Short Communication

Effects of centrifugation and removal of seminal plasma on motility of fresh boar sperm

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Abstract

Extended semen from 6 boars was centrifuged and then gently resuspended (CR), had the supernatant decanted to remove residual seminal plasma and resuspended in new extender (CD), or served as non-treated controls. Measures of sperm motility were assessed immediately after centrifugation and then every 24 h until 96 h using a computer-assisted sperm analyser. Total and progressive sperm motilities were reduced ($P<0.05$) by 48 h in CD. Total motility was not affected in CR sperm but progressive motility was reduced ($P<0.05$) at 72 h. Changes with time were very similar for VSL, VAP, VCL and BCF. Control values remained relatively stable but VCL, VSL, VAP and ALH were lower within minutes of centrifugation and decanting of CD sperm. Both CR and CD treatment values for all variables were lower ($P<0.05$) from 3 d. These data indicate that centrifugation can negatively affect boar sperm motility and that even the small amount of residual seminal plasma in fresh-extended boar semen has a protective role. Sperm age-associated reduced motility can be countered with caffeine.

Keywords: boar, sperm, centrifugation, seminal plasma removal, caffeine, motility

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Introduction

Artificial insemination (AI) is the most common reproductive technology employed in swine production (Bortolozzo et al., 2015) and appropriate processing of semen is essential for optimal reproductive outcomes. Boar semen undergoes centrifugation prior to cryopreservation to allow the removal of seminal plasma, performed in order to improve post-thaw fertility (Okazaki et al., 2009). In contrast, the presence of seminal plasma at thawing may actually improve boar sperm fertility (Garcia et al., 2005; Okazaki et al., 2009) but with fresh liquid semen processing, seminal plasma is diluted but not removed. Stallion ejaculates are cooled to 5°C for transportation although some stallions produce ejaculates that do not tolerate cooling very well. Interestingly, centrifugation and removal of seminal plasma improve sperm motility characteristics at 48 h (Brinsko et al., 2000). Further, stallion sperm were not damaged when centrifuged at 900 x g but were when centrifuged at 4,500 x g (Len et al., 2010). This suggests species differences in the tolerance of sperm to centrifugation.

Adequate progressive sperm motility is presumably essential for establishment of an appropriate sperm reservoir in the oviduct. It is established that the addition of caffeine to boar sperm increases their motility (Miyamoto and Nishikawa, 1980) and has been used to improve insemination results in gilts and sows (Yamaguchi et al., 2009, 2013), likely due to caffeine's property of increasing sperm numbers in the uterus by reducing the intra-uterine inflammatory reaction in sows (Yamaguchi et al., 2013). The effect of caffeine on sperm motility after several days of storage needs further investigation; if motility is needed for establishment of a sperm reservoir, it is possible that enhancing motility of aged sperm will improve their apparent fertility.

The use of thawed sperm in AI has been found to reduce both litter size and farrowing rates by 20-30% as compared to insemination using fresh cooled sperm (Johnson et al., 2000). While the process of cryopreservation is known to be detrimental, resulting in cryoinjury including a capacitation-like effect (Kirkwood et al., 2008), to our knowledge the effects of individual components of the sperm processing, including centrifugation and near-total removal of seminal plasma, have not been explored. Such knowledge could be useful since centrifugation has been shown to induce sub-lethal damage to human sperm (Alvarez et al., 1993) and seminal plasma is known to modulate sperm function (Caballero et al., 2012). The aim of the current study was to determine the impact of residual seminal plasma in fresh semen doses on sperm motility. We hypothesised that neither centrifugation *per se*, nor removal of residual seminal plasma, would affect sperm motility in fresh semen doses and any sperm age-dependant reduction in motility will be countered by caffeine supplementation.

Methodology

Fresh liquid semen doses from six boars were obtained from a commercial stud (SABOR, Claire, SA) with each dose containing 3 x 10^9 motile sperm diluted to 80 mL in Androstar® extender prior to dispatch. The raw ejaculates each provided about 20 semen doses so each dose would contain 5% seminal plasma. On arrival and during all subsequent testing the semen samples were maintained at 20°C. Three aliquots (10 mL) from each of the semen doses were allocated to treatment as follows:

1. Centrifugation for 10 min at 1,000 x g and then gently agitated to resuspend the sperm (CR)
2. Centrifugation for 10 min at 1,000 x g, decanting of supernatant to remove residual seminal plasma and resuspension in fresh extender (CD)
3. No centrifugation or decanting (Control)

The CD semen group had the seminal plasma and extender decanted until 2.5 mL of volume remained, after which 7.5 mL of Androstar® extender at 20°C was added and the contents were mixed by gentle inversion.

