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Effect of resveratrol treatment on apoptosis and apoptotic pathways during boar semen freezing*

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Abstract: Resveratrol (3,5,4'-trihydroxystilbene, RSV) has been widely used in mammalian cells, but whether it can be used during freezing boar semen is still unknown. The effects of RSV treatment during boar semen freezing on its anti-freezing ability, apoptosis, and possible apoptotic pathways were observed in this study. Sperm motility, mitochondrial membrane potential ($\Delta\Psi_m$), adenosine triphosphate (ATP) content, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL)-positive apoptotic state, and messenger RNA (mRNA) expression levels of apoptotic genes involved in different apoptotic pathways after freezing with or without RSV treatment were tested. The results showed that: (1) Compared with fresh sperm, the motility, normal acrosome rate, and plasma membrane integrity rate of frozen boar sperm decreased significantly (P<0.05), and RSV did not significantly increase the sperm motility (0.44 vs. 0.40, P>0.05), but it did significantly improve the normal acrosome rate (57.65% vs. 47.00%, P<0.05) and plasma membrane integrity rate (46.67% vs. 38.85%, P<0.05). (2) After freezing, most boar sperm showed low mitochondrial $\Delta\Psi_m$. RSV treatment could increase the rate of high mitochondrial $\Delta\Psi_m$ of boar sperm. (3) RSV treatment significantly decreased reactive oxygen species (ROS) levels (58.65% vs. 88.41%, P<0.05) and increased the ATP content (0.49 μmol/L vs. 0.25 μmol/L, P<0.05) of boar sperm during freezing. (4) The apoptotic rate of the freezing group (80.41%) with TUNEL detection increased significantly compared to the fresh group (9.70%, P<0.05), and RSV treatment greatly decreased the apoptotic rate (68.32%, P<0.05). (5) Real-time polymerase chain reaction (RT-PCR) showed that not only the genes from the death receptor-mediated apoptotic pathway (tumor necrosis factor-α (*TNF-α*), Fas ligand (*FasL*), and *Caspase-8*), but also the genes from the mitochondria-mediated apoptotic pathway (manganese superoxide dismutase (*MnSOD*), B-cell lymphoma 2 (*Bcl-2*), Bcl-2-associated X protein (*Bax*), and *Caspase-9*) were both significantly changed after freezing. RSV treatment during freezing greatly changed their expression levels. Although RSV treatment during boar semen freezing did not significantly increase motility after thawing, it still played an efficient antioxidant role, which could enhance the mitochondrial function and decrease the apoptotic level induced by both the death receptor- and mitochondria-mediated apoptotic pathways.

Key words: Resveratrol (RSV); Boar semen freezing; Antioxidant; Mitochondrial function; Apoptotic pathway
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1 Introduction

Sperm freezing is a modern common assistive reproductive technology, which could conserve valuable genetic resources over a long period of time, and increase utilization efficiency and inter-regional exchange of excellent sires. Boar sperm membranes are richer in polyunsaturated fatty acids than those of other farm livestock. This makes them very susceptible

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to oxygen-induced damage mediated by lipid peroxidation (LPO) (Awda et al., 2009). Moreover, because of the multiparous nature of the animal and large doses of each ejaculate and for each artificial insemination, great difficulties in boar semen freezing have been observed by pig breeders. Present boar semen freezing technology cannot fully meet the needs of actual production, and further studies on boar sperm damage mechanisms should be carried out.

It has been confirmed that sperm freezing could damage sperm mitochondrial function and stimulate the sperm to produce excessive reactive oxygen species (ROS) (Gürler et al., 2016). Excessive ROS consume the sperm antioxidant defense system, and this causes the sperm to enter an oxidative stress state. ROS also directly oxidize sperm DNA bases or covalent bonds to DNA through their lipid peroxide malondialdehyde (MDA), causing DNA double-strand breaks (DSBs) and the occurrence of apoptosis (Aitken and Koppers, 2011; Tao et al., 2019).

Antioxidants have been widely used in the cryopreservation of mammalian embryos and sperm. Many studies have demonstrated that the treatment of antioxidants in cryoprotective agents increased sperm viability and fertility after thawing by decreasing sperm DNA damage. These antioxidants include ascorbate, catalase, vitamin E (VE), vitamin C (VC), brass wood, rosmarinic acid, and Rhodiola. Li et al. (2010) showed that the application of ascorbate or catalase during semen freezing significantly reduced the ROS levels of frozen-thawed sperm and improved sperm quality. Martinez-Soto et al. (2010) evaluated the effects of genistein supplementation in a thawing extender on frozen-thawed human semen parameters and found that it could reduce ROS levels and decrease membrane lipid disorder and DNA damage caused by cryopreservation. During boar semen freezing, Luño et al. (2014) suggested that rosmarinic acid provided protection for boar spermatozoa against oxidative stress during cryopreservation because of its antioxidant properties.

