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The Effect of Freeze Drying Using Different Media and Storage Temperatures on Some Parameters of Buffalo Bull Spermatozoa

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THE PRESENT study was designed to display the effect of freeze-drying (lyophilization) technique using different freeze-drying media and storage temperatures on some parameters of buffalo bull spermatozoa. The semen samples were collected once weekly from five mature buffalo bulls maintained in Animal Reproduction Research Institute, Ministry of Agriculture, Al- Haram, Giza, Egypt. Samples were allocated into two portions; the first one was cryopreserved in Tris-Fructose-Egg yolk-Glycerol, while the second portion was immediately freeze-dried. These two portions were exposed to the same technical procedures. The freeze-dryer set was programmed at -55°C and 0.001 mbar pressure. The media tested were: EGTA solution, EDTA solution, TCM199 with Hanks salts enriched with 10% fetal calf serum (FCS) and TCM199 with Hanks salts enriched with 10% FCS and 0.2 M trehalose. The storage temperatures experienced were (4, - 20, - 80 ºC). The efficiency of each medium and storage temperature on some sperm parameters was explored. Our results revealed no motility either in raw or frozen-thawed spermatozoa after freeze-drying. In case of raw and frozen-thawed semen; with no respect to storage temperatures, the best freeze-dried sperm parameters were observed in TCM-Trehalose medium, while the best storage temperature was (-80°C) followed by (-20°C) with no respect to the type of freeze-drying media. From the present study, it was concluded that the freeze-drying medium containing trehalose could preserve efficiently components of buffalo bull spermatozoa, especially the acrosome, while the storage temperature (-80°C) and (-20°C) were the best for storage of freeze-dried buffalo bull spermatozoa.

Keywords: Buffalo, freeze-drying, EGTA, EDTA, Trehalose, acrosome.

The first trial to conserve sperm using dehydration was documented by Polge *et al.* (1949) using fowl sperm; although sperm appeared motile after rehydration, their fertilizing capacity was not assessed. Trials had been proceeded to freeze-dry mammalian sperm without any satisfactory results (Sherman, 1954 and Bialy &

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Smith, 1957). The first recorded birth following AI with freeze-dried sperm was reported in rabbit (Yushchenko, 1957). Development of freeze-drying aimed to preserve biologically active materials such as enzymes, pharmaceutical materials (e.g. antibiotics) and others (Keskintepe *et al*., 2002, Kusakabe *et al*., 2004). Furthermore, it has been used to conserve cells, owing to its capability to hinder water via ice sublimation (Polge *et al*., 1949). Although, more than 40-50% of sperms are sensitive to damage during the freezing process, freeze-drying could be considered as an alternative process for preservation of sperms. Species diversity in susceptibility to preservation methods must be taken in consideration during our judgment on novel methods used for preservation of buffalo bull spermatozoa. For that, the sperm evaluation parameters outputs as a result of freeze-drying process may differ from one species to another. Our study on freeze-drying of buffalo bull spermatozoa misses the available literatures. Nowadays, a great research attention has been paid to freeze-drying of sperm. As compared to ordinary cryopreservation methods, Freeze-drying offers low cost, no requirement to liquid nitrogen, little space needs for sperm storage and a reliable and low costly method of sperm transport (Gil *et al.,* 2014). There's obvious success in production of offspring with freeze-dried sperm following the application of ICSI in animals (Wakayama *et al.,* 1998, Liu *et al.,* 2004). Freeze-drying provided a new approach for storage and transportation of freeze-dried sperm at 4ºC or room temperature (25 ºC), with many benefits for preservation of spermatozoa from animals (Kwon *et al.,* 2004). 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 (Critser *et al.,* 1987). 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 (Choi *et al.,* 2011). 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 (Wakayama and Yanagimachi, 1998, Kusakabe *et al.,* 2001). Many trials were carried out to protect sperm structures during freeze-drying via various protecting substances, e.g. albumin (Wakayama and Yanagimachi, 1998, Wakayama *et al.,* 1998), EGTA (Kusakabe *et al.,* 2001 and Kaneko *et al.,* 2003), EDTA (Kaneko and Serikawa, 2012) and trehalose (McGinnis *et al.,* 2005). The main target of the current study was to investigate the effect of freeze-drying using different media and storage temperatures on some parameters of buffalo bull spermatozoa.

