Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa

A Heise ^{a,*}, PN Thompson ^a, D Gerber ^a

^a Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, 0110 Onderstepoort, South Africa

*Corresponding author at: Section of Reproduction, Department of Production Animal Studies, Private bag x 04, Faculty of Veterinary Science, 0110 Onderstepoort, South Africa.

Tel.: +27 12 5298259; fax: +27 12 5298314; email address: annett.heise@gmx.de

Abstract

Fresh and post-thaw parameters (motility, morphology and viability) of stallion epididymal spermatozoa that have been and have not been exposed to seminal plasma were evaluated, and directly compared to fresh and post-thaw parameters of ejaculated spermatozoa.

Six sperm categories of each stallion (n= 4) were evaluated for motility, morphology and viability. These categories were fresh ejaculated spermatozoa (Fr-E), fresh epididymal spermatozoa that had been exposed to seminal plasma (Fr-SP+), fresh epididymal spermatozoa that had never been exposed to seminal plasma (Fr-SP-), frozen-thawed ejaculated spermatozoa (Cr-E), frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing (Cr-SP+) and frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma (Cr-SP-).

Results show that seminal plasma stimulates initial motility of fresh epididymal stallion spermatozoa while this difference in progressive motility is no longer present post-thaw; and that progressive motility of fresh or frozen-thawed ejaculated stallion spermatozoa is not always a good indicator for post-thaw progressive motility of epididymal spermatozoa.

This study shows that seminal plasma has a positive influence on the incidence of overall sperm defects, midpiece reflexes and distal cytoplasmic droplets in frozen-thawed stallion epididymal spermatozoa while the occurrence of midpiece reflexes is likely to be linked to distal cytoplasmic droplets. Furthermore, seminal plasma does not have an influence on viability of fresh and frozen-thawed morphologically normal epididymal spermatozoa.

We recommend the retrograde flushing technique using seminal plasma as flushing medium to harvest and freeze stallion epididymal spermatozoa.

Keywords: stallion, epididymal spermatozoa, seminal plasma, motility, morphology, viability

1. Introduction

Cryopreservation of epididymal spermatozoa may be the only opportunity to preserve valuable genetics of males in cases of unforeseen injury or death. Stallion epididymal spermatozoa have been cryopreserved successfully (Bruemmer et al. 2002; Cary et al. 2004; Heise et al. 2010; Jimenez 1987; Melo et al. 2008; Morris et al. 2002; Neild et al. 2006; Papa et al. 2008; Tiplady et al. 2002). Different methods for collection of stallion epididymal spermatozoa have been described (Bruemmer 2006; Cary et al. 2004) and their cryopreservation after 24 hours of ground transportation has been shown to be successful (Neild et al. 2006). It has been hypothesised that epididymal spermatozoa are more resistant to cold shock and should therefore be more suitable for cryopreservation than ejaculated spermatozoa (Johnson et al. 1980) but only one report has shown post-thaw motility of epididymal spermatozoa that was equal to or better than that of frozen-thawed ejaculated spermatozoa (Volkman et al. 2001).

It has been demonstrated that stallion epididymal spermatozoa are fertile and pregnancies as well as live foals have been produced (Barker and Gandier 1957; Heise et al. 2010; Melo et al. 2008; Morris et al. 2002; Papa et al. 2008). Pregnancy rates after conventional AI with fresh and frozen-thawed epididymal spermatozoa have been low (Morris et al. 2002) but higher pregnancy rates using frozen-thawed epididymal spermatozoa have only been reported recently (Heise et al. 2010; Melo et al. 2008; Papa et al. 2008). The effects of seminal plasma on pre-freeze and post-thaw spermatozoal motility of epididymal spermatozoa have been investigated but the results were contradictory. Most papers report a beneficial effect of seminal plasma on freshly harvested epididymal spermatozoa (Braun et al. 1994b; Stout et al. 1999; Tiplady et al. 2002) but reports on the effect of seminal plasma on post-thaw quality variables are not as clear. While Stout et al. (1999) found a beneficial effect of seminal plasma on post-thaw motility, others (Bruemmer et al. 2002; Cary et al. 2004; Morris et al. 2002; Papa et al. 2008; Tiplady et al. 2002) could not demonstrate a

difference. In contrast, Volkmann et al. (2002) reported a negative effect of seminal plasma on post-thaw motility of stallion epididymal spermatozoa.

To compare existing publications with regards to post-thaw spermatozoal motility and exposure or no exposure of equine epididymal spermatozoa to seminal plasma is difficult as experimental designs differ significantly which may have an influence on the outcome and may help to explain contradictory findings. The timing of addition and volume of seminal plasma vary greatly between studies. Epididymal spermatozoa were just exposed to seminal plasma during collection (Braun et al. 1994b; Stout et al. 1999; Tiplady et al. 2002), after initial exposure to semen extender (Braun et al. 1994a; Cary et al. 2004; Papa et al. 2008) or seminal plasma was a component of freezing extender (Bruemmer et al. 2002). Major differences are also present for the harvesting of the testes and epididymides. Some studies used epididymides obtained after slaughter at an abattoir (James et al. 2002; Magistrini et al. 1988), while others preferred castrations under general anesthesia (Braun et al. 1994a; Morris et al. 2002), as opposed to standing castrations, because there are concerns regarding possible effects of a local anaesthetic on subsequent spermatozoal viability (Morris et al. 2002). Other reports did not specify the castration method (Braun et al. 1994b; Bruemmer et al. 2002; Tiplady et al. 2002).

