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Swim-Up Procedure in Boar Semen Improves Motility and Viability but Recovered Sperm Could Carry Active Caspases and Chromatin Damage

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Abstract: Different procedures to obtain sperm of quality are routinely used. However, the collected sperm may carry alterations not detected by standard semen parameters. In this study, researchers used flow cytometry and fluorescence microscopy to analyze the effects of the Swim-Up (SU) procedure on selected parameters of boar sperm quality i.e., viability, mitochondrial function, chromatin abnormalities and active caspases-3 and -7 expression. Increases in percentages of sperm motility (18.3±4.2 vs. 31.9±4.7), sperm with mitochondrial function (16.1±3.6 vs. 25.9±4.1) and sperm with chromatin abnormalities (293.6±9.2 vs. 384.7±2.2, DFI values) were observed after SU. Active caspases were located at specific places on the head and flagellum of the sperm and the percentage of sperm showing expression of this proteases was higher after SU (12.4±3.0 vs. 29.7±3.8). The SU procedure allowed the isolation of motile and metabolically active sperm subpopulations however, these subpopulations carried chromatin alterations and sperm that showed caspases expression. It is possible that the increase in the percentage of sperm with active caspases was related to the use of centrifugation during SU. This study may help to demonstrate the low predictive value of traditional quality parameters such as motility when studying fertility potential even when SU is used.

Key words: Boar, sperm, viability, apoptosis markers, swim-up, semen quality

INTRODUCTION

Ejaculated sperm form a heterogeneous cell population with different functional characteristics. Motility is an evident feature of sperm that has been used to indicate vitality (Ricci *et al*., 2009) and the Swim-Up (SU) procedure permits the separation of a subpopulation of motile sperm in an ejaculate. Other than sperm motility, indicators of sperm function or sperm quality have been observed in sperm subpopulations isolated by the SU procedure. For example, cytoplasmic membrane integrity (Ricci *et al*., 2009) and positive mitochondrial function (Pichardo *et al*., 2010) are widely used. The integrity of sperm DNA is essential for embryonic development (Fatehi *et al*., 2006) however, DNA and chromatin abnormalities can be present in ejaculated sperm of normal individuals (Evenson *et al*., 1980; Chohan and Hunter, 2004). Relevant events during apoptosis are related to caspase activation (Johnson *et al*., 2000), further extrinsic and intrinsic pathways of apoptosis converge with the activation of caspases (Chang and Yang, 2000). Active caspases have been detected in the sperm of many species including humans (Weng *et al*., 2002; Paasch *et al*., 2003; Kotwicka *et al*., 2008), ovine (Marti *et al*., 2008) and recently, boars (Moran *et al*., 2008). Presence of active caspases in ejaculated sperm has been negatively correlated with semen quality (Marti *et al*., 2008; Paasch *et al*., 2003) and fertility (Fatehi *et al*., 2006). In addition, active caspases were found in a lower percentage of sperm obtained after the SU procedure (Weng *et al*., 2002; Almeida *et al*., 2005). Early and late markers of apoptosis such as phosphatidylserine translocation (Weng *et al*., 2002; Kotwicka *et al*., 2008; Marti *et al*., 2008) and DNA fragmentation (Sakkas *et al*., 2002; Weng *et al*., 2002; Marti *et al*., 2008) have been used to evaluate sperm quality under different conditions. Fragmentation of spermatic DNA may originated from processes other than apoptosis (Sakkas *et al*., 1999) but then since intrinsic and extrinsic paths of apoptosis converge in caspase activation, it is a more reliable marker of cell death.

This study was intended for analyze whether SU procedure improves sperm motility and functional parameters of boar semen when that semen samples have

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an initial low motility. Functional parameters include integrity of cytoplasmic membrane and mitochondrial function, chromatin integrity and presence of active caspase-3 and -7.

MATERIALS AND METHODS

Sample collection: All experiments were performed using neat or SU-processed semen. The boars were 1.5 years of age, weighed 100-115 kg and were kept at the Facultad de Medicina Veterinaria y Zootecnia under uniform nutritional conditions. Semen samples were collected from four mature Pelon Mexicano boars using a collection dummy by the gloved hand technique and filtered to remove the gel. Boars used in this study were selected with basis in their low percentages of sperm motility. Ejaculates were obtained from every 2nd day, four ejaculates were obtained of each boar. With the aim to minimize boars' stress the technician in charge of the ejaculates obtaining was always the same. One aliquot of each ejaculate was processed by SU and stained as described below. The protocol of this study follow the Guide for the Care and Use of Laboratory Animals of the National Research Council and was approved by Bioethics Committee of the Faculty.