Material and Methods

Semen collection and evaluation

Five mature buffalo bulls, kept at Animal Reproduction Research Institute, Ministry of Agriculture, were implemented in this study. Semen was collected by using the artificial vagina once weekly (n= 3, within 24 replicate). Immediately after collection, semen was evaluated. Only semen samples with >80% motility and <10% morphologically abnormal sperm were used for this study.

Freeze-drying media

Medium1: 10 mmol/L Tris–HCl buffered supplemented with 50 mmol/L of each NaCl and EGTA [ethyleneglycol-bis (b-aminoethyl ether)-*N, N, N′ N′*-tetraacetic acid] with a final pH adjusted to 8.2.

Medium 2: 10 mmol/L Tris and 1 mmol/L EDTA with a final pH adjusted to 8.2.

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.).

Medium4: TCM 199 with Hank's salts supplemented with 10% (v/v) FCS and 0.2 mol/L trehalose.

All the media were designed according to Martins *et al.* (2007) except medium 2 was designed according to Kaneko and Serikawa (2012).

Experimental design

Ejaculates collected weekly were pooled, and processed in two portions: First one was cryopreserved with Tris-Fructose-Egg yolk-Glycerol extender as described by Foote (1970) with a total concentration of 30×10^6 sperm / 0.5 mL to be freeze-dried with the different media used in this study. The second one was freeze-dried immediately with the different media used in this study. According to Abdalla *et al*. (2009) 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) (Parrish *et al.*, 1995) to remove percoll remains and allocated into the four media 1- 4, respectively.

Sperm freeze-drying

Samples were diluted in all experimental media, placed in tubes of 1.5 ml and kept at room temperature for 30 min. Sperm cell suspensions were cooled in liquid nitrogen vapor (approximately –80ºC 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 hrs 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 .

Rehydration

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

Sperm assessment

Sperm motility

A drop of sperm suspension from each treatment was placed on a pre-warmed slide (37°C) and covered with cover slip. Individual progressive forward motile sperm subjectively was assessed by using phase contrast hot stage (37°C) microscope set at magnification of 400 × Visual motility was assessed microscopically with closed circuit television in 5-6 fields each slide.

Sperm morphology

Smears were stained with Eosin-Nigrosin (Campbell *et al.,* 1956) morphologically abnormal sperm percent. A total of 200 sperm cells will examined randomly.

Sperm membrane integrity

The hypo osmotic swelling test (HOST) was performed as described by (Jeyendran *et al.,* 1984). The swollen spermatozoa characterized by coiling of the tail are considered to have an intact plasma membrane.

Sperm viability and Percentage of normal acrosomes

The dual staining procedures with Trypan Blue Giemsa stain (TBG) was performed as described by (Kovács and Foote, 1992). Sperm with no blue staining and a purple acrosome were considered viable (Way *et al.,* 1995).

Statistical analysis

The obtained data were tabulated and computed for statistical analysis using the SAS computerized program v. 9.2 (SAS, 2008) to calculate the analysis of variance (ANOVA) for the different parameters. The obtained results, for the different semen parameters in different experiments, were expressed in mean ± standard errors of mean. Differences between means were compared with the LSD procedure at least at $P<0.05$.

TABLE 1. Effect of freeze-drying media and storage temperature on morphology (%) of raw buffalo spermatozoa after freeze-drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	24.67 ± 1.76	21.67 ± 2.90	23.33 ± 2.85	27.33 ± 2.90	24.17^{A}	0.0032
-20	18.67 ± 1.76	21.33 ± 2.60	19.33 ± 1.20	19.00 ± 3.21	19.58^{B}	
-80	28.00 ± 3.60	25.67 ± 3.18	28.00 ± 3.21	27.00 ± 3.46	27.58^{A}	
Overall mean	23.78^{a}	22.89^{a}	23.56^{a}	24.44^{a}		
Significance	0.9251					

Interaction significance LSD = 0.8372

Values within the same column with different letters differed significantly at least at $p < 0.05$

