Paper

Comparison of cushioned centrifugation and SpermFilter filtration on longevity and morphology of cooled-stored equine semen

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This study compares two methods for seminal plasma removal by evaluating sperm recovery rates, and motility and morphology of cooled-stored semen. Ejaculates were divided into three groups: control, filtration and cushioned centrifugation. Semen was extended to 25 million sperm/ml using a skim-milk-based extender and stored at 5°C for all groups. Sperm motility (total motility (%TM) and progressive motility (%PM)) was determined at 0, 24, 48 and 72 hours by a computer-assisted sperm analyser. Sperm morphology was assessed using differential interference microscopy. Overall, %TM of the centrifugation group was significantly higher than the filter group, but not significantly different than the control. No significant difference in %TM or %PM was detected for the control group and filter. Cushioned centrifugation was a superior method to obtain progressively motile sperm compared with control (P=0.03) and filter groups (P<0.001). No significant difference was found for the per cent of normal sperm cells and detached heads between the groups. This study demonstrated that cushioned centrifugation was a superior method to remove seminal plasma while preserving %TM and enhancing %PM for stallions under cooled storage over three days. However, as the differences appear to be negligible, the SpermFilter may represent an alternative for farms lacking a centrifuge.

During natural mating, semen (i.e. sperm cells and seminal plasma) is deposited directly into the mare's reproductive tract, which minimises the exposure of spermatozoa to seminal plasma (Loomis 2006). Seminal plasma has been proven to increase pregnancy rates in semen deposited into an inflamed uterus through the action of suppressing sperm-polymorphonuclear neutrophil binding and phagocytosis (Troedsson and others 1995a, b, Alghamdi and others 2004). However, when spermatozoa are subjected to high concentrations of seminal plasma for prolonged periods of time, a deleterious effect on fertility and motility was found (Jasko and others 1992, Akcay and others 2006). Upon standard semen collection and processing using an artificial vagina, sperm cells exposure to seminal plasma is greater than natural mating (Loomis 2006).

An increase in artificial breeding practices using cooled-stored semen and cryopreservation has driven the need for semen processing techniques that maximise spermatozoa longevity and fertility under artificial conditions (Loomis 2006). Removal of seminal plasma from stallion ejaculates prior to storage of cooled or cryopreserved semen has been shown to increase the percentage of progressively motile spermatozoa as well as minimise the decrease in per cent progressive motility over time (Brinsko and others 2000, Moore and others 2005). Jasko and others (1992) reported that the best longevity and least deleterious effects on sperm motility parameters were achieved with a volume of seminal plasma of 5–20 per cent under cooled semen storage conditions.

Centrifugation was first introduced in the 1970s as a method to concentrate spermatozoa into a sperm pellet and separate the seminal plasma, which allows the pellet to be re-extended for cryopreservation (Martin and others 1979). Centrifugation is widely used in equine practice as a method to remove seminal plasma prior to semen cryopreservation and for cooling and shipping semen, particularly for stallions presenting low sperm concentration (e.g. <100 million/ml) and for stallions presenting seminal plasma toxicity (Loomis 2006, Hoffmann and others 2011). This method, while effective, is not innocuous as centrifugation of semen can result in decreased total sperm number (poor sperm recovery), mechanical damage to the sperm

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membrane resulting in decreased sperm viability and reported increase in morphological defects at certain settings (Sieme and others 2003, Weiss and others 2004, Aurich 2008). Cushioned centrifugation (i.e. 60 per cent wt/vol. iodixanol in water placed at the bottom of the conical tube) provides an interface where the sperm pellet is collected, allowing higher velocities (e.g. 1000 g) and longer centrifugation times (e.g. 20–25 minutes) compared with traditional non-cushioned centrifugation (e.g. 400–800 g for 5–10 minutes). The cushion technique has resulted in reportedly greater sperm recovery and apparently less damage to the spermatozoa in comparison to traditional non-cushioned centrifugation (Knop and others 2005, Loomis 2006, Waite and others 2008, Hoogewijs and others 2010).

