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Original article

The effect of cholesterol loaded cyclodextrins on post-thawing quality of buffalo semen in relation to sperm DNA damage and ultrastructure

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ABSTRACT

The cryopreservation of germ cells is a major tool for the propagation of animals with desired genetic traits. Although cryopreservation of spermatozoa in some animals is effective, its effectiveness is variable. For example, cryopreservation efficiency of buffalo bull spermatozoa remains very poor. In this study, we evaluated sperm DNA damage and ultrastructure in buffalo bull spermatozoa vitrified in the presence or absence of cholesterol-loaded cyclodextrins (CLC). Our results showed that cryopreserved buffalo spermatozoa had elevated levels of deteriorated plasma and mitochondrial membranes, which are the likely causes of DNA damage after vitrification. Accordingly, the levels of the activity of Alanine Aminotransferase (ALT), Alkaline phosphatase (ALP) and Aspartate Aminotransferase (AST) were also elevated following exposure of buffalo bull spermatozoa to a cycle of freezing-thawing. Importantly, supplementation of Tris-Egg Yolk-Glucose (TEYG) extender with (CLC) improved the quality of buffalo spermatozoa following cryopreservation. This protective effect of CLC is likely due to decreasing mitochondrial and plasma membrane deterioration with subsequent inhibition of DNA damage. These results suggest that cholesterol loss is the likely reason for poor semen quality in buffaloes following cryopreservation, and provide evidence that manipulating lipid content during cryopreservation is a promising strategy to improve the quality of buffalo semen.

1. Introduction

The population of buffaloes is increasing gradually in South Asia and the Mediterranean regions [1]. This increase reflects the growing demand for buffalo products as well as their substantial contribution to the economy of animal production. Despite the importance of water buffaloes (*Bubalus bubalis*), they have not received sufficient attention regarding the improvement of their reproductive performance and genetic quality [2].

Development of different assisted reproductive technologies (ARTs) can improve the genetic quality of the livestock species. Artificial insemination (AI) is the most widely used technique for rapid genetic improvement through propagation of desired genes from animals with high genetic qualities [3]. The benefits of the AI technique can be fully achieved by successful freezing and subsequent thawing of semen without compromising sperm quality and diminishing its ability to fertilize eggs [4]. Unfortunately, the fertility rate with frozen-then-thawed buffalo semen under field conditions is poor, the consequence being that only a small percentage of buffaloes are bred using AI techniques [5]. Cryopreservation of mammalian spermatozoa is a complex process influenced by several inter-related factors. Consequently, many strategies have been employed to improve the efficiency of cryopreservation process [6]. However, the overall success to improve the efficiency of the cryopreservation of buffalo bull semen remains low. Therefore, understanding the molecular mechanism by which freezing-followed by thawing compromises the quality of buffalo semen is a prerequisite to improve its efficiency.

The freeze-thaw cycle compromises the fertility of buffalo bull semen by perturbing sperm motility and viability likely through

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promoting plasma membrane deterioration and DNA apoptosis [4,7–10]. Deterioration of the sperm plasma membrane is one of the first responses to cryodamage [11]. Therefore, plasma membrane composition is an important determinant for sperm freezability. In particular, the levels of cholesterol and cholesterol/phospholipid ratio are related to the susceptibility of the sperm membranes to cryodamage [12,13]. Interestingly, sperm cells lose about 14% of its cholesterol content after cryopreservation [14–17]. Therefore, manipulating the lipid profile of sperm plasma membrane improves the cryosurvival of buffalo spermatozoa [18]. However, the underlying mechanism of cholesterol-induced cryotolerance of buffalo spermatozoa remains largely unknown.

Apoptosis is a reversible process initiated by different mechanisms including TNF, Fas, caspase 3 and caspase-independent pathways [19–23]. During vitrification, phosphatidylserine is translocated to the outer leaflet of the plasma membrane [24]. This translocation is likely caused by the disruption of membrane phospholipids asymmetry, which is the key step in apoptosis [25,26]. Interestingly, a membrane-nanodomain-enriched 1-stearoy-2-oleoyl phosphatidylserine can bind cholesterol [27], an indispensible component of the plasma membrane that maintains the membrane structural integrity [28]. Although cholesterol supplementation improved the quality of spermatozoa in different species [13,29–31], the effect of cholesterol supplementation on cryopreservation-induced DNA damage in mammalian spermatozoa remains unclear.

The present study aimed to investigate the effect of supplementation of semen extenders with CLC on the post-thawing quality of buffalo semen in relation to sperm ultrastructure and DNA damage.

2. Materials and methods

2.1. Experimental animals

This study was carried out on 6 buffalo-bulls (Egyptian water buffalo) of 4–6 years old (400–600 kg weight) belonging to the farm of Animal Reproduction Research Institute (ARRI), Giza, Egypt. The animals were maintained under optimum nutritional and managemental practices as per the standard criteria fixed for maintenance of breeding bulls in Egyptian bull stations.

