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Assessment of Morphological Features of Bull Semen Thawed at Various Temperatures and Periods of Time with CASA

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ABSTRACT

Thawing condition is one the most important factors affecting the re-animation of the spermatozoon in order to fertilise the oocyte. For that matter, we aimed to evaluate the morphological features of the head and midpiece of cryopreserved spermatozoa thawed at different temperatures and for various durations, with using CASA (Computer aided sperm analyser). Frozen semen samples belonging to the same batch, collected from three different bulls were grouped as; control group thawed for 20 seconds at 37 ℃; and experimental groups were thawed for 30, 40, 50 and 60 s at 25 ℃ and 37 ℃; for 10, 15, 20 and 25 s at 40 ℃; for 3, 6, 9, 12 s at 70 ℃. Morphometrical features of the samples were evaluated by using CASA system with nine repetitions. As a result, straws thawed at 25℃ for 40 s had the highest average length of head (6.22 ± 0.09 µm), and the width of mid-piece (0.68 ± 0.01 µm). It was concluded that the thawing temperature and duration has affected/altered the morphometry of the sperm head and midpiece, although the results were not statistically significant (p> 0.005).

Keywords: Bull sperm, CASA, morphometry, sperm head, sperm mid-piece

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Farklı Süre ve Sıcaklıklarda Çözdürülen Boğa Spermalarının Morfolojik Fonksiyonlarının CASA Cihazı ile Değerlendirmesi

ÖZ

Suni tohumlamada kullanılan dondurulmuş spermanın çözüm sonu parametrelerini belirleyen faktörlerin başında çözdürme koşulları gelmektedir. Yapılan araştırma ile Simental, Holstein ve Brown Swiss ırkı boğaların donmuş spermalarının, deneysel olarak belirlenen farklı sıcaklık ve sürelerde çözdürülmesi sonucu CASA cihazı ile spermatozoon başı ve orta kısmına ait parametreler bakımından değerlendirilmesi amaçlanmıştır. Çalışmada gruplar; kontrol grubu 37 ℃'de 20 saniye; deney grupları ise, 25 ℃'de 30, 40, 50 ve 60 sn.; 37 ℃'de 30, 40, 50 ve 60 sn.; 40 ℃'de 10, 15, 20 ve 25 sn.; 70 ℃'de 3, 6, 9, 12 sn. olarak belirlenmiştir. Üç farklı ırk boğaya ait, tek ejakülasyonda elde edilen dondurulmuş boğa spermaları, 9 tekrarda CASA parametreleri açısından değerlendirilmiştir. 25 ℃'de 40 sn.'de çözdürülen spermaların baş uzunluklarının (6.22 ± 0.09), orta kısım genişliğinin (0.68 ± 0.01) diğer sıcaklık ve sürelere göre daha yüksek ortalamaya sahip olduğu saptanmıştır. Çözdürme sıcaklık ve süresinin spermatozoon baş ve orta kısmına dair parametreleri değiştirdiği ancak elde edilen sonuçların istatiksel olarak anlamlı olmadığı belirlenmiştir (p> 0.005).

Anahtar Kelimeler: Boğa spermatozoonu, CASA, morfoloji, spermatozoon başı, spermatozoon orta kısmı

INTRODUCTION

In general, the length and width of the sperm head varies depending on the changes in the size and organization of the acrosome and nuclei (Mortimer, 2018, Gu et al. 2019). The relationship of sperm head and midpiece morphometry with fertility has been clearly established (Gil et al., 2009; Valverde et al., 2016; Yaniz et al., 2015), indicating that males with high fertility rates produce longer and conical spermatozoa (Beletti et al. 2005; Gravance et al. 2010). However, cryopreservation and thawing procedure causes acrosome, midpiece and tail abnormalities that are primarily originated by plasma membrane damage, resulting in rendering sperm cells with impaired fertilization ability. This indicates the presence of variation both in the membrane properties of individuals and their responses to freeze-thawing (Valverde et al. 2016) conditions and osmotic stress is related differences in the plasma membrane volume and shrinkage.