Two experiments were conducted. For the first, membrane integrity, mitochondrial function and chromatin structure were analyzed by flow cytometry in semen samples before and after SU. For the second, the presence of active caspases was evaluated in semen samples before and after SU. In addition to detection of active caspases by flow cytometry it is important to know their location in the sperm however, for the first experiment researchers had the not access to a fluorescence microscopy with the proper cube filters due this we realized the second experiment. The same number of samples was evaluated in each experiment. For both experiments motility was an indicator of SU procedure repeatability.

SU procedure: For this research we designed a micro swim-up procedure. Aliquots (100 μL) of neat semen were carefully pipetted on a column containing 400 μL of dextran (30 mg mL^{-1} Human Tubal Fluid (HTF) medium (200 mmol L^{-1} sucrose, 50 mmol L^{-1} NaCl, 18.6 mM sodium lactate, 21 mmol L^{-1} HEPES, 10 mmol L^{-1} KCl, 2.8 mmol L^{-1} glucose, 0.4 mmol L^{-1} $MgSO_4$, 0.3 mmol L^{-1} sodium pyruvate, 0.3 mmol L^{-1} K_2HPO_4, 1.5 UI mL^{-1} penicillin and 1.5 mg mL^{-1} streptomycin, pH 8) and 300 μL of bovine serum albumin (5 mg mL^{-1} in HTF medium). The tubes were centrifuged at 8.5×g for 3 min (previously researchers observed in the laboratory that this regime of centrifugation permits recovery a good number sperm without cytoplasmic membrane damage) then 100 μL were carefully obtained from the top of the column.

Sperm motility: Fresh samples were diluted in HTF medium at 37°C before evaluate sperm motility for SU samples an aliquot was directly analyzed. Sperm motility was subjectively assessed with aid of a clear field microscope (ECLIPSE 90i, Nikon Instruments Co. Melville, USA) at 100X. About 15 μL of fresh or SU sperm suspension were put on a clean slide prewarmed to 37°C and covered gently with a coverslip. Only sperm swimming in a progressive manner were considered as motile. For each sample two hundred sperm cells were counted and the percentage of motile cells was obtained. Motility of fresh and SU samples was evaluated quickly and the same person perform the evaluation throughout the study.

Sperm viability assays: Membrane integrity and mitochondrial function were considered as viability factors for sperm in fresh and SU-processed semen samples. Two assays were used for test membrane integrity: SYBR in conjunction with Propidium Iodide (PI) for a simple test of exclusion stain and calcein in conjunction with Ethidium homodimers (EthD1) for identify esterases present in the cytoplasm and to stain the nucleus, respectively.

Aliquotes of 25 μL of neat or SU semen samples were stained with SYBR14 and PI contained in the LIVE/DEAD® Sperm Viability kit (Molecular Probes Inc., Eugene, OR, USA) according to the manufacturer's instructions. Final concentrations of SYBR 14 and PI were 100 nmol L^{-1} and 12 μmol L^{-1}, respectively and samples were incubated during 10 min.

Calcein-acetoxymethylester (calcein-AM) is a non-fluorescent membrane-permeable molecule that is reduced to fluorescent calcein by intracellular esterases (Uggeri *et al*., 2004). In this study, calcein AM and EthD1 contained in the LIVE/DEAD® viability/cytotoxicity kit for mammalian cells (Molecular Probes) were used as follow: sperm suspension at a concentration of 25 μmol L^{-1} of calcein AM and 1 μmol L^{-1} of EthD1 were mixed and incubated during 15 min in the dark at room temperature prior to flow cytometry analysis.

Metabolic function in sperm was evaluated by mitochondrial dehydrogenase activity by using resazurin. The non-fluorescent resazurin is reduced by dehydrogenases in functional mitochondria to fluorescent

resorufin molecule (Zrimsek *et al*., 2006). The LIVE/DEAD® Cell Viability Assay kit (Molecular Probes) was used as follows: sperm suspension was incubated with resazurin and SYTOX green at a final concentration of 50 and 1 μmol L^{-1}, respectively during 15 min at room temperature.

