33342, oocytes were analyzed using fluorescence microscopy at 200x magnification to determine the percentage cleavage rate (> 1 cell embryos/number of inseminated oocytes) and percentage rate of embryo development to the blastocyst stage (number of blastocysts/number of inseminated oocytes).

2.9. Statistical analysis

Statistical analyses were conducted using the SPSS software version 21.0 (SPSS, Inc., Chicago, IL, USA). First, data were evaluated for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test). When required, data were transformed using arcsine $\sqrt{x}$ or log(x) to meet parametric assumptions (Puig-Timonet et al., 2018). Data, including those on sperm motility, acrosome integrity, plasma membrane integrity, mitochondrial membrane potential, T-AOC, MDA content, and ROS concentrations, were subsequently analyzed using the repeated-measures analysis of variance, followed by use of the Sidak's *post-hoc* test (except ROS concentrations). The Friedman and Wilcoxon tests were used as nonparametric alternatives for data analyses. Time of liquid storage was considered a within-subjects factor and IRPS concentration was considered a between-subjects factor. Furthermore, an independent-samples Student's *t*-test was performed to assess the difference in sperm-oocyte binding, percentage cleavage rate of oocytes fertilized, and the percentage of inseminated oocytes developing to the blastocyst stage. Results are presented as mean ± standard deviation,

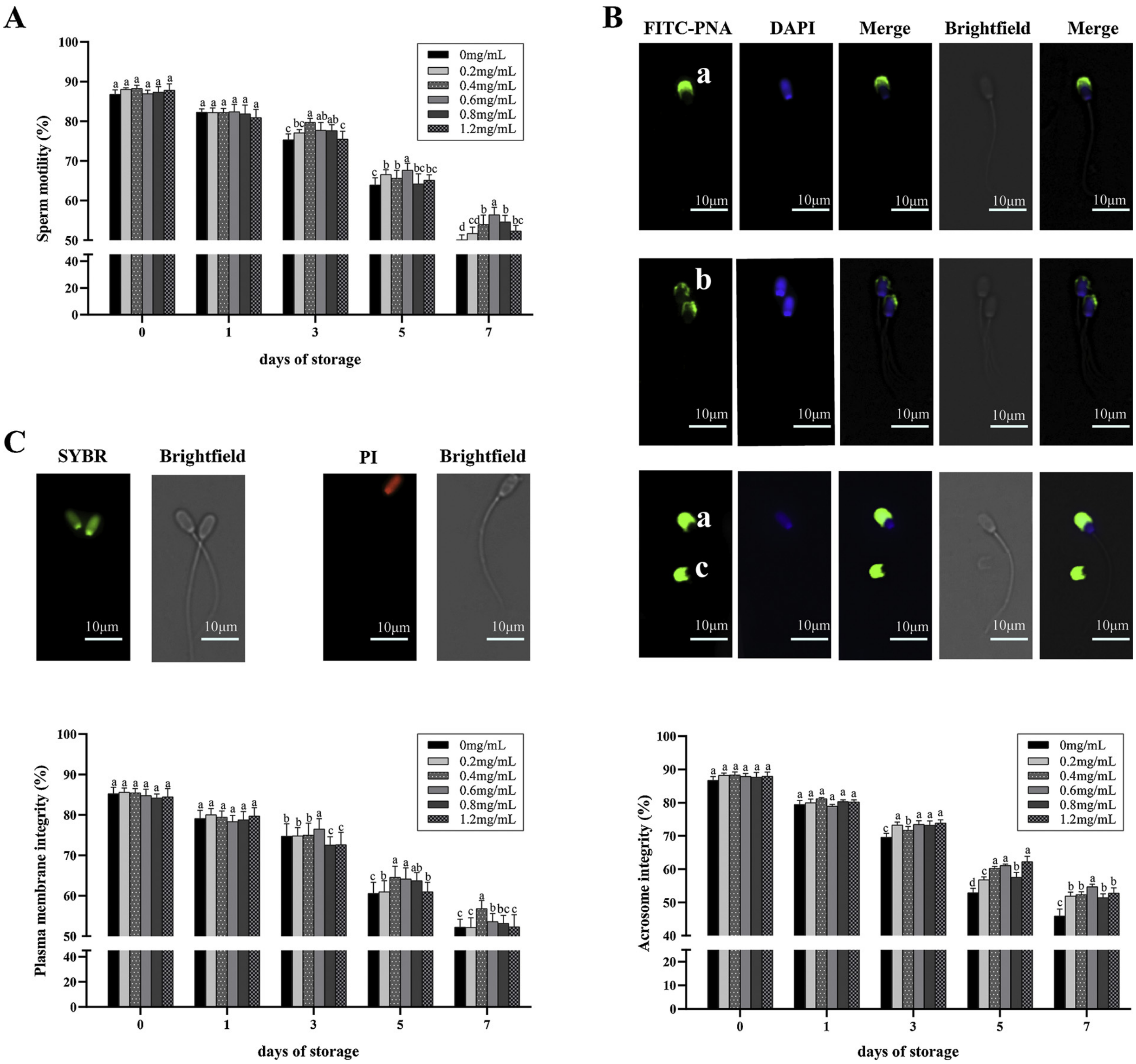

Fig. 1. Effect of different concentrations of IRPS on boar sperm quality variables; (A) Sperm motility, (B) Fluorescent patterns of sperm acrosome (Upper) and percentage of acrosome intact sperm (Lower) (a) Sperm with intact acrosome, (b) Sperm with disrupted acrosome and (c) exocytosed acrosome; (C) Fluorescent patterns of sperm plasma membrane (Upper) and percentage of plasma membrane intact sperm (Lower) (plasma membrane integrity: green fluorescence, plasma membrane damage: red fluorescence); Scale bars = 10 µm; Values are presented as mean ± SD (n = 5); [a–d] Different letters indicate significant differences between values ($P < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and $P < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. Effects of IRPS supplementation on sperm quality variables

