Efficiency of Ringer B. Braun solution on stallion epididymal sperm motility and viability compared to the commercial extender within 72 hours of storage

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Abstract

Collection of epididymal stallion sperm offers the opportunity to retain and use genetic material from the males after elective castration or even *post mortem*. The aim of the present study was to evaluate the effect of the Ringer B. Braun solution on stallion epididymal sperm viability and motility during liquid storage for 72 h at 4 °C and at 20 °C. Eight stallions (3–10 years old) were used in the study. Sperm from the cauda epididymis was harvested immediately after routine castration. The sperm from epididymis was washed out and diluted with Ringer B. Braun solution and with a commercial semen extender which was used as a control. Samples from each horse were divided into two parts: one part of samples was stored at 4 ± 1 °C as recommended for the commercial semen extender, the other one was stored at 20 ± 1 °C. Sperm viability, motility, and pH were checked one hour after collection and dilution, and after 24, 48 and 72 h of incubation. The results of sperm viability, subjective and progressive sperm motility showed slightly higher results in semen samples diluted with Ringer B. Braun solution in one hour compared to the commercial extender, by 2.40 ± 0.49% for viability ($P > 0.05$), by 0.30 ± 9.92% for subjective motility ($P > 0.05$) and by 5.70 ± 6.07% for progressive motility ($P > 0.05$). We suggest that Ringer solution could be used for a short term storage (1–24 h) of equine epididymal semen at a 4 °C temperature.

Horse, semen extender, semen preservation, semen quality

Collection of epididymal sperm allows saving genetic material of valuable breeding animals or endangered species in case of unexpected death or emergency castration. The collection of epididymal spermatozoa has been reported in different species (Martinez-Pastor et al. 2005; Monteiro et al. 2011; Toyonaga et al. 2011; Martins et al. 2012; Neuhauser et al. 2019). The death of a valued stallion can be devastating and unexpected, as can a castration be necessary for the stallion's health and wellbeing. Collection of sperm from the epididymis can be the last option to secure the stallion's genetic material (Cary et al. 2004; Martinez-Pastor et al. 2005; Toyonaga et al. 2011). Collection, preservation, and artificial insemination of mares with epididymal sperm has become more and more popular in equine breeding (Bruemmer 2006; Melo et al. 2010). However, the success of epididymal semen collection, preservation, and fertilization ability is highly dependent on the speed and temperature at which the testes are transported to the centre, from the time the animal was castrated or the time of its death (Monteiro et al. 2013a,b).

The epididymis is crucial for sperm maturation and sperm storage (Brito 2007). Epididymal spermatozoa remain quiescent in the cauda epididymis and most spermatic cells are immotile after harvesting. The estimated capacity of spermatozoa stored in the epididymis corresponds to more than 10 ejaculates (Sostaric et al. 2008). Biochemical studies have shown that the development of motility from the caput to the cauda epididymis is associated with increased levels of intraspermatic pH, adenosine monophosphate (cAMP) and calcium ions, mediators responsible for the activation of sperm kinetics (Kann and Serres 1980).

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After collection, semen must be diluted with an extender to maintain sperm viability until its use for artificial insemination. With an appropriate extender, diluted epididymal sperm can be successfully stored under cooled conditions for more than 24 h (Neuhauser et al. 2019). According to manufacturer's instructions in such commercial extenders the refrigerated semen can be stored for 72 h. The extender is a medium which contains protective compounds that allow sperm survival outside the reproductive tract, protecting sperm against temperature fluctuations, maintaining sperm motility and fertilizing capacity, and controlling bacterial growth (Pinto et al. 1999). The presence of specific substrates inside extenders may allow for better survival of spermatic cells (Guasti et al. 2013). Therefore, choosing an extender with optimal properties for preserving sperm viability, motility, and fertilizing ability may be the key to successful sperm fertilizing ability preservation (Martins et al. 2012; Neuhauser et al. 2018). Although there are many commercial extenders available for semen storage, there is no special standardized extender for epididymal semen storage.

