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ORIGINAL ARTICLE

In vivo fertilizing ability of stallion spermatozoa processed by single layer centrifugation with Androcoll-E™

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Abstract A colloid with a species specific silane-coated, silica-based formulation, optimized for stallion (Androcoll-E™), enables a better sub-population of spermatozoa to be selected from stallion ejaculates. However, such a practice has not been critically evaluated in stallions with fertility problems. In this study we evaluate whether single-layer centrifugation (SLC) through Androcoll-E™ could be used to enhance fertility rates in a subfertile stallion. Ejaculates were obtained from two different stallions, one Lusitano (fertile) and one Sorraia (subfertile), with distinct sperm characteristics and fertility. Motility, morphology, plasma membrane structural (eosin-nigrosin) and functional integrity (HOS test), mitochondrial functionality ($\Delta\psi m$; JC-1) and longevity (motility after 72 h cooling) after centrifugation in Androcoll-E™, as well as pregnancy rates obtained after artificial insemination (AI), with and without (control group) SLC-treated sperm were assessed. The effect of SLC on sperm characteristics, and fertility results were evaluated by ANOVA and Fisher procedures, respectively. Our results showed that SLC-selected sperm did not differ from the raw semen in terms of viability, morphology, response to hypo-osmotic conditions (HOS test) and mitochondrial membrane potential ($\uparrow\Delta\Psi$mit; JC-1). Sperm motility in cooled samples was not improved

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by SLC treatment. Our data show that SLC through Androcoll-E™ has no effect on pregnancy rates in the stallions used in this trial.

1. Introduction

Artificial insemination (AI) in farm animals has been used through decades, but the expansion in horses was lower for several reasons (Loomis, 2001), including specie-specific constraints related to sperm conservation techniques. Several sperm quality studies in mammalian species have been made demonstrating how it can be defined and measured despite some lack of correlation with fertility (Colenbrander et al., 2003). Mammalian ejaculates are characterized by several sperm populations, not only related with motility, morphology and viability but also with acrosome and DNA integrity, mitochondrial membrane potential, among others. Nowadays, in equine breeding industry, the goal is to select the best sperm population to develop AI techniques which have additional benefits in terms of efficiency in the use of semen and improvement of fertility's potential of stallions. The main challenge is to improve the technique by minimizing seminal plasma and bacterial carryover. Morrell and Wallgren (2011) showed that this can be achieved by sperm selection using single layer centrifugation (SLC) procedures. In fact, the use of SLC through a colloid with a species specific silane-coated, silica-based formulation optimized for stallion spermatozoa (Androcoll-E™), enables a sub-population of highly motile spermatozoa with normal morphology and good chromatin integrity to be selected from stallion ejaculates (Johannisson et al., 2009; Morrell et al., 2010). The work developed by our team and others (Costa et al., 2012) showed that SLC with Androcoll-E™ improved progressive motility and percentage of live cells with intact acrosome, in fertile stallions, but did not have an effect on DNA integrity.

The use of SLC to select the best spermatozoa from ejaculates of low quality and/or low fertility for subsequent use in AI was reported by Morrell et al. (2011) and Mari et al. (2011). However, strong evidence on the benefits of AI SLC-treated sperm in stallions with fertility problems is lacking. Therefore, in this assay, the effect of treatment of sperm with Androcoll-E™ on per cycle fertility was studied using ejaculates from two stallions of two Portuguese autochthonous breeds – Lusitano, a male already proven to be fertile, and Sorraia, known to have very different sperm quality (Gamboa et al., 2009). Sorraia is a critically endangered breed, with an extremely reduced effective population, high level of inbreeding (Luís et al., 2007) and low fertility rates (Oom et al., 1991; Gamboa et al., 2009). A conservation plan for this endangered breed is ongoing and strategies for breeding management are under study (Pinheiro et al., 2013). For this reason, fertile mares were artificial inseminated with SLC and non-SLC samples to determine if pregnancy rates could be improved by SLC-selected sperm used in AI programs. Viability, morphology, resistance to hypo-osmotic conditions and mitochondrial membrane potential were evaluated. Sperm survival rate of Androcoll-E™ treated semen cooled and stored at 4 °C for 24 h, 48 h and 72 h was also analyzed.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.1. Semen collection and evaluation

This study was undertaken in two consecutive reproductive seasons (2012 and 2013) using two healthy adult male horses (*Equus caballus*) from different Portuguese breeds: a Lusitano (13 years old; 2012 season) with proven fertility and a Sorraia (4 years old; 2013 season), of unknown fertility. The animals were located at the Coimbra College of Agriculture, Polytechnic Institute of Coimbra (Coimbra, Portugal—40°12′54.3″ N and 00°41′ E) and were kept in boxes with straw bedding, water freely available, and fed with hay and concentrates three times a day.