3.1.1. Sperm motility

As depicted in Fig. 1A, the total sperm motility gradually decreased during the 7 days of storage but was greater with IRPS supplementation of media than in the control group. Total sperm motility on the first day of preservation was not different among all the groups. After 3 days of storage, however, the groups supplemented with 0.4, 0.6, and 0.8 mg/mL IRPS had a greater total sperm motility compared to the control ($P < 0.05$). Notably, there was the greatest total sperm motility after 5 or 7 days in the group treated with 0.6 mg/mL IRPS ($P < 0.05$), indicating that the addition of IRPS could suppress the decrease in total sperm motility

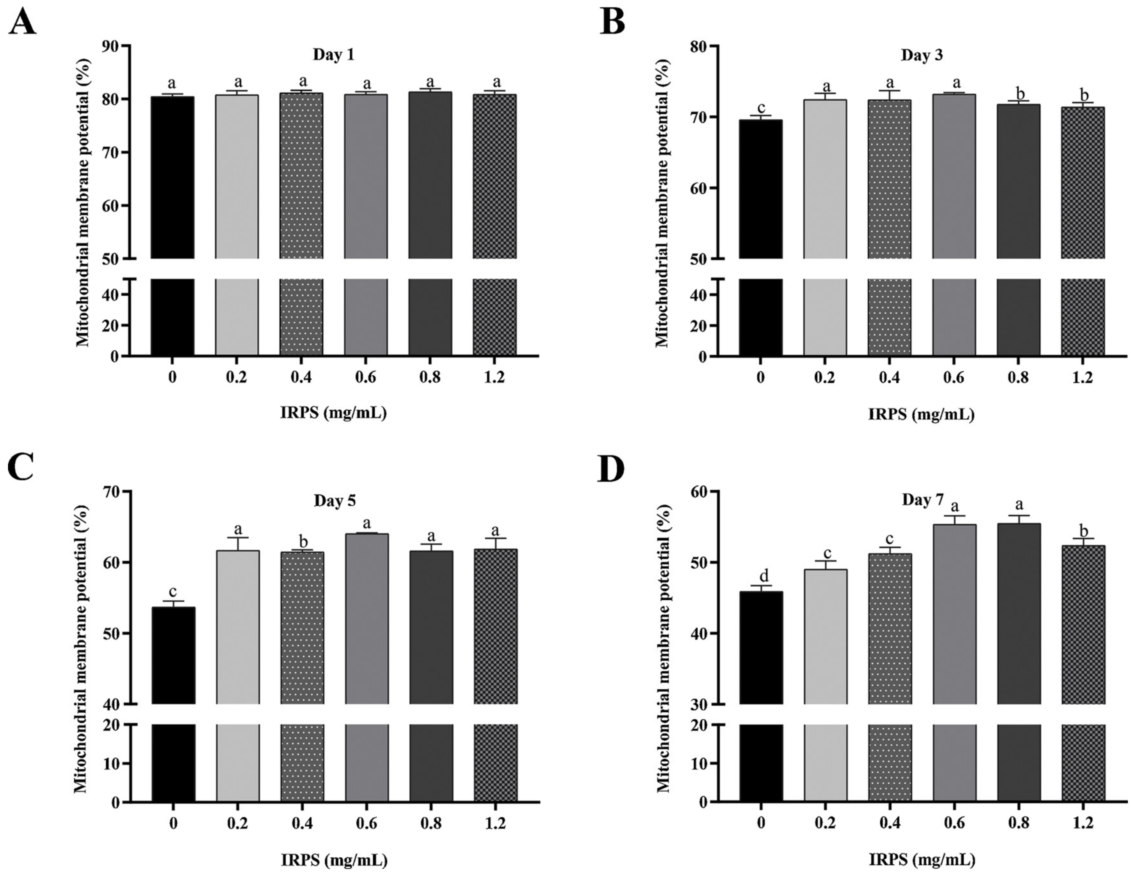

Fig. 2. Effect of different concentrations of IRPS on mitochondrial membrane potential of sperm during different storage periods; (A) Day 1, (B) Day 3, (C) Day 5, (D) Day 7; Values are presented as mean ± SD (n = 5); [a–d] Different letters indicate significant differences between values ($P < 0.05$).

during storage.