Results

Our results revealed no motility either in raw or frozen-thawed spermatozoa after freeze-drying. In case of raw semen; with no respect to storage temperatures, the best freeze-dried sperm parameters were observed in TCM-Trehalose medium as represented by sperm membrane integrity (Table 2) and acrosome status (Table 4) (64.56 and 66.22) respectively and with no respect to type of (-80°C) followed by (-20°C) as observed in sperm

membrane integrity (Table 2) (68.92 and 58.50) respectively and sperm viability (Table 3) (82.75 and 76.92) respectively. In case of frozen-thawed semen; with no respect to storage temperatures the best freeze-dried media was TCM-Trehalose medium as represented by sperm morphology (Table 5) and viability (Table 7) (21.33 and 74.78) respectively while the best storage temperature was (-80°C) followed by (-20°C) with no respect to the type of media as showed in sperm membrane integrity (Table 6) (62.17 and 56.83) respectively and acrosome status (Table 8) (66.17 and 56.33) respectively.

TABLE 2. Effect of freeze - drying media and storage temperature on membrane integrity (%) of raw buffalo spermatozoa after freeze – drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	56.67 ± 2.40	54.67 ± 2.02	51.33 ± 1.76	58.00 ± 2.64	55.17^{B}	0.0001
-20	53.67 ± 3. 48	62.33 ± 4.05	54.67 ± 2.33	63.33 ± 3.18	58.50^{B}	
-80	67.33 ± 3.75	69.67 ± 4.05	66.33 ± 4.05	72.33 ± 3.75	68.92^{A}	
Overall mean	59.22^{ab}	62.22^{a}	57.44^{b}	64.56^{a}		
Significance	0.0579					

Interaction significance LSD = 0.7802
Values within the same column with different letters differed significantly at least at P < 0.05.

TABLE 3. Effect of freeze-drying media and storage temperature on viability (%) of raw buffalo spermatozoa after freeze-drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	72.33 ± 2.96	73.67 ± 2.60	79.00 ± 4.04	77.00 ± 3.46	75.50^{B}	0.0025
-20	81.00 ± 2.08	71.00 ± 2.88	81.67 ± 2.60	73.67 ± 2.73	76.92^{B}	
-80	85.00 ± 0.00	85.00 ± 0.00	82.67 ± 2.33	78.33 ± 3.28	82.75^{A}	
Overall mean	79.44^{a}	76.55^{a}	81.11^{a}	76.44^{a}		
Significance	0.1332					

Interaction significance LSD = 0.0996
Values within the same column with different letters differed significantly at least at *P* < 0.05.

TABLE 4. Effect of freeze - drying media and storage temperature on acrosome integrity (%) of raw buffalo spermatozoa after freeze-drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	55.67± 5.04	59.33 ± 4.63	72.33 ± 2.02	59.33 ± 3.48	61.67^{A}	0.2176
-20	47.67 ± 4.41	66.67 ± 3.48	51.00 ± 2.64	68.00 ± 3.46	58.33^{A}	
-80	58.67 ± 5.78	67.00 ± 3.21	56.00 ± 3.78	71.33 ± 3.76	63.25^{A}	
Overall mean	54.00^{b}	64.33^{a}	59.78^{ab}	66.22^{a}		
Significance	0.0042					

Interaction significance LSD = 0.0060

Values within the same column with different letters differed significantly at least at $P < 0.05$.

TABLE 5. Effect of freeze drying media and storage temperature on morghology (%) of frozen-thawed buffalo spermatozoa after freeze-drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	36.33 ± 2.33	33.33 ± 4.41	26.00 ± 6.66	17.00 ± 2.08	28.17^{A}	0.0625
-20	24.00 ± 3.78	18.33 ± 2.33	18.66 ± 3.28	27.00 ± 3.78	22.00^{B}	
-80	24.66 ± 3.48	20.00 ± 2.08	30.66 ± 3.53	20.00 ± 2.64	23.83^{AB}	
Overall mea	28.33^{a+}	23.89^{ab}	25.11^{ab}	21.33^{b}		
Significance	0.1451					

Interaction significance LSD = 0.0111

Values within the same column with different letters differed significantly at least at $P < 0.05$

