Original Article

Comparison of the effects of different thawing methods on post-thaw sperm characteristics of buffalo bull semen

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Abstract

Application proper procedures for thawing of cryopreserved sperm are required for successful artificial insemination. The study was aimed to compare the effects of watery and dry thawing systems on motility, acrosome integrity, plasma membrane integrity, morphologic and kinematic characteristics of buffalo bull spermatozoa. Anatolian Buffalo bull spermatozoa, cryopreserved in volumes of 0.25 mL straws, were thawed by using 1) watery thawing system; a total of 10 straws were thawed in a water bath at 37 °C for 30 sec and 2) dry thawing system; a total of 10 straws were thawed in a dry thawing device at 37 °C for 30 sec. There was no difference between in watery and dry thawing system for the percentages of hypo-osmotic swelling test of post-thaw buffalo spermatozoa (78.70±1.13 vs. 77.20±0.98%) and acrosome damages of spermatozoa (16.70±1.02 vs. 16.00±0.59). Total abnormal spermatozoa rate of dry thawing system was lower (*P*<0.05) than that of watery thawing system. There was no difference between thawing methods for all kinematic characteristics except for STR value (*P*<0.05) resulted in a decrease in dry thawing system. In conclusion, the current study showed that the use of dry thawing system could be used as an alternative to watery thawing system because most post-thaw buffalo bull sperm values in dry thawing were similar to those in watery thawing system. Furthermore, dry thawing system has some advantages compared to watery thawing system because of portable, practical, easily using in farms or barns and no water necessity.

Keywords: Buffalo, sperm, dry thawing, watery thawing

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Introduction

In many developing countries of the World, buffalo products (milk and meat) have major contributions to the rural economy (Warriach *et al.*, 2015; Devkota, 2018). Apart from milk and meat productions, buffaloes also provide draught powder (Warriach *et al.*, 2015). The World buffalo population is about 206 600 676 (FAO, 2019), and most population is located in Asia. Recently, Turkey's buffalo population is approximately 184 192 in 2019 (TUIK, 2019). Although buffaloes can utilize poor quality roughages and adapt to harsh environmental conditions, they have poor reproductive efficiency independently of their location Worldwide (Warriach *et al.*, 2015).

Artificial insemination is an important tool for assisted reproductive technology due to the improvement of the multiplication of buffaloes with high genetic potential (Baruselli and Carvalho, 2005). Freezing-thawing procedures of semen for artificial insemination can result in low post-thaw motility, viability, acrosome integrity (Kumar *et al.*, 2011). In artificial insemination, applying proper procedures for thawing of cryopreserved sperm is essential for maintaining optimum sperm parameters and fertilization capacity. Some researchers (Pace *et al.*, 1981; Dhami and Sahni, 1993; Sarıözkan *et al.*, 2009) have revealed significant correlations between post-thaw sperm characteristics and fertility of cryopreserved spermatozoa. It is well known that besides the many factors that affected the post-thaw semen characteristics, thawing procedure (time and temperature) is crucial for post-thaw semen parameters. In other words, thawing procedure is as important as freezing procedure in terms of its impact on the survival of spermatozoa (DeJarnette *et al.*, 2000).

Raizada *et al.*, (1990) and Andrabi *et al.*, (2008) reported that buffalo bull spermatozoa were more vulnerable than cattle bull spermatozoa to potential detrimental effects of freezing and thawing procedure on spermatozoa fertilization. Difficulty in freezability of buffalo bull semen compared to cattle bulls may be caused by the differences between lipid ratio of the spermatozoa of bulls (Jain and Anand, 1976; Tatham, 2000). Spermatozoa plasma membrane integrity is assessed through the hypo-osmotic swelling test (HOST) that has been recognized as a reliable procedure for the evaluation of the functional status of the sperm plasma membrane. This technique indicates both the functional and structural status of the plasma membrane and is highly related to fertility (Tartaglione and Ritta, 2004).