Measures of sperm motility, including total motility (TM), progressive motility (PM), velocity curvilinear (VCL), velocity straight line (VSL), velocity average path (VAP), beat cross frequency (BCF) and amplitude of lateral head displacement (ALH), were assessed within 3 min after centrifugation (0 h) and at 24, 48, 72 and 96 h using a computer-assisted sperm analyser (CASA; AndroVision®, Minitube, Smythesdale, Vic. Australia). These variables were chosen as associations with farrowing rate (PM, VCL, BCF) and litter size (TM, VAP, VSL, ALH) have been documented previously (Broekhuijse et al., 2012). Following the 96 h assessment, the semen doses were incubated in extender with 10 mM caffeine and motilities were reassessed 30 min later. For sperm motility analyses, the semen (1 mL) was warmed at 37°C in a water bath for 5 min before two 10 µL drops were placed on a slide using a pipette and covered with glass cover slips, with both the slide and cover slips warmed to 37°C. For each boar, the various CASA determinations were calculated at each time point from the average of three fields in each of the two drops.

Statistical analyses: The effect of time of count (0, 24, 48, 72 and 96 h) and treatment (CR, CD and Control), and the interaction between these effects on total and progressive motility were analysed in Genstat 17th Edition (VSN International, Hemel Hempstead, UK) using a linear mixed model after adjustment for the individual by fitting boar identification as a random term. Results were deemed significant if $P<0.05$ and pairwise comparisons were performed using a Fishers least significant difference test.

Results and Discussion

Total motility in the control semen was maintained above 70% throughout the experiment (Table 1). However, the addition of caffeine at 96 h resulted in an increase from 75.6% to 88.8%. For CR sperm, TM was maintained at control levels but was 18.2% and 15.0% lower than the controls at 72 and 96 h ($P<0.05$; Table 1). Due to a laboratory error, the response to caffeine at 96 h for CR sperm was not

obtained. For CD sperm, TM was lower (P<0.05) than the controls at 48 h with the difference becoming progressively larger until 96 h; following the addition of caffeine at 96 h, TM increased from 22% to 48.9%.

Progressive motility in the control semen was maintained above 70% except at the 96 h measurement when it was 67.3% (Table 1). Sperm PM at 96 h was increased from 67.3% to 80.2% in response to caffeine. Compared to the control values, PM of the CD sperm was reduced by 48 h (P<0.05) and, at 96 h, was increased from 9.8% to 35.8% by the addition of caffeine. For CR sperm, PM was significantly lower than that of the controls from 72 h (Table 1).

Changes with time were very similar for VSL, VAP, VCL and BCF. The control values remained relatively stable but VCL, VSL, VAP and ALH were lower within minutes of centrifugation (Table 1). Both CR and CD treatment values for all variables were lower (P<0.05) from 3 d (Table 1).

Table 1 Effect of centrifugation and resuspension (CR) or centrifugation and seminal plasma removal (CD) on measures of sperm motility over 96 h

	Time from centrifugation (h)					
Motility variable*	0**	24	48	72	96	SEM
Total (%)						5.80
Control	85.2	82.8	85.5^{a}	86.4^{a}	75.6^{a}	
CR	82.2	85.2	80.6^{a}	68.2^{b}	60.6^{a}	
CD	72.9	69.3	66.3^{b}	54.9^{b}	22.0^{b}	
Progressive (%)						6.12
Control	76.6	72.4	77.6^{a}	78.5^{a}	67.3^{a}	
CR	73.9	73.8	73.3^{a}	53.4^{b}	45.8^{b}	
CD	62.2	57.5	52.8^{b}	40.8^{b}	9.8^{c}	
VCL (µm/s)						7.50
Control	106.3^{a}	69.4^{ab}	75.9^{ab}	90.3^{a}	74.2^{a}	
CR	90.7^{ab}	81.1^{a}	87.3^{a}	55.4^{b}	50.1^{b}	
CD	82.2^{b}	59.5^{b}	62.7^{b}	50.5^{b}	18.2^{c}	
VSL (µm/s)						2.84
Control	31.2^{a}	24.3	26.2^{a}	32.2^{a}	28.5^{a}	
CR	27.2^{ab}	25.9	26.1^{a}	15.2^{b}	14.1^{b}	
CD	22.6^{b}	18.7	17.4^{b}	13.6^{b}	3.5^{c}	
VAP (µm/s)						3.63
Control	47.4^{a}	31.8	35.0^{ab}	41.8^{a}	36.5^{a}	
CR	38.8^{ab}	34.7	36.2^{a}	21.5^{b}	20.5^{b}	
CD	33.8^{b}	25.0	25.1^{b}	19.7^{b}	6.3^{c}	
BCF (Hz)						1.49
Control	13.9	14.2^{ab}	15.8^{a}	18.6^{a}	16.3^{a}	
CR	15.1	15.9^{a}	13.9^{a}	9.8^{b}	8.9^{b}	
CD	11.3	11.6^{b}	9.6^{b}	8.0^{b}	2.4^{c}	
ALH (µm)						0.08
Control	1.42a	0.83	0.84	0.96^{a}	0.84^{a}	
CR	1.15b	0.93	1.05	0.73^{b}	0.67^{a}	
CD	1.08b	0.72	0.84	0.68^{b}	0.32^{b}	

