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Cryopreservation of bull semen is associated with carbonylation of sperm proteins

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ABSTRACT

Artificial insemination with cryopreserved semen enables affordable, large-scale dissemination of gametes with superior genetics. However, cryopreservation can cause functional and structural damage to spermatozoa that is associated with reactive oxygen species (ROS) production, impairment of sperm motility and decreased fertilizing potential, but little attention has been paid to protein changes. The goal of this study was to investigate the oxidative modifications (measured as carbonylation level changes) of bull spermatozoa proteins triggered by the cryopreservation process. Flow cytometry and computer-assisted sperm analysis were used to evaluate changes in viability, ROS level and motility of spermatozoa. Western blotting, in conjunction with two-dimensional electrophoresis (2D-oxyblot) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight spectrometry, was employed to identify and quantify the specifically carbonylated spermatozoa proteins. Cryopreservation decreased motility and viability but increased the number of ROS-positive cells. We identified 11 proteins (ropporin-1, outer dense fiber protein 2, glutathione S-transferase, triosephosphate isomerase, capping protein beta 3 isoform, actin-related protein M1, actin-related protein T2, NADH dehydrogenase, isocitrate dehydrogenase, cilia- and flagella-associated protein 161, phosphatidylethanolamine-binding protein 4) showing differences in protein carbonylation in response to cryopreservation. The identified proteins are associated with cytoskeleton and flagella organization, detoxification and energy metabolism. Moreover, almost all of the identified carbonylated proteins are involved in capacitation. Our results indicate for the first time that cryopreservation induces oxidation of selected sperm proteins via carbonylation. We suggest that carbonylation of sperm proteins could be a direct result of oxidative stress and potentially lead to disturbances of capacitation-involved proteins or could indicate cryopreservation-induced premature capacitation.

1. Introduction

Bull sperm cryopreservation is an invaluable technique for artificial insemination that allows affordable, worldwide dissemination of gametes with superior genetics [1]. However, cryopreservation is known to damage bull sperm in a variety of ways resulting in sub-lethal damage and modifications responsible for a shortened lifespan of sperm cells [2]. Oxidative and osmotic stresses during the freezing and thawing processes alter lipid and protein composition; decrease motility and viability; cause damage to mitochondria, acrosomes and sperm tails; and increase sperm DNA fragmentation [3–5]. Moreover, the freezing and thawing processes induce rearrangement of lipid membranes, resulting in increased fluidity and intracellular calcium, which eventually initiates the capacitation-like changes in cryopreserved spermatozoa (cryocapacitation) [3,6]. Studies of cryogenic injuries are important for better understanding of the mechanism of sperm cryogenic damage and for improvement of cryopreservation protocols.

Cryopreservation induces a variety of alterations in sperm structure and function leading to the generation of reactive oxygen species (ROS) that can react with sperm lipids, DNA and proteins, causing multiple disturbances of cell physiology [7]. Spermatozoa are especially susceptible to injury caused by oxidative stress, as sperm plasma membranes are rich in polyunsaturated fatty acids that readily undergo peroxidation, forming secondary products that can oxidize sperm proteins [8,9]. The influence of ROS on

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spermatozoa lipids and DNA is well known [10], but less attention has been paid to protein oxidative damage due to cryopreservation. It was reported that cryopreservation induces prominent phosphorylation and redox changes in sperm proteins [11]. However, it is still unknown which proteins become oxidized and what the physiological consequences of this process are.

There are numerous types of protein oxidative modifications that can be measured with a variety of methods, but the most common methods are based on determination of sulfhydryl and carbonyl (C–O) groups of proteins [12,13]. The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and relative stability of carbonylated proteins [8]. Carbonylation of proteins has been used as an indicator of sperm oxidation in the pathology of fresh human semen. It was earlier shown that protein carbonylation level in sub-fertile men is higher than in the control group [18]. Another studies showed that abnormal semen exhibits significantly higher levels of protein carbonylation in comparison to the control group [19]. However, to our knowledge, carbonylation of proteins has not been determined yet as a result of cryopreservation in mammals. Carbonylation of sperm proteins after freezing and thawing can be predicted because cryopreservation itself induces ROS production. This prediction is supported by results of Chatterjee et al. [11], who showed that cryopreservation triggers sperm protein oxidative changes, measured by sulfhydryl group determination.

The objective of this study was to compare protein carbonylation levels between fresh-diluted and cryopreserved bull semen. Using a combination of two-dimensional electrophoresis, western blotting and mass spectrometry, we identified the proteins showing increased carbonyl levels after cryopreservation. It is worth emphasizing that semen was diluted with an extender deprived of egg yolk, which is still frequently used ingredient of semen extenders [12]. Application of egg yolk-free extender prevents contamination with proteins from other sources, which is crucial in proteomic studies.