Rao *et al.* (1986) stated that the optimal thawing rate was 37 °C for the 30 sec for the best sperm quality and fertility rate after thawing. When access to warm water is not available, alternative thawing procedures such as air thawing, artificial insemination gun thawing, or thawing in the water at different temperatures have been performed (Rao *et al.*, 1986; Correa *et al.*, 1996). The semen should be rapidly thawed because of increasing sperm motility (Lyashenko and Bashchenko, 2014). Dry thawing system may be easily used in farms and barns because it is portable, water is not needed for its operation, and it works with 12-13.6 V. It keeps the temperature at 37 °C for about 10 min for thawing procedure. There are holes on its surface for both 0.25 and 0.50 mL straws, and also the catheter of artificial insemination.

The more number of motile spermatozoa providing an insemination dose means the more chances of spermatozoa reaching the oviduct for the selection of spermatozoa by the oviduct for fertilizing the oocyte. To the best of our knowledge the present study was the first study designed to compare the effects of watery and dry thawing systems at 37 °C for 30 sec on motility, acrosome integrity, plasma membrane integrity, morphologic and kinematic parameters of buffalo bull spermatozoa.

Materials and Methods

Frozen semen: Frozen Anatolian Buffalo bull spermatozoa (name of bull: Karahisar, identify number: TR30001442002, freezing date and place: 22.11.2018, Republic of Turkey Ministry of Agriculture and Forestry, International Center for Livestock Research and Training, Lalahan, Turkey) collected at the same date from the same bull and cryopreserved in volumes of 0.25 mL straws, kept in liquid nitrogen (standard storage procedure), were used in the study.

Semen thawing: A total of twenty straws (ten per each thawing procedure) were used for watery thawing system consisting of a water bath or for dry thawing system consisting of the dry thawing device. For both thawing systems, thawing procedure was applied at 37 °C and 30 sec. Whereas the first method is a conventional method for thawing of spermatozoa, the other is newly developed for thawing of spermatozoa (Figure 1). This system is portable and can be worked by a lighter socket of a car or vehicle (12-13.6 V).

Thawing procedure:

I. Watery thawing system: To thaw the semen samples, the straws (n=10) were immersed in water at 37 ºC for 30 sec in a waterbath.

II. Dry thawing system: To thaw the semen samples, the straws (n=10) were thawed by placing them in the oppropriate holes at 37 ºC for 30 sec. There are four round holes (the first for warming of catheter before AI, the second for straw by volume 0.50 mL, the third and fourth for straws by volume 0.25 mL) on top surface of the device of dry thawing system.

Hypo-osmotic swelling test: Hypo-osmotic swelling test was used to evaluate post-thaw functionality of the buffalo spermatozoa plasma membrane. HOST was performed using a 190 mOsm/kg hypo-osmotic solution (0.735 g sodium citrate and 1.351 g fructose in 100 ml distilled water). 500 µL of HOS solution prepared at 37°C was placed into eppendorf tube. 50 µL of semen sample was added to the HOS solution, and the mixture was incubated for 30 min at 37°C (Rasul *et al.*, 2001a). After incubation, a 5- µL semen sample drop was examined under a phase-contrast microscope (400×) assisted with a heating plate (37°C). A total of 200 spermatozoa were classified according to swelling and spiral tail status in spermatozoon (Figure 2).

Figure 1 Device of dry thawing method.

