

Effects of riboflavin on boar sperm motility, sperm quality, enzyme activity and antioxidant status during cryopreservation

Ruilan Dong | Lan Luo | Xiaobin Liu | Guanghui Yu 

College of Animal Science and Technology,
Qingdao Agricultural University, Qingdao,
Shandong, P. R. China

Correspondence

Guanghui Yu, College of Animal Science and
Technology, Qingdao Agricultural University,
No. 700 Changcheng Road, Chengyang
District, Qingdao, Shandong 266109, China.
Email: guanghuiyu@qau.edu.cn

Funding information

Agricultural Animal Breeding Project of
Shandong Province, Grant/Award Numbers:
2019LZGC019, 2020LZGC012

Abstract

Objectives: This study was conducted to evaluate the effect of adding riboflavin to boar sperm freezing extender on the challenge of cryopreservation.

Methods: Different concentrations (0, 5, 10, 15, 20 or 25 μM) of riboflavin were added to the freezing extender. Spermatozoa motility, membrane integrity, acrosomal integrity, mitochondrial membrane potential and enzyme activities were analysed once 10 min after thawing. Q-PCR was used to detect the mRNA expression of *Caspase3*, *Bcl-2* and *Bax*.

Results: The results showed that the addition of 10 μM riboflavin to boar sperm freezing extender significantly increased the frozen-thawed sperm progressive motility compared with the control group ($p < 0.05$). Activities of superoxide dismutase, glutathione peroxidase and catalase improved after adding riboflavin to the extender ($p < 0.05$). During freezing-thawing, the boar sperm mitochondrial membrane potential, acrosomal integrity, plasma membrane and DNA at 10 μM in the riboflavin group increased by 6.6%, 9.6%, 5.49% and 5.62% ($p < 0.05$), respectively, compared with the control group. The addition of 10 μM riboflavin to the extender significantly decreased the malondialdehyde ($p < 0.05$) content, whereas it increased the ATP content ($p < 0.05$) of boar sperm during freezing-thawing. Furthermore, the expression of *Caspase-3* and *Bax* ($p < 0.05$) were significantly lower, whereas the expression of *BCL-2* ($p < 0.05$) was greater than the control group when adding 10 μM riboflavin to the extender.

Conclusions: Riboflavin showed cryoprotective capacity to the freezing extender used for boar sperm during the process of freezing-thawing, and the optimal concentration of riboflavin for the frozen extender was 10 μM .

KEYWORDS

antioxidant, boar sperm cryopreservation, riboflavin, sperm quality

1 | INTRODUCTION

Sperm cryopreservation is used for storage of superior genetic resources, improvement of breeding, preservation of genetic resources

of endangered species (Yeste, 2016). Moreover, fertility of frozen-thawed boar sperm is still very variable compared with other animal species (Bolarín et al., 2006). Boar sperm cryopreservation has not been used under production conditions as efficiently as liquid preserved sperm, due to the high susceptibility of boar spermatozoa to damage during cryopreservation (Waberski et al., 2019; Yeste et al.,

Ruilan Dong and Lan Luo contributed equally to this work of this article.

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2017). The plasma membrane of boar sperm is rich in polyunsaturated fatty acids with low cholesterol to phospholipids compared with other animals (Wathes et al., 2007). Thus, the membrane structure injury and lipid peroxidation would lead to the boar sperm damage during cryopreservation (Pezo et al., 2021).

Free radicals are essential substances for maintaining normal life, as well as 'dangerous killers' of macromolecules and cells (Halliwell, 2009). When oxygen free radicals and by-products of the metabolic process are in excess, the body will suffer different degrees of oxidative damage (Qian et al., 2014). Therefore, adding antioxidants to the freezing process of boar sperm may avoid such oxidative damages. Reactive oxygen species (ROS) is generated during the cryopreservation process resulting in lipid peroxidation, which leads to irreversible damage of sperm cells in the freezing-thawing process (Brouwers et al., 2005) and the sperm membrane, acrosome and DNA damage (Bennetts & Aitken, 2005). A large number of studies have reported that adding exogenous antioxidants into cryopreservation medium is an effective strategy to resist oxidative stress and improve sperm quality during cryopreservation (Amidi et al., 2016; Taylor et al., 2009; Zhu et al., 2015). Antioxidant addition to the sperm extender can be helpful to the sperm survival after freezing and thawing (de Albuquerque Lagares et al., 2020).

Riboflavin, also called vitamin B₂, is a kind of water-soluble vitamins. There are two kinds of the coenzyme forms: flavin mononucleotide and flavin adenine dinucleotide. Riboflavin plays an important role in the enzyme reaction (Saedisomeolia & Ashoori, 2018). It also participates in some vitamin metabolism (Balasubramaniam et al., 2019). The lack of riboflavin can damage the oxidative folding of the stress response system (Manthey et al., 2005). Flavin adenine dinucleotide is incorporated into the acrosomal matrix during its formation. Lack of flavin adenine dinucleotide impairs sperm function and male reproductive advantage (Yen et al., 2020). Riboflavin was associated with spermatogenesis in male Wistar rats (Tumkiratiwong et al., 2006). Riboflavin carrier protein is a phosphoglycoprotein and has the fertility function appearance during spermatogenesis and role in sperm function (Sreekumar et al., 2005). At present, there is no evidence reporting the effects of supplementing of riboflavin in sperm extenders on boar sperm quality. Therefore, the purpose of this study was to evaluate whether riboflavin could protect boar sperm during cryopreservation.

2 | MATERIALS AND METHODS

2.1 | Animals and sperm collection

Sperm was collected from 8 boars (Duroc, aged 20 ± 4 months). Boar sperm was collected using the gloved-hand technique, then filtered and incubated in an incubator at 17°C, and subsequently delivered to the laboratory within 30 min for sperm quality evaluation. Sperm concentration was determined using a haemocytometer. Only ejaculate sperm samples with more than 90% motility were used for further study. To exclude individual differences, each sample was made from a mixed sample from 8 different boar sperm.

2.2 | Sperm freezing

The basic medium for boar sperm freezing was Tris-citric acid-glucose solution (100 ml) which contained 46.6 mM Tris, 15.1 mM citric acid, 153 mM glucose, 26.7 mM trisodium citrate, 11.9 mM sodium hydrogen carbonate, 6.3 mM EDTA-2Na and 100,000 IU penicillin-streptomycin solutions. The extender I (80% lactose and 20% egg yolk; volume fraction) contained different concentrations of riboflavin including 0 μM (control group), 5 μM (group I), 10 μM (group II), 15 μM (group III), 20 μM (group IV) and 25 μM (group V), respectively.

