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Short Communication / *Kısa Bilimsel Çalışma*

Effect of alternative cryopreservation procedures on bull semen*

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Summary: The aim of this study was to assess the use of - 152°C ultra freezer for freezing and storing bull semen as an alternative to freezing over the liquid nitrogen vapor and storing in liquid nitrogen in terms of certain post-thaw spermatological parameters. Two ejaculates, one per each bull, were used without pooling. Four different freezing and storing protocols were tested. At 1 week, 2 months and 6 months after cryopreservation, the frozen semen was thawed and the seminal parameters were determined. As a conclusion, this study has confirmed that the use of -152 °C ultra-low freezer for freezing and storing bull semen for 6 months is a viable alternative to liquid nitrogen.

Key words: Bull, cryopreservation, semen, storage, ultra-low freezer.

Alternatif Dondurma ve Saklama Yöntemlerinin Boğa Sperması Üzerine Etkisi

Özet: Bu çalışmanın amacı, boğa spermasının -152 °C derin dondurucuda dondurulup saklanmasının sıvı azot buharında dondurma ve sıvı azotta saklamaya çözüm sonu kimi spermatolojik parametreler bakımından bir alternatif olup olmayacağının araştırılmasıdır. Bu çalışmada iki adet boğanın birer ejakulatı miks yapılmadan kullanıldı. Araştırmada dört farklı dondurma ve saklama protokolü denendi. Dondurma işleminden bir hafta, iki ve altı aylık periyotlar sonrasında payetler çözdürüldü ve çözüm sonu bazı spermatolojik özellikler değerlendirildi. Sonuç olarak bu çalışma -152°C derin dondurucunun boğa spermasının dondurulması ve altı ay süreyle saklanmasında sıvı azota bir alternatif olarak kullanılabilirliğini ve boğa spermasının dondurulması ve saklanmasında iyi bir ortam sağladığını doğrulamaktadır.

Anahtar sözcükler: Boğa, derin dondurucu, dondurma, saklama, sperma.

Bull semen can be frozen in pellets, ampoules or straws and then stored in liquid nitrogen (13). In addition to the traditional method for freezing semen in straws, freezers operating with liquid nitrogen are also used (2,3,4,11). However, with the changes in life sciences, liquid nitrogen-based systems have faced with some problems, the most important two being the contamination risk and temperature change up to 90 °C (4,6,7,8,10). Therefore, there is a need to investigate feasible alternatives for semen cryopreservation which can prevent the complications of traditional methods (2). However, there are also other studies confirming that another viable alternative may be the use of ultra-low freezers at -152 °C, not too far from liquid nitrogen temperature at -196 °C (2,3,4,11). It remains to be established mechanical freezers which can provide air phase at -150°C without the need for liquid nitrogen can prevent cross-contamination of germ-plasm over a long period of storage (6,7). The aim of this experiment was to assess the use of an ultra-low freezer at -152 °C for freezing and storage of bull semen for 6 months. Post-thawing seminal parameters were evaluated to see whether an ultra-low freezer at -152 °C could be a feasible alternative to cryopreservation in liquid nitrogen.

Two Holstein bulls were used in this experiment. Semen was collected using an artificial vagina. The ejaculates from each bull were not pooled but processed individually. Following semen collection the semen volume, concentration, pH, sperm motility and the percentages of abnormal and dead sperm cells were determined. Following the seminal analysis, the ejaculates were diluted with Bioxcell, packaged in 0.25 ml plastic straws (9) and equilibrated. Following equilibration, the sperm motility and the percentages of dead spermatozoa and abnormal spermatozoa rate were determined. Four different freezing and storing protocols were tested: I- Straws were frozen over the liquid nitrogen vapor and then plunged into and stored in liquid nitrogen. II- Straws were frozen over liquid nitrogen vapor, plunged into liquid nitrogen and then stored in

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ultra-low freezer at -152 °C. III- Straws were frozen and stored in ultra-low freezer. IV- Straws were frozen in ultra-low freezer and stored in liquid nitrogen. In the first protocol, following the equilibration, semen was frozen over the liquid nitrogen vapor at -120 to -140 °C for 10 minutes and then plunged into and stored in liquid nitrogen at -196 °C. In the second protocol, the equilibrated semen was frozen over the liquid nitrogen vapor at -120 to -140 °C for 10 minutes and plunged into liquid nitrogen. The straws were then moved from liquid nitrogen to ultra-low freezer at -152 °C to be stored. In the third protocol, semen was frozen and stored in ultra-low freezer. In the forth protocol, semen was frozen in ultra-low freezer at -152 °C for 10 minutes and then frozen straws were moved to liquid nitrogen container to be stored. Frozen semen was stored in ultra-low freezer and liquid nitrogen for six months. Thawing was carried out at 1 week, 2 months and 6 months after cryopreservation. 10 frozen straws per each protocol were plunged into water bath at 37 °C for 20 seconds. After thawing, the sperm motility and the percentages of abnormal and dead sperm cells were determined. Results are presented as mean ± standard error of the mean (SEM). Data were analyzed using the general linear model procedure (ANOVA) of SPSS 15.0. Values were considered to be statistically significant when $p < 0.05$.

