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# Effects of glycerol on apoptotic signaling pathways during boar spermatozoa cryopreservation ☆

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ABSTRACT

Artificial insemination (AI) with post-thawed boar spermatozoa results in low farrowing rates and reduced litter sizes mainly due to cryoinjury or damages to spermatozoa during cryopreservation. Low viability and motility of post-thawed boar spermatozoa are highly associated with apoptosis during cryopreservation. Although glycerol is widely used a cryoprotectant (CPA) for boar spermatozoa cryopreservation, the mechanism and relationship between glycerol and apoptosis-related gene expression needs to be clarified. In this study, we treated boar spermatozoa with different concentrations of glycerol in lactose egg yolk (LEY) extender to evaluate the apoptosis-related gene expression and protease activities of caspases. These results show that: (1) low concentrations of glycerol (2% and 3%) were more suitable for boar spermatozoa cryopreservation; (2) apoptosis-related genes involved in intrinsic mitochondrial and extrinsic death receptor apoptotic signaling pathways were widely expressed in different concentrations of glycerol treated boar spermatozoa; (3) there was a significant positive correlation ($r = 0.840$, $P = 0.037$) between the percentage of Annexin $V^+/PI^+$ staining spermatozoa and caspase-6/9 protease activity. In conclusion, 2% and 3% glycerol have the best anti-apoptotic effects, and the expression of Fas/FasL and Bcl-2/Bax have a strong correlation with spermatozoa parameters.



## Introduction

Artificial insemination (AI) with frozen/thawed semen results in lower conception rates and smaller litter sizes compared to insemination with liquid or extended boar spermatozoa [41]. Cryopreservation causes damage to the plasma and mitochondrial membranes of the spermatozoa [56,59] and triggers molecular changes that interfere with fertilization and regulate molecular signals of apoptosis [7]. During the process of cryopreservation, temperature changes induce a rapid phase transition and drastic increases in reactive oxygen species (ROS), resulting in poor motility of frozen/thawed spermatozoa [15].

Apoptosis, also known as programmed cell death (PCD), is essential in tissues containing somatic cells as well as for normal spermatogenesis in mammals and plays a vital role in maintaining cellular homeostasis and eliminating a surplus of harmful cells [2]. Apoptosis or apoptotic-like changes increase during the cryopreservation spermatozoa, which acts to induce apoptosis in boar [13,34], bovine [49], human [56,74], equine [12] and mouse spermatozoa [17]. The predominant characteristics of apoptosis are loss of motility, phosphatidylserine exteriorization (PS), lipid peroxidation, caspase activation, oxidative DNA damage, and the activation of free radicals generated by the mitochondria during spermatozoa cryopreservation [3,62,69].

Caspases cleave a variety of cellular substrates after aspartic acid residues, a characteristic that is central to their role in mammalian apoptosis, and are considered to be active factors in the apoptotic pathway of spermatozoa [62,68]. Caspases are synthesized in the cytosol of mammalian cells as inactive zymogens, which are then activated by intracellular caspase cascades [18]. Caspase activity is usually measured as a characteristic of apoptosis and correlates with other apoptotic makers [68]. Activation of receptors on the plasma membrane (type I, extrinsic apoptosis) represents one of the major pathways for caspase activation [70]. Both the death receptor signal pathway (Fas/FasL) and the mitochondria signaling pathway (Bcl-2/Bax) trigger caspase initiators (caspase-8,-9) and downstream caspase executioners (caspase-3,-6,-7) giving rise to apoptosis [18,29]. Caspase-1 and -8 are involved in Fas-mediated apoptosis [77]. As an activator, caspase-8 is associated with the death-effector domain (DED) of Fas-associated proteins with death domains (FADD), and links upstream Fas/FasL proteins with

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downstream executioner proteins. In addition, procaspase-8 can be activated through a cytochrome C-dependent pathway [29]. Caspase-13, which is involved in receptor-initiated death pathways, can also be activated by caspase-8 [38]. Similar to caspase-8, the function of caspase-15 is to cleave Bid and activate caspase-3 [25], while caspase-2 provides an important link between etoposide-induced DNA damage and the engagement of the mitochondrial apoptotic pathway by triggering cytochrome C release and apoptosis [57]. As an executioner, caspase-6 plays an important role in the apoptotic process and interacts with caspase-8 [29].

Mitochondria play a cardinal role in the control of apoptosis. After induction of apoptosis, opened mitochondrial pores lead to decreased mitochondrial membrane potential, followed by the release of pro-apoptotic factors into the cytoplasm, resulting in the degradation phase. The pro-apoptotic protein Bax, translocates to the mitochondria and is cross-linked as a homodimer, which promotes the release of cytochrome C. The release of cytochrome C activates apoptotic protease-activating factor-1 (Apaf-1), which in turn activates downstream caspase cascades. Conversely, the anti-apoptosis protein Bcl-2 is an integral membrane protein which is heavily localized to mitochondria [1]. Bcl-2 can prevent the release of cytochrome C and apoptosis, suggesting that Bcl-2 maintains the integrity of the mitochondrial outer membrane by inhibiting cytochrome C release [43,80]. Pro-apoptotic and anti-apoptotic family members can heterodimerize, and their relative concentrations may act as a rheostat for the suicide program [65]. In PCD events, the Bcl-2/Bax ratio may be of primary importance to determine whether cells are undergoing apoptosis [71].