3.1.2. Acrosome integrity

To determine whether the addition of IRPS affects the sperm acrosome integrity during semen preservation at 17 °C, there was evaluation of spermatozoa after FITC-PNA and DAPI staining. The results are depicted in Fig. 1B. There was no difference in acrosome integrity at the initiation of storage; however, there were changes starting on day 3, when all IRPS-treated groups had greater acrosome integrity than the control group ($P < 0.05$). Importantly, after 7 days of storage, the group treated with 0.6 mg/mL IRPS had a greater acrosome integrity than all other groups ($P < 0.05$).

3.1.3. Plasma membrane integrity

In the process of semen preservation, the plasma membrane integrity of the sperm was substantially greater in the 0.6 mg/mL IRPS than control group (Fig. 1C). When semen aliquots, however, were supplemented with 0.8 or 1.2 mg/mL IRPS, sperm plasma membrane integrity was less than in the control group on day 3 ($P < 0.05$). Although both 0.4 and 0.6 mg/mL concentrations of IRPS suppressed the decrease in the sperm plasma membrane integrity on day 5, treatment with 0.4 mg/mL IRPS for 7 days resulted in the greatest plasma membrane integrity ($P < 0.05$). These data indicate IRPS exerted protective effects on the plasma membrane of the sperm preserved at 17 °C.

3.2. Effects of IRPS on sperm mitochondrial membrane potential

The changes in the mitochondrial membrane potential of the sperm treated with different concentrations of IRPS are depicted in Fig. 2. The data indicate mitochondrial membrane potential of the sperm was markedly affected by supplementation of the extender with IRPS during semen preservation. There were no notable differences in the mitochondrial membrane potential of the sperm among the 0.2, 0.4, and 0.6 mg/mL IRPS treatment groups during the first 3 days of preservation; however, the values were greater than those in the control group ($P < 0.05$). In addition, there was a greater mitochondrial membrane potential maintained in the group treated with 0.6 mg/ml IRPS throughout the storage period, indicating this was a concentration range for IRPS that could be used to maintain sperm quality during periods when there was preservation occurring.

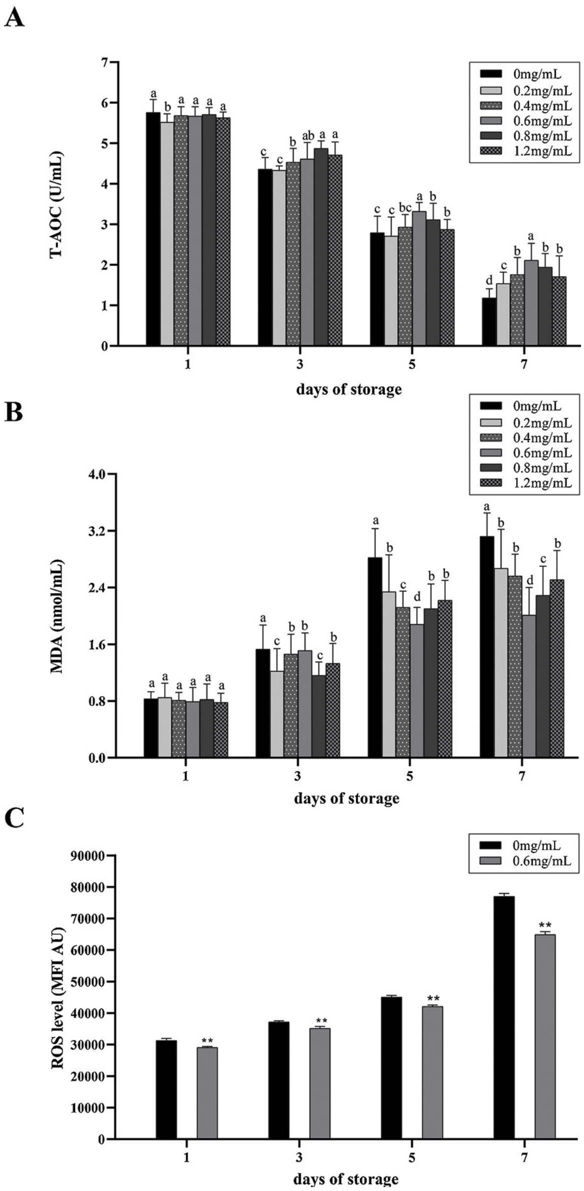

Fig. 3. T-AOC, MAD content, and ROS concentrations on different days during liquid storage of boar sperm with different concentrations of IRPS; (A) Total antioxidant capacity, (B) Malondialdehyde content, (C) Reactive oxygen species concentrations; Values are presented as mean ± SD (n = 5); [a–d] Different letters indicate differences between values ($P < 0.05$); *$P < 0.05$, **$P < 0.01$.

3.3. Effects of IRPS on T-AOC, MDA content, and ROS concentrations

To further explore the antioxidant properties of IRPS during semen storage at 17 °C, there was examination of T-AOC, the MDA content, and ROS concentrations, and the data are depicted in Fig. 3. After 1 day of preservation, there were no obvious differences in the MDA content among the groups (Fig. 3B), but T-AOC was less in the 0.2 mg/mL IRPS group ($P < 0.05$; Fig. 3A). Differences in T-AOC and the MDA content among the groups, however, gradually increased during the period of semen preservation. On days 5 and 7 of storage, there was a markedly lesser MDA content in the 0.6 mg/mL IRPS treatment group compared with that in the other groups, which was consistent with maintaining T-AOC at the greatest concentration with the 0.6 mg/mL IRPS supplementation of semen medium ($P < 0.05$). Importantly, compared with that in the control group, the ROS production was less ($P < 0.01$) on days 1, 3, 5, and 7 of storage as a result of supplementation of the semen extender with 0.6 mg/mL IRPS (Fig. 3C).