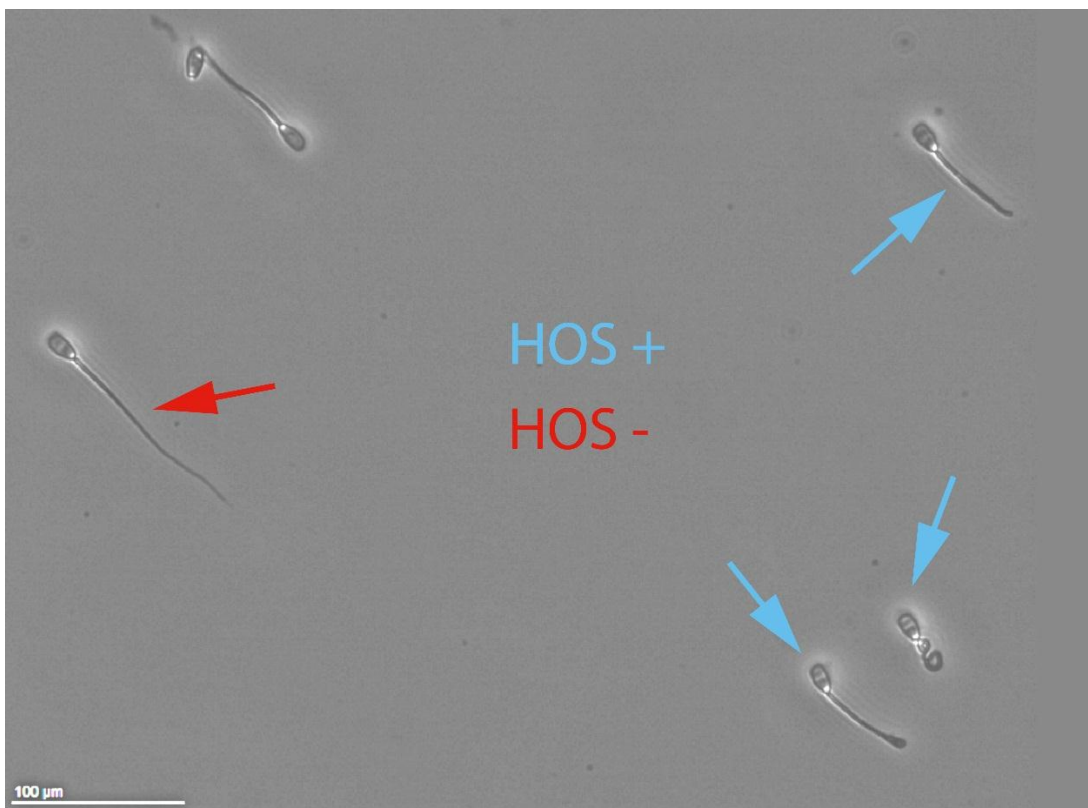

Figure 2 Evaluation of plasma membrane integrity through HOST.

Acrosomme integrity: Fluorescein conjugated lectin Pisum sativum agglutinin (FITC-PSA) staining method described by Kumar *et al*., (2016) was used for determining of damage of acrosome integrity in the study. For this, 20 µl semen was placed in 500 µl PBS and centrifuged at 1,500 rpm for 10 min. After centrifugation, the supernatant was discarded. The spermatozoa pellet was transferred into 250 µl PBS. One drop of it was dropped on a microscope slide for the smear. After allowed the smear dried, it was fixed with paraformaldehyde (4%) at room temperature for 45 min. Dried slides were stained with FITC-PSA (50 µg / mL in PBS solution) in a dark place at room temperature for 20 min. After, the excess stain was washed off with bidistilled water, allow the slide to dry. A thin layer of glycerol was coated on the slide and examined under a fluorescence microscope (Figure 3).

