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THE EFFECTS OF THAWING TIME, POST-THAWED THERMAL APPLICATIONS AND RESISTANCE TEST ON SEMEN CHARACTERISTICS IN BULLS*

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Boğalarda Eritme Süresi, Eritme Sonrası Isı Uygulamaları ve Dayanıklılık Testinin Spermatolojik Özelliklere Etkileri

Özet: Sunulan çalışmada, kısa ve uzun süreli eritme teknikleri ile erime sonrası uygulanan ısı değişiklikleri arasındaki ilişkinin ortaya konması amaçlandı. Çalışmada siyah alaca ırkı 4 baş boğaya ait 0.25 ml.'lik payetlerde dondurulmuş sperma örnekleri kullanıldı. Payetler 37°C su banyosunda 12 ve 30 saniyelik sürelerle eritilerek 2 ayrı eritme tekniği (A ve B grupları) ve her bir teknik için 6 alt grup oluşturuldu. Alt gruplardan birinci gruptaki spermalar kontrol grubunu oluşturdu ve eritme sonrası hiç bir işlem yapılmaksızın spermatolojik testler uygulandı. İkinci gruptakiler eritme sonrası 2±1°C su banyosunda 300 saniye tutuldu. Üçüncü ve dördüncü gruplardaki örnekler eritmeyi takiben sırasıyla 45 ve 150 saniye sürelerle 5°C çevre ısına maruz bırakıldı. Beşinci ve altıncı gruplardakiler ise eritmenin ardından 20°C çevre ısısına sırasıyla 45 ve 150 saniye süreyle bırakıldı. Spermatolojik incelemeler (motilite, akrozomal ve membran bütünlüğü), yukarıdaki ısı uygulamalarını takiben gerçekleştirildi. Sonra sperma örnekleri, tamponlanmış modifiye hepes ile sulandırılarak 37°C'deki etüvde 2 saat inkübe edildi. İnkübasyonu takiben motilite ve akrozomal bütünlük incelemeleri tekrarlandı.

İnkübasyon öncesi ve sonrasındaki veriler incelendiğinde, motilite, membran bütünlüğü ve morfolojik anomalilerde her iki eritme tekniğinde de farklılık bulunmamıştır. Tek fark inkübasyon sonrasında 20°C'de 45 saniye bekletilen A5 ve B5 eritme grupları arasında, motilite değerlerinde tespit edilmiştir. Eritme sonrası belirtilen koşullar altında, 12 saniyelik eritme sonrası (A5) sperma motilitesi, 30 saniyelik (B5) eritmeye kıyasla önemli derecede daha yüksek bulunmuştur (P<0.01).

Potansiyel fertilitenin belirlenmesinde kullanılan rutin testlerin inkübasyon sonrasında tekrarlanması yararlı olabilir. Eritme işleminin tamamlanmasından sonra tohumlama mümkün olduğu kadar kısa sürede gerçekleştirilmeli ve bu süreye 20°C'nin altındaki çevre ısılarında daha da dikkat edilmelidir. Daha kısa teknik olan 12 saniyede eritme, özellikle hava sıcaklığının 20°C ve daha altında olduğu günlerde rahatlıkla kullanılabilir.

Anahtar Kelimeler: Boğa sperması, eritme süresi, soğuk şoku, ozmotik şok, HOST, dayanıklılık testi

Abstract: The aim of the present study was to demonstrate the relationship between short- and long-thawing procedures and post-thawing thermal conditions. Semen samples of 4 Holstein bulls, frozen in straws of 0.25 ml, were used. Straws were thawed in water baths at 37°C using two different thawing techniques, the 12- (Group A) and 30-second (Group B) thawing, and 6 subgroups (A1-A6 / B1-B6) were established for each technique. Semen samples of the first subgroup were used as control, which were subjected to spermatological tests without any post-thawing processes. Those in the second group were kept in 2±1°C water bath for 300 seconds after thawing. Following the thawing, samples in the third and the fourth groups were kept at 5°C for 45 and 150 seconds, respectively. Samples in the fifth and the sixth groups were also kept at 20°C for 45 and 150 seconds, respectively. Spermatological evaluations (motility, acrosomal and membrane integrity) were carried out after the applications. Then, in order to perform resistance test, the semen samples were diluted with modified buffered hepes and incubated at 37°C for 2 hours. Following the incubation, motility and acrosomal integrity were re-evaluated.