Changes in the temperature during the freezing and thawing of the sperm reduce the rate of motile spermatozoa and cause ultra-structural, biochemical and functional damage (Senger 1980). The fertilization ability of spermatozoa after thawing is greatly affected by the thawing temperature and duration (Senger 1980, İleri and Ak 1993, Nur et al. 2003). Subtle differences in shape, volume or area between spermatozoa are likely to be responsible for differences in water inlet and outlet microfluidic channel velocity throughout the plasmalemma during cryopreservation as well as the thawing process (Tekin and Daşkın 2019, Nur et al. 2003), which may be the source of morphologically different sperm subpopulations (Nunez-Martinez et al. 2007). Thus, leads to sperm chromatin changes during these processes (Karabinus et al. 1997). These changes are responsible for a decrease in the surface area of the sperm head, which may be caused by excessive condensation of spermatozoon nucleochromatin (Royere et al. 1998). Changes in chromatin have been associated with a reduced fertility in bulls (Sailer et al. 1996; Ostermeier et al., 2001; Januskauskas et al. 2003). As a result of the chromatin structure alterations and the decrease at the surface area of the sperm head, abnormal morphological structure may occur in the sperm head. Abnormal chromatin structure and head related morphological abnormalities of the spermatozoon are responsible for the loss of fertility in bulls (Saacke and White 1972; Gravance et al. 1998).

The midpiece morphology has a key importance for the mobilization of the spermatozoon due to phospholipid hydroperoxide glutamate peroxide and tyrosine phosphoryl proteins, which both have a primary role in sperm motility (Shivaji et al. 2009) that generates the ATP synthase beta subunit. Mitochondria are the source of adenosine triphosphate (ATP) in the midpiece of the sperm. It transmits energy to axonemia located in the principal part that produces the driving force of sperm (Piomboni et al. 2012, Gu et al. 2019). Therefore, the lengths of the sperm midpiece and the principal part can be critical in determining the sperm swimming rate and fertilization rate (Firman et al. 2010, Bennison et al. 2015; Gu et al. 2019). In house mice and primates, the length of the sperm midpiece was largely considered a reasonable predictor of sperm swimming rate/velocity (Anderson and Dixson 2002, Firman et al. 2010, Gu et al. 2019). With regard to sperm competition, beneficial changes in sperm morphology can increase swimming speed of the sperm cells, which is the main determinant of fertilization success (Ramm et al. 2005, Fisher et al. 2016, Gu et al. 2019). Interestingly, Non-Return Rates (NRR) in cows inseminated with the cryopreserved semen in which mid-length was measured, resulted with up to a 5% reduction in NRR in those with a longer midpiece (Shahani et al. 2010). Moreover, alterations in midpiece has a negative impact on the normal product of cellular respiration, thus creates Reactive Oxygen Species (ROS) that subsequently accumulates in the sperm midpiece and mitochondria (Desai et al. 2010).

Since the fertilising ability of sperm is highly affected by environmental conditions, the success of artificial insemination depends on providing the optimum conditions (Leticia, et al., 2013). While routine laboratory studies use scientific guidelines for thawing temperature and time values; indeed field conditions are usually different than those of the laboratory. The alterations in the morphometrical feauters of the sperm that could occur during the different thawing conditions can directly affect the result of insemination. Owing to the recent development of digitalized microscopic evaluation of spermatozoa with CASA (Computer Assisted Semen Analysis) technology, morphometric measurement of a spermatozoon became optimized and thus, introduced new possibilities in the spermatology assessment (Garcia-Herreros et al. 2007, Yaniz et al. 2015).

In this study, it was aimed to evaluate the morphological alterations in the head and midpiece of the spermatozoa subjected to different thawing time and temperatures in order to reflect various possible field conditions prior to artificial insemination practice.

MATERIALS and METHODS

Selection of Semen
For this study, a total of 153 commercially frozen semen straws from three different bull breeds

(Holstein, Simmental and Brown Swiss) were used. All the semen samples were selected from a single semen batch to eliminate individual differences, diluted, packed in 0,25 ml/straw, frozen and stored at -196℃ under the commercial standard cryopreservation process by Supergenetics® company, Konya/Turkey. Only the frozen semen samples were accepted with the following criteria met; motility with 70%, morphologically normal spermatozoa rate and viability with 80 % were used.