3.4. Effects of adding IRPS during semen liquid storage on IVF

Considering the results when there was evaluation of the effects of IRPS concentrations on sperm viability in the present study,

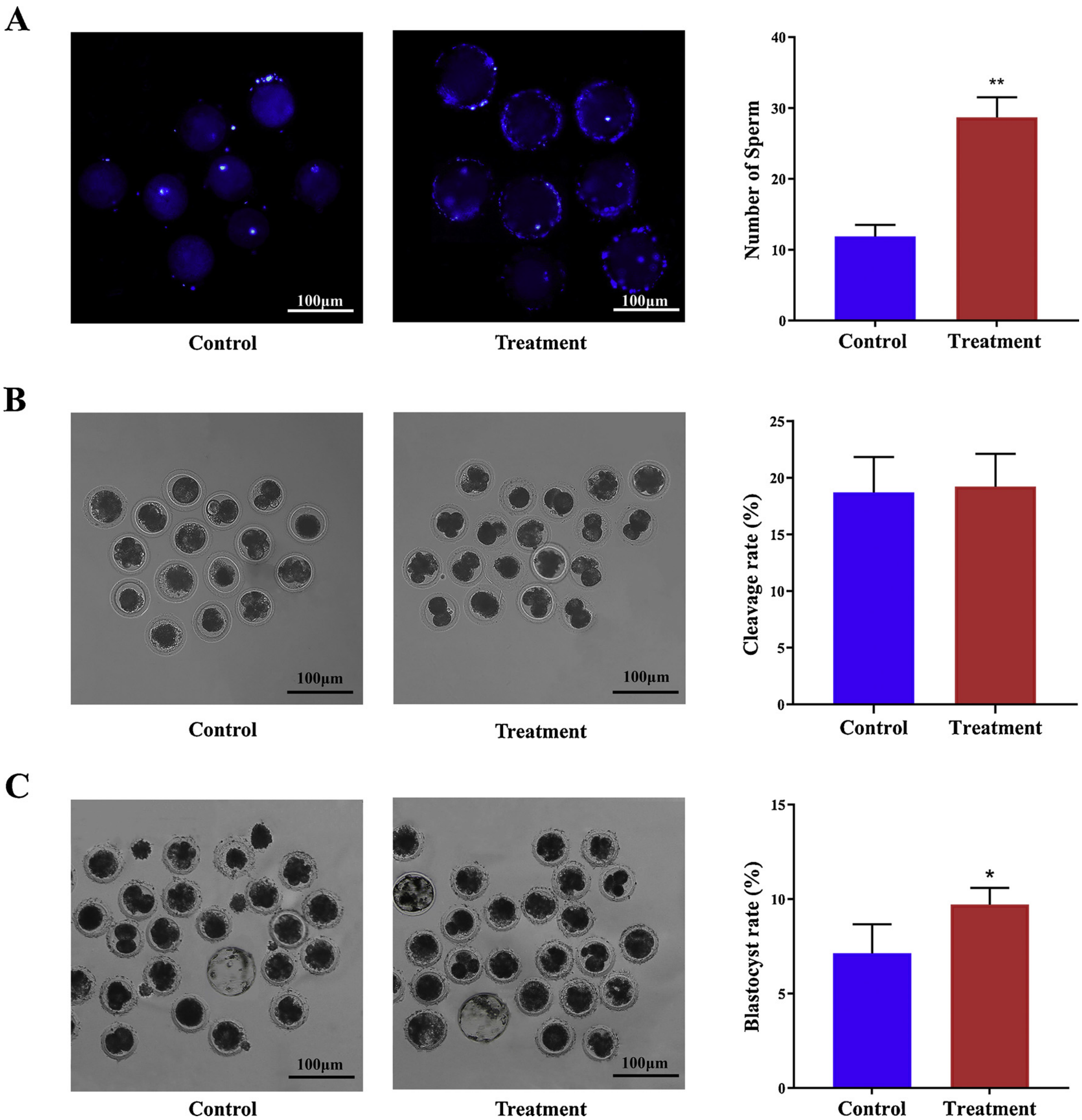

Fig. 4. Effect of adding 0.6 mg/mL IRPS on sperm-oocyte binding and embryo development; (A) Fluorescent patterns of sperm-oocyte binding and its quantification, (B) Fluorescent patterns of Cleavage and its quantification (cleavage rate: > 1 cell embryos/number of inseminated oocytes), (C) Fluorescent patterns of Blastocyst and its quantification (blastocyst rate: number of blastocysts/number of inseminated oocytes); Control: 0 mg/mL IRPS, Treatment: 0.6 mg/mL IRPS; Scale bars = 100 µm; Values are presented as mean ± SD (n = 5); Different letters indicate differences between values $*P < 0.05$, $**P < 0.01$.

adding 0.6 mg/mL IRPS to the suspension medium for *in vitro* sperm preservation was beneficial for sperm quality and antioxidant capacity; therefore, this concentration of IRPS was selected for the IVF experiment of this study.