Semen was routinely collected (at least three collections/week) from May to July, using a phantom (Hannover model) and an artificial vagina (INRA model). Immediately after dismount the raw ejaculates were brought to the laboratory and filtered through a sterile gauze to remove the gel and any large particles of debris. The gel-free semen was immediately assessed for color, smell and general appearance and maintained at 35 °C in a water-bath during seminal evaluation and handling. Semen volume and pH were also registered. Sperm motility was assessed using a phase contrast microscope (Laborlux, Leica; equipped with a heating stage), at the time of collection, and the percentage of total progressive motile spermatozoa (PMSAC) was estimated visually by the observation of 5–10 microscopy fields in each of two drops (5.5 μl each drop) of raw semen. Filtered semen was diluted 40× in formal saline solution to assess sperm concentration using a photocolorimeter (λ = 546 nm; Colorimeter 254, Ciba-Corning). Sperm viability and morphology were accessed as previously described (Gamboa and Ramalho-Santos, 2005). Briefly, an aliquot of sperm was mixed with eosin-nigrosin on a slide for viability evaluations (Bloom, 1950), as well as with India ink for sperm morphology analysis (Foote, 2003). Two hundred cells were assessed for each slide using bright-field microscopy (Laborlux, Leica; ×1000) in 20–50 microscopy fields.

2.2. Semen treatment

Before semen collection, 15 mL of Androcoll-E™ (available from J.M. Morrell, SLU, Uppsala, Sweden) was transferred to a 50 mL Falcon tube and maintained at r.t. (20–23 °C) for 15 min. After collection, semen was diluted at a final concentration of 100×10^6 sperm/mL, to a final volume of 18 mL, in INRA96 extender (IMV technologies, L'Aigle, France) maintained at 35 °C prior to semen collection. This volume of extended semen was carefully transferred to the top of the colloid and centrifuged at 500*g* for 20 min. Above the pellet,

more than one "phase supernatant" (or layer) was distinguished (Fig. 1) and each one of them was separately removed and stored in different tubes. The pellet was re-suspended to a final volume of 5 mL and sperm concentration was determined using a Neubauer chamber. Sperm doses, both for AI and for motility evaluation after cooling, were prepared in INRA96 extender to a final concentration of 20×10^6 spz/mL. For AI, 15 mL of diluted semen was packaged in an Air-Tite type syringe with no air and used within the first 30 min after collection. For sperm motility analysis, 10 mL of diluted semen was fractionated in 10 mL centrifuge tubes that were then packed into 50 mL Falcon tubes and stored under anaerobic conditions in a refrigerator (4 °C) for 24 h, 48 h and 72 h. Anaerobic conditions were reached by loading the tubes with 10 ml of diluted sperm and no air. Raw semen was also diluted in INRA96 and the sperm doses, both for AI and for motility evaluation after cooling, were also prepared to a final concentration of 20×10^6 spz/mL.

Raw semen, the sperm treated and non-treated with Androcoll-E™, as well as each layer, were studied in relation to volume, sperm concentration and viability, HOS test, and mitochondrial membrane potential.

2.3. Hypo-osmotic swelling test (HOS test)

The hypo-osmotic swelling test (HOS test) was used to evaluate plasma membrane functionality. The HOS test determines the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment. Influx of the fluid due to hypo-osmotic stress causes the sperm tail to coil and balloon or "swell". A higher percentage of swollen sperm indicates the presence of sperm having a functional and intact plasma membrane (Ramu and Jeyendran, 2013). Semen samples were subjected to a hypo-osmotic medium (Hank's balanced salt solution with 26 mM Hepes buffer (HHBS): 1,3 mm $CaCl_2{\cdot}2H_2O$; 0,3 mM $Na_2HPO_4{\cdot}12H_2O$; 0,4 mM KH_2PO_4; 5,4 mM KCl; 0,8 mM $MgSO_4{\cdot}7H_2O$; 26 mM $C_8H_{18}N_2O_4S$; 5,5 mM $C_6H_{12}O_6$; 417 mM $NaHCO_3$, 50 mOsm; 1:40 dilution) and, in order to eliminate potential misinterpretation of the results, the incidence of spermatozoa with a bent flagellum prior to HOS was also evaluated in isotonic media (HHBS, 300 mOsm; 1:40 dilution). Samples were count using a hemocytometer (Neubauer chamber).

2.4. Mitochondrial membrane potential

Inner mitochondrial membrane potential (ΔΨmit) was monitored with the cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrae thylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Invitrogen, CA, USA), as previously described (Gamboa et al., 2010). JC-1 is a slow-response probe that, when electrophoretically translocated across membranes, shows fluorescence changes associated with the inner mitochondrial membrane transmembrane potential (IMM). Thus, JC-1 depicts orange-red fluorescence in fully functional (polarized) mitochondria; on depolarization, the orange-red punctate staining is replaced by diffuse green monomer fluorescence (Reers et al., 1991). Briefly, semen was diluted to 20×10^6 cells/mL and then incubated for 20 min at 35 °C with JC-1 in DMSO (2 μM). Sperm samples (5.5 mL) were placed on glass micro-