Table 1. Thawing temperatures and durations of the groups

Groups	Thawing temperature and times
Control	37 ℃ 20 s
Group 1	25 ℃ 30 s
Group 2	25 ℃ 40 s
Group 3	25 ℃ 50 s
Group 4	25 ℃ 60 s
Group 5	37 ℃ 30 s
Group 6	37 ℃ 40 s
Group 7	37 ℃ 50 s
Group 8	37 ℃ 60 s
Group 9	40 ℃ 10 s
Group 10	40 ℃ 15 s
Group 11	40 ℃ 20 s
Group 12	40 ℃ 25 s
Group 13	70 ℃ 3 s
Group 14	70 ℃ 6 s
Group 15	70 ℃ 9 s
Group 16	70 ℃ 12 s

Thawing of the Semen

Frozen semen straws from Simental, Holstein and Brown Swiss were divided into 17 groups according to the thawing time and temperature. Each semen straw (0.25 ml) was thawed at the temperatures and times given in the groups (Table-1). The straws were thawed in a water bath (Memmert, MiniTube®, Germany), taken to the Eppendorf tubes (1.5 ml.) previously warmed at heating table (Leica, HI 1220, Germany) then immediately used for morphological staining.

Sperm Preparation and Morphological Staining

To examine the morphology, smear samples were taken and dried in the air at least for 30 s with previously cleaned slide to eliminate the foreign particles during analysis. Then, smear samples were covered with fixation solution for 1 minutes, and excessive part was drained. Thereafter, the fixed smears placed horizontally onto staining tray, and covered with Sperm Blue stain (Microptics®, Spain) for 3 minutes. Once stained, all the slides were permanently sealed with Eukitt® mounting medium and a coverslip.

Spermatozoa Morphometry

The measurement of spermatozoa head and midpiece morphometric analysis were done with SCA morphological software (Microptics®, Spain). A phase-contrast microscope (Nikon, Eclipse 50i) with a camera attachment (Basler, Scout Specific digital Basler camera with 1280×1024 resolution, 60 frame per sec.) were used under x60 magnification objective. SCA® Morphology module were used for automatic morphometry analysis under bright-field microscopy. Spermatozoa were randomly selected and captured from different slide fields until 100 spermatozoa were reached. The selection of those spermatozoa with foreign particles and overlapped ones were discarded manually. The dimensions of the head and midpiece were manually selected, measured simultaneously by the software system and recorded as µm unit into a separate excel file.

Statistical Analyses

Descriptive statistics for the data were calculated and shown as "Arithmetic Mean ± Standard Error". For each variable, the group effect is examined with the help of mixed models; in the model created, repetitions of semen measurements are random; degrees, duration basic effects are included as fixed effects. In cases where the interaction term was found meaningful, simple effects analysis with Bonferroni correction was applied. For all statistical analysis, $p < 0.05$ criterion was used. The analyzes were carried out through the Stata 12 / MP4 (License No: 50120500264) statistical package program.

RESULTS

The parameters related to the head of sperm, thawed at different times and temperatures are given in Table-2 while the parameters related to the mid-piece are given in Table-3. When the parameters were evaluated statistically, no significant difference was found ($p > 0.005$).

The lowest head width was obtained as 2.89 ± 0.05 µm in semen thawed for 60 s at 37 °C ($p > 0.005$). The head widths of sperm, which were thawed at 70 °C for both 6, 9 and 12 s, was determined as 2.96 ± 0.06 µm. Sperm thawed for 3 s at the same temperature were found to have a head width of 2.91 ± 0.05 µm.

The lowest head length measurement was found to be 6.04 ± 0.09 µm in group thawed at 70 °C for 3 s. It was determined that the head perimeter at the same temperature and time had the lowest value with 13.03 ± 0.19 µm. The head area was the highest as 17.42 ± 0.49 µm^2 in sperms thawed for 12 s at 70 °C. Sperm in the group thawed at 25°C for 50 s have the lowest head area, 16.54 ± 0.43 µm^2 ($p > 0.005$).

When the midpiece widths were evaluated, the semen thawed at 25°C 40 s were found to have the highest value of 0.68 ± 0.01 µm, and the lowest value of 0.6 ± 0.03 µm at 25 °C 50 s ($p > 0.005$). It was determined that the semen thawed at 25 °C 40 s in the middle part area had the numerically highest with 5.26 ± 1.58 µm^2.