Evaluation of sperm morphology is part of the breeding soundness examination of stallions. Sperm morphology greatly impacts fertility in all species, and it would not be expected to be any different in horses (Brito 2007). Some studies report decreased fertility due to stallion sperm abnormalities (Bielanski 1975; Jasko et al. 1990) whereas others have found no relationship between morphology and fertility of fresh stallion sperm (Bielanski and Kaczmariski 1975; Voss et al. 1981). At this stage, there are no publications available describing stallion epididymal spermatozoal morphology or giving a general overview about stallion epididymal spermatozoal quality parameters with or without exposure to seminal plasma in direct comparison to ejaculated stallion spermatozoa. As spermatozoal

quality parameters like motility, morphology and viability have a major influence on fertility and pregnancy rates, it is of great interest to describe these and investigate the influence of seminal plasma on these parameters.

The aims of this study were 1) to investigate the effect of seminal plasma on spermatozoal motility, morphology and viability of equine epididymal spermatozoa and 2) to directly compare sperm parameters of fresh and frozen-thawed stallion epididymal spermatozoa to sperm parameters of fresh and frozen-thawed ejaculated spermatozoa.

2. Materials and methods

2.1 Animals and experimental design

Four stallions were used. Stallion A was a Warmblood (5 years old), stallion B and C were Boerperds (4 years old) and stallion D was a Welsh (4 years old). Stallions were housed in stables and fed concentrates and hay three times daily. Water was freely available to all horses.

Stallions used in this study were selected on results of breeding soundness examinations including semen evaluations for fresh and frozen-thawed ($\geq 30\%$) progressive spermatozoal motility, spermatozoal concentration and total spermatozoal numbers.

Six sperm categories of each stallion were evaluated for motility, morphology and viability. These categories were fresh ejaculated spermatozoa (Fr-E), fresh epididymal spermatozoa that had been exposed to seminal plasma (Fr-SP+), fresh epididymal spermatozoa that had never been exposed to seminal plasma (Fr-SP-), frozen-thawed ejaculated spermatozoa (Cr-E), frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing (Cr-SP+) and frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma (Cr-SP-).

Data for motility, morphology and viability of fresh and frozen-thawed epididymal spermatozoa that had and had not been exposed to seminal plasma was compared directly.

Data for motility, morphology and viability of fresh and frozen-thawed epididymal spermatozoa that had and had not been exposed to seminal plasma was also compared to semen parameters of fresh and frozen-thawed ejaculated spermatozoa.

Fresh semen of all stallions was collected and evaluated for spermatozoal motility, morphology, viability and concentration every second to third day for approximately four weeks. A part of each ejaculate was frozen and stored. Castrations to harvest the epididymides were performed three to four weeks after the last semen collection. The epididymal semen was evaluated in the same way as the ejaculated semen. Spermatozoal concentration was determined for epididymal sperm categories to establish total sperm numbers recovered per epididymis.

2.2 Semen collection of ejaculated spermatozoa and harvesting of epididymal spermatozoa

Semen of all stallions was collected on a teaser mare in oestrus using a Hannover Model artificial vagina. Castrations were performed three to four weeks after the last semen collection and both testes were recovered via open castration under general anaesthesia. The cauda epididymis and the vas deferens were separated from each testis. Connective tissue was removed from the cauda epididymis. The epididymal duct was cut at the site in the tail of the epididymis where the tubular diameter became distinctly narrower. A blunted 18G four cm hypodermic needle connected to a 20 ml syringe filled with flushing medium was inserted into the open end of the vas deferens. Spermatozoa were then flushed in a retrograde direction from the vas deferens through the cauda epididymis into a sterile 50 ml plastic tube.

For each stallion one epididymal tail was flushed with 20 ml seminal plasma and the other with 20 ml freezing medium. Left and right epididymides were randomly allocated to the different treatment groups.

Each epididymal semen sample was divided into two parts. One part was used for the evaluation of spermatozoal motility and the preparation of eosin-nigrosin smears for evaluation of sperm morphology of fresh epididymal spermatozoa. The other part was cryopreserved. The aliquot of epididymal spermatozoa flushed with seminal plasma and destined for cryopreservation was diluted with centrifugation medium to a concentration of 100×10^6 spermatozoa/ml and further treated like ejaculated semen (see semen freezing). Epididymal semen flushed with freezing medium was further diluted with freezing medium to a final concentration of 500×10^6 spermatozoa/ml and then cryopreserved as described below without centrifugation.

2.3 Harvesting of seminal plasma

Seminal plasma was harvested from all stallions involved in the project during the same breeding season. The collections to harvest seminal plasma were done inbetween the collections to freeze semen.

To harvest seminal plasma from the stallions, semen was collected and the sperm rich fraction was separated from the gel fraction using a sterile gauze filter and then centrifuged at 600 g for 15 min. The supernatant was placed into a sterile 50 ml plastic tube and the centrifugation was repeated twice more. Seminal plasma of all four stallions was pooled and frozen at -18°C in aliquots of 50 ml. Aliquots of seminal plasma were thawed in a water bath at 37°C before use.

2.4 Evaluation of spermatozoal motility and concentration

An aliquot of semen (0.5 ml for fresh semen evaluation; 50 µl for frozen-thawed semen) was transferred into a 4 ml plastic tube, diluted with 2 ml skim milk extender (see semen extender: centrifugation medium) and kept in a 37°C water bath. Evaluation took place immediately. A drop (7 µL) of diluted semen was placed on a pre-warmed glass slide and covered with a 22 x 22 mm cover slip. The percentage of motile spermatozoa was determined by eyeball assessment by two independent examiners at x 200 magnification using a phase contrast microscope with a heated microscope stage (37°C). Motility assessment was done completely blind to the evaluator as to treatment group. Percentages of progressively motile, aberrantly motile and immotile spermatozoa were recorded; sperm concentration was determined by using a hemacytometer. The sperm count was done on a one in 80 dilution, counting 20 squares of 0,004 mm³ (each consisting of 16 small squares) after allowing the filled hemacytometer chamber to settle for 5 min. This protocol gave the concentration in million spermatozoa per ml.