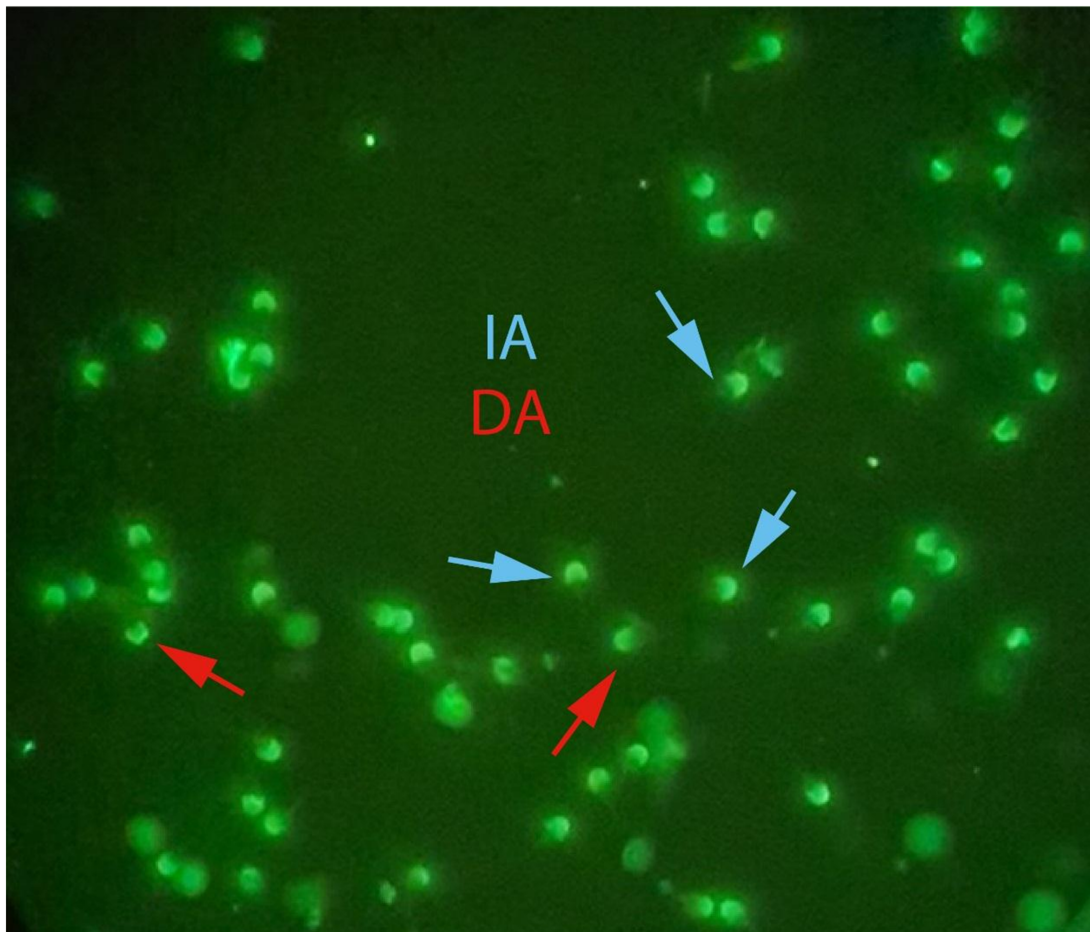

Figure 3 Evaluation of acrosome integrity through FITC-PSA staining (IA ; Intact acrosome, DA ; Damage acrosome).

Assessment of sperm motility and kinematic characteristics: Total motility and progressive motility were evaluated with a CASA (Sperm Class Analyzer®, version 6.3.0.59, Microptic, Barcelona, Spain). The slide (Leja 20 µm) was placed onto a stage warmer set at 37 °C. At least five microscopic fields and 500 spermatozoa were analyzed for each sample. The instrument settings were 25 Hz frame rate and 25 frames captured per sample, with sperm cell detection based on head area of 25 μm^2 to 90 μm^2. For kinematic characteristics of sperm movement; VSL (straight-line velocity, µm/s), VCL (curvilinear velocity, µm/s), VAP (average path velocity, µm/s), ALH (amplitude of lateral head displacement, µm), LIN (linearity, VSL/VCL × 100), WOB (wobble, VAP / VCL × 100), STR (straightness, VSL/VAP × 100), and BCF (beat-cross frequency, hertz) were determined with the software system. Total motility (MOT) was defined as sperm cells with VCL > 15 mm/s, progressive motility (PROG) was defined as sperm cells with STR > 70 mm/s.

Assessment of sperm morphology: The morphology of spermatozoa was evaluated with a Spermac® (Stain Enterprise, P.O. Box12421, 0110, Onderstepoort, Republic of South Africa) stain kit with a 400x objective, blue filter microscope (Nikon, Eclipse, Tokyo, Japan). Briefly, a drop of semen was placed on a glass slide, and a thin smear was prepared and allow to air dry for about 5 min on a warm plate at 37°C. The slide was then fixed for 5 min and washed with distilled water 5-6 times. Excess water was removed with a piece of filter paper, and the slide was placed into stain solution A for 2 min before being totally dried. This procedure was repeated for solutions B and C excepting the slide was placed into stain solutions B and C for 1 min. Finally, the slide was air-dried. After staining procedure, a total of 200 spermatozoa were evaluated for abnormal acrosome, head, mid-piece, and tail forms.

Statistical analysis: The data were summarized with describing as means ($\bar{X}$) ± their standard error of means ($S_{\bar{X}}$). Comparisons of the groups were made by The Least Square Method. All statistic analysis and evaluations were made using SAS, (2009) statistic suits. The value of $P<0.05$ was considered statistically significant.