Sperm was diluted with Tris-citric acid-glucose solution (v:v = 1:1) and incubated for 2 h at 17°C. It was centrifuged at 800 × g for 10 min. The supernatant was then removed and the sediments were diluted to 3 × 10⁹ cells/ml with a mixture of extender I (80% lactose and 20% egg yolk; volume fraction) and different concentrations of riboflavin, which was cooled slowly to 4°C in a refrigerator for 2 h. After cooling, then sperm was resuspended 1:1 with extender II (including 95.5% extender I containing different concentrations of riboflavin, 3% glycerol and 1.5% Orvus Ex Paste; volume fraction) at 4°C. The diluted spermatozoa were placed in a 4°C refrigerator for another 30 min. The sperm was transferred into 0.5 ml straws. Sealed straws were placed horizontally on a rack and frozen in a vapour 3 cm above liquid nitrogen for 10 min. After freezing, the straws were immersed in liquid nitrogen for storage.

2.3 | Sperm thawing

Thawed sperm (42°C for 10 s in a water bath) were extended at 37°C with Tris-citric acid-glucose (4:1). After incubation for 10 min (He et al., 2020; Shen et al., 2016), sperm quality was determined for the next experiments.

2.4 | Assessments of sperm motility and velocity after freezing-thawing

Sperm motility was analysed using a computer-assisted sperm analysis (CASA) system (QH-III, Spain). Briefly, sperm samples were suspended in Tris-citric acid-glucose to a concentration of 2–3 × 10⁷ sperm/ml. For each evaluation, a 4 μl aliquot of sperm sample was placed in a pre-warmed (37°C) counting chamber and three fields were analysed at 37°C, assessing a minimum of 100 sperm per sample. The values for the following variables were recorded: curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %) and straightness (STR, %).

2.5 | Assessments of sperm membrane integrity, acrosome integrity and DNA integrity after freezing-thawing

Membrane integrity and acrosome integrity were detected using the sperm fluorescein isothiocyanate-peanut agglutinin (PNA-FITC,

GENMED SCIENTIFICS INC, USA). Briefly, 100 μ l of sperm sample were transferred into culture tubes, and dyes were added at a final concentration of 100 nM SYBR-14, with 1 μ g/ml of peanut agglutinin conjugated with phycoerythrin solution and 12 μ M propidium iodide (PI). Samples were mixed and incubated for 10 min at 37°C in darkness. Just before analysis, 400 μ l phosphate-buffered saline (PBS) was added to each sample and remixed before they were detected through a flow cytometer. Analysed sperm were allocated into the following four categories: viable with intact acrosome (PNA-FITC-/PI-); viable with acrosomal exocytosis (PNA-FITC+/PI-) and nonviable cells with damaged acrosome (PNA-FITC+/PI+). Only viable spermatozoa were analysed.

DNA integrity was measured using the acridine orange (Solarbio, China) staining (Zribi et al., 2012). Preliminary sample processing was the same as described above. Appropriate amount of spermatozoa cell suspension and acridine orange stain (19:1) were collected, mixed and incubated for 10 min at 37°C in darkness. Just before analysis, 400 μ l PBS was added to each sample and remixed before they were detected using a flow cytometer. Analysed sperm were allocated into the following two categories: damaged sperm DNA and intact double-stranded DNA. Damaged sperm DNA will be denatured into single-stranded under the action of acid, emitting red or blue fluorescence, while intact double-stranded DNA will emit green fluorescence.

2.6 | Assessments of sperm mitochondrial membrane potentials and ROS content after freezing-thawing

The mitochondrial membrane potentials of the sperm were detected using JC-1 (lipophilic cation 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-benzimidazolylcarbocyanine iodide) by Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, China). Briefly, sperm samples were stained with JC-1 working extender, and incubated for 20 min at 37°C in darkness. Subsequently, samples were washed twice with JC-1 buffer and resuspended in JC-1 buffer. Samples were analysed using a flow cytometer (FACS Calibur, BD Biosciences). Sperm with high mitochondrial membrane potentials that showed red fluorescence were measured in the FL2 channel (585 nm). Sperm with low mitochondrial membrane potentials that showed green fluorescence were measured in the FL1 channel (530 nm). Data were processed using the FCS Express 5.0 program (De Novo Software) and 10,000 sperm-specific events were evaluated.

ROS content was measured using a Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, China), as previously described by Zhu et al. (2015). Briefly, sperm samples were suspended in Tris-citric acid-glucose extender containing 10 μ M DCFH-DA and suspensions (10^7 sperm/ml) were incubated for 20 min at 37°C in darkness. After incubation, the sample was centrifuged to wash away dihydrodichlorofluorescein diacetate (H₂DCF-DA). Samples were analysed using the flow cytometer. The fluorescent signal detected at 488 nm excitation and 525 nm emission is expressed with flow cytometry. The experiment was replicated three times.

2.7 | Assessment of sperm SOD, CAT and GPx activities after freezing-thawing

The post-thaw sperm samples (about 10^8 sperm each group) were centrifuged at $1200 \times g$ for 5 min at 4°C. After removing the supernatant, the sperm sediment was resuspended with 400 μ l of PBS for extracting enzymes. Subsequently, the lysates resulting from sperm cell disruption using an ultrasonic homogeniser (JY92-IIN, Ningbo Xinzhi Biotechnology Co., Ltd, China) in a cooling bath were centrifuged at $4000 \times g$ for 10 min at 4°C, and its supernatant was used for the SOD, CAT and GPx analysis. The antioxidant enzyme activities of SOD, CAT and GPx were detected by commercial enzyme kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The absorbance at 532 nm (SOD), 405 nm (CAT) and 412 nm (GPx) was recorded by a microplate reader (Infinite M Nano, TECAN, Switzerland), respectively.

2.8 | Assessments of sperm intracellular ATP content and lipid peroxidation after freezing-thawing

Adenosine triphosphate (ATP) content in sperm was measured by an ATP assay kit (Beyotime Institute of Biotechnology, China). The luminescence was immediately measured using a luminometer (GloMax 20/20, Promega, USA). A standard curve of ATP content was detected from five ATP-gradient concentrations ranging from 0.1 to 1.0 μ mol/L. The experiment was repeated three times.

Lipid peroxidation of spermatozoa was analysed by determining the malondialdehyde content with the lipid peroxidation malondialdehyde assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol. The sperm samples (about 10^8 sperm each group) were lysed in 100 μ l of cell lysis reagent. After centrifuging at $10,000 \times g$ for 10 min, the supernatant was used to detect the malondialdehyde. Subsequently, the samples were incubated for 40 min at 95°C, and 200 μ l of each incubated sample was added to 96-well plates. The absorbance at 532 nm was recorded by a microplate reader (Infinite M Nano, TECAN, Switzerland), and malondialdehyde content of the samples was calculated using the formula derived from the standard curve.