Resveratrol (3,5,4'-trihydroxystilbene, RSV) is a non-flavonoid polyphenol found mainly in grapes. It has pleiotropic effects, including anti-cancer, anti-aging, anti-inflammatory, and antioxidant actions, as well as cardioprotective and neuroprotective properties (Shimizu et al., 2016). The effects of RSV during the freezing of sperm have been studied in recent work. Different studies obtained different results. During the freezing of human spermatozoa, RSV treatment could prevent cryopreservation-induced lipid damage (Garcez et al., 2010) and DNA damage (Branco et al., 2010); however, RSV treatment in both studies did not prevent the observed reduction in sperm motility after thawing. During ram semen freezing, RSV treatment in the cryopreservation medium did not produce positive effects on spermatozoa motility nor the integrity of the acrosome or plasma membrane (Silva et al., 2012). However, during bull sperm freezing, the results demonstrated that RSV supplementation offered protection for sperm motility, high mitochondrial activity, and DNA integrity (Bucak et al., 2015).

The possible effects of RSV treatment to cryopreservation extenders on boar sperm parameters following the freezing-thawing process have not been reported. This study evaluated the effects of RSV supplementation on sperm motility, integrity of the acrosome and plasma membrane, mitochondrial function, apoptotic level, and gene expression levels of apoptotic-related genes from the death receptor- and mitochondria-mediated apoptotic pathways.

2 Materials and methods

2.1 Animals

Three mature boars (Meishan pigs) kept in individual pens were used for the collection of boar semen once a week during a two-month period.

2.2 Semen collection

Gloved-hand method was used for boar semen collection. Only ejaculate sperm samples with more than 90% motility were used for the next study. Collected semen was diluted with Beltsville thawing solution (BTS; 1:1, v/v) at the same temperature, and transported to the lab in a 17 °C refrigerator within 2 h.

2.3 Semen freezing

The basic medium for boar semen freezing was Tris-citric-glucose (TCG). TCG solution (100 mL) contained 2.42 g Tris, 1.48 g citric, 1.10 g glucose, and 100000 IU penicillin-streptomycin solutions. RSV concentration in TCG was 1 mmol/L.

Semen was centrifuged at 800*g* for 10 min, and then, the pellets were diluted in extender I (80% TCG and 20% egg yolk; volume fraction) to 3×10^9 cells/mL,

and were placed in a refrigerator (4 °C) with more than ten layers of gauze for 2 h. Then semen was resuspended 1:1 with extender II (71% TCG, 20% egg yolk, 8% glycerol, and 1% Orvus Ex Paste; volume fraction) at 4 °C. Diluted spermatozoa were placed in a refrigerator (4 °C) for another 1 h.

The semen was loaded with 0.5 mL straws. Sealed straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen for 10 min. After freezing, the straws were plunged into liquid nitrogen for storage.

2.4 Semen thawing

Thawed sperm (52 °C for 12 s in water bath) were extended at 37 °C with BTS (1:1). When RSV was used in the thawing, its concentration in BTS was 1 mmol/L. After incubation for 15 min, sperm quality was determined for the next experiments.

2.5 Assessments of sperm motility, normal acrosome rate, and plasma membrane integrity rate

The assessment methods of the sperm motility, normal acrosome rate, and plasma membrane integrity rate refer to the study of Dai et al. (2009).

Briefly, sperm motility was analyzed by computer-assisted sperm analysis (CASA; Minitube, Germany). Spermatozoa acrosome was stained in 2.2 g/L Coomassie blue G250 for 30 min and was checked for the percentage of acrosome-intact spermatozoa in at least 200 spermatozoa under a bright field. An easily identifiable swelling and coiling of sperm tails was used to evaluate plasma membrane integrity. Boar spermatozoa were incubated at 37 °C for 60 min in a mixture of 13.5 g/L fructose and 7.3 g/L Na-citrate; those with swelling and coiling tails were of normal plasma membrane integrity (Qiu et al., 2011).

2.6 Mitochondrial function with JC-1 staining

The mitochondrial function of the sperm was detected with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1) staining. Centrifugal sperm were stained with 2 μmol/L JC-1 in a CO_2 incubator at 37 °C for 30 min and washed twice with phosphate-buffered saline (PBS). At least 200 sperm cells per sample were examined under a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan; excitation at 450–490 nm, emission at 520 nm) to assess mitochondrial activity. Those sperm with high mitochondrial membrane potential ($\Delta\Psi_m$) had yellow/orange fluorescence, and those with low mitochondrial $\Delta\Psi_m$ had green fluorescence (Schäfer and Holzmann, 2000). The experiment was replicated three times.

