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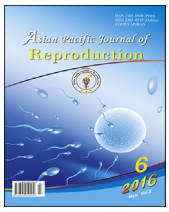

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The effect of freeze-drying media and storage temperature on ultrastructure and DNA of freeze-dried buffalo bull spermatozoa

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ABSTRACT

Objective: The present study intended for displaying the effect of different freeze-drying media and temperature of storage on the ultrastructure and DNA of freeze-dried buffalo bull spermatozoa. The semen samples undergone freeze-drying were raw semen and frozen-thawed semen extended in Tris-Fructose-Egg yolk-Glycerol.

Methods: Semen samples were processed in two portions: First portion was cryopreserved with Tris-Fructose-Egg yolk-Glycerol extender to be freeze-dried with the different media used in this study. Second portion was freeze-dried immediately with the different media used in this study. Semen samples were centrifuged in a percoll gradient (45–90%) for 20 min at 700 × g to remove seminal plasma. Subsequently, sperms were washed twice in Tyrode's albumen lactate pyruvate (TALP) to remove percoll remains, and allocated into the four freeze-drying media (media 1, 2, 3 and 4) respectively. The media tested were: medium 1 (EGTA solution), medium 2 (EDTA solution), medium 3 (TCM199 with Hanks salts and 10% FCS) and medium 4 (TCM199 with Hanks salts and 10% FCS and trehalose). For all the media used, samples were diluted, placed in tubes of 1.5 ml and kept at room temperature for 30 min. Then sperm cell suspensions were cooled in liquid nitrogen vapor (approximately −80 °C for 1 h), by keeping the tubes at a distance of 5 cm from liquid nitrogen surface before plunged into it. Frozen samples were immediately inserted into the freeze-drying machine, previously stabilized at (−40 °C) and 350 × 10^{-3} Mbar pressure. After 12–16 h of freeze-drying, the tubes containing the samples were covered with aluminum foil and stored for 3 months at different temperatures; 4 °C, −20 °C and −80 °C. Freeze-dried sperm samples were re-hydrated by adding 100 μL of milli-Q water at room temperature. To evaluate sperm ultrastructure, transmission electron microscopy was done. For detection of DNA fragmentation, commet assay was performed.

Results: Electron microscopy showed that the sperm cell component most affected by freeze-drying was the plasma membrane, which was destroyed in all media either in raw or frozen thawed sperm. Microtubules organization was also disorganized in the majority of the sperm from freeze-drying medium 2 and 3, diverging from freeze-drying media 1 and 4, in which microtubules were intact. Conversely, the acrosome and mitochondria were well protected in all media. However, the storage temperature has no effect. The freeze-drying medium with EDTA solution exhibited and the lowest percent of DNA damage (9.3) while the freeze-drying medium (TCM with trehalose) exhibited the highest percent of DNA damage (13.6) at temperature of storage (4 °C). In contrast, the freeze-drying medium with EGTA solution exhibited the lowest percent of DNA damage (15.3) while the freeze-drying medium (TCM with trehalose) exhibited the highest percent of DNA damage (18) at temperature of storage (−20 °C). On the other hand, the freeze-drying medium with EDTA solution exhibited the lowest percent of DNA damage

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(11.3) while the freeze-drying medium (TCM with trehalose) exhibited the highest percent of DNA damage (20) at temperature of storage (−80 °C). Consequently, the freeze-drying medium (TCM without trehalose) exhibited the lowest percent of DNA damage (6.5) while the freeze-drying medium with EDTA solution exhibited the highest percent of DNA damage (13.9) at temperature of storage (4 °C). On the contrary, the freeze-drying medium with EGTA solution exhibited the lowest percent of DNA damage (13.2) while the freeze-drying medium (TCM without trehalose) exhibited the highest percent of DNA damage (20.8) at temperature of storage (−20 °C). Conversely, the freeze-drying medium with EDTA solution exhibited the lowest percent of DNA damage (6) while the freeze-drying medium (TCM with trehalose) exhibited the highest percent of DNA damage (12) at temperature of storage (−80 °C).

