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Loss of protein kinase 2 subunit alpha 2 (CK2α') effect ram sperm function after freezing and thawing process

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ABSTRACT

Protein kinase 2 subunit alpha 2 (CK2α'), a serine/threonine-selective protein kinase, is associated with sperm apoptosis. However, the presence of CK2α' in ram sperm during the freezing-thawing process has not been previously reported. Thus, this study was conducted to determine the effect of cryopreservation on the association between CK2α' and ram sperm function. Sperm variables, including sperm motility, DNA damage and acrosome integrity, were measured in fresh (F), cooled (CO) and freeze-thawed (FT) sperm. Sperm proteins and total mRNA were extracted form cells of each group, and subjected to western blot and real-time PCR analysis for detection of CK2α' proteins and relative abundance of mRNA. The distribution pattern of CK2α' protein in ram sperm was also monitored in each group using an immunofluorescence technique. The results provided evidence that the freeze-thaw process has an impact on ram sperm variables, and the normalized CK2α' protein and relative abundance of CK2α' mRNA were both significantly less in FT than F sperm. The amount of CK2α' in FT extended seminal plasma was increased as compared with F samples. Furthermore, immunofluorescence revealed that CK2α' was distributed throughout the acrosome of ram sperm. The association of CK2α' with DNA damage and acrosome integrity was confirmed using Pearson's linear correlation. In conclusion, the understanding the molecular effects of cryopreservation on CK2α' in ram sperm could provide insight into methods for improving fertility associated with frozen-thawed ram sperm.

1. Introduction

Sperm cryopreservation is an effective method to improve brood stock and increase the population of offspring produced from artificial insemination (Liu et al., 2015; Liu et al., 2016a,b). However, cryopreservation might cause damage to sperm compared with fresh samples (Alvarez et al., 2012; Liu et al., 2016a,b). In most of the surviving sperm, cryopreservation cause sub-lethal cryodamage, resulting in reductions in sperm viability in the female reproductive tract or *in vitro* (de Vasconcelos Franco et al., 2016). Cryopreservation also causes an increase in sperm DNA damage, reduction or elimination of certain transcripts, and production of apoptosis-related changes (García-Herrero et al., 2011; Nekoonam et al., 2016). Moreover, cryopreservation leads to formation of intracellular ice and results in cell dehydration, causing protein denaturation, which is a major damaging factor associated with

Abbreviations: ROS, reactive oxygen species; ATP, Adenosine triphosphate; 2-DE, two-dimensional gel electrophoresis; IVF, *in vitro* fertilization.

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cell injury due to freezing (Osipova et al., 2016). Although different cryoprotectants were used to avoid these damages, the cryoprotectants are inadequate to completely prevent sperm cryodamage. Damage at the molecular level involved the plasma membrane, acrosome, mitochondria, increases in reactive oxygen species (ROS), decreased DNA integrity, and reduced sperm motility and survival (Gürler et al., 2016). Cryopreservation at the molecular level also results in reduced metabolic activity and loss of cytoplasmic proteins, membrane-bound proteins, enzymes, and other cellular components, as well as incorporation of defects in sperm proteins that might impair sperm motility, fertilization, and early events following fertilization (Zilli et al., 2005).

Therefore, a more complete understanding of cellular and subcellular alterations to sperm induced by freezing and thawing is important to improving the process of sperm cryopreservation. However, limited reference information exists regarding the effects of the freezing-thawing processes on ram semen. The CK2α' genesis preferentially expressed in the late stages of spermatogenesis, and male mice with decreased CK2α' gene expression have oligospermia and globozoospermia, and are, thus, are infertile (Xu et al., 1999). The role of CK2α' in ram sperm during the freeze-thawing process was not confirmed. In a previous study, two-dimensional electrophoresis (2-DE) was used to determine proteomic changes in ram sperm during the freezing-thawing process, and it was shown that CK2 content decreased in the thawed sperm, and it has been verified that casein kinase II subunit alpha 1 (CK2α) gene expression was decreased in the thawed sperm (He et al., 2016). Hence, the objective of this study was to determine the effect of cryopreservation on ram sperm motility, DNA damage, acrosome integrity, and CK2α' gene expression following freezing and thawing processes.