2.7 Detection of ROS level using flow cytometry

Sperm ROS levels were stained with 10 μg/mL dihydrodichlorofluorescein diacetate (H_2DCF-DA) for 15 min at room temperature in the dark (Li et al., 2010). After incubation, the sample was centrifuged with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered human tubal fluid (HTF-HEPES) to wash away H_2DCF-DA. The fluorescent signal detected at 488 nm excitation and 525 nm emission is expressed with flow cytometry (FCM). The experiment was replicated three times.

2.8 Measurement of the ATP content in sperm

Adenosine triphosphate (ATP) content in sperm was measured by an ATP assay kit (Roche, Mannheim, Germany). The luminescence was immediately measured using a luminometer (Synergy 2, BioTek, VT, USA). A standard curve of ATP content was detected from five ATP-gradient concentrations ranging from 0.1 to 1.0 μmol/L. The experiment was repeated three times.

2.9 Apoptotic rate with TUNEL analysis

The apoptotic level of sperm was detected with a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) apoptotic detection kit (Beyotime, Nanjing, China). After fixation with 40 g/L paraformaldehyde for 1 h at room temperature, the sperm were washed with 1× PBS and permeabilized using 0.1% (1 g/L) Triton X-100 for 2 min on ice. After washing twice with PBS, the sperm DNA was labeled by incubating the sperm with 50 μL of TUNEL detection mixture (2 μL of TdT enzyme and 48 μL of fluorescein isothiocyanate (FITC)-labeled nucleotides) for 60 min at 37 °C in the dark. After the reaction, the samples were washed twice and resuspended in 1 mL of PBS. Sperm DNA was examined under a fluorescence microscope (Nikon Eclipse E600) for preliminary observation; green fluorescence shows a TUNEL-positive result and no fluorescence shows a TUNEL-negative result. In the FCM assessment, the apoptotic rate of sperm was detected at a 515–565 nm excitation wavelength and a

450–500 nm emission wavelength. The experiment was replicated three times.

2.10 RNA extraction, reverse transcription, and RT-PCR

Boar sperm were collected with or without RSV treatment during freezing. Three pools each containing 1 mL of semen (1.5×10^6 spermatozoa) were used to carry out the RNA extraction and real-time polymerase chain reaction (RT-PCR) analysis. RNA was extracted by the guanidinium thiocyanate phenol chloroform method (Toni et al., 2018). Reverse transcription was carried out with a FastQuant RT kit (with genomic deoxyribonuclease (gDNase); Tiangen, Beijing, China), and complementary DNA (cDNA) samples from the reverse transcription reaction were diluted with nuclease-free water to 60 μL. The quantification of all gene transcripts (Fas ligand (*FasL*), *p53*, tumor nuclear factor-α (*TNF-α*), B-cell lymphoma 2 (*Bcl-2*), Bcl-2-associated X protein (*Bax*), manganese superoxide dismutase (*MnSOD*), Cu-Zn-superoxide dismutase (*CuZnSOD*), *Survivin*, *Caspase-3*, *Caspase-8*, *Caspase-9*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)) was performed by quantitative RT-PCR (qRT-PCR) using the SYBR Green I chimeric fluorescence method (SYBR® Premix Ex Taq™ II, TaKaRa, Dalian, China). Gene annotations were obtained from GenBank. The accession number and primer sequence are presented in Table 1. Each reaction mixture (20.0 μL) consisted of 10.0 μL of SYBR® Premix Ex Taq™ II, 0.8 μL of each of forward (10 μmol/L) and reverse (10 μmol/L) primers, 0.4 μL of Rox Reference Dye II, 2.0 μL of cDNA, and 6.0 μL of dH_2O. cDNA was conducted using the 7500 RT-PCR system (Applied Biosystems, CA, USA) under the following conditions: 95 °C for 30 s and 35 cycles of 95 °C for 5 s and 72 °C for 30 s. The relative expression levels of genes analyzed using porcine *GAPDH* as the housekeeping gene and Safety Data Sheet (SDS) software for relative quantification (Applied Biosystems). The experiment was repeated three times for each sample (Dai et al., 2015).

2.11 Statistical analysis

Experiments were repeated at least three times, and the data were pooled for statistical analysis. The percentages were subjected to an arcsine transformation, and the transformed values were analyzed by analysis of variance (ANOVA). The level of significance was set at $P<0.05$.

