Single layer density centrifugation improved semen quality in a stallion with hydrocele

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Abstract

Hydrocele was diagnosed in a 21 year old stallion with a history of subfertility. Subjective progressive motility and normal sperm from ejaculates (n = 12) were (mean ± SEM) 25 ± 11% and 24 ± 7%, respectively. From each ejaculate,1 sample was centrifuged through a silica colloid and refrigerated for 72 hours. An uncentrifuged sample served as control. Morphologically normal sperm was higher ($p < 0.05$) in semen centrifuged through the silica colloid. Sperm motility in cool-stored semen was higher ($p < 0.05$) in silica colloid centrifuged semen compared to control at 24, 48, and 72 hours postrefrigeration. Percent viable sperm and sperm with high mitochondria membrane potential were higher ($p < 0.05$) at 24 hours of postrefrigeration for semen centrifuged through silica colloid. Three mares were inseminated at alternate estrus, either with semen centrifuged through the silica colloid or with control. Inseminations (n = 3) with uncentrifuged semen ($\geq 500 \times 10^6$ motile sperm) did not result in pregnancy. However, inseminations with fresh semen (n = 2) or 24 hours chilled semen (n = 2) obtained after centrifugation with single layer colloid ($\geq 170 \times 10^6$ motile sperm) resulted in pregnancies. We concluded that hydrocele had apparently contributed to decreased semen quality and fertility in this stallion, and centrifugation of the ejaculate through a silica colloid improved semen quality.

Keywords: Stallion, semen, hydrocele, single layer centrifugation

Introduction

Hydrocele refers to accumulation of serous fluid between the visceral and parietal layers of tunica vaginalis. It can be either permanent or may appear in periods of high ambient temperature, resolving after temperature decreases. It is usually bilateral and may be accompanied by scrotal edema.[1,2] Relationship between hydrocele and fertility is known.[2,3] Impact on spermatogenesis (decline in semen quantity and quality) was apparently due to fluid accumulation, resulting in heat-induced testicular degeneration.[2,4] Treatment of hydrocele may at best provide only transient improvement.[2] However, it is possible to improve the quality and fertilizing ability of some poor quality ejaculates through density centrifugation with silica colloids.[5]

A 21 year old Lusitano dressage stallion with a history of subfertility was presented. The stallion was intensively utilized in a dressage school. Detailed information on his reproductive use could not be obtained. Stallion produced 11 registered foals: 9 in 1999, 1 in 2013, and 1 (last one) in 2014. Existence of a subfertility problem and a genital "swelling" was recognized by the referring veterinarian. Stallion appeared normal on physical examination except for the enlarged scrotum (Figure 1). Procedures conducted adhered to the ethical principles of equine semen collection centers (EU Directive 92/65/EEC). Materials used were obtained with the knowledge and authorization of stallion owner.

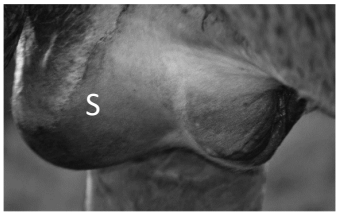

Figure 1. Bilateral diffused enlargement of the scrotum (S)

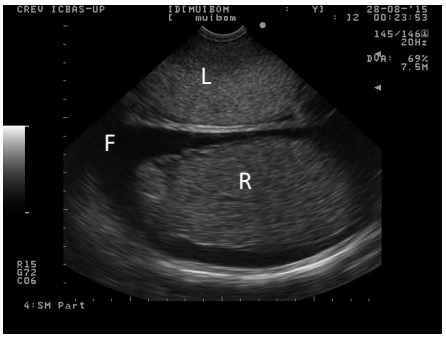

Figure 2. Ultrasonographic images of left (L) and right (R) testes; note the presence of hypoechogenic fluid (F)

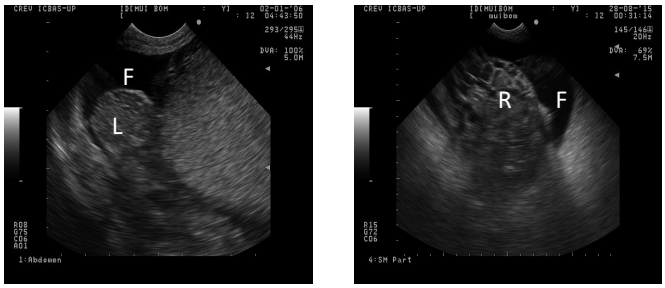

Figure 3. Ultrasonographic image of left (L) and right (R) epididymides; note the presence of hypoechogenic fluid (F)

