# Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS

B. Macías-García[a,b,c,*], L. González-Fernández[b,c], J.M. Gallardo-Bolaños[a], F.J. Peña[a], A. Johannisson[d], J.M. Morrell[b]

[a] *Veterinary Teaching Hospital, Laboratory of Equine Reproduction, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain*
[b] *Division of Reproduction, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden*
[c] *Department of Veterinary Physiology and Pharmacology, TAMU 4466, College Station, TX 77843-4466, United States*
[d] *Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden*

*Keywords:*
Osmotic shock
Equine
Androcoll-E large
ROS production

A B S T R A C T

The aim of this study was to elucidate if SLC after 24 h storage selects the subpopulation of spermatozoa that better withstands osmotic shock. To test this hypothesis, viability, mitochondrial membrane potential (MMP) and superoxide anion ($O_2^{\cdot -}$) production of uncentrifuged (UC) and single layer centrifugation (SLC) – selected spermatozoa were analyzed following SLC after storage of the semen. An aliquot of the extended ejaculate ($100 \times 10^6$ spermatozoa/mL) was centrifuged through a single layer of a silane-coated silica based colloid formulation optimized for equine spermatozoa (Androcoll-E large, SLU, Sweden) and the rest was used as control. UC and SLC-sperm samples were subjected to osmotic challenges (75 and 900 mOsm) with a subsequent return to isosmolarity (300 mOsm) using Biggers–Whitten–Whittingham (BWW) medium. Viability and MMP decreased after the different osmotic stress in UC and SLC spermatozoa, and return to isosmolarity did not reverse these effects. $O_2^{\cdot -}$ production was enhanced after SLC in all osmolarities tested. Interestingly, the percentage of living spermatozoa showing $O_2^{\cdot -}$ production was increased after 900 mOsm stress in UC spermatozoa, this increase being more evident in SLC spermatozoa. Returning spermatozoa to 300 mOsm enhanced this percentage in UC viable cells but not in SLC spermatozoa. The scenario observed for UC spermatozoa shows that $O_2^{\cdot -}$ is produced in response to isolated hyperosmolarities and subsequent osmotic excursions. As the viability, MMP and cell volume remained the same between SLC and UC spermatozoa, we conclude that Androcoll-E large is likely selecting a higher percentage of physiologically $O_2^{\cdot -}$ producing spermatozoa.



*Abbreviations:* SLC, single layer centrifugation; SL, single layer; UC, uncentrifuged; BWW, Biggers–Whitten–Whittingham; ROS, reactive oxygen species; mOsm, milliosmolar; MMP, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine; PI, propidium iodide; AI, artificial insemination; H33258, Hoechst 33258; HE, hydroethidine; DCFDA, dichlorodihydrofluorescein diacetate; $O_2^{\cdot -}$ +, viable spermatozoa producing superoxide anion; $O_2^{\cdot -}$ −, viable non superoxide anion producing spermatozoa.

## 1. Introduction

Artificial insemination (AI) is a widely used technique in equine reproduction that has the advantage, among others, of allowing a better distribution of ejaculates. The doses are usually supplied cooled or frozen, and are used successfully in field conditions by many veterinarians. However, one of the problems associated with cryopreservation of equine spermatozoa is that the technique is not available on all breeding farms. Furthermore, not all breeding farms satisfy European regulations and are therefore not allowed to distribute frozen semen (92/65/EWG). For these reasons, stallions must be sent to specified facilities where this freezing service is offered, which is inconvenient for both the owner and the stallion. A good solution would be to send the cooled ejaculates overnight to a more specialized facility and freeze them there, this choice being less expensive for the owner and less stressful for the stallions.

This approach was attempted before by Backman et al., who stored spermatozoa at 5 °C for 18 h before freezing; after such storage, spermatozoa showed a decrease in the percentage of total and progressive motility after thawing, although fertility remained the same (Backman et al., 2004). The decrease in motility parameters is not surprising due to the damage that the cooling process exerts on spermatozoa (Aurich, 2005). There are many different procedures aimed at increasing equine spermatozoa quality after cooling that could be attempted before freezing spermatozoa. These maneuvers include different centrifugation protocols (Hoogewijs et al., 2010), glass–wool filtration (Sieme et al., 2003) or multiple gradient centrifugation such as Percoll (Trein et al., 2005).

In the present work, single layer large centrifugation (SLC) of extended stallion spermatozoa through Androcoll-E (SLU, Sweden) was used. Androcoll-E large is a silane-coated silica colloid formulation optimized for stallion spermatozoa, allowing the centrifugation of up to 18 mL of extended ejaculates on 15 mL of a single layer of colloid in a 50 mL tube (Morrell et al., 2009). Androcoll-E has previously been shown to select high quality spermatozoa in frozen (Macias-Garcia et al., 2009) and fresh equine spermatozoa (Johannisson et al., 2009; Morrell et al., 2008, 2010), increasing the pregnancy rate in subfertile stallions (Morrell et al., 2011b). Single layer centrifugation (SLC) with Androcoll-E, performed after storage of the cooled semen for 24 h, has also been shown to select motile spermatozoa with good chromatin integrity (Morrell et al., 2011a). Furthermore, a recent study has proven that centrifugation of equine ejaculates using Androcoll-E prior to freezing, improves the post-thaw quality of extended equine spermatozoa (Hoogewijs et al., 2011). These results open the possibility of shipping cooled sperm, processing it using SLC through Androcoll-E on arrival at the freezing facility, and subsequently freezing the SLC-samples.

During the freezing process, spermatozoa are subjected to different conditions that lead to damage such as cold shock, increase in ROS production, and osmotic shock (Burnaugh et al., 2010; Cole and Meyers, 2011; Macias et al., 2011). Among these sources of damage, osmotic shock is claimed to be the most damaging one by far, inducing oxidative damage and apoptosis in spermatozoa (Ball, 2008; Burnaugh et al., 2010; McCarthy et al., 2010). Therefore, in the present study, the aim was to elucidate if SLC with Androcoll-E of cooled stallion spermatozoa after 24 h storage at 5 °C, selects the subpopulation of spermatozoa that better withstands osmotic shock. For this purpose, spermatozoa were subjected to different anisosmolar shocks and then returned to isosmolarity in SL and non-SL centrifuged samples; viability, mitochondrial membrane potential, spermatozoa volume and ROS production were then evaluated.

