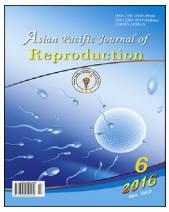
Contents lists available at ScienceDirect

Asian Pacific Journal of Reproduction

journal homepage: www.apjr.net



Original research http://dx.doi.org/10.1016/j.apjr.2016.11.002

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

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ARTICLE INFO

Article history:
Received 27 Jun 2016
Received in revised form 3 Nov 2016
Accepted 4 Nov 2016
Available online 15 Nov 2016

Keywords:
Buffalo
Semen
Freeze-drying
Sperm preservation
Commet assay

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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Peer review under responsibility of Hainan Medical College.

(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.

Medium 2: 10 mmol/L Tris and 1 mmol/L EDTA and pH of final solution adjusted to 8.0.

Medium 3: TCM 199 with Hank's salts (Gibco Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% (v/v) FCS (Gibco Life Technologies Inc.).

Medium 4: TCM 199 with Hanks salts supplemented with 10% (v/v) fetal calf serum and 0.2 mol/L trehalose.

All the media were designed according to Martins *et al.* [22] except for medium 2 was designed according to Kaneko *et al.* [20].

2.3. Experimental design

Ejaculates collected weekly were pooled, and processed in two portions: First portion was cryopreserved with Tris-Fructose-Egg yolk-Glycerol extender as described by Foote [23] with a total concentration of 30 × 10^6 sperm/0.5 mL 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. According to Abdalla *et al.* [24] 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) [25] to remove percoll remains, and allocated into the four freeze-drying media (1, 2, 3 and 4 respectively).

2.4. Sperm freeze-drying

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 a programmable freeze-dryer stabilized at (−55 °C) and 0.001 mbar pressure. After 24 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.

2.5. Rehydration

Freeze-dried sperm samples were rehydrated by adding 100 µL of milli-Q water at room temperature.

2.6. Ultrastructural assessment

To evaluate sperm ultrastructure, transmission electron microscopy was done on samples from all freeze-drying media [22]. Sperms of each freeze drying media were fixed for 3 h at room temperature in a solution containing 2% glutaraldehyde, 2% paraformaldehyde, 5% sucrose and 5 mM, $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, the specimens were rinsed in buffer, and post-fixed (1 h) in 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Dehydration was carried out in acetone and embedding in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 1011 (JEOL, Tokyo, Japan) transmission electron microscope, operating at 80 kV. All electron microscopy reagents were purchased from Electron Microscopy Sciences (Ft. Washington, PA, USA).

2.7. Detection of DNA fragmentation by comet assay

According to Singh *et al.* [26], freeze-dried spermatozoa from all media (10 µL of 1 × 10^6 cells/mL suspension) were mixed with 1% (w/v) low-melting agarose gel (90 µL), added onto agarose-covered slides, treated with lysis solution for 3 h (including 10 mM dithiothreitol for 0.5 h and 4 mM lithium diiodosalicylate for 1.5 h in the latter two-thirds) and then processed with electrophoresis under a pH > 13 alkaline condition (10 V, 20 min). Half of the sperm suspension was treated with 10 mM H_2O_2 for 20 min at 4 °C before being mixed with low melting agarose gel. Sperm DNA were stained with SYBR Green, and the captured BMP images of the comet by fluorescence microscope (E600; Nikon, Tokyo, Japan) were analyzed by the Comet Score software (>100 comets per sample). The DNA fragmentation index (tail moment) was calculated as the length of comet tail (pixel) × the percent DNA librated.

3. Results

Regardless to semen status and storage temperature, results of transmission electron microscopy showed that in the sperm cell components, the most affected part by freeze-drying was the plasma membrane (Figures 1–8A–H). Microtubules organization