Centrifugation is not always practical; it requires specialised costly equipment and takes additional time compared with routine semen processing, precluding its use for most clinicians in ambulatory practice or farms lacking a centrifuge and semen processing area. Therefore, the need for an inexpensive, practical, time-efficient method for removal of seminal plasma has driven the development of the SpermFilter (Botu-Pharma, Botucatu, Brazil) (Alvarenga and others 2010, Neto and others 2013). This disposable hydrophilic synthetic membrane filter uses surface tension to draw the smaller molecules of seminal plasma through the filter, leaving the spermatozoa concentrated above the membrane (Alvarenga and others 2010, Neto and others 2013). The suggested benefits of the SpermFilter include a higher sperm recovery rate compared with traditional centrifugation, decreased cost, readily useable with minimal training and is thought to be less detrimental to sperm viability (Neto and others 2013). However, anecdotal experiences suggest that filtering the sperm with SpermFilter appears to be associated with alterations in sperm morphology (particularly detached heads). Studies comparing cushioned centrifugation and SpermFilter on sperm longevity upon cooled storage, recovery rates and sperm morphology are lacking. Therefore, the authors hypothesised that cushioned centrifugation and sperm filter are equally effective methods to concentrate sperm and remove seminal plasma as judged by sperm recovery rates, sperm motility and sperm morphology. The objectives of this study were to compare cushioned centrifugation and the SpermFilter, through sperm longevity under cooled-shipped conditions, sperm morphology and sperm recovery between the two treatments.

Materials and methods

Stallion semen collection

Fifteen sexually experienced stallions, ages ranging from 5 to 22 years old (eight quarter horses, 4 standardbreds, 2 thoroughbreds and 1 paint horse) had semen collected once every other day for a total of three ejaculates per stallion. Each ejaculate was collected in a Missouri model artificial vagina (Nasco, Fort Atkinson, Wisconsin, USA), with the stallion mounted on the phantom, and a teaser mare present in the breeding shed. Stallions were collected at 2–3-day intervals in October of 2014, until a total of 45 ejaculates were obtained. The artificial vagina was lubricated (Priority Care1, First Priority, Elgin, Illinois, USA) and fitted with a clean inline sperm filter (Har-Vet, Spring Valley, Wisconsin, USA) for each semen collection.

Semen processing

Following collection, the total gel-free volume of the ejaculate was weighed and sperm concentration determined using a spectrophotometer (Equine Densimeter, Animal Reproduction Services, Chino, California, USA) (n=30 ejaculates) or an automated cell counter (NucleoCounter SP-100, Chemometric, Germany) (n=15 ejaculates). The entire ejaculate was then extended to a 1:1 volume using temperature-matched skim-milk-based extender (BotuSemen, Botu-Pharma, Botucatu, Brazil). The extended ejaculate was then split into three equal volumes, each third was allocated to one of the following groups: the control group, the filtered group (SpermFilter) and the centrifugation group.

The control group was extended to 25 million sperm/ml using temperature matched BotuSemen extender. An aliquot (1 ml) of raw semen was obtained and added to temperature-matched buffered formol saline for later sperm morphology evaluation. The second third of the ejaculate was filtered (SpermFilter group), using a commercially available product (SpermFilter), according to previously established methodology introduced by Alvarenga and others (2010). Here briefly, 24 ml of extended semen (1:1v/v) was poured into the filter and seminal plasma was removed until 7.5 ml of filtrate was obtained. This filtrate was resuspended using temperature-matched BotuSemen extender to a final concentration of 25 million sperm/ml. On a subset of animals (n=5 stallions, 15 ejaculates), the per cent of recovery following filtration was calculated and recorded. Concentration was determined using a Nucleo-Counter SP-100 before and after recovering. A 1 ml aliquot of semen after processing was added to temperature-matched buffered formol saline for later morphological analysis.

The centrifugation group semen was placed in a 50 ml conical tube (Corning, Centristar, Corning, New York, USA) with a 1 mL centrifugation cushion (Minitube Centrifugation Cushion, Minitube, Germany). The sample was centrifuged for 20 minutes at 1000 g as previously described (Ecot and others 2005, Sieme and others 2006, Waite and others 2008). Following centrifugation, the supernatant and cushion was removed, leaving a remaining volume of approximately 7.5 ml. The remaining portion was then re-extended using BotuSemen to a final concentration of 25 million sperm/ml. The supernatant volume and concentration was recorded to calculate a percentage recovery rate. A 1 ml aliquot of the re-extended sample was obtained and added to temperature-matched buffered formol saline for later morphological analysis.