The volume of all samples was adjusted to 500 μL with TNE buffer (0.01 mol L^{-1} Tris-HCl, 0.15 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA, pH 7.4) prior to flow cytometry analysis. List mode data of 10,000 events were collected for each sample using CellQuest™ 3.3 software (BDIS) in a Power Mac G4 with Mac OS 9. Percentages of sperm positives to each fluorescent marker in dot plots were obtained with WinList 3.0 (Verity Software House, Inc, Topsham, Maine) software in a PC with Windows XP.

Sperm chromatin structure assay (SCSA®): Neat or SU-processed ejaculates were placed in TNE buffer, frozen and kept in liquid nitrogen until analysis. All of samples were thawed and processed for SCSA as described by Evenson and Jost (2000). Briefly, the frozen sperm samples were thawed in a water bath at 37°C and aliquots of the sperm suspension (200 μL) were mixed with 400 μL of acid solution (Triton X-100 0.1%, NaCl 0.15 mol L^{-1}, Hcl 0.08 N, pH 1.2). About 30 sec later, 1.2 mL of a solution containing 6 μg mL^{-1} of acridine orange (molecular probes) in staining buffer (citric acid 0.1, 0.2 mol L^{-1}, EDTA 1 mmol L^{-1}, NaCl 0.15 mol L^{-1}, pH 6.0) were added and flow cytometry began 3 min after adding the acid solution. All samples were evaluated the same day and a re-calibration with a standard sample was performed after five to ten samples. A FACS can flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) equipped with an argon laser (488 nm) was used to evaluate the sperm parameters. A live gate was used in the FSC and SSC parameters to exclude aggregates and debris thereby restricting data acquisition to an almost pure population of sperm. List mode data of 10,000 events were collected for each sample using CellQuest™ 3.3 software and the DNA Fragmentation Index (DFI) was obtained with WinList 3.0 software.

Detection of active caspases: Active caspases-3 and -7 were labeled *in situ* with a fluorescent inhibitor of caspases (FLICA™) provided with the Image-iT™ LIVE Red Caspase-3 and -7 Detection kit (Molecular Probes), according to the manufacturer's instructions. Briefly, FLICA 150X was diluted with DMSO to 30X then a dilution 1:30 with sperm suspension was incubated during 1 h. Counterstaining was done with SYTOX (SYTOX is a fluorochrome used to detect cells with membrane damaged). To demonstrate FLICA specificity controls were made as follow: positive control was done by incubating a sperm sample with H_2O_2 (Sigma, St Louis, MO, USA) at a final concentration of 50 μmol L^{-1} during 40 min (Bejarano *et al*., 2008) and the sample was processed.

For negative control fresh semen samples were incubated during 20 min with Z-DEVD-FMK (SIGMA, St. Louis, MO, USA) which is a cell-permeable inhibitor of caspase-3, 6, -7, 8 and 10 which competitively and irreversibly inhibits the caspases. The inhibitor was diluted in dimethyl sulfoxide (SIGMA) and added to the samples to reach a final concentration of 40 μmol L^{-1} and incubated during 20 min before addition of FLICA.

To identify location of caspase-3 and -7 in sperm, two smears were made for each semen sample. The cells were examined under a fluorescence microscope (ECLIPSE 90i, Nikon Instruments, Melville, New York) with a standard G2-A filter cube at a 1000X magnification. Micrographs were taken with a digital-cooled camera (DS-5Mc, Nikon) coupled to the microscope and captured with NIS-Elements F 2.30 software (Nikon). The images were processed with ImageJ 1.38r software (Rasband, 2005).

All of results are expressed as mean±SEM. For all of results Kolmogorov-Smirnov test was used to determine normality. A paired two tailed t-test for paired samples was performed to compare percentages of motility, viability, mitochondrial function and DFI values for neat or SU-processed semen samples. The relationships among the viability tests, caspase activity and SCSA were analyzed by Spearman correlation. All analysis were performed with the R 2.9.0 software (R Development Core Team, 2009) running in a macbook with Mac OS x 10.4. $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Sperm motility: Swim-up procedure designed for this research permits to obtain a subpopulation of motile sperm. For the first and second experiments percentage of sperm motility increased significantly after SU (18.3±4.2 vs. 31.9±4.7 and 12.4±4.2 vs. 29.7±6.6, respectively) (Fig. 1). There was a relation for the sperm motility before and after SU for the first ($r = 0.70$, $p = 0.00251$) and second ($r = 0.84$, $p = 0.00015$) experiments.

