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Effects of Different Equilibration Conditions on Cryopreserved Bovine Sperm Quality

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ABSTRACT

Equilibration is the one of the important steps of semen cryopreservation and it is the stage in which semen is cooled and phase changes occur. The condition of equilibration is mostly regarded; and success of the equilibration conditions depend on empirical experiences. The aim of this study was to investigate the effects of three different equilibration methods on post-thaw total motility (TMOT), progressive motility (PMOT), kinetic parameters of spermatozoa (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF), plasma membrane and acrosome integrity (PMAI) and DNA fragmentation index (DFI). In each of 4 bulls; two ejaculates were split into three aliquots, diluted by Andromed® and equilibrated for 24h at 4°C in Drawed Straw (Experiment 1), Cups (Experiment 2) and Shaker (Experiment 3). After thawing, sperm quality was determined by examination of TMOT, PMOT, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, PMAI and DFI. There were no differences ($p>0.05$) depending on equilibration conditions for TMOT, PMOT, VSL, VAP, BCF as kinetic parameters and for PMAI and DFI as morphological parameters. The highest VCL value was observed in experiment 3, when the highest LIN, STR, WOB and ALH values were observed in experiment 2 ($p<0.05$). The results show that changes in equilibrating condition have no detrimental effect on post-thaw bull semen quality. In addition, it can be said that equilibrating bull semen in a shaker presents some kinetic benefits in contrast with cup or straw equilibration methods.

Key Words: Bull Semen, CASA, Equilibration, Equilibration Condition, Flow Cytometry.

Farklı Ekilibrasyon Ortamlarının Çözüm Sonu Boğa Sperması Kalitesi Üzerine Etkisi

ÖZ

Ekilibrasyon, spermanın sıcaklığının düşürüldüğü ve faz değişimlerinin meydana geldiği aşama olarak, sperma dondurulmasında en önemli basamaklardan biridir. Ekilibrasyonun gerçekleştirildiği ortam çoğu zaman göz ardı edilmekte, ekilibrasyon koşullarının başarısı ampirik tecrübelerle dayanmaktadır. Bu çalışmanın amacı farklı ekilibrasyon ortamlarının toplam motilite (TMOT), progresif motilite (PMOT), spermatozoa hareket parametreleri (VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF), plazma membran ve akrozom bütünlüğü (PMAI) ve DNA fragmentasyon indeksi (DFI) üzerine etkisinin araştırılmasıdır. Çalışmada 4 baş boğadan iki kez sperma alınarak üç eşit hacme bölündü. Andromed® ile sulandırılan spermalar 24 saat 4°C'de payet içinde (Deneme 1), kapta (Deneme 2) ve çalkalayıcıda (Deneme 3) ekilibrasyona bırakıldı. Çözüm sonu sperma kalitesi TMOT, PMOT, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, PMAI ve DFI parametreleri değerlendirilerek belirlendi. Farklı ekilibrasyon koşulları arasında, hareket özellikleri olan TMOT, PMOT, VSL, VAP, BCF ve morfolojik özellikler olan PMAI ve DFI açısından istatistiki bir farklılık bulunamadı ($p>0,05$). En yüksek VCL değeri Deneme 3'te, en yüksek LIN, STR, WOB ve ALH değerleri Deneme 2'de gözlemlendi ($p<0,05$). Bu sonuçlar farklı kolullarda yapılan ekilibrasyonun çözüm sonu spermatolojik değerler üzerinde herhangi bir zararlı etkisi olmadığını göstermektedir. Ayrıca boğa spermasının çalkalayıcıda ekilibre edilmesinin kap ve payet içinde ekilibre edilmesine göre spermatozoa hareket özellikleri açısından daha yararlı olduğu söylenebilir.

Anahtar Kelimeler: Boğa Sperması, CASA, Ekilibrasyon, Ekilibrasyon Ortamı, Flow Sitometri.

