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RESEARCH ARTICLE

Use of ultra-freezers of -152 °C as a viable alternative to liquid nitrogen in storing

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Depolamada sıvı nitrojene bir alternatif olarak -152°C ultra dondurucuların kullanımının araştırılması

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Öz

Amaç: Bu çalışmanın amacı, dondurulmuş spermanın -152 °C derin dondurucuda sıvı azot içinde depolanmasının geleneksel yöntemlerde kullanılan depolama yöntemine alternatif bir çözüm olup olmayacağının araştırılmasıdır.

Gereç ve Yöntem: Farklı günlerde iki boğadan toplam 80 payet toplandı. Aynı günde toplanan spermalar kontrol ve deney grubu olmak üzere iki farklı gruba ayrıldı. Çalışmanın deney grubunu bir hafta boyunca -152 °C'de saklanan spermalar oluştururken, kontrol grubunu ise geleneksel şekilde -196 °C'de saklanmış spermalar oluşturdu Deney süresinin sonunda donmuş spermalar, 30 saniye boyunca 37 °C su banyosunda çözdürüldü. Sonra CASA sistemi kullanılarak spermanın hareket parametreleri ve morfolojik özelikleri incelendi.

Bulgular: CASA sistemi kullanılarak yapılan değerlendirmenin sonucunda, -196 °C'de dondurulmuş ve saklanmış spermanın hareketi ve ilerleme hareketi (%69.66, %37.61) -152 °C'de (% 54.54,% 28.72) saklanan değerlerden daha yüksek bulundu (p <0.001). Hiperaktiviteye sahip spermaların yüzdesi -196 ve -152 gruplarında sırasıyla % 30.79 ve % 25.61 (p<0.05) olarak saptandı. Kinetik (hareket) değerler arasında istatistiksel bir fark olmadığı ve sayısal değerlere yakın olduğu görüldü (p>0.05). Anormal sperma oranı göz önüne alındığında, sıvı nitrojende saklanan spermlar için %32 ve -152°C'de buzdolabında saklanan spermanın %45 olduğu saptandı (p <0.001).

Öneri: Sperma değerlendirilmesinden sonra alınan verilerin istatistiksel analizi sonucunda, -152°C derin dondurucuda saklanan spermaların çözüm sonu spermatolojik parametrelerinin kabul edilebilir aralıklarda olduğu saptandı.

Anahtar kelimeler: Boğa sperması, depolama, -152°C derin dondurucu

Abstract

Aim: This study aims to investigate the utilization of the ultra-freezer to store the semen at a -152 °C as an alternative method to traditional semen storage methods

Materials and Methods: A total of 80 straws were collected from two bulls on different days. The semen collected on the same day were sorted into two different groups. The experimental group of the study was composed of sperm stored at -152 °C for one week, while the control group was composed of sperm stored at -196 °C using the traditional storage method. At the end of the experimental period, frozen semen at -196 °C and -152 °C were thawed at a temperature of 37 °C water bath for 30 seconds. Then, the spermatological parameters and morphological properties of the sperm were examined by using a computer-assisted sperm analysis system (CASA).

Results: The evaluation of semen samples yielded the following results; the motility and progressive motility of the frozen and stored sperm (69.66%; 37.61%) at -196 °C were higher than the values (54.54%; 28.72%) stored at -152 °C (p<0.001). The percentage of sperm with hyperactivity in group -196 and -152, was found to be 30.79% and 25.61%, respectively, (p<0.05). There was no statistical difference between kinetic values, and it was close to numerical values (p>0.05). Moreover, it was determined that the abnormal sperm rate was at 32% for the semen stored in liquid nitrogen and was 45% for the semen stored at -152 °C (p <0.001).

Conclusion: As a result of the experiment, it was determined that the post-thawing parameters of the sperms stored in -152 °C freezer were within acceptable ranges.