2.9 | The expression of apoptosis-related genes after freezing-thawing

Boar sperm were collected with or without riboflavin treatment during freezing-thawing. Three pools each containing 1 ml of sperm (1.5×10^6 sperm) were used to carry out the RNA extraction and Q-PCR analysis. RNA was extracted by the RNA Prep Pure animal tissue extraction kit (TaKaRa, Dalian, China). Reverse transcription was carried out with a EvoM-MLV reverse transcription kit (TaKaRa, Dalian, China), and complementary DNA (cDNA) samples from the reverse transcription reaction were diluted with nuclease-free water to 60 μ l. The quantification of all gene transcripts of *Caspase-3*, B-cell lymphoma 2 (*Bcl-2*),

TABLE 1 Primers used for Q-PCR

Gene name	Primer sequence (5'-3')	Annealing temperature	Fragment size
<i>GAPDH</i>	F:CCCCAACGTGTCGGTTGT R:CTCGGACGCCTGCTTCAC	60	91
<i>Caspase-3</i>	F:CAGACAGTGGTCTGAGGATGA R:GCTACCTTTCGGTTAACCCGA	60	73
<i>Bcl-2</i>	F:AGGGCATTTCAGTGACCTGAC R:CGATCCGACTCACCAATACC	60	193
<i>Bax</i>	F:TGCCTCAGGATGCATCTACC R:AAGTAGAAAAGCGCGACCAC	60	199

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *Bcl-2*, B-cell lymphoma 2; *Bax*, Bcl-2-associated X protein.

TABLE 2 Effects of riboflavin treatments on boar sperm motility after freezing-thawing

Sperm motility	Riboflavin					
	Control	5 μ M	10 μ M	15 μ M	20 μ M	25 μ M
VCL (μ m/s)	95.1 \pm 0.7f	104.9 \pm 1.9e	134.6 \pm 0.9 ^a	128.5 \pm 1.1b	123.3 \pm 1.9c	112.1 \pm 1.3d
VSL (μ m/s)	40.7 \pm 0.62 ^f	53.0 \pm 0.6 ^d	69.1 \pm 0.8 ^a	64.4 \pm 0.4 ^b	59.9 \pm 0.9 ^c	50.3 \pm 0.9 ^e
VAP (μ m/s)	73.8 \pm 0.3 ^d	87.2 \pm 0.7 ^c	108.7 \pm 0.7 ^a	106.5 \pm 0.6 ^{ab}	103.5 \pm 2.7 ^b	90.6 \pm 0.6 ^c
LIN (%)	42.8 \pm 0.4 ^b	50.6 \pm 1.5 ^a	51.3 \pm 0.6 ^a	50.1 \pm 0.2 ^a	48.6 \pm 1.4 ^a	44.8 \pm 0.5 ^b
STR (%)	55.2 \pm 1.4 ^c	60.8 \pm 1.1 ^{ab}	63.5 \pm 1.4 ^a	60.5 \pm 0.9 ^{ab}	58.0 \pm 3.31 ^{bc}	55.5 \pm 1.9 ^c

Note: Values denote mean \pm standard error (SEM). Different superscript letters in the same line indicate significant difference ($p < 0.05$). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness.

Bcl-2-associated X protein (*Bax*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed by Q-PCR using the SYBR Green I chimeric fluorescence method (SYBR[®] Premix Ex Taq[™] II, TaKaRa, Dalian, China). The accession number and primer sequence were presented in Table 1. Each reaction mixture (20.0 μ l) consisted of 10.0 μ l of SYBR[®] Premix Ex Taq[™] II, 0.8 μ l of each of forward (10 μ mol/L) and reverse (10 μ mol/L) primers, 0.4 μ l of Rox Reference Dye II, 2.0 μ l of cDNA and 6.0 μ l of dH₂O. Q-PCR was conducted using the CFX Manager 3.0 system (Bio-Rad, USA) under the following conditions: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. The relative expression levels of genes analysed using porcine *GAPDH* as the housekeeping gene and Safety Data Sheet (SDS) software for relative quantification (Bio-Rad CFX Manager 3.0 system).

2.10 | Statistical analysis

Values were expressed as mean \pm standard error of the mean (SEM). Normality of data was checked using the Shapiro–Wilk test. The effects of riboflavin treatment were analysed by one-way ANOVA analysis using GLM procedure in SAS 9.4 (SAS Institute, Inc., Cary, NC). Multiple comparisons were performed using the Tukey's HSD test of SAS 9.4 (SAS Institute, Inc., Cary, NC). The level of statistical significance was set to 5%.

3 | RESULTS

3.1 | Effects of riboflavin on sperm motility after freezing-thawing

The motility of frozen-thawed sperm including straight-line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) were significantly improved by the addition of riboflavin (from 5 to 25 μ M) compared with the control group (Table 2, $p < 0.05$). Sperm motility in the extender containing 10 μ M of riboflavin is significantly greater than the control group ($p < 0.05$).

3.2 | Effects of riboflavin on sperm SOD, CAT and GPx enzyme activity after freezing-thawing

The extender containing 10 μ M of riboflavin showed greater SOD and GPx enzyme activities than other treatment groups and the control group (Table 3, $p < 0.05$). The CAT and GPx activities were significantly improved by the addition of riboflavin compared with the control group (Table 3, $p < 0.05$). The extenders with 10 and 15 μ M of riboflavin showed significantly improved CAT activities as compared with other treatment groups and the control group ($p < 0.05$).

TABLE 3 Effects of riboflavin on sperm enzyme activities of SOD, CAT and GPx after freezing-thawing

Enzyme activities	Riboflavin					
	Control	5 μ M	10 μ M	15 μ M	20 μ M	25 μ M
SOD (nmol/ml)	68.82 \pm 1.05 ^c	71.33 \pm 0.54 ^{bc}	75.63 \pm 0.39 ^a	72.67 \pm 0.53 ^b	68.63 \pm 0.58 ^c	70.34 \pm 0.52 ^c
CAT (nmol/ml)	1.33 \pm 0.26 ^c	2.26 \pm 0.21 ^b	3.84 \pm 0.20 ^a	3.40 \pm 0.16 ^a	2.39 \pm 0.12 ^b	2.67 \pm 0.09 ^b
GPx (nmol/ml)	44.20 \pm 0.85 ^e	48.67 \pm 0.64 ^c	53.55 \pm 0.55 ^a	51.03 \pm 0.45 ^b	48.46 \pm 0.36 ^c	46.14 \pm 0.24 ^d

Note: Values denote mean \pm standard error (SEM). Different superscript letters in the same line indicate significant difference ($p < 0.05$). SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