## 2. Materials and methods

### 2.1. Animals and husbandry

#### 2.1.1. Osmotic shock

Semen (4 ejaculates per stallion) was collected from 3 fertile warmblood stallions 4–15 years old at two commercial studs in Sweden (Västerbo Stuteri, Heby; and Gurresta Stuteri, Vassunda). Semen was collected regularly from the stallions (up to three times per week) throughout the breeding season according to standard practice on the commercial stud. The stallions mounted a phantom and ejaculated into a Colorado model artificial vagina. Gel was removed with an in-line filter. Sperm concentration was measured immediately after ejaculation using a Spermacue photometer (Minitüb, Tiefenbach, Germany), and warm semen extender (INRA96®; IMV, l'Aigle, France) was added to provide a 20 mL final volume reaching a final concentration of $100 \times 10^6$ spermatozoa/mL.

#### 2.1.2. Cellular volume measurement

9 ejaculates (three ejaculates per stallion) were obtained from 3 fertile Andalusian stallions individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations. Ejaculates were collected using a Missouri model artificial vagina with an inline filter to separate the gel fraction, lubricated and pre-warmed to 45–50 °C. The collected ejaculate was immediately transported to the laboratory for evaluation and processing. All the collected ejaculates used in this work were evaluated using a CASA system (ISAS® Proiser, Valencia, Spain) and showed at least 85% of total motile sperm.

### 2.2. Experimental treatments

#### 2.2.1. Androcoll-E large centrifugation

Each ejaculate was split into 2 aliquots. The first aliquot was used as control (2 mL of $100 \times 10^6$ spermatozoa/mL) and was not centrifuged. The remaining 18 mL of extended spermatozoa ($100 \times 10^6$ spermatozoa/mL) were layered on top of Androcoll-E-large (15 mL) in 50 mL Falcon tubes and were centrifuged at $300 \times g$ for 20 min. After aspiration of the supernatant (semen extender, seminal plasma and colloid), the spermatozoa pellet was recovered and transferred to a clean tube containing INRA96® at room temperature. Spermatozoa concentration was measured using a Nucleocounter (Chemometic, Allerød, Denmark), and was then adjusted to $100 \times 10^6$/mL.

*2.2.2. Osmotic shock*

Once processed, the SLC and uncentrifuged (UC) samples were subjected to different anisosmotic shocks. Anisosmolar solutions of 75 mOsm and 900 mOsm and the isosmolar 300 mOsm solutions (control) Biggers–Whitten–Whittingham (BWW) were prepared (91.06 mM NaCl, 4.78 mM KCL, 2.44 mM $MgSO_4$, 1.17 mM $KPO_4$, 21.0 mm HEPES, 5.5 mM glucose (anhydrous), 0.25 mM sodium pyruvate, 1.71 mM lactic acid hemicalcium salt, and 21.55 mM sodium lactate) at pH 7.4 as previously described (Macias et al., 2011, 2012). For each ejaculate, 500 μL of SLC (Androcoll-E large centrifuged samples) and UC (control) spermatozoa at $100 \times 10^6$ spermatozoa/mL were placed in 6 tubes (3 tubes per treatment), the tubes were centrifuged at $800 \times g$ for 5 min at room temperature (23 °C). The supernatant was discarded and the pellets were resuspended in 500 μL of 75 mOsm, 300 mOsm and 900 mOsm of pre-warmed (37 °C) BWW medium for the SLC and UC samples. The tubes were placed in an air incubator for 10 min, after which, 100 μL aliquots were used for the different assays. The remainder of each sample was returned to isosmotic conditions as follows: after centrifugation at $800 \times g$ for 5 min at room temperature, the supernatant was discarded, the sperm pellet was resuspended in 400 μL of 300 mOsm BWW and the resulting suspension was incubated at 37 °C for 10 min. The same assays were repeated. All osmolarities were checked using a freeze point digital micro-osmometer Type 5 R (Hermann Roebling Messtechnik, Berlin, Germany).

*2.2.3. Cellular volume measurement*

Cellular volume was measured using a Multisizer$^3$ Coulter Counter (Coulter Corporation Inc., Miami, FL, USA). Spermatozoa were suspended in the corresponding BWW medium and drawn through a cylindrical opening (2–60 μm) separating two electrodes between which a small electrical current flows. As each particle passes through the aperture, it displaces its own volume of conducting liquid, momentarily increasing the impedance of the aperture. This change produces a pulse that is digitally processed in real time and is proportional to the tridimensional volume of the particle that produced it. The analysis of the pulses was digitally converted to volume ($\mu m^3$). For each sample, 30,000 events were ana- lyzed and particles below 6 and above 60 $\mu m^3$ were discarded.

*2.2.4. Spermatozoa viability*

A combination of the fluorochromes SYBR-14 and propidium iodide (PI) (Live-Dead® Sperm Viability Kit; Invitrogen, Eugene, OR, USA) was used. After processing, $3 \times 10^6$ spermatozoa were placed in 270 μL Cellwash (Becton Dickinson). To this suspension, 1.5 μL of SYBR-14 stock solution (1:50 in Cellwash) and 1.5 μL of PI were added before incubating the mixture for 10 min at 37 °C. Flow cytometry analysis was performed using an argon-ion laser FACStar Plus flow cytometer (Becton Dickinson, San José, CA, USA), equipped with standard optics. From each sample a total of 10,000 events were collected and quantified as percentages. Three categories of spermatozoa could be described: live spermatozoa with an intact membrane emitting green fluorescence (SYBR-14+/PI–), moribund showing green and red fluorescence (SYBR-14+/PI+) and dead emitting red fluorescence (SYBR-14-/PI+), according to the degree of intactness of the plasma membrane. When excited using an Argon Laser (488 nm), SYBR-14 emitted at 519 nm (detected through FL1 fotodetector) and PI at 617 nm (detected through FL3 fotodetector).