In the death receptor signaling pathway, the binding of the surface protein Fas ligand (FasL) to the Fas receptor triggers apoptosis in Fas-expressing cells [20]. Ligation of FasL to Fas in the cellular membrane also triggers the activation of caspase-8. Once activated, caspase-8 transduces a signal to effector caspases-3, -6, and -7 leading to the degradation of the cellular substrates of these caspases [53]. The expression of Fas has been reported in apoptotic spermatocytes, spermatids and Sertoli cells, and its expression in ejaculated sperm was reported first by Sakkas et al. [58].

Glycerol is one of the permeating cryoprotectants (CPAs) and is widely used to freeze spermatozoa of different species, including the boar [8,14]. Glycerol can effectively improve post-thawed motility and viability of spermatozoa [19,35]. However, depending on the concentration, glycerol can have chemical and osmotic toxicities on the plasma membrane [36,48]. Although effects of glycerol on spermatozoa parameters have been clearly studied, the exact cryoprotective or anti-freezing mechanism of glycerol remains unknown. In present study, from the point view of apoptosis, we systematically evaluated the effects of different concentrations of glycerol on post-thawed spermatozoa parameters as well as gene expression in apoptosis-related signaling pathways during boar spermatozoa cryopreservation. Identification of the caspase-mediated signaling pathways of apoptosis may lead to a better understanding of the molecular mechanism of cryoinjury and apoptosis and help to improve or optimize freezing extender of boar spermatozoa.

## Materials and methods

### *Sperm collection and treatment*

Sperm-rich fractions of ejaculates were collected from three sexually mature Landrace boars. Concentration, motility and viability of spermatozoa were measured by SQA-V (MES, Israel). Only morphologically normal spermatozoa with at least 80% motility and concentrations greater than $1 \times 10^8$ mL$^{-1}$ were used.

Spermatozoa were diluted (1:1 v/v) with Beltsville Thawing Solution (BTS, 3.7 g glucose, 0.3 g $Na_3$ citrate, 0.125 g $NaHCO_3$, 0.125 g $Na_2$–EDTA, 0.075 g KCL, 0.6 g/L penicillin G Na, 1.0 g/L dihydroestreptomicin, all diluted to 100 mL) and cooled slowly to 17 °C for 1–2 h. The extended spermatozoa were centrifuged at 800*g* for 15 min. Then, the pellets were resuspended with a lactose-egg yolk (LEY) extender (80 mL (80%, v/v), 310 mM betalactose, 20 mL hen's egg yolk). After thoroughly mixing, spermatozoa were slowly cooled to 5 °C for 2 h in the refrigerator. At this temperature, a third extender was added to the sperm to yield a final concentration of 0%, 2%, 3%, 5%, 7%, 10% and 14% glycerol and equivalented at 5 °C for 30 min. 0% glycerol was used as the control. Then, spermatozoa were loaded into 0.25 mL straws (FHK, Japan) and frozen using a controlled-rate freezing instrument (CryoMed controlled-rate freezer, Thermo Fisher, USA). The spermatozoa were cooled to −5 °C at a rate of 6 °C min$^{-1}$ and then from −5 to −80 °C at a rate of 40 °C min$^{-1}$, maintained at −80 °C for 30 s, cooled to −150 °C at a rate of 70 °C min$^{-1}$, and then plunged into liquid nitrogen for storage at −196 °C. One ejaculate directly cooled to −80 °C was also used as the control (direct freezing treatment, no any extender).

Three straws of frozen spermatozoa were thawed by immersing the straws at 37 °C in a water-incubator for 8–10 s. Spermatozoa were diluted with BTS (1:10 v/v) at room temperature. Post-thawed spermatozoa were incubated at 37 °C for 5 min before analysis. Spermatozoa motility was evaluated via SQA-V (MES, Israel). For sperm viability, diluted spermatozoa were stained using Typan Blue Staining Cell Assay Kit (Beyotime, China) according to manufacturer's instructions.

### *RNA extraction and quantitative real-time PCR (qRT-PCR) analysis*

Post-thawed spermatozoa were homogenized in Trizol LS reagent (Invitrogen, USA), and the total RNA was extracted. To avoid contamination with somatic cells, spermatozoa were treated with a hypotonic solution with 0.5% of Triton X-100 (Roche, Germany). The concentration and quality of total RNA were measured with a Nanodrop 2000 (Thermo Scientific, USA). For cDNA synthesis, 1 μg total RNA in a final volume of 20 μL was reverse-transcribed by TaKaRa PrimeScript RT Reagent Kit (TaKaRa Biotech, China) according to the manufacturer's instructions.

qRT-PCR was performed using SYBR® Premix Ex Taq II (TaRaKa Biotech, China) on the CFX manager PCR System (Bio-Rad, USA). Briefly, a total volume of 10 μL contains 6 μL SYBR Green I Premix and 0.5 μL each of forward and reverse primers, 1.0 μL of cDNA. The thermal cycling profile was the following: dwell temperature of 95 °C for 3 min, and 40 cycles of 94 °C for 10 s, and the primer specific annealing temperature for 30 s, and a final step to acquire the fluorescence.

### *Primer design*

All primers were cited from the literature and designed with homologous counterparts in the GenBank Database by Premier Primer 5.0 software. To determine the expression pattern of caspases in cryopreserved boar spermatozoa, we cloned, sequenced and detected the caspase expression patterns of spermatozoa in the direct freezing treatment. The results showed that the caspase-1, 2, 6, 8, 13, 15 can be detected by qRT-PCR (Unpublished data). Therefore, only caspase-1, 2, 6, 8, 13 and 15 were used for qRT-PCR in this study (Table 1).

### *Measurement of caspase protease activity*

Protease activities of caspase-3, caspase-8 and caspase-9/6 were determined using the ApoAlert Caspase Fluorescent Assay Kits