No significant difference was observed between two different thawing techniques, with respect to all criteria, prior to and after the incubation. The only exception was found in the motility in groups A5 and B5 kept at 20°C for 45 second post-incubation. When thawed semen is kept under the mentioned thermal conditions; sperm motility at 12-second technique (A5) is higher than the 30-second technique (B5, P < 0.01).

Following the incubation, repetition of routine tests used to determine the potential fertility may be useful. Special attention should be paid in performing the artificial insemination immediately after the thawing, especially at seasonal temperatures below 20°C. The shorter technique of thawing in 12 seconds can be easily used especially at 20°C and lower temperatures.

Key words: Bull semen, thawing time, cold shock, osmotic shock, HOST, resistance test

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Introduction

In the artificial insemination procedure, during the thawing and after the insemination process, semen is exposed to different temperatures and osmotic pressures. Exposure of semen to sudden temperature changes causes cold shock, which negatively influences the motility and morphology of spermatozoa (**14, 23, 24**). Additionally, bull semen, frozen in hypertonic solutions, is subject to different osmotic pressures during the thawing process and in the fluids of the female genital tract after the insemination process. Osmotic shock decreases the vitality potential of spermatozoa and causes membrane defects (**7, 8, 10, 14**).

It has been reported that different thawing methods provide a gradual reversion to pre-freezing conditions, which can minimize membrane defects and sudden changes during osmotic pressure alterations (**9**). Some authors have reported that osmotic stress can be reduced and membrane integrity can be protected after thawing by using of different thawing methods (**4, 7**).

Damage to semen, caused by possible thermal changes after thawing, depends on seasonal temperature and experience of the performer (thawing time, compliance with the insemination procedure etc). Negative effects to semen caused by temperature alterations, after the thawing, are known. Yavasca et al (**23**) thawed semen samples at 37°C for 30 seconds and exposed them to different thermal conditions at different times. Researchers have reported that pregnancy rates decrease while the environmental temperature decreases and also the time between thawing and insemination increases. Two separate studies were carried out by Correa et al., in which they thawed the semen samples at 37°C, incubated at 37°C, 21°C and 5°C. They have reported that, compared to thawing at 37°C, the osmotic change during thawing at 21°C and lower temperatures developed slower and thus caused less semen damage (**7, 9**). Researchers have expressed that this gradual temperature decrease lower the transition rate from hypertonic medium to hypotonic medium and may thus prevent osmotic shock (**7**). In another study, it has been reported that the temperature in the straw approximately approaches 0°C after thawing at 37°C for 12 seconds. Therefore, it has been suggested that short time thawing techniques are more successful especially in cold seasons (**21**).

In the studies up to date, the effects of different thawing techniques and post-thawing thermal conditions on semen have been generally examined but the number of studies evaluating the relationship between each other is very limited. It has not been studied in detail yet whether or not different thawing techniques strengthen the spermatozoa against harmful effects of post-thawing conditions.

In the present study, semen samples were thawed at 37°C at different times (12 and 30 seconds) and exposed to different thermal conditions. The aims of our study were:

i. to determine the effects of experimental post-thawing temperature differences on the spermatological characteristics;

ii. to demonstrate the relationship between short- and long-thawing procedures and post-thawing thermal conditions, and;

iii. to determine the optimal thawing time.

Materials and Methods

In the study, semen samples of 4 Holstein bulls (0.25 ml straws), frozen in the Department of Reproduction and Artificial Insemination of Faculty of Veterinary Medicine, Istanbul University, were used.

Semen Thawing: Frozen semen samples were thawed in water baths at 37°C for 12 seconds (group A) and for 30 seconds (group B).