On palpation, thickened scrotal wall, and presence of scrotal and preputial edema were felt. Scrotal width, as measured with a caliper, was 12 cm. The fluid-filled scrotum made palpation of the freely movable testis difficult. It was not possible to differentiate head and body of the epididymides, and only the epididymal tails could be felt. The stallion showed no sign of discomfort during palpation. Ultrasonography (Aloka Prosound 2 with 5 - 7.5 MHz probes, Aloka Holding Europe, Steinhausertrasse, Switzerland) revealed hypoechoic fluid accumulation within the vaginal cavity (Figures 2 and 3) leading to a clinical diagnosis of bilateral hydrocele. There were no noticeable changes observed in relation to the amount or echogenicity of the fluid throughout the study. The stallion demonstrated good libido, displaying an immediate full erection of penis when teased in the presence of a mare in estrus. The erect penis appeared normal and there was no evidence of pathology on palpation.

Method

Twelve ejaculates were collected using a Missouri artificial vagina (IMV Technologies, L'Aigle, France) at biweekly intervals, using a phantom as a mount and a mare in estrus. Subjective semen progressive motility was assessed by examining 10 µl of semen placed on a prewarmed (37ºC) slide covered by a cover slip, using a light microscope with a built-in stage warmer at 200 x magnification. Sperm concentration was estimated using a spectrophotometer (SpermaCue®, Minitub Ibérica, Tarragona, Spain) and sperm morphology was evaluated by examining 100 unstained sperm in wet mounts, using a phase-contrast light microscope (Axiostar 4X105TAR, Carl Zeiss Microscopy GmbH, Jena, Germany) at 1000 x magnification.

Raw semen had the following values (mean ± SEM) for sperm parameters: subjective sperm progressive motility 25 ± 11% (range; 15 - 50); gel-free semen volume 99 ± 38 ml (range; 40 - 150); sperm concentration, 184 ± 50 x 10^6/ml (range; 112 - 310); and morphologically normal sperm, 24 ± 7% (range; 13 - 38). Ejaculates (n = 12) were filtered and gel-free semen was diluted to a concentration of 100 x 10^6 sperm/ml in a prewarmed (37ºC) antibiotic-free extender (EquiPlus ref. #13570/0201; Minitub Ibérica; Tarragona, Spain). Processed ejaculates were used either as control (C) or centrifuged through single layer silica colloid (SLC).

Group C Ejaculates were further diluted in antibiotic-free EquiPlus to a final concentration of 50 x 10^6 sperm/ml and refrigerated for 72 hours in an Equitainer® (Minitub Ibérica; Tarragona, Spain). Group SLC ejaculates were prepared for centrifugation by carefully pipetting 15 ml of extended ejaculate (100 x 10^6 sperm/ml) over 15 ml of Androcoll-E® (Minitub Ibérica) in a 50 ml sterile falcon centrifuge tube. After centrifugation at 600 x g for 20 minutes, the supernatant was removed using a sterile Pasteur pipette. Sperm remaining in the pellet were resuspended in antibiotic-free EquiPlus to a final concentration of 50 x 10^6 sperm/ml and refrigerated for 72 hours in an Equitainer®.

Sperm kinetics of C and SLC samples were evaluated using a computer-assisted sperm analysis system (ISAS®; Proiser, Valencia, Spain) before refrigeration and at 24, 48, and 72 hours postrefrigeration. For each sample, a 5 chambered semen slide (ISAS-D4C20, Proiser, Valencia, Spain) was filled with extended semen and 5 fields were analysed for percentage of total motility (TM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity index (LIN) and straightness index (STR). The settings used for the computer-assisted semen analysis were as follows: 25 frames/second, cell size 4 µm to 75 mm, velocity of rapid cells > 90 µm/second, and straightness 35%.