*2.2.5. Evaluation of mitochondrial membrane potential (MMP)*

MMP was evaluated using the lipophilic cationic compound 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine (JC-1) (Molecular Probes Europe BV, Leiden, The Netherlands). This dye has the ability to differentially label mitochondria with low and high membrane potential. If MMP is high, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm (being the emission detected through FL3 fotodetector), when excited at 488 nm. At the same excitement wavelength (488 nm), JC-1 forms monomers emitting in the green wavelength (525–530 nm; FL1 detector) when labeling mitochondria with low membrane potential. For staining, a 3 mM stock solution of JC-1 was prepared in DMSO. From each spermatozoa suspension, $5 \times 10^6$ spermatozoa were placed in 1 mL of Cellwash (Becton Dickinson) and stained with 0.5 μL of JC-1 stock solution. The samples were incubated at 38 °C in the dark for 40 min before flow cytometric analysis. For each sample, a total of 30,000 events were evaluated.

*2.2.6. ROS staining*

From each sperm suspension, $3 \times 10^6$ spermatozoa were placed in 240 μL Cellwash (Becton Dickinson) and stained with 9 μL of Hoechst 33258 (H33258) (stock solution concentration 40 μM), 9 μL of Hydroethidine (HE) (stock solution concentration 40 μM) for detection of superoxide anion ($O_2^{\bullet -}$) and 9 μL of dichlorodihydrofluorescein diacetate (DCFDA) (stock solution concentration 2 mM) for detection of $H_2O_2$. The H33258 was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA), and HE and DCFDA from Molecular Probes (Eugene, OR, USA). DCFDA and HE when excited at 488 nm emit fluorescence at 530 nm (detected through FL1 fotodetector) and 610 nm respectively (detected through FL3 fotodetector). H33258 was excited using an UV laser (350 nm) and emitted at 461 nm (detected through FL4 fotodetector). All stock stain solutions were diluted in DMSO.

The samples were incubated at 38 °C for 30 min before analyzing and a total of 100,000 events were assessed. The method is a modification of one previously described (Guthrie and Welch, 2006), the modification being the use of Hoechst 33258 as an independent analysis of dead spermatozoa based on previous reports (de Leeuw et al., 1991; Fraser et al., 2001).

*2.2.7. Confocal microscopy*

Spermatozoa were subjected to the different osmotic shocks and loaded with DCFDA, H33258 and HE using the same protocol previously described for ROS detection. A small volume of the cell suspension (10 μL at

100 $\times 10^6$ spermatozoa/mL) was placed on microscope slides, gently mixed with 10 μL of anti-fade solution and a slide cover was gently placed on the drop. Confocal laser scanning microscopy was performed on a Zeiss LSM 510 system (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). A Zeiss Plan-pochromat 63× (NA1.4) oil immersion objective was used for focusing the excitation beam and collecting signal. Cells were sequentially excited with a 488 nm argon laser. Emission from DCFDA and HE was collected with confocal acquisition via a 505–550 nm band-pass filter and a 560–615 nm band-pass filter, respectively. The H33258 signal was obtained through UV excitation with a mercury lamp (HBO 103, Carl Zeiss, Jena, Germany) and emission was collected with half confocal acquisition (pinhole size of 1.5 airy unit). All acquisitions were performed sequentially in order to prevent potential bleed-through. All images were processed using ZEN 2008 Light Edition (Carl Zeiss, Jena, Germany) software.

### 2.3. Statistical analysis

For the flow cytometry experiments, 4 different ejaculates from 3 different stallions were used ($n$ = 12); in the cellular volume experiments 3 ejaculates from 3 different stallions were tested ($n$ = 9). All the ejaculates were subjected to 2 treatments (control vs. SLC) and 3 different osmolarities (75, 300 and 900 mOsm), and were returned afterwards to a 300 mOsm solution. The data were firstly examined using the Kolmogorov–Smirnov test to determine their distribution. In view of the non-Gaussian distribution of most of the data gathered, multivariate analysis of variance was performed, and when significant differences were found, the non-parametric Mann–Whitney $U$-test was used to directly compare pairs of values. Analyses were performed using SPSS ver. 15.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Viability assay

UC and SLC spermatozoa did not show significant differences between homologous treatments for viability ($p > 0.05$) i.e. the centrifugation was neither beneficial nor detrimental to sperm membrane integrity (Table 1). Spermatozoa subjected to all anisosmolar conditions showed decreased viability for UC and SLC treatments. For uncentrifuged spermatozoa viability was decreased by 47.05% for the 75 mOsm of BWW medium and 71.6% for the 900 mOsm of BWW medium, compared to UC 300 mOsm. Sperm samples processed by SLC using Androcoll-E showed a decrease in viability of 25.3% for the 75 mOsm and 63.99% for the 900 mOsm solutions when compared to 300 mOsm SLC spermatozoa control. After returning spermatozoa to isosmolar conditions (300 mOsm), the observed decrease in viability was shown to be irreversible (Table 1).

**Table 1**
Equine sperm plasma membrane integrity assessed by SYBR-14 and propidium iodide (PI).

| | SYBR living | SYBR dying | SYBR dead |
|---|---|---|---|
| 75 UC | 28.7 ± 3.5** | 1.3 ± 0.3 | 66.9 ± 3.9** |
| 300 UC | 54.2 ± 2.8 | 1.9 ± 0.3 | 42 ± 2.9 |
| 900 UC | 15.4 ± 3.9** | 1.4 ± 0.3 | 80 ± 4.1** |
| 75–300 UC | 31 ± 4.1** | 0.7 ± 0.7* | 67 ± 4** |
| 300–300 UC | 50 ± 3 | 1.5 ± 0.2 | 47.2 ± 3 |
| 900–300 UC | 18.6 ± 2.5** | 1.5 ± 0.3 | 78.2 ± 2.3** |
| 75 SLC | 36.3 ± 4* | 1.7 ± 0.6 | 60.6 ± 4.4* |
| 300 SLC | 48.6 ± 4.6 | 2.7 ± 0.7 | 47.3 ± 4.7 |
| 900 SLC | 17.5 ± 2.7** | 1.7 ± 0.6 | 80 ± 2.8** |
| 75–300 SLC | 38 ± 4.4* | 1.8 ± 0.5 | 59 ± 4.5* |
| 300–300 SLC | 50 ± 4.7 ** | 2 ± 0.5 | 47 4.7 ** |
| 900–300 SLC | 25.2 ± 3.5 | 1.6 ± 0.5 | 72.1 ± 3.7 |

Uncentrifuged (UC) and single layer centrifuged (SLC) spermatozoa (4 different ejaculates from 3 stallions) using Androcoll-E, were exposed to different anisosmolar solutions and returned to isosmolar conditions (mean ± standard error of the mean; $n$ = 12).
Values bearing * or ** within a column differ statistically from the 300 mOsm uncentrifuged control (300 UC). No statistically significant differences were found between SLC and UC paired treatments or after returning sperm to isosmolar conditions.
* $p < 0.05$.
** $p < 0.001$.