Packaging and storage

Three 5 ml aliquots from each group were packaged into Whirl-Pak (Nasco) bags. These were then stored in an Equitainer as previously described (Douglas-Hamilton and others 1984, Varner and others 1987) for 24 hours and then maintained refrigerated at 5°C for the rest of the experiment.

Sperm motility and morphology

The sperm motility for each group (control, filtered and centrifugation) was determined at four time points: immediately after packaging (T0), 24 hours (T24), 48 hours (T48) and 72 hours (T72) after semen storage. A small aliquot (10 μL) of extended semen was placed on a heated slide with a coverslip, and assessed for the per cent of total sperm motility (%TM) and per cent progressive sperm motility (%PM) using a computer–assisted sperm analyser (SpermVision II, Minitube of America, Vernon, Wisconsin, USA). At least 1000 cells were evaluated across seven different fields. Before each evaluation, the semen was warmed to 37°C for five minutes.

Samples stored in buffered formol saline were prepared as a wet mount slide with coverslip for morphological analysis. Differential interference microscopy was used at ×1000 magnification to classify the morphology of 200 sperm cells. Cells were categorised as normal, abnormal heads, abnormal acrosomes, proximal cytoplasmic droplets, distal cytoplasmic droplets, abnormal midpieces, abnormal tails and coiled tails. The per cent of total normal cells and detached heads was accounted for the statistical analyses.

Statistical analysis

Motility parameters were analysed by analysis of variance repeated measures and when significant by Tukey's test. Mann-Whitney rank sum test was used to compare per cent of normal sperm cells and per cent of detached heads between groups. Recovered rates between the filtered and centrifugation groups were performed by paired *t* test, whereas concentrations in the discarded fluid for groups filtered and centrifugation were

TABLE 1: Mean±sem for total (TM) and progressive sperm motility (PM) in cooled-stored semen (n=45 ejaculates) from 15 light breed stallions at four time points following semen collection

	Control		Filtered		Cushioned centrifugation	
Time (hours)	TM%	PM%	TM%	PM%	TM%	PM%
0	70±4†*	61.3±4.5†*╪	67±4.3†*	57.5±5.1†*	70.2±4†*	63.2±4.3†*╪
24	61±5‡*	52.1±5.2†‡*	58±4.2‡*	48.3±4.5‡*	61±3.9‡*	51.5±4.2‡*
48	54±5.7§¶*	44.3±5.6§*	55±4‡§*	46.5±4.1‡§*	59±4.1‡§ᶜ╪	51±4‡╪
72	53±5.7¶*╪	43.3±5.1§*	51.6±3.8§*	42.6±3.8§*	56.7±4.3§¶╪	48.1±4.5‡╪

Each ejaculate was processed as control group, filtered or centrifuged
Different letters within columns and characters between columns (* or ╪) for each parameter denote statistical significance with Tukey's test (P<0.05)
†SpermFilter, Botu-Pharma, Botucatu, Sao Paulo 18603-495, Brazil
‡BotuSemen, Botu-Pharma, Botucatu, Sao Paulo 18603-495, Brazil
§Cushion Fluid, Minitube of America, Verona, WI 53593, USA
¶SpermVision, Minitube of America, Verona, WI 53593, USA

compared by *t* test. Significance was set at P<0.05. The data are expressed as means and sem (mean±sem).

Results

Overall, the mean for all the different time points evaluated, total motility for the centrifugation group was higher (P<0.0001) than the filtration group (Table 1). No difference was found between total motility for the control and centrifugation group (P>0.3). Total motility between the filtration group and the control group was not different (P=0.1). However, cushioned centrifugation was found to be a superior method to obtain progressively motile sperm compared with the control (P=0.03) and filter groups (P<0.001). There was no difference between the control and filter group for progressive motility (P=0.18). There were no differences for the per cent of normal sperm cells and detached heads between the three groups (P>0.05) (Table 2).