3 Results

3.1 Effects of RSV treatment during freezing on sperm motility, normal acrosome rate, and plasma membrane integrity rate

The results for sperm motility, normal acrosome rates, and plasma membrane integrity rates of the three

Table 1 Primers used for qRT-PCR

Gene name	Accession number	Primer sequence (5'→3')	
		Forward primer	Reverse primer
GAPDH	NM_001206359.1	CACGATGGTGAAGGTCGGAG	TTGACTGTGCCGTGGAACTT
FasL	NM_213806.1	CGTGAGGGTCAATTCTGCTGT	CTTGTCTGTGTAATCCTCCCCC
p53	NM_213824.3	GAACAGCTTTGAGGTGCGTG	GCCATCCAGTGGCTTCTTCT
TNF-α	NM_214022.1	ATTCAGGGATGTGTGGCCTG	CCAGATGTCCCAGGTTGCAT
Bax	XM_003355975.2	GCCGAAATGTTTGCTGACGG	CGAAGGAAGTCCAGCGTCCA
Bcl-2	XM_003121700.3	GGCAACCCATCCTGGCACCT	AACTCATCGCCCGCCTCCCT
MnSOD	NM_214127.2	CCCAAAGGGGAATTGCTGGA	AACAAGCGGCAATCTGCAAG
CuZnSOD	NM_001190422.1	GTGCAGGGCACCATCTACTT	TCTTGATCCTTTGGCCCACC
Survivin	NM_214141.1	ACCACCGCATCTCCACATTT	TGGGACAGTGGATGAAACCG
Caspase-3	NM_214131.1	CCGAGGCACAGAATTGGACT	TTTCAGCGCTGCACAAAGTG
Caspase-8	NM_001031779.2	AGGCCCTGCTGAAGAAAATCT	CCTGTTCTCCCAGACAGTCC
Caspase-9	XM_003127618.2	AACTTCTGCCATGAGTCGGG	CCAAAGCCTGGACCATTTGC

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; *FasL*: Fas ligand; *TNF-α*: tumor nuclear factor-α; *Bcl-2*: B-cell lymphoma 2; *Bax*: Bcl-2-associated X protein; *MnSOD*: manganese superoxide dismutase; *CuZnSOD*: Cu-Zn-superoxide dismutase; qRT-PCR: quantitative real-time polymerase chain reaction

groups are shown in Table 2 (n=4). Freezing significantly decreased the sperm apparent indices (P<0.05). RSV treatment during freezing partially increases sperm motility, but there were no significant differences (0.44 vs. 0.40, P>0.05). The normal acrosome rate (57.65%) and plasma membrane integrity rate (46.67%) of the frozen samples with RSV treatment were much higher than those of the frozen group without RSV treatment (47.00% and 38.85%, respectively, P<0.05).

3.2 Effects of RSV treatment during freezing on mitochondrial $\Delta\Psi_m$ of boar sperm

Representative images of $\Delta\Psi_m$ are shown in Fig. 1. Sperm with high $\Delta\Psi_m$ show an orange color (arrows) and sperm with low $\Delta\Psi_m$ show a green color under a fluorescence microscope. The effect of RSV treatment during freezing on mitochondrial $\Delta\Psi_m$ of boar sperm is shown in Fig. 2 (n=3). Approximately 87.22% of boar sperm from fresh semen had high mitochondrial $\Delta\Psi_m$, and this was significantly higher than those of the two frozen groups (P<0.05). The treatment of RSV significantly improved the mitochondrial $\Delta\Psi_m$ after thawing (41.05% vs. 26.16%, P<0.05).

3.3 Effects of RSV treatment during freezing on boar sperm ROS levels and ATP content

The effects of RSV treatment on ROS levels are shown in Fig. 3 (n=3). The ROS-positive rate from fresh sperm was 3.26%. After freezing, the ROS-positive rate increased greatly, and RSV treatment during freezing could significantly decrease the ROS levels in boar sperm (88.41% vs. 58.65%, P<0.05).

The results of ATP content from the RSV treatment during freezing of boar semen are shown in Fig. 4 (n=3). Compared with the fresh group ((0.93±0.19) μmol/L), the ATP content of the freezing group ((0.25±0.04) μmol/L) decreased significantly (P<0.05). The ATP content of the RSV treatment group was (0.49±0.09) μmol/L, which was much higher than that of the freezing group without RSV treatment, but was still much lower than that of the fresh group (P<0.05).

Table 2 Effects of RSV treatment during freezing on boar sperm motility, normal acrosome rate, and plasma membrane integrity rate

Group	Sperm motility (%)	Normal acrosome rate (%)	Plasma membrane integrity rate (%)
Fresh	93.20±0.05^a	83.56±0.04^a	82.41±0.10^a
Freezing	40.12±0.08^b	47.00±0.17^c	38.85±0.06^c
RSV treatment	44.07±0.08^b	57.65±0.12^b	46.67±0.07^b

Data are expressed as mean±standard error of mean (SEM), n=4. Means in the same column with different small letter superscripts mean significant difference (P<0.05). RSV: resveratrol