### 3.2. Mitochondrial membrane potential (MMP)

Androcoll-E large centrifugation did not affect mitochondrial function, since UC and SLC samples homologous treatments did not show statistically significant differences ($p > 0.05$). On the other hand, MMP dramatically decreased for both hyperosmolar and hyposmolar osmotic shocks, (Table 2). The percentage of spermatozoa showing high MMP was decreased after hypo- and hyperosmotic shock for UC and SLC spermatozoa. Returning spermatozoa to isosmolar conditions increased the percentage of the spermatozoa subpopulation depicting high and low MMP in UC spermatozoa for 75 mOsm and 900 mOsm of BWW medium. For SLC spermatozoa this percentage increased in the 900 mOsm solution (13.7% ±3.7 UC vs. 33.7% 5.5 SLC) but not for the 75 mOsm (34.4% ±5.5 UC vs. 45% 5.5 SLC) (Table 2).

### 3.3. Reactive oxygen species (ROS) production

In the flow cytometry assays, only the fluorescence from HE and H33258 probes were taken into account, since DCFDA fluorescence did not show appreciable changes. Therefore, the combination of the 3 probes was also examined in a different experiment using confocal laser microscopy (Section 3.4). The percentage of viable (H33258-excluding) $O_2^{\bullet -}$ producing spermatozoa ($O_2^{\bullet -}$+ was calculated, and for this purpose, the percentage of live $O_2^{\bullet -}$+ spermatozoa was divided by the sum of percentages of live $O_2^{\bullet}$+ and live $O_2^{\bullet -}$ – (non $O_2^{\bullet -}$ producing) spermatozoa (Fig. 1). This proportion clearly showed that a higher percentage of cells produced superoxide anion radical in the SLC spermatozoa sample compared to the UC control (42.5 ±4 vs. 21.9 ±5 respectively; Mean SEM). Moreover, in all the different osmolarities tested, a higher percentage of live spermatozoa producing $O_2^{\bullet -}$ was detected for the SLC vs. UC treatments (Fig. 1). When returning

**Table 2**
Horse spermatozoa mitochondrial membrane potential (MMP) assessed using JC-1.

| | High MMP | High/low MMP | Low MMP |
|---|---|---|---|
| 75 UC | 5.32 ± 1.5* | 26.22 ± 3.6**,o | 65 ± 3.6**,o |
| 300 UC | 13.73 ± 2 | 39.6 ± 3 | 42.7 ± 2.7 |
| 900 UC | 0.7 ± 0.2** | 10.2 ± 2.2**,o | 85.7 ± 2.5**,o |
| 75–300 UC | 7.5 ± 4.1* | 41.6 ± 5.5 | 47.5 ± 6.3 |
| 300–300 UC | 14 ± 3.5 | 44.45 ± 3.5 | 37.8 ± 3.6 |
| 900–300 UC | 5.5 ± 2* | 23.9 ± 5.5** | 67.9 ± 5.8** |
| 75 SLC | 2.7 ± 0.7* | 34.4 ± 5.5* | 59.8 ± 6* |
| 300 SLC | 15.8 ± 2.4 | 35 ± 3 | 46 ± 4 |
| 900 SLC | 1.6 ± 0.4** | 13.7 ± 3.7**,o | 83 ± 4.2**,o |
| 75–300 SLC | 2.8 ± 0.6* | 45 ± 5.5 | 50.3 ± 6 |
| 300–300 SLC | 9 ± 2.2 | 40.3 ± 2.5 | 48 ± 2.8 |
| 900–300 SLC | 3.2 ± 1.9* | 33.7 ± 5.5* | 63 ± 5.8* |

Uncentrifuged (UC) and single layer centrifuged (SLC) spermatozoa using Androcol-El (4 ejaculates from 3 different stallions), were exposed to different anisosmolar solutions and returned to isosmolar conditions (mean ± standard error of the mean; $n = 12$).
High MMP: % of spermatozoa with all their mitochondria showing high membrane potential.
High/low MMP: percentage of spermatozoa depicting simultaneously mitochondria with low and high potential. Low MMP: percentage of spermatozoa depicting low potential.
Values bearing * or ** within a column differ statistically from the 300 mOsm uncentrifuged control (300 UC). No statistically significant differences were found between SLC and UC homologous treatments. Statistically significant differences were found when returning sperm to isosmolar conditions, being the control 300 UC.
° $p < 0.05$ when returning to isosmolarity.
* $p < 0.05$.
** $p < 0.001$.

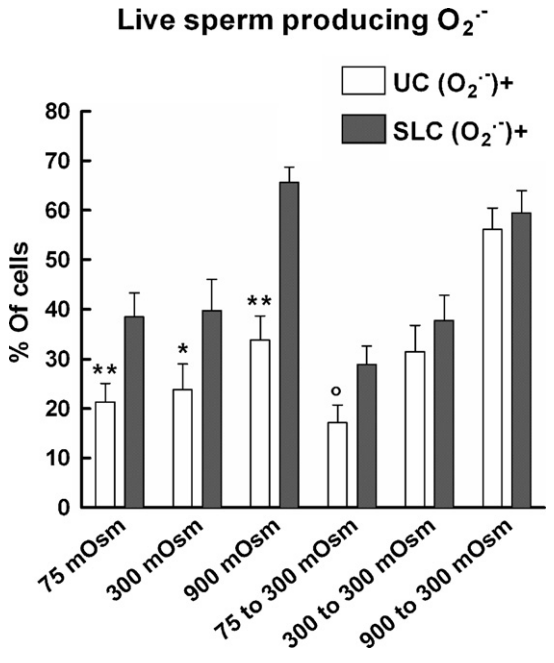


**Fig. 1.** Percentage of viable $O_2^{\cdot-}$ spermatozoa in UC and SLC sperm (mean; error bar represents standard error of the mean; $n = 12$). Values bearing * differ statistically from the SLC homologous treatment. *$p < 0.05$. **$p < 0.001$. °$p < 0.05$ when compared to 300 SLC.