Velocity curvilinear (VCL), Velocity straight line (VSL), Beat cross frequency (BCF), Amplitude of lateral head displacement (ALH)
*Determined by computer-assisted sperm analysis
**The initial time zero motility determinations were made immediately after centrifugation.
CR: Centrifugation for 10 min at 1,000 x g and then gently resuspended
CD: Centrifugation for 10 min at 1,000 x g, decanting of supernatant and resuspension in Androstar®
Control: No centrifugation or decanting
Superscript letters (^{a,b,c}) indicate a significant difference (P<0.05) between treatments within time.

The present data clearly indicate that centrifugation does decrease boar sperm motility although effects are not immediately apparent. This is consistent with results from human sperm indicating that centrifugation induced sub-lethal sperm damage since effects on motility were not evident until after 24 h (Alvarez et al., 1993). In apparent contrast, centrifugation of stallion sperm at up to 900 x g was without effect, although sperm recoveries were low and the sperm were assessed only to 48 h (Len et al., 2010). Alvarez et al. (1993) have suggested that human sperm recovery methods avoiding centrifugation might alleviate cryoinjury occurring during sperm cryopreservation. Suggestions as to how centrifugation causes a reduction in motility include stress imposed on the sperm plasma membrane (Alvarez et al., 1993) as well as the production of reactive oxygen species that would result in indirect damage to the sperm membrane (Am-in et al., 2011).

Centrifugation and resuspension in seminal plasma-free extender resulted in a more rapid and more severe negative impact on both total and progressive sperm motilities. Seminal plasma contains antioxidants and the total antioxidant status positively correlates with sperm motility, morphology and plasma membrane integrity (Am-in et al., 2011). Seminal plasma functions include stimulating sperm motility during ejaculation, maintaining sperm membrane integrity, and preventing premature capacitation (Maxwell and Johnson, 1995; Am-in et al., 2011). The absence of seminal plasma can also be expected to result in a more severe impact of centrifugation, assuming reactive oxygen species are generated. Interestingly, removal of seminal plasma

prior to freezing resulted in an increased post-thaw sperm motility of poor freezability boars but had no effect on sperm motility of good freezability boars (Okazaki et al., 2009). Indeed, if consistently expressed, post-thaw motility of sperm frozen with or without seminal plasma might provide a simple screening test for good freezability boars. A dose-dependent reduction in cryoinjuries and increased conception rates to breeding from thawing sperm in media with seminal plasma were also noted and the impact on conception rate was possibly due to effects of seminal plasma on the sperm and also on the uterus (Okazaki et al., 2009).