Post-Thawing Thermal Applications: Six sub-groups were generated after thawing. Post-thawing direct examinations were carried out in control groups (A1-B1). In treatment groups, semen samples were exposed to different post-thawing thermal conditions. In order to generate cold shock, straws were kept in the water bath at 2±1°C for 300 seconds (A2-B2). Straws of the other groups were kept for different times at different temperatures. In these groups, after the thawing, straws were placed in an insemination catheter that was taken from 5±1°C cold cabinet and were kept at the same environment for 45 seconds (A3-B3) or 150 seconds (A4-B4) after fitting the catheter sheath. Similarly, some of the straws were kept at room temperature (20±1°C) for 45 seconds (A5-B5) or 150 seconds (A6-B6).

Resistance Test: After the thawing process, semen samples from the control and treatment groups were placed in an incubator (after thermal applications), taking into consideration the medium conditions and the time needed for spermatozoa to reach the fertilization site. “Modified Buffered Hepes Medium (Oocyte Washing Medium) was used as the incubation medium (**13**). Having examined the spermatological qualities in all groups, semen samples were diluted to 1/9 (semen/medium) ratio and incubated at 37°C for 2 hours for the resistance test.

Spermatological Evaluations: Prior to the resistance test, the motility, membrane and acrosomal integrity were investigated, and just after the resistance test, the motility and acrosomal integrity were re-examined. Membrane integrity was not re-examined because the dilution of semen was increased in the resistance test, too much. Therefore semen samples could not be re-diluted for conduction of the HOS test.

Motility: This quality was examined by 20x objective of a phase-contrast microscope. The mean of the 3 different preparations was calculated.

Membrane Integrity (Hypoosmotic Swelling Test, HOST): 960µl of HOST incubation solution (**15**) was added to 40 µl of thawed semen and incubated at 37°C for 60 minutes. Osmolarity of the solution was adjusted to 100±5 mOsm by means of an osmometer (Knauer, D-14163 Berlin). After the incubation, a drop of the mixture was placed on the slide and covered with cover glass and the tail structure of spermatozoa was examined under the phase-contrast microscope at 1000x magnification. Membranes of cells with a swollen or thickened tail, beginning from the middle part, were

considered as intact and functionally active. Two hundred cells were evaluated in each preparation.

Acrosomal Integrity: For acrosomal examinations, Fast Green FCF –Eosine B stain combination, modified from Aalseth and Saacke (**1**), was used. 5 µl semen was mixed with 15 µl stain and incubated at 37°C for 3 minutes. A thin-layered smear of the mixture was prepared on the de-oiled glass slide. Examinations were performed under a phase contrast microscope at 1000x magnification. Two hundred cells were evaluated in each sample. Spermatozoa with unstained acrosomal region were considered as cells having intact acrosome.

Statistical Analyses: In the statistical comparisons between the groups, one-way analysis of variance (ANOVA) was used. The significance of the differences between the groups were determined by Duncan's test (**12**). The correlations of the data were analyzed by Pearson Correlation Test. Averages of the data were calculated. All statistical analyses were carried out using SPSS program v. 10.0 for Windows.

R e s u l t s

Table 1 summarizes the findings. No significant difference was observed between two different thawing techniques, with respect to both motility and morphological abnormalities, prior to and after the incubation. The only exception was found in the motility in groups A5 and B5 kept at 20°C for 45 second post-incubation. When thawed semen was kept under the mentioned thermal conditions; sperm motility at the 12-second technique (A5) was higher than the 30-second technique (B5, $P < 0.01$).

Comparison of the sub-groups (A1-A6 or B1-B6) among each other;

When the treatment groups are compared with the control groups before and after the incubation, motility and intact acrosome rates significantly decreased only in those kept in cold water (A2 and B2, $P < 0.01$).

In comparison with the control group in both thawing techniques, no significant difference was detected before the incubation, with respect to acrosomal defects detected in other groups. After the incubation, with respect to acrosomal defects in both thawing techniques, a significant increase was observed in 5°C applications (A3-A4 and B3-B4) in comparison with the control groups ($P < 0.01$).

No significant difference was founded in the membrane integrity between two different thawing techniques.