The results of the IVF experiment are depicted in Fig. 4. There was a difference ($P < 0.01$) in sperm binding to oocytes when semen samples of the two groups (with and without semen pretreatment with IRPS during storage) were used for insemination of oocytes. With the IRPS-pretreated sperm, there was greater sperm binding to oocytes than with samples not pretreated with IRPS (Fig. 4A). Interestingly, with IRPS treatment there was an increased ($P < 0.05$) percentage of embryos developing to the blastocyst stage; however, there was no effect of IRPS treatment on percentage cleavage rate (Fig. 4B, C).

4. Discussion

Oxidative damage has been recognized as one of the major factors contributing to sperm dysfunction after *in vitro* storage, and thus, finding an optimal antioxidant is the important for maintaining sperm quality (Tremellen, 2008; Tuncer et al., 2010; Basioura et al., 2018). The IRPS compound, extracted from Isatis root, is a natural macromolecular substance composed of different proportions of xylose, arabinose, glucose, rhamnose, mannose, and galactose. Polysaccharides have antioxidant activity not only because these compounds scavenge ROS free radicals but also because of the increased antioxidant enzyme activity in the body. In some studies there have been benefits of IRPS in ameliorating lipid peroxidation; however, its potential application as an antioxidant in semen preservation remain unknown (Du et al., 2013; Li et al., 2019). With this study, there was exploration of the antioxidant properties of IRPS as an additive for semen storage at 17 °C. As expected, IRPS, especially at a concentration of 0.6 mg/mL in a liquid storage system, positively affected the preservation of semen quality during storage, resulting in greater values for sperm quality variables, T-AOC, and fertilization capacity than values for the control group. Furthermore, the reduction in the concentrations of ROS and MDA in stored semen clearly indicated that IRPS had a marked antioxidant capacity.

The sperm motility variable is indicative of metabolic homeostasis and membrane integrity and is an important variable for assessing quality and predicting fertilization success (Johnson et al., 2000; Sutkeviciene et al., 2009). Results of other studies indicate there are some antioxidants that effectively protect boar sperm against oxidative stress, with sperm viability being maintained during periods of storage (Mendez et al., 2013; Pintus et al., 2018; Wang et al., 2018). Consistently, results of the present study indicated that supplementation with IRPS resulted in a maintenance of sperm motility until the third day of storage. Furthermore, as the storage time increased, the sperm motility decreased in the control group to less than 50% on day 7 of preservation, whereas adding IRPS reversed this trend of decreased motility as storage time advanced. In contrast to results of the present study, resveratrol, a natural antioxidant in a wide variety of plant species does not affect sperm motility when added to boar semen (Martin-Hidalgo et al., 2013; Bucci et al., 2018). This difference in maintaining sperm motility during storage may be due to the protection of sperm metabolism by IRPS which is related to mitochondrial function.

It is currently well accepted that in the case of boar sperm, ATP generated in the process of glycolysis is very important for sperm viability during liquid storage (Rodriguez-Gil and Bonet, 2016). A decrease in sperm motility also occurs after inhibition of either the mitochondrial respiratory chain complex I or oxidative phosphorylation, suggesting that mitochondria-transformed energy also has an important function in maintaining boar sperm metabolism (Guo et al., 2017). Mitochondria are the main site for oxidative phosphorylation to form ATP, but ROS are also mainly produced in mitochondria, where oxygen is combined with electrons in electron transport chain complexes I and III to form superoxide anions (Ford, 2004; Orrenius et al., 2007). When ROS excessively accumulates, oxidative stress leads to a suppression of mitochondrial function and DNA content, leading to a range of adverse consequences, including decreased mitochondrial activity and sperm motility. The mitochondrial functions are related to the mitochondrial membrane potential. In the present study, IRPS had a pronounced effect on maintaining the mitochondrial membrane potential of the sperm for 3 to 7 days of storage, indicating IRPS could allow for sustaining normal mitochondrial function during periods of sperm preservation. Similar findings have been reported from previous studies, wherein other antioxidants, such as astragalus polysaccharide or oligomeric proanthocyanidins, were added to boar semen extenders (Li et al., 2018; Weng et al., 2018). Collectively, results from the present study provide convincing evidence that IRPS maintains the normal function of sperm mitochondria, which is beneficial for the metabolism of sperm cells and for sperm motility.

The sperm plasma membrane is responsible for maintaining homeostasis between sperm intracellular and extracellular environments, and is involved in sperm capacitation and the acrosome reaction (de Andrade et al., 2007; Sutkeviciene et al., 2009). Results of previous studies indicate the plasma membrane of boar spermatozoa is rich in polyunsaturated fatty acids susceptible to the effects of ROS, resulting in lipid peroxidation and impairing membrane integrity and sperm motility (Vernet et al., 2004; Awda et al., 2009; Orzolek et al., 2013). In the present study, the sperm membrane integrity was the highest on days 3 and 5 of storage at a concentration of IRPS of 0.6 mg/mL. Meanwhile, after 3 days of storage, the plasma membrane integrity in the 0.8 and 1.2 mg/mL IRPS treatment groups was markedly less than that of the control group. Consistent with this finding, Wang et al. (2018) reported the addition of L-glutamine at an optimal concentration was beneficial for the plasma membrane integrity of the sperm, while relatively greater concentrations had the opposite effect. Considering these previous and present findings, the concentrations of antioxidants for semen liquid preservation must be considered carefully.