Spermatological parameters of fresh semen of donor bulls are presented in Table1. Evaluation of sperm characteristics of fresh semen showed similar mean values compared to those obtained in other studies (1,12) (Table1). After equilibration and before freezing, the semen samples were analyzed in terms of sperm motility and percentages of dead spermatozoa and abnormal spermatozoa (Table 2). The percentages of sperm motility (mean ± SEM) observed in bull 1 and bull 2 throughout the experimental period are shown in Table 3. The mean percentages of sperm motility of donor bulls did not show any significant differences in four freezing protocols (LN-LN, LN-ULF, ULF-ULF and ULF-LN) at 1 week, 2 months and 6 months after cryopreservation (p>0,05) (Tablo 3). Table 4 shows the percentages (mean ± SEM) of dead spermatozoa observed in two bulls throughout the experimental period. The mean percentages of dead spermatozoa of donor bulls did not show any significant differences in four freezing protocols (LN-LN, LN-ULF, ULF-ULF and ULF-LN) at 1 week, 2 months and 6 months after cryopreservation (p>0,05) (Table 4). The percentages of abnormal

Table 1 : Spermatological parameters of fresh semen of donor bulls.
Tablo 1: Nativ boğa spermalarında belirlenen spermatolojik parametreler.

Bull	Volume (mL)	Motility (%)	Concentration 10^9/mL	Abnormal spermatozoa (%)	Dead spermatozoa (%)	pH
1	4.0	80.0	1.29	11	15	6.2
2	4.5	80.0	1.27	13	17	6.4

Table 2: Spermatological parameters after equilibration(2 hours in +4°C)(X ± Sx; n=7).
Tablo 2: Alışım (+4°C'de iki saat) sonrası spermatolojik parametreler (X ± Sx; n=7).

Bull	Motility (%)	Dead spermatozoa (%)	Abnormal spermatozoa (%)
1	77.14 ± 2.67	16.28 ± 1.80	13.85 ± 1.35
2	76.42 ± 3.78	17.28 ± 1.11	14.71 ± 1.50

Table 3: Percentages of sperm motility after thawing in four freezing protocols at 1 week, 2 months and 6 months after cryopreservation (X ± Sx; n = 10).
Tablo 3: Dondurma işleminden bir hafta, iki ay ve altı ay sonra elde edilen çözüm sonu motilite değerleri (X ± Sx; n = 10).

Experimental periods	Bull	Group 1 (LN-LN)[a]	Group 2 (LN-ULF)[b]	Group 3 (ULF-ULF)[c]	Group 4 (ULF-LN)[d]
Day 7	1	54.00 ± 6.15	54.50 ± 5.99	55.50 ± 3.69	55.00 ± 4.08
	2	52.00 ± 5.37	51.00 ± 4.59	52.50 ± 5.40	50.00 ± 3.33
Day 60	1	55.00 ± 4.71	55.50 ± 4.97	56.00 ± 4.59	54.00 ± 3.94
	2	51.50 ± 4.12	50.50 ± 4.97	51.00 ± 4.59	51.50 ± 4.12
Day 180	1	54.50 ± 4.97	55.50 ± 6.85	55.50 ± 5.50	55.00 ± 4.08
	2	51.00 ± 4.59	50.00 ± 3.33	51.50 ± 5.30	50.50 ± 4.97

[a] LN-LN: freezing and storing in liquid nitrogen
[b] LN-ULF: freezing in liquid nitrogen and storing in ultra-low freezer
[c] ULF-ULF: freezing and storing in ultra-low freezer
[d] ULF-LN: freezing in ultra-low freezer and storing in liquid nitrogen

Table 4 : Percentages of dead sperm cells of after thawing in four freezing protocols at 1 week, 2 months and 6 months after cryopreservation (X ± Sx; n = 10).
Tablo 4: Dondurma işleminden bir hafta, iki ay ve altı ay sonra elde edilen çözüm sonu ölü spermatozoa yüzdeleri (X ± Sx; n = 10).