Results

The rates of abnormal spermatozoa, HOST positive and acrosome integrity of post-thaw buffalo spermatozoa thawed in watery and dry thawing system were presented in Table 1. There was no difference between in watery and dry thawing system for the percentages of HOST positive of post-thaw buffalo spermatozoa (78.70±1.13 vs. 77.20±0.98%) and acrosome damages of spermatozoa (16.70±1.02 vs. 16.00±0.59). Total abnormal spermatozoa rate of dry thawing system was lower ($P<0.05$) than that of watery thawing system. Motility rates and kinematic values of buffalo bull spermatozoa thawed in watery and dry

systems were given in Table 2. There was no difference between thawing methods for all kinematic characteristics except for STR value ($P<0.05$) resulted in a decrease in dry thawing system.

Table 1 Values (mean ± standard error of means) for buffalo bull sperm characteristics in watery and dry thawing systems.

	Parameters	Watery Thawing System $\bar{X} \pm S_{\bar{X}}$	Dry Thawing System $\bar{X} \pm S_{\bar{X}}$	P value
Abnormal spermatozoa (%)	**Acrosome**	1.90±0.31	1.70±0.33	0.668
	Head	4.70±0.59	3.50±0.60	0.173
	Middle part	1.70±0.15	0.80±0.32	0.022
	Tail	18.10±0.90	17.50±0.71	0.608
	Total	26.40±1.07	23.50±0.71	0.038
HOST Positive (%)		78.70±1.13	77.20±0.98	0.332
Acrosome damages of spermatozoa (%)		16.70±1.02	16.00±0.59	0.564

Table 2 Motility rates (mean ± standard error of means) and kinematic values of buffalo bull sperm in watery and dry thawing system.

Parameters	Watery Thawing System $\bar{X} \pm S_{\bar{X}}$	Dry Thawing System $\bar{X} \pm S_{\bar{X}}$	P value
TM (%)	79.23±1.78	74.30±1.58	0.054
PM (%)	57.44±2.48	53.15±2.00	0.195
VCL (µm/s)	90.83±6.52	90.58±4.73	0.975
VAP (µm/s)	79.25±6.38	76.97±5.05	0.782
VSL (µm/s)	65.96±5.75	63.30±4.80	0.726
STR (%)	72.25±0.87	68.98±1.02	0.025
LIN (%)	61.66±1.34	57.47±1.56	0.058
WOB (%)	78.78±0.96	75.75±1.24	0.069
ALH (µm)	2.22±0.08	2.38±0.04	0.108
BCF (Hz)	6.47±0.16	6.13±0.09	0.094

TM: Total motility, PM: Progressive motility, VCL: Curvilinear velocity, VAP: Average path velocity, VSL: Straight-line velocity, STR: Straightness (VSL/VAP × 100), LIN: Linearity (VSL/VCL × 100), WOB: Wobble (VAP / VCL × 100), ALH : Amplitude of lateral head displacement, BCF : Beat-cross frequency.

Discussion

It has been known that a structural and biochemical active plasma membrane of spermatozoa required to accomplish capacitation, acrosome reaction and oocyte penetration processes. In the present study, the sperm plasma membrane integrity was assessed through the HOST recognized as a reasonable procedure for the evaluation of the functional status of the sperm plasma membrane. Tartaglione and Ritta (2004) mentioned that the functional and structural status of the plasma membrane of spermatozoa is highly related to fertility. Ansari *et al.* (2011) stated that the plasma membrane integrity rate of buffalo bull semen cryopreserved in 0.25 mL straw and thawed at 37 °C within 30 sec was 59.0%. Rastegarnia *et al.* (2013) reported that plasma membrane integrity of buffalo bull semen, thawed at 37 °C within 30 sec, was 61.3%. Gangwar *et al.* (2018) stated that post-thaw plasma membrane integrity of buffalo bull semen was 54.30%. In the present study, the results of the HOST values of spermatozoa in watery and dry thawing systems were similar to each other and higher than that of Ansari *et al.* (2011), Rastegarnia *et al.* (2013) and Gangwar *et al.* (2018).

