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Research Article

In vitro Evaluation of Short-term Preserved Stallion Semen in Different Doses and Temperatures [1] [2]

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Abstract

This study was conducted to evaluate the effects of different temperatures and doses on stallion sperm survival after 24 h and 48 h short-term storage. Ejaculates from four warm-blooded stallions were used in this study. Ejaculates were collected by an open-ended artificial vagina which separates fractions of semen into sterile plastic cups. Only the second and third fractions (spermatozoa-rich fractions) were used to obtain spermatozoa for the experiment. Semen was diluted with INRA 96 extender to final concentrations of 50, 100, 200 and 400x10^{6}sp/ml. Diluted semen were stored at different temperatures (2, 4 or 8°C) for 24-48 h. After 24 h and 48 h cooled storage, spermatozoon motility, abnormal spermatozoon ratio and membrane integrity were evaluated. According to results the highest motility values (52.5% and 55%) were determined at 50 and 100 x10^{6}sp/ml concentration at 4°C after 24 h cooled storage. After 48 h of storage, the highest motility was obtained from semen stored at 8°C. Abnormal spermatozoon and membrane integrity values were not significantly different after cooled storage. Consequently, it was determined that semen with 50 and 100 million spermatozoa/ml concentration stored at 4°C for 24 h and semen with 50, 100, 200, 400 million spermatozoa/ml concentration stored at 8°C for 48 h gave better results with respect to motility than the others.

Keywords: *Stallion semen, Doses, Temperature, Short-term preservation, In vitro evaluation of semen*

Farklı Doz ve Sıcaklıklarda Kısa Süreli Saklanan Aygır Spermasının *in vitro* Değerlendirilmesi

Özet

Bu araştırma, farklı doz ve sıcaklıkların (24-48 saat) aygır spermatozoonlarının yaşam kabiliyetleri üzerine etkisini ortaya koymak amaçlanmıştır. Çalışmada dört sıcakkanlı aygırdan alınan ejekülatlar kullanılmıştır. Ejekülatların alınması için açık uçlu suni vagina kullanılmış ve bu yolla sperma fraksiyonlar halinde cam kaplara alınmıştır. Deney süresince sadece 2. ve 3. fraksiyonlar (spermatozoondan zengin fraksiyonlar) çalışmada kullanılmıştır. Sperma INRA 96 sulandırıcısı ile final yoğunluk 50, 100, 200, 400x10^6 spermatozoa/ml (sp/ml) olacak şekilde sulandırılmıştır. Sulandırılan sperma 2°C, 4°C ve 8°C lik sıcaklıklarda 24-48 saat süreler ile saklanmıştır. 24-48 saatlik saklama sonrası spermatozoon motilitesi, anormal spermatozoa ve membran bütünlüğü yönünden muayene edilmiştir. Elde edilen sonuçlara göre kısa süreli saklanan spermada 24. saat sonunda en yüksek motilite değerleri (%52.5 ve %55) 4°C'de 50 ve 100 milyon yoğunlukta elde edilmiştir, 48. saat sonunda ise en yüksek motilite değerleri 8°C'de saklanan spermadan elde edilmiştir. Anormal spermatozoa ve membran bütünlüğü değerleri farklılık göstermemiştir. Sonuç olarak, 4°C'de 50 ve 100 milyon dozlarında 24 saat saklamanın ve 8°C'de 50, 100, 200 ve 400 milyon dozlarında 48 saat saklamanın diğerlerine göre daha iyi sonuçlar verdiği tespit edilmiştir.

Anahtar sözcükler: *Aygır sperması, Doz, Isı, Kısa süreli saklama, Spermanın in vitro değerlendirilmesi*

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INTRODUCTION

Horse breeders rarely give importance to fertility as selection criteria in mating, although many stallions do not have enough features to be used for breeding. This has resulted in stallions' transferring undesirable performance qualities to their offspring in the gene pool. Therefore, significant differences were formed in semen qualities of the stallions used for breeding. A winner stallion may also be in the gene pool even though the stallion has poor semen quality or the semen is not appropriate to use with the advanced technology of semen processing techniques. This has led the scientists' dealing with stallions, to come a long way in terms of assessment of all possibilities to increase reproductive performance [1].