spermatozoa to 300 mOsm, the differences between homologous UC and SLC osmotic treatments were not statistically different. The percentage of live $O_2^{\cdot-}$ producing spermatozoa was enhanced in both UC and SLC spermatozoa in the 900 to 300 mOsm treatment compared to 300 UC and 300 SLC controls respectively. Regarding the 75 to 300 mOsm treatment, only the UC spermatozoa showed significantly less $O_2^{\cdot-}$ production than the 300 mOsm SLC treated spermatozoa. In the hypotonic condition (75 mOsm), a lower percentage of spermatozoa showed $O_2^{\cdot-}$ production, and returning them to 300 mOsm did not exert an increase in $O_2^{\cdot-}$ production (Fig. 1).

### 3.4. Confocal laser microscopy

Sperm cells analyzed by flow cytometry were studied by confocal microscopy (Fig. 2). The results showed that hydrogen peroxide was localized in the midpiece in most spermatozoa (Fig. 2, panels $B_{1-3}$), although some spermatozoa showed green fluorescence in the acrosome region too (Fig. 2, panels $B_2$ and $B_3$). A different intensity of fluorescence was found in the acrosomal region: some spermatozoa showed intense fluorescence (Fig. 2, panel $B_3$) whereas in some others the acrosome showed little fluorescence (Fig. 2, panel $B_2$). The superoxide radical was distributed in the head with different red fluorescence intensity (Fig. 2, panels $C_{1-3}$). The non-viable spermatozoa showed blue fluorescence in the nucleus (Fig. 2, panel $A_1$ and $A_3$) whereas the viable spermatozoa showed no blue fluorescence (Fig. 2, panel $A_2$). In some spermatozoa the pattern was different, with a narrow band of fluorescence appearing in the region between the head and tail (Fig. 2, panel $A_2$). This weak fluorescence pattern was not considered a sign of non-viability (de Leeuw et al., 1991).

### 3.5. Cell volume measurement

Although all the anisosmolarities studied yielded statistically different changes in cellular volume when compared to their controls ($p < 0.05$), no differences were found between the UC and SLC homologous treatments ($p > 0.05$) (Fig. 3). When returned to 300 mOsm, all spermatozoa recovered their original volume.

## 4. Discussion

The aim of the study was to elucidate if SLC with Androcoll-E of cooled stallion spermatozoa after 24 h storage at 5 °C selects the subpopulation of spermatozoa that better withstands osmotic shock, by evaluating viability, mitochondrial membrane potential, spermatozoa volume and ROS production in the treated sperm samples. No statistically significant differences were found in sperm viability between treatments comparing the paired SLC and UC samples at all the osmolarities and osmolarity challenges tested. Although previous studies have reported an increase in the percentage of viable spermatozoa using Androcoll-E of fresh semen (Morrell et al., 2010), this was not found in the current study where stored semen was used for SLC.