Ringer B. Braun solution is commonly used as a suitable media for epididymal semen collection and transportation to artificial insemination centre for further semen evaluation, dilution, liquid storage, or cryopreservation (Martins et al. 2009; Martins et al. 2012; Stawicki et al. 2016). As Ringer B. Braun solution is normally not intended as semen extender, it might be used when no other extenders and refrigerators for semen cooling are available. The aim of the present study was to evaluate the effect of the Ringer B. Braun solution on stallion epididymal sperm viability and motility during liquid storage for 72 h at 4 °C and at 20 °C.

Materials and Methods

Eight horses of 3–10 years of age without a breeding history were used in the study. The testicles and epididymides from all horses, without pathologies, were obtained in the same way via routine surgical castration at the clinic. After surgery, the testicles were immediately taken to the laboratory and the sperm from the epididymal cauda was harvested within one hour after castration. The epididymis was cleaned and the cauda with vas deferens were isolated from the rest of the epididymis by making a cut near the junction of the corpus and the proximal cauda. Retrograde washing was performed of the vas deferens and cauda epididymis. We used a syringe loaded with 5 ml of extender and cannulated the vas deferens using a blunted 21G needle. The content of the right epididymis was washed out using Ringer B. Braun solution without additives (B. Braun Melsungen AG, Germany); the content of the left epididymis was washed out using the commercial extender Equiplus for stallion semen without antibiotics (Minitube, GmbH, Tiefenbach, Germany) which was used as a control. The vas deferens and cauda were perfused with the extender, injecting air afterwards, until all the contents were flushed out of the cauda epididymis. The samples were collected in a plastic tube. Both extenders were prepared beforehand and warmed to a temperature of 37 ± 0.5 °C before use.

All samples were initially evaluated for sperm viability, subjective and objective sperm motility, semen pH, concentration and morphology evaluation. Primarily semen quality assessment was performed during the first hour after semen collection and was named R0 hour (for Ringer) and E0 hour (for commercial extender).

After initial semen quality evaluation, samples in Ringer and commercial extender from each horse were divided into two parts (each tube 2.5 ml aliquots). One part of samples (Ringer 4 °C [R4] and commercial extender 4 °C [E4]) were kept at 4 ± 1 °C in a refrigerator (Eiron, Latvia) as recommended by the commercial extender manufacturer. The second part of samples (Ringer 20 °C [R20] and commercial extender 20 °C [E20]) were incubated at 20 ± 1 °C ("room temperature") in the acclimatization box (Friocell, Germany). Sperm viability, subjective and objective motility and pH were checked after 24, 48, and 72 h of incubation. In total, 140 tests for sperm viability, subjective and objective motility and pH were performed.

Sperm viability was detected using eosin-nigrosin staining (Minitube, Tiefenbach, Germany) according to the manufacturer's instructions. Motility of spermatozoa was assessed in two ways: subjectively by visual inspection and objectively using software (Sperm Class Analyzer, Microptic, Spain). Motility of spermatozoa was examined subjectively by visual inspection at 37 °C under phase-contrast microscope Olympus BH2 with a pre-warmed 37 °C stage (Olympus Optical Co., Ltd., Japan) using × 400 magnification. Motility was analysed on 5-µl aliquots of semen. Whole samples in tubes (2.5 ml) were pre-warmed in a 37 °C water bath (Memmert, Schwabach, Germany) for 5 min before analysis. The percentage of progressive spermatozoa in three–five fields of the microscopic view was determined as subjective motility. For objective motility, the sperm sample was prepared as described above. Objective sperm motility was analysed by the Sperm Class Analyzer (SCA) software. At least 1500 cells in one sample were analysed. The total general and the progressive sperm motility by percent

were fixed. Sperm pH value was analysed by an electronic pH meter (AB150, Fisher Scientific Accumet®, USA) according to the manufacturer's instructions. Sperm concentration and morphology of diluted stallion epididymal semen were assessed using conventional semen evaluation methods (Januskauskas 2010). Sperm concentration was assessed in Neubauer improved (Germany) blood cell counting chamber. A total number of abnormal spermatozoa were determined in dry preparations, stained with SpermBlue (Microptic, Spain). After the dilution, the final sperm concentration was 233.7 ± 30.1 million/ml in the Ringer samples and 220.8 ± 31.1 million/ml in commercial extender samples. The total amount of abnormal spermatozoa was 69.84 ± 10.02% for Ringer and 68.25 ± 12.16% for commercial extender.