Sperm viability: Percentages of sperm with membrane integrity in semen samples were not modified after SU

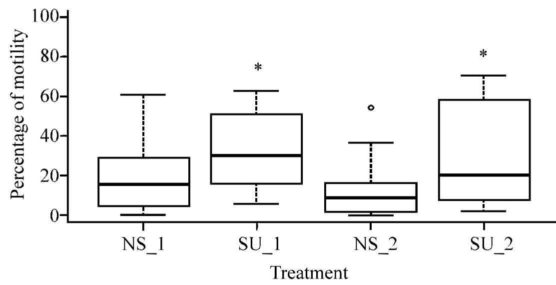

Fig. 1: Distribution of the percentage of motility in neat or SU semen of boars. NS_1, sperm motility of neat semen for experiment one; SU_1, sperm motility of SU semen for experiment one; NS_2, sperm motility of neat semen for experiment two; SU_2, sperm motility of SU semen for experiment two. Values are medians (horizontal bar in boxes) with 25-75% interquartile ranges (boxes), dotted vertical lines indicate minimum and maximum values; circle indicate an outlier. *Indicates differences of SU versus NS samples of respective experiment. $p<0.05$, Student t-test, n = 16

when were evaluated by SYBR/PI nor calcein/EThD1 (37.9±3.4 vs. 31.8±2.9 and 25.9±4.0 vs. 26.2±2.9, respectively). However, the percentage of sperm with active mitochondria increased significantly after SU procedure (16.1±3.6 vs. 25.9±4.1) (Fig. 2a).

Chromatin structure: The values of DFI in sperm recovered after SU procedure was significantly higher than in neat samples (293.6±9.2 vs. 384.7±2.2) (Fig. 2b).

Detection of sperm with active caspases: For the second experiment, presence of sperm with active caspases were evaluated before and after SU procedure. To verify the specificity of FLICA reagent to detect active caspases H_2O_2 was used as inducer of active caspases. Histograms for positive and negative controls are shown in Fig. 3. Fluorescence intensity of FLICA was higher when samples were treated with H_2O_2 however, fluorescence intensity was lower when an inhibitor of caspases activation was added before H_2O_2. The mean percentage of sperm with active caspases was significantly higher after SU-procedure (12.4±3.0 vs. 29.7±3.8) (Fig. 4).

For the head active caspases were located in the acrosomal region, equatorial segment or postacrosomal region whereas for flagellum FLICA fluorescence was observed in middle and principal section (Fig. 5). In most of the sperm cells the active caspases were located in the middle piece or middle piece plus principal piece. A variable fluorescence intensity was observed in cytoplasmic droplet when it was present (Fig. 5).

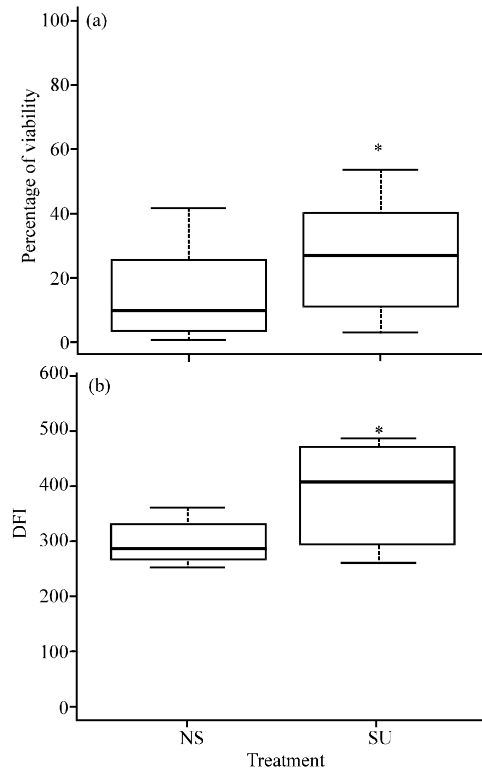

Fig. 2: Distribution of the percentage of viable sperm a) and DFI b) of neat or SU-processed semen. As viable sperm were considered sperm positives to resazurin and negatives to SYTOX; DFI = Denaturation Fluorescence Index. NS = Neat Semen; SU = Swim-Up semen. Values are medians (horizontal bar in boxes) with 25-75% interquartile ranges (boxes), dotted vertical lines indicate minimum and maximum values. *$p<0.05$, Student t-test, n = 16

Swim-up procedure is routinely used for preparing motile sperm subpopulations for ART in humans and other species (Almeida *et al*., 2005) however, this procedure is not extended to the boar sperm. In this study researchers observed that the percentage of motile sperm increased significantly after the SU procedure and this increase was constant in both experiments. Other studies using boar sperm have reported no changes in motility after the SU procedure (Carvajal *et al*., 2004; Park *et al*., 2008).