Stallions' semen quality analyses are essential for fertility to be maximised. There can also be changes from poor to medium degree in ejaculates of stallions. These changes are mainly caused by; season, semen collection and processing techniques, semen collection frequency and problems in spermatogenesis. Factors such as maintenance-supply, semen collection procedures and number of mares to be artificially inseminated; as well as semen quality may effect fertility. The conventional parameters in evaluation of unprocessed semen quality are; amount, concentration, total number of spermatozoa in ejaculate, motile spermatozoa rates, morphology of spermatozoa and seminal pH [2].

Although short-term stored semen has wide range of usage in livestock animals and horses, sufficient implementation and optimum fertilisation rates have not been reached yet. In studies performed, the main step is related with storage of semen with an appropriate extender and additives (antioxidants) in 4°C for 24 h [3-6]. However, quite different and conflicting results are obtained in motility, abnormal spermatozoa, membrane integrity and fertilisation rates after storage [7,8]. Seminal plasma components provide membrane stability which protects semen during cooling, freezing and thawing. Changes in spermatozoon membrane reduce fertilising ability of frozen thawed semen. Stabilising substances in seminal plasma maintain membrane integrity and thus freezability of spermatozoa. Addition of seminal plasma from stallions with high post-thaw motility to semen from stallions with low post-thaw motility improves membrane integrity and progressive motility. Seminal plasma from different stallions contain different amounts of components which may be considered as a determining factor for the ability of spermatozoa from each stallion to survive cryopreservation. Individual differences influence seminal plasma, causing diversity in cooling and freezing processes by effecting the spermatozoa membrane integrity. Cryopreserving spermatozoa induces changes in the plasma membrane. Removal of seminal plasma by centrifugation before cryopreservation causes a premature capacitation reaction when the semen is frozen. For that reason, spermatozoa are unable to undergo the acrosome reaction and complete fertilisation [3,9,10]. Different diluents (INRA 82, lactose-EDTA, skimmed milk, skimmed milk-glucose, sugar based or ionic diluents), different cryoprotectants (Glycerol, Ethylene glycol, DMSO), different packaging methods (ampoule, vial, 0.25 and 0.5 ml sequins, 4 ml macrotube, 2.5 ml flat macrotube) and storage methods (short and long-term) were used for storage of stallion semen [10-12].

This study contains reproductive biotechnologies and preservation techniques. High fertility rates are expected, by using biotechnological methods such as transporting stored semen and artificial insemination applications, in mares. Study is directed to cryopreservation biotechnologies of sperm cells. Compared to other farm animals, sufficient fertilisation rates cannot be obtained from cooled-stored or frozen-stored stallion semen [13]. Fertilisation ability could be changed by semen concentration and storage temperature [5]. Thus, to determine the most effective semen concentrations and storage temperatures, and accordingly to increase fertility; different methods and techniques should be developed. In this study, spermatological parameters of short-term stored stallion semen with different doses at different temperatures were obtained. For this reason, spermatozoa integrity and fertilisation ability can vary in accordance with spermatozoa concentration and temperature of storage. With this respect, in vitro integrity and fertilization ability of stallion semen was aimed to be evaluated.

MATERIAL and METHODS

This study was performed in horse farms in Mahmudiye-Eskişehir and the laboratory of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine Ankara University. Materials for the study were obtained from the horse farms around the area and Dörtnal Veterinary Clinic's horses and 4 European warm-blooded stallions were used. The stallions were fed twice a day with 12 h intervals. Depending on the stallions' performance and the number of copulations, an average of 5 kg of pellet feed were given to each stallion. In addition, 2 kg clover and 2 kg dry grass were given per day. Stallions were allowed to wonder around the paddock approximately 8 h per day, no additional exercises were done. The experimental procedures were approved by the Animal Experiments Local Ethics Committee of Ankara University (No: 2008-29-138).