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INTRODUCTION

Cryopreservation of semen results with reduced functionality and viability because spermatozoon cannot endure low temperatures. Cellular defects, in various grades, can arise in each step of cryopreservation and quality of post-thaw semen can change according to these defects. However, achieving semen without time and place is possible with only cryopreservation (Bailey et al., 2000). Cryopreservation also has a key role in developing assisted reproductive techniques like artificial insemination and in vitro fertilization. The widespread use of assisted reproductive techniques allows acceleration of the genetic selection and development in production (Watson, 2000). Invention of cryoprotectants such as glycerol and egg yolk is known as a milestone of semen cryopreservation. After this important invention, semen preservation protocols and extending methodology have been tried to be improved in various species; and obtaining high quality post-thaw semen has been aimed for ages. Although there have been many developments; post-thaw viability, fertility of frozen semen and decrease of cellular defects during freezing process could not reach the expected range. However, this kind of laboratory studies helped classification of detrimental effects in freezing and determination of cases causing these defects (Watson, 2000; Aires et al., 2003; Stadnik et al., 2015). Cryopreservation consists basically of lowering temperature, cellular dehydration, freezing and thawing steps. Because of the high sensitivity of mammalian spermatozoa to temperatures below body heat, decreasing the temperature of semen causes irreversible losses in post-thaw spermatological parameters, initially in motility. Defect named cold shock also occur in different cellular patterns and functionalities, such as membranous structure and permeability, acrosomal integrity in addition to motility (Bailey et al., 2000; Tartaglione and Ritta, 2004; Leite et al., 2010).

The harmful effect of low temperature differs due to cholesterol/phospholipid rate in plasma membrane, the number of fatty acids, saturated hydrocarbon chains and protein/phospholipid rate. Because of the plasma membrane contents that differ among species, the effect of low temperature is observed in different types. Bull semen is known to be very sensitive to cold shock. The occurrence of cold shock can be decreased with cryoprotectants and controlled during lowering the temperature (Hammerstedt et al., 1990; Watson, 2000). In cryopreservation process, extended semen is cooled first to 4°C. This step, named equilibration, is carried out in order to distribute glycerol and other osmotically active diluent components, both in and out of the cell, as slowly as possible. Otherwise,

osmotically active materials rush into the cell and plasma membrane can be diminished. Equilibration is performed slowly to cool semen to 4-5°C and hold the semen at this temperature for 30 minutes to 24 hours. Basically, equilibration is made in order to adjust the plasma membrane to low temperatures and to decrease the metabolic activity of the spermatozoa controlledly (Bailey et al., 2000; Kumar et al., 2003; Layek et al., 2016). Equilibration is one of the important steps of cryopreservation in terms of post-thaw quality and fertility of semen. Up to now, a number of strategies were developed as equilibration methods by numerous of studies. Packaging extended semen at the end of equilibration and packaging before equilibration are distinguished as the mostly used two methods (Thun et al., 2002; Amirat et al., 2004; Leite et al., 2010). However, strategies like these are developed by individual experience. No study has been done with the aim to determine the best equilibration method for obtaining high quality post-thaw semen. In this research, evaluation of the effects of 3 different equilibration methods on cryopreservation of bull semen in terms of post-thaw spermatological parameters was aimed.

MATERIALS and METHODS

Ejaculates were collected from four fertile bulls that were already held and being used for routine examinations. The bulls were kept under standard conditions of feeding and management. The animals showed no disturbances in general conditions and had no sexual dysfunction or disease of the sexual organs during the period of investigations. Semen was collected using an artificial vagina (Model Hannover, Minitüb, Tiefenbach, Germany). Sperm was collected two times per bull. Totally eight ejaculates were used in study. The percentage of progressively motile sperm was determined objectively using a phase contrast microscope. Ejaculates with >70% progressively motile sperm were used in the experiments. Each ejaculate was split in to three aliquots and diluted with Andromed® extender. A portion of each ejaculate was diluted to a final concentration of 60×10^6 sperm/ml. Each aliquot of the diluted sperm were equilibrated at +4°C for 24 hours in 3 experiments.

Experiment 1: Extended semen samples were equilibrated in drawn straws.

Experiment 2: Extended semen samples were equilibrated in cups.

Experiment 3: Extended semen samples were equilibrated on shaker.

After 24 hours, Experiment 2 and Experiment 3 were packaged in 0.25 ml straws, all groups were frozen to -150°C with a computer cooling program and stored in liquid nitrogen.