Statistical analysis was performed using the SPSS statistical analysis software, version 25 (SPSS Inc., Chicago, IL, USA). The results were produced as the mean ± standard error of the mean (M ± SEM). Multiple comparisons of group means were calculated using LSD method. The differences were considered to be significant when *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Analysis of the linear relationship between the dependent variables was evaluated by Pearson correlation coefficient.

Results

We found significant differences in epididymal sperm viability, motility and pH results in two tested dilution media after 72 h of liquid storage at temperatures of 4 °C and 20 °C. As shown in Table 1, only R0 (compared to E0) test of sperm viability showed higher results by 2.4 ± 0.49% ($P > 0.05$) in semen samples diluted with Ringer B. Braun solution. The solution was less effective for protection of the functionality of sperm plasma membrane during the liquid storage at 4 °C and 20 °C for 72 h. However, results of sperm viability at 24 h remained still high and stable.

Table 1. The effect of dilution media (R – Ringer B. Braun solution, E – commercial semen extender) on sperm viability results in liquid epididymal equine semen stored at 4 °C (R4 and E4) and at 20 °C (R20 and E20) for 0, 24 (a), 48 (b) and 72 (c) h.

Treatment	Viability, % Storage time (h)			
	0	24[a]	48[b]	72[c]
R	90.90 ± 1.77	-	-	-
R4	-	83.50 ± 3.58*[c]	78.90 ± 3.29	70.20 ± 3.85*[a]
R20	-	82.10 ± 3.03*[c]	77.50 ± 3.6	66.70 ± 5.32*[a]
E	88.50 ± 2.26	-	-	-
E4	-	87.00 ± 1.70	83.10 ± 2.53	80.30 ± 3.55
E20	-	86.90 ± 1.85*[c]	80.80 ± 2.47	76.10 ± 3.9*[a]

Letters [a, b, c] indicate significant differences between the means in the same semen dilution media over different storage time (* $P < 0.05$).

The motility assay showed lower sperm motility in samples of both dilution media during longer storage time (Figs 1–3). Significant differences were detected in the results of subjective and objective motility between storage times in all tested samples ($P < 0.05$). The only tests of subjective and progressive sperm motility by Sperm Class Analyzer (SCA) software performed one hour after equine epididymal semen dilution with two different diluents (R0 and E0) showed higher sperm motility results by 0.30 ± 9.92% for subjective motility ($P > 0.05$) and by 5.70 ± 6.07% for progressive motility ($P > 0.05$) in semen samples diluted with Ringer B. Braun solution.

Analysis of the effect of storage temperature showed a negative effect of "room temperature" (20 °C) on sperm motility in the samples diluted with Ringer B. Braun solution or commercial extender. Sperm motility in samples diluted with Ringer B. Braun solution or commercial extender and stored at 20 °C temperature decreased rapidly and differ significantly from the results in samples stored at 4 °C. The subjective sperm motility,

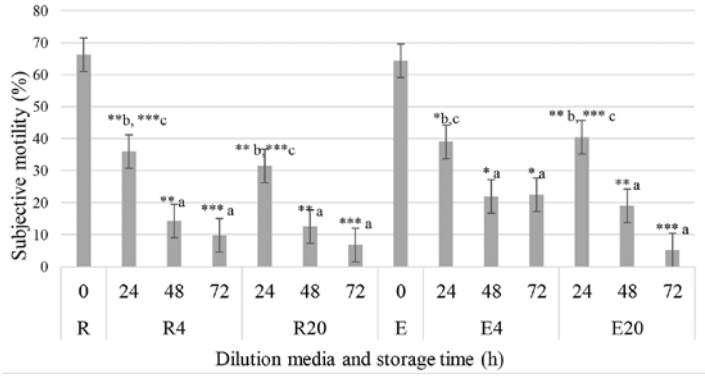

Fig. 1. The effect of dilution media (R – Ringer B. Braun solution, E – commercial semen extender) on sperm subjective motility results in liquid epididymal equine semen stored at 4 °C (R4 and E4) and at 20 °C (R20 and E20) for 0, 24 (a), 48 (b) and 72 (c) h.