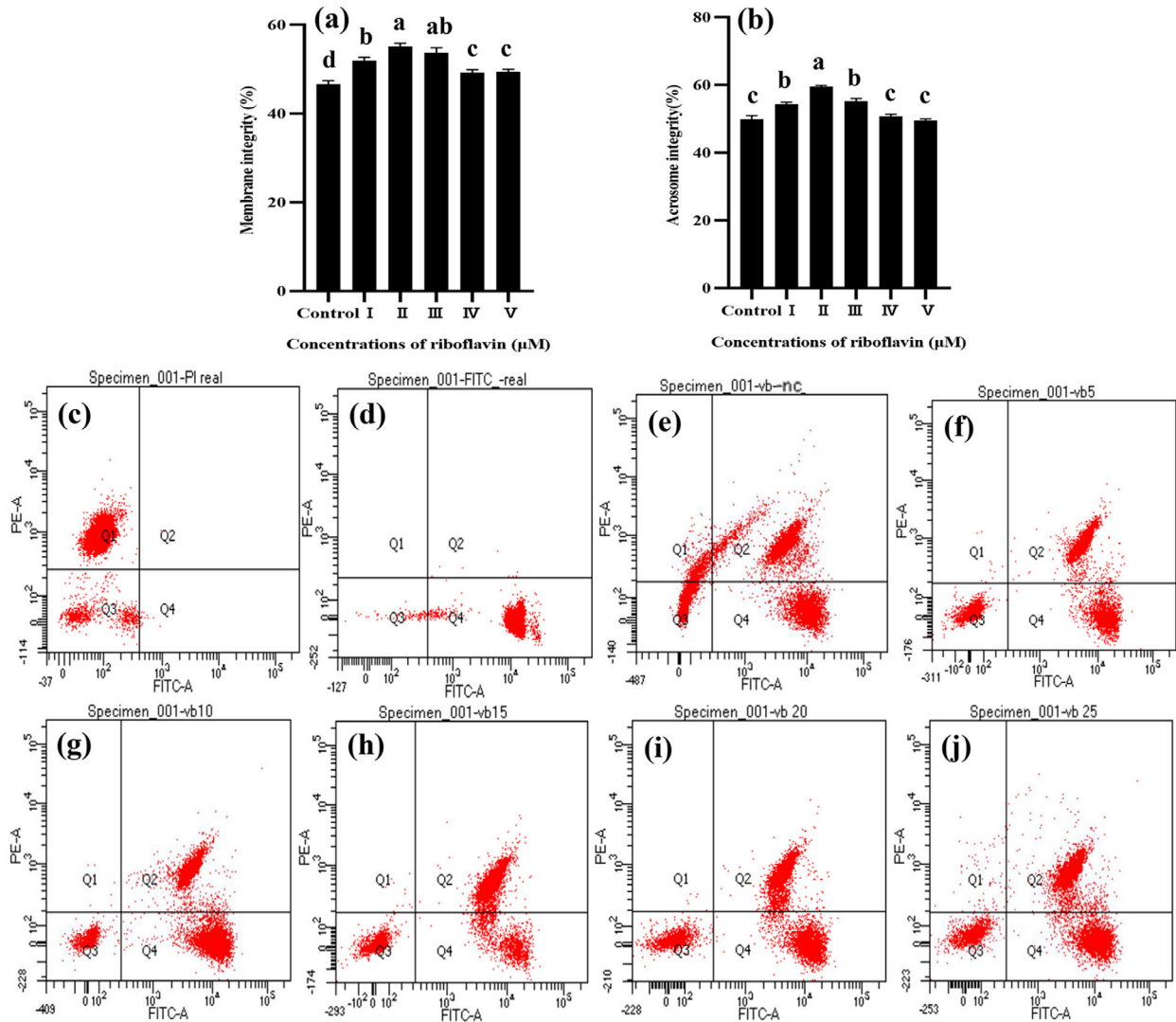


FIGURE 1 Effects of riboflavin on sperm membrane integrity and acrosome integrity after freezing-thawing. (a) Membrane integrity, (b) acrosome integrity, (c)–(j) flow cytometer quadrant categories. (c)–(j) Quadrants: Left lower quadrant means sperm live with intact acrosome and intact membrane. Left upper quadrant means sperm dead with intact acrosome and damaged membrane. Right lower quadrant means sperm live with damaged acrosome and intact membrane. Right upper quadrant means sperm dead with damaged acrosome and damaged membrane

3.3 | Effects of riboflavin on sperm membrane integrity, acrosome integrity and DNA integrity after freezing-thawing

Supplementation of riboflavin to the extender significantly improved membrane integrity of boar sperm compared with the control group

(Figure 1a, $p < 0.05$) during freezing-thawing. The sperm membrane integrity of frozen-thawed sperm with 10 and 15 μ M riboflavin treatment groups were much higher than other treatment groups and the control group (Figure 1a, $p < 0.05$). However, there was no significant difference for 10 and 15 μ M riboflavin treatments ($p > 0.05$). In addition, the sperm membrane integrity declined from the riboflavin

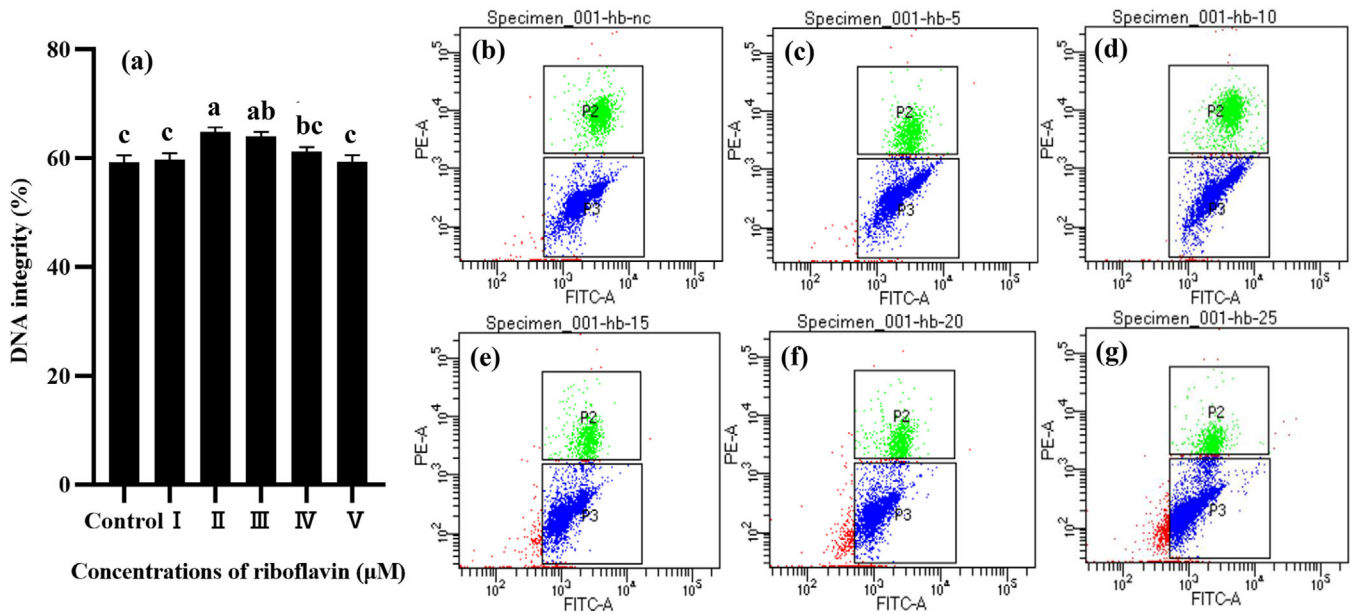


FIGURE 2 Effects of riboflavin on sperm DNA integrity after freezing-thawing. (a) DNA integrity, (b)–(g) flow cytometer categories. (b)–(g) Green fluorescence means intact double-stranded DNA. Blue fluorescence means damaged sperm DNA will be denatured into single-stranded under the action of acid