D i s c u s s i o n

Exposure of bull spermatozoa to different osmotic pressures (**7, 8, 10, 15**) during the thawing process of semen, frozen in hypertonic solutions and in the fluids of female genital tract after artificial insemination, as well as sudden post-thawing thermal changes (**14, 23, 24**) damage the motility and morphology of spermatozoa. Some authors have reported that different thawing techniques may reduce the negative effects of osmotic shock (**4, 7, 9, 23**). It was determined that by thawing in a water bath at 37°C, the temperature of the semen in the 0.3 ml plastic straws reached to 32°C in the 30-second thawing technique, and to 0°C in the 12-second technique (**2**). This situation connotes that thermal conditions in different degrees can negatively affect semen thawed by different techniques. An interaction between different post-thawing thermal conditions and thawing techniques is possible.

The 12-second thawing is recommended due to it being protective against thermal decreases in the thawed semen, especially in cold weathers (**19, 21**). Almquist et al (**2**) have reported that 30-second thawing at 35°C is better than 12-second thawing. In our study, no significant difference was observed between these two different thawing techniques, with respect to both motility and acrosomal integrity, prior to and after the incubation (Table 1). The sole exception was found in the motility in groups A5 and B5 kept at 20°C for 45 seconds post-incubation. When thawed semen is kept under the mentioned thermal conditions; it can be observed that the 12-second technique (A5, 51.63%±1.51) is better than the 30-second technique (B5, 45.00%±2.73) for protection of spermatozoa vitality ($P < 0.01$). In 12-second thawing, the straws reached 0°C and when they were exposed to 20°C, they continued to thaw and so to say, they underwent a gradual thawing. However, those thawed in 30 seconds reached 32°C, and when exposed to 20°C, they re-cooled to 12°C. These thermal descents and ascents can be suggested to damage the motility. Therefore, it can be emphasized that shorter thawing technique should be used especially at cold weather. Meanwhile, in our study, detection of no difference between the two techniques in any other groups has led to the suggestion that the difference found was coincidental.

When the treatment groups are compared with the control groups before and after the incubation, it can be said that the motility scores of the treatment groups showed a significant decrease only in those kept in cold water (A2 and B2), while remaining unaffected in other groups. Acrosomal defects were also found to be significantly high in the groups kept in colder water ($P < 0.01$). In most studies, the cold shock was generated by different thermal (0°C, 2°C, 5°C) and time (1, 5, 10, 15 min) applications and it has been mainly determined that motility and acrosomal integrity are damaged (**3, 6, 14, 18**). Our results are also in line with the results of these authors.

In comparison with the control group, no significant difference was detected before the incubation, with respect to acrosomal defects detected in other groups. However, after the incubation, regarding acrosomal defects in both thawing techniques, a significant increase was observed in 5°C applications (A3-A4 and B3-B4) in comparison with the control groups (Table 1, $P < 0.01$). It can be stated that acrosomal integrity is more sensitive to thermal changes, as opposed to motility, and such sensitivity can be put forth after the incubation. Also in various studies, it has been reported that in samples thawed at different temperatures and subjected to different thermal applications, after the inoculation, acrosomal defects increase as the temperature decreases (**11, 14**). DeJarnette et al (**11**) have claimed that acrosomal integrity is more sensitive than motility in the determination of fertilization capacity of the spermatozoon, in samples exposed to 3-hour post-thawed incubation.

In our study, we have determined that membrane integrity (HOST) is not influenced by different thermal conditions (Table 1). It can be said that the HOS test is not sensitive enough for use in the determination of damages arising from post-thawing thermal changes. Likewise, some researchers have reported that the HOS test is not sensitive enough for use in the determination of damages due to sudden thermal changes and of fertility especially in *in-vitro* (IVF) conditions (**16, 17, 20**). Some authors, however, have expressed that thermal differences damage the cellular membrane that can be detected by HOST (**7, 9**). In literature surveys, a limited number of studies investigating the use of the HOS test in the determination of effects of post-thawing thermal changes and cold shock could be found. In these studies, generally the relationship between HOST and fertility is investigated (**5, 8, 15, 17, 22**).