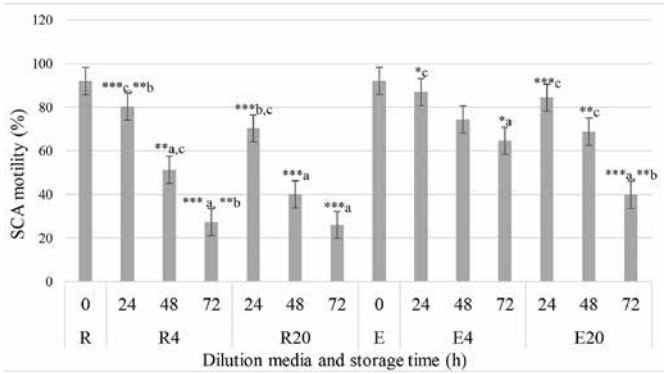

Fig. 2. The effect of dilution media (R – Ringer B. Braun solution, E – commercial semen extender) on sperm subjective motility results in liquid epididymal equine semen stored at 4 °C (R4 and E4) and at 20 °C (R20 and E20) for 0, 24 (a), 48 (b) and 72 (c) h.

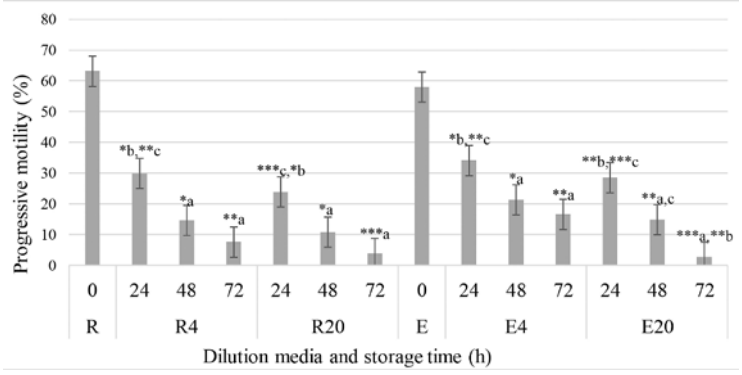

Fig. 3. The effect of dilution media (R – Ringer B. Braun solution, E – commercial semen extender) on sperm objective progressive motility results in liquid epididymal equine semen stored at 4 °C (R4 and E4) and at 20 °C (R20 and E20) for 0, 24 (a), 48 (b) and 72 (c) h.

total motility, and progressive sperm motility in samples diluted with Ringer B. Braun solution at 72 h of storage at 4 °C temperature were higher than in samples stored at 20 °C temperature by 3 ± 0.28% ($P < 0.001$), 1.3 ± 0.47% ($P < 0.01$) and by 3.7 ± 1.76% ($P < 0.001$), respectively. As expected, sperm motility results, assessed in three ways, were higher at 72 h in samples diluted with commercial extender and stored at 4 °C temperature (Figs 1–3).

Semen pH was measured to evaluate ionic conditions in equine epididymal semen during storage. Semen pH detected one hour after semen collection and dilution was almost equal (pH 6.90 ± 0.10/7.00 ± 0.00) in both R0/E0 semen samples ($P > 0.05$). The results showed decreased sperm pH in all samples stored in liquid for the whole 72 h. A significant difference in semen pH change during storage was observed in E20 group. The pH changed from 7.00 ± 0.00 after 24 h to 6.10 ± 0.10 after 72 h ($P < 0.05$). Semen samples diluted with Ringer B. Braun solution and stored for 72 h at 20 °C and at 4 °C, showed lower pH by 0.8 ($P < 0.05$). Slight reduction of semen pH by 0.30 ± 0.06 was also detected in semen samples diluted with commercial extender and stored at 4 °C temperature for 72 h ($P < 0.05$).