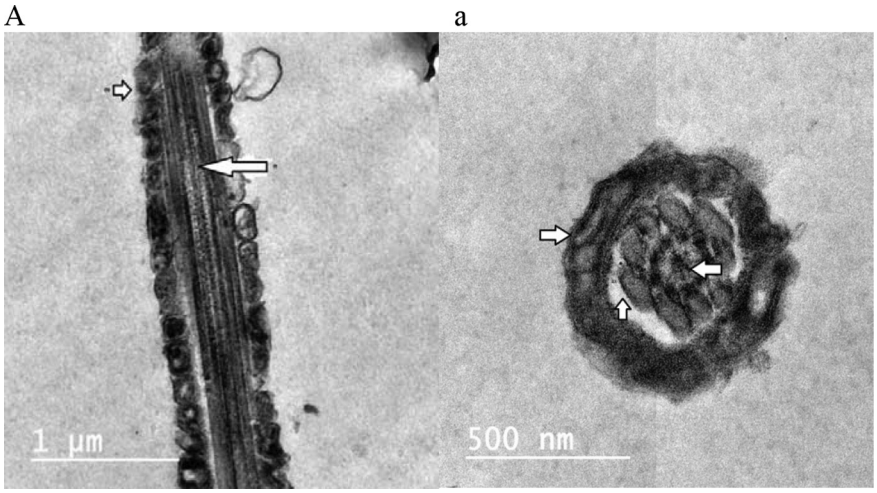

Figure 1. Electron micrographs showing raw buffalo bull spermatozoa after freeze-drying in medium 1.
(A) Sperm with injured plasma membrane (arrowhead) and intact acrosome (a) and nucleus (n); (a) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax).

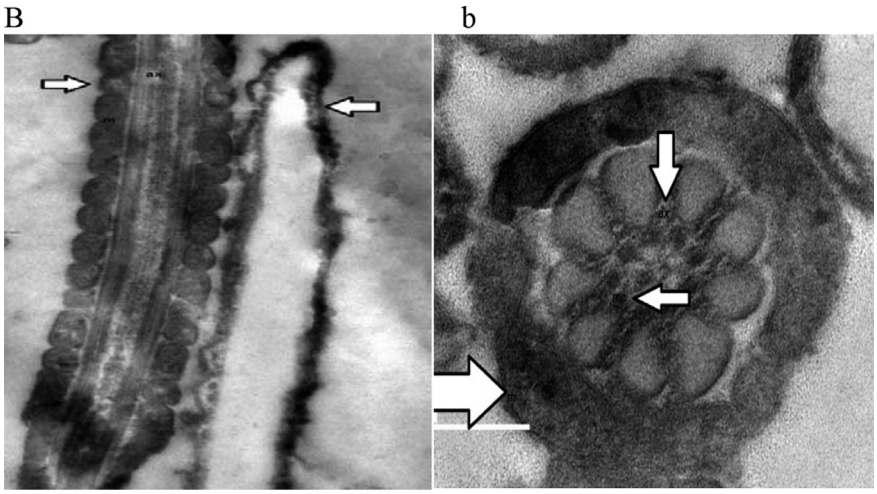

Figure 2. Electron micrographs showing raw buffalo bull spermatozoa after freeze-drying in medium 2.
(B) Sperm with injured plasma membrane (pm, arrowhead), existence of intact mitochondria (m), coarse fibers (cf) and axoneme (ax); (b) sperm with disordered microtubules (mc).

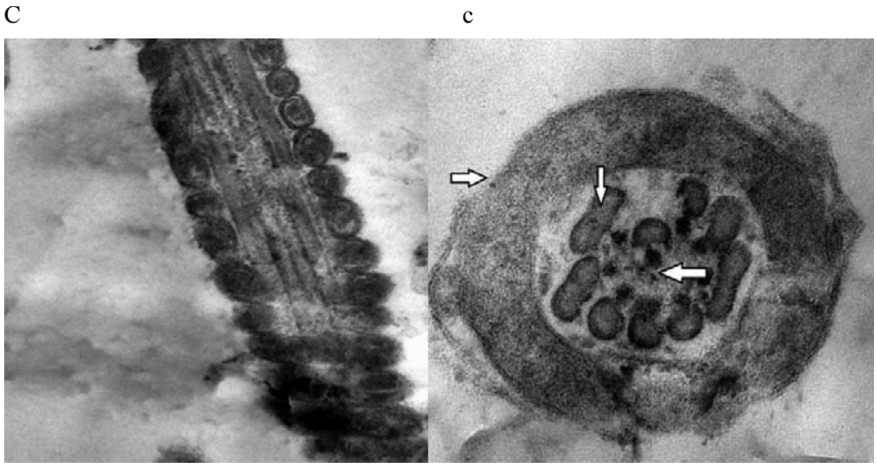

Figure 3. Electron micrographs showing raw buffalo bull spermatozoa after freeze-drying in medium 3.
(C) Sperm with injured plasma membrane (arrowhead) and integrated acrosome (a) and nucleus (n); (c) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and damaged axoneme (ax); disordered microtubules (mc).