2. Materials and methods

2.1. Semen collection, cryopreservation and thawing

Animal care and sample-collection procedures used in this study were approved and conducted according to standards established by the Animal Husbandry and Veterinary Research Institute of Gansu Province (Pingliang, China). Five mature and healthy Dorset rams (*Ovis aries*) in good body condition were maintained with a uniform feeding and management regimen, with three rams being randomly selected for the present study. Semen was collected twice weekly from each ram using an artificial vagina maintained at 37 °C during the breeding season (Autumn). In 1 day, two ejaculates were collected from each ram after an interval of ~20 min to eliminate individual differences, and a hematocytometer was used to evaluate sperm motility, sperm concentration, and sperm abnormality by light microscopy (Nikon 80i; Nikon, Tokyo, Japan) at 400 × magnification (He et al., 2016). Ejaculates (5 μL) were diluted to 3×10^7 sperm/mL using diluent buffer consisting of 2.4 g Tris, 1.3 g citric acid, 0.5 g fructose, 500 IU benzylpenicillin, and 500 IU streptomycin in 100 mL deionized water (Hafez, 2000). The percentage of sperm motility was calculated as the number of motile sperm divided by the total number of sperm (both motile and non-motile). Ejaculates containing sperm exhibiting motility of >80% in a volume ranging from 0.75 mL to 2 mL, sperm concentrations $>3 \times 10^9$ sperm/mL, and sperm abnormalities of <10% were pooled, with this constituting one semen sample. Each pool was protected from light, incubated at 37 °C for 30 min, thoroughly mixed, and divided into two fractions. One fraction was used as a fresh sample and was centrifuged at 275*g* for 10 min at room temperature to separate fresh seminal plasma (FSP) and sperm pellet. The proteins from the sperm pellet were extracted as per the method described in the Section 2.3 of this manuscript. These sperm samples were stored at −20 °C until Western analysis of cryopreserved samples until further use. The other fraction was diluted at a ratio of one part semen to five parts extender (at 37 °C), and cryopreserved using the conventional methods of the Animal Husbandry and Veterinary Research Institute of Gansu Province. The main cryopreservation procedure was as follows: The extender (main components: 2.422 g Tris, 1.34 g citric acid, 0.5 g fructose, 2 mL M199 medium, 500 IU benzylpenicillin, 500 IU streptomycin, 0.25% (v/v) soybean Lecithin and 5.6% (v/v) glycerol in 100 mL deionized water; Hafez, 2000; Endang et al., 2010; Triwulanningsih et al., 2010) was added to the semen samples at a 5 × volume. The pH of the extender was adjusted to 7 using Tris. The final sperm concentration in diluted samples was approximately 5×10^8/mL. Diluted samples were divided into three parts. One-third of the samples was processed for separation of the sperm pellet and semen extender, thus this sample was not subjected to the cryopreservation procedure and was considered to be non-cryopreserved (NC) samples. Two-thirds of samples were kept in a refrigerator and were slowly cooled from 37 °C to 5 °C for at least 3 h. One-half of the samples was processed for separation of the sperm pellet and semen extender, thus this sample was considered to be the cooled (CO) sample. The other one-half of the aliquot was loaded into 0.25 mL straws and suspended at 4 cm above the liquid nitrogen vapor and kept 10 min, then submerged in liquid nitrogen and stored in a liquid nitrogen tank for >72 h. Immediately before evaluation, frozen samples in each straw were thawed in a 37 °C water bath for 30 s and sperm motility was evaluated (Varela Junior et al., 2009) and the sample was processed for separation of the sperm pellet and semen extender, thus, this sample was considered to be the frozen-thawed (FT) sample.

2.2. Sperm function tests

2.2.1. Motility

Percent sperm motility was calculated as the number of motile sperm divided by the total number of sperm (both motile and non-motile; He et al., 2016).

2.2.2. DNA damage

Sperm DNA damage was evaluated using acridine orange (AO) according to the method described by Nur et al. (2010). At least 200 sperm were evaluated using a fluorescence microscope at 400 × magnification. Percentages of