2.5 Evaluation of spermatozoal morphology and viability

Recording of spermatozoal morphology and viability was performed by evaluating eosin-nigrosin stained semen smears. The eosin-nigrosin stain was prepared by adding 2 g eosin and 5 g nigrosin to 100 ml of a water based buffer with a pH of less than 6.8 and an osmolality of 293. After adding eosin and nigrosin the solution was sonicated and filtered. The eosin-nigrosin stain had a pH of 8.49 and an osmolality of 420 .

A phase-contrast microscope at 1000 x magnification was used for the evaluation; 500 spermatozoa per smear of each sample (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP-) were evaluated. Data was captured using the data capture sheet and method described for bull semen morphology by Nöthling and Irons (2008).

2.6 Semen freezing

Immediately after collection the sperm rich fraction was separated from the gel fraction using a sterile gauze filter. Gel-free semen was evaluated for volume, concentration and percentage of progressively motile spermatozoa. Semen was diluted in skim milk centrifugation medium to a concentration of 100×10^6 spermatozoa/ml and centrifuged for 10 min at 600 g. After removal of the supernatant, sperm pellets were re-suspended in skim milk freezing extender to a final concentration of 500×10^6 spermatozoa/ml. Semen was packaged in 0.5 ml straws (0.5 cc semen straws, Minitüb, Tiefenbach, Germany) and equilibrated for 60 min at 4°C (straws placed on a rack with a distance of 10 mm between straws). Straws on the rack were then placed 3.5cm above liquid nitrogen for 20 min before being plunged into liquid nitrogen. Straws were thawed in a 37°C water bath for 30 s.

2.7 Semen extender

Semen extenders used were skim milk based (modified INRA 82)(Palmer 1984). The centrifugation medium contained glucose (25g), lactose (1.5g), raffinose (1.5g), sodium citrate (dehydrate) (0.25g), potassium citrate (0.41g), HEPES (4.76g), UHT sterilised skim milk (500ml), deionised water (500ml), Penicillin G (50 000IU), Gentamycin sulfate (5mg). The freezing medium contained the same ingredients but also egg yolk (40ml) and glycerol (25ml). For the preparation of egg yolk, 40 ml egg yolk were mixed with 40 ml deionised water and centrifuged at 15000 g for 10 min. Forty ml of the liquid component was harvested and used in the extender.

2.8 Statistical analysis

The effects of flushing medium and of freezing on percentage progressively motile spermatozoa were estimated using Tukey-Kramer Multiple-Comparison Test. The test was done on combined motilities for all four stallions. Multiple logistic regression models were used to estimate the effect of flushing medium and freezing on morphological sperm

defects. The main predictors to interact were flushing medium (skim milk extender or seminal plasma) and semen treatment (fresh or frozen). The clustering of observations within stallions was accounted for by including stallions as a fixed effect. Data were analysed in Stata 10.1 (StataCorp, College Station, TX, USA).

Effect of semen extender and treatment on occurrence of sperm defects was described according to the data capture sheet used by Nöthling and Irons (2008): overall sperm defects (nuclear as well as acrosome and tail defects), nuclear defects, knobbed acrosomes, midpiece reflexes, distal droplets and damaged/degenerate acrosomes.

Only specific defects that constituted more than 2% of all defects were taken into consideration for evaluation.

Separate models were used for each of the following outcomes: overall sperm defects, nuclear defects, knobbed acrosomes, midpiece reflexes, distal droplets and damaged/degenerate acrosomes. Comparisons between the 6 sperm categories (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP-) regarding sperm defects were done by Fisher's Exact tests. Fisher's Exact test was also used to test viability (live/ dead ratio) for morphologically normal sperm.

3. Results

3.1 Number of spermatozoa recovered from the epididymides

Number of spermatozoa recovered from the epididymides varied between 8×10^9 and 28×10^9 spermatozoa per epididymis. Stallion A had: 22×10^9 , 22×10^9 ; stallion B: 19×10^9 , 28×10^9 ; stallion C: 8×10^9 , 8×10^9 ; stallion D: 18×10^9 , 17×10^9 .

3.2 Effect of seminal plasma on spermatozoal motility

Results are summarised in table 1. For all stallions, Fr-SP+ contained more progressively motile spermatozoa than Fr-SP- ($P < 0.05$) and was similar to Fr-E. However, after freezing

and thawing, this difference in progressive motility between epididymal spermatozoa exposed and not exposed to seminal plasma was no longer present, even though it still appeared to persist in stallion D. For all stallions, post-thaw progressive motilities of epididymal spermatozoa that had been exposed to seminal plasma (Cr-SP+) and that had never been exposed to seminal plasma (Cr-SP-) were both lower than that of frozen-thawed ejaculated spermatozoa (Cr-E) ($P<0.05$).

3.3 Effect of seminal plasma on spermatozoal morphology and on spermatozoal viability

Only defects that constituted more than 2% of all defects were taken into consideration for evaluation. Table 2 shows the percentage of morphological sperm defects per sperm category combined for all stallions, calculated on the total number of sperm cells ($n=2000$) evaluated per category. Sperm morphology of all stallions was within normal limits for fresh ejaculated spermatozoa as described by Palmer (1984). Looking at all defects in the individual multiple logistic regression models, flushing medium and freezing had a significant influence on the occurrence of overall sperm defects (nuclear as well as acrosomal and tail defects), midpiece reflexes, distal droplets and damaged/degenerate acrosomes. Results are listed in tables 3, 4, 5 and 6. Flushing medium and freezing did not have a significant influence on the occurrence of nuclear defects and knobbed acrosomes. Results on spermatozoal viability are summarised in table 7.