Table 2: The morphometrical features related to the head of sperm, thawed at different temperatures and time periods

Groups (n=9)	Arithmetic mean ± Standard error			
	Head Length (µm) (p=0.930)	Head Width (µm) (p=0.994)	Head Area (µm²) (p=0.993)	Head Perimeter (µm) (p= 0.997)
Control (37°C 20 s)	6.15 ± 0.07	2.92 ± 0.04	16.88 ± 0.38	13.28 ± 0.17
37°C 30 s	6.21 ± 0.11	2.95 ± 0.06	17.27 ± 0.50	13.36 ± 0.24
37°C 40 s	6.11 ± 0.08	2.90 ± 0.05	16.70 ± 0.39	13.21 ± 0.15
37°C 50 s	6.16 ± 0.12	2.93 ± 0.06	16.90 ± 0.64	13.22 ± 0.23
37°C 60 s	6.07 ± 0.09	2.89 ± 0.05	16.65 ± 0.42	13.15 ± 0.21
25°C 30 s	6.10 ± 0.06	2.93 ± 0.03	16.69 ± 0.41	13.18 ± 0.12
25°C 40 s	6.22 ± 0.09	2.95 ± 0.06	17.33 ± 0.53	13.38 ± 0.18
25°C 50 s	6.11 ± 0.08	2.92 ± 0.05	16.54 ± 0.43	13.23 ± 0.18
25°C 60 s	6.15 ± 0.08	2.93 ± 0.05	17.11 ± 0.48	13.31 ± 0.17
40°C 10 s	6.15 ± 0.06	2.93 ± 0.04	16.93 ± 0.33	13.23 ± 0.14
40°C 15 s	6.13 ± 0.07	2.92 ± 0.05	16.86 ± 0.39	13.32 ± 0.15
40°C 20 s	6.15 ± 0.09	2.96 ± 0.07	17.09 ± 0.49	13.32 ± 0.22
40°C 25 s	6.18 ± 0.09	2.94 ± 0.05	17.16 ± 0.50	13.37 ± 0.23
70°C 3 s	6.04 ± 0.09	2.91 ± 0.05	16.78 ± 0.44	13.03 ± 0.19
70°C 6 s	6.12 ± 0.07	2.96 ± 0.06	17.01 ± 0.43	13.29 ± 0.16
70°C 9 s	6.21 ± 0.09	2.96 ± 0.06	17.33 ± 0.48	13.39 ± 0.19
70°C 12 s	6.30 ± 0.09	2.96 ± 0.06	17.42 ± 0.49	13.43 ± 0.22

Table 3: The morphometrical features related to the midpiece of sperm. thawed at different temperatures and time periods.

Groups (n=9)	Arithmetic mean ± Standard error	
	Midpiece Width (µm) (p=0.514)	Midpiece Area (µm²) (p=0.381)
Control (37°C 20 s)	0.64 ± 0.02	3.53 ± 0.12
37°C 30 s	0.65 ± 0.01	3.81 ± 0.08
37°C 40 s	0.67 ± 0.01	3.65 ± 0.10
37°C 50 s	0.65 ± 0.02	3.61 ± 0.18
37°C 60 s	0.65 ± 0.01	3.76 ± 0.10
25°C 30 s	0.64 ± 0.02	3.48 ± 0.18
25°C 40 s	0.68 ± 0.01	5.26 ± 1.58
25°C 50 s	0.60 ± 0.03	3.34 ± 0.12
25°C 60 s	0.66 ± 0.01	3.73 ± 0.15
40°C 10 s	0.65 ± 0.02	3.60 ± 0.14
40°C 15 s	0.63 ± 0.01	3.62 ± 0.05
40°C 20 s	0.66 ± 0.03	3.52 ± 0.17
40°C 25 s	0.67 ± 0.01	3.66 ± 0.17
70°C 3 s	0.65 ± 0.02	3.79 ± 0.12
70°C 6 s	0.65 ± 0.03	3.45 ± 0.18
70°C 9 s	0.65 ± 0.01	3.70 ± 0.12
70°C 12 s	0.64 ± 0.01	3.52 ± 0.08