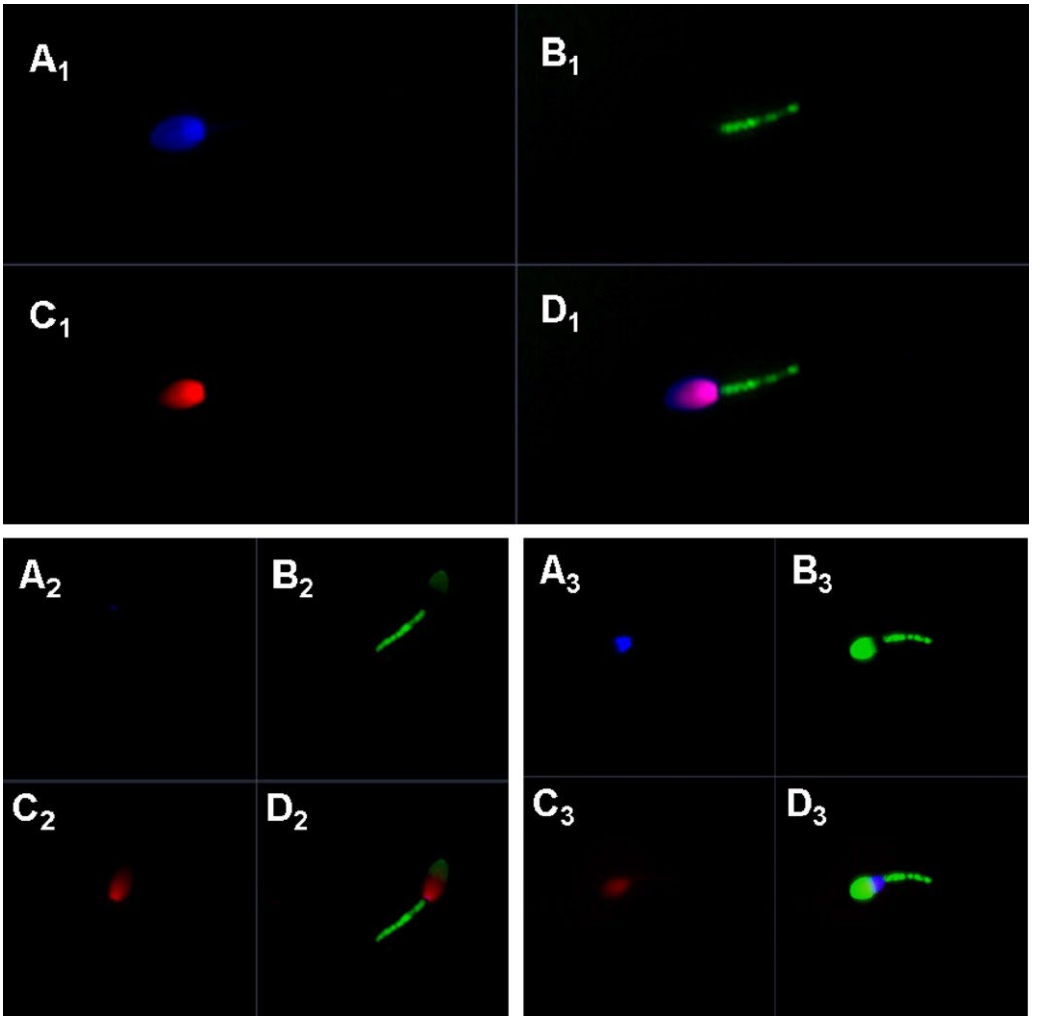


**Fig. 2.** Confocal laser microscopy images of stallion sperm loaded with Hoechst 33258, DCFDA and HE. (A) Blue fluorescence associated with dead sperm, (B) green fluorescence associated with spermatozoa producing significant amounts of $H_2O_2$, (C) red fluorescence associated with $O_2^{\bullet -}$ production (D) overlaid images showing the subcelullar distribution of all probes. Top: dead spermatozoa showing $O_2^{\bullet -}$ production in the head and $H_2O_2$ production in the midpiece. Bottom left: live spermatozoa showing red fluorescence in the head and green fluorescence in the midpiece and acrosome. Bottom right: dead spermatozoa showing red fluorescence in the head and green fluorescence in the midpiece and acrosome. Original magnification, 63×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Concerning the effects of anisosmolarity on sperm membrane status, previous results have shown that the sperm plasmalemma is very susceptible to osmotic shock damage (Ball and Vo, 2001; Gonzalez-Fernandez et al., in press), and that stallion spermatozoa plasma membrane is more sensitive to osmotic excursions than to a given osmolarity (Macias et al., 2011). However, in our results we found that the change in osmolarity itself is enough to exert irreversible damage on spermatozoa plasma membrane since the return to isosmolarity did not have a beneficial effect. These differences may be explained because in the present work SYBR-14/PI was used to evaluate membrane integrity, while in the previously mentioned works, viability was measured using different staining protocols (Yo-Pro/ethidium homodimer) (Macias et al., 2012) and analytical methods (flow cytometry vs. epifluorescence microscopy) (Macias et al., 2011; Yeste et al., 2010). This result suggest that possibly the permeability of the probes differs depending on the osmolarity of the media used, producing apparent differing results as shown for isolated membranes (Madhavarao et al., 2001).

Mitochondrial membrane potential was also clearly affected by osmolarity changes, with no differences being found between SLC and UC spermatozoa. These organelles seem to be more sensitive to osmotic shock than plasma membrane as previously shown for Rhesus monkey and horse spermatozoa (Correa et al., 2007; Macias et al., 2011; Pommer et al., 2002; Rutllant et al., 2003). When spermatozoa were returned to the isosmolar conditions, a decrease in the percentage of low MMP and an increase of the subpopulation of spermatozoa showing high and low MMP were observed. Once again, this change could indicate that the permeability of the probe is affected by the osmotic strength of the solution, rather than a real improvement of

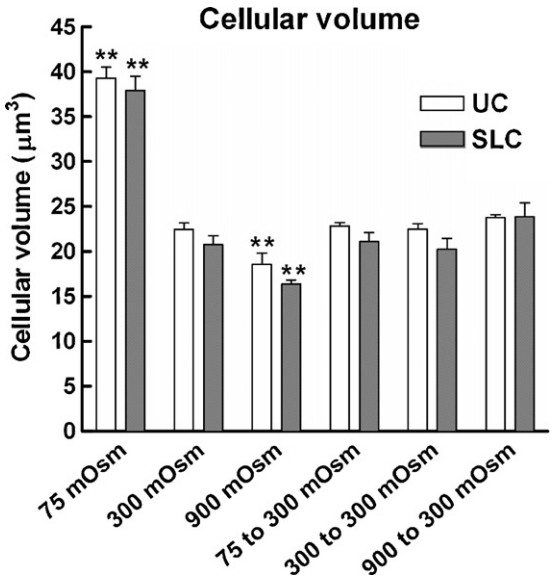


**Fig. 3.** Cellular volume ($\mu m^3$) of spermatozoa subjected to different osmolarities and returned to 300 mOsm (mean; error bar represents standard error of the mean; $n = 9$). Cellular volume ($\mu m^3$) of spermatozoa did not show any statistically significant difference between UC and SLC homologous treatments. Values bearing * differ statistically from the 300 mOsm control UC or SLC. Each sample was compared to its own control. $^{**}p < 0.001$.

the mitochondrial membrane condition, as permeability of JC-1 is affected by mitochondrial volume and morphology (Perry et al., 2011).

The most interesting finding in this study is that SLC of equine spermatozoa using Androcoll-E large selects a spermatozoa subpopulation that clearly responds differently to osmotic shock, in terms of superoxide anion production, than UC spermatozoa. Other preparation methods, such as Percoll gradients, have been shown to select boar spermatozoa with higher basal ROS production (Matas et al., 2010). A similar result has been reported in equine spermatozoa (Baumber and Ball, 2005) and has been attributed to the fact that Percoll centrifugation removes the seminal plasma contained in the ejaculate. Seminal plasma is the major source of antioxidant enzymes for mammalian spermatozoa, keeping ROS production under a controlled threshold (Ws et al., 2006). A controlled ROS production is required in the fertilization process, being necessary for spermatozoa to acquire hyperactivated motility and to undergo capacitation and the acrosome reaction (Baumber et al., 2003a,b; Griveau and Le, 1997). Specifically, a positive correlation between protein oxidation, motility and viability has been found in spermatozoa from fertile stallions during the breeding season (Morte et al., 2008). On the other hand, increased ROS production, or excessive ROS amounts in the external milieu, have been shown to be detrimental to spermatozoa, increasing DNA fragmentation (Baumber et al., 2002, 2003a,b), impairing motility (Baumber et al., 2000) and decreasing fertility (Ball et al., 2001). Therefore spermatozoa must maintain a balance between the oxidative and anti-oxidative processes: the threshold between pathologic and physiologic ROS production has not been clearly established yet.