Conclusion: From the present study, we demonstrated that the freeze-drying medium containing EGTA and EDTA solution were more efficient in avoiding damage to components of buffalo bull sperm, especially the nuclei. Therefore, the medium used for freeze-drying process directly affected sperm nuclear integrity. Also, the storage temperature of freeze-dried sperm affects sperm nuclear integrity.

1. Introduction

Application of artificial insemination (AI) with frozen-thawed semen has been stated on a restricted level in buffalo due to poor preservability and fertility of buffalo bull spermatozoa. Also, more than 40%–50% of sperms are sensitive to damage during the freezing process, in addition to species diversity in susceptibility to cryopreservation methods. For the aforementioned information, the sperm DNA outputs as a result of freeze-drying process (lyophilization) may differ from one species to another. Our study on freeze-drying of buffalo bull spermatozoa supplied the available literature. Sperm freeze-drying is considered as an alternative technique to cryopreservation. Development of freeze-drying was aimed to preserve biologically active materials such as enzymes, pharmaceutical materials (*e.g.* antibiotics) and others [1,2]. Furthermore, it has been used to conserve cells, awing to its capability to hinder water via ice sublimation [3]. Nowadays, a great deal of research attention has paid to freeze-drying of sperm. Compare to ordinary cryopreservation, freeze-drying needs lower cost, no liquid nitrogen, little space for sperm storage, and it is a more reliable method of sperm shipping. Even though sperm freeze-drying in different species has been documented, there are scattered reports for buffalo bull sperm. The first trial to conserve sperm using dehydration was documented by Polge *et al.* [3] using fowl sperm; although sperm appeared motile after rehydration, their fertilizing capacity was not assessed. After then, trials to freeze-dry mammalian sperm exhibited unsatisfactory results [4,5]. The first recorded birth following AI with freeze-dried sperm was reported in rabbit [6]. There's obvious success in production of offspring with freeze-dried sperm following the application of intracytoplasmic sperm injection (ICSI) [7,8]. Freeze-drying provided new potentials for storage and transportation of freeze-dried sperm at room temperature or at 4 °C, with many benefits for preservation of spermatozoa from animals [9]. One of the important challenges with any preservation method is the degree of cellular damage. Regardless of the protocol applied, cryopreservation has a damaging effect on sperm, resulting in reduction of both motility and fertilizing capacity [10]. Therefore, in spite of apparent reduction in motility, cells still viable and characterized by normal nucleus and centrosome integrity which are essential for the success of ICSI [11]. Although freeze-drying was focused on proper preservation of structural and functional sperm characteristics, an intact sperm nucleus is a necessary part for success of embryo production [7,12]. Nuclei of sperm are highly stable and concentrated with DNA organization [13]; 6-time more compact and 40-time lower than somatic cells [14,15]. This DNA packing is important to protect the cell and reduce injuries caused by external factors before fertilization. DNA of sperm can injure during freeze-drying and particularly during storage when inadequate protection is given. It is established that DNA injures could be due to activation of endogenous nucleases, oxidative stresses and storage conditions which takes place after freeze-drying [16,17]. Many trials were carried out to protect sperm structures during cryopreservation via various protecting substances, albumin [7,18], EGTA [12,19], EDTA [20] and trehalose [21]. The main target of the current study is to investigate the effect of various freeze-drying media and different storage temperatures on the ultrastructural components and DNA of freeze-dried buffalo bull sperm.

2. Materials and methods

2.1. Semen collection and evaluation

Five mature bulls, kept at Animal Reproduction Research Institution, Agriculture Research Center, Ministry Agriculture, were implemented in this study. Semen was collected by using the artificial vagina once a week. Immediately after collection, semen was evaluated. Only semen samples with >80% motility and <10% morphologically abnormal sperm were used for this study.

2.2. Freeze-drying media

Medium 1: 10 mmol/L Tris–HCl buffered supplemented with 50 mmol/L of each of NaCl and EGTA [ethyleneglycol-bis (b-aminoethyl ether)–*N, N, N′ N′*-tetraacetic acid] and pH of final solution adjusted to 8.2.