TABLE 6. Effect of freeze - drying media and storage temperature on membrane integrity (%) of frozen-thawed buffalo spermatozoa after freeze- drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	51.00 ± 3.21	46.66 ± 10.14	48.33 ± 8.82	49.33 ± 8.68	48.83^{B}	0.0119
-20	53.33 ± 5.21	58.33 ± 5.04	54.67 ± 3.38	61.00 ± 1.53	56.83^{AB}	
-80	62.67 ± 3.28	64.00 ± 3.46	61.67 ± 4.33	60.33 ± 5.36	62.17^{A}	
Overall mean	55.67^{a}	56.33^{a}	54.89^{a}	56.89^{a}		
Significance	0.9771					

Interaction significance LSD = 0.9615

Values within the same column with different letters differed significantly at least at $P < 0.05$.

TABLE 7. Effect of freeze- drying media and storage temperature on viability (%) of frozen- thawed buffalo spermatozoa after freeze- drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	54.33 ± 2.96	66.67 ± 6.67	63.33 ± 4.41	73.33 ± 4.41	64.42^{A}	
-20	58.67 ± 3.84	63.33 ± 5.81	65.67 ± 4.70	73.67 ± 2.73	65.33^{A}	
-80	72.33 ± 2.96	52.67 ± 8.19	72.00 ± 3.46	77.33 ± 2.33	68.58^{A}	0.4305
Overall mean	61.78^{b}	60.89^{b}	67.00^{ab}	74.78^{a}		
Significance	0.0049					

Interaction significance LSD = 0.0739

Values within the same column with different letters differed significantly at least at $P < 0.05$.

TABLE 8. Effect of freeze- drying media and storage temperature on acrosome integrity (%) of frozen- thawed buffalo spermatozoa thawed buffalo spermatozoa after freeze-drying.

Storage temperature (°C)	Type of media				Overall mean	Significance
	Tris-EGTA	Tris-EDTA	TCM	TCM-Trehalose		
+4	45.67 ± 2.73	65.33 ± 2. 96	54.00 ± 3.46	62.00 ± 3.21	56.75^{B}	
-20	64.00 ± 3.60	55.33 ± 5.61	49.67± 3.53	56.33 ± 3.84	56.33^{B}	0.0013
-80	65.67 ± 3.48	74.67 ± 3.76	76.00 ± 3.21	48.33 ± 4.63	66.17^{A}	
Overall mean	58.56^{b}	65.11^{a}	59.89^{ab}	55.56^{b}		
Significance				0.0331		

Interaction significance LSD = 0.0001

Values within the same column with different letters differed significantly at least at $P < 0.05$

Discussion

The sperm plasma membrane and acrosome integrities are highly susceptible to injuries, due to loss of water during dehydration (Sherman, 1954, Yushchenko, 1957, Kusakabe *et al.*, 2001, McGinnis *et al.*, 2005 and Kawase & Suzuki, 2011). Water loss from phospholipid head groups in cell membranes could be due to lateral phase separation resulting in extravasation of intracellular contents (Crowe *et al.*, 1997). In the current study, we found out new data concerning the degree of protection of structural and functional characteristics of the buffalo bull sperm using various freeze-drying media and storage temperatures. We investigated that medium containing trehalose sufficiently protected the membrane and acrosome of buffalo bull sperm. This come in agreement with Martins *et al.* (2007) in bovine, who stated that freeze-drying medium containing EGTA or trehalose adequately preserved bovine sperm viability during the freeze-drying process, though of the mechanism and function of these solutions in protecting the sperm

are not clear till now. Our results also are in accordance with Hirabayashi *et al.* (2005) in rats and Zăhan *et al*. (2014) in boar. In addition, we found that storage temperature (-80 and -20°C) were the best in preservation of acrosome and sperm membrane comparing to storage at (4°C). These findings are in accordance with Kawase and Suzuki (2011) who stated that freeze dried sperm stored at -80°C with or without transportation can maintain their ability to generate viable offsprings for long time. Our investigation on sperm freeze-drying revealed no motility. These results come in accordance with Martins *et al.*, 2007, Magalhães *et al.*, 2012, Hara *et al.*, 2014 and Zăhan *et al.*, 2014. In conclusion, we demonstrated that the freeze-drying medium containing trehalose and the storage temperature (-80°C) and (- 20°C) could preserve efficiently buffalo bull sperm vitality, especially the acrosome, sperm membrane integrity and morphology.