4. Discussion

4.1 Effect of seminal plasma on motility of equine epididymal spermatozoa

Progressive motility of fresh epididymal spermatozoa that had been exposed to seminal plasma was higher than that of fresh epididymal spermatozoa that had not been exposed to seminal plasma. These findings agree with those of Braun et al. (1994b) and Stout et al. (1999) who found that seminal plasma significantly increased initial spermatozoal motility,

which supports the conclusion that seminal plasma induces motility in live, but quiescent, spermatozoa (Stout et al. 1999). However, the post-thaw progressive motility of epididymal spermatozoa that had been exposed to seminal plasma prior to freezing and that had not been exposed was similar and poor in three of the four stallions. A difference in post-thaw motilities for Cr-SP+ and Cr-SP- only persisted in one stallion. The combined progressive motilities for Cr-SP+ and Cr-SP- for all four stallions were similar.

While some earlier studies (Bruemmer et al. 2002; Cary et al. 2004; Morris et al. 2002; Papa et al. 2008; Tiplady et al. 2002) also failed to find a beneficial effect of seminal plasma on post-thaw spermatozoal motility, others did detect differences due to the addition of seminal plasma to epididymal spermatozoa prior to freezing (Stout et al. 1999; Volkmann et al. 2001). Whereas Stout et al. (1999) showed a positive influence of seminal plasma on post-thaw motility, Volkmann et al. (2001) reported a negative influence of seminal plasma on cryopreservation of stallion epididymal spermatozoa. In the latter study (Volkmann et al. 2001) post-thaw progressive motility of epididymal spermatozoa not exposed to seminal plasma was not only better than that of epididymal spermatozoa that had been exposed to seminal plasma, but also better than that of frozen-thawed ejaculated spermatozoa. The current study showed a significantly higher progressive motility in frozen-thawed ejaculated spermatozoa than in Cr-SP+ and Cr-SP- which contradicts the hypothesis that epididymal spermatozoa are less susceptible to cold shock and therefore more resistant to freezing and thawing than ejaculated spermatozoa (Johnson et al. 1980). Similar findings regarding poor post-thaw motilities for epididymal spermatozoa have been published previously (Bruemmer et al. 2002; Jimenez 1987; Tiplady et al. 2002).

A difference in post-thaw spermatozoal motility between Cr-SP+ and Cr-SP- was evident in one stallion, with higher post-thaw motility for Cr-SP+. As the same pool of seminal plasma was used to flush the epididymides for all four stallions, the composition of seminal plasma will not have influenced the post-thaw progressive motility of epididymal spermatozoa in

this study. Instead, we suspect that stallion difference was responsible for differences in the freezability of epididymal spermatozoa, just as it is for ejaculated spermatozoa (Magistrini et al. 1988; Vidament et al. 1997). In an extensive French study, 19% and 30% of stallions were considered to have unfreezable and poorly freezable semen, respectively (Vidament et al. 1997).

All four stallions chosen for the project produced post-thaw progressive motilities of at least 30% during a test freezing. Therefore, progressive motility of fresh or frozen-thawed ejaculated spermatozoa is not necessarily a good predictor for post-thaw epididymal spermatozoal motility in the same stallion. Magistrini et al. (1988) have however reported a strong correlation between motility of frozen-thawed ejaculated and epididymal spermatozoa of 19 stallions.

Looking at the motility results from a different angle, it can also be seen that the decrease in sperm motility between fresh and frozen-thawed spermatozoa differs for ejaculated spermatozoa and epididymal spermatozoa, even though the difference was only statistically significant ($p < 0.05$, paired t-test) for ejaculated spermatozoa and epididymal spermatozoa that had been exposed to seminal plasma. For ejaculated spermatozoa, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 25%, 30%, 47% and 48% for stallions A, D, B and C, respectively. These decreases in sperm motility after cryopreservation are in agreement with findings for human spermatozoa where sperm cryopreservation was reported to result in a 45% decrease in the average velocity of sperm movement (Donnelly et al. 2001). For epididymal spermatozoa that had been exposed to seminal plasma, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 63%, 87%, 88% and 88% for stallions D, A, B and C, respectively, which is a more pronounced decrease than for the ejaculated spermatozoa of the same stallions. For epididymal spermatozoa that had not been exposed to seminal plasma, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 0%, 67%, 87% and

88% for stallions B, A, D and C, respectively. Here it is interesting to notice that for stallion B who showed a very low initial motility (Fr-SP-, table 1) the decrease in motility after cryopreservation was 0%. These findings further contradict the hypothesis that epididymal spermatozoa are less susceptible to cold shock and should therefore be more resistant to freezing and thawing than ejaculated spermatozoa (Johnson et al. 1980).

A positive relationship exists between post-thaw motility and fertility of frozen-thawed semen (Vidament 2005). Improving post-thaw motility of epididymal spermatozoa could improve its fertility as shown recently (Melo et al. 2008; Papa et al. 2008). For ejaculated spermatozoa, seminal plasma of stallions with good post-thaw spermatozoal motility improves post-thaw motility of stallions with poor post-thaw motility (Aurich et al. 1996). Therefore, seminal plasma of stallions with excellent post-thaw motility of ejaculated spermatozoa should be collected and stored for use when epididymal spermatozoa need to be harvested in an emergency situation. Furthermore, the post-thaw addition of seminal plasma may be beneficial for post-thaw epididymal spermatozoa motility as well as for longevity, as it has been shown in dogs (Nöthling et al. 2007).