Semen was collected daily from stallions for 3 days in order to empty the extragonadal semen reserves at the beginning of the research and during breeding season (April-May). After 2 days of sexual resting, a total of 16 ejaculates, separated into fractions, were collected by open-ended artificial vagina, from each stallion twice a week.

The spermatozoa used for short-term storage were taken from the second and third fractions (spermatozoa rich fractions). For this purpose, only the second and third fractions were collected into the collection tube. The collected semen was diluted with skimmed-milk glucose extender with a ratio of 1:1. Extended semen was centrifuged twice at room temperature at 500 G for 10 min to separate seminal plasma. With the pellet that was obtained, concentrations were determined and using the INRA 96 extender, the pellets were extended to 50, 100, 200, 400x10^6 sp/ml.

The semen that was extended with INRA 96 extender was separated into 3 equal parts to be short-term stored and they were kept at 2, 4 and 8°C for 48 h. With reference to this, 12 trial groups including different semen doses (50, 100, 200, 400x10^6) and different temperatures (2, 4 and 8°C) were formed. After the dilution process, the semen was put in equal volume closed-end glass tubes and these were stored for 48 h. Evaluations of spermatological parameters were conducted at 0., 24., and 48. h in order for the spermatozoa fertilization abilities to be presented. Before the spermatological inspections, the short-term stored semen was put into 37°C water for 5 min. After that process, spermatozoa motility, abnormal spermatozoa ratio and membrane integrity were evaluated. Spermatozoa motility was evaluated subjectively and in percentage by 3 microscope fields scanned by 2 different observers on a microscope with heating plate (10x20) [5].

Abnormal spermatozoa ratios were determined by eosin staining method. For this purpose, samples taken from the semen were put into 3% sodium citrate, which has 2% eosin in dilution. Smears were taken from each sample and examined with a light microscope (400x). In different microscopic area, at least 200 spermatozoa were counted and abnormal spermatozoa ratio were noted as percentage [5].

Hypoosmotic swelling test (HOS) was performed in order to determine membrane integrity. For this purpose, 100 mikroliters semen was added to 1 ml lactose solution and then incubated at 37°C for 30 min. After the incubation, at least 100 spermatozoa were evaluated at the microscope according to their tail curling and membrane integrity. Each experimental group (dilution ratios and temperatures) were evaluated 3 times.

The data was analysed with General Linear Model (GLM) method in order to reveal the effect of different temperatures and different semen concentrations on some of the spermatological properties. Analysis of the temperature and concentration of each spermatological parameter were carried out with one way analysis of variance (ANOVA). The degree of significance, among experimental groups, was determined as $P<0.05$. SPSS 10.0 programme which runs under Windows software was used for the analysis.

RESULTS

Semen's fertilization ability was presented by biometrical evaluation of the data. The effect of different temperatures and different spermatozoa concentrations on some spermatological parameters were presented in *Table 1*.

Accordingly, significant differences in spermatozoa motility, abnormal spermatozoa ratio and membrane integrity were determined right after dilution (0. hour); and similar results were obtained for all 4 concentrations. Similar to this, even though no significant differences were observed after 24 h of storage in all 3 different temperatures; the different temperatures displayed differences in motility within themselves and these differences were found statistically significant. Accordingly, highest motility (50.00%) was observed in semen containing 100 million spermatozoa after storage in 2°C for 24 h. However, abnormal spermatozoa and membrane integrity was not significantly different. Similarly, when the properties of semen stored at 4°C were evaluated, significant decrease of motility from 30.00% to 24.00% was determined in concentrated semen at a concentration of 200 and 400x10^6 sp/ml. Whereas, semen storage performed at 8°C for 24 h, at a concentration of 50 and 100x10^6 sp/ml has been detected to have higher motility values with respect to more concentrated semen samples. No significant differences in abnormal spermatozoa ratio and membrane integrity were observed.

When the data obtained from storage of semen for 48 h was evaluated, in all 3 temperature degrees the semen quality was found to be decreased; resulting in decrease of spermatozoa motility, increase of abnormal spermatozoa ratio and decrease of membrane integrity.

