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Trehalose improves rabbit sperm quality during cryopreservation

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ABSTRACT

High levels of reactive oxygen species are associated with spermatozoa cryopreservation, which bring damage to functional spermatozoa. The aim of the present study was to investigate whether and how the freezing extenders supplemented with trehalose was beneficial for the survival of rabbit spermatozoa. semen was diluted with Tris-citrate-glucose extender addition of different concentrations of trehalose. Addition of 100 mM trehaose significantly improved post-thaw rabbit sperm parameters, such as motility, acrosome integriy, membrane integrity and mitochondrial membrane potential. Moreover, when freezing extenders supplemented with trehalose, activities of catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) of post-thaw spermatozoa were enhanced, meanwhile, reactive oxygen species (ROS) level and Malondialdehyde (MDA) content were decreased. The results suggest that freezing extenders supplemented with 100 mM trehalose resulted in less ROS level and MDA content, higher motility and mitochondrial membrane potential as well as the integrity of acrosome and plasma membrane. Supplementation of trehalose with freezing extenders is beneficial to the rabbit breeding industry.

1. Introduction

Cryopreservation of spermatozoa is an important technique for preservation of male fertility. However, the cryopreserved sperm has not been applied in rabbit breeding industry owing to the lower survival and fertility, smaller litter sizes and higher production cost [23]. High levels of reactive oxygen species is produced during cryopreservation, which is detrimental to spermatozoa, resulted in loss of motility and damages of structural membrane [8,33]. Thus, supplementation of antioxidants with freezing extender may protect spermatozoa against oxidative stress to improve the quality of post-thaw sperm.

Trehalose, known as α-D-glucopyranosyl α-D-glucopyranoside, is a non-reducing disaccharide of glucose. Trehalose could be utilized to stabilize simple systems, such as lipids and proteins, as well as more complex biologicals [25]. As trehalose could reduce intracellular ice crystal formation and maintain the protein structural during sperm cryopreservation [20], it has been used for improving the quality of post-thaw spermatozoa in mammals animals including rams [4], boars [13], goats [1], bulls [30]. Dalimata and Graham (1997) [11] reported a significant improvement in rabbit sperm quality when sperm were frozen in an egg yolk-acetamide extender supplemented with trehalose. However, Kozdrowski et al. (2009) [18] stated that addition of trehalose to the extender did not have a favourable effect on viability and fertility of post-thaw European brown hare spermatozoa. Thus, it is unclear whether the freezing extenders supplemented with trehalose is beneficial to post-thaw rabbit sperm or not. The aim of the study was to assess whether and how the trehalose protect rabbit sperm during cryopreservation.

In our previous study [33], ROS was accumulated during cryopreservation and post-thaw incubation in rabbits. Unfortunately, spermatozoa are rich in polyunsaturated fatty acids which is susceptible to lipid peroxidation, and Tuncer et al. (2013) [31] reported that trehalose protected spermatozoa by reducing lipid peroxidation. Therefore, we hypothesized that exposed of rabbit spermatozoa to trehalose before freezing could improve the post-thaw spermatozoa quality by its antioxidative capacity. The present study was conducted to examine (i) activities of catalase, superoxide dismutase and T-AOC of post-thaw spermatozoa; (ii) intracellular ROS, MDA content of post-thaw spermatozoa; (iii) motility, membrane integrity, acrosome intactness, mitochondrial membrane potential of post-thaw spermatozoa.

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2. Materials and methods

2.1. Animals selection

All experimental procedures involving animals were approved by the Northwest A&F University's Institutional Animal Care and Use Committee. Seventeen mature rabbits (fifteen males, two females) were selected for the present study. Rabbits were individually housed and maintained under uniform feeding and managemental conditions, and fed in a commercial standard diet and water ad libitum.

2.2. Semen collection and evaluation

An artificial vagina was used to collect semen twice for a week. Each ejaculate was shifted to water bath maintained at 37 °C and sent back to the Lab less than 30 min for evaluation. Sperm concentration of each sample was evaluated by hemocytometer. Motility was assessed with a phase-contrast microscope (Olympus, Japan) at 400 × magnification. Ejaculates with over 90% visual motility and more 2×10^8 sperm/mL were mixed to avoid individual differences for cryopreserving in this study.

2.3. Semen extension and freezing

Tris-citric-based extender (TCG) was the same with our previous study [33]. The freezing extenders were TCG with egg yolk (20%, v/v), DMSO (4%, v/v) and different concentrations of trehalose (0, 50, 75, 100,150, 200 mM).