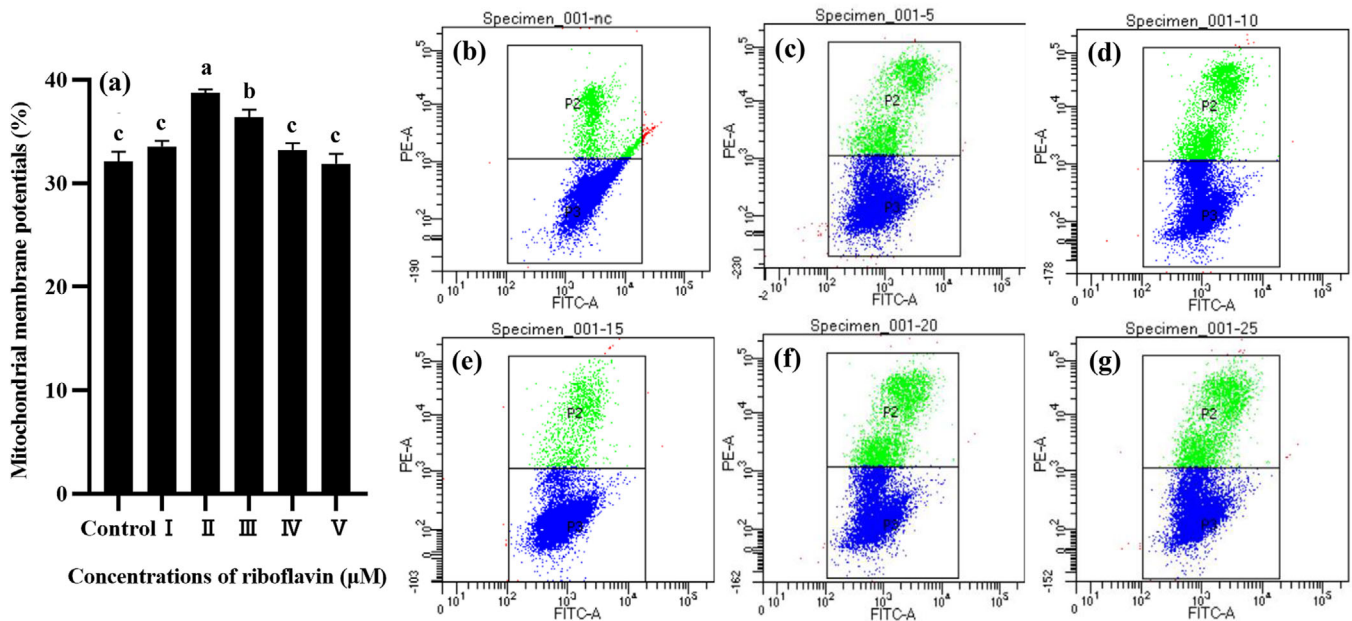


FIGURE 3 Effects of riboflavin on sperm mitochondrial membrane potentials after freezing-thawing. (a) Mitochondrial membrane potentials, (b)–(g) flow cytometer categories. (b)–(g) Green fluorescence means sperm with low mitochondrial membrane potentials. Blue fluorescence means sperm with high mitochondrial membrane potentials

concentration of 15 to 25 μM (Figure 1a, $p < 0.05$). The acrosomal integrity of frozen-thawed sperm with 10 μM riboflavin treatment was significantly higher than other treatment groups and the control group (Figure 1b, $p < 0.05$). DNA integrity in the 10 μM riboflavin treatment was significantly higher than the control group (Figure 2a, $p < 0.05$). The extenders with 10 μM of riboflavin showed significantly improved integrities of membrane, acrosomal and DNA (Figures 1 and 2).

3.4 | Effects of riboflavin on sperm mitochondrial membrane potential after freezing-thawing

The mitochondrial membrane potential of frozen-thawed sperm with 10 and 15 μM riboflavin treatment groups were significantly higher than other treatment groups and the control group (Figure 3a, $p < 0.05$). There was no difference between the control group and the extenders with 5 μM , 20 μM and 25 μM of riboflavin (Figure 3, $p > 0.05$).

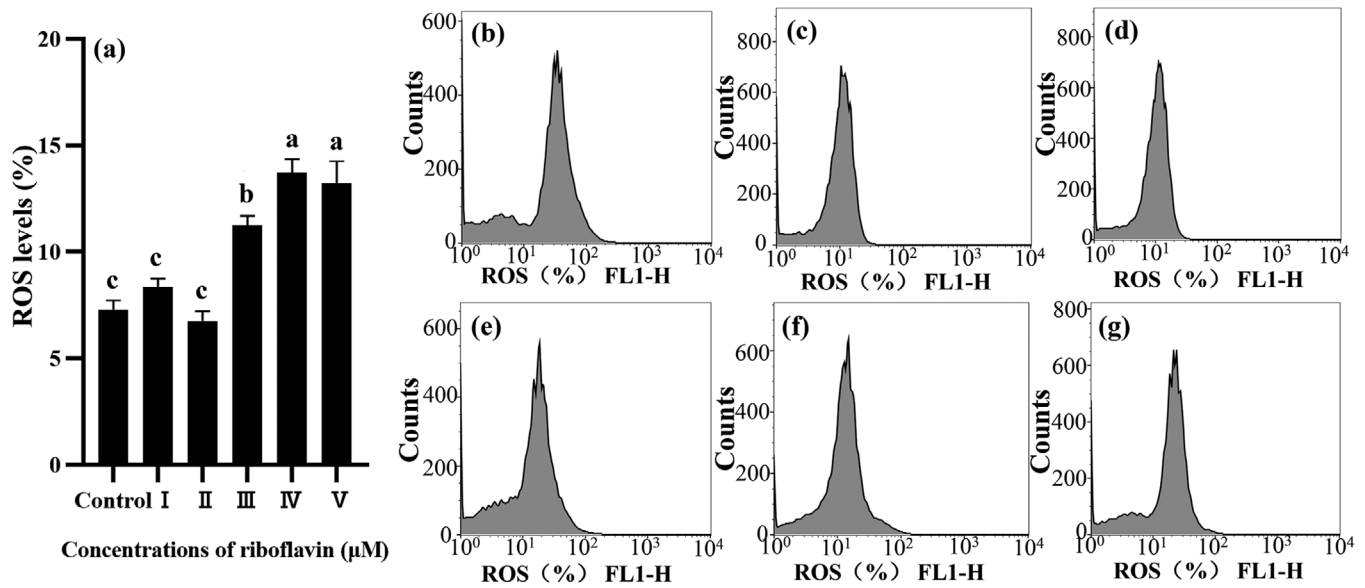
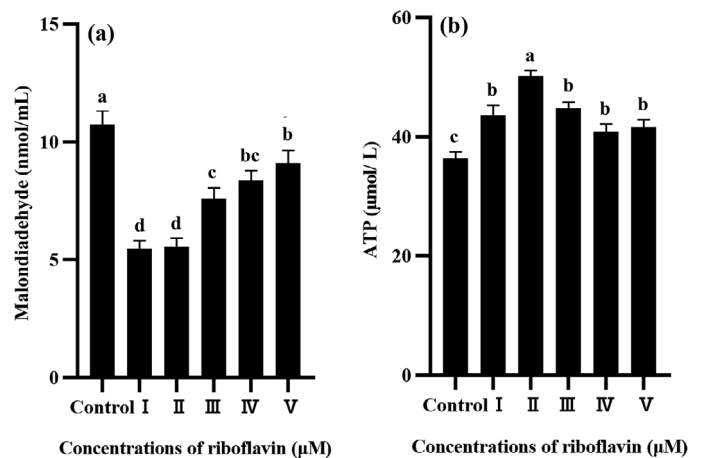