When the temperatures were considered, in contrast to what was expected after storage for 48 h, the highest motility values were obtained from semen stored at 8°C. Similarly, membrane integrity values were higher compared to the other temperatures. However, no difference in abnormal spermatozoa ratios was observed. When all temperature values were evaluated, there was no difference in spermatological properties of different concentrations of semen stored at 2°C, semen including 100 million spermatozoa was found to have the highest motility value (30%). On the other hand, even though semen stored at 8°C shows differences when compared with other temperatures, samples of different concentrations had no differences in spermatological properties. However, after 48 h of storage the highest motility and membrane integrity values were obtained with 8°C.

DISCUSSION

In this study, after storage at 4°C for 24 h the highest motility values of 52.5% and 55% were recorded. The highest

Table 1. *Effect of different storage temperatures and spermatozoa concentrations on quality of semen*
Tablo 1. *Farklı saklama sıcaklıklarının ve spermatozoon yoğunluklarının sperma kalitesi üzerine etkisi*

Time	Temperature	Concentration (x10^6 sp/ml)	Spermatozoa Motility (%)	Abnormal Spermatozoa (%)	Membrane Integrity (%)
0. hour	Room temperature after dilution (20°C)	50	67.5±0.10	46.5±2.12	51.3±8.83
		100	62.5±0.10	48.0±1.41	50.7±6.15
		200	67.5±0.35	52.5±3.53	57.9±1.27
		400	68.0±0.01	47.5±0.70	52.9±1.55
24. hour	2°C a	50	47.5±0.35 b	76.5±2.12	36.5±12.0
		100	50.0±0.07 b	75.0±1.41	31.0±8.48
		200	40.0±0.01 a	77.0±4.24	33.5±7.77
		400	40.0±0.01 a	80.5±6.36	32.5±17.6
	4°C a	50	52.5±0.10 a	73.0±0.01	27.5±9.19
		100	55.0±0.21 a	75.0±2.01	31.0±5.65
		200	30.5±0.17 b	74.0±1.65	21.0±7.07
		400	24.5±0.24 b	79.0±3.76	24.5±13.4
	8°C a	50	35.0±0.07 a	82.0±4.24	35.5±3.53
		100	40.0±0.07 a	75.5±7.77	39.3±9.47
		200	45.0±0.21 b	81.5±3.53	32.5±7.77
		400	47.5±0.03 b	77.0±1.41	34.5±0.70
48. hour	2°C a	50	30.0±0.00	91.5±2.12	11.5±4.94
		100	25.0±0.07	87.5±0.70	18.5±9.19
		200	25.0±0.00	91.0±8.48	19.5±2.12
		400	30.0±0.00	97.0±4.24	15.0±9.89
	4°C a	50	22.5±0.03 a	92.0±4.24	18.0±2.10
		100	30.0±0.14 b	84.0±1.41	13.0±3.36
		200	20.0±0.28 a	94.0±2.94	17.0±3.03
		400	22.5±0.31 a	97.0±2.02	18.0±5.20
	8°C b	50	35.0±0.07	91.0±7.07	25.0±4.44
		100	30.0±0.10	96.0±2.82	21.5±12.0
		200	40.0±0.15	97.0±1.41	24.0±8.48
		400	40.0±0.22	96.0±5.65	28.0±11.3

a, b: *The difference of group averages in the same column between different letters are significant (P<0.05)*

motility rates of the groups stored for 48 h were noted at 8°C. Among the groups, abnormal spermatozoa and membrane integrity values were not significantly different. According to results, semen stored at 4°C in 50 and 100 million doses for 24 h and semen stored at 8°C in 50, 100, 200, 400 million doses for 48 h were observed to have better results compared to the other groups.

Many methods on short-term storage of stallion semen have been tested and it was notified that especially genetic biological differences and differences depending on the method effect spermatozoa's fertilisation ability. Some parameters such as extenders, dilution ratios and storage temperature differences have been reported to effect success by different researchers [3-8,10,11]. Similar to this, the research determined that in examinations performed after dilution ratio or spermatozoa concentration does not effect spermatological properties.