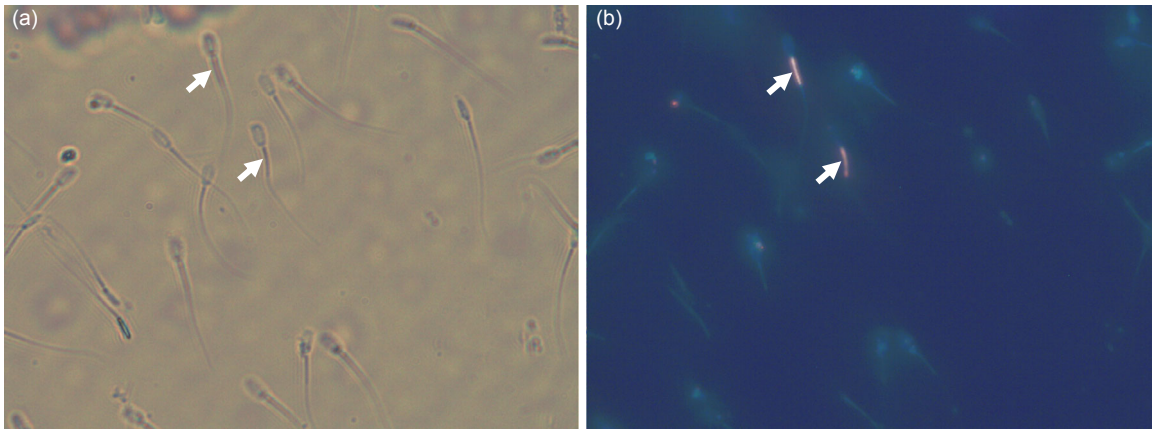

Fig. 1 JC-1 staining of spermatozoa under fluorescent microscope

(a) Under bright field; (b) Fluorescence exposed. Spermatozoa with orange fluorescence (arrows) showed high mitochondrial $\Delta\Psi_m$. JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; $\Delta\Psi_m$: membrane potential

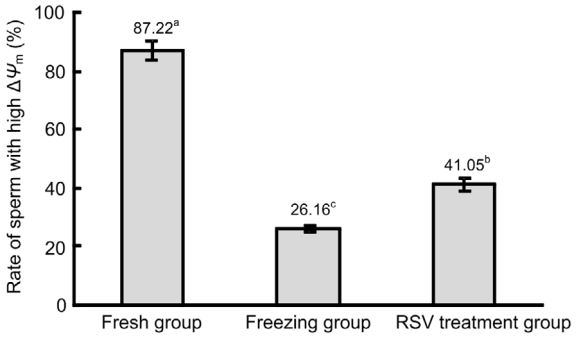

Fig. 2 Effect of RSV treatment on $\Delta\Psi_m$ of boar frozen sperm

Data are expressed as mean±standard error of mean (SEM), n=3. Different letters indicate a significant difference between treatments (P<0.05). RSV: resveratrol; $\Delta\Psi_m$: membrane potential

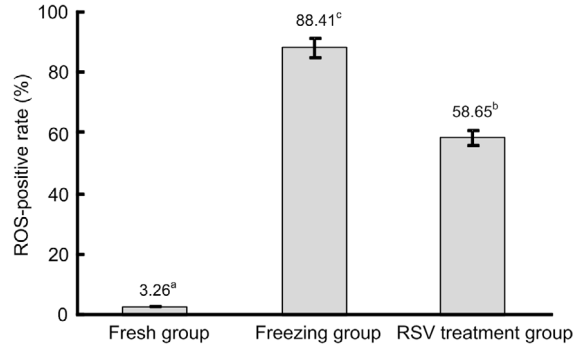

Fig. 3 Effect of RSV treatment on ROS level of boar frozen sperm

Data are expressed as mean±standard error of mean (SEM), n=3. Different letters indicate a significant difference between treatments (P<0.05). RSV: resveratrol; ROS: reactive oxygen species

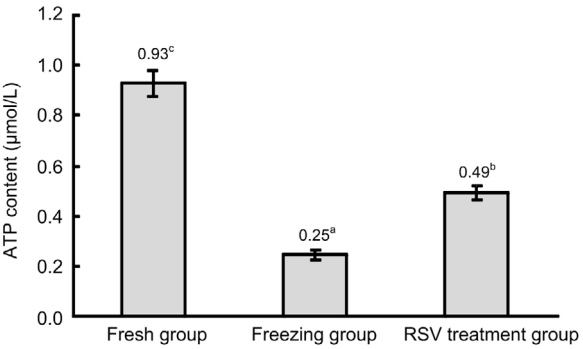

Fig. 4 Effect of RSV treatment during freezing on ATP content of boar sperm

Data are expressed as mean±standard error of mean (SEM), n=3. Different letters indicate a significant difference between treatments (P<0.05). RSV: resveratrol; ATP: adenosine triphosphate

3.4 Effects of RSV treatment during freezing on apoptotic status of boar sperm

The results of the TUNEL-positive apoptotic rates of boar sperm with fluorescence microscopy observation and FCM detection are shown in Table 3 (n=3). From fluorescence microscopy observation and FCM detection, the TUNEL-positive apoptotic rates of fresh boar sperm were 5.81% and 9.70%, respectively, which were significantly higher than those of the frozen sperm (69.52% and 80.41%, respectively, P<0.05). RSV treatment during freezing could significantly decrease the apoptotic levels from 69.52% to 51.61% with fluorescence microscopy observation and from 80.41% to 68.32% with FCM detection (P<0.05).

