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Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights

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ABSTRACT

Semen cryopreservation is a well-established procedure used in veterinary assisted reproduction technology applications. We investigated damaging effects of cryopreservation on the structural and ultrastructural characteristics of bull sperm induced at different temperatures and steps during standard cryopreservation procedure using transmission (TEM) and scanning electron microscopy. We also examined the effect of cryopreservation on sperm DNA and chromatin integrity. Five healthy, fertile Friesian bulls were used, and the ejaculates were obtained using an artificial vagina method. The semen samples were pooled and diluted in a tris-yolk fructose (TYF) for a final concentration of 80×10^6 spermatozoa/ml. The semen samples were packed in straws (0.25 ml), and stored in liquid nitrogen (−196°C). Samples were evaluated before dilution, just after dilution (at 37°C), at 2 h and 4 h during equilibration, and after thawing (37°C for 30 s in water bath). In association with step-wise decline in motility and viability, our results showed that the plasma membrane surrounding the sperm head was the most vulnerable structure to cryo-damage with various degrees of swelling, undulation, or loss affecting about 50% of the total sperm population after equilibration and freezing. Typical acrosome reaction was limited to 10% of the spermatozoa after freezing. We also observed increased number of mitochondria with distorted cristae (15%). Chromatin damage was significantly increased by cryopreservation as evident by TEM (9%). This was mainly due to DNA breaks as confirmed by Sperm Chromatin Structure Assay (SCSA) (8.4%) whereas the chromatin structure was less affected as evaluated microscopically by toluidine blue staining. We concluded that, using standard cryopreservation protocol, the most pronounced damage induced by cryopreservation is observed in the plasma membrane. Further improvement of cryopreservation protocols should thus be targeted at reducing plasma membrane damage. Acrosomal, mitochondrial and chromatin damage are also evident but appear to be within acceptable limits as discussed.

1. Introduction

Semen cryopreservation is a well-established procedure used in human and veterinary assisted reproduction technology (ART) applications. Over the last 50 years, it was used for genetic improvement of beef and dairy cattle. It is also used to control venereal diseases and facilitate management of cattle herd fertility. In human, it is usually associated with male fertility preservation which is usually required prior to cancer therapy [1].

Spermatozoa are characterized by plasma membrane fluidity and low water content which make it more resistant to cryodamage compared to other cell types [2]. However, cryopreservation have been shown to induce deleterious changes of sperm structure and function [3]. This involves thermal stress due to the change in temperature during cooling, freezing and thawing as well as the osmotic stress caused by addition of high concentrations of cryoprotective agents and by crystallization [4]. This results in protein denaturation, shrinkage and irreversible membrane collapse [3]. Therefore, phospholipids and cryoprotective agents, as well as optimal dilution, equilibration and cooling procedures are required to avoid cold shock, reduce crystallization and minimize sperm damage.

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Conventional sperm evaluation parameters used in AI centers for sire semen evaluation and post-thawing assessment of cryopreserved semen are usually limited to examining post-thawing motility and morphology. Evaluation of sperm motility either subjectively or by computerized sperm analysis has been used as the main parameter to determine sperm quality and predict fertility in humans and animals [5,6]. However, motility shows a high degree of biological variability and in some cases are found to be fair measures of fertility. For example, variation in sperm motility from 60 to 90% had very low correlation to pregnancy rates in swine [7]. In cattle and sheep, a wide variation (about 20%) in post-thaw motility percentage was not correlated to the fertility [8]. Therefore, it is important to use more efficient tests for better evaluation.

Several structural and ultrastructural sperm components have been reported to be affected by cryopreservation, however their relative importance for routine semen evaluation is not clear. For example, acrosome and plasma membrane integrity are critical for the process of acrosome reaction (AR) and their damage may cause premature AR leading to reduced fertilizing capacity [9]. Mitochondria provide ATP which determines sperm motility [10] and are also vulnerable to cryo-damage. In addition, DNA damage has been recognized as an important indicator of sperm quality and has a great clinical significance in assessment of sperm selection in human [11]. However, in cattle, the effect of cryopreservation on sperm chromatin integrity has not been extensively examined. Assessment of sperm DNA damage is widely determined using Sperm Chromatin Structure Assay (SCSA) which require assessment by flow cytometry and therefore not routinely used [12]. Instead, Toluidine Blue (TB) test is a less expensive staining that is used to estimate chromatin integrity which has been shown to be correlated to SCSA results [13], and therefore may be a good evaluation tool for post thawing sperm quality.