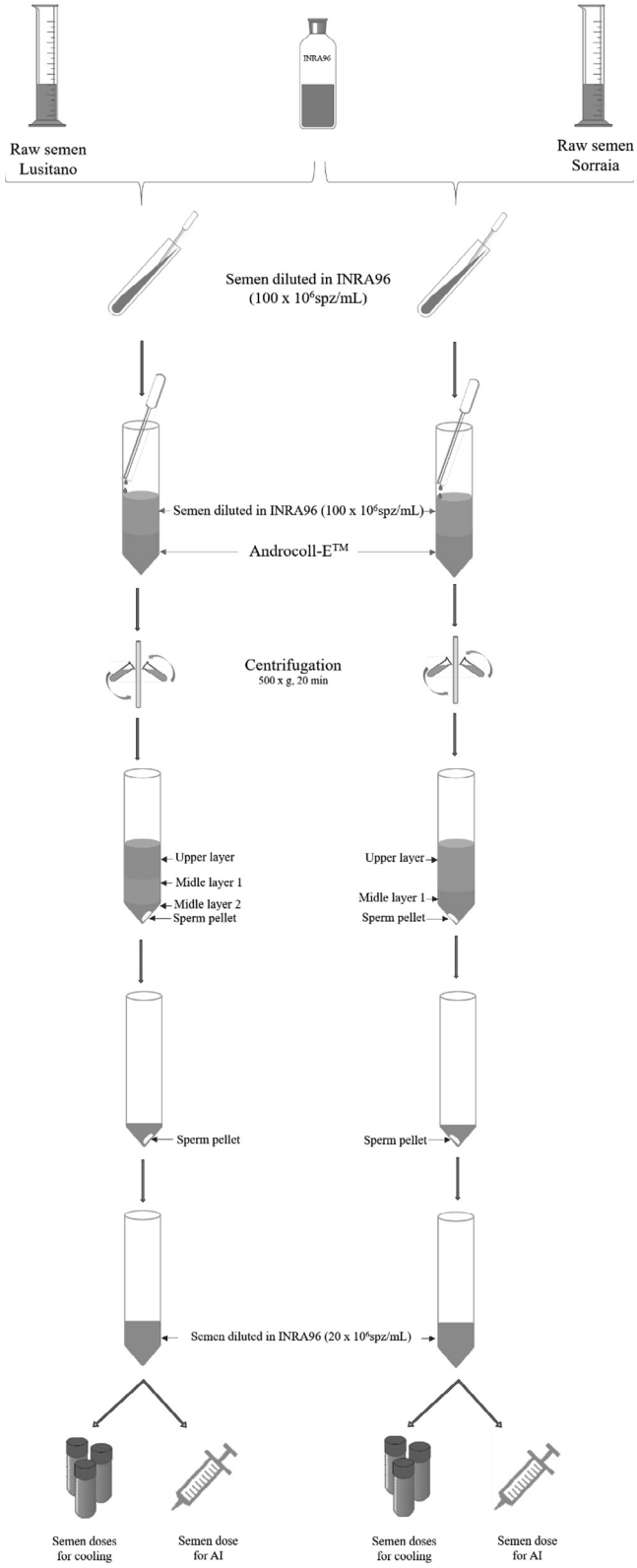

Figure 1 Schematic representation of the processing of the ejaculates using the single-layer centrifugation (SLC) with Androcoll-E™, respectively for Lusitano (PSL; left panel) and Sorraia (right panel) sperm. The position of the different layers are shown (black arrows).