3.5 Effects of RSV treatment during freezing on the mRNA expression levels of apoptosis-related genes

The results of RT-PCR are shown in Fig. 5 (n=3). Compared with the fresh group, the relative mRNA expression levels of *MnSOD*, *CuZnSOD*, and *Survivin* in the frozen group significantly decreased (P<0.05), and the levels of *FasL*, *p53*, *TNF-α*, *Bax*, *Caspase-3*, *Caspase-8*, and *Caspase-9* significantly increased (P<0.05). When the semen was treated with RSV during freezing, the mRNA expression levels of *FasL*, *p53*, *TNF-α*, *Bax*, *Caspase-3*, and *Caspase-9* significantly decreased, and the levels of *Bcl-2*, *MnSOD*, and *Survivin* significantly increased compared with the frozen group (P<0.05).

4 Discussion

As an effective antioxidant, RSV has been shown to have a protective effect on the in vivo oxidative damage repair of some mammalian sperm, on in vitro embryo development, and on oocyte and embryo cryopreservation. This study found that RSV treatment could partly improve motility of sperm after thawing, but there was no significant difference (P>0.05). This was similar to the results of RSV treatment during human (Pasqualotto et al., 2006) and bull (Bucak et al., 2015) semen freezing. Spermatozoa and seminal plasma contain several antioxidants that provide protection against the toxic effects of free radicals (Jeulin et al., 1989). However, following the freezing-thawing process, this antioxidant system fails

Table 3 Effects of RSV treatment during boar semen freezing on TUNEL-positive apoptotic rates

Group	TUNEL-positive apoptotic rate	
	Fluorescence microscope observation	FCM analyses
Fresh	5.81±1.22[a]	9.70±1.50[a]
Freezing	69.52±3.80[c]	80.41±6.10[c]
RSV treatment	51.61±3.20[b]	68.32±3.90[b]

Data are expressed as mean±standard error of mean (SEM), n=3. Means in the same column with different small letter superscripts mean significant difference (P<0.05). RSV: resveratrol; TUNEL: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling; FCM: flow cytometry

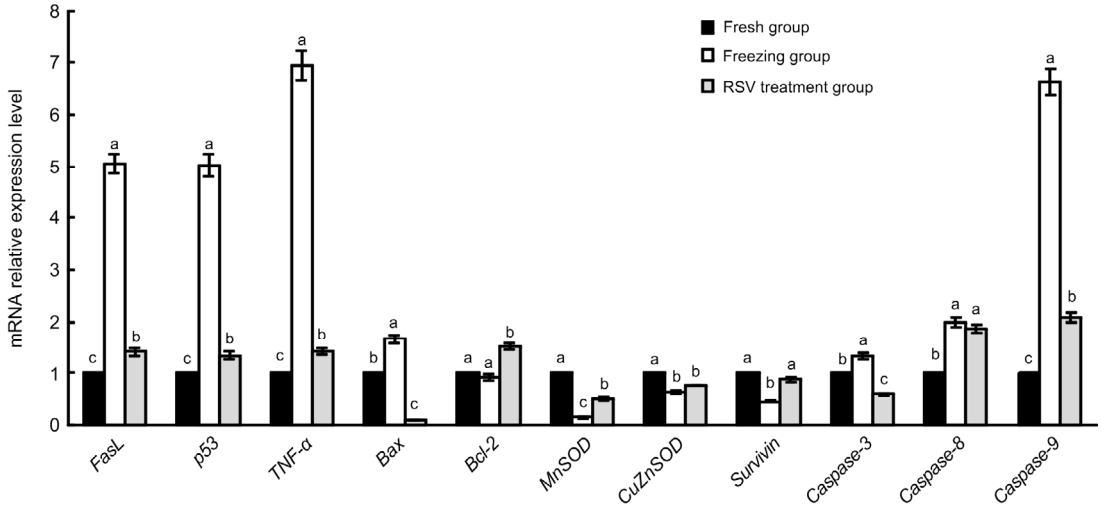

Fig. 5 Effects of RSV treatment on mRNA expression levels of boar frozen sperm

Data are expressed as mean±standard error of mean (SEM), n=3. The result of the fresh group is set as 1. Different letters for the same gene indicate a significant difference between groups (P<0.05). RSV: resveratrol; *FasL*: Fas ligand; *TNF-α*: tumor necrosis factor-α; *Bcl-2*: B-cell lymphoma 2; *Bax*: Bcl-2-associated X protein; *MnSOD*: manganese superoxide dismutase; *CuZnSOD*: Cu-Zn-superoxide dismutase

to protect spermatozoa against oxidative damage and the toxic effects of free radicals (Gadea et al., 2004). A study showed that RSV reaches peroxidized rigid membranes and increases membrane fluidity such that it interacts more efficiently with radicals in the disordered lipid bilayer (Collodel et al., 2011). For this reason, the treatment of RSV during freezing significantly increased boar sperm acrosome and plasma membrane integrity rates against LPO in the present study. Additional studies should be carried out to elucidate the non-conformance between the significant increase in the normal acrosome and plasma membrane integrity rates and the weak improvement in sperm motility after RSV treatment.

Apoptosis has three stages: induction, execution, and degradation. After the induction of apoptotic factors, the mitochondrial permeability transition pore (mPTP) opens, and the $\Delta\Psi_m$ decreases. Then, the cells undergo apoptosis caused by the decrease in mitochondrial function (Green and Reed, 1998). JC-1 can accumulate in mitochondrial matrices and form aggregates from monomers, which show different colors under epifluorescence microscopy, and this is widely used in the detection of mitochondrial function. The orange cells show a high $\Delta\Psi_m$ and the green cells show a low $\Delta\Psi_m$ (Ou et al., 2012). In the present study, we found that the rates of high $\Delta\Psi_m$ of the two frozen groups significantly decreased compared with the fresh group, and RSV treatment during freezing could greatly improve the rate of high $\Delta\Psi_m$ (P<0.05). Our results were similar to the study of Bucak et al. (2015) with bull semen freezing but were not consistent with the study of Silva et al. (2012) on goat semen freezing. The disparity can probably be attributed to the different species, extender compositions, and antioxidant doses used in the previous studies.

Mitochondria are the major source of energy in eukaryotic cells, producing ATP via oxidative phosphorylation and the citric acid cycle. ATP content in sperm is directly related to its motility (Perchec et al., 1995). ROS is a double-edged sword. On one hand, a moderate amount of ROS is involved in the normal physiological functions of sperm, such as sperm capacitation, acrosome reaction, and binding to the zona pellucida at physiological concentrations (Awda et al., 2009). On the other hand, ROS play a significant role in male infertility (Kefer et al., 2009), especially during the freezing of sperm (Gürler et al., 2016). The present study found that the ATP content in frozen sperm was significantly decreased and the ROS level was significantly increased compared with fresh sperm. Sperm freezing can cause mitochondrial damage. On one hand, damaged mitochondria reduce sperm ATP content and cause the rapid decrease in motility. On the other hand, damaged mitochondria release a large amount of ROS, which causes oxidative damage to sperm and induces apoptosis. RSV treatment during freezing can significantly improve the ATP content and reduce ROS levels. This study pointed out that RSV can protect mitochondrial function and reduce ROS production during the freezing process. This conclusion is very similar to the results from human (Pasqualotto et al., 2006) and bull (Bucak et al., 2015) semen freezing.

During semen freezing of animals, apoptotic-like changes were widely found. These mainly manifested as losses in sperm motility, activation of caspases, phosphatidylserine extemalization, DNA oxidative damage, and so on. In the present study, the apoptotic rate of frozen sperm with TUNEL staining significantly increased, which was similar to the above findings. TUNEL-positive cells are regarded as DNA-damaged and apoptotic cells. It has been proven that freezing can induce DNA damage, and antioxidant treatment can reduce this damage (Trzcińska et al., 2015). During the freezing of human (Branco et al., 2010) and bull sperm (Bucak et al., 2015), RSV has been shown to have antioxidant ability and can improve the DNA integrity of frozen sperm. Our study also showed that RSV treatment during freezing can significantly reduce the TUNEL-positive apoptotic rate and decrease DNA damage in frozen sperm. This result was consistent with the above studies.

Apoptosis is a gene-controlled, autonomous, programed cell death process. At least two pathways are involved in the apoptosis of eukaryotic cells: the mitochondria-mediated intrinsic apoptotic pathway and the death receptor-mediated extrinsic apoptotic pathway (Hsuuw et al., 2013). In the mitochondria-mediated intrinsic apoptotic pathway, *MnSOD* and *CuZnSOD* are the genes related to the removal of oxygen free radical synthesis in the body, which can represent the antioxidant capacity of the cells. This study found that RSV treatment during freezing could partly recover the gene expression levels of *MnSOD* and *CuZnSOD*. This indicates that RSV has antioxidant capacity during boar sperm freezing and can take part in the mitochondria-mediated intrinsic apoptotic pathway. *Caspase-9*, *Bcl-2*, *Bax*, and *Caspase-3* are the most important genes in the mitochondria-mediated intrinsic apoptotic pathway (de Oliveira et al., 2016). After freezing, the gene expression level of *Bcl-2* in the present study decreased, and the gene expression levels of *Caspase-9*, *Bax*, and *Caspase-3* increased significantly. RSV treatment during freezing improved the gene expression level of *Bcl-2* and decreased the gene expression levels of *Caspase-9*, *Bax*, and *Caspase-3*. This result indicates that RSV can reduce the apoptotic level of frozen boar sperm by the mitochondria-mediated intrinsic apoptotic pathway. This is similar to the results of Mukherjee et al. (2014) in goat embryo in vitro development after parthenogenetic activation or nuclear transfer.