Post-thaw examinations were done with the CASA system Sperm Class Analyser (SCA® v.4.2, Barcelona, Spain), with an attached phase-contrast microscope (Olympus BX41, Olympus Europe GmbH, Hamburg, Germany). Chambers of 20 µm (Leja; Nieuw Venne, The Netherlands) were loaded with semen and were kept at 37°C. The percentage of spermatological parameters such as; TMOT, PMOT, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF were determined in at least ten different microscopic fields per sample, with a frequency of 60 frames per second. Plasma membrane and acrosome intact sperm (PMAI) were stained by FITC-PNA (fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin)/PI (propidium-iodide) assay. Sperm samples were diluted to a concentration of 5x10⁶ sperm/mL with Tyrode's medium. Five microliters of FITC-PNA (100µg/mL) and 3 µl PI (2.99mM) were added to 492 µL of diluted sperm suspension. Sperm samples were incubated at 37°C for 30 min and remixed just before measurement. The percentage of sperm with a high DNA fragmentation index (DFI) was assessed by the SCSA™ as described by Evenson and Jost (2001). Before performing the statistical analysis, data were examined with Shapiro-Wilk test for normality and

Levene test for homogeneity of variances as parametric test assumptions. One way ANOVA was used to evaluate the differences between groups for the variables. Tukey's test was used as post hoc test for the variables that were found statistically significant. For all comparisons, differences were considered with a minimum of 0.05 significance level. All statistical analysis were performed by using SPSS 14.01 package programme for Windows.

RESULTS

As a result, experiment 2 showed increase in TMOT (73,78±3,68) and PMOT (53,08±2,59) although there was no statistical significance (p>0,05). Considering the equilibration conditions, there were differences (p<0.05) in VCL, LIN, STR, WOB and ALH. While the highest VCL value (87,4±3,03) was found in experiment 2, the highest LIN (43,99±1,36), STR (71,98±1,22), WOB (61,06±1,16) and lowest ALH (3,08±0,11) values were observed in experiment 3. According to the results, the other kinetic traits were found similar; as well as PMAI and DFI values (p>0,05). All results are shown in Table 1 and Table 2.

Table 1: Results of CASA evaluation of post-thaw bull semen equilibrated in three different conditions

Tablo 1: Üç farklı koşulda ekilibre edilen boğa spermasının çözümsonu CASA değerlendirmesi sonuçları

	Experiment 1	Experiment 2	Experiment 3
TMOT (%)	69,52±2,93	73,78±3,68	66,06±3,33
PMOT (%)	49,89±1,96	53,08±2,59	51,70±2,72
VCL (µm/s)	84,13 ^a ±2,82	87,4 ^a ±3,03	72,03 ^b ±2,57
VSL (µm/s)	28,84±1,29	29,25±0,92	31,63±1,33
VAP (µm/s)	45,30±1,38	46,58±1,33	43,94±1,63
LIN (%)	34,35 ^b ±1,35	33,55 ^b ±0,60	43,99 ^a ±1,36
STR (%)	63,56 ^b ±1,61	62,83 ^b ±0,81	71,98 ^a ±1,22
WOB (%)	53,94 ^b ±0,94	53,4 ^b ±0,59	61,06 ^a ±1,16
ALH (µm)	3,73 ^a ±0,13	3,86 ^a ±0,13	3,08 ^b ±0,11
BCF (Hz)	8,51±0,39	8,59±0,27	9,49±0,32

a,b: Averages in groups in the same row with different superscripts are statistically important (p<0,05).

a,b: Aynı satırdaki farklı harfleri taşıyan grup ortalamaları arasındaki farklar istatistiksel açıdan önemlidir (p<0,05).

Table 2: Post-thaw PMAI and DFI averages of bull semen equilibrated in three different conditions

Tablo 2: Üç farklı koşulda ekilibre edilen boğa spermalarının çözüm sonu PMAI ve DFI ortalamaları

	PMAI (%)	DFI (%)
Experiment 1	61,75±3,08	6,23±0,62
Experiment 2	61,69±2,71	5,58±0,47
Experiment 3	55,60±3,77	6,53±0,58

Differences between values are not statistically significant (p>0,05).

Gruplar arası farklılıklar istatistiksel olarak önemsizdir (p>0,05).