Evaluations after 24 h of storage for semen revealed that high dilution rates and low concentrations gave high motility values at 2°C and 4°C, in this case fertility rates can be higher at 2°C or 4°C with concentrations of 50 and 100 million semen. In contrast it was determined that 24 h of storage in low dilution rates at 8°C have more successful results. According to these results, high fertility rates can be obtained from semen stored at 8°C with 200 million or 400 million concentrations. Evaluations after 48 h of storage indicate that semen samples at 2°C and 4°C have low motility values. Conversely, semen stored at 8°C with low dilution rates or high semen concentirations (200 and 400×10^6 sp/ml) gave high motility and membrane integrity values. It was concluded that high fertility rates could be obtained at 8°C compared to 2°C and 4°C at 48 h storage of semen.

Results suggest that there may be an interaction

between semen concentration and storage temperature. Because high concentrated samples have low motility values at low temperatures (2°C and 4°C) whereas same samples have high motility values at a higher temperature (8°C).

Stallion ejaculation consists of 5 to 8 fractions and these fractions have different compositions because they contain secretions from different accessorial glands [14,15]. It has been reported that the reduce of seminal plasma rates increase the motility in semen cryopreservation [13,16,17]. It has been found that seminal plasma proteins increase the resistance of spermatozoa [18]. Similarly, Iwamoto et al.[19] detected that seminal plasma protects semen more efficiently against cold shock. Besides, structural differences in seminal plasma, which consists of individual differences between stallions, increase resistance of spermatozoa [18,20]. Likewise, Aurich et al.[10] stated that addition of seminal plasma from stallions with high post-thaw motility to semen from stallions with low post-thaw motility significantly improved membrane integrity and progressive motility. Varner et al.[17] reported that seminal plasma obtained from spermatozoon-rich fraction reaped a benefit more than whole ejaculates. A similar result was obtained by Katila et al.[11] although different ejaculations' effect on spermatozoon have not been revealed yet. Akçay et al.[9] indicated that last fraction (gel fraction) was essentially important for spermatozoa and also stated that, sperm plasma membranes suffer no obvious damage as the highest membrane integrity values were obtained in skim milk glucose extender. The results in the study suggest that sperm from different stallions differ in their sensitivity to the absence of seminal plasma in skim milk extender. The results also indicate that skim milk glucose extender provides protection for sperm membrane integrity but does not effectively protect motility. Conversely Moore et al.[3] reported that, seminal plasma did not affect sperm motility, viability or acrosomal integrity.

In this study, it was observed that stallion semen samples stored at 4°C for 24 h, containing the last fraction of seminal plasma, have better spermatozoa parameters. The seminal plasma proteins increase spermatozoa resistance and protect the spermatozoa against cold shock.

As a result, after the examination of samples immediately after dilution (0 h), no significant difference was observed. Additionally when analyzing the 24 h data, only temperatures among themselves, showed statistically differences in motility ($P<0.05$). After the evaluation of 48 h stored semen samples, decrease in semen quality in all three temperatures, and accordingly, reduction in semen motility, increase in the rate of abnormal spermatozoa and membrane integrity have been observed. However, after 48 h storage, some interesting results were obtained; when temperatures were compared, highest values of motility and membrane integrity were seen at 8°C. If semen is to be kept for 48 h, 8°C should be preffered in order to increase fertility rates.

In this context, the current research will be an initial step for the next studies on short-term storage of semen in horses. However, this research is based on *in vitro* examinations. Considerably actual results may be taken after *in vivo* evaluations. Thus, studies must be repeated in different ways for a successful storage protocol. Beside increasing the number of animals, storage of different breeds' stallion semen is important for future studies. More reliable results can be obtained, by having fertility rates of artificial insemination done with fresh or short-term stored semen and comparison of horse breeds and horses individually. If the semen is to be kept for a longer time, higher temperatures (i.e. 8°C) should be preferred. There are no differences among temperature rates when semen is stored for 24 h, even though it can be observed that higher temperatures (i.e. 8°C) give better results when storage period lengthens.

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