At 1 and 24 hours postrefrigeration, sperm (10,000 per sample) were subjected to dye-sperm coincubation and evaluated for membrane and acrosome integrity[6] and mitochondrial membrane potential utilizing an EPICS XL flow cytometer (Beckman Coulter, Brea, CA), equipped with the EXPO32ADC software (Beckman Coulter). Samples (50 µl) were stained with 5 µl of *Arachis hypogaea* lectin conjugate (FITC/PNA; 100 µg/ml; (catalog # L 7381, Sigma-Aldrich, Munich, Germany) and incubated with propidium iodide (PI; 2.99 mM; catalog # P 4170, Sigma-Aldrich). This stain combination allowed for identification of 4 sperm populations: minimal green and red fluorescence (sperm with intact plasma and intact acrosomal membranes); minimal green and enhanced red fluorescence (sperm with damaged plasma membrane and intact acrosomal membrane); minimal red and enhanced green fluorescence (sperm with intact plasma membrane and damaged acrosomal membrane); and enhanced red and green fluorescence (sperm with damaged plasma membrane and acrosomal membranes). Propidium-iodide-negative sperm with either intact or damaged/reacted acrosome were considered viable (viability). Mitochondrial membrane potential was assessed by flow cytometry after staining with 0.5 µl of JC-1 (5,5', 6,6'–tetrachloro-1,1', 3,3' tetraethylbenzymidazolyl

carbocianyne iodide). Stock solution had 3 mM JC-1 (catalog # T4069, Sigma-Aldrich) in DMSO (catalog # D8418, Sigma-Aldrich)[7] with a final concentration of 1.5 µM. DMSO in the semen samples at analysis was ~ 0.05%.

Data analyses

Levene's Test was applied to test for equality of variances) and independent 2-sample Student's t-tests were applied to compare semen parameters between groups, using SPSS Version 24 for Windows (IBM® SPSS® Statistics, New York, NY). Significance was set at $p < 0.05$.

Breeding management

Three mares with endometrial biopsies graded as IIA[8] were artificially inseminated at 24 hours after intravenous treatment with 1500 IU of hCG (Chorulon®, MSD Animal health, Paço de Arcos, Portugal) on detection of a follicle ≥ 35 mm. Three mares were randomly assigned to receive their first insemination with either C semen or SLC semen. Insemination dose of C semen contained ≥ 500 x 10^6 motile sperm (TM ranged from 22 to 33% and normal sperm ranged from 13 to 38%). Insemination dose of SLC semen (either fresh [n = 2] or 24 hours refrigerated [n = 2]) contained ≥ 170 x 10^6 motile sperm, (TM ranged from 30 to 55% and normal sperm ranged from 36 to 50%). Two mares were inseminated twice with fresh and refrigerated SLC semen. A third mare was inseminated with fresh SLC semen, with C semen and with 24 hours refrigerated SLC semen at subsequent estruses. Pregnancy diagnosis was performed on days 12 - 14 postovulation via transrectal ultrasonography and fetal heartbeat was assessed on day 25 postovulation. Luteolysis was induced in nonpregnant mares (on days 14 - 16 days postovulation) and in pregnant mares (on day 25 postovulation) with intramuscular treatment of 10 mg of prostaglandin $F_{2\alpha}$ analog (Dinoprost; Dinolytic®, Zoetis, Porto Salvo, Portugal).

Results

Morphologically normal sperm were higher ($p < 0.05$) in SLC group compared to C group (44 ± 4 versus 24 ± 2%). Before refrigeration, differences ($p > 0.05$) were observed in TM (40% ± 5 versus 33% ± 3%). TM was higher ($p < 0.05$) in SLC group for all duration of refrigeration (Table 1). Higher ($p < 0.05$) LIN, VSL, and STR were observed at 24 hours after refrigeration in SLC group compared to C group (40 ± 1 versus 34 ± 1%, 32 ± 2 versus 25 ± 2 µm/second, and 74% ± 2 versus 64 ± 2%, for SLC and C groups, respectively). At 48 hours and 72 hours after refrigeration, STR was higher ($p < 0.05$) in SLC group (68 ± 3 versus 58 ± 2% and 70 ± 3 versus 59 ± 4%, respectively) compared to C group. There were no differences ($p > 0.05$) between 2 groups for the remaining kinetic parameters analyzed.