Keywords: Bull semen, storage, -152°C ultra -low temperature freezer

Introduction

The main objective of modern livestock breeding is to achieve maximum efficiency per animal. This aim can be achieved by preserving and spreading the genetic structures of the animals with high yields. One of the most important biotechnological methods used for this objective is the artificial insemination method. The main purpose of artificial insemination, whose history is dates back many years, is to ensure that offspring are obtained from males and females that are superior in terms of genetic character by eliminating the risk of infectious diseases. Many factors affect the success of artificial insemination. However, semen supply, freezing and storage of semen are the most important elements affecting the success of artificial insemination. Semen quality varies according to various criteria (Foote 1978).

Recent studies have shown limited important progress in the preservation of sperm. However, important information has been obtained regarding the effect the structure and function protection of spermatozoa. It is evident that protection greatly affects many sperm characteristics such as motility, respiratory activity, membrane status, and DNA quality. Thus, compared to fresh semen; the viability rate of frozen semen decreases, the transplantation in the female reproductive system decreases, the fertilization timing changes, and the post-fertilization embryo development is affected (Gillan et al 2004). Furthermore, it is thought that approximately 50% of the sperm is damaged due to the physical and chemical processes occurring during cooling, freezing, and thawing (Watson 2000). Many studies have reported changes in the membrane during cryopreservation. Additionally, cold shock during freezing and temperature shock during thawing have been shown to cause significant changes in the permeability of the plasma membrane (Ricker et al 2006).

For this reason, many studies on sperm freezing and storage were carried out using different animal species and are still being ongoing. Various methods have been tried and developed for long-term storage and preservation of sperm in various studies, conducted to date. Currently, it is believed that one of the best ways to store semen and preserve it for extended periods is to store it in liquid nitrogen (Akhter et al 2010). It is known that storage and storage conditions need to be eased and improved due to the increased need for frozen semen, for use in artificial insemination applications. Similarly, liquid nitrogen, nitrogen tanks, and technical support are among factors that cause semen storage costs to increase. Therefore, new studies are needed to overcome the difficulty of accessing liquid nitrogen to protect frozen sperms and to develop new alternative methods that can provide similar results to the traditional method. In this study, it has been proposed to use freezers that store at a temperature of -152 ° C, whose temperature is close to the liquid nitrogen (-196 °C) used in traditional methods (Watson 2000). It is believed that these freezers will increase storage capacity, reducing the risk of accidents in liquid nitrogen use, as well as reducing the cost of liquid nitrogen supplements.

This study aims to investigate the utilization of the ultra-freezer to store semen at -152 °C as an alternative method to traditional semen storage methods in terms of some spermatological parameters.

Material and Methods

Animal material

Imported semen of 40 Holstein and 40 Simmental breeds were used for the study.

Freezing and storage

Semen with the same production date for each breed was divided into two groups, to compare the storage conditions of frozen semen. A total of 40 semen straws were kept for one week at -152 °C, while other straws with the same production date were stored in a liquid nitrogen tank.

Evaluation of the sperm

0.5 µl thawed semen belonging to these groups were dropped on a slide, the coverslip was closed and analysed in the (CASA) system integrated with a heated plate to evaluate the motility and kinetic parameters of the semen in each group (Inanç et al 2018a). Sperm morphology kit (Sperm Blue ®, Microptics ®, Spain) was used to evaluate the abnormal spermatozoa ratio of sperm belonging to each group. The investigation was made in Computerized Sperma Analyser (CASA), Sperm Class Analyser (SCA ® v.4.2) under a bright field microscope with a magnification of 600 x. For abnormal spermatozoa examination from the thawed straws belonging to each group; 10 µl of sperm was dropped and smeared on a glass microscope slide, then left to air-dry. The dried preparation was placed horizontally on the dyeing tray, and the fixation solution was applied to it. Preparation was left for 10 minutes for fixation. Then, fixation fluid was carefully removed from the preparation, and the preparation was stained by sperm blue for 15 minutes. Finally, the sperm blue was removed, and the cover glass was placed on the slide. The prepared preparation was evaluated to examine at least 200 spermatozoa in the (CASA) system (Inanç et al. 2018b).

Statistical analysis

Descriptive statistics for the data were calculated. Paired Sample t-test was used to examine the differences between temperatures in terms of measurements. The ($p<0.05$) criterion was used in the evaluation of the data. SPSS 14.01 (Sta-

tistical Package for the Social Sciences) software program was used for statistical analysis.