Acrosome integrity is another important indicator of sperm quality, which markedly benefited from IRPS supplementation during the entire storage period. To elucidate the potential mechanism of the protective effects of IRPS on sperm membrane and acrosome integrity, there was evaluation of T-AOC of the sperm and the content of MDA, an oxidative stress product. During the storage period, IRPS-treated samples had a greater T-AOC and lesser MDA content, compared to untreated groups. Furthermore, the addition of 0.6 mg/mL IRPS inhibited the formation of ROS in sperm throughout the storage period. The consistency of these results indicated that the addition of IRPS could effectively enhance the antioxidant capacity of stored boar semen. Regarding the physiological mechanism, IRPS exerts its antioxidant activity against ROS in semen, thereby protecting the sperm mitochondrial function, plasma membrane integrity, and acrosomal integrity, which helps preserve sperm motility. The underlying mechanism by which IRPS penetrates the sperm membrane to reach mitochondria and exerts its scavenging action, however, remains unclear and requires further investigation.

It is not sufficient to simply evaluate the quality variables of the sperm for predicting the outcome of fertilization, because the fertilization capacity of sperm is regulated by various factors (Weng et al., 2018). To further verify the effects of IRPS, there was an IVF experiment conducted to evaluate the effects of semen supplemented with 0.6 mg/mL IRPS and an un-supplemented control group on fertilization and embryo development to the blastocyst stage. Results from this IVF experiment indicated that fertilization capacity was also enhanced with the addition of IRPS, where semen treated with 0.6 mg/mL had a greater sperm-oocyte binding rate. Additionally, even though the percentage cleavage rate during embryo development was unaffected by the IRPS treatment, the percentage development to the blastocyst stage in the IRPS group was markedly enhanced. This phenomenon may be explained by oxidative stress, which damages sperm mitochondrial DNA. It has been reported that early embryonic development is affected by the mitochondrial DNA content (Fragouli and Wells, 2015; Wu et al., 2019). The consequences of mitochondrial DNA damage may have a minor effect on the cleavage process but will eventually affect the development of the embryo, thereby reducing the percentage development of embryos to the blastocyst stage. To fully confirm the effect of IRPS on artificial insemination outcomes, therefore, fertility should be conducted in the future.

In summary, in the present study, IRPS, as an antioxidant was effective in enhancing values for sperm quality variables and antioxidant capacity during liquid storage at 17 °C, with an optimum concentration of 0.6 mg/mL. Furthermore, the addition of IRPS increased the number of spermatozoa bound to oocytes as well as the percentage of embryos developing to the blastocyst stage when IRPS-treated semen was used for oocyte inseminations. These findings are indicative of the considerable potential of IRPS, as a semen supplement, in enhancing reproductive performance; however, further exploration of the molecular mechanism of IRPS and verification of its effects are necessary.

Authors contribution

ZQR and WKSY designed and performed the experiments, interpreted the results and wrote the manuscript. QL and LM contributed to most analyzing the data. JYX and JHJ recorded data and performed partial data analysis. GSY revised the manuscript. WJP designed and financed the experiments. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest in this study.

Acknowledgments

This research was financially supported by the Key Research and Development Program of Shaanxi Province (2018ZDXL-NY-02-03), the National Key Research and Development Program of China (2017YFD0502002), and the National Swine Industry Technology System (CARS-36).