Discussion

Findings of the current study indicated that prolonged storage time significantly reduced sperm viability and motility in samples with both dilution media. We detected a significant difference in lowering sperm quality indices in subjective and objective motility results between storage times in all tested samples ($P < 0.05$). Sperm motility reduction may be associated with the enhancement in the generation of free radicals and their negative impact on the plasma membrane lipid and protein distribution and loss of physiological function during semen dilution and storage (Baumber et al. 2000). As expected, after 72 h of storage, sperm viability and motility indices, assessed in all three ways, were higher in samples diluted with the commercial extender, which is in agreement with results of studies by other authors (Pagl et al. 2006; LeFrapper et al. 2010; Neuhauser et al. 2017; Alamaary et al. 2019).

While the commercial extender showed better results in sperm preservation during the longer period (72 h), Ringer B. Braun solution was better during short-term storage (24 h at 4 °C). We found that the subjective and progressive sperm motility one hour after epididymal semen dilution showed higher sperm motility results by 0.30 ± 9.92% for subjective motility and by 5.70 ± 6.07% for progressive motility ($P > 0.05$) in semen samples diluted with Ringer B. Braun solution. We assume that better preservative properties in the protective effect on cell membrane integrity and motility of Ringer B. Braun solution in this situation is the result of mineral composition which may have offered protection of sperm from cold stress. In contrast, the commercial extender is based on caseinates derived from milk casein and highly purified proteins, buffer, and sugars.

Another finding in this study was the lack of positive effect of room temperature (20 °C) on sperm viability and motility using both dilution media. We wanted to verify the effect of room temperature on semen preservation because there are many random situations when no special refrigerators for semen cooling are available on the farm. Our results showed that the viability results in semen samples diluted with Ringer B. Braun solution or in commercial extender and stored at 4 °C temperature were higher than samples stored at 20 °C temperature ($P > 0.05$). Another study also suggests the optimal semen storage temperature of 4–6 °C if stored for 24 h and more (Varner et al. 1989). Almost no loss of fertility was noted during storage at 5 °C for 24 h in studies by Aurich (2005) and Price et al. (2008). Additionally, studies in rats, goats and cattle have shown that most of these species demonstrated superior seminal viability when the epididymides were stored

between 4 °C and 5 °C compared with those kept at room temperature (James 2004). Many studies support the results of this study of 4 °C being the optimal storage temperature for epididymal spermatozoa (Bruemmer 2006; Monteiro et al. 2011; Vieira et al. 2013).

Sperm motility was also affected during storage at 20 °C. It decreased rapidly and differed significantly compared to results of samples stored at 4 °C. As expected, sperm motility results, assessed in three ways, were higher at 72 h in samples diluted with commercial extender and stored at 4 °C temperature than in Ringer B. Braun solution. Studies in testis-epididymis storage complex at 5 °C provided better preservation of epididymal sperm than storage at room temperature. Regardless of the temperature, progressive motility is the sperm indicator that is most sensitive to storage time (Monteiro et al. 2013a).

The contact of extended semen with environmental air is an additional factor that could modify stored semen quality (Ribeiro et al. 2016). The authors observed that the pH of semen changed as the amount of air increased within the tubes; however, only a few studies have analysed the effect of air presence on semen pH and semen quality indicators. In our study, semen pH was measured for the evaluation of ionic conditions in equine epididymal semen. The pH value slightly decreased during storage in all samples; however, the most stable pH remained in semen samples diluted with the commercial extender and stored at 4 °C for 72 h. Our results support the results of other studies that the addition of sugars and special substances in the commercial extender can control the pH better and provide the energy which enhances sperm motility and viability (Alamaary et al. 2019).

The results of the present study suggest that the commercial extender, as a product used to preserve cooled stallion semen, has better suitability for epididymal sperm preservation in stallions for 72 h of liquid storage at 4 °C compared to Ringer B. Braun solution. However, sperm viability, subjective motility and progressive motility results were higher in semen samples diluted with Ringer B. Braun solution when stored for a shorter period (24 h). The study suggest that Ringer B. Braun solution could be used for short-term storage (1–24 h) of equine epididymal semen at 4 °C. However, no protective effect of Ringer B. Braun solution or commercial extender on the functionality of sperm plasma membrane has been observed at room temperature (20 °C) for 72 h.

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