4.2 Effect of seminal plasma on morphology of equine epididymal spermatozoa

4.2.1 Effect of seminal plasma on occurrence of overall sperm defects

Evaluating the different logistic regression models separately, it can be seen that fresh epididymal sperm (SP- as well as SP+) had similar numbers of overall sperm defects (nuclear as well as acrosome and tail defects, see table 3). Comparing fresh ejaculated spermatozoa with fresh epididymal sperm (SP- as well as SP+) shows that fresh ejaculated spermatozoa have significantly lower numbers of defects for this category. Freezing significantly increased the number of overall defects (nuclear as well as acrosome and tail defects) for Cr-SP- and Cr-E but significantly decreased numbers of defects for Cr-SP+.

Frozen-thawed ejaculated spermatozoa still had significantly lower numbers for this category than Cr-SP- or Cr-SP+. The increase in overall defects (nuclear as well as acrosome and tail defects) after freezing for Cr-SP- and Cr-E is expected as membrane and other cryoinjuries to spermatozoa are always encountered due to the freezing process. The decreased overall defects for Cr-SP+ are somewhat surprising but it can be seen that this is due to the fact that the number of distal droplets for Cr-SP+ is dramatically decreased after freezing as compared to Fr-SP+. This explains the general decrease of defects as distal droplets constitute the major portion of tail defects for Fr-SP+.

4.2.2 Effect of seminal plasma on occurrence of distal droplets

The occurrence of distal droplets (table 5) was significantly higher for fresh and frozen-thawed epididymal spermatozoa (SP+ and SP-) than for fresh and frozen-thawed ejaculated spermatozoa. Interestingly, before freezing Fr-SP+ showed more distal droplets than Fr-SP- whereas after freezing Cr-SP+ had less distal droplets than Cr-SP-. This indicates that seminal plasma influences the shedding of distal droplets during the cryopreservation process but that it is not absolutely necessary for the process.

It has been shown previously that seminal vesicular fluid in bulls has a droplet removing effect (Bialy and Smith 1958) and a haemolytic factor, phospholipid-binding protein (PBP), in bull vesicular gland fluid has been shown to support droplet removal (Matousek and Kysilka 1980). In our study, even though the exposure time to seminal plasma was comparable for ejaculated as well as epididymal spermatozoa that had been exposed to seminal plasma, ejaculated spermatozoa had significantly less distal droplets than epididymal spermatozoa that had been exposed to seminal plasma. This occurred for fresh and frozen-thawed spermatozoa. The reason for this remains unclear but one possible explanation could be that ejaculated spermatozoa have spent a longer time period in the epididymis than epididymal spermatozoa that were flushed from the epididymis. Looking at

all four stallions used in this experiment average total spermatozoal numbers in the ejaculates were 6×10^9 per collection and $16\text{--}47 \times 10^9$ for epididymal sperm recovery per stallion. Assuming that around 54×10^9 spermatozoa are stored in the cauda epididymides as part of the spermatozoal reserve, 2×10^9 spermatozoa are stored in the ampullae of the deferent ducts and 2×10^9 in the remainder of the deferent ducts (McKinnon and Voss 1992) it can be seen that the latter two fractions constitute a major part of the ejaculated sperm numbers whereas the sperm numbers recovered from the epididymides via flushing were much higher. Only a minor portion of ejaculated spermatozoa originates from the cauda epididymis. To harvest epididymal spermatozoa we flushed further proximal in the epididymal tail, closer to the epididymal body, than where ejaculated spermatozoa would have been released from during a normal ejaculation. Therefore, ejaculated spermatozoa would have spent a prolonged time period in the epididymides moving from the tail into the ductus deferens and would have been exposed longer to epididymal secretions which are thought to play a role in the droplet shedding process (Barth and Oko 1989).

Another explanation could be that for ejaculated spermatozoa the seminal plasma was excreted during ejaculation from only the specific stallion, while pooled seminal plasma was used for the flushing of the epididymal spermatozoa. This could possibly indicate that stallion-specific factors in seminal plasma help shedding of “own” distal cytoplasmic droplets. Pooling of seminal plasma might have decreased these factors through dilution for the individual stallions and therefore decreased the number of distal droplets that were shed. Freezing decreased the numbers of distal droplets for ejaculated spermatozoa, epididymal spermatozoa that had been exposed to seminal plasma (SP+) and epididymal spermatozoa that had never been exposed to seminal plasma (SP-). Our results confirm that the sperm maturation processes in the epididymis regarding cytoplasmic droplets, including migration of the proximal cytoplasmic droplet from the proximal neck location to the distal portion of the midpiece, were normal for all four stallions but that possibly the time that the milieu in

the cauda epididymis has to influence spermatozoa was insufficient for complete shedding of all distal cytoplasmic droplets in epididymal spermatozoa. As distal cytoplasmic droplets do not have a special significance or a negative impact on fertility, it can be concluded that even though the number of epididymal spermatozoa with distal cytoplasmic droplets was high, it should not impair the use of epididymal spermatozoa for artificial insemination.

4.2.3 Effect of seminal plasma on occurrence of midpiece reflexes

Evaluation of midpiece reflexes (table 4) showed that for fresh as well as for frozen-thawed epididymal spermatozoa SP+ had significantly less midpiece reflexes than Fr-SP- or Fr-E. At the same time, fresh and frozen-thawed Epidid-SP- had significantly more midpiece reflexes than ejaculated spermatozoa. Freezing significantly decreased the numbers of midpiece reflexes for epididymal spermatozoa that had been exposed to seminal plasma and for ejaculated spermatozoa, but increased these numbers for epididymal spermatozoa that had never been exposed to seminal plasma.