DISCUSSION

Routine assessment of sperm, including the assessment of normal spermatozoa morphology, has long been used to assess the effects of the semen freezing process and thawing procedures. Abnormal spermatozoa morphology is an important indicator of decreased fertility in stallions and bulls. Spermatozoa head abnormalities have been associated with early embryonic loss, decreased embryo quality, and reduced capacity to bind to the ova (Maroto-Moralesa et al. 2010). Studies investigating chromatin condensation and the morphology of spermatozoa suggest that abnormal chromatin condensation may be affected by or lead to morphological abnormalities. The change in sperm head parameters revealed that it was associated with fertility potential and abnormal chromatin structure in bulls (Sailer et al. 1996). In addition, changes in spermatozoon chromatin structure after scrotal isolation of bulls were found to be parallel to an increase in morphologically abnormal spermatozoa percentage (Karabinus et al. 1997). Chromatin condensation in epididymal cat spermatozoa assessed by aniline blue and acridine orange staining was determined to be significantly higher than spermatozoa with tail defects only in spermatozoa with head abnormalities (Hingst et al. 1995). Abnormal chromatin condensation was observed in about 95% of human spermatozoa with some abnormalities. The percentage of spermatozoa showing normal chromatin condensation was found to be lower than those with abnormalities in the spermatozoa head (Dadoune et al. 1998).

The present study showed unexpected correlation between sperm thawing temperature and time on sperm morphometric parameters even if it is not statistically significant. The control group (37 °C 20 s) and 70 °C 12 s head area determined as 16.88 ± 0.38 µm²; and 17.42 ± 0.49 µm² respectively (Table 2). It is known that spermatozoon morphology is also negatively affected at this temperature. In parallel with the studies mentioned above, the change in the head area draws attention. Even though the thawing temperature is set for 70 °C in 12 s perfectly, the cooling time from 70 °C to heating stage temperature should be taking into consideration. In line with above mentioned statement, our preliminary result for intact membrane rate conversely showed the highest abnormality. Based on this, it is determined that the changes occurring per sperm also show negativities related to morphology. The head width of sperm thawed in 70 °C 6, 9 and 12 s was found to be the same as 2.96 ± 0.06 µm. The same temperature value was found to be 2.91 ± 0.05 µm in semen thawed for 3 s (Table 2). When the parameters on the head perimeter is taken into account, the lowest value

was determined as 13.03 ± 0.19 μm² in the semen thawed for 3 s at 70 (Table 2). The lowest value of the head length ratio of the sperms that were kept for 3 s at 70 °C was determined as 6.04 ± 0.09 μm (Table 2), but no significant difference was found between the parameters (p> 0.005). However, this results could be interpret as the timing and temperature is take a importance place for optimised osmotic swelling at right ionic equilibrium. Gravance et al. (2009) compared the length and width of the head before and after semen cryopreservation, it was determined that the head length before cryopreservation was 8.67 μm, while it decreased to 8.45 μm after freezing and thawing. The head width was decreased from 4.55 μm to 4.39 μm. Higher thawing temperatures should provide even faster heating, but are not usually prefered due to the perceived risk of cell injury above 37 °C (Calamera et al. 2010).

Sperm are exposed to changes in lipid composition of the sperm plasma membrane (Buhr et al. 1989), externalization of phosphatidylserine, physical and chemical stress (Schiller et al. 2000), externalization of phosphatidylserine (Glander and Schaller 1999) and thus reduction in head size (Gravance et al. 1998) during freezing. Human spermatozoa has also been reported to be particularly sensitive to lipid peroxidation caused by the oxygen radical (Jones et al. 1979, Alvarez et al. 1987), membrane phospholipid damage (Alvarez and Storey 1995, Aitken 1995). ATP produced by thermal flagellum for maintaining sperm motility (Calamera et al. 1982) and ATP loss due to membrane damage (Calamera et al. 2010). In addition to membrane-bound cryo-damage, oxygen radicals have been found to damage peripheral axonemia and nuclear DNA (Sailer et al. 1995). The production of superoxide anion (O_2^-) has been shown to increase during thawing in bovine sperm (Chatterjee and Gagnon 2001). This may partially explain why DNA damage has increased immediately after it has been resolvedChatterjee and Gagnon (2001) have determined that the potential for increased motility recovery observed after thawing at 40 °C will be with a faster recovery rate of sperm enzymatic antioxidant activity. During thawing, there are two processes that terminate the degree of cell damage: [1] the amount of oxygen radical production and [2] recovery rate of enzymatic antioxidant activity. As principle, the higher the temperature rises, the faster the recovery of enzymatic antioxidant activity occurs. Therefore, it has been reported that after thawing at 40 °C, the increase in the production of oxygen-radical that occurs during thawing can be neutralized more efficiently than at 37 °C. When sperm DNA integrity and ATP content were evaluated to detect sperm damage due to oxygen-free radical origin at different thawing temperatures tested, the thawing at 40 °C had no observable determinative effect on DNA integrity. No remarkable difference in ATP content

