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Research Article

The Effects of Seminal Plasma, Skim Milk, and Tyrodes Solutions on Survival of Stallion Sperm Stored at 4 °C

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Abstract: This study was conducted to evaluate the effects of skim milk, skim milk extender supplemented with modified Tyrode's medium, and seminal plasma (obtained from the sperm-rich fraction) on stallion sperm survival after 24 h cooled storage at 4 °C. Ejaculates from 3 Finnhorse stallions were used in this study. Ejaculates were collected by an automated phantom (Equidame) which separates fractions of semen into 5 cups. Only the second cup (sperm-rich fraction) was used to obtain spermatozoa and seminal plasma for the experiment. Semen was diluted with the stallion's own second cup seminal plasma and the extenders to a final concentration of 50×10^6 sperm/ml. After 24-h cooled storage in Equitainer, spermatozoal motion characteristics were evaluated using a computerized motility analyzer. Spermatozoal viability was assessed by staining spermatozoa with CAM/PI (calcein AM / propidium iodide). Membrane integrity was evaluated using a fluorometer and staining with Hoechst 33258. After cooled storage, seminal plasma showed significantly lower viability and membrane integrity than the extenders and differences were significant, but total motility and progressive motility in seminal plasma and Kenney extender were not significantly different. The best total motility, progressive motility, VCL (velocity curve linear) and DCL (distance curve linear) values were observed in skim milk extender supplemented with Tyrode's medium. The results of this study demonstrated that; 1) exposure to seminal plasma for long periods is detrimental to sperm survival, 2) skim milk glucose extender provides good protection for sperm viability and membrane integrity, but not for motility, 3) on the contrary, skim milk glucose extender supplemented with modified Tyrode's medium is more effective in maintaining motility, presumably because of its ionic balance.

Key Words: Stallion semen, cooled storage, seminal plasma, semen extenders

4 °C'de Saklanan Aygır Spermatozoonlarının Yaşam Kabiliyetleri Üzerine Seminal Plazma, Yağsız Süt ve Tyrodes Solusyonlarının Etkisi

Özet: Bu araştırma, 4 °C'de 24 saat saklanan aygır spermatozoonlarının canlılıkları üzerine yağsız süt, Tyrode solusyonu ile desteklenmiş yağsız süt ve seminal plazmanın (spermatozoonlardan zengin fraksiyondan elde edilen) etkilerini degerlendirmek amacıyla yapıldı. Araştırmada üç adet Finlandiya ırkı aygırdan alınan ejekülatlar kullanıldı. Ejekülatlar, sperma fraksiyonlarını 5 ayrı kap içerisine ayıran otomatik fantom (Equidame) kullanılarak alındı. Spermatozoa ve seminal plazmanın elde edilmesinde sadece 2. kapdan (spermatozoondan zengin fraksiyon) elde edilen sperma kullanıldı. Alınan sperma, final yoğunluk 50×10^6 spermatozoon/ml olacak şekilde aygırların kendi seminal plazmaları ve sulandırıcılar ile sulandırıldı. Equitainer içinde 24 saat saklamadan sonra, spermatozoonların hareket özellikleri bilgisayar destekli motilite analizörü ile degerlendirildi. Spermatozoa canlılığı CAM/PI (calcein AM / propidium iodide) ikili boyama yöntemi ile, membran bütünlüğü ise fluorometre yardımıyla ve Hoechst 33258 boyası kullanılarak tesbit edildi. Soğukta saklama sonrası, seminal plazma sulandırıcılara oranla daha düşük canlılık ve membran bütünlüğü değerleri gösterdi ve farklılıklar önemli bulundu. Ancak seminal plazma ve yağsız süt sulandırıcısından elde edilen toplam motilite ve ileri yönlü motilite oranları arasında farklılıklar anlamlı bulunmadı. En iyi toplam motilite, ileri yönlü motilite, VCL (velocity curve linear) ve DCL (distance curve linear) değerleri Tyrode solusyonu ile desteklenmiş yağsız süt sulandırıcısında elde edildi. Araştırmadan elde edilen sonuçlara göre; 1) spermanın, uzun süre seminal plazmaya maruz kalmasının spermatozoon canlılığı üzerine olumsuz etkisi olduğu, 2) yağsız süt glukoz sulandırıcısının spermatozoon canlılığı ve membran bütünlüğü üzerine iyi bir koruma sağladığı, fakat motilite için aynı durumun söz konusu olmadığı, 3) buna karşılık, Tyrode solusyonu ile desteklenmiş yağsız süt glukoz sulandırıcısının muhtemelen iyonik dengesinden dolayı spermatozoa motilitesinin devamında daha etkili olduğu gözlenmiştir.

Anahtar Sözcükler: Aygır sperması, soğukta saklama, seminal plazma, sperma sulandırıcıları

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Introduction

In the horse industry, milk or milk-based extenders are used routinely for dilution and storage of semen cooled to 4–8 °C. Although artificial insemination (AI) with chilled and transported semen has been in use for several years, pregnancy rates are still low and variable related to variable semen quality of stallions. Over the years, a variety of extenders have been proposed for cooling, storage, and transport of stallion semen (1).

Motility and fertility of cooled semen vary greatly among stallions, extenders, or techniques. Differences in seminal plasma might be one cause of this variation. Stallions ejaculate in 5 to 8 jets, the composition of which differ (2). Presumably seminal plasma contains substances that are beneficial to spermatozoal transport and survival in the female genital tract (3,4). On the other hand, it is known to contain factors that are harmful for viability and motility during semen storage (5). Therefore, seminal plasma is removed when semen is frozen or its proportion reduced by adding extender for cooled semen storage.

A large variety of extenders combining various components (sugars, electrolytes, buffers, egg yolk, milk, and milk products) have been proposed for cooling sperm. Milk and milk-based extenders are known to be practical and efficient in protecting equine spermatozoa during storage before AI (6,7). However, milk is a biological fluid with a complex composition (more than 100,000 molecules) and contains components that could be beneficial or detrimental to sperm survival. Moreover, the concentration of protective milk components may be optimized to improve long term sperm storage at positive temperatures. Centrifugation and removal of all seminal plasma results in significant reductions in motility of stored sperm when standard extenders are used (8) suggesting that a low concentration of seminal plasma or a seminal plasma substitute is essential for maintenance of spermatozoal motion.

Studies on the effect of seminal plasma fractions and extenders on sperm function are contradictory (7-11). For cooled storage, dilution of semen to reduce the proportion of seminal plasma increases sperm motility (8,9). Varner et al. (7) reported the sperm-rich fraction to be superior to the whole ejaculate, and similar results have been reported by Katila et al. (12) in semen freezing. However, experiments using fractioned semen collection and cooled storage yielded surprising results: last fractions at the ejaculate maintained motility better than sperm-rich fractions (11,13). Individual variation in seminal plasma composition may have a significant role in spermatozoal motility after cooled storage (14-16). Webb and Arns (15) reported that total removal of seminal plasma and dilution of semen with skim milk extender containing modified Tyrode's medium improved motion characteristics following cooled storage of spermatozoa. However, modified Tyrode's medium is detrimental to sperm motility when seminal plasma is present (17).

Padilla and Foote (18) centrifuged stallion semen to remove all seminal plasma and re-suspended spermatozoa in a skim milk-glucose extender that had been supplemented with a high-potassium modified Tyrode's medium. The motility of spermatozoa extended in the modified Tyrode's medium was much improved with cooled storage when compared to centrifuged semen that was extended in a non-supplemented skim milk-glucose extender with no seminal plasma. However, spermatozoa extended in the modified Tyrode's medium in the presence of seminal plasma had reduced motility, suggesting an apparent interaction between seminal plasma and extender composition.

The objective of the present study were to investigate the effects of skim milk extender, skim milk extender supplemented with modified Tyrode's medium and seminal plasma (obtained from the sperm-rich fraction) on stallion sperm survival after 24 h cooled storage at 4 °C.

Materials and Methods

Three Finnhorse stallions were used in this study. During the week preceding the experiment, they were collected daily to deplete extragonadal sperm reserves. Totally 9 ejaculates, 3 ejaculates from each stallion, were collected on consecutive days in February using an automated phantom (Equidame® phantom, Haico Oy, Loimaa, Finland). Ejaculates were fractioned into 5 cups. Only the second cup (sperm-rich fraction) without pre-sperm fluid was used to obtain spermatozoa and seminal plasma for the experiment. The contents of the cup were centrifuged for 15 min at 500 g. After removal of the supernatant, skim milk extender (6) was added to make a final concentration of 100×10^6 sperm/ml.

To obtain seminal plasma in the experiment, the supernatant of cup 2 were centrifuged twice for 15 min at 3000 g and the last supernatant was filtered using 0.22

µm Millex-GP filter unit (Millipore, Billerica, MA, USA). Extended sperm was mixed with the stallion's own seminal plasmas from the second cup, skim milk extender (4.9 g glucose, 2.4 g skim milk, 100 ml dist. water, pH 6.8) and skim milk extender supplemented with modified Tyrode's medium (65:35, v/v) in a ratio of 1:1 to a final concentration of 50×10^6 sperm/ml. Modified Tyrode's medium was prepared as described by Padilla and Foote (18). The samples were cooled in an Equitainer (Hamilton-Thorne Research, South Hamilton, MA, USA) for 24 h and transported to the laboratory by bus. After storage, aliquots of each sample were incubated in a water bath for 10 min at 37 °C prior to analysis. Spermatozoal motion characteristics were evaluated using a computerized motility analyzer (Sperm Vision Minitub, Tiefenbach b. Landshut, Germany). Calibrating and operating parameters had been optimized for stallion sperm. A video digitizing rate of 60 frames/sec was used to gather data; spermatozoa tracked for 60 consecutive frames were used for calculating kinematics and for determining motility. The analyses were performed on 2 drops per sample and 7 fields per drop. At least 200 spermatozoa were examined in each sample. For the analysis, a 300-µl aliquot of the thoroughly but gently mixed semen sample was placed into an open 3-ml tube. The tube was kept in a 37 °C water bath (Grants Instruments Ltd., Cambridge, UK) for 5 min before analysis. A 5-µl aliquot was placed on a pre-warmed 38 °C microscope slide and overlaid with a cover slip (24 mm × 24 mm × 1.5 mm).

Spermatozoal viability was assessed by a dual staining method with CAM/PI (calcein AM / propidium iodide) and counting 200 cells in fluorescence microscopy as described by Katila et al. (19). Briefly, 10 µl of CAM [1 mg/ml in dimethylsulphoxide (DMSO)] and 500 µl of PI [0.02 mg/ml in Beltsville Thawing Solution (BTS; USDA, Beltsville, MD, USA)] were mixed with 500 µl of BTS. For staining, 100 µl-aliquots of semen were placed into 3-ml tubes, and 100 µl of CAM/PI solution was added. Each sample was further incubated for 10 min in the dark at 35 °C. Subsamples of the stained suspension (5 µl) were placed on clean microscopic slides and overlaid carefully with cover slips. The smears were evaluated under an epifluorescence microscope (Olympus BH2 with epifluorescence optics, Olympus Optical Co., Ltd., Japan) using 500× magnification. For each semen sample, 200 spermatozoa were differentiated into green (live) and red (dead) cell categories.

For evaluation of plasma membrane integrity, spermatozoa were stained with Hoechst 33258 and measured in a fluorometer developed for reading the fluorescence output of 96-well plates (Fluoroscan Ascent, Thermo Labsystems Oy, Vantaa, Finland). Analysis was done according to Katila et al. (19). Six mg of Hoechst 33258 were diluted in 200 ml of BTS, mixed for 30 min in the dark, and stored in aliquots of 2 ml at -20 °C. Before use, the dye was thawed in a dark chamber at 35 °C (Thermax, B8000, Bergen, Norway). Briefly, 500-µl aliquots of fresh mixed semen were placed into 3 ml tubes and capped. The tubes were rapidly frozen by immersion directly into liquid nitrogen for 1 min. The tubes were taken to room temperature for 30 sec before being placed in a 35 °C water bath for 3 min to cause disruption of plasma membranes. For the analysis, equal 50-µl aliquots of fresh mixed semen sample and Hoechst 33258 were dispensed into the wells of the plate (Black Microtiter Plate 96 wells, Thermo Labsystems Oy, Vantaa, Finland) in 3 replicates. The rapidly frozen subsamples were then analyzed in the same manner as non-treated samples. Blanks containing 50 µl of the respective extender and 50 µl of Hoechst 33258 were dispensed in 4 replicates. The plate was gently shaken for 2 min. Before analysis, the plate was incubated in the fluorometer for 8 min. Eleven samples and their blanks were analyzed simultaneously. The interference filter at the excitation path and the emission filter had maximum transmissions at 355 nm and 460 nm for Hoechst 33258.

Data are expressed as a mean ± standard error of mean (SEM). Difference in the means between treatments and stallions was tested by 1-way analysis of variance (ANOVA). Tukey's multiple comparison test was used for the post-hoc comparisons. Differences were considered significant at the probability level of 0.05.

Results

One way analysis of variance indicated significant differences between seminal plasma and extenders in viability, membrane integrity, and motion characteristics and differences were significant (Tables 1 and 2). However, total motility and progressive motility in seminal plasma and skim milk extender were not significantly different. The best total motility, progressive motility, VCL (velocity curve linear) and DCL (distance curve linear) values were observed in skim milk extender supplemented

Table 1. Obtained spermatological parameters from each stallion (n = 3)

Stallion Number	Extender	Viability (%)	Membrane Integrity (%)	Total Motility (%)	Progressive Motility (%)	VCL (μm/s)	DCL (μm)
	Skim milk	68.0 ± 1.6	72.3 ± 2.2	39.5 ± 2.8	35.1 ± 1.1	136.2 ± 5.5	62.4 ± 3.3
1	Skim milk Tyrode	74.0 ± 2.5	57.5 ± 1.8	43.6 ± 2.6	37.0 ± 1.5	174.5 ± 7.1	76.8 ± 3.2
	Seminal Plasma	58.0 ± 1.1	38.8 ± 1.4	19.3 ± 1.8	10.2 ± 3.6	186.5 ± 7.2	80.1 ± 4.1
	Skim milk	80.0 ± 1.8	78 ± 2.3	20.5 ± 1.5	12.4 ± 0.9	83.6 ± 4.8	38.5 ± 3.9
2	Skim milk Tyrode	82.0 ± 1.8	62.2 ± 1.8	67.1 ± 2.0	56.5 ± 2.2	170.9 ± 8.1	75.3 ± 2.8
	Seminal Plasma	77.0 ± 0.8	38.2 ± 1.5	46.1 ± 1.1	27.5 ± 1.8	138.2 ± 4.3	63.5 ± 3.0
	Skim milk	83.0 ± 2.6	85.4 ± 3.8	56.3 ± 2.1	48.5 ± 2.6	110.1 ± 9.9	55.0 ± 1.8
3	Skim milk Tyrode	81.0 ± 2.9	75.3 ± 3.6	78.4 ± 4.2	71.4 ± 3.8	183.2 ± 8.7	82.3 ± 3.3
	Seminal Plasma	66.0 ± 1.9	51.4 ± 3.1	50.5 ± 1.6	43.1 ± 1.8	167.6 ± 4.5	76.4 ± 5.1

with Tyrode's medium. The best viability and membrane integrity percentages were observed in skim milk extender in all samples. In contrast, total and progressive motility were best maintained in Tyrode's medium, and the differences were significant ($P < 0.05$) (Table 2).

When individual stallions were examined, semen from stallion 1 showed poor results, but as for the extenders, the stallion gave same results similar to the others.

Discussion

The most important finding of the present study was that sperm kept in seminal plasma from sperm-rich fraction showed worse motility values compared to sperm in skim milk extender and skim milk extender supplemented Tyrode's solution. In all samples, skim milk extender supplemented Tyrode's solution were superior to the others. The results of the present study are in accordance with the previous studies (9,10).

Various components within seminal plasma may be stimulatory or deleterious to sperm motility (5,7). Contribution of the accessory sex glands is different in successive fractions, and therefore, seminal plasma composition is also different (20). The sperm-rich fraction that originates from ampulla and bulbourethral glands contains 80%-90% of the biochemical components of semen and is relatively low in citric acid and sodium chloride. This experiment was not designed to study components of seminal plasma from different fractions, but it seems that ionic constitution of seminal plasma is

Table 2. Averaged spermatological parameters from different extenders (n = 9).

Extender	Viability (%)	Membrane Integrity (%)	Total Motility (%)	Progressive Motility (%)	VCL (μm/s)	DCL (μm)
Skim milk	77.5 ± 3.1^{b}	78.5 ± 2.8^{c}	40.4 ± 5.5^{b}	32.0 ± 1.2^{b}	109.9 ± 7.1^{a}	51.9 ± 6.6^{b}
Skim milk Tyrode	79.2 ± 2.3^{b}	65.0 ± 2.9^{b}	63.3 ± 4.8^{a}	54.9 ± 4.7^{a}	176.2 ± 10^{a}	78.1 ± 6.8^{a}
Seminal Plasma	63.0 ± 1.1^{a}	42.8 ± 4.1^{a}	38.6 ± 3.0^{b}	26.9 ± 5.5^{b}	164.1 ± 8.7^{a}	73.3 ± 7.1^{a}

$^{a-c}$: Values with different superscripts within rows are significantly different ($P < 0.05$).

important in cooled storage. A relationship between spermatozoal motility and electrolytes (Na, K, Cl) has been assumed by several authors (14,15,18,21).

A second important finding was that total removal of seminal plasma was better than presentation of seminal plasma. The highest viability and membrane integrity values were obtained in skim milk glucose extender and the best motion characteristics in skim milk glucose extender supplemented with modified Tyrode's medium without seminal plasma. Seminal plasma is not considered as an appropriate medium for storage of stallion spermatozoa. Many researchers have reported that exposure to seminal plasma for long periods of time is detrimental to sperm survival, similarly most of these experiments were carried out using either sperm-rich fraction or total ejaculate (7-9,15,17). A limited amount of data is available for cooled storage in seminal plasma from sperm-poor fractions. Total ejaculate contains pre-sperm fluid which has been shown to lower motility (10). Post-cooling motility is an important indicator of the success of a cooling protocol. It is generally accepted that the presence of a small percentage of seminal plasma is required to optimize spermatozoal motility following cooled storage (8,9). Jasko et al. (8) recommend the inclusion of 5% to 20% of seminal plasma. Sieme et al. (22) found that storage of semen at 5 °C resulted in a significant increase of motility when semen was centrifuged and addition of seminal plasma did not improve motility. Removal of seminal plasma and dilution of semen using skim milk extender supplemented with modified Tyrode's medium during cooled storage has been reported to maintain well spermatozoal motility and fertility (15,17). These results agree with our results. Webb and Arns (15) noted that removal of seminal plasma did not affect post-storage motility of sperm in skim milk glucose extender. Our results do not agree with the findings of Padilla and Foote (18) who reported that motility of sperm in skim milk extender containing seminal plasma was superior to motility of sperm in skim milk extender without seminal plasma. According to our results, skim milk glucose extender provides good protection for sperm viability and membrane integrity, but not for motility. On the contrary, skim milk glucose extender supplemented with modified Tyrode's medium is more effective in maintaining motility, presumably because of its ionic balance.

Seminal plasmas were not exchanged between stallions, but it seems that seminal plasma from stallion 1 were not good quality because of semen quality. In the study of Katila et al. (11), seminal plasma exchange between stallions changed some motility parameters. Aurich et al. (23) reported that addition of seminal plasma from stallions with high post-thaw motility of sperm to ejaculates from stallions with low post-thaw motility increased motility and membrane integrity. The opposite effect was detected when seminal plasma from stallions with low sperm motility was added to stallion sperm with high motility. Similarly, according to Brinsko et al. (16) individual variability of stallions has an important role in motility after cooled storage.

As reported by Pickett et al. (9) and Katila (4), numerous studies have been carried out to compare extenders, temperature of storage, etc. Unfortunately, the majority of studies are based on motility evaluations, which do not always reflect true fertilizing capacity. So, it is quite difficult to compare our results to the literature.

The results of the present study indicate that 1) skim milk glucose extender provides good protection for sperm viability and membrane integrity, but not for motility, 2) on the contrary, skim milk glucose extender supplemented with modified Tyrode's medium is more effective in maintaining motility, presumably because of its ionic balance, 3) exposure to seminal plasma for 24 h is detrimental to sperm survival.

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