FIGURE 4 Effects of riboflavin on sperm ROS content after freezing-thawing. (a) ROS content, (b)–(g) flow cytometer analysed results

FIGURE 5 Effects of riboflavin on sperm malondialdehyde and ATP content after freezing-thawing. (a) Malondialdehyde content, (b) ATP content



3.5 | Effects of riboflavin on sperm ROS, malondialdehyde and ATP content after freezing-thawing

There was no difference for the ROS level of frozen-thawed sperm between the control group and riboflavin treatment groups at the concentrations of 5 and 10 μM (Figure 4a, $p > 0.05$). The lowest ROS level was observed in the 10 μM riboflavin supplementation in the extender among riboflavin groups. However, 15, 20 or 25 μM riboflavin supplementation exerted a negative effect on the ROS levels as compared to the control group (Figure 4, $p < 0.05$). The malondialdehyde of frozen-thawed sperm was significantly decreased by the riboflavin treatments (Figure 5a, $p < 0.05$). The 5 and 10 μM riboflavin treatments showed the lowest malondialdehyde levels compared with other treatment groups and the control group (Figure 5, $p < 0.05$). The ATP content of boar sperm by riboflavin treatment during cryopreservation were shown in Figure 5b. The ATP content of frozen-thawed sperm with 10 μM

riboflavin group was significantly greater than other treatment groups and the control group (Figure 5b, $p < 0.05$).

3.6 | Effects of riboflavin on the sperm apoptosis-related genes mRNA expression after freezing-thawing

The addition of 5, 10 and 15 μM riboflavin to the extender significantly decreased the expression level of *Caspase-3* of the frozen-thawed sperm. The lowest expression level of *Caspase-3* was observed in the 10 μM riboflavin supplementation in the extender among riboflavin groups (Figure 6a, $p < 0.05$). The expression level of *BCL-2* of the frozen-thawed sperm was significantly greater in the 10 μM riboflavin group compared with other treatment groups (Figure 6b, $p < 0.05$). The level of *Bax* of the frozen-thawed sperm was significantly decreased by increasing riboflavin concentration in the extender, especially for the

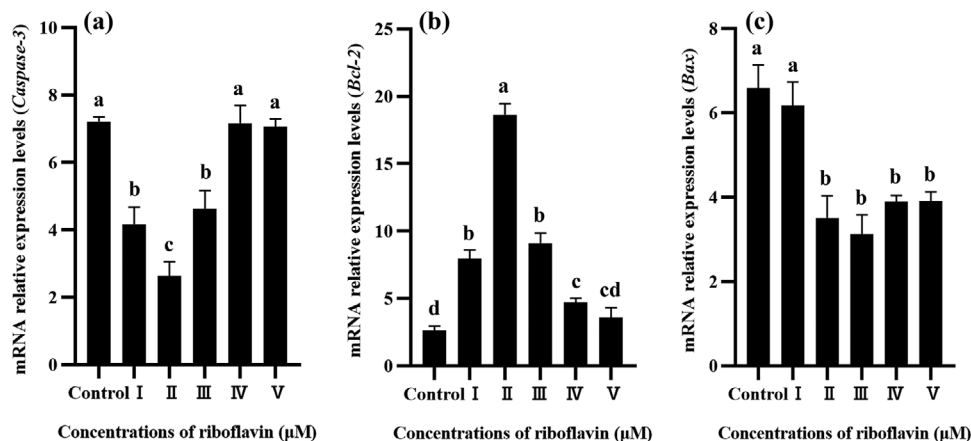


FIGURE 6 Effects of riboflavin on mRNA expressions after freezing-thawing. (a) *Caspase-3* expression, (b) *Bcl-2* expression, (c) *Bax* expression. *Bcl-2*, B-cell lymphoma, *Bax*, Bcl-2 associated X protein

riboflavin treatment groups at the concentrations of 10 and 15 μM to the extender (Figure 6c, $p < 0.05$). There was no difference among the groups with 10, 15, 20 and 25 μM of riboflavin (Figure 6c, $p > 0.05$).

4 | DISCUSSION

Boar sperm cryopreservation is associated with sperm damage via different levels of plasma membrane injury and oxidative stress (Saadeldin et al., 2020). Such damage is caused by drastic temperature changes during the freeze-thawing process (Lee & Kim, 2018). Addition of antioxidants to freezing extenders could help spermatozoa against oxidative stress and improve the viability of sperm after freeze-thawing (Naijian et al., 2013). Some researchers have demonstrated the antioxidant function of riboflavin (Tang et al., 2014), and show that an altered riboflavin level causes spermatozoa morphological and bioenergetic defects in mice (Kuang et al., 2021). Riboflavin has recently been used to assist cryopreservation of platelets (Jimenez-Marco et al., 2021). However, their functions in boar sperm are not well known. This is the first report on the effect of riboflavin on boar spermatozoa during the freezing-thawing process. In the present study, the effects of different concentrations of riboflavin in extenders were evaluated. Our results showed that sperm motility was well protected by riboflavin treatment. The velocity was significantly improved by adding riboflavin to the extender (Table 2). In the current study, the optimum concentration of riboflavin was 10 μM .

Riboflavin deficiency is associated with reduced antioxidant enzymes activity, and the addition of riboflavin can enhance the activity of these enzymes (Tumkiratiwong et al., 2003). In this study, SOD, GPx and CAT activity of the frozen-thawed sperm was greater in the groups supplemented with 10 μM of riboflavin as compared with the control group ($p < 0.05$). This is in agreement with previous research (Chen et al., 2015), in which riboflavin deficiency can lead to decreased activity of SOD, GPx and glutathione reductase. The reason may be that riboflavin provides protection for boar spermatozoa against oxidative stress because of its antioxidant properties, which

can reduce the oxidative damage by scavenging ROS or inhibiting the generation of ROS. Riboflavin status has an effect on reduced GPx content in tissues, since the reduction of oxidised glutathione is a riboflavin dependent process (Al-Harbi et al., 2015). However, the antioxidant activities of riboflavin decreased at high concentrations. The possible reason may be the greater amounts of antioxidant additive destroyed the mitochondria of the spermatozoa (Hu et al., 2013). Reportedly, when the riboflavin concentration is exceeded 12.76 nmol/L, the oxidative stress is weakened (Ashoori & Saedisomeolia, 2014).