Experimental periods	Bull	Group 1 (LN-LN)[a]	Group 2 (LN-ULF)[b]	Group 3 (ULF-ULF)[c]	Group 4 (ULF-LN)[d]
Day 7	1	32.80 ± 2.10	31.20 ± 1.69	33.80 ± 2.82	33.60 ± 3.57
	2	34.50 ± 2.79	33.30 ± 3.34	34.10 ± 4.75	34.80 ± 2.90
Day 60	1	31.80 ± 1.62	31.30 ± 2.41	32.40 ± 2.07	33.00 ± 3.80
	2	32.20 ± 3.08	32.90 ± 4.01	35.10 ± 3.14	33.70 ± 3.23
Day 180	1	31.30 ± 2.11	30.30 ± 1.89	31.50 ± 2.55	32.10 ± 3.38
	2	33.80 ± 3.36	33.00 ± 3.59	32.30 ± 3.27	32.90 ± 2.69

[a] LN-LN: freezing and storing in liquid nitrogen
[b] LN-ULF: freezing in liquid nitrogen and storing in ultra-low freezer
[c] ULF-ULF: freezing and storing in ultra-low freezer
[d] ULF-LN: freezing in ultra-low freezer and storing in liquid nitrogen

Table 5: Percentages of abnormal sperm cells of after thawing in four freezing protocols at 1 week, 2 months and 6 months after cryopreservation (X ± Sx; n = 10).
Tablo 5: Dondurma işleminden bir hafta, iki ay ve altı ay sonra elde edilen çözüm sonu anormal spermatozoa oranları (X ± Sx; n = 10).

Experimental periods	Bull	Group 1 (LN-LN)[a]	Group 2 (LN-ULF)[b]	Group 3 (ULF-ULF)[c]	Group 4 (ULF-LN)[d]
Day 7	1	21.80 ± 1.81	21.10 ± 1.20	20.90 ± 1.79	19.80 ± 2.15
	2	22.10 ± 2.13	20.90 ± 1.37	21.30 ± 1.83	21.30 ± 1.89
Day 60	1	22.00 ± 1.56	20.40 ± 2.37	20.10 ± 1.52	20.30 ± 1.83
	2	21.30 ± 2.63	21.30 ± 2.00	21.10 ± 1.29	22.90 ± 1.91
Day 180	1	21.30 ± 2.16	20.80 ± 1.87	21.80 ± 1.69	19.70 ± 1.64
	2	22.00 ± 1.56	21.90 ± 2.13	21.80 ± 1.69	21.40 ± 1.58

[a] LN-LN: freezing and storing in liquid nitrogen
[b] LN-ULF: freezing in liquid nitrogen and storing in ultra-low freezer
[c] ULF-ULF: freezing and storing in ultra-low freezer
[d] ULF-LN: freezing in ultra-low freezer and storing in liquid nitrogen

spermatozoa (mean ± SEM) observed in donor bulls throughout the experimental period are shown in Table 5. No significant differences were found between the four freezing protocols (LN-LN, LN-ULF, ULF-ULF and ULF-LN) at 1 week, 2 months and 6 months after cryopreservation (Table 5). Finally, According to the statistical analysis, no significant difference was observed between the four groups in the two bulls in terms of seminal parameters (p > 0,05). In addition the sperm motility and percentages of dead and abnormal spermatozoa for each individual bull did not change significantly in four groups at 1 week, 2 months and 6 months after cryopreservation, showing that storage time neither decreased sperm motility nor increased dead and abnormal sperm cells. As a conclusion, this study has confirmed that the use of -152 °C ultra-low freezer for freezing and storing bull semen for 6 months is a viable alternative to liquid nitrogen.

This study assesses, for the first time, the use of ultra-low freezer at -152 °C for the cryopreservation of bull semen. However, there are a few studies investigating the ultra-low freezer at -152 °C for freezing and storing canine and goat semen (2,3,4,11).One of the studies on the cryopreservation of canine semen assessed the post-thawing seminal parameters obtained by similar protocols including the use of ultra-low freezer at -152 °C and liquid nitrogen, finding no significant differences between both protocols in terms of sperm motility and percentages of dead and abnormal spermatozoa (2). When compared, the results of our study also detected no statistically significant differences between the similarly applied protocols (freezing over liquid nitrogen vapor and storing in liquid nitrogen, freezing over liquid nitrogen vapor and storing in ultra-low freezer, and freezing and storing in ultra-low freezer) in terms of sperm motility and percentages of dead and abnormal spermatozoa. In another study on the cryopreservation of canine semen, semen was frozen in ultra-low freezer at -152 °C and liquid nitrogen, and stored for 12 months, reporting no significant differences between cryopreservation in the two freezing protocols in terms of sperm motility and percentages of live and abnormal spermatozoa (3). Similarly, our study on the cryopreservation of bull semen detected no significant differences between the ultra-low freezer and liquid nitrogen protocols in terms of seminal parameters at 6