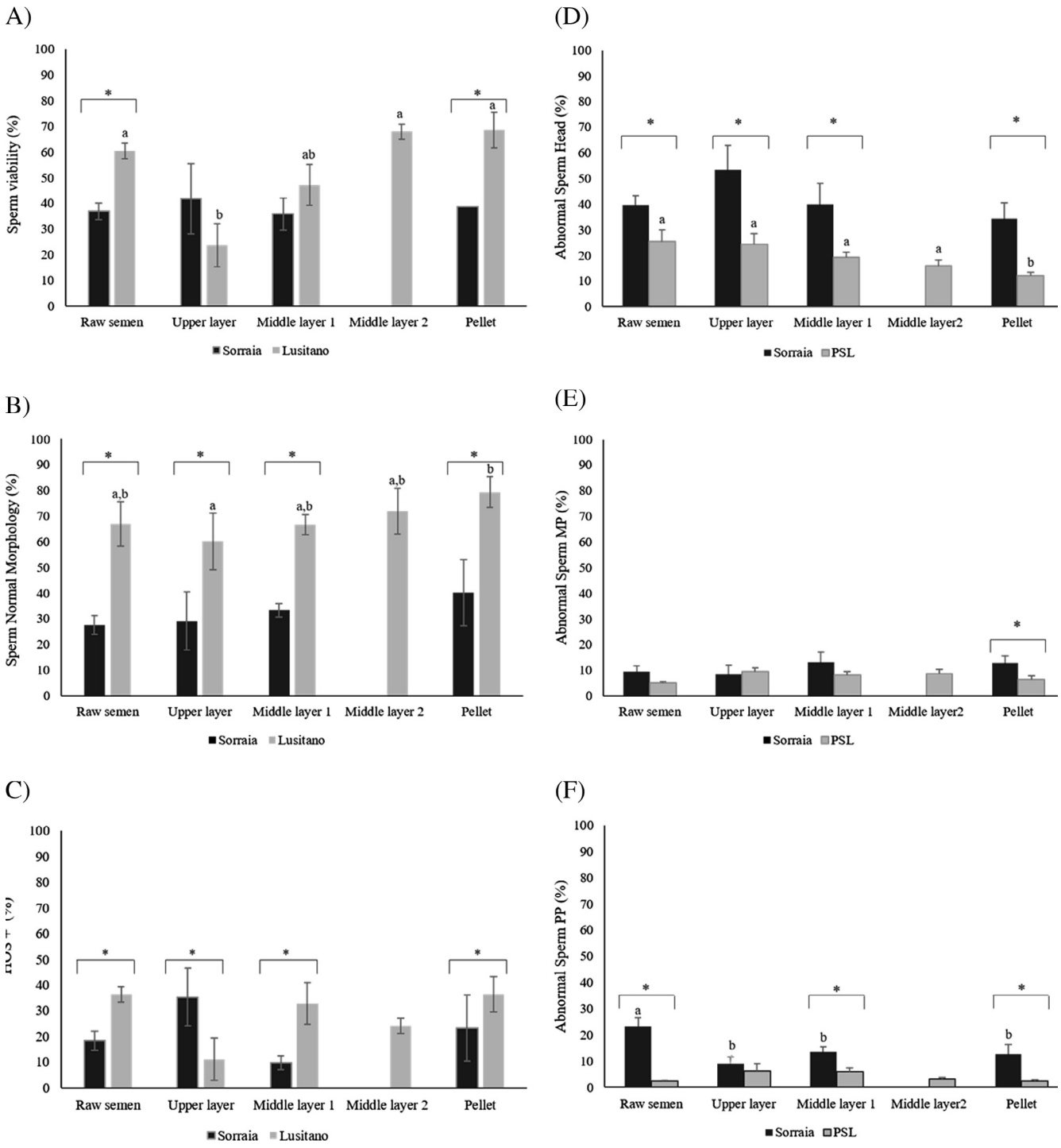

Figure 2 Sperm characteristics in raw semen and in the different layers after SLC-treatment. Proportion (mean ± SEM) of sperm (A) vitality, (B) morphology, (C) HOS+, abnormalities in (D) head, (E) midpiece (MP) and (F) principal piece (PP). Different superscript in mean values represents significant differences ($P < .05$) between layers (a, b) and between stallions (*).

scope slides with coverslips, and 200 cells/sample were analyzed with a HUND H 600 AFL (Helmut Hund GmbH, Wetzlar, Germany) fluorescence microscope.

2.5. Artificial insemination

Thirteen (13) fertile mares (4–20 years old), belonging to the Coimbra College of Agriculture, were used in this study. Mares reproductive management was as follows: after detecting the mare's estrus (mare reproductive behavior assessed in the presence of an intact stallion), the mare's reproductive status was observed regularly by ultrasonographic scanning (Falco–Esaote, Probe 5 MHZ transducer – Pie Medical Equipment BV); when a follicle $\geqslant$35 mm in diameter was observed, females were artificially inseminated with 300×10^6 spz (20×10^6 sperm/mL; 15 mL) per AI, every other day until

ovulation. Insemination was performed, using a sterile uterine catheter, within the first 60 min after semen collection. The time-interval "last AI-ovulation" was determined. For diagnosis of pregnancy and twining inspection ultrasonographic images of the conceptus at 13 days after last AI were taken. To calculate the fertility per cycle (FC) we utilized the estimations and rules commonly used in French National Stud Farms (France, 1996) and previously described (Gamboa and Ramalho-Santos, 2005).

In the first estrous cycle explored, mares were assigned to semen treatment at random (SLC or non-SLC) and if they did not conceive, another cycle was explored in order to alternate IA/SLC-treated with IA/non-SLC-treated sperm and vice versa. If they conceive the embryonic loss was induced by the use of Dinolytic (Pfizer Animal Health, Louvain-la-Neuve, France). In 2012s' breeding season, 10 mares were inseminated with the Lusitano sperm in 18 cycles: in nine cycles Androcoll-E™ treated semen was used and, in the other nine, non-treated doses. In 2013s' breeding season 11 mares were used to study Sorraia sperm: from 15 cycles, in six we used Androcoll-E™ treated semen was tested and in the other nine, non-treated doses. Four mares were inseminated over two consecutive cycles.