Differences among studies may be attributed to changes in parameters such as centrifugal force and centrifugal duration used in the SU procedure. In previous studies on the SU procedure, a high centrifuge force was used whereas in the study we used low centrifugal force and short centrifugal duration. In this sense, sperm damage induced by the duration of centrifugation applied

to the semen sample during the SU procedure is more dangerous than the centrifugal force applied to the semen samples (Carvajal *et al*., 2004).

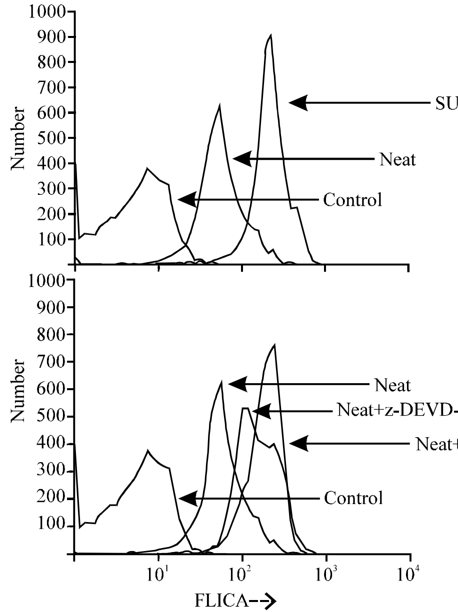

Fig. 3: Representative histograms of the active caspases in sperm of neat or SU-processed semen; a) Control (Neat semen without FLICA); Neat semen and SU semen and b) Controls for the FLICA experiment. Control (Neat semen without FLICA); Neat semen; Neat semen plus FLICA; Neat+H_2O_2, neat semen plus H_2O_2 plus FLICA; Neat+z-DEVD-FMK+H_2O_2, neat semen plus z-VAD (20 min before adding H_2O_2) plus FLICA

Therefore, the use of a low centrifugal force and a short duration of application in the present study can explain the changes in the percentage of motile sperm after the SU procedure that we observed.

SYBR/PI assay permits to detect viable sperm cells (Garner and Johnson, 1995). Researchers detected no differences in the percentage of viable sperm between SYBR/PI and calcein/EthD1 assay techniques. This result

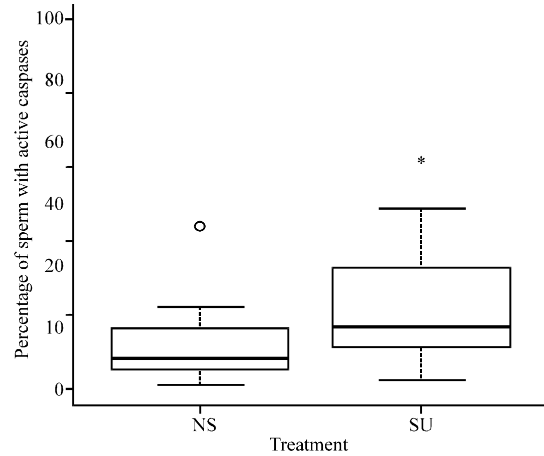

Fig. 4: Distribution of the percentage of sperm expressing active caspase-3 and -7 detected in sperm of neat or SU-processed semen. NS = Neat Semen; SU = Swim-Up semen. Values are medians (horizontal bar in boxes) with 25-75% interquartile ranges (boxes), dotted vertical lines indicate minimum and maximum values; circle indicate an outlier. *$p<0.05$, Student t-test, n = 16