Results

The motility and progressive motility of the frozen and stored sperm at -196 °C (69.66±2.08, 37.61±1.6) were higher than the values (54.54±2.24, 28.72±1.6) stored at -152 °C (p<0.001). The hyperactivity in group -196 and -152, was determined as; 30.8±1.6and 25.61±1.4, respectively (p<0.05) (Table 1).

It was observed that the values of straight line velocity (VSL), curvilinear velocity (VCL), and velocity average path (VAP) rates were similar for frozen and stored sperm at -196 °C (55.94 ± 1.72, 96.30 ± 1.43, 71.08 ± 1.58). For semen stored

Table 1. The Casa Motility and Kinetic parameters of the sperm samples stored at -196 ºC and -152 ºC

Parameters	Temperature	n	Mean	SEM	Std. Deviation	P
Motility %	-196	40	69.660	2.088	13.207	<0.001
	-152	40	54.543	2.242	14.180	
Progressive %	-196	40	37.6	1.6	10.0	<0.001
	-152	40	28. 7	1.6	10.1	
VCL (µm/s) (Micrometer / second)	-196	40	96.304	1.438	9.094	0.149
	-152	40	93.332	1.548	9.788	
VSL (µm/s) (Micrometer / second)	-196	40	55.946	1.720	10.876	0.408
	-152	40	53.769	1.705	10.786	
VAP (µm/s) (Micrometer / second)	-196	40	71.083	1.580	9.991	0.137
	-152	40	67.443	1.588	10.045	
LIN %	-196	40	57.908	1.387	8.773	0.691
	-152	40	57.203	1.112	7.033	
STR %	-196	40	78.190	0.908	5.741	0.571
	-152	40	78.839	0.712	4.501	
WOB %	-196	40	73.680	0.978	6.184	0.282
	-152	40	72.300	0.808	5.113	
ALH (µm) (Micrometer)	-196	40	3.390	0.076	0.481	0.386
	-152	40	3.448	0.054	0.341	
BCF (Hz) (Hertz)	-196	40	8.605	0.124	0.784	0.322
	-152	40	8.777	0.140	0.884	
Hyperactive %	-196	40	30.8	1.6	0.099	0.002
	-152	40	25.6	1.4	0.089	

ALH: Amplitude of lateral head displacement, BCF: Beat cross frequency, VAP: Velocity average path, VCL: Curvilinear velocity, VSL: Straight line velocity, WOB: Wobble
LIN: Linearity of movement, STR: Straightness

at -152 °C, the values of the kinetic parameters were determined as (53.76 ± 1.70, 93.33±1.54, 67.44±1.58) (p>0.05). Also, It was observed that, despite there was no statistical difference between kinetic (activity) values, the numerical values were close in Amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and wobble (WOB) (3.39 ± 0.076, 8.60 ± 0.12, 73.68 ± 0.978) for group -196 and; (3.44 ± 0.054, 8.77 ± 0.14, 72.30 ± 0.808) for group -152 (p>0.05) (Table 1).

Abnormal spermatozoa ratio for sperm stored in liquid nitrogen (-196) and sperm stored in the freezer at -152 °C were; 32.0±2.1 and 45.2±2.2, respectively (p<0.001).

Furthermore, the head, mid-piece, and tail abnormality values, which are among the formal characteristics of semen, were recorded as 10.77 ± 0.57, 11.38 ± 0.65, 8.66 ± 0.56, respectively, for the sperm stored in the ultra-freezer. The values were recorded as 8.61 ± 0.56, 6.21 ± 0.52, 5.93 ± 0.48, respectively, for the sperm stored in liquid nitrogen.
While there was no statistical difference between the acrosome abnormality observed in spermatozoon between storage temperatures (p>0.05); Abnormalities due to the cytoplasmic droplet, head, mid-piece, and tail were found statistically significant (p<0.001) (Table 2).

Discussion

Techniques such as dry freezing (desiccation and freeze-drying) that were studied in recent years and applied to sperm, are a possible alternative to the traditional method (Holt 1997 and Ward et al 2003). Nevertheless, the use of freezers that can drop to temperatures as low as -152 ° C, close to the temperature of -196 °C of liquid nitrogen is considered as an alternative method (Medrano et al 2002).