DISCUSSION

In this study, it was aimed to evaluate the effects of different equilibration conditions on motility (TMOT), kinetic traits (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF), progressive motility (PMOT), plasma membrane and acrosome integrity (PMAI) and DNA fragmentation index (DFI) of frozen-thawed bull semen. When the results were evaluated, beneficial effects were observed in terms of conditions. However these differences were not caused only by one condition. Therefore, it is not possible to say which equilibration method is better than the others. Besides, the results show that changes in equilibration conditions have no detrimental effect on post-thaw bull semen quality. On the contrary, most of the beneficially increased or decreased values were observed in the shaker method. In previous studies (Thun et al., 2002; Amirat et al., 2004; Anzar et al., 2011), progressive motility of frozen bull semen with values within a range of 50%, gave similar results to experiment group 2 with the highest rate of 53.08% in this research. Likewise, in the same studies (Hering et al., 2015; Moallem et al., 2015; Murphy et al., 2017) the total motility of the cryopreserved bull semen was around 50-75%. When our results were taken into consideration (66,06-73,78%), it was seen that they were consistent with the other studies. These results show that equilibrating bull semen in straws, cup or shaker do not have any detrimental effect in terms of progressive and total motility. Also cup and shaker methods have better values than equilibration in straw, although the difference is not statistically significant. Different sperm kinetic traits evaluated by CASA, velocity parameters like VCL, VSL and VAP can be useful for prediction of fertility. There is a positive correlation between these parameters and percentage of pregnancy (Fetterolf and Rogers, 1990; Liu et al., 1991). However, the other parameters STR, LIN, WOB were not found highly correlated in some researches, although they observed positive correlation with in vitro fertilization rates (Sukcharoen et al., 1996; 1998). ALH reflects the distance of head movement laterally and it has poor negative correlation with spermatozoa fertility similar to BCF. In the study, the only benefit of experiment 2 was found in terms of VCL value. VSL, STR, LIN and WOB were increased and ALH was decreased in shaker as beneficial effects. The other kinetic parameters were found similar in all groups. In the light of all these information, it can be said that equilibration in shaker has more benefits on sperm motion traits than the other equilibration conditions. Plasma membrane and acrosome integrity is in charge of healthy semen production, and they are mostly overlooked. On the other hand, crystallization is archenemy for this parameter. So, semen freezing and thawing protocols, especially

equilibration, may be the most important step for this type of morphological integrity. Ahmad et al. (2015), measured the percentage of PMAI as 60%. In studies Anzar et al (2011) determined that 71% of frozen thawed bull sperm have intact plasma membrane and acrosome. On the other hand, Almadaly et al. (2014) found 91.6% of bull sperm with intact acrosome when 60% of those have intact plasma membrane determined by subjective method. In a research conducted by Nagy et al. (2003) with flow cytometer, the percentage of plasma membrane and acrosome integrity was found as 59.3% in frozen thawed bull semen. The PMAI results of the study were found in the same range as the previous studies mentioned above. Although the shaker method gave lower value than the others, there is no statistical significance between differences. In the study, it can be seen that equilibration of the bull semen in different conditions have no negative effects on plasma membrane and acrosomal status. Kinetic and structural qualities of spermatozoa are essential for prediction of fertility. However, DNA fragmentation analysis can be useful to differentiate infertility with normal spermatological characteristics (D'Occhio et al., 2007). Although, there is low correlation between DNA fragmentation and the current parameters, it is known that the sperm chromatin status can influence fertilization and embryonic survival rate (Bochenek et al., 2001). Sperm DNA fragmentation index is a valuable measurement for semen analyses and it has great potential to become part of the routine examination. DFI has low average in bulls while individual variations are disregarded. And it is realised that if DFI values are above 7% to 10%, fertility index will decrease (Karabinus et al., 1990; Karoui et al., 2012). In the study, DFI was between 5.58% and 6.53%. These values are below the subfertility limit and similar to bull DFI average. So, it can be said that the equilibration conditions have no detrimental effect in terms of DNA fragmentation.

CONCLUSIONS

In conclusion, according to these results, equilibrating bull semen in straws, cup or on shaker do not differ in terms of post-thaw bull sperm quality. In addition, equilibration on shaker can increase some sperm movement characteristics that are associated with in vivo and in vitro fertility. Finally, these three equilibration methods can be used in bull semen cryopreservation protocols conveniently. However, more studies, supported with the fertility datas, must be done in order to understand the effects of equilibration conditions in bull semen cryopreservation exactly.

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