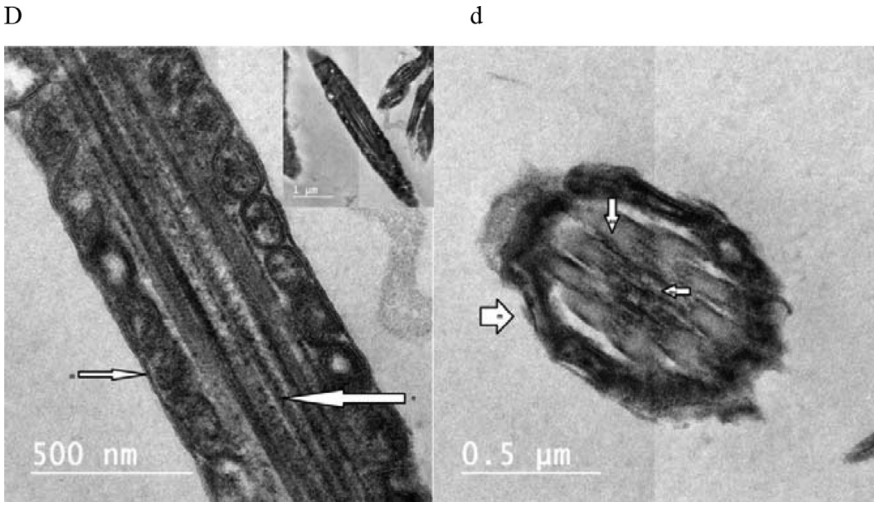

Figure 4. Electron micrographs showing raw buffalo bull spermatozoa after freeze-drying in medium 4.
(D) Sperm with injured plasma membrane (arrowhead) and integrated acrosome (a) and nucleus (n); (d) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax).

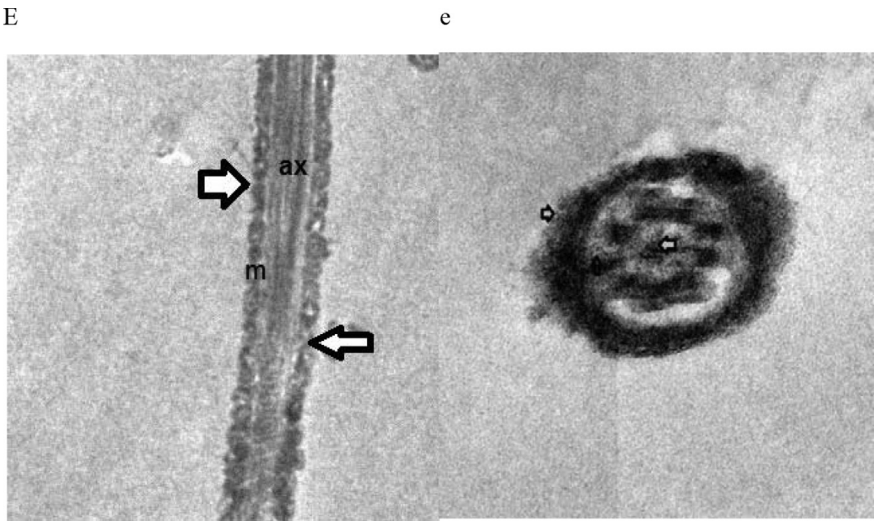

Figure 5. Electron micrographs showing frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 1.
(E) Sperm with injured plasma membrane (arrowhead) and intact axoneme (ax) and nucleus (n); (e) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax).

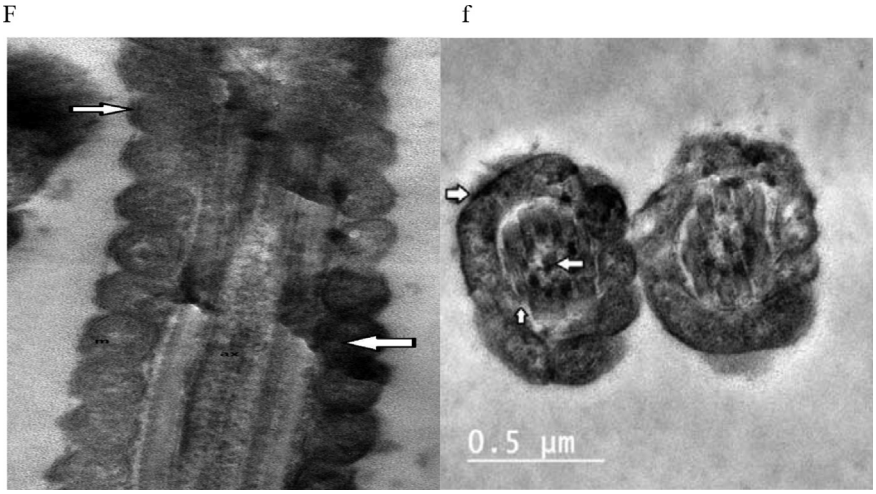

Figure 6. Electron micrographs showing frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 2.
(F) Sperm with injured plasma membrane (arrowhead) and integrated axoneme (ax) and nucleus (n); (f) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax), and disordered microtubules (mc).