Table 1. Total motility (TM) in percent for control (C) and Androcoll-E® centrifuged (SLC) semen at 24, 48, and 72 hours of refrigeration

Duration of refrigeration in hours	C	SLC
	TM	TM
24	15 ± 2[a]	30 ± 3[b]
48	10 ± 2[a]	25 ± 3[b]
72	13 ± 1[a]	30 ± 4[b]

[a,w]Within row, means without a common superscript differed ($p < 0.05$)

Cytometry analyses revealed viability of SLC semen to be higher ($p < 0.05$) compared to C semen after 24 hours of refrigeration (74% ± 3 versus 57% ± 5%, respectively). Acrosome reaction (PNA+PI-) was not different. After 24 hours of refrigeration, percentage of sperm with higher mitochondrial membrane potential was 59 ± 2% in SLC samples and 47 ± 4% in the C group ($p < 0.05$).

None of the inseminations with C semen resulted in pregnancies. All inseminations with SLC semen resulted in pregnancies. Fetal heartbeat was detected in 3 pregnancies, and 1 embryo was lost between days 14 - 25 postovulation.

Discussion

Although hydrocele is not always associated with decreased fertility[2] it can affect spermatogenesis.[2,4] In this case, apparently, age-related testicular degeneration contributed to the low quality of the ejaculates. However, no evidence of testicular degeneration was noted 3 months before, during or after the study. Higher incidence of abnormal sperm and poor semen motility are compatible with disrupted spermatogenesis, possibly due to testicular heat stress.[2,4] Improvement of semen quality is possible after centrifugation with single layer colloid centrifugation.[9,10,11] To authors' best knowledge, this is the first report of the use of this technique in poor quality ejaculates collected from a stallion with hydrocele. Although the number of inseminations was limited, pregnancies were established in mares inseminated with treated semen that had a lower number of motile sperm.

Conclusion

Hydrocele possibly contributed to poor sperm quality in this stallion. Single layer density centrifugation of the ejaculate improved semen quality by decreasing the number of abnormal sperm and increasing the number of sperm with high mitochondrial membrane potential. Apparently, improved semen quality resulted in higher pregnancy rate.

Acknowledgement

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Conflict of interest

No conflict of interest to declare.

References

1. Ball BA: Diagnostic methods for evaluation of stallion subfertility: a review. J Equine Vet Sci 2008;28:650-665.
2. Varner DD, Schumacher J, Blanchard TL, et al: In: Pratt PW: editor. Diseases and Management of Breeding Stallions. Goleta, California; American Veterinary Publications: 1991. p. 242-244.
3. Henry M, Amaral D, Tavares FF, et al: Hydrocele of the vaginal cavity of stallions. J. Reprod Fertil Suppl 2000;56:13-21.
4. Schumacher J, Varner DD: Abnormalities of the spermatic cord. In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine Reproduction. 2nd edition, Ames; Wiley-Blackwell: 2011. p. 1145-1155.
5. Morrell JM, Mari G, Kútvölgyi G, et al: Pregnancies following artificial insemination with spermatozoa from problem stallion ejaculates processed by single layer centrifugation with Androcoll-E Reprod Dom Anim 2011;46:642-645.
6. Ortgies F, Klewitz J, Görgens A, et al: Effect of procaine, pentoxifylline and trolox on capacitation and hyperactivation of stallion spermatozoa. Andrologia 2012;44:130-138.
7. Morillo-Rodriguez A, Ortega-Ferrusola C, Macías-Garcia B, et al: Freezing stallion semen with the new Cáceres extender improves post thaw sperm quality and diminishes stallion-to-stallion variability. Anim Reprod Sci 2011;127:78-82.
8. Kenney RM, Doig PA: Equine endometrial biopsy. In: Youngquist RS, Threlfall WR: editors. Current Therapy in Theriogenology. 2nd edition, Philadelphia; Elsevier Saunders: 1986. p. 723-729.
9. Costa AL, Martins-Bessa A, Rebello de Andrade A, et al: Single layer centrifugation with Androcoll-E™ improved progressive motility and percentage of live spermatozoa with intact acrosome of chilled stallion semen but did not have an effect on DNA integrity. Open J Anim Sci 2012;2:159-165.
10. Morrell JM, Rodriguez-Martinez H: Biomimetic techniques for improving sperm quality in animal breeding: a review. Open Androl J 2009;1:1-9.
11. Johannisson A, Morrell JM, Thorén J, et al: Colloidal centrifugation with Androcoll-E prolongs stallion sperm motility, viability and chromatin integrity, Anim Reprod Sci 2009;116:119-128.