Discussion

This is apparently the first study to compare longevity, sperm morphology and recovery rates between cushioned centrifugation and SpermFilter as means to remove seminal plasma and concentrate sperm. In this study, cushioned centrifugation resulted in higher per cent of progressive motile sperm upon cooled storage over 72 hours and superior yields compared with the SpermFilter. As expected, cooling and time resulted in a reduction in total and progressive motility across groups. It is worth noting that a previous study carried out in Brazil did not find differences for sperm motility parameters for non-cushioned traditional centrifugation and SpermFilter for stallions classified as normal 'coolers'; however, the filtered increased progressive sperm motility for stallions classified as 'poor coolers' (Neto and others 2013). While it is possible, though very unlikely, that results for sperm motility parameters would have been different if the autors had used stallions classified as 'bad coolers', it is more likely that cushioned centrifugation would result in even superior results for progressive motility compared with the SpermFilter. However, such hypothesis has not been evaluated.

Despite the fact that the SpermFilter presented lower recovery rates (Table 3) and lower progressive motile sperm, SpermFilter still is a viable option for farms and ambulatory practitioners lacking a centrifuge to process the semen. It is worth noting that SpermFilter should only be used for one single stallion due to risk of disease transmission across stallions; if only one ejaculate is going to be processed, the cost of acquisition of the SpermFilter may discourage its purchase. The differences in progressive motility and inferior sperm yield would likely have minimal impact on the reproductive outcome in most clinical situations. The SpermFilter provides an affordable, practical option for veterinarians or clients with smaller breeding operations or where location or circumstance means that cushioned centrifugation is not readily available. Contrary to anecdotal experiences, neither method of semen processing affected the total per cent of morphologically normal sperm and detached heads. The authors' results do not support the unfounded anecdotal claim that the SpermFilter alters sperm morphology grossly.

It is worth noting that neither the sperm ultrastructure nor fertility were evaluated herein. Previous studies have evaluated the effects of cushioned centrifugation on semen quality, and one study found that while sperm membrane intactness (SMI) decreased with centrifugation, sperm quality was similar between control and centrifuged groups at 24 hours (Bliss and others 2012). That particular study attributed the small differences seen in SMI and Sperm DNA quality (COMPαT (cells outside the main population)) to methodology used for analysis. It is unlikely that filtering the sperm would affect the sperm ultrastructure or fertility; however, this has not been evaluated.

In conclusion, upon the conditions of the present study, cushioned centrifugation appeared to be a superior method for seminal plasma removal in equine sperm compared with filtration using the SpermFilter. Neither cushioned centrifugation nor SpermFilter is associated with reduction in normal sperm or detached sperm heads. The SpermFilter may represent an alternative option for practitioners with ambulatory practices and

TABLE 2: Mean±sem for the per cent of normal sperm cells and detached heads in raw or processed semen (n=45 ejaculates) from 15 light breed stallions

	Morphologically normal cells (%)	Detached heads (%)
Control	70.7±2.9	1.34±0.33
Filtered	70.8±4.6	1.41±0.29
Cushioned centrifugation	73.8±3.1	1.32±0.36

Each ejaculate was processed as control group, filtered or cushioned centrifugation
No statistical differences were noted based on Mann–Whitney rank sum test

TABLE 3: Mean±sem for total sperm numbers (TSN) pre- and post-processing and per cent recovery in filtered and cushioned centrifugation for stallion (n=15 ejaculates)

	Pre TSN billion	Post TSN billion	Per cent recovery	Discarded fluid
Filtered	1.97±0.3	1.86±0.28*	93.0±2.7	9.5±1.5*
Cushioned centrifugation	1.97±0.3	1.98±0.29†	100.5±0.3	1.6±0.4†

Concentration was determined by the use of Nucleo-Counter SP-100
Different letters within columns denote statistical differences based on *t* test (P<0.05)
*SpermFilter, Botu-Pharma, Botucatu, Sao Paulo 18603-495, Brazil
†BotuSemen, Botu-Pharma, Botucatu, Sao Paulo 18603-495, Brazil

farms lacking a centrifuge as the differences appear to be small enough to be negligible.

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