Midpiece reflexes are common sperm defects in stallions (Brito 2007) and develop in the corpus and cauda epididymis (Barth and Oko 1989). It has been hypothesised that the susceptibility to bending in that area may be due to an increased sensitivity to ions that is acquired in the epididymis (Barth and Oko 1989; Swanson and Boyd 1961). It was also shown that frequent ejaculations decreased the numbers of midpiece reflexes in some bulls indicating that the incidence of midpiece reflexes is related to the length of time spermatozoa spent in the epididymis (Swanson and Boyd 1961). However, this does not explain our results where epididymal spermatozoa that have been exposed to seminal plasma (fresh and frozen) have significantly less midpiece reflexes than epididymal spermatozoa that have not been exposed to seminal plasma (fresh and frozen). The castration and removal of both epididymides was performed exactly at the same time and the spermatozoa recovered from the epididymides have been exactly the same time in the

epididymis prior to the procedure. It does indicate though that seminal plasma exerts a positive effect on epididymal spermatozoa preventing midpiece reflex formation. In bulls, midpiece reflexes can be induced in vitro by rapid cooling of semen, exposure of semen to hypotonic solutions, treatment of bulls with estradiol and scrotal insulation (Barth and Oko 1989). This could possibly explain why Fr-SP- had significantly more midpiece reflexes than Fr-SP+ as the different flushing media (freezing extender vs. seminal plasma) created different ionic environments for the epididymal spermatozoa at the time of flushing.

In some cases the underlying cause for midpiece reflexes has been thought to be a weakness in the midpiece caused by abnormal development in spermiogenesis (Barth and Oko 1989). This cause for midpiece reflexes can be excluded in our case as all sperm categories (Fr-E, Fr-SP+ and Fr-SP-) should have had the same incidence of midpiece reflexes if it was due to spermiogenesis.

Ejaculated spermatozoa and epididymal spermatozoa that had been exposed to seminal plasma both showed significantly less midpiece reflexes after freezing. A possible explanation for that could be that the freezing process helped to shed the cytoplasmic droplets trapped in the bend of midpiece reflexes and consequently helped to straighten the midpieces. Such a bending of sperm tails followed by resuming of a straight conformation has been thought to be possible previously (Dickey 1965) but was not supported by others (Barth and Oko 1989). This could possibly also explain why the percentage of midpiece reflexes increased after freezing for epididymal spermatozoa that had never been exposed to seminal plasma as it might indicate a protective effect of seminal plasma on midpieces during cryopreservation. Furthermore, epididymal spermatozoa that had never been exposed to seminal plasma did not experience the effect of seminal plasma on distal droplet shedding which might predispose them further to midpiece reflex formation during cryopreservation due to a higher percentage of retained distal droplets.

Spermatozoa with midpiece reflexes display a reverse swimming motion in a circular pattern and are therefore not able to fertilise an oocyte as they do not reach the site of fertilization. Midpiece reflexes have been classified as minor defects with no or little effect on fertility (Barth and Oko 1989). It can probably be assumed for our experiment that the effect of the observed midpiece reflexes on fertility would be low.

4.2.4 Effect of seminal plasma on occurrence of damaged/ degenerate acrosomes

The occurrence of damaged/degenerate acrosomes (table 6) was similar for Fr-SP- and Fr-SP+. Fresh ejaculated spermatozoa had significantly more damaged/degenerate acrosomes than Fr-SP- and Fr-SP+. Freezing significantly increased the incidence of damaged/degenerate acrosomes for ejaculated as well as epididymal spermatozoa (SP+ and SP-). After freezing, Cr-SP+ had significantly more damaged/degenerate acrosomes than Cr-SP- whereas the number of spermatozoa with damaged/ degenerate acrosomes was similar for Cr-SP- and Cr-E.

These findings show that the percentage of damaged/ degenerate acrosomes is significantly increased for frozen-thawed spermatozoa (ejaculated and epididymal spermatozoa) which is most likely due to cryoinjury during the freezing process. Our findings also show that the exposure of epididymal spermatozoa to seminal plasma prior to freezing increases the chances for cryoinjury regarding the acrosome. Even though the percentage of spermatozoa with damaged/degenerate acrosomes increased after freezing and thawing, the values were still within normal limits expected for fertile stallions as described by Palmer (1984) and should therefore not have a major impact on fertility.

4.2.5 Effect of seminal plasma on occurrence of nuclear defects and knobbed acrosomes

The occurrence of nuclear defects and knobbed acrosomes in epididymal spermatozoa was not influenced by flushing medium or freezing. This finding was to be expected as nuclear

defects in general and knobbed acrosomes specifically have been shown to be developed during spermiogenesis (Barth and Oko 1989) and should not be affected by sperm maturation, exposure to different flushing media or cryopreservation.

4.3 Effect of seminal plasma on viability of equine epididymal spermatozoa

Table 7 shows that there is no difference in viability for morphologically normal fresh epididymal spermatozoa that had been exposed to seminal plasma and fresh epididymal spermatozoa that had never been exposed to seminal plasma. Furthermore, the percentage of viable morphologically normal spermatozoa is higher for epididymal spermatozoa (Fr-SP+ and Fr-SP-) than for fresh ejaculated spermatozoa. After freezing however, there is no difference in percentage viable morphologically normal spermatozoa for epididymal spermatozoa (Cr-SP+ and Cr-SP-) and ejaculated spermatozoa. This shows that seminal plasma does not influence the viability of morphologically normal epididymal spermatozoa (fresh and frozen-thawed) and that the viability of morphologically normal frozen-thawed epididymal spermatozoa (Cr-SP+ and Cr-SP-) is similar to frozen-thawed ejaculated spermatozoa.