2.6. Statistical analysis

Data were analyzed with the SPSS Statistic software (version 20, IBM). Differences in spermatozoa quality in the several fractions after SLC-treatment were tested by ANOVA. Bonferroni post hoc tests were performed only if the initial test result was significant at $P < .05$. Sperm motility differences between treated and non-treated Androcoll-E™ samples were analyzed by the independent-samples T test procedure. A significant difference was reported at $P < .05$ (Maroco, 2007).

3. Results

Stallions sperm characteristics (mean value ± SEM) are presented in Fig. 2. For the Lusitano stallion, from a total of 19 ejaculates, seven of them were non-SLC-treated, eight were both treated and non-treated and four were SLC-treated. For the Sorraia horse, from a total of 13 ejaculates, five of them were non-SLC-treated and eight were treated and non-treated. As previously reported (Gamboa et al., 2009), seminal traits differed significantly between stallions except for ΔΨmit. Significant differences ($P < .05$) between stallions were also observed for semen volume (34.2 ± 17.3 and 9.3 ± 9.5, PSL and Sorraia stallions, respectively) and sperm concentration (156.0 ± 54.6 and 454.0 ± 280.0, PSL and Sorraia stallions, respectively).

For the Lusitano stallion, 12 ejaculates treated with Androcoll-E™ showed 4 layers after centrifugation: upper layer (seminal plasma plus extender with some spermatozoa), middle layers 1 and 2 (Androcoll-E™ plus spermatozoa, debris and extender) and pellet (rich in spermatozoa). In the Sorraia stallion, 8 ejaculates treated with Androcoll-E™ showed only 3 layers after centrifugation (Fig. 1). The sperm characteristics from each layer are shown in Figs. 2 and 3. In relation to initial sperm diluted in INRA96, there was 46.7% ± 8.4 and 54.0 ± 9.0 of sperm loss with SLC-treatment in Lusitano and Sorraia stallions, respectively. The percentage of spermatozoa retained in the colloid layer for Lusitano and Sorraia stallions were, respectively, 43.8% ± 8.3 and 48.9% ± 10.3.

Both in Lusitano and Sorraia stallions, raw semen was characterized by a low percentage of spermatozoa with a positive result for HOS test (HOS+), and the mean values did not differ ($P > 0.05$) from the percentage of sperm with membranes osmotically active, recovered after SLC-treatment (Fig. 2). For the HOS test, no differences were evident between middle layers and pellet, in both stallions. Nevertheless, in the Lusitano samples SLC-recovered sperm presented the highest percentage of live cells with membranes osmotically active, contrasting with Sorraia samples where the highest percentage of live cells with membranes osmotically active was retained in the upper layer (Fig. 2).

When sperm morphological quality was analyzed, the SLC-selected pellet presented a better population of spermatozoa regarding normal head in Lusitano samples ($P < 0.05$), while in Sorraia stallion no difference was found (Fig. 2).

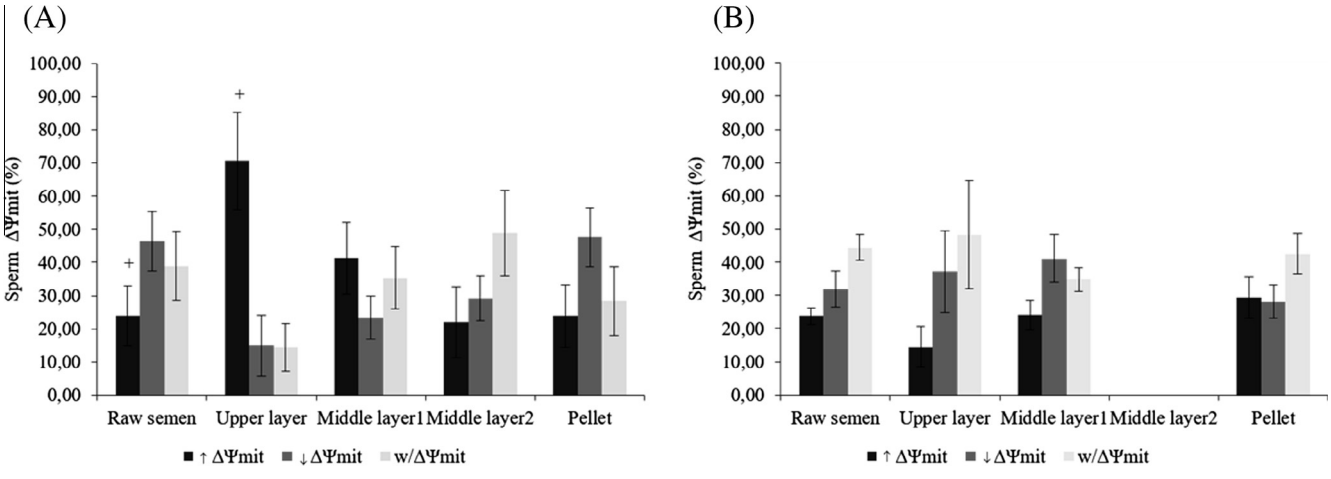

Figure 3 Proportion (mean ± SEM) of sperm mitochondrial membrane potential (ΔΨmit) in raw semen and in the different layers after single-layer centrifugation (SLC) on the sperm from Lusitano (A) and Sorraia (B) stallions. ↑ΔΨmit, high mitochondrial membrane potential; ↓ΔΨmit, low mitochondrial membrane potential; w/ΔΨmit, without mitochondrial membrane potential. Values bearing + possible differ statistically ($P < 0.1$).