References

Abeydeera, L.R., Day, B.N., 1997. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol. Reprod. 57, 729–734.
Aitken, R.J., 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol. Reprod. Dev. 84, 1039–1052.
Awda, B.J., Mackenzie-Bell, M., Buhr, M.M., 2009. Reactive oxygen species and boar sperm function. Biol. Reprod. 81, 553–561.
Basioura, A., Boscos, C.M., Parrilla, I., Tsousis, G., Tsakmakidis, I.A., 2018. Effect of astaxanthin on the quality of boar sperm stored at 17 °C, incubated at 37 °C or under in vitro conditions. Reprod. Domest. Anim. 53, 463–471.
Bucci, D., Spinaci, M., Yeste, M., Mislei, B., Gadani, B., Prieto Martinez, N., Love, C., Mari, G., Tamanini, C., Galeati, G., 2018. Combined effects of resveratrol and epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters. Theriogenology 117, 16–25.
de Andrade, A.F., de Arruda, R.P., Celeghini, E.C., Nascimento, J., Martins, S.M., Raphael, C.F., Moretti, A.S., 2007. Fluorescent stain method for the simultaneous determination of mitochondrial potential and integrity of plasma and acrosomal membranes in boar sperm. Reprod. Domest. Anim. 42, 190–194.
Du, Z., Liu, H., Zhang, Z., Li, P., 2013. Antioxidant and anti-inflammatory activities of Radix isatidis polysaccharide in murine alveolar macrophages. Int. J. Biol. Macromol. 58, 329–335.
Fang, Q., Wang, J., Hao, Y.Y., Li, H., Hu, J.X., Yang, G.S., Hu, J.H., 2017. Effects of iodine methionine on boar sperm quality during liquid storage at 17 °C. Reprod. Domest. Anim. 52, 1061–1066.
Ford, W.C., 2004. Regulation of sperm function by reactive oxygen species. Hum. Reprod. Update 10, 387–399.
Fragouli, E., Wells, D., 2015. Mitochondrial DNA assessment to determine oocyte and embryo viability. Semin. Reprod. Med. 33, 401–409.
Fu, J., Yang, Q., Li, Y., Li, P., Wang, L., Li, X., 2018. A mechanism by which Astragalus polysaccharide protects against ROS toxicity through inhibiting the protein dephosphorylation of boar sperm preserved at 4 °C. J. Cell. Physiol. 233, 5267–5280.
Garner, D.L., Johnson, L.A., 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 53, 276–284.
Gomez-Fernandez, J., Gomez-Izquierdo, E., Tomas, C., Moce, E., de Mercado, E., 2013. Is sperm freezability related to the post-thaw lipid peroxidation and the formation of reactive oxygen species in boars? Reprod. Domest. Anim. 48, 177–182.
Guo, H., Gong, Y., He, B., Zhao, R., 2017. Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. Theriogenology 87, 276–283.
Guthrie, H.D., Welch, G.R., 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J. Anim. Sci. 84, 2089–2100.
Han, J., Jiang, X., Zhang, L., 2011. Optimisation of extraction conditions for polysaccharides from the roots of Isatis tinctoria L. By response surface methodology and their in vitro free radicals scavenging activities and effects on IL-4 and IFN-γ mRNA expression in chicken lymphocytes. Carbohydr. Polym. 86, 1320–1326.
Hu, J., Geng, G., Li, Q., Sun, X., Cao, H., Liu, Y., 2014. Effects of alginate on frozen-thawed boar spermatozoa quality, lipid peroxidation and antioxidant enzymes activities. Anim. Reprod. Sci. 147, 112–118.
Johnson, L.A., Weitze, K.F., Fiser, P., Maxwell, W.M., 2000. Storage of boar semen. Anim. Reprod. Sci. 62, 143–172.
Knox, R.V., 2015. The fertility of frozen boar sperm when used for artificial insemination. Reprod. Domest. Anim. 50 (Suppl 2), 90–97.
Kumaresan, A., Kadirvel, G., Bujarbaruah, K.M., Bardoloi, R.K., Das, A., Kumar, S., Naskar, S., 2009. Preservation of boar semen at 18 °C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim. Reprod. Sci. 110, 162–171.
Li, J.P., Yuan, Y., Zhang, W.Y., Jiang, Z., Hu, T.J., Feng, Y.T., Liu, M.X., 2019. Effect of Radix isatidis polysaccharide on alleviating insulin resistance in type 2 diabetes mellitus cells and rats. J. Pharm. Pharmacol. 71, 220–229.
Li, Q., Shaoyong, W., Li, Y., Chen, M., Hu, Y., Liu, B., Yang, G., Hu, J., 2018. Effects of oligomeric proanthocyanidins on quality of boar semen during liquid preservation at 17 °C. Anim. Reprod. Sci. 198, 47–56.
Li, X., Hou, R., Yue, C., Liu, J., Gao, Z., Chen, J., Lu, Y., Wang, D., Liu, C., Hu, Y., 2016. The selenylation modification of Epimedium polysaccharide and Isatis root polysaccharide and the immune-enhancing activity comparison of their modifiers. Biol. Trace Elem. Res. 171, 224–234.
Malo, C., Gil, L., Gonzalez, N., Martinez, F., Cano, R., de Blas, I., Espinosa, E., 2010. Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (Rosmarinus officinalis). Cryobiology 61, 142–147.
Martin-Hidalgo, D., de Llera, A.H., Henning, H., Wallner, U., Waberski, D., Bragado, M.J., Gil, M.C., Garcia-Marin, L.J., 2013. The effect of resveratrol on the quality of extended boar semen during storage at 17 °C. J. Agric. Sci. 5, 231.