Our results agree with previous findings (Moore et al. 2005) which showed that seminal plasma did not affect post-thaw viability of ejaculated spermatozoa. It is possible that no difference in post-thaw viability was seen for ejaculated and epididymal spermatozoa (SP+ and SP-) due to the pre-freeze centrifugation process which removed most of the seminal plasma from ejaculated as well as epididymal sperm (SP+).

4.4 Epididymal sperm numbers

Expected sperm numbers that can be harvested from both cauda epididymides of a stallion should be about 54×10^9 sperm or approximately 61% of the sperm in the excurrent duct system (Amann et al. 1979). Epididymal sperm numbers recovered in this experiment were

close to these expected numbers for three of the four stallions and much higher than in previously published reports (Bruemmer et al. 2002; Cary et al. 2004; James et al. 2002) but quite consistent with the numbers provided by Bruemmer (2006). Our results support the use of retrograde flushing to harvest epididymal spermatozoa as opposed to float-up methods.

5. Conclusions

The authors realise that the outcome regarding the occurrence of certain spermatozoal defects might have been accentuated due to the fact that only four stallions were used for this study. Nevertheless, our study guides the way to a possible larger scale trial investigating the effects of seminal plasma on post-thaw parameters of stallion epididymal spermatozoa. This study confirms findings of previous reports that seminal plasma stimulates initial motility of fresh epididymal stallion spermatozoa while this difference in progressive motility is no longer present post-thaw. Our results indicate that progressive motility of fresh or frozen-thawed ejaculated stallion spermatozoa is not always a good indicator for post-thaw progressive motility of epididymal spermatozoa. Our study shows that seminal plasma has a positive influence on the incidence of overall sperm defects, midpiece reflexes and distal cytoplasmic droplets in frozen-thawed stallion epididymal spermatozoa while the occurrence of midpiece reflexes is likely to be linked to distal cytoplasmic droplets. Furthermore, we show that seminal plasma does not have an influence on viability of fresh and frozen-thawed morphologically normal epididymal spermatozoa. In conclusion, based on the results of this study and taking recent findings concerning fertility of epididymal spermatozoa (Heise et al. 2010) into consideration, we recommend the retrograde flushing technique using seminal plasma as flushing medium to harvest and freeze stallion epididymal spermatozoa.

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Table 1

Effect of seminal plasma on spermatozoal motility

		Fr-E	Fr-SP+	Fr-SP-	Cr-E	Cr-SP+	Cr-SP-
Stallion A	PMS (%)	67	75	30	50	10	10
	AMS (%)	18	10	10	15	20	10
	IMS (%)	15	15	60	35	70	80
Stallion B	PMS (%)	66	80	10	35	10	10
	AMS (%)	19	10	10	20	20	10
	IMS (%)	15	10	80	45	70	80
Stallion C	PMS (%)	77	80	75	40	10	10
	AMS (%)	12	5	5	20	20	35
	IMS (%)	11	15	20	40	70	55
Stallion D	PMS (%)	71	80	40	50	30	5
	AMS (%)	14	10	10	5	20	5
	IMS (%)	15	10	50	45	50	90

Stallion A: Warmblood PMS (%): Progressively motile spermatozoa

Stallion B: Boerperd AMS (%): Aberrantly motile spermatozoa

Stallion C: Boerperd IMS (%): Immotile spermatozoa

Stallion D: Welsh

Fr-E fresh ejaculate

Fr-SP+ fresh epididymal spermatozoa that had been exposed to seminal plasma

Fr-SP- fresh epididymal spermatozoa that had never been exposed to seminal plasma

Cr-E frozen-thawed ejaculated spermatozoa

Cr-SP+ frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing

Cr-SP- frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma

Table 2

Percentage of morphological sperm defects for all sperm categories (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP)

combined for all stallions, calculated on the total number of sperm cells (n=2000) evaluated per category.

Different letters in a row show significant differences between sperm categories for a specific defect ($P \leq 0.05$)

Sperm category						
(%)	Fr-E	Fr-SP+	Fr-SP-	Cr-E	Cr-SP+	Cr-SP-
Normal sperm	68.15 ^a	34.1 ^d	35.42 ^d	63.30 ^b	49.00 ^c	29.90 ^e
<i>Main groups of defects</i>						
Sperm with defects (nuclear as well as acrosome and tail defects)	31.85 ^e	65.90 ^b	64.58 ^b	36.70 ^d	51.00 ^c	70.10 ^a
Sperm with nuclear defects	2.95 ^{ab}	3.55 ^a	2.20 ^b	2.85 ^{ab}	3.15 ^b	3.45 ^{ab}
<i>Sub-groups of defects</i>						
Knobbed acrosome	2.60 ^a	1.55 ^b	1.35 ^{bc}	2.25 ^{ac}	2.25 ^{bc}	1.65 ^{ac}
Midpiece reflex	7.90 ^c	3.75 ^d	10.75 ^b	5.00 ^b	2.15 ^e	20.50 ^a
Distal cytoplasmic droplet	9.10 ^e	46.25 ^a	38.30 ^b	7.65 ^e	25.65 ^d	31.25 ^c
Sperm with damaged and/or degenerate acrosomes	4.20 ^c	2.30 ^d	2.30 ^d	7.70 ^b	9.20 ^a	5.80 ^b