In a recent study, sperm stored at - 80 °C for a period of one month provided was observed to yield similar results to those stored in liquid nitrogen (Buranaamnuay et al 2016). The results of the aforementioned study differ from the results obtained by this study. It believed that the differences in results of the storage in ultra-freezer (-152 °C) and liquid nitrogen (-196 °C) was due to the alteration in the storage time. According to the fertility results, the post-inseminating pregnancy rates in females were found to be similar to those rates, when using sperm stored in -196 °C liquid nitrogen and dry ice with -79 °C (Larson and Graham 1958 and Macpherson 1960). The rates in this study were found to be different when using sperm stored in the ultra-freezer at -152 °C compared to those stored at -196 °C liquid nitrogen. A decrease

Table 2. The Casa Abnormal Parameters of the sperm samples stored at -196 ºC and -152 ºC

Parameters	N	Mean	SEM	Std. Deviation	P
NORMAL -152 %	40	55.4	2.2	13.7	<0.001
NORMAL -196 %	40	67.8	2.0	12.4	
ABNORMAL -152 %	40	45.2	2.2	13.8	<0.001
ABNORMAL -196 %	40	32.0	2.1	13.1	
ACROSOME -152 %	40	5.600	0.367	2.324	0.337
ACROSOME -196 %	40	5.213	0.380	2.404	
CYTOPLASMIC D -152 %	40	7.813	0.417	2.638	<0.001
CYTOPLASMIC D -196 %	40	6.250	0.489	3.093	
HEAD -152 %	40	10.775	0.570	3.604	<0.001
HEAD -196 %	40	8.613	0.569	3.596	
TAIL -152 %	40	11.388	0.653	4.132	<0.001
TAIL -196 %	40	6.213	0.528	3.338	
MID-PIECE -152 %	40	8.663	0.562	3.556	<0.001
MID-PIECE -196 %	40	5.938	0.489	3.095	
ONE DEFECT -152 %	40	20.275	1.094	6.922	<0.001
ONE DEFECT -196 %	40	14.600	0.970	6.134	
MULTI-DEFECT -152 %	40	23.838	1.137	7.192	<0.001
MULTI-DEFECT -196 %	40	17.525	1.222	7.731	

was observed in the sperm parameters (motility and abnormality) that were stored in the freezer, compared to liquid nitrogen. It is thought that the factors causing decrease are due to the difference in animal species and storage time that was used in both studies. For this purpose, a desire to search for new methods that can be used as an alternative to traditional freezing and storage methods are needed for the development of new technologies that would replace traditional methods (Alamo 2005). In recent years, techniques such as dry freezing (drying and freeze-drying) applied to semen are thought to be an alternative to the traditional method (Holt 1997 and Ward 2003). Deep freezers with temperatures that can drop to -152 °C are offered as an alternative to the traditional method (Medrano et al 2002). The temperatures of those freezers do not differ from the temperature of -196 °C of liquid nitrogen. In order to determine, whether this type of storing can be used as a long-term sperm bank or as a short-term warehouse, it is necessary to examine long-term cryopreservation.

In this study, semen was used from two different bulls on different dates, and frozen in liquid nitrogen between 2012 and 2014 and then stored in an ultra-freezer. The motility, morphologic qualities, as well as normal and abnormal sperm ratios of sperms stored at -152 °C were investigated and evaluated. The study showed that, the motility rate of the sperm stored at -152 °C was found as 54.54 ± 2.24. This rate is close to the motility rate of 55% obtained in four protocols in the study conducted by Yavaş and Daşkın (2012). This result is also similar to the rate (%57-75) found by Batista et al (2006) after storing at -152 ° C at three different periods (60 days, 120 days, 365 days). However, the rate found in this study is different from the rates of the study done by Medrano et al (2002) Obtained after two-days and two-month storage at -150 °C (33%, 44.8% respectively).

The reasons for the decrease observed in the values in this experiment, are believed to be the differences in storage time and accepted temperature. The values of (VSL) , (VCL) and (VAP) rates for semen frozen and stored at -196 °C were found (55.94 ± 1.72, 96.30 ± 1.43, 71.08 ± 1.58) respectively. It is considered that when sperm stored at -152 ° C, the values of the sperm kinetic parameters decrease (53.76 ± 1.70, 93.33 ± 1.54, 67.44 ± 1.58) sighting the decrease or increase in the storage temperature. The numerical values of the kinetic parameters were similar for both storage temperatures in Amplitude of (ALH), beat cross frequency (BCF), and WOB (3.39 ±0.076, 8.60 ±0.12, 73.68 ± 0.978).

In this study, using two different storage methods (ultra-freezer at -152 °C, liquid nitrogen -196 °C), the rate of abnormal semen showed an increase of 13% after storage in the ultra-freezer (- 152 °C) (ratio in liquid nitrogen is 32 ± 2.1, and rate in ultra-freezer is 45±2.2). The reason for this increase is attributed to the increase in the temperature of the samples taken during storage.

When compared in terms of sperm abnormality, the results obtained in this study were found similar to the results of the study on goat sperm done by Medrano et al (2002). In the study of Medrano et al (2002) it was observed that when the sperm was stored at -152 °C for two days, the abnormality rate of the sperm reached 46%. Additionally, it was found that it reached 45% when it stored for two months (60 days) at the same temperature (Medrano et al 2002). The results of this study are different from the results obtained by Yavaş and Daşkın (2012), who studied using four different protocols mentioned earlier. In the study of Yavaş and Daşkın (2012), the rate of abnormality ranged between 19% and 22% after storing sperm in 3 different periods (1 week - 2 months - 6 months). The reason for this difference arises from the difference in storage time-examined between the two studies.

The abnormality in the study was much higher compared with the results (the abnormality ranged between 9.7% and 10%) in the study performed by Batista (2006) on 5 dogs in 3 different periods (2 months-4 months-one year) using the same storage temperature (-152 °C). The reason for that difference can be explained as the difference in the animal material or length of the time of the storage. The study showed that, the length of the head, the body part, and tail values of the sperm were recorded as %10.77, %11.38, %8.66, respectively, after storage in an ultra-freezer. These values were recorded as %8.61, %6.21, and %5.93 after storing the sperm in liquid nitrogen. It has been determined that the rates of acrosome disorder are similar to each other in both storage methods (Table 2). The reason for the increase of abnormality in sperm organelles can be explained by the 44-degree difference in the temperature.

Until today, no scientific studies investigating the storage of sperm in liquid nitrogen and storage at -152 °C have been found in literature. With that in mind, it can be said that this is the first research of its kind, done on this subject. Nonetheless, a similar study was carried out on bulls using a freezer that provides cooling, freezing, and storage at -152 °C (Yavaş and Daşkın 2012).

According to statistical analysis, there are no significant differences in semen parameters between the four groups of bulls. Moreover, it was observed that there were no significant differences in the freezing periods between the groups. Similarly, there was no decrease in semen movements or an increase in dead and abnormal semen. In this study, it was determined that using the -152 °C freezer for freezing and storage of sperm for 6 months as an alternative to liquid nitrogen is believed to provide an ideal environment for freezing and storing bull semen (Yavaş and Daşkın 2012).

Conclusion

This study was performed to evaluate the use of freezers at -152 °C as an alternative for storing frozen sperm at -196 °C. Despite the simplicity and mechanical reliability of the use of liquid nitrogen in sperm storage, liquid nitrogen-based systems face several problems such as; temperature changes, need for regular nitrogen support, high cost of samples and contamination. Thus, there is a need to find alternative method to avoid classic technical complications.

It was observed that the sperm values stored at -152 °C in the freezer contrasted from the sperm values stored in liquid nitrogen at -196 °C. It was determined that frozen bull semen has usable limits after storage and thawing in ultra-freezers. However, more studies are required to further investigate this subject in the future.

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Conflict of Interest

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During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study or no moral support.

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Ethical Approval

Approval of the study was obtained from Ankara University,Animal Experiments Local ethics Committe (29.11.2017 - 2017/24/191) Number Ethics Committee Decision

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