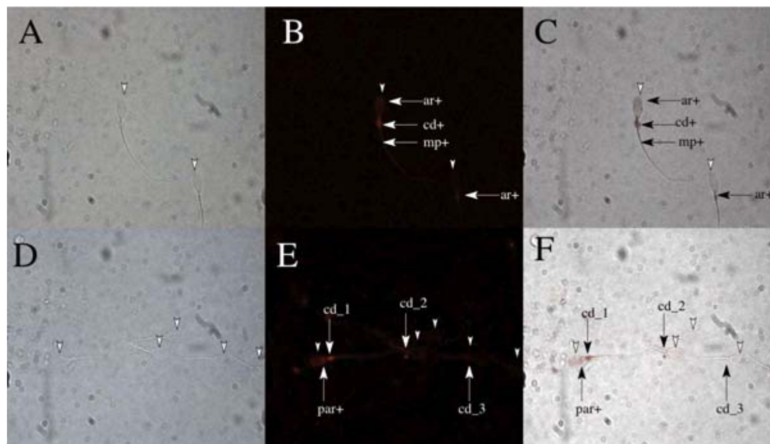

Fig. 5: Location of active caspase-3 and -7 in boar sperm. The montage illustrates the clear-field view (A and D), the fluorescence view (SR-DEVD-FMK FLICA) (B and E) and an overlay of both (C and F). Note that FLICA fluorescence could be intense, medium and even absent in the cytoplasmic drop (cd_1, cd_2 and cd_3, respectively in F). Arrowheads indicate the head of sperm; white and black arrows indicate the sperm segments with active caspases. Cd+: cytoplasmic drop staining positive; par+: post-acrosomal region staining positive; ar+: acrosomal region staining positive; mp+, middle piece staining positive; pp+, principal piece staining positive

indicates that both of these assays are reliable when detecting membrane damage. However, we also found that the percentage of sperm with metabolic activity (resazurin/SYTOX assay) was lower than when using other viability assays. This demonstrates that although, the spermatic subpopulation obtained after SU, presents no damage in the cytoplasmic membrane, not all sperm cells have functional mitochondria. The positive relationship between mitochondria activity and motility observed in the present study is corroborated by the fact that mitochondria produce the energy necessary for flagellum to beat. The reduction of resazurin is a reliable method to evaluate metabolic function in boar sperm. In addition, when this method is evaluated by flow cytometry, their values are approximately the same that those of motility (Zrimsek *et al*., 2006) as we observed in this study.

Recently, Moran *et al*. (2008) detected the presence of active caspases in boar spermatozoa using flow cytometry. Although, to the knowledge, researchers are the first to report the location of active caspases-3 and -7 in boar sperm. The presence of caspase activity in cytoplasmic droplets suggests a relationship between the presence of caspases and cytoplasm in sperm. Possible errors during differentiation and sperm detachment from Sertoli cells in the seminiferous epithelium may be the origin of the sperm subpopulations with an abnormal quantity of cytoplasm. This idea is supported by observations of Blanco-Rodriguez and Martinez-Garcia (1999) which describe the detachment of apoptosis markers with the cytoplasmic droplet and the presence of those markers in residual bodies. The functions if any, of active caspases in ejaculated sperm and its relation with other parameters of quality are not clear. For example, we do not observe a relationship between motility and active caspases which corresponds with recent observations of sperm expressing active caspases (Kotwicka *et al*., 2008; Pichardo *et al*., 2010).

DNA damage does not block fertilization and early embryonic development but does induce apoptosis after the first cleavages (Fatehi *et al*., 2006). There is a strong relationship between the results of SCSA and other tests designed to identify DNA damage (Sailer *et al*., 1995; Chohan *et al*., 2006). Researchers found an increase in the DFI values in SU samples. Some studies have indicated low DFI values after SU processing (Spano *et al*., 2000) while others have indicated no changes (Sakkas *et al*., 2000). We observed that although, SU processing improves motility, the chromatin is damaged which can result in early development risks (Fatehi *et al*., 2006). We do not know the cause of the high DFI values in the sperm but the fact that DFI values increase in a concomitant manner with the percentage of sperm with active caspases may indicate that caspases function in the nucleus as suggested early (Weng *et al*., 2002). Previous research from the laboratory demonstrated an increase in DFI values of ovine sperm selected by the SU procedure (Pichardo *et al*., 2010). However, in the same study, the percentage of active caspases decreased. Apart of the species studied, the difference in the results between the previous and this study could be the use of centrifugation during the SU procedure. In line with this, it has been demonstrated that other physical factors such as freezing/thawing induce caspases activation in sperm (Martin *et al*., 2007).

CONCLUSION

The results may help to explain the low fertility potential of semen samples when motility and viability are used as the principal parameters of quality and may be used as evidence for the necessity to evaluate DNA integrity and apoptosis markers. In summary, when used the SU procedure for separate motile sperm of boar, the procedure improves the sperm quality by increasing the percentage of motile and metabolically active sperm although, an increase of sperm with caspases activity and alterations in chromatin have also been observed.

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