Effective removal of seminal plasma has been previously documented with extensive dilution of seminal plasma associated with flow-cytometric sex-sorting of sperm (>3,000-fold). This level of dilution negatively impacts the sperm, leading to loss of membrane integrity, sperm agglutination, capacitation, and premature death and this effect has been termed the "dilution effect" (Maxwell and Johnson, 1999). In the present study, it is possible that the near total removal of seminal plasma from CR semen resulted in a dilution effect on the sperm. However, while motilities were clearly reduced, membrane integrity or capacitation status was not examined. In CD sperm, at 96 h after seminal plasma removal, total and progressive motilities of 22 and 9.8%, respectively, were noted. However, not all immotile sperm were dead as when stimulated by 10 mM caffeine, they immediately increased the total and progressive motilities to 48.9 and 35.8%, respectively. The duration of the effect of caffeine was not determined, although we have previously shown that, compared to non-treated sperm, caffeine increased total and progressive motilities by 10 to 15% for at least 2 h but that these motilities were 40% lower than control by 24 h, presumably due to sperm exhaustion (data not shown). However, a 2 h increase in motility would suffice to ensure sperm entry to the oviduct sperm reservoir prior to the uterine immune response to the sperm. Although speculative, a short-term increase in motility may enhance the breeding outcome if aged sperm are inseminated. Such a situation could arise if wean to mating intervals are prolonged, such as during the hotter months, and fresh semen doses are not available. Previous experience with aged sperm has indicated improved sow fertility to post-cervical insemination (Am-in et al., 2011), which suggests that aged semen doses contain viable sperm, just fewer of them. It is possible that enhancing sperm motility, such as with caffeine, would enhance the establishment of the sperm reservoir and limit adverse effects on fertility. This is worthy of further study.

The data from the current study refute our hypothesis as motility was reduced consequent to centrifugation. However, we support earlier data indicating the effect to be sub-lethal as impact on motility was not observed until 72 h. Further, assuming 20 doses per ejaculate, extended fresh semen can be expected to have only about 5% seminal plasma; even this small amount of seminal plasma appears to play a protective role in boar semen. This effect must be considered if future semen doses are produced with fewer sperm and so less seminal plasma. The effect of volume of such doses would need to be evaluated.

The present data indicate that centrifugation can damage boar sperm. However, the damage does not become apparent until 72 h; therefore, fertility would not be affected if semen is used prior to this time. However, the near-total removal of seminal plasma from boar semen appears to induce a dilution effect with sperm motility decreasing within 24 h.

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บทคัดย่อ

ผลของการปั่นเหวี่ยงเพื่อแยกน้ำเลี้ยงเชื้อต่อการเคลื่อนไหวของตัวอสุจิสุกร

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น้ำเชื้อที่ได้มาจากพ่อสุกร 6 ตัวถูกปั่นเหวี่ยงเพื่อแยกน้ำเลี้ยงเชื้อและอสุจิออกจากกันและทำการผสมกลับไปดังเดิม (CR) หรือถูกปั่นเหวี่ยงและรินน้ำเลี้ยงเชื้อทิ้งและนำตัวอสุจิไปผสมกับสารละลายน้ำเชื้อชนิดใหม่ (CD) หรือเป็นน้ำเชื้อปกติกลุ่มควบคุม ทำการตรวจวัดการเคลื่อนไหวของตัวอสุจิทันทีหลังจากปั่นแยกและทุกๆ 24 ชั่วโมงจนถึง 96 ชั่วโมงโดยใช้เครื่องตรวจประเมินคุณภาพน้ำเชื้อด้วยคอมพิวเตอร์ การเคลื่อนไหวภาพรวมและการเคลื่อนไหวไปข้างหน้าของตัวอสุจิลดลง ($P<0.05$) หลังจาก 48 ชั่วโมงในกลุ่ม CD การเคลื่อนไหวภาพรวมไม่ได้รับผลกระทบในกลุ่ม CR แต่การเคลื่อนไหวไปข้างหน้าของตัวอสุจิลดลง ($P<0.05$) หลังจากผ่านไป 72 ชั่วโมง การเปลี่ยนแปลงของ VSL VAP VCL และ BCF ในแต่ละเวลาใกล้เคียงกัน ค่าพารามิเตอร์ของกลุ่มควบคุมคงที่ แต่ VCL VSL VAP และ ALA มีค่าต่ำกว่าในเวลาเดียวกันหลังจากปั่นแยก (ตารางที่ 1) ค่าพารามิเตอร์ของกลุ่ม CR และ CD ลดลงเมื่อผ่านไป 3 วันหลังจากปั่นแยก ($P<0.05$) ข้อมูลนี้บ่งชี้ว่าการปั่นแยกสามารถสร้างความเสียหายให้กับตัวอสุจิสุกร และสารที่มีอยู่ในน้ำเลี้ยงเชื้อของพ่อสุกรสามารถทำหน้าที่ปกป้องตัวอสุจิได้ การเคลื่อนไหวของตัวอสุจิที่ลดลงเมื่อเก็บรักษานานขึ้นสามารถต้านได้ด้วยกาเฟอีน

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