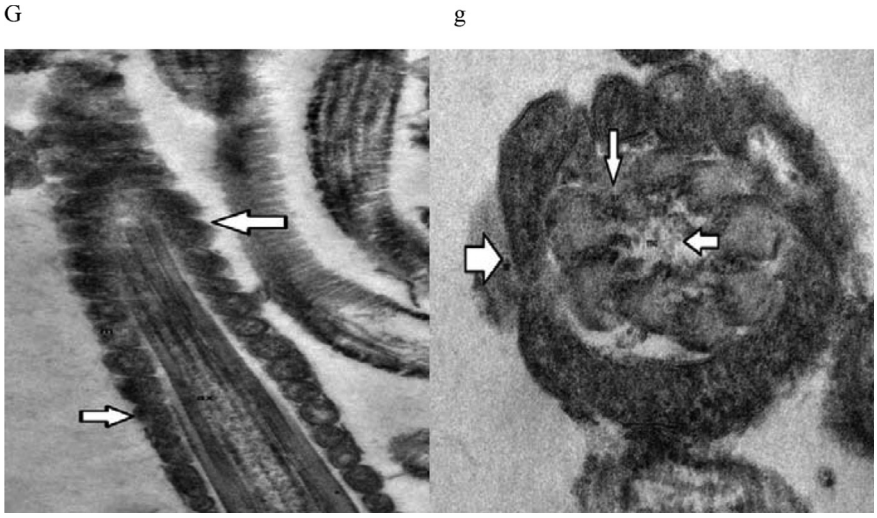

Figure 7. Electron micrographs showing frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 3.
(G) Sperm with injured plasma membrane (arrowhead) and integrated axoneme (ax) and nucleus (n); (g) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax), and disordered microtubules (mc).

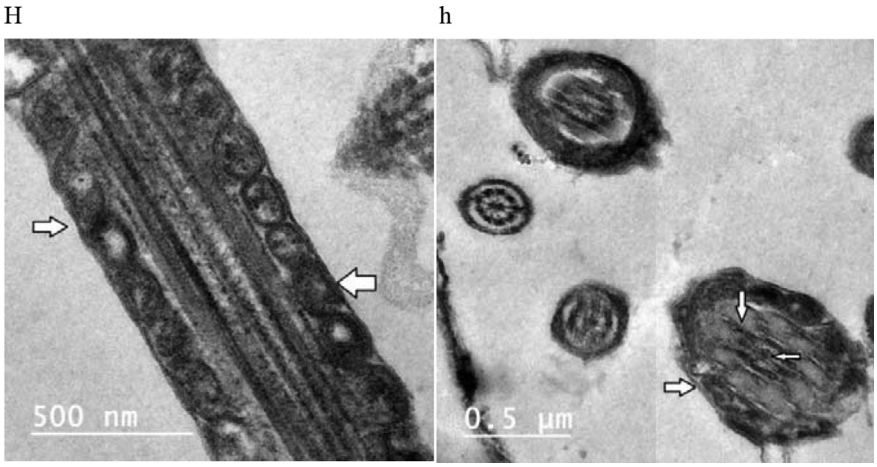

Figure 8. Electron micrographs showing frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 4.
(H) Sperm with injured plasma membrane (arrowhead) and intact axoneme (ax) and nucleus (n); (h) sperm with injured plasma membrane (arrowhead), existence of integrated mitochondria (m) and axoneme (ax).

was also disordered in the majority of sperms freeze-dried in medium 2 and 3 (Figures 2b, 3c, 6f, 7g), diverging from freeze-drying media 1 and 4 (Figures 1a, 4d, 5e, 8h), in which microtubules were intact. Conversely, the acrosome and mitochondria were well protected in all media.

The present results of (Table 1) showed that the freeze-drying medium (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) (see Figures 9 and 10,

Table 1
Comet results of raw buffalo bull spermatozoa after freeze-drying.

Storage temp	Media	% of damage	Head diameter	DNA% in head	Tail length	DNA% in tail	Tail moment
4 °C	1	10.40	23.31	84.42	6.46	15.58	1.19
	2	9.30	18.49	78.39	4.87	21.61	1.31
	3	10.90	16.02	81.34	4.90	18.66	1.03
	4	13.60	16.22	79.53	5.49	20.47	1.29
−20 °C	1	15.30	18.70	80.59	4.00	19.41	0.94
	2	17.90	15.32	76.16	3.97	23.84	1.22
	3	15.70	18.09	80.25	5.30	19.75	1.23
	4	18.00	18.46	77.06	5.44	22.94	1.54
−80 °C	1	14.80	21.09	79.70	6.01	20.30	1.37
	2	11.30	24.22	78.04	7.26	21.96	1.76
	3	13.40	23.12	78.74	7.73	21.26	1.81
	4	20.00	21.28	75.21	5.53	24.79	1.55

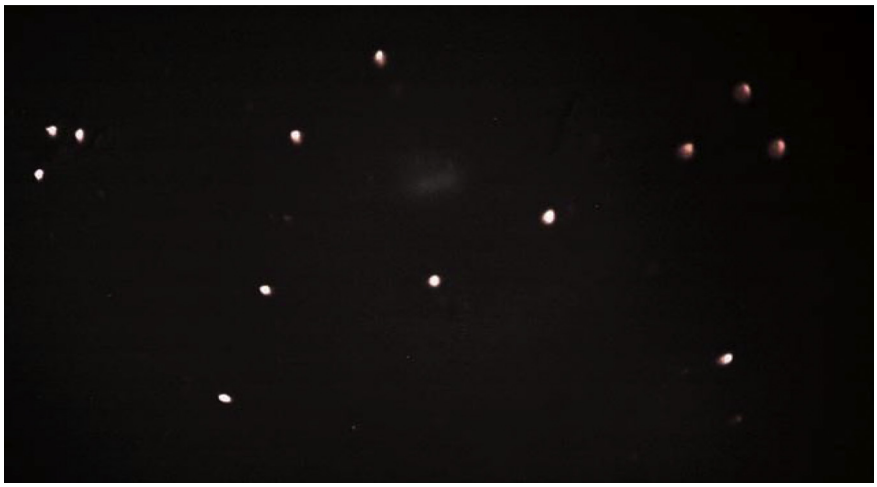

Figure 9. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 2 and storage at 4 °C. The presence of comet tails indicates fragmented DNA in these sperms.

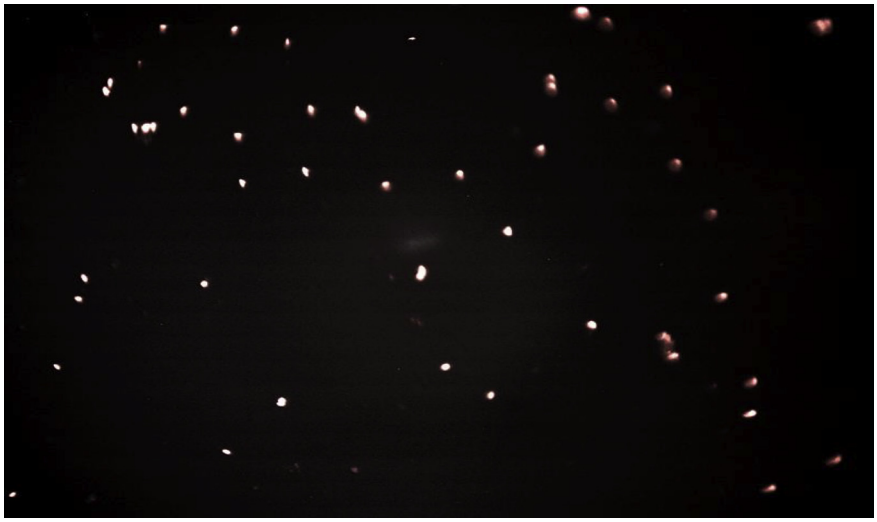

Figure 10. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 4 and storage at 4 °C. The presence of comet tails indicates fragmented DNA in these sperms.

respectively). In contrast, the freeze-drying medium (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) (see Figures 11 and 12, respectively). On the other hand, the freeze-drying medium (EDTA solution) exhibited the lowest percent of DNA damage (11.3), while the freeze-drying medium (TCM with trehalose) exhibited the highest

Figure 11. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 1 and storage at −20 °C. The presence of comet tails indicates fragmented DNA in these sperms.

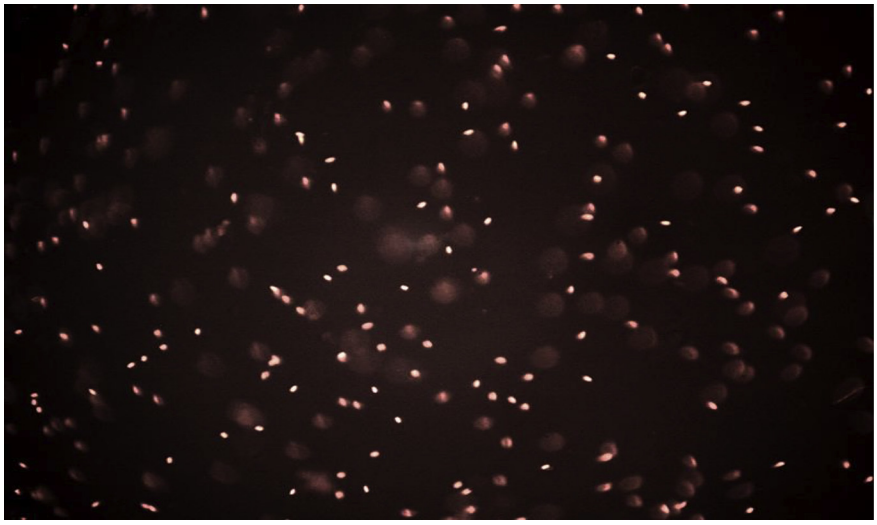

Figure 12. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 4 and storage at −20 °C. The presence of comet tails indicates fragmented DNA in these sperms.

percent of DNA damage (20%) at temperature of storage (−80 °C) (see Figures 13 and 14, respectively).

Consequently, the present results of (Table 2) revealed that the freeze-drying medium (TCM without trehalose) exhibited the lowest percent of DNA damage (6.5%) while the freeze-drying medium (EDTA solution) exhibited the highest percent of DNA damage (13.9%) at temperature of storage (4 °C) (see Figures 15 and 16, respectively). On the contrary, the freeze-drying medium (EGTA solution) exhibited the lowest percent of DNA damage (13.2%) while the freeze drying medium

Figure 13. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 2 and storage at −80 °C. The absence of comet tails indicates intact DNA in these sperms.

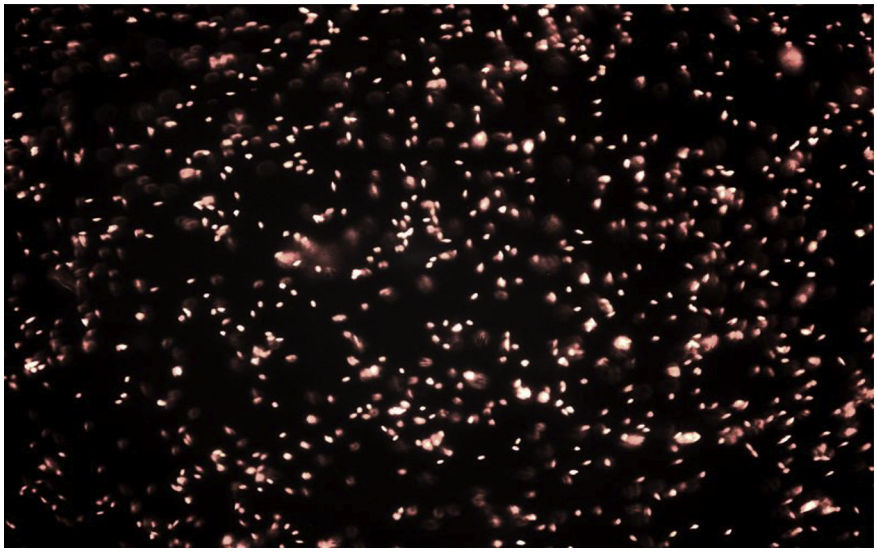

Figure 14. Comet assay of raw buffalo bull spermatozoa after freeze-drying in medium 4 and storage at −80 °C. The presence of comet tails indicates fragmented DNA in these sperms.

Table 2

Comet results of frozen-thawed buffalo bull spermatozoa after freeze-drying.

Storage temp	Media	% of damage	Head diameter	DNA% in head	Tail length	DNA% in tail	Tail moment
4 °C	1	11.00	24.92	83.06	5.15	16.94	0.93
	2	13.90	27.74	78.05	67.57	21.95	1.91
	3	6.50	22.29	80.25	4.84	19.75	1.10
	4	9.00	23.52	80.14	5.73	19.86	1.35
−20 °C	1	13.20	21.11	82.32	5.58	17.68	1.11
	2	14.20	32.67	83.04	7.67	16.96	1.33
	3	20.80	20.00	79.45	4.29	20.55	1.07
	4	14.60	18.97	80.90	4.10	19.10	0.85
−80 °C	1	7.80	25.85	74.52	6.52	25.47	1.87
	2	6.00	23.75	75.24	5.58	24.76	1.62
	3	9.60	21.72	83.73	4.25	16.27	0.77
	4	12.00	27.64	77.45	6.36	22.55	1.54

Medium 1: Tris–HCl buffer supplemented with NaCl and EGTA, Medium 2: Tris buffer supplemented with EDTA, Medium 3: TCM 199 with Hank's salts supplemented with 10% (v/v) FCS, Medium 4: TCM 199 with Hanks salts supplemented with 10% (v/v) FCS and Trehalose.

Figure 15. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 3 and storage at 4 °C. The absence of comet tails indicates intact DNA in these sperms.

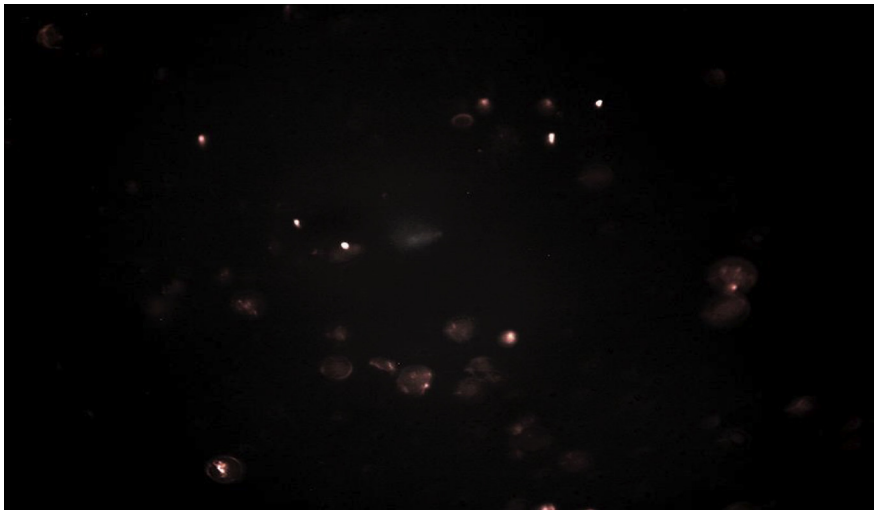

Figure 16. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 3 and storage at 4 °C. The presence of comet tails indicates fragmented DNA in these sperms.

(TCM without trehalose) exhibited the highest percent of DNA damage (20.8%) at temperature of storage (−20 °C) (see Figures 17 and 18, respectively). Conversely, the freeze-drying medium (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) (see Figures 19 and 20, respectively).

Figure 17. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 1 and storage at −20 °C. The absence of comet tails indicates intact DNA in these sperms.

Figure 18. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze drying in medium 3 and storage at −20 °C. The presence of comet tails indicates fragmented DNA in these sperms.

Figure 19. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 2 and storage at −80 °C. The presence of comet tails indicates fragmented DNA in these sperms.

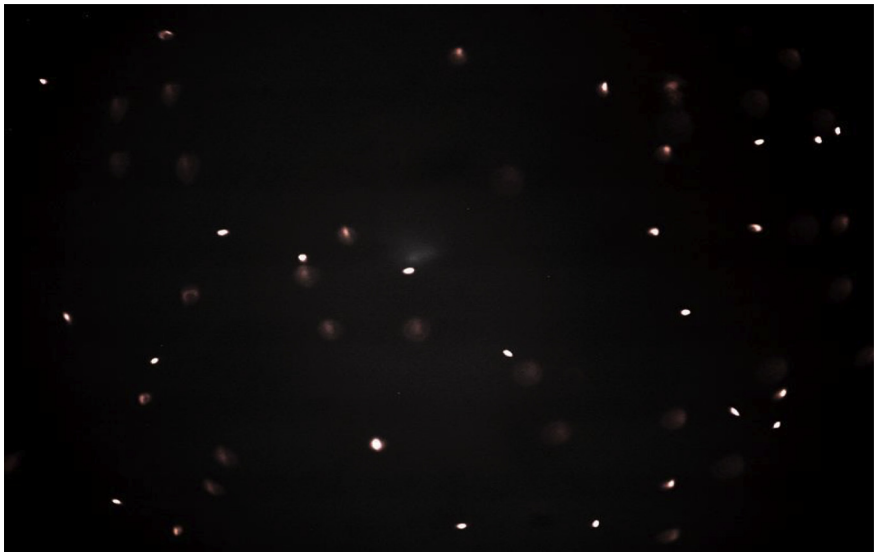

Figure 20. Comet assay of frozen-thawed buffalo bull spermatozoa after freeze-drying in medium 4 and storage at −80 °C. The presence of comet tails indicates fragmented DNA in these sperms.

4. Discussion

In the current study, we found out new data concerning on the degree that various freeze-drying media protected the structural and functional characteristics of the buffalo bull sperm. We investigated that EGTA solution sufficiently protected the nucleus, acrosome and mitochondria of buffalo bull sperm. This come in agreement with Olaciregui *et al.* [27] in stallion and Kusakabe *et al.* [12] in mice, who stated that freeze-drying medium with EGTA, better maintains chromosome integrity of spermatozoa during freeze-drying than does ordinary cell culture medium. In addition, we found that presence of EDTA exhibited the lowest percent of DNA damage in both raw at (4 and −80 °C) and frozen semen at (−80 °C). These findings are in accordance with Kaneko, *et al.* [20] who stated that integrity of chromosome could be maintained well by adding a small amount of EDTA into the solution during freeze-drying. Our results also, revealed that TCM without trehalose gave the lowest percent of DNA damage in frozen-thawed semen (4 °C). However, TCM with trehalose gave the highest percent of DNA damage in raw and frozen-thawed semen. It is well known that both EGTA and EDTA, chelate calcium (inhibits or reduces the activity of calcium-dependent endonucleases [12] through reducing the availability of the circulating calcium; therefore, EGTA is advised to minimize chromosome injuries [28]. The main objective of investigations on sperm freeze-drying is to conserve its motility and maintain fertilizing capacity, enabling the sperm to be used AI or *in vitro* fertilization. However, this objective has not been achieved in any species. In the present exploration, sperms were immotile after freeze-drying in all media. The sperm plasma membrane injuries were established by electron microscopy. In this respect, the plasma membrane is highly susceptible to injuries, due to loss of water during dehydration [4,6,12,21,29]. Water loss from phospholipid head groups in cell membranes could be due to lateral phase separation resulting in extravasation of intracellular contents [30]. Acrosome is the main second part of sperm which is highly affected by freeze-drying. In spite of >70% of the sperm had an intact acrosome (Figures 1–8A–H), in all media tested regardless to semen status or storage temperature. In this regard, Martins *et al.* [22] recorded that freeze-drying media conserved acrosome integrity. In spite of the slight differences among media used, in general we demonstrated a significant disorganization in microtubules with media containing EDTA and TCM without trehalose (Figures 2b, 3c, 6f, 7g), Media containing EGTA and trehalose had the lowest rate of tail separation. Martins *et al.* [22] in bovine and Hirabayashi *et al.* [31] in rats obtained similar results. These results are in disagreement with those reported in pigs [9], mice [7,12,19] and rabbits [8]. However, Men *et al.* [32] suggested that freeze-drying medium supplied with considerable amounts of trehalose could maintain DNA integrity but not fertilizing ability before ICSI. Maybe bovine sperm has better steadiness in the connection region than other species, which could give an explanation for the low rate of loss of tail after freeze-drying when compared to data from other species [22]. According to electron microscopy, in all media the mitochondria were conserved after freeze-drying. Therefore, Martins *et al.* [22] hypothesized that the medium with EDTA, due to its hypertonicity, could affect microtubule integrity. In conclusion, we demonstrated that the freeze-drying medium containing EGTA and EDTA solution were more sufficient in avoiding injuries to constituents of buffalo bull sperm, especially the nuclei. Also, the storage temperature of freeze-dried sperm affects sperm nuclear integrity.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are greatly indebted to the National Research Centre and Animal Reproduction Research Institute for sponsoring this work and also greatly indebted to RSTDG for their.

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