2. Materials and methods

2.1. Study design

This study aimed to investigate the oxidative modifications of bull spermatozoa proteins triggered by the cryopreservation process. Two groups of semen were compared: fresh-diluted and cryopreserved, obtained from the same ejaculate. We determined the quality and oxidative status of sperm by performing analyses of sperm motility, viability, ROS production, total protein carbonylation and identification of specifically carbonylated sperm proteins.

2.2. Semen collection

The experiment was carried out on semen collected from five mature Holstein Friesian bulls provided by the SHiUZ (Animal Breeding and Insemination Center, Olecko, Poland). Semen samples were collected using an artificial vagina. Cryopreservation and sperm quality assessment were conducted in accordance with the standard commercial procedure. The criteria for cryopreservation were following: a concentration at least 1×10^9 spermatozoa/mL, sperm motility at least 70%, and the quality of mass movement of the spermatozoa graded at least 2 on a 3-degree scale as described earlier in detail [5].

Average spermatozoa concentration of fresh semen collected from five bulls used in the experiment was $1.58 \pm 0.28 \times 10^9$/mL and percentage of motile sperm was 75± 5%, as assessed with light microscope by experienced operator of SHiUZ.

2.3. Preparation of fresh-diluted semen

Each ejaculate was divided into two parts designed for either preparing fresh-diluted or cryopreserved semen samples. The fresh-diluted semen samples were obtained by dilution of fresh semen with protein-free commercial BIOXcell extender, deprived of egg yolk (IMV Technologies, L'aigle, France) in a 1:1 ratio. The semen samples were transported on ice to a laboratory of the Institute of Animal Reproduction, Polish Academy of Sciences (Olsztyn, Poland). The procedure of semen dilution was recommended by the personnel of SHiUZ as a standard for handling fresh semen. Moreover, this procedure is also recommended by manufacturer of the extender (BIOXcell) used in the study [1A]. Since extender compounds were present in both, fresh-diluted and cryopreserved samples, we assume that extender components didn't influence oxidative status of proteins. All analyzes (sperm motility, viability, ROS production, total protein carbonylation and identification of specifically carbonylated sperm proteins) were performed after transporting the semen samples on ice (4 °C) to a laboratory of the Institute of Animal Reproduction, Polish Academy of Sciences. The transportation lasted for 3 h and analyses were made within 5 h from semen collection.

2.4. Preparation of cryopreserved semen

Before cryopreservation semen was diluted with BIOXcell extender to a final concentration of 48×10^6 spermatozoa/mL. Our flow cytometry measurements of cryopreserved semen showed $50.00 \pm 7.28 \times 10^6$ spermatozoa/mL (see below). Diluted semen aliquots were automatically packed into 250-μL straws and equilibrated for 2.5 h at 4 °C. The straws were subsequently frozen in liquid nitrogen vapor using a computer-controlled automatic freezer Digitcool 5300 (IMV technologies), following the freezing curve presented in the supplementary data (Supplemental Fig. 1S). The cryopreserved semen was transported and stored in liquid nitrogen containers. The straws were thawed for 1 min at 37 °C prior to analysis.

2.5. Computer-assisted sperm analysis

Spermatozoa motility parameters of fresh-diluted and cryopreserved bull semen were determined with computer-assisted sperm analysis using a Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The cryopreserved semen was diluted with BIOXcell extender at a ratio from 1:19 to 1:44 depending on initial concentration of semen and the fresh-diluted samples were diluted with BIOXcell extender at a ratio 1:20 prior to motility measurements. Thus, the concentration of fresh-diluted and cryopreserved semen used to assess sperm motility was similar. Semen concentrations were consistent with the requirements of Hobson Sperm Tracker analyzer that we used in our study. Video recordings of motile sperm were captured using a microscope with a 4× negative phase objective and a Sony CCD black-and-white video camera (SPTM108CE). MicroCell 50-μm-deep, two-chamber slides (Conception Technologies, San Diego, USA) mounted on a heated stage (37 °C) were used. The sperm motility parameters were as follows: MOT – percentage of motile sperm (%), VCL – curvilinear velocity (μm/s), VSL – straight line velocity (μm/s), VAP – average path velocity (μm/s), LIN – linearity (%, 100 × VSL/VCL) and BCF – beat cross frequency (Hz). Each sample was analyzed in duplicate.