Fr-E fresh ejaculate

Fr-SP+ fresh epididymal spermatozoa that had been exposed to seminal plasma

Fr-SP- fresh epididymal spermatozoa that had never been exposed to seminal plasma

Cr-E frozen-thawed ejaculated spermatozoa

Cr-SP+ frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing

Cr-SP- frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma

Table 3

Effect of epididymal semen extender, freezing and animal on occurrence of sperm defects (nuclear as well as acrosomal and tail defects) in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Fresh			
	Epidid-SP- vs. ejac	4.27	3.73, 4.89	<0.001
	Epidid-SP+ vs. ejac	4.54	3.96, 5.20	<0.001
	Epidid+SP vs. Epidid-SP	1.06	0.93, 1.22	0.369
	Frozen			
	Epidid-SP- vs. ejac	4.46	3.89, 5.11	<0.001
	Epidid-SP+ vs. ejac	1.86	1.63, 2.12	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.42	0.37, 0.48	<0.001
	Ejaculated: Frozen-thawed vs. fresh	1.25	1.10, 1.43	0.001
Freezing	Epidid-SP-: Frozen-thawed vs. fresh	1.31	1.14, 1.50	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.51	0.45, 0.59	<0.001
Animal (4 levels)		—	—	<0.001
Extender*Freezing interaction		—	—	<0.001

Ejac: ejaculated spermatozoa

Epidid-SP+: epididymal spermatozoa that had been exposed to seminal plasma

Epidid-SP-: epididymal spermatozoa that had never been exposed to seminal plasma

Table 4

Effect of epididymal semen extender, freezing and animal on occurrence of midpiece reflexes in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Fresh			
	Epidid-SP- vs. ejac	1.43	1.15, 1.79	0.001
	Epidid-SP+ vs. ejac	0.44	0.33, 0.59	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.31	0.23, 0.41	<0.001
	Frozen			
	Epidid-SP- vs. ejac	5.49	4.34, 6.95	<0.001
	Epidid-SP+ vs. ejac	0.41	0.28, 0.59	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.07	0.05, 0.10	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	0.60	0.46, 0.78	<0.001
	Epidid-SP-: Frozen-thawed vs. fresh	2.30	1.91, 2.78	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.56	0.38, 0.82	0.003
Animal (4 levels)		—	—	<0.001
Extender*Freezing interaction		—	—	<0.001

Ejac: ejaculated spermatozoa

Epidid-SP+: epididymal spermatozoa that had been exposed to seminal plasma

Epidid-SP-: epididymal spermatozoa that had never been exposed to seminal plasma

Table 5

Effect of epididymal semen extender, freezing and animal on occurrence of distal droplets in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Fresh			
	Epidid-SP- vs. ejac	6.32	5.29, 7.55	<0.001
	Epidid-SP+ vs. ejac	8.81	7.38, 10.52	<0.001
	Epidid-SP+ vs. Epidid-SP-	1.39	1.23, 1.58	<0.001
	Frozen			
	Epidid-SP- vs. ejac	5.57	4.60, 6.74	<0.001
	Epidid-SP+ vs. ejac	4.21	3.47, 5.11	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.76	0.66, 0.87	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	0.83	0.66, 1.03	0.097
	Epidid-SP-: Frozen-thawed vs. fresh	0.73	0.64, 0.83	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.40	0.35, 0.45	<0.001
Animal (4 levels)		–	–	<0.001
Extender*Freezing interaction		–	–	<0.001

Ejac: ejaculated spermatozoa

Epidid-SP+: epididymal spermatozoa that had been exposed to seminal plasma

Epidid-SP-: epididymal spermatozoa that had never been exposed to seminal plasma

Table 6

Effect of epididymal semen extender, freezing and animal on occurrence of damaged/degenerate acrosomes in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Fresh			
	Epidid-SP- vs. ejac	0.52	0.36, 0.76	0.001
	Epidid-SP+ vs. ejac	0.52	0.36, 0.76	0.001
	Epidid-SP+ vs. Epidid-SP-	1	0.66, 1.52	1.000
	Frozen			
	Epidid-SP- vs. ejac	0.72	0.56, 0.93	0.013
	Epidid-SP+ vs. ejac	1.2	0.98, 1.57	0.074
	Epidid-SP+ vs. Epidid-SP-	1.72	1.34, 2.21	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	1.99	1.50, 2.64	<0.001
	Epidid-SP-: Frozen-thawed vs. fresh	2.73	1.92, 3.90	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	4.70	3.36, 6.59	<0.001
Animal (4 levels)		—	—	<0.001
Extender*Freezing interaction		—	—	<0.001

Ejac: ejaculated spermatozoa

Epidid-SP+: epididymal spermatozoa that had been exposed to seminal plasma

Epidid-SP-: epididymal spermatozoa that had never been exposed to seminal plasma

Table 7

Table 7 shows the percentage of morphologically normal spermatozoa per sperm category that are viable (i.e. with intact cell membranes). Different letters in a row show significant differences between sperm categories for viability ($P \leq 0.05$).

	Sperm categories					
	Fr-E	Fr-SP+	Fr-SP-	Cr-E	Cr-SP+	Cr-SP-
Live morphologically Normal spermatozoa (%)	84 ^b	91 ^a	91 ^a	64 ^c	60 ^c	60 ^c

Fr-E fresh ejaculate

Fr-SP+ fresh epididymal spermatozoa that had been exposed to seminal plasma

Fr-SP- fresh epididymal spermatozoa that had never been exposed to seminal plasma

Cr-E frozen-thawed ejaculated spermatozoa

Cr-SP+ frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing

Cr-SP- frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma