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Effect of Seminal Plasma Removal on Cell Membrane, Acrosomal Integrity and Mitochondrial Activity of Cooled Stallion Semen

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Abstract

Fresh semen samples were collected from 11 warm blood stallions, each ejaculate was distributed into three equal parts. The first part was diluted in a skim milk-glucose diluent (SMG), the second part was diluted in a skim milk-glucose supplemented with Tyrode's medium (SMG-T), the third part was centrifuged to remove the seminal plasma, then the sperm was resuspended in the second diluent (SMG-T-C). The diluted semen were evaluated immediately after dilution (0 hour) and at 24, 48, 72, and 96 hours of storage at 5°C. Flow cytometry was performed to determine sperm viability, mitochondrial activity and acrosomal integrity. Immediately after dilution the tested parameters of sperms that diluted in SMG-T was significantly ($P<0.001$) higher than those diluted with SMG and SMG-T-C, and with SMG-T-C were higher significantly ($P<0.05$) than those diluted with SMG. The decreasing rate in tested sperm parameter was greater significantly ($P<0.001$) in semen samples which were diluted with SMG than those diluted with SMG-T and SMG-T-C. In conclusion, the present study indicated that viability, acrosomal integrity, and mitochondrial activity of stallion sperms were better preserved in SMG-T in comparison with SMG, also centrifugation and removal of the seminal plasma have an adverse effect on these three sperm parameters.

*Keywords:*Stallion; sperm viability; mitochondrial activity; acrosome integrity; diluents; seminal plasma.

Introduction

The most common assisted reproductive technique world wide applied in the equine industry is artificial insemination, either with fresh or frozen semen. Good quality semen is the most important factor to implement breeding programs (Pickett, 1993; Magistrini et al., 1996; Parlevliet and Colenbrander, 1999). Many problems are implicated in the limited use of artificial insemination with frozen equine semen (Klug *et al*., 1975; Tischner, 1979; Muller, 1987; Volkman and Zyl, 1987; Palmer and Magistrini, 1992). Therefore, over the past years, technology for cooling semen has been developed. The main cause of the increased use of cooled semen is the high proportion of stallions (20–40%) whose sperm responds poorly to cryopreservation (Vidament *et al*., 1997).

Few studies have been carried out the effects of diluents on quality of equine semen stored at 5°C. Varner *et al*. (1989) demonstrated that storage of spermatozoa at 5°C was superior to storage at 20°C and that storage of spermatozoa between 4 and 6°C was also superior to storage between 0 and 2°C for preserving spermatozoal motility using milk-based extenders. Semen stored for 24 hours maintains fertility similar to that of fresh semen (Francl *et al*., 1987), but when stored at 5°C for 48 hours, sperm motility, velocity (Jasko *et al.*, 1992), and fertilization rates generally (Pickett *et al.*, 1989) decline.
A large variety of extenders combining various components such as sugars, electrolytes, buffers, egg yolk, milk and milk products, have been proposed for cooling sperm. A skim milk extender, originally described by Kenney *et al*. (1975) has been used in a slightly modified formula for preservation of equine semen at 5°C (Rota *et al*., 2004). Braun *et al*. (1993) evaluated the motility of equine semen stored in a skim milk extender, slightly modified Kenney extender and those stored in an egg yolk extender at 5°C. Masuda *et al*. (2004) was used a modified boar semen extender supplemented with 2% casein for preservation of equine semen during the storage at 5°C.

Some of the previous studies indicated that sem-

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inal plasma in a high concentration can reduce sperm motility when stored at 5°C (Varner *et al*., 1987; Brinsko *et al*., 2000); while others found that complete removal of seminal plasma maximizes longevity of sperm motility (Jasko *et al*., 1991). Love *et al*. (2005) demonstrated a reduction effect of centrifugation and removing of the seminal plasma in DNA integrity, in spite of maintenance of sperm motility.

In previous studies, motility and fertility of equine semen stored in skim milk based extender were examined, but there was no information about the effect of these diluents on other sperm parameters such as cell membrane integrity (viability), acrosomal integrity, and mitochondrial activity of stallion sperms. Therefore, the present study was conducted to estimate the influence of diluents and dilution method on these sperm parameters during the storage at 5°C.

Materials and methods

Diluents

Two diluents in three dilution were used in this study, the first one consisted of skim milk and glucose and the second one consisted of a skim milk, glucose and Tyrode's medium (65%:35%, vol:vol). Both diluents were prepared as described by Padilla and Foote (1991).

Semen samples

Fresh semen samples from 11 warm blood stallions (Equine Health Service, Westphalian Chamber of Agriculture, Münster, Germany) were collected using a pre warmed artificial vagina. A nylon mesh filter was used to eliminate the gel fraction, and only allow the sperm-rich fraction of the ejaculate to enter into the collection bottle. Following the determination of ejaculate volume, and concentration of spermatozoa, each ejaculate was distributed into three equal parts. The first part was immediately diluted in a skim milk-glucose diluent and the second part was diluted in a skim milk-glucose supplemented with Tyrode's medium, the third part of the ejaculate was centrifuged to remove the seminal plasma, then the sperm was resuspended in the second diluent. The final concentration of sperm in semen of the three groups was 25 million cells/ml. Both media were warmed to 37°C prior to semen collection. The diluted semen samples were cooled slowly to about 5°C and stored for 96 hours.

Semen analysis

All the laboratory examinations were conducted in the physiology laboratory of the Institute of the Anatomy, Physiology and Hygiene of Domestic Animals, University of Bonn (Bonn, Germany).

The diluted semen samples were evaluated immediately after dilution (0 hour) and at 24, 48, 72, and 96 hours of storage after cooling at 5°C. Flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) was performed to determine sperm viability, mitochondrial activity and acrosomal integrity. A total of 10000 sperms were analysed for each sample.

To evaluate the sperm viability, LIVE/DEAD sperm viability kit (L-7011, Molecular Probes, Eugene, OR, USA) was used. A staining solution was prepared that contained 0.8 µl SYBR-14 and 9 µl PI/ml HEPES-0.1% BSA. 100 µl of the diluted semen was stained with 300 µl of the staining solution of SYBR-14. The samples were incubated at 37°C for 15 min prior to flow cytometric examination (Garner *et al*., 1997).

To estimate the sperm mitochondrial activity, stock solutions of 0.53 mM Rhodamine 123 (R-302, Molecular Probes, Eugene, OR, USA) were prepared in DMSO and 2.99 mM Propidium Iodide (PI) (P-4170, Sigma, Deisenhofen, Germany) in Tyrode's Salt Solution (T-2397, Sigma, Deisenhofen, Germany). Final staining solution contained 3 µl of R123 stock solution and 12 µl PI stock solution/ml Hepes-BSA (Ball *et al.*, 2000). 150 µl of the diluted semen was stained with 300 µl of the final staining solution of R123. The samples were incubated at 37°C for 30 min before flow cytometric examination.

The acrosomal integrity of spermatozoa was analysed using a stock solution of 1 mM LYSO-G (L-7526, Molecular Probes, Eugene, OR, USA), which was prepared in DMSO (D-8779, Sigma, Deisenhofen, Germany), and 2.99 mM Propidium Iodide (PI) in Tyrode's Salt Solution. Final staining solution contained 5 µl of LYSO-G stock solution and 12 µl PI stock solution/ml Hepes-BSA (Garner *et al*., 1997). 150 µl of the diluted semen was stained with 300 µl of the final staining solution of LYSO-G. The samples were incubated at 37°C for 30 min before flow cytometric examination.

Statistic analysis

At first the data were tested for normal distribution using a Normality test. Normal distributed data were compared by ANOVA. Significant differences were determined by Duncan's Multiple Range Test. The data sets, that had not fulfilled the assumption of normal distribution, were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks. Significant differences were determined by Dunn's Test. Pearson correlation coefficients were used to evaluate the correlation between the tested sperm parameters and time of semen storage. All statistical analyses were performed by SigmaStat (Jandel scientific software V3.1). $P<0.05$ was considered as statistically significant.

Results

Immediately after dilution, viability, acrosomal integrity and mitochondrial activity of sperms that diluted with SMG-T was significantly ($P<0.001$) higher than those diluted with SMG and SMG-T-C, also the tested sperm parameters of semen diluted with SMG-T-C were significantly ($P<0.05$) higher than those diluted with SMG, Table 1.

Table 1. Results of semen evaluation which was evaluated immediately after dilution (0 hour) a

Sperm Parameters	Semen diluents and dilution methods		
	SMG	SMG-T	SMG-T-C
Sperm viability	72.5±15.1a	84.7±4.7b	81.6 ± 6.1c
Acrosomal integrity	64.2±12.4a	82.0±5.1b	76.1±7.2c
Mitochondrial activity	70.0±15.3a	83.1±5.2b	79.4±6.8c

a,b,c Values with a different superscript within rows were significantly different ($P<0.05$).

Through the 96 hours of storage at 5°C, the three tested sperm parameters of semen, that diluted by examined dilution methods, were reduced gradually with the time of the storage. There was a significant negative ($-0.989> r <-0.96$, $P<0.01$) correlation between the storage time and the tested parameters. The reduction rate in tested sperm parameter was significantly ($P<0.001$) higher in semen samples which were diluted with SMG than those diluted with SMG-T and SMG-T-C, while no differences in reduction rate of sperm parameters were observed between the last two methods of dilution.

In both SMG-T and SMG-T-C dilution methods, sperm viability, acrosomal integrity and mitochondrial activity were reduced gradually during the 96 hours of storage at 5°C; while in SMG the sperm parameters were reduced sharply especially at the first 24 hours of storage at 5°C (Fig. 1-3).

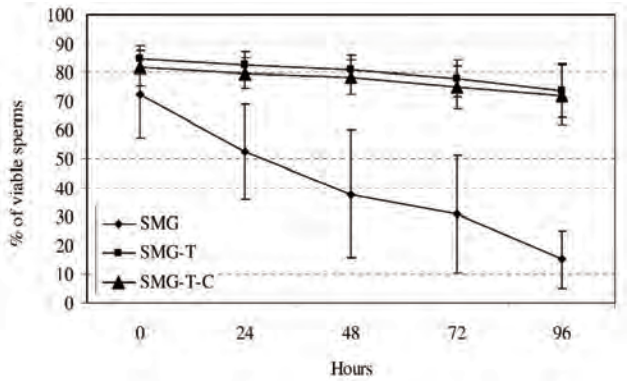

Fig. 1. Sperm viability of the three groups of diluted semen that stored for 96 hours at 5°C.

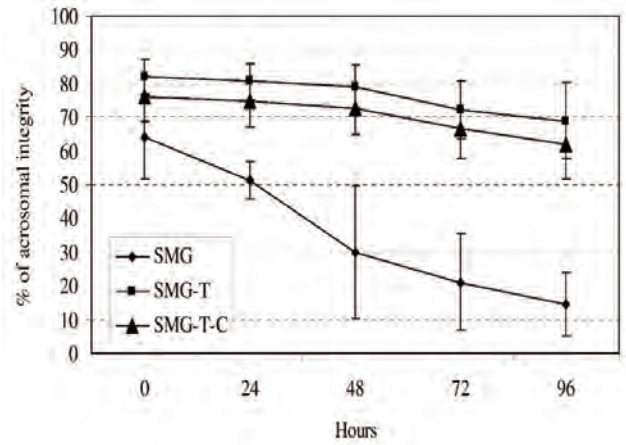

Fig. 2. Acrosomal integrity of the three groups of diluted semen that stored for 96 hours at 5°C.

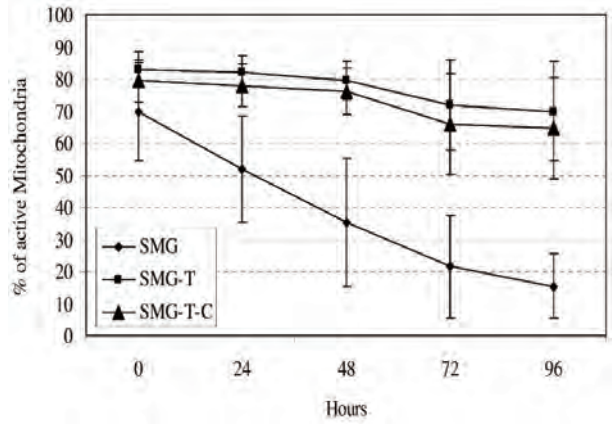

Fig.3. Mitochondrial activity of the three groups of diluted semen that stored for 96 hours at 5°C.

Discussion

Numerous previous studies reported a marked effect of extender, centrifugation and seminal plasma removal on preservation of stallion semen that

stored at 5°C (Padilla and Foote, 1991; Braun *et al*., 1993; Ball *et al.*, 2000; Brinsko *et al*., 2000; Love *et al*., 2005). The majority of these studies were based on motility evaluation which did not always reflect the effects on other sperm parameters such as viability, acrosomal integrity and mitochondrial activity, therefore the present study was planed.

Results of the present study indicated that a significant difference in sperm parameters, after dilution and storage at 5°C, among the stallions, this variation occurred due to the individual differences in susceptibly of equine semen to extension and storage (Bedford *et al*., 1995), similar observation in sperm motility was reported previously by Padilla and Foote (1991).

In this study, the tested parameters were also maintained in the SMG-T treatment. This method of dilution was superior to dilute the semen with SMG in preserving the sperm parameters. As in other species, the electrolytes such as Potassium and Sodium in equine semen plasma are important in regulation of sperm functions (Pesch et al., 2006). Padilla and Foote (1991) reviewed that the ratio of Sodium:Potassium in stallion seminal plasma was 4.6:1, while in SMG and SMG-T the ratio were 1:1 and 4.3:1, respectively. The great difference in Sodium:Potassium ratio between the SMG and the original seminal plasma may be the main cause of reduction in sperm parameters immediately after dilution. While this ratio in SMG-T was very close to the ratio in seminal plasma, therefore the tested sperm parameters were not affected after dilution. This finding agreed with Rota *et al.* (2004) who suggested that milk and glucose alone might not be sufficient for the optimal spermatozoal preservation.

Seminal plasma is very important for sperm metabolism as well as sperm function and survival and transport in the female genital tract (Pesch *et al*., 2006). While long-term seminal plasma exposure results in irreversible loss of motility (Baas *et al*., 1983). The previous studies showed that the addition of modified Tyrode medium to the SMG extender may improve spermatozoal motility after cooled preservation when seminal plasma has to be completely removed (Padilla and Foote, 1991; Rigby *et al*., 2001). Our results demonstrated that centrifugation and removal of the seminal plasma (dilution in SMG-T-C) had a reverse effect on sperm parameters especially on acrosomal integrity in comparison with dilution in SMG-T. The explanation of this result depends on two factors; the first is centrifugation of the semen and the other one is the removal of seminal plasma. Padilla and Foote (1991) were reported that centrifugation of stallion semen reduced the sperm motility immediately after dilution and during the storage at 5°C. In other hand, Ball *et al*. (2000) found that removal of seminal plasma increased the susceptibility of stallion spermatozoa to oxidative stress. This result agreed with Love *et al*. (2005) where found that centrifugation and removal of the seminal plasma may enhance sperm motility of cooled-stored stallion semen, but also had an adverse effect on sperm DNA integrity.

Conclusion

The present study indicated that viability, acrosomal integrity, and mitochondrial activity of stallion sperms were better preserved in a skim milk-glucose diluent that supplemented with Tyrode's medium in comparison with skim milk-glucose diluent alone, also centrifugation and removal of the seminal plasma have an adverse effect on these three sperm parameters.

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