The assessment of sperm morphology in different thawing methods is of particular importance due to the proper fertilization process. The intact acrosome has an

importance for acrosome reaction, and it is required at the right time to facilitate fertilization process (Oura and Toshimori, 1990; Thomas *et al*., 1997). Ahmed *et al.* (2017) stated that there was a positive correlation between fertility of preserved semen and the percentage of viable spermatozoa with intact acrosome. Medeiros *et al*. (2002) stated that the presence of intact acrosome was the key for fertilization and highly correlated with frozen semen fertility. Acrosome integrity may negatively be affected by thawing procedure of semen of buffalo (Rasul *et al*. 2001a). In the current study, there were no differences in the percentages of spermatozoa with intact acrosome among different thawing methods. The damages of acrosome integrity were mimicked that of previous studies reporting acrosome damages below 20% of buffalo bull semen (Rasul *et al*. 2001a; Kumar *et al*. 2016). The results for the post-thaw middle part defect and total abnormal spermatozoa rates thawed in watery system were higher compared to dry system. Sperm abnormality rates in the present study were compatible with the results of El-Sheshtawy *et al*. (2009); Scholkamy *et al*. (2009); El-Sheshtawy *et al*. (2010).

Mahmoud *et al.* (2013), who investigated the relationship between frozen-thawed sperm characteristics and fertility in buffalo bulls, reported that the motility and abnormality rates of post-thawed buffalo bull spermatozoa were 42.51 and 15.19%, respectively. El-Sheshtawy *et al.* (2010) stated that motility rates in buffalo bull sperm were between 36.0 and 43.0%. Ansari *et al.* (2011) noted that the motility of buffalo semen cryopreserved in 0.25 mL straw and thawed at 37 °C within 30 sec was 61.7%. Rastegarnia *et al.* (2013) determined that the motility and progressive motility of buffalo bull spermatozoa after thawing at 37 °C within 30 sec were 62.7 and 25.4%, respectively. Singh *et al.* (2017) reported that the spermatozoa motility of post-thawed semen ranged from 33.3 to 67.6% (mean 43.6%) in buffalo bulls. Post-thawed sperm motility of the current study, including different thawing methods, was higher than that of Mahmoud *et al.* (2013), El-Sheshtawy *et al.* (2010), Ansari *et al.* (2011), Singh *et al.* (2017). The rate of abnormality of buffalo bull spermatozoa thawed in watery and dry thawing system was higher than that of Mahmoud *et al.* (2013). Progressive motility rate of post-thawed buffalo bull sperm of the different thawing methods of the present study was higher than that of Rastegarnia *et al.* (2013).

Gillan *et al.* (2005) reported that motility and progressive motility, kinematic characteristics, acrosome integrity, mitochondrial function, morphology, and morphometrics may be associated with fertility. Total and progressive sperm motility may be considered two major factors for accurate fertility predictors, but there are many other factors that cause influence on fertility (Rodríguez-Martínez, 2007). For this reason, CASA technology, developed in the mid-1980s (Bompart *et al.,* 2018; Gallagher *et al.,* 2018), provides more useful information about the specific sperm quality variables based on objective measurements (Valverde and Madrigal-Valverde, 2018; Yániz *et al*., 2018a; Yániz *et al*., 2018b). The pattern of spermatozoa motion reflects exposure to the biochemical environment and physical conditions. Freezing and thawing of spermatozoa can cause damages to motion characteristics (visual or computerized motility and curvilinear velocity), plasma membrane integrity, and the acrosomal ridge of buffalo spermatozoa (Rasul *et al*., 2001b). Some researchers (Kumar *et al*., 2014; Singh *et al*., 2017) reported that the relationship between sperm kinematics and their relationship with fertility was not clearly established in buffalo bulls. In the present study, the rates of spermatozoa motility and progressive motility and all kinematic parameters except for STR value were not affected by different thawing methods. Singh *et al.* (2017) reported that spermatozoa STR value from high fertile bulls was about 70%. Although it was observed that sperm STR value was higher in the watery thawing method than the dry thawing system, the STR values of buffalo bull sperm obtained from both the watery and dry thawing systems in our study were compatible with the result of Singh *et al.* (2017).

In conclusion, the current study showed that dry thawing as an alternative to watery thawing may be used for frozen buffalo bull semen because most the frozen-thawed sperm values in dry thawing were similar to those in watery thawing. Dry thawing has some advantages compared to watery system because of portable, practical, easily using farms and barns, and no water necessity.

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