During standard cryopreservation procedure, different processing steps are involved, namely, dilution of semen at 37°C with Tris-based diluents containing egg yolk and glycerol, followed by cooling to 4°C and equilibration. Rapid freezing is then performed to avoid crystallization. It is also not clear how each processing step contributes to the overall damage observed post-thawing. Understanding when the damage is induced can help in improvement of the most critical steps in the future.

Therefore, this study aims to 1) screen several structural and ultrastructural assessment tools to evaluate sperm quality during and after cryopreservation (plasma membrane and acrosome integrity, mitochondrial structure, chromatin damage, in addition to motility, viability and morphological abnormalities) 2) define which tests are more valuable for routine use, and 3) identify the damage caused by each processing step during cryopreservation (before dilution, after dilution, 2 h equilibration, 4 h equilibration and after thawing).

2. Materials and methods

All chemicals were purchased from (Sigma Pharmaceuticals, UK) unless otherwise stated.

2.1. Collection and selection of semen samples

Ejaculates were collected from five healthy, fertile Friesian bulls, 4–8 years old, raised at the international livestock management training center, Skha, Kafr El-Sheikh, Egypt. Semen was collected twice a week for 5 weeks. The bulls were kept under standard conditions of feeding and management. Semen was collected using an artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany) pre-warmed to 42°C. The percentage of progressive motility for each sample was determined subjectively by two experienced researchers using a phase contrast microscope with 200× magnification. Ejaculates with ≥70% motility were selected for cryopreservation experiments.

2.2. Cryopreservation procedures

Semen was cryopreserved using standard production procedures in our AI centers according to Chen *et al.* [14] with some modifications. Briefly, semen was gradually diluted at 37°C with Tris-yolk fructose (TYF) extender containing 30.28 mg/mL tris aminomethane, 16.75 mg/mL citric acid anhydrous, 12.5 mg/mL fructose, 7% (v/v) glycerol, 20% (v/v) egg yolk, 100 IU/mL penicillin and 100 μg/mL streptomycin. The extension rate was 1 semen: 20 extender to bring the sperm concentration to 80×10^6 sperm/mL. Diluted semen samples were kept at 4°C in a cooling chamber for 4 h as an equilibration period then automatically filled in 0.25 mL French straws (IVM technologies, L' Aigle, France), placed 4 cm above liquid nitrogen for 10 min then frozen in liquid nitrogen (−196°C) as described by Salisbury *et al.* [15]. Samples were evaluated before dilution, just after dilution (at 37°C), at 2 h and 4 h during equilibration, and after thawing (37°C for 30 s in water bath).

2.3. Assessment of sperm progressive motility

Percentage of progressive sperm motility in each semen sample (10 μL) was determined using phase contrast microscope (Olympus, Tokyo, Japan) supplied with a warm stage adjusted to 37°C.

2.4. Assessment of sperm viability and abnormalities

A smear from diluted semen was made on a glass slide and was stained by eosin (1.67%) and nigrosin (10%) stain [16]. A total of 300 sperm were examined in each sample at 400× under light microscope (Olympus). The number of dead spermatozoa (red stained) were counted. The number of sperm cells bearing head and tail morphological abnormalities were also recorded as previously described [17].

2.5. Scanning electron microscope evaluation of semen samples

To assess the structural damage induced by each step of cryopreservation, sperm samples were examined by a scanning electron microscopy. Samples (3 replicates) were centrifuged at 500×*g* for 20 min, and the sperm pellets were collected. Samples were fixed in a solution containing 2.5% (w/v) buffered glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (Sorensen buffer) pH 7.4 at 4°C overnight [18]. Fixed sperm pellets were then washed three times for 15 min each in 0.1 M sodium phosphate and treated in 2% sodium phosphate buffered osmium tetroxide pH 7.4 for 90 min. Pellets were finally washed in 0.1 M sodium phosphate buffer pH 7.4 and dehydrated in an increasing gradient of ethanol. Three drops of 100% acetone were added to the pellets on small glass plates before gluing them onto the specimen stubs of the microscope. The specimens obtained after the acetone had evaporated were coated with gold-palladium membranes and observed using a scanning electron microscope (Jeol JSM-6510 L.V). The microscope was operated at 30 kV. Only the central areas of the glass plates were examined (100 sperm per sample per replicate). The occurrence of detached and cracked heads and damage in the tail region was examined.

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