Semen samples were divided into six parts, diluted with the extender containing different concentrations of trehalose at 37 °C, and cooled to 5 °C. After that, the cooled semen was mixed with the freezing extenders and equilibration at 5 °C for 30 min. Then, diluted semen samples were packed and sealed in 0.25 mL-straws immediately. Sperm were frozen and thawed as described by Zhu et al. (2015) [33].

2.4. Sperm motility

As described with Zhu et al. (2015) [33], 10 μL of post-thawed semen was placed on a pre-warmed clean glass slide. Sperm sample was evaluated under a phase-contrast microscope (Olympus, Japan) at 400 × magnification. After viewing five different fields of the sample, sperm motility were estimated and noted by one observer (ZZD).

2.5. Sperm membrane integrity

Membrane integrity were assessed by LIVE/DEAD Sperm Viability Kit (Leiden the Netherlands, L7011) [33]. Briefly, sperm supernatant were stained with SYBR-14 (100 μM in DMSO) and propidium iodide (PI) (2.4 mM in water) at 36 °C in the dark. Then, an epifluoresence microscope (Nikon 80i; Tokyo, Japan) was used to monitor and photograph the stained sperm in a set of filters (400×). 200 sperm were counted, and the treatments were replicated 5 times. All samples were evaluated by one observer (ZZD).

2.6. Acrosome integrity

Acrosome integrity was assessed by fluorescein isothiocyanate-peanut agglutinin (FITC-PNA, Sigma) solution (100 μg/mL) stained [33]. Briefly, sperm samples were fixed with absolute methanol for 10 min, and incubated with FITC-PNA solution (100 μg/mL) in dark at 37 °C for 30 min. Subsequently, samples were rinsed with PBS for three times prior to air-dried in dark. An epifluoresence microscope (Nikon 80i; Tokyo, Japan) with a set of filters (400×) were used to assess samples immediately. Photographs were also taken with a phase-contrast microscope in the same field, 200 sperm were counted, and the treatments were replicated 5 times. All samples were evaluated by one observer (ZZD).

2.7. Mitochondrial membrane potentials (ψm)

JC-1 (lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, Haimen, China) was used to analyze the changes of sperm mitochondrial membrane potential (ΔΨm) [9]. There are two types of JC-1 in stained mitochondrial plasma, one is a monomer, which emits green fluorescence in a low mitochondrial membrane potential, and the other is an aggregates, which emits red fluorescence in a high mitochondrial membrane potential. Briefly, sperm samples (2×10^6/mL) were stained with JC-1 working solution (5 μM) at 37 °C for 30 min in the dark, centrifuged (600 × g, 5 min) and washed with JC-1 buffer and placed on ice. The stained samples were immediately estimated under a fluorescence microscope (Nikon 80i; Tokyo, Japan) with a set of filters (400×). High membrane potential was associated with emission at red (590 nm), low membrane potential was green (at 530 nm). At least 200 of spermatozoa were counted in each field. The treatments were replicated 5 times. All samples were evaluated by one observer (ZZD).

2.8. Intracellular ROS measurement

The intracellular ROS level was measured using the probe of 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Institute of Biotechnology, Nanjing, China), according to Zhu et al. (2015) [33], intracellular DCFH-DA was deesterified to dichlorodihydrofluorescein which is oxidized by ROS to produce the fluorescent compound dichlorofluorescein. Sperm suspensions (10×10^6 cells/mL) were incubated with 10 μM DCFH-DA at 37 °C for 30 min in the dark. The fluorescence intensity was measured using the fluorescence plate reader (Synergy HT, BioTek, USA) at Ex./Em. = 485/535 nm. The treatments were replicated 5 times.

2.9. Intracellular MDA measurement

Malondialdehyde (MDA) content was quantified by a commercial kit according to the manufacturer's protocol [26]. In brief, sperm cellular extracts were prepared by sonication (20 KHz, 750 W, operating at 40%, on 3s, off 5s, 5 cycles) in ice-cold buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT), lysed cells were centrifuged at 12,000 × g for 10 min to remove debris after sonication. The supernatant was subjected to the measurement of MDA (at 532 nm) levels with a microplate reader. MDA levels were then normalized to milligram protein.

2.10. T-AOC activity

T-AOC activity was measured by using a T-AOC assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). According to the manufacturer's instructions, sperm samples were washed three times with TCG, then sperm pellets were resuspended, lysed ultrasonically (20 KHz, 750 W, operating at 40%, on 3s, off 5s, 5 cycles) on ice, then centrifuged at 12,000 × g for 10 min at 4 °C, supernatants were mixed with the reaction buffer, then measured at 520 nm a microplate reader.