In our study the percentage of living spermatozoa producing $O_2^{\cdot -}$ measured by flow cytometry was increased after Androcoll-E large centrifugation as previously described for Percoll gradients (Baumber and Ball, 2005). This finding could be, in part, related to the ability of Androcoll-E to remove the seminal plasma as previously described for Percoll gradient (Baumber and Ball, 2005). Another plausible explanation is that Androcoll-E is also selecting a subset of live sperm that are more metabolically active and thus more capable of producing physiological ROS levels. This assumption is based on previous reports showing that Androcoll-E selected spermatozoa exhibit better motility parameters and less DNA fragmentation (Morrell et al., 2011a), and increased spermatozoa fertilizing ability (Morrell et al., 2011b) than uncentrifuged sperm samples. Furthermore, our study shows an increase in the percentage of live sperm producing $O_2^{\cdot -}$ in centrifuged spermatozoa using Androcoll-E large after the osmotic challenges compared to the uncentrifuged controls (Fig. 1). The $O_2^{\cdot -}$ production was significantly enhanced by hyperosmolar conditions as previously reported for Rhesus Macaque and horse spermatozoa (Burnaugh et al., 2010; McCarthy et al., 2010). Our results confirm a link between osmotic shock and ROS production, previously reported in other cellular types (Lambert, 2003; Lambert et al., 2006) and in spermatozoa (Burnaugh et al., 2010; McCarthy et al., 2010).

When spermatozoa were returned to 300 mOsm BWW after incubation in the 900 mOsm solution, an increase in the HE fluorescence was observed for the UC spermatozoa but not for SLC ones. This result indicates that $O_2^{\cdot -}$ is produced during osmotic stress and volume excursions responses in equine spermatozoa. The fact that the SLC spermatozoa subjected to 900 mOsm do not experience a similar increase, suggests that Androcoll-E selects a subset of live spermatozoa that cope with osmotic excursions differently. Interestingly, Hoogewijs et al. (2011) found that centrifugation of equine ejaculates using Androcoll-E large prior cryopreservation allows the recovery of spermatozoa with superior motility and viability parameters, and less DNA fragmentation compared to cushion centrifugation. The fact that frozen-thawed spermatozoa quality is improved after Androcoll-E large centrifugation indicates that the observed increase in $O_2^{\cdot -}$ after osmotic challenges is not adversely affecting spermatozoa quality over that of regularly processed semen. Moreover, this increase in endogenous $O_2^{\cdot -}$ after SLC appeared not to be deleterious for the spermatozoa as there were no statistically significant differences between the SLC and control spermatozoa in terms of membrane integrity or mitochondrial status ($p > 0.05$). Interestingly, spermatozoa volume remained the same for UC and SLC spermatozoa in homologous treatments. This result shows that the difference in the osmotic shock response for Androcoll-E selected spermatozoa is not related to cellular volume changes, but rather to biochemical differences.

In an attempt to localize where $H_2O_2$ and $O_2^{\cdot -}$ were produced, confocal microscopy was used. In these experiments we observed that, as expected, the superoxide

anion radical was localized in the head of the spermatozoa. Hydroethidine, when oxidized by superoxide radical, is transformed to ethidium, which has a high binding affinity to DNA (Gomes et al., 2005). Due the similarity existing between the different ROS and the rapid degradation of these molecules, we cannot rule out the possibility that the probes used in this study can be oxidized by other ROS such as $HO^{\bullet}$ and $ROO^{\bullet}$, and also by reactive nitrogen species (RNS) such as $^{\bullet}NO$ and $ONOO^{-}$ (Gomes et al., 2005). Hydrogen peroxide associated fluorescence was seen in the midpiece, suggesting that hydrogen peroxide is produced in the mitochondria as previously reported in human spermatozoa (Koppers et al., 2010) and, in some spermatozoa, DCFDA fluorescence was also detected in the acrosomal region, possibly due to the ability of the acrosome to store ROS (Fatimah et al., 2010), or due to their involvement in the acrosome reaction.

## 5. Conclusions

Although strong conclusions cannot be extracted about the possible physiological or pathological role of ROS production in equine spermatozoa during osmotic shock, the present study highlights the fact that ROS are produced in response to osmotic shock and to changes in osmolarity in live sperm. Androcoll-E large selects a subset of live sperm capable of producing $O_2^{\bullet -}$ in isosmolar conditions and after a hyperosmotic shock. Further studies need to be performed to establish the limit between physiology and pathology in regard to ROS production.


## Funding

Supported by Ministerio de Ciencia e Innovación Madrid, Spain. Project: AGL 2007-60598 [GAN], Junta de Extremadura-FEDER (GR 10010 and PCE1002) and the Swedish Foundation for Equine Research (Stiftelsen Svensk Hästforskning), Stockholm (project number H1047052). The first author holds a research grant from the Ministerio de Ciencia e Innovación Madrid, Spain (grant: BES-2008-001880).

## Acknowledgments

The collaboration of Västerbo and Gurresta Studs is acknowledged. The authors also thank the collaboration of Equine Breeding Service of the Spanish Army.


## References


Aurich, C., 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim. Reprod. Sci. 89, 65–75.

Backman, T., Bruemmer, J.E., Graham, J.K., Squires, E.L., 2004. Pregnancy rates of mares inseminated with semen cooled for 18 h and then frozen. J. Anim. Sci. 82, 690–694.

Ball, B.A., Vo, A., 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22, 1061–1069.

Ball, B.A., Vo, A.T., Baumber, J., 2001. Generation of reactive oxygen species by equine spermatozoa. Am. J. Vet. Res. 62, 508–515.

Ball, B.A., 2008. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim. Reprod. Sci. 107, 257–267.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J. Androl. 21, 895–902.

Baumber, J., Vo, A., Sabeur, K., Ball, B.A., 2002. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. Theriogenology 57, 1025–1033.

Baumber, J., Ball, B.A., Linfor, J.J., Meyers, S.A., 2003a. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24, 621–628.

Baumber, J., Sabeur, K., Vo, A., Ball, B.A., 2003b. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology 60, 1239–1247.

Baumber, J., Ball, B.A., 2005. Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. Am. J. Vet. Res. 66, 1415–1419.

Burnaugh, L., Ball, B.A., Sabeur, K., Thomas, A.D., Meyers, S.A., 2010. Osmotic stress stimulates generation of superoxide anion by spermatozoa in horses. Anim. Reprod. Sci. 117, 249–260.