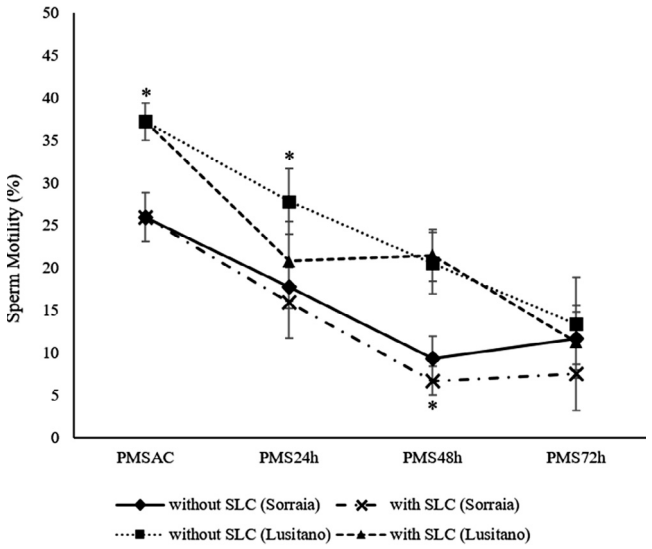

Figure 4 Proportion (mean) of motile spermatozoa after collection (PMSAC) and after dilution in IRA96, with (SLC-selected) and without (non SLC-selected) centrifugation on Androcoll-E™ , cooled (4 °C) and stored for 24 h (PMS24 h), 48 h (PMS48 h) and 72 h (PMS72 h). Values bearing * differ significantly between stallions ($P < 0.05$).

Sperm processing by SLC does not significantly increase the percentage of spermatozoa with high mitochondrial membrane potential. In fact, no differences were observed between raw semen and sperm SLC-selected from both Lusitano and Sorraia stallions (Fig. 3).

Sperm motility after collection (PMSAC; mean ± SEM) in samples subject to centrifugation in Androcoll-E™ was 37.1% ± 2.1 and 25.9% ± 2.9, respectively for Lusitano and Sorraia stallions (Fig. 4). In Sorraia samples this value does not differ from that observed after SLC-treatment (25.% ± 2.6) and after 24 h of conservation at 4 °C (17.7% ± 2.6 and 15.8% ± 4.2, respectively, with and without SLC treatment). In contrast, Lusitano sperm motility after SLC (47.5% ± 1.9) was significantly higher than in raw semen and in 24 h stored (4 °C) sperm with (20.7% ± 4.7, $n = 7$; $P < 0.005$) or without (27.8% ± 3.9, $n = 10$; $P < 0.001$) SLC-treatment. Although sperm motility decreased with time independent of the semen treatment, no differences were observed between SLC-selected and non-SCL selected sperm in samples conserved at 4 °C for 24 h, 48 h and 72 h.

The inferential statistical analysis (Fisher test) (Maroco, 2007) showed that, in the Lusitano stallion, per cycle fertility obtained with Androcoll-E™ treated semen did not differ (the Fisher exact test statistic value is 1. The result is not significant at $P < 0.05$) from per cycle fertility obtained with non SLC-treated sperm. For the Sorraia stallion, per cycle pregnancy rate was equal to zero for both treatments (Table 1).

4. Discussion

Single-layer colloid centrifugation (SLC) of semen is used to select the best spermatozoa from sperm samples in a variety of mammals (Henkel and Schill, 2003; Samardzija et al., 2006; Dorado et al., 2011a,b; Nicolas et al., 2012) including horses (Morrell et al., 2010). In order to evaluate if SLC through Androcoll-E™ can be helpful in horse's reproduction, we used sperm samples obtained from a fertile stallion (Lusitano) and a Sorraia horse of unknown fertility. The Sorraia breed is characterized by high levels of inbreeding (Luís et al., 2007; Pinheiro et al., 2013), poor seminal traits and low fertility (Gamboa et al., 2009; Oom et al., 1991).

Considering sperm membrane structural and functional integrity, morphology and mitochondrial membrane potential, no differences were found between raw semen and the sperm recovered after SLC. Moreover, fertility rates were not improved with SLC treatment using Androcoll-E™. The sperm quality results obtained contrast with other results carried out in horses (Johannisson et al., 2009; Morrell et al., 2010; Costa et al., 2012). Nevertheless, at the head level, morphologically significant differences were observed for the sperm population recovered by SLC in the Lusitano stallion but not for the Sorraia one. The Sorraia breed is characterized by polymorphic spermiogram, with macrocephalic and microcephalic spermatozoa present and the SLC procedure seems not sufficiently efficient in selecting the best sperm.