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تأثير التجميد المجفف للسائل المنوي لطلائق الجاموس على بعض خصائص الحيوان المنوي

محمد اسماعيل شهبه* ، **رضا ابراهيم الششتاوي*** ، **عبد السلام ابراهيم العزب**** ، **علاء السيد عبد الغفار**** ، **مها سليمان زيادة***** و **عادل أنيس زكي*****
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تم تجميع السائل المنوي من خمس طلائق تخص مزرعة معهد بحوث التناسليات الحيوانية بالهرم التابع لمركز البحوث الزراعية بوزارة الزراعة بواسطة المهبل الصناعي أسبوعيا وتم تقييم السائل المنوي مبدئيا إعتمادا على التركيز ، الحركة الفردية لتقدير معدل التخفيف. العينات التى احتوت على ٧٠٪ حركة فردية ، ٨٠٪ شكل طبيعي للحيوان المنوي تم استخدامها في الدراسة. تم خلط عينات السائل المنوي من الطلائق لتجنب الإختلافات الفردية والحصول على قدر كافي من السائل المنوي .ثم تم تقسيم السائل المنوي الى جزئين: الجزء الاول تم فيه تجميد السائل المنوي بإستخدام مخفف تريس حامض الستريك والفركتوز ثم بعد الإذابة تم فصل البلازما منه بإستخدام gradient percoll (٤٥-٩٠٪) . الجزء الثانى تم تجميده ثم تجفيفه مباشرة بإستخدام المخففات الأربعة المستخدمة في الدراسة.المخففات المستخدمة في التجميد المجفف(التجفيد) : EGTA-Tris(10 مل مول تريس هيدروكلورايد ، ٥٠ مل مول EGTA ، ٥٠ مل مول كلوريد الصوديوم مع ضبط الأس الهيدروجيني عند ٨.٠(و EDTA-Tris(١٠ مل مول تريس و ١ مل مول EDTA مع ضبط الأس الهيدروجيني عند ٨.٠(وTCM 199) مزودة بـ١٠ ٪ (حجم / حجم) fetal calf serum وTrehalose-TCM (TCM 199 مزودة بـ ١٠٪ (حجم / حجم) fetal calf serum و٢., مول/ لتر من التريهالوز). بعد التخفيف تم الحصول على تركيز ٥-٣×١٠^٦ حيوان منوي / مل من كل معاملة، ثم تمت التعبئة في انابيب 1,5 مل عند درجة حرارة الغرفة لمدة ٣٠ دقيقة ثم بعد ذلك تم تعريض الانابيب لبخار النيتروجين السائل من على بعد ٥ سم لمدة ساعة (تقريبا عند -80 درجة مئوية) وأخيرا تم غمس الانابيب في النيتروجين السائل(LN_2) عند (-١٩٦) بعمق ١ سم لمدة ٢٠ ثانية بمجرد التجميد تم وضع الانابيب في جهاز التجميد المجفف (التجفيد) المبرمج عند (-٥٥ درجة مئوية) و ضغط ١٠^-٣ مل بار، وبعد ٢٤ ساعة تم حفظ الانابيب في ورق الالومنيوم لمدة ٣ شهور عند٤ ، -٢٠ و-٨٠ درجة مئوية. تم إعادة اسالة العينات باضافة ١٠ ٠ ميكروليتر محلول متعادل التوتر في درجة حرارة الغرفة. تم تقييم السائل المنوي لكل مخفف ودرجة حفظ على حدة من خلال تقييم الحركة الفردية للسائل المنوي، نسبة الحيامن الغير طبيعية ، نسبة الحيامن الحية، سلامة جدار الحيامن، سلامة الاكروزوم. من النتائج يمكن استنتاج أن المخفف الـTrehalose-TCM عند درجة الحفظ-٨٠ ثم -٢٠ درجة مئوية كان الأفضل في حفظ الحيامن بعد التجفيد.