Cole, J.A., Meyers, S.A., 2011. Osmotic stress stimulates phosphorylation and cellular expression of heat shock proteins in rhesus macaque sperm. J. Androl. 32, 402–410.

Correa, L.M., Thomas, A., Meyers, S.A., 2007. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. Biol. Reprod. 77, 942–953.

de Leeuw, A.M., Den Daas, J.H., Woelders, H., 1991. The fix vital stain method. Simultaneous determination of viability and acrosomal status of bovine spermatozoa. J. Androl. 12, 112–118.

Fatimah, I.S., Iswadi, I.M., Khairul, O., Nurhazilah, M., Fadzilah, M.S., Padzil, A.R., Srijit, D., Mazni, O.A., 2010. A preliminary study of reactive oxygen species activity within motile Boer buck sperm using confocal laser scanning microscope (CLSM). Clin. Ter. 161, 125–128.

Fraser, L., Gorszczaruk, K., Strzezek, J., 2001. Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. Reprod. Domest. Anim. 36, 325–329.

Gomes, A., Fernandes, E., Lima, J.L., 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45–80.

Gonzalez-Fernandez, L., Morrell, J.M., Peña, J.M., Macias-Garcia, B., in press. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology, http://dx.doi.org/10.1016/j.theriogenology.2012.02.021.

Griveau, J.F., Le, L.D., 1997. Reactive oxygen species and human spermatozoa: physiology and pathology. Int. J. Androl. 20, 61–69.

Guthrie, H.D., Welch, G.R., 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J. Anim. Sci. 84, 2089–2100.

Hoogewijs, M., Rijsselaere, T., De, V.S., Vanhaesebrouck, E., De, S.C., Govaere, J., Thys, M., Hoflack, G., Van, S.A., de, K.A., 2010. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 74, 118–126.

Hoogewijs, M., Morrell, J., Van, S.A., Govaere, J., Johannisson, A., Piepers, S., De, S.C., de, K.A., De, V.S., 2011. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. Equine Vet. J. 43 (Suppl. 40), 35–41.

Johannisson, A., Morrell, J.M., Thoren, J., Jonsson, 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.

Koppers, A.J., Garg, M.L., Aitken, R.J., 2010. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic. Biol. Med. 48, 112–119.

Lambert, I.H., 2003. Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. J. Membr. Biol. 192, 19–32.

Lambert, I.H., Pedersen, S.F., Poulsen, K.A., 2006. Activation of PLA2 isoforms by cell swelling and ischaemia/hypoxia. Acta Physiol. (Oxford) 187, 75–85.

Macias-Garcia, B., Morrell, J.M., Ortega-Ferrusola, C., Gonzalez-Fernandez, L., Tapia, J.A., Rodriguez-Martinez, 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.

Macias, G.B., Miro, M.A., Gonzalez, F.L., Ortega, F.C., Morillo, R.A., Gallardo Bolanos, J.M., Balao da Silva, C.M., Rodriguez, M.H., Tapia, J.A., Peña, F.J., 2011. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic induced stress: role of c-Jun N-terminal kinase (JNKs) pathway. J. Androl. 33, 105–113.

Macias, G.B., Ferrusola, C.O., Aparicio, I.M., Miro-Moran, A., Rodriguez, A.M., Bolanos, J.M., Fernandez, L.G., da Silva, C.M., Martinez, H.R., Tapia, J.A., Pena, F.J., 2012. Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology 77, 1280–1289.

Madhavarao, C.N., Sauna, Z.E., Srivastava, A., Sitaramam, V., 2001. Osmotic perturbations induce differential movements in the core and periphery of proteins, membranes and micelles. Biophys. Chem. 90, 233–248.

Matas, C., Sansegundo, M., Ruiz, S., Garcia-Vazquez, F.A., Gadea, J., Romar, R., Coy, P., 2010. Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. Theriogenology 74, 1327–1340.

McCarthy, M.J., Baumber, J., Kass, P.H., Meyers, S.A., 2010. Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa. Biol. Reprod. 82, 644–651.

Morrell, J.M., Dalin, A.M., Rodrigez-Martinez, H., 2008. Prolongation of stallion sperm survival by centrifugation through coated silica colloids: a preliminary study. Anim. Reprod. Sci. 5, 121–126.

Morrell, J.M., Johannisson, A., Dalin, A.M., Rodriguez-Martinez, H., 2009. Single-layer centrifugation with Androcoll-E can be scaled up to allow large volumes of stallion ejaculate to be processed easily. Theriogenology 72, 879–884.

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., Garcia, B.M., Peña, F.J., Johannisson, A., 2011a. Processing stored stallion semen doses by single layer centrifugation. Theriogenology 76, 1424–1432.

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

Morte, M.I., Rodrigues, A.M., Soares, D., Rodrigues, A.S., Gamboa, S., Ramalho-Santos, J., 2008. The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. Anim. Reprod. Sci. 106, 36–47.

Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B., Gelbard, H.A., 2011. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques 50, 98–115.

Pommer, A.C., Rutllant, J., Meyers, S.A., 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology 58, 1373–1384.

Rutllant, J., Pommer, A.C., Meyers, S.A., 2003. Osmotic tolerance limits and properties of rhesus monkey (*Macaca mulatta*) spermatozoa. J. Androl. 24, 534–541.

Sieme, H., Martinsson, G., Rauterberg, H., Walter, K., Aurich, C., Petzoldt, R., Klug, E., 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reprod. Domest. Anim. 38, 134–140.

Trein, C.R., Brito, E.L., Neves, A.P., Jobim, M.I., Ribeiro, L.A., Mattos, R.C., 2005. Cooling rate, centrifugation and percoll selection in equine semen stored at +4 degrees C. Anim. Reprod. Sci. 89, 226–228.

Ws, O., Chen, H., Chow, P.H., 2006. Male genital tract antioxidant enzymes—their ability to preserve sperm DNA integrity. Mol. Cell Endocrinol. 250, 80–83.

Yeste, M., Briz, M., Pinart, E., Sancho, S., Bussalleu, E., Bonet, S., 2010. The osmotic tolerance of boar spermatozoa and its usefulness as sperm quality parameter. Anim. Reprod. Sci. 119, 265–274.