Concerning the effect of SLC on sperm membrane functionality, some authors (Shekarriz and DeWire, 1995) consider that time of centrifugation, more than g-force, induces ROS formation in semen. Reactive oxygen species (ROS) are associated with sperm membrane injury through spontaneous lipid peroxidation, which may change sperm function (Agarwal and Allamaneni, 2004). Previous work carried out in horses showed that Androcoll-E™ selects a subset of live sperm capable of producing superoxide anion in isosmolar conditions (Macías-García et al., 2012). However, in our study, if ROS were produced during SLC-centrifugation, it is reasonable to assume that their level was not deleterious enough to be associated with sperm membrane injury, as suggested by viability and HOS data. Besides, the functional integrity of sperm membrane is a prerequisite to fertilization and fertility rates

Table 1 Per cycle fertility rate (FC) diagnosed on day 13 after last artificial insemination (AI) with Androcoll-E™ treated (SLC-treated) or non-treated (non-SLC-treated).

Sperm	Stallion	No of mares	AI/mare	DG^+	FC (%)	AI/pregnant mare
SLC-treated	Lusitano	9	1.3 ± 0.2	7	78	1.4 ± 0.3
	Sorraia	6	1.2 ± 0.2	0	0	0.0
Non-SLC-treated	Lusitano	9	1.6 ± 0.2	8	89	1.6 ± 0.3
	Sorraia	9	1.3 ± 0.2	0	0	0.0
Total	Lusitano	18	1.4 ± 0.2	15	88	1.5 ± 0.2
	Sorraia	15	1.3 ± 0.1	0	0	0

DG^+ -positive pregnancy diagnosis; DG^- -negative pregnancy diagnosis.

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obtained with sperm SLC-treated do not differ from non-SLC-treated rates.

The HOS test evaluates the functional integrity of plasmalemma (Ramu and Jeyendran, 2013). It is a different approach to eosin-nigrosin, which evaluates the structural integrity of plasma membrane (Zhu and Liu, 2000). In this study, it seems that, in both stallions, the sperm regulatory volume mechanism is not affect by SLC-treatment since that sperm plasmalema remains functional as we have shown by the HOS test results. Apparently, the sperm extender compensates both the loss of osmolytes of low molecular weight and the antioxidant defenses offered by the seminal plasma. This assumption seems to be supported by results obtained for sperm motility in cooled samples over time. Indeed, sperm motility in SLC-selected samples did not differ significantly from that observed in non-SLC-selected samples.

It has been believed that the function of the mitochondria in the midpiece is to provide ATP for sperm movement through oxidative phosphorylation. In stallions, sperm mitochondrial activity was correlated with sperm viability and progressive motility in raw semen (Foote, 2003). In frozen-thawed sperm (Macías-García et al., 2009), SLC through Androcoll-E™ improved the percentage of spermatozoa depicting high $\Delta\Psi$mit. To our knowledge, this is the first report where mitochondrial membrane potential was evaluated in fresh SLC-selected stallions' sperm. The ejaculate of Lusitano horse was characterized by a good viability but only satisfactory motility and low mitochondrial membrane potential, even in conditions where oxygen was present, contrasting with Sorraia's semen, where poor viability and motility were observed but mitochondrial membrane potential was higher. However, AI of fertile mares with sperm doses prepared following SLC did not result in any pregnancy.

In our study, there was no significant difference in pregnancy rates between SLC and non-SLC sperms for either the fertile or subfertile stallion tested. Overall, the SLC-centrifugation seems not to affect sperm plasmalema functionality, but did not select for other evaluated characteristics of sperm quality.

5. Conclusion

This technique did not improve pregnancy rates in either the fertile or the infertile stallions used in this trial. The results of the present study demonstrate that any benefits of the SLC technique may be stallion-dependent.

Author contributions

S.G. planned the experiment; S.G., A.Q. and F.C. performed the experiments; S.G. and A.Q. analyzed the data and wrote the manuscript; P.B. performed the ultrasonographic examinations of the mares; M.R.R. contributed to discussion and reviewed/edited manuscript; M.M.O. assistance with the Sorraia horse and reviewed manuscript; A.R. is the PI of the project PTDC/CVT/108456/2008.