2.2. Semen collection, processing and preservation

Two successive ejaculates, twice a week, were collected from each bull using the Artificial Vagina (AV) method as described by Abd El-Malak [32]. Immediately after semen collection, each ejaculate was evaluated [33]. Only semen samples of at least 70% individual motility and 800.00×10^6 sperm cells/ml were pooled together for semen processing and preservation. Out of 288 ejaculates, 265 ejaculates were used for further processing and analysis.

Pooled semen samples were divided into 4 equal portions and diluted with Tris-Egg Yolk-Glucose (TEYG) extender only (control) or TEYG supplemented with 1, 5 or 10 mM of cholesterol-loaded cyclodextrins (CLC). Methyl-β-cyclodextrin (MβCD) was pre-loaded with cholesterol as per the method described by Purdy and Graham [29]. In brief, a 0.45 ml aliquot of cholesterol dissolved in chloroform (200 mg cholesterol per 1 ml chloroform) was added to 2 ml of methanol containing 1 g of methyl-β-cyclodextrin. After mixing, the solution was poured into a glass Petri dish. After drying, the crystals were removed from the Petri dish and stored at 22 °C until use. A working solution of CLC was prepared by adding 50 mg of CLC to 1 ml of Tris diluent. The diluted semen was then examined for sperm motility followed by cryopreservation in liquid nitrogen (LN) as described previously [34].

Semen straws were stored in a LN tank for at least 24 h before evaluation. Randomly selected 3 frozen straws from each treatment were removed and thawed in a water bath at 37 °C for 30 s. The frozen-thawed semen was then evaluated.

2.3. Semen quality assessments

2.3.1. Microscopy evaluation

Post-thawed samples were classically assessed for post-thaw motility (PTM), viability index and acrosomal integrity. Immediately after thawing of frozen semen, individual motility and acrosomal integrity were examined. To evaluate post-thawing motility, a diluted semen sample using sodium citrate dihydrate 2.9% solution was examined on a hot stage (37 °C) phase contrast microscope and examined at high power (40×). Individual motility was recorded as the percentage of the anterior forward progressive motility of spermatozoa [35]. For detection of sperm acrosomal integrity, sperm cells were stained with silver nitrate stain and the percentages of acrosomal abnormalities were recorded as described [36]. Frozen-thawed semen was then incubated at 37 °C for 3 h and individual motility percentages were regularly recorded at interval of 1 h to calculate the viability index (V. I). The viability index was calculated using the following formula: V. I = [PTM (0 h) ÷ 2] + PTM (1 h) + PTM (2 h) + PTM (3 h) [37].

2.3.2. Biochemical evaluation

Fresh and frozen-thawed semen were centrifuged at $1000 \times g$ for 10 min to separate the seminal plasma from spermatozoa. The supernatant was collected and then centrifuged at $3000 \times g$ for 15 min at 4 °C followed by freezing at −20 °C until evaluation. The activity of Alkaline Phosphatase (ALP; K412-500, BioVision, Milpitas, California, USA), Alanine Aminotransferase (ALT; K752-100, BioVision), Aspartate Aminotransferase (AST; K753-100, BioVision) was measured in the supernatants using the colorimetric method, according to the manufacturers' instructions. To measure the level of lipid peroxidation, Malondialdehyde (MDA) in the sample was reacted with Thiobarbituric Acid (TBA) to generate the MDA-TBA product. The MDA-TBA was then quantified colorimetrically following manufacturer's instructions (K739-100, BioVision). Total Antioxidant Capacity, which includes either the combination of both small molecule antioxidants and proteins or small molecules alone, was colorimetrically measured (TAC; K274-100, BioVision) [38]. Glutathione Reductase (GSH) content was also measured as described previously [39].

2.3.3. Hypo-osmotic swelling test

The hypo-osmotic swelling test was conducted as an in-vitro assay to evaluate the effect of CLC supplementation on the functional integrity of frozen-thawed spermatozoa [40]. The percentage of spermatozoa with tail swelling was determined using a phase contrast microscope. A minimum of 300 sperm were counted twice across five slides. The proportion of spermatozoa showing a positive response of hypo-osmotic swelling was presented as a percent [40].

2.3.4. Alkaline comet assay

Single-cell gel electrophoresis assay was carried out as previously described [41]. In brief, frosted slides were covered with 100 μl of 0.5% low melting point agarose followed by solidification in a chilled metal tray for 30 min. Then, 1×10^5 spermatozoa in 10 μl of Biggers- Whitten- Whittingham (BWW) medium were mixed with 75 μl of 0.5% low melting point agarose at 37 °C and mounted on top of the first agarose layer prior to solidification at room temperature. Sperm cells were exposed to lysis solution including 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, with 1% Triton X-100 for 1 h at 4 °C. Slides were then further