was observed between semen thawed at 40 °C and thawed at 37 °C. In the present study, the head length parameters of the control group, which thawed in 37 °C 20 s, and the sperms thawed in 40 °C 10 s, were found to be very close to each other with the values of 6.15 ± 0.07 μm and 6.15 ± 0.06 μm, respectively. In length, perimeter and area of the head parameters, 37 °C 30 s (6.21 ± 0.11 μm /13.36 ± 0.24 μm /17.27 ± 0.5 μm) and 40 °C 25 s (6.18 ± 0.09 μm /13.37 ± 0.23 μm /17,16 ± 0,5 μm) have the highest values among the same temperatures. In this study, morphometrical values of sperm head were similar between 37 °C and 40 °C. It was observed that the width of the midpiece at 37 °C 40 s and 40°C 25 s were uniform as 0,67 ± 0,01 μm. Based on these parameters, 37 °C and 40 °C temperature values appear to have a similar effect on sperm morphology.

In mammals, the variability in total sperm length has been stated to occur mainly due to differences in the length of the flagellum and the mid-piece (Cummins and Woodall 1985). The spermatozoa mid-piece contains a dense helix array mitochondria that provides energy to push the cell that determines the flagellar beat frequency (Cardullo and Baltz 1991). The size of the mid-piece of the spermatozoa can be important in determining the outcome of sperm competition. Comparative studies between vertebrates showed that the sperm size correlated positively with swimming speed (Fitzpatrick et al., Lüpold et al. 2009). Studies show that increases in the mid-piece volume can turn into higher swimming speed and thus provide an advantage in sperm competition (Anderson and Dixson 2002). As a contradiction to this hypothesis, a study in red deers (Cervus elaphas) revealed that there was a negative correlation between sperm mid-piece length and sperm swimming speed. Spermatozoa, which have long heads and shorter mid-piece, swim faster. However, no relation was found between sperm velocity and flagellum or total sperm lengths (Malo et al. 2006). This highlights the need for investigation on these relationships in other mammals. In general, spermatozoa with longer tails have been found to have a tendency to reach the ovum faster while swimming in the female genital tract and to swim faster than those with a shorter tail (Gomendio and Roldan 1991). In the present study, semen thawed in 25 °C 40 s were determined to have the numerically greatest midpiece width with a value of 0.68 ± 0.01 μm as well as midpiece area with 5.26 ± 1.58 μm² (Table 3). The group with the lowest mid-piece area was found to be 3.34 ± 0.12 μm² in the sperm thawed at 25 °C for 50 s.

In conclusion, when the head and mid-piece parameters of the sperm thawed at different times and temperatures were evaluated, the changing time and temperature caused different results in the

parameters. The thawing conditions of semen are a factor that directly affects spermatozoa. Since changes in time and temperature affect the morphology of spermatozoa, changes occur in the mid-piece and head sizes. These changes have positive or negative effects on the fertilization ability of spermatozoa. When performing insemination in field conditions, it has been observed that paying attention to the dissolution time and temperature of the semen is important for the positive functioning of the insemination process and obtaining pregnancies. Any setbacks that occur during this process affect spermatozoa negatively and decrease their fertilization abilities. Especially in high temperature values, it was seen in the parameters related to the head and mid-piece that morphology was negatively affected. It should be noted that the cause of unsuccessful insemination may be due to changes in temperature and time that will occur during the thawing of the semen.

Conflict of Interest: The authors declare that they have no conflict of interest.

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