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References

Agarwal, A., Allamaneni, S., 2004. Oxidants and antioxidants in human fertility. Middle East Fertil. Soc. J. 9, 187–197.
Bloom, E., 1950. A one minute live–dead sperm stain by means of eosin-nigrosin. Fertil. Steril. 1, 176–177.
Colenbrander, B., Gadella, B.M., Stout, T.A., 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Domest. Anim. 38, 305–311.
Costa, A., Martins-Bessa, A., Andrade, A., Guimarães, T., Rebordão, M., Gamboa, S., Bravo, P., Correia, M., Colaço, J., Gaivão, I., Rocha, A., 2012. 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. OJAS 2, 159–165.
Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., Hidalgo, M., 2011a. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm(®) gradient. Anim. Reprod. Sci. 125, 211–218.
Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., Demyda, S., Muñoz-Serrano, A., Hidalgo, M., 2011b. Centrifugation on PureSperm(®) density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 76, 381–385.
Foote, R.H., 2003. Effect of processing and measuring procedures on estimated sizes of bull sperm heads. Theriogenology 59, 1765–1773.
Gamboa, S., Ramalho-Santos, J., 2005. SNARE proteins and caveolin-1 in stallion spermatozoa: possible implications for fertility. Theriogenology 64, 275–291.
Gamboa, S., Machado-Faria, M., Ramalho-Santos, J., 2009. Seminal traits, suitability for semen preservation and fertility in the native Portuguese horse breeds Puro Sangue Lusitano and Sorraia: implications for stallion classification and assisted reproduction. Anim. Reprod. Sci. 113, 102–113.
Gamboa, S., Rodrigues, A.S., Henriques, L., Batista, C., Ramalho-Santos, J., 2010. Seasonal functional relevance of sperm characteristics in equine spermatozoa. Theriogenology 73, 950–958.
Henkel, R.R., Schill, W.B., 2003. Sperm preparation for ART. Reprod. Biol. Endocrinol. 14, 108–129.
Johannisson, A., Morrell, J., Thorén, J., Jönsson, M., Dalin, A.M., Rodriguez-Martinez, H., 2009. Colloidal centrifugation with Androcoll-E prolongs stallion sperm motility, viability and chromatin integrity. Anim. Reprod. Sci. 116, 119–128.
Loomis, P.R., 2001. The equine frozen semen industry. Anim. Reprod. Sci. 68, 191–200.
Luís, C., Cothran, E., Oom, M., 2007. Inbreeding and genetic structure in the endangered Sorraia horse breed: implications for its conservation and management. J. Heredity 98, 232–237.
Macías-García, B., Morrell, J.M., Ortega-Ferrusola, C., González-Fernández, L., Tapia, J.A., Rodriguez-Martínez, H., Peña, F.J., 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim. Reprod. Sci. 114, 193–202.
Macías-García, B., González-Fernández, L., Gaallardoo-Bolaños, J. M., Peña, F.J., Johannisson, A., Morrel, J.M., 2012. Androcoll-E-large selects a subset of live stallion spermatozoa capable of producing ROS. Anim. Reprod. Sci. 132, 74–82.
Mari, G., Castagnetti, C., Rizzato, G., Mislei, B., Iacono, E., Merlo, B., 2011. Density gradient centrifugation of sperm from a subfertile stallion and effect of seminal plasma addition on fertility. Anim. Reprod. Sci. 126, 96–100.

Maroco J., 2007. Análise Estatística com a utilização do SPSS, 3ª ed., Silabo, Lisboa.

Morrell, J.M., Wallgren, M., 2011. Colloid centrifugation of boar semen. Reprod. Domest. Anim. 46, 18–22.

Morrell, J.M., Rodriguez-Martinez, H., Johannisson, A., 2010. Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. Equine Vet. J. 42, 579–585.

Morrell, J.M., Mari, G., Kútvölgyi, G., Meurling, S., Mislei, B., Iacono, E., Rodriguez-Martinez, H., 2011. Pregnancies following artificial insemination with spermatozoa from problem stallion ejaculates processed by single layer centrifugation with Androcoll-E. Reprod. Domest. Anim. 46, 642–645.

Nicolas, M., Alvarez, M., Borragán, S., Martinez-Pastor, F., Chamorro, C.A., Alvarez-Rodriguez, M., de Paz, P., Anel, L., 2012. Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine, and PureSperm 100) in preparation of fresh or frozen-thawed brown bear spermatozoa. Theriogenology 77, 1119–1128.

Oom, M.M., Costa-Ferreira, J., Cothran, E.G., 1991. Inbreeding, reproductive success and genetic variation in the Sorraia horse of Portugal. Anim. Genet. 11, 22–23.

Pinheiro, M., Kjöllerström, H., Oom, M., 2013. Genetic diversity and demographic structure of the endangered Sorraia horse breed assessed through pedigree analysis. Livestock Sci. 152, 1–10.

Ramu, S., Jeyendran, R.S., 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods Mol. Biol. 927, 21–25.

Reers, M., Smith, T.W., Chen, L.B., 1991. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 30 (18), 4480–4486.

Samardzija, M., Karadjole, M., Matkovic, M., Cergolj, M., Getz, I., Dobranic, T., Tomaskovic, A., Petric, J., Surina, J., Grizelj, J., Karadjole, T., 2006. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. Anim. Reprod. Sci. 91, 237–247.

Shekarriz, M., DeWire Jr, D.M., 1995. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. Eur. Urol. 28, 187–197, TA..

Zhu, W.J., Liu, X.G., 2000. Cryodamage to plasma membrane integrity in head and tail regions of the human sperm. Asian J. Androl. 2, 135–138.

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