Mendez, M.F., Zangeronimo, M.G., Rocha, L.G., Faria, B.G., Pereira, B.A., Fernandes, C.D., Chaves, B.R., Murgas, L.D., Sousa, R.V., 2013. Effect of the addition of IGF-I and vitamin E to stored boar semen. Animal 7, 793–798.
Menegat, M.B., Mellagi, A.P., Bortolin, R.C., Menezes, T.A., Vargas, A.R., Bernardi, M.L., Wentz, I., Gelain, D.P., Moreira, J.C., Bortolozzo, F.P., 2017. Sperm quality and oxidative status as affected by homogenization of liquid-stored boar semen diluted in short- and long-term extenders. Anim. Reprod. Sci. 179, 67–79.
Moraes, C.R., Meyers, S., 2018. The sperm mitochondrion: organelle of many functions. Anim. Reprod. Sci. 194, 71–80.
Orrenius, S., Gogvadze, V., Zhivotovsky, B., 2007. Mitochondrial oxidative stress: implications for cell death. Annu. Rev. Pharmacol. Toxicol. 47, 143–183.
Orzolek, A., Wysocki, P., Strzezek, J., Kordan, W., 2013. Superoxide dismutase (SOD) in boar spermatozoa: purification, biochemical properties and changes in activity during semen storage (16 °C) in different extenders. Reprod. Biol. 13, 34–40.
Pei, Y., Yang, L., Wu, L., He, H., Geng, G., Xu, D., Chen, H., Li, Q., 2018. Combined effect of apigenin and ferulic acid on frozen-thawed boar sperm quality. Anim. Sci. J. 89, 956–965.
Pintus, E., Kadlec, M., Jovicic, M., Sedmikova, M., Ros-Santaella, J.L., 2018. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress. Pharmaceutics 10, 212.
Puig-Timonet, A., Castillo-Martin, M., Pereira, B.A., Pinart, E., Bonet, S., Yeste, M., 2018. Evaluation of porcine beta defensins-1 and -2 as antimicrobial peptides for liquid-stored boar semen: effects on bacterial growth and sperm quality. Theriogenology 111, 9–18.
Roca, J., Parrilla, I., Bolarin, A., Martinez, E.A., Rodriguez-Martinez, H., 2016. Will AI in pigs become more efficient? Theriogenology 86, 187–193.
Rodriguez-Gil, J.E., Bonet, S., 2016. Current knowledge on boar sperm metabolism: comparison with other mammalian species. Theriogenology 85, 4–11.
Ros-Santaella, J.L., Pintus, E., 2017. Rooibos (Aspalathus linearis) extract enhances boar sperm velocity up to 96 hours of semen storage. PLoS One 12, e0183682.
Shaoyong, W., Li, Q., Ren, Z.Q., Wei, C.S., Chu, G.Y., Dong, W.Z., Yang, G.S., Pang, W.J., 2019. Evaluation of ε-polylysine as antimicrobial alternative for liquid-stored boar semen. Theriogenology 130, 146–156.
Spinaci, M., Muccilli, V., Bucci, D., Cardullo, N., Gadani, B., Tringali, C., Tamanini, C., Galeati, G., 2018. Biological effects of polyphenol-rich extract and fractions from an oenological oak-derived tannin on in vitro swine sperm capacitation and fertilizing ability. Theriogenology 108, 284–290.
Sutkeviciene, N., Riskeviciene, V., Januskauskas, A., Zilinskas, H., Andersson, M., 2009. Assessment of sperm quality traits in relation to fertility in boar semen. Acta Vet. Scand. 51, 53.
Tremellen, K., 2008. Oxidative stress and male infertility–a clinical perspective. Hum. Reprod. Update 14, 243–258.
Tuncer, P.B., Bucak, M.N., Buyukleblebici, S., Sariozkan, S., Yeni, D., Eken, A., Akalin, P.P., Kinet, H., Avdatek, F., Fidan, A.F., Gundogan, M., 2010. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. Cryobiology 61, 303–307.
Vernet, P., Aitken, R.J., Drevet, J.R., 2004. Antioxidant strategies in the epididymis. Mol. Cell. Endocrinol. 216, 31–39.
Wang, S., Sun, M., Wang, N., Yang, K., Guo, H., Wang, J., Zhang, Y., Yue, S., Zhou, J., 2018. Effects of L-glutamine on boar sperm quality during liquid storage at 17 °C. Anim. Reprod. Sci. 191, 76–84.
Wei, Z.Y., Wang, X.B., Zhang, H.Y., Yang, C.H., Wang, Y.B., Xu, D.H., Chen, H.Y., Cui, B.A., 2011. Inhibitory effects of indigowoad root polysaccharides on porcine reproductive and respiratory syndrome virus replication in vitro. Antivir. Ther. (Lond.) 16, 357–363.
Weng, X.G., Cai, M.M., Zhang, Y.T., Liu, Y., Gao, Z.L., Song, J., Liu, Z.H., 2018. Effect of Astragalus polysaccharide addition to thawed boar sperm on in vitro fertilization and embryo development. Theriogenology 121, 21–26.
Wu, H., Whitcomb, B.W., Huffman, A., Brandon, N., Labrie, S., Tougias, E., Lynch, K., Rahil, T., Sites, C.K., Pilsner, J.R., 2019. Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting. Hum. Reprod. 34, 163–170.
Yamashiro, H., Abe, Y., Fukuda, T., Kino, Y., Kawaguchi, I., Kuwahara, Y., Fukumoto, M., Takahashi, S., Suzuki, M., Kobayashi, J., Uematsu, E., Tong, B., Yamada, T., Yoshida, S., Sato, E., Shinoda, H., Sekine, T., Isogai, E., Fukumoto, M., 2013. Effects of radioactive caesium on bull testes after the Fukushima nuclear plant accident. Sci. Rep. 3, 2850.
Zhang, X.G., Li, H., Wang, L., Hao, Y.Y., Liang, G.D., Ma, Y.H., Yang, G.S., Hu, J.H., 2017. The effects of different levels of superoxide dismutase in Modena on boar semen quality during liquid preservation at 17 °C. Anim. Sci. J. 88, 55–62.
Zhang, X.G., Liu, Q., Wang, L.Q., Yang, G.S., Hu, J.H., 2016. Effects of glutathione on sperm quality during liquid storage in boars. Anim. Sci. J. 87, 1195–1201.