Oxidative stress is one of the deleterious effects occurred during freezing-thawing damage caused by the increase of free radicals in the body, which leads to changes in the structure and function of cells, and finally leads to apoptosis and even necrosis of cells (Gong et al., 2014). In this study, integrity of the plasma membrane, acrosome and DNA of frozen-thawed boar sperm was improved by the addition of riboflavin compared with the control group. Results indicate that riboflavin could reduce DNA damage caused by cryopreservation. Riboflavin effectively resists oxidative stress caused by the increase of ROS, thus protecting the internal structure of sperm cells and reducing sperm apoptosis in the freeze-thawing process. The addition of riboflavin in the extenders resulted in increased vitality and decreased apoptosis in spermatozoa. Similar to the measurement of only one time point after freezing-thawing reported in other studies (He et al., 2020; Shen et al., 2016), the limitation of this study was that the boar sperm was only tested once 10 min after thawing.

5 | CONCLUSION

In conclusion, supplementation with riboflavin could provide cryoprotective capacity for frozen-thawed boar sperm. The addition of riboflavin to the sperm extender can increase the activities of SOD, CAT, GPx and enhance the antioxidant defence system of sperm. Thus, it improves sperm motility, plasma membrane integrity, acrosomal integrity and mitochondrial activity. Adding riboflavin to the

sperm extender can also reduce sperm apoptosis and DNA damage. This research helps us understand how riboflavin affects sperm quality, which will provide new insights into the field of swine reproduction.

ACKNOWLEDGEMENTS

This research was funded by the Agricultural Animal Breeding Project of Shandong Province, grant number 2019LZGC019 and 2020LZGC012.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Ruilan Dong: conceptualisation; software; writing – original draft. Lan Luo: methodology; writing – review & editing. Xiaobin Liu: formal analysis; project administration. Guanghui Yu: conceptualisation; funding acquisition; project administration; resources; validation; writing – review & editing.

ETHICAL STATEMENT

The trial was conducted according to the animal care and use guidelines of the Animal Care and Use Committee of Animal Science and Technology College, Qingdao Agricultural University (Qingdao, China).

DATA AVAILABILITY STATEMENT

The people who want to get the original data can contact with the corresponding author by e-mail.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/vms3.833>.

ORCID

Guanghui Yu  <https://orcid.org/0000-0002-2068-5487>

REFERENCES

- Al-Harbi, N. O., Imam, F., Nadeem, A., Al-Harbi, M. M., Korashy, H. M., Sayed-Ahmed, M. M., Hafez, M. M., Al-Shabanah, O. A., Nagi, M. N., & Bahashwan, S. (2015). Riboflavin attenuates lipopolysaccharide-induced lung injury in rats. *Toxicology Mechanisms and Methods*, 25, 417–423. <https://doi.org/10.3109/15376516.2015.1045662>
- Amidi, F., Pazhohan, A., Shabani Nashtaei, M., Khodarahmian, M., & Nekoonam, S. (2016). The role of antioxidants in sperm freezing: A review. *Cell Tissue Bank*, 17, 745–756. <https://doi.org/10.1007/s10561-016-9566-5>
- Ashoori, M., & Saedisomeolia, A. (2014). Riboflavin (vitamin B₂) and oxidative stress: A review. *British Journal of Nutrition*, 111, 1985–1991. <https://doi.org/10.1017/s0007114514000178>
- Balasubramaniam, S., Christodoulou, J., & Rahman, S. (2019). Disorders of riboflavin metabolism. *Journal of Inherited Metabolic Disease*, 42, 608–619. <https://doi.org/10.1002/jimd.12058>
- Bennetts, L. E., & Aitken, R. J. (2005). A comparative study of oxidative DNA damage in mammalian spermatozoa. *Molecular Reproduction and Development*, 71, 77–87. <https://doi.org/10.1002/mrd.20285>
- Bolarin, A., Roca, J., Rodríguez-Martínez, H., Hernández, M., Vázquez, J. M., & Martínez, E. A. (2006). Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used. *Theriogenology*, 65, 669–680. <https://doi.org/10.1016/j.theriogenology.2005.06.006>
- Brouwers, J. F., Silva, P. F., & Gadella, B. M. (2005). New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. *Theriogenology*, 63, 458–469. <https://doi.org/10.1016/j.theriogenology.2004.09.046>
- Chen, L., Feng, L., Jiang, W. D., Jiang, J., Wu, P., Zhao, J., Kuang, S. Y., Tang, L., Tang, W. N., Zhang, Y. A., Zhou, X. Q., & Liu, Y. (2015). Dietary riboflavin deficiency decreases immunity and antioxidant capacity, and changes tight junction proteins and related signaling molecules mRNA expression in the gills of young grass carp (*Ctenopharyngodon idella*). *Fish and Shellfish Immunology*, 45, 307–320. <https://doi.org/10.1016/j.fsi.2015.04.004>
- de Albuquerque Lagares, M., Silva, G. C. D., Cortes, S. F., Luz, S. B., de Resende, A. C., Alves, N. C., Wenceslau, R. R., & Stahlberg, R. (2020). Does coenzyme Q10 exert antioxidant effect on frozen equine sperm? *Journal of Equine Veterinary Science*, 88, 102964. <https://doi.org/10.1016/j.jevs.2020.102964>
- Gong, G., Xiang, L., Yuan, L., Hu, L., Wu, W., Cai, L., Yin, L., & Dong, H. (2014). Protective effect of glycyrrhizin, a direct HMGB1 inhibitor, on focal cerebral ischemia/reperfusion-induced inflammation, oxidative stress, and apoptosis in rats. *PloS One*, 9, e89450. <https://doi.org/10.1371/journal.pone.0089450>
- Halliwell, B. (2009). The wanderings of a free radical. *Free Radical Biology and Medicine*, 46, 531–542. <https://doi.org/10.1016/j.freeradbiomed.2008.11.008>
- He, W. H., Zhai, X. H., Duan, X. J., & Di, H. S. (2020). Effect of resveratrol treatment on apoptosis and apoptotic pathways during boar semen freezing. *Journal of Zhejiang University Science B*, 21, 485–494. <https://doi.org/10.1631/jzus.B1900520>
- Hu, J. H., Sun, X. Z., Li, Q. W., Zhang, T., Hu, X. C., Hu, J. H., & Wang, L. Q. (2013). The effect of *Laminaria japonica* polysaccharide on sperm characteristics and biochemical parameters in cryopreserved boar sperm. *Animal Reproduction Science*, 139, 95–100. <https://doi.org/10.1016/j.anireprosci.2013.03.015>
- Jimenez-Marco, T., Ballester-Servera, C., Quetglas-Oliver, M., Morell-García, D., Torres-Reverte, N., Bautista-Gili, A. M., Serra-Ramon, N., & Girona-Llobera, E. (2021). Cryopreservation of platelets treated with riboflavin and UV light and stored at –80°C for 1 year. *Transfusion*, 61, 1235–1246. <https://doi.org/10.1111/trf.16324>
- Kuang, W., Zhang, J., Lan, Z., Deepak, R., Liu, C., Ma, Z., Cheng, L., Zhao, X., Meng, X., Wang, W., Wang, X., Xu, L., Jiao, Y., Luo, Q., Meng, Z., Kee, K., Liu, X., Deng, H., Li, W., & Chen, L. (2021). SLC22A14 is a mitochondrial riboflavin transporter required for sperm oxidative phosphorylation and male fertility. *Cell Reports*, 35, 109025. <https://doi.org/10.1016/j.celrep.2021.109025>
- Lee, E., & Kim, D. (2018). Effects of astaxanthin on miniature pig sperm cryopreservation. *BioMed Research International*, 2018, 6784591. <https://doi.org/10.1155/2018/6784591>
- Manthey, K. C., Chew, Y. C., & Zempleni, J. (2005). Riboflavin deficiency impairs oxidative folding and secretion of apolipoprotein B-100 in HepG2 cells, triggering stress response systems. *Journal of Nutrition*, 135, 978–982. <https://doi.org/10.1093/jn/135.5.978>
- Naijian, H. R., Kohram, H., Shahneh, A. Z., Sharafi, M., & Bucak, M. N. (2013). Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. *Cryobiology*, 66, 151–155. <https://doi.org/10.1016/j.cryobiol.2012.12.010>
- Pezo, F., Yeste, M., Zambrano, F., Uribe, P., Risopatrón, J., & Sánchez, R. (2021). Antioxidants and their effect on the oxidative/nitrosative stress of frozen-thawed boar sperm. *Cryobiology*, 98, 5–11. <https://doi.org/10.1016/j.cryobiol.2020.11.007>
- Qian, Y., Li, X.-F., Sun, C.-X., Jiang, G.-Z., Xu, W.-N., Wang, Y., & Liu, W.-B. (2014). Dietary biotin requirement of juvenile blunt snout bream,