months after cryopreservation (motility and percentages of dead/live and abnormal spermatozoa). In another study (11), semen taken from bucks were frozen and stored using liquid nitrogen and ultra-low freezer at -152 °C to compare sperm cryosurvival between freezing methods. The results of that study showed similar mean values for the freezing methods at 2 days and 2 months after cryopreservation. Similarly, the findings of our study showed no significant differences between the two protocols in terms of seminal parameters at 1 week and 2 months after cryopreservation. In another study on the cryopreservation of goat semen in ultra-low freezer at -152 °C (4), the in vitro quality of semen was reported to be not modified after cryopreservation for up to 1 year. Our study on bulls also confirmed that ultra-low freezer did not change the post-thawing parameters observed at 6 months after cryopreservation. The results of this study confirmed that the use of ultra-low freezers at -152 °C for freezing and storing bull semen for 6 months is a viable alternative to cryopreservation in liquid nitrogen. However, before recommending the routine use of this freezer for long-term cryopreservation as semen banks, further studies should be done to assess cryopreservation for 1 year and more. It is also necessary to assess the fertility of bull semen frozen and stored in ultra-low freezer.

References

1. **Ak K**.(1994): *Spermanın muayenesi. Reprodüksiyon ve Suni Tohumlama*.İstanbul Üniversitesi Veteriner Fakültesi Masaüstü Yayımcılık Ünitesi, Ders Notu No:23, 6. Bölüm.
2. **Alamo D, Batista M, González F, Rodrígez N, Cruz G, Cabrera F, Garcia A.** (2005): *Cryopreservation of semen in the dog: use of ultra freezers of -152°C as a viable alternative to liquid nitrogen.* Theriogenology, **63**, 72-82.
3. **Batista M, Alamo D, González F, Cruz MG, Gracia A**. (2006): *Influence of the freezing technique (nitrogen liquid vs ultrafreezer of -152°C)andmale-to-male variation over the semen quality in Canarian Mastiff breed dogs*. Reprod Domest Anim, **41**, 423-8.
4. **Batista M, Niño T, Alamo D, Castro N, Santana M, González F, Cabrera F, Gracia A.** (2009): *Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed*. Theriogenology, **71**, 1307-15.
5. **Bielanski A, Bergeron H, Lau PCK, Devenish J**.(2003): *Microbial contamination of embryosandsemenduring long termbankinginliquid nitrogen*. Cryobiology,**46**,146-52.
6. **Bielanski A**. (2005): *Non-transmission of bacterial and viral microbes to embryos and semen stored in the vapour phase of liquid nitrogen in dry shippers*. Cryobiology, **50**, 206-10.
7. **Burden DW.** (1999): *Issues in contamination and temperature variation in the cryopreservation of animal cells and tissues.* Application Note:99-08 Erişim Adresi: http://www.revco-sci.com/documents/Cryo%20Paper.doc. Erişim Tarihi: 12.01.2009.
8. **Fountain D, Ralston M, Higgins N, Gorlin JB, Uhl L, Wheeler C, Antin JH, Churchill WH, Benjamin RJ.**(1997): *Liquid nitrogen freezers: a potential source of microbial contamination of hematopoietic stem cell components*. Transfusion, **37,** 585-91.
9. **Hansen D** (2009): *Boğa spermasının sulandırılması ve dondurulması aşamaları*. Erişim Adresi: http://www.sarkkemikal.com/hayvansagligi/pdf/spermahazirlama.pdf, Erişim Tarihi: 12.01.2009.
10. **Könirsch HL, Collin C, Sifer C, Devaux A, Kuttenn F, Madelenat P, Brun-Vezinet F, Feldmann G, Benifla J-L**. (2003): *Safety of cryopreservation straws for human gamete or embryos: a study with human immunodeficiency virus-1 under cryopreservation conditions.* Human Reproduction, **18**,140-4.
11. **Medrano A, Cabrera F, González F, Batista M, Gracia A.** (2002): *Is sperm cryopreservation at –150°C a feasible alternative?* Cryoletters, **23,** 167-172.
12. **Özkoca A.** (1984): *Çiftlik hayvanlarında reprodüksiyon ve suni tohumlama.* İ Ü Vet Fak Yay.
13. **Sevinç A.** (1984): *Dölerme ve suni tohumlama*. Ankara Üniversitesi Veteriner Fakültesi Yayınları: 397, Third Edition.

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