In the correlation analyses, a significant correlation was found between HOST and motility ($r=0.34$, $P < 0.001$). In addition, a significant negative correlation was found between acrosomal abnormalities and motility ($r=-0.19$, $P < 0.001$). In post-incubation assessments, it has been determined that the negative correlation between acrosomal abnormalities and motility became more evident ($r=-0.31$, $P < 0.001$). Also in various studies, significant correlations between HOST and motility ($r=0.94$, $r=0.70$, $r=0.61$) and HOST and fertility ($r=0.57$, $r=0.43$) were found (**8, 10, 15**). Researchers have generally determined a negative but a lower correlation between the response to HOST and morphology (**15, 16, 22**). Nur (**17**) has found significant correlations between HOST data and motility, but insignificant correlations with acrosomal morphology after thawing. Similarly, in our study, we found significant correlations between HOST scores and motility but insignificant correlation with acrosomal integrity.

In conclusion,

Hypoosmotic Swelling Test is not as sensitive as the motility and acrosomal integrity in determination of the effects of thermal conditions.

Artificial insemination procedure should begin as soon as possible after completion of thawing process. Special care should be paid to the time especially at 20°C and lower temperatures.

After the resistance test, repetition of routine tests used in determination of potential fertility may be useful.

The shorter thawing technique in 12 seconds can be used especially at 20°C and lower temperatures.

Table 1: Post-Thawed Spermatological Characteristics in Two Different Thawing Groups and Six Thermal Application Sub-Groups at Pre- and Post-Resistance Test Incubation

Tablo 1: Dayanıklılık Testi Öncesi ve Sonrasında, 2 Farklı Eritme ve 6 farklı Isı Uygulaması Alt-grubunda Eritme Sonrası Spermatolojik Özellikler

Treatment Groups		Pre-Resistance Test Incubation			Post-Resistance Test Incubation	
		HOST (%)	Motility (%)	Acrosomal Defects (%)	Motility (%)	Acrosomal Defects (%)
A (Thawed for 12 second at 37°C)	**A1** Control	48.10±1.68	55.00±1.07^{abc}	47.38±1.57^{bc}	49.89±1.33^{ab}	53.70±1.52^{d}
	A2 (300 sec / 2°C)	41.25±1.93	47.00±1.36^{d}	58.28±1.56^{a}	32.63±2.04^{c}	69.15±1.66^{a}
	A3 (45 sec / 5°C)	45.10±1.51	54.25±0.86^{abc}	51.45±1.80^{b}	48.88±1.24^{ab}	59.95±1.82^{bc}
	A4 (150 sec / 5°C)	44.90±1.85	52.38±1.16^{c}	50.38±1.94^{bc}	47.00±1.73^{ab}	60.38±2.14^{b}
	A5 (45 sec / 20°C)	46.58±1.81	57.38±0.89^{a}	50.18±1.56^{bc}	51.63±1.51^{aA}	53.55±1.47^{d}
	A6 (150 sec / 20°C)	43.83±2.02	55.88±1.07^{ab}	52.78±1.57^{b}	47.63±1.87^{ab}	57.28±1.55^{bcd}
B (Thawed for 30 second at 37°C)	**B1** Control	48.65±1.82	56.38±1.01^{ab}	45.30±1.94^{c}	46.75±2.06^{ab}	55.08±1.47^{cd}
	B2 (300 sec / 2°C)	44.78±1.41	48.75±0.99^{d}	58.30±1.55^{a}	30.88±2.32^{c}	67.13±1.32^{a}
	B3 (45 sec / 5°C)	44.98±1.62	53.75±0.99^{bc}	50.30±1.80^{bc}	46.13±2.28^{ab}	60.80±1.76^{b}
	B4 (150 sec / 5°C)	46.98±1.61	54.63±0.78^{abc}	45.33±1.85^{c}	47.63±1.61^{ab}	59.73±1.61^{bc}
	B5 (45 sec / 20°C)	46.25±1.95	57.25±0.69^{a}	49.70±1.84^{bc}	45.00±2.73^{bB}	54.95±1.79^{cd}
	B6 (150 sec / 20°C)	46.42±1.73	56.88±0.55^{a}	52.65±1.96^{b}	51.63±1.10^{a}	53.15±1.59^{d}

^{abcd} (within groups): Mean values in the same column with different superscripts differ significantly ($P<0.01$).

$^{A, B}$ (inter groups): Mean values in the same column with different superscripts differ significantly ($P<0.01$).

Aynı sütunda farklı üst karakterlere sahip ortalama değerler önemli oranda farklıdır (P<0.01).

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