- Megalobrama amblycephala*. *Aquaculture Nutrition*, 20, 616–622. <https://doi.org/10.1111/anu.12114>
- Saadeldin, I. M., Khalil, W. A., Alharbi, M. G., & Lee, S. H. (2020). The current trends in using nanoparticles, liposomes, and exosomes for semen cryopreservation. *Animals (Basel)*, 10. <https://doi.org/10.3390/ani10122281>
- Saedisomeolia, A., & Ashoori, M. (2018). Chapter two – Riboflavin in human health: A review of current evidences. In N. A. M. Eskin (Ed.), *Advances in food and nutrition research* (Vol. 83, pp. 57–81). Academic Press.
- Shen, T., Jiang, Z. L., Li, C. J., Hu, X. C., & Li, Q. W. (2016). Effect of alpha-lipoic acid on boar spermatozoa quality during freezing-thawing. *Zygote*, 24, 259–265. <https://doi.org/10.1017/s0967199415000155>
- Sreekumar, A., Acharya, K. K., Lalitha, H. S., Indi, S. S., Bali, P., & Seshagiri, P. B. (2005). Germ cell-specific localization of immunoreactive riboflavin carrier protein in the male golden hamster: Appearance during spermatogenesis and role in sperm function. *Reproduction*, 129, 577–587. <https://doi.org/10.1530/rep.1.00512>
- Tang, J., Wen, Z. G., Guo, Z. B., Huang, W., Guo, Y. M., Xie, M., & Hou, S. S. (2014). Dietary riboflavin supplementation improve the growth performance and antioxidant status of starter white Pekin ducks fed a corn-soybean meal diets. *Livestock Science*, 170, 131–136. <https://doi.org/10.1016/j.livsci.2014.10.016>
- Taylor, K., Roberts, P., Sanders, K., & Burton, P. (2009). Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. *Reproductive BioMedicine Online*, 18, 184–189. [https://doi.org/10.1016/S1472-6483\(10\)60254-4](https://doi.org/10.1016/S1472-6483(10)60254-4)
- Tumkiratiwong, P., Sukkachart, H., Buapuan, T., Tungtrongchitr, R., & Tungtrongchitr, A. (2006). Riboflavin-deficient and *Trichinella spiralis*-induced stresses on plasma corticosterone associated with spermatogenesis in male Wistar rats. *Southeast Asian Journal of Tropical Medicine Public Health*, 37, 250–256.
- Tumkiratiwong, P., Tungtrongchitr, R., Migasena, P., Pongpaew, P., Rojekittikhun, W., Vudhivai, N., Tungtrongchitr, A., Phonrat, B., & Nuamtanong, S. (2003). Antioxidant enzyme levels in the erythrocytes of riboflavin-deficient and *Trichinella spiralis*-infected rats. *Southeast Asian Journal of Tropical Medicine Public Health*, 34, 480–485.
- Waberski, D., Riesenbeck, A., Schulze, M., Weitze, K. F., & Johnson, L. (2019). Application of preserved boar semen for artificial insemination: Past, present and future challenges. *Theriogenology*, 137, 2–7. <https://doi.org/10.1016/j.theriogenology.2019.05.030>
- Wathes, D. C., Abayasekara, D. R., & Aitken, R. J. (2007). Polyunsaturated fatty acids in male and female reproduction. *Biology of Reproduction*, 77, 190–201. <https://doi.org/10.1095/biolreprod.107.060558>
- Yen, C. A., Ruter, D. L., Turner, C. D., Pang, S., & Curran, S. P. (2020). Loss of flavin adenine dinucleotide (FAD) impairs sperm function and male reproductive advantage in *C. elegans*. *Elife*, 9, e52899. <https://doi.org/10.7554/eLife.52899>
- Yeste, M. (2016). Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology*, 85, 47–64. <https://doi.org/10.1016/j.theriogenology.2015.09.047>
- Yeste, M., Rodríguez-Gil, J. E., & Bonet, S. (2017). Artificial insemination with frozen-thawed boar sperm. *Molecular Reproduction and Development*, 84, 802–813. <https://doi.org/10.1002/mrd.22840>
- Zhu, Z., Fan, X., Lv, Y., Zhang, N., Fan, C., Zhang, P., & Zeng, W. (2015). Vitamin E analogue improves rabbit sperm quality during the process of cryopreservation through its antioxidative action. *PLoS One*, 10, e0145383–e0145383. <https://doi.org/10.1371/journal.pone.0145383>
- Zribi, N., Chakroun, N. F., Ben Abdallah, F., Elleuch, H., Sellami, A., Gargouri, J., Rebai, T., Fakhfakh, F., & Keskes, L. A. (2012). Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. *Cryobiology*, 65, 326–331. <https://doi.org/10.1016/j.cryobiol.2012.09.003>

How to cite this article: Dong, R., Luo, L., Liu, X., & Yu, G. (2022). Effects of riboflavin on boar sperm motility, sperm quality, enzyme activity and antioxidant status during cryopreservation. *Veterinary Medicine and Science*, 8, 1509–1518. <https://doi.org/10.1002/vms3.833>