FasL, *TNF-α*, and *Caspase-8* are the three important genes from the death receptor-mediated extrinsic apoptotic pathway (Scott et al., 2009). In the present study, all these genes changed greatly in boar sperm after freezing, which indicated that the death receptor-mediated apoptotic pathway could take part in the apoptosis induced by freezing. This result was consistent with the studies of Dai et al. (2016) in porcine vitrified oocytes and Zeng et al. (2014) during boar semen freezing. Our study also found that the gene expression levels of these three genes decreased significantly after RSV treatment during freezing, which also showed that RSV treatment during boar semen freezing could be involved in the regulation of the death receptor-mediated apoptotic pathway in yet unknown ways.

Freezing can cause oxidative damage to sperm. RSV treatment can reduce such oxidative damage and improve the normal acrosome rate and plasma membrane integrity rate, thereby partially improving sperm motility.

The integrity of plasma membrane and acrosome is an important indicator of sperm fertilization ability. Whether the improvement of the integrity of plasma membrane and acrosome in the RSV group can significantly improve the pregnancy rate after artificial insemination in pigs still needs further study.

In conclusion, RSV treatment during boar semen freezing can improve boar sperm mitochondrial function, reduce oxidative damage, change gene expression levels of apoptosis-related genes from both mitochondria- and death receptor-mediated apoptotic pathways, and then achieve the effects of decreasing the apoptotic level and partly increasing motility after thawing.

Contributors

Wei-hua HE, Xiu-jun DUAN, and He-shuang DI performed the experimental research and data analysis, wrote and edited the manuscript. Xiao-hu ZHAI participated in the study design, data analysis, and writing and editing of the manuscript. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Compliance with ethics guidelines

Wei-hua HE, Xiao-hu ZHAI, Xiu-jun DUAN, and He-shuang DI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：猪精液冷冻过程中白藜芦醇处理对其凋亡和凋亡途径的影响

目　的：阐明白藜芦醇（RSV）在猪精液冷冻保存中的应用效果及作用机制。

创新点：在冷冻过程中添加 RSV，观察其对猪冻后精子抗冻能力的影响，并阐明其细胞凋亡作用机理。

方　法：在猪精液的冷冻和解冻过程中添加 1 mmol/L RSV，解冻后检测精子活力、线粒体膜电位、腺苷三磷酸（ATP）含量、凋亡水平和凋亡通路中相关基因的表达情况。

结　论：（1）与鲜精相比，冷冻精液的活力、顶体完整性和质膜完整性均显著降低，冷冻前后 RSV 处理未能显著提高精子活力（0.44 vs. 0.40，$P>0.05$），但能显著提高顶体完整性（57.65% vs. 47.00%，$P<0.05$）和质膜完整性（46.67% vs. 38.85%，$P<0.05$）。（2）解冻后精子线粒体膜电位显著下降，RSV 的添加能提高精子膜电位水平。（3）冷冻解冻过程中添加 RSV 能显著降低精子的活性氧（ROS）水平（58.65% vs. 88.41%，$P<0.05$），增加精子的 ATP 含量（0.49 μmol/L vs. 0.25 μmol/L，$P<0.05$）。（4）TUNEL 凋亡检测后，冷冻精子的凋亡率（80.41%）与鲜精组（9.70%）相比显著增加（$P<0.05$），RSV 处理能显著降低冻精的凋亡比例（68.32%，$P<0.05$）。（5）实时荧光定量聚合酶链反应（qRT-PCR）的结果显示，猪精液冷冻后，无论是死亡受体介导凋亡途径中的相关基因（肿瘤坏死因子 α（*TNF-α*）、TNF 受体超族配体（*FasL*）和半胱氨酸的天冬氨酸蛋白水解酶 8（*Caspase-8*）），还是线粒体介导凋亡途径中的相关基因（锰超氧化物歧化酶（*MnSOD*）、B 淋巴细胞瘤-2（*Bcl-2*）、*Bcl-2* 相关 X 蛋白质（*Bax*）和半胱氨酸的天冬氨酸蛋白水解酶 9（*Caspase-9*）），均产生明显变化，RSV 添加亦能显著改变其表达水平。综上所述，猪精液冷冻解冻过程中 RSV 添加虽未能显著提高精子活力，但仍表现为抗氧化保护效果，体现在改善了线粒体功能，并通过改变死亡受体和线粒体介导的凋亡途径中相关基因的表达水平，降低了冻后精子的细胞凋亡。

关键词：白藜芦醇（RSV）；猪精液冷冻；抗氧化剂；线粒体功能；凋亡途径