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Akt preserves sperm viability and motility in stallion sperm by inhibiting caspases 3 and 7.

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This paper is dedicated to the memory of Dr. Fernando Peña-Martín (1922-2013)

ABSTRACT

Akt, also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis. In order to gain insight of mechanisms regulating sperm survival after ejaculation, the role of Akt was investigated in stallion spermatozoa using a specific inhibitor and phosphoflow approach. Stallion spermatozoa were washed and incubated in BWW media supplemented with 1% PVA in presence of 0, 10, 20, or 30μM of the Akt inhibitor SH5. The inhibitor resulted in a reduction of the percentage of sperm showing phosphorylated Akt that reached a maximum after one hour of incubation, attributable either to dephosphorylation or prevention of an active phosphorylation pathway. During in vitro incubation stallion spermatozoa de-phosphorylated spontaneously, so after 4 hours of incubation treated samples did not differ from controls. Akt inhibition decreased the proportion of motile spermatozoa (total and progressive), and also sperm velocity. Likewise, Akt inactivation reduced membrane intactness leading to enhanced membrane permeability, as well as a reduction in mitochondrial membrane potential concomitant with more spermatozoa depicting active caspases 3 and 7. On the other hand, neither the percentage of spermatozoa suffering oxidative stress, the production of mitochondrial superoxide radical, DNA oxidation or sperm DNA fragmentation changed when Akt was inhibited. It is concluded that Akt is needed for intactness of membrane and motility in ejaculated stallion spermatozoa, presumably acting by inhibition of caspases 3 and 7, which would prevent progression of spermatozoa to an incomplete form of apoptosis, for which we hereby propose the term *spermptosis.*

Key words: stallion, sperm, Akt, phosphoflow, pannexin, mitochondria, caspases 3 and 7

INTRODUCTION

Artificial insemination using chilled transported semen is, with most breeding registries now accepting this reproductive technology, widely used by the equine industry (Pena FJ 2012). However, the viability and potential fertilizing capacity of cooled stallion spermatozoa last only for a few days or, for some individuals even only hours (Macias Garcia *et al.* 2012). Apoptosis, is a form of programmed cell death

executed by a family of cysteine proteases termed caspases acting via two major pathways; the intrinsic activated by cellular stress and originating by liberation of mitochondrial pro-apoptotic proteins that activates caspase 9. The other pathway is extrinsic, triggered by death receptors that after activation by their ligands leads to cleavage of caspase 8 (Aitken *et al.* 2011). Occurrence of apoptosis in ejaculated spermatozoa remains under debate. The theory of abortive apoptosis considers apoptosis in spermatozoa as remnants from previous spermatogenesis (Sakkas *et al.* 2002), on the basis that spermatozoa are terminal, transcriptionally silent cells. Such theory is in turn questioned by facts emanating from the finding that biotechnological procedures such as cooling storage and cryopreservation, induce features typical of apoptosis in ejaculated spermatozoa (Moran *et al.* 2008, Ortega-Ferrusola *et al.* 2008, Ortega Ferrusola *et al.* 2009, Said *et al.* 2010, Aitken *et al.* 2012) thus suggesting that apoptotic phenomena can be triggered after ejaculation, particularly those related to its intrinsic pathway (Pena *et al.* 2003b). Unraveling whether apoptosis does indeed occur in ejaculated spermatozoa may have tremendous implications for reproductive biotechnologies in humans or animals where sperm manipulation for ART´s is rather extended (Pena *et al.* 2006, Ortega-Ferrusola *et al.* 2009). Akt , also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis in somatic cells (Hers *et al.* 2011), and growing evidence suggests that is also involved in the regulation of sperm motility and survival (Aparicio *et al.* 2005, Aquila *et al.* 2007, Koppers *et al.* 2011, Parte *et al.* 2012). Although the first component of this pathway, PI3K has been the subject of several studies in sperm biology, Akt has received less attention despite that recent research points out Akt as a key factor for sperm survival after ejaculation; acting suppressing sperm capacitation and preventing the spermatozoa to enter in an apoptotic cascade leading to sperm senescence and finally death (Pujianto *et al.* 2010). Moreover, we have recently demonstrated that sperm death occurring during conservation of stallion spermatozoa also involves apoptotic related phenomena, both after cryopreservation (Ortega-Ferrusola *et al.* 2008, Ortega Ferrusola *et al.* 2010) and chilling (Macias Garcia *et al.* 2012). In view of these evidences and in order to determine the role of Akt in the regulation of survival of ejaculated stallion spermatozoa, we investigated whether Akt affected stallion sperm survival using a specific inhibitor and phosphoflow cytometry approach, that is the use of antibodies against phosphorylated forms of a particular protein and detecting those using flow cytometry (Tazzari *et al.* 2002, Krutzik *et al.* 2004, Schulz *et al.* 2007, Schulz *et al.* 2012). The results indicated that survival of ejaculated spermatozoa was regulated by Akt through the inhibition of an apoptosis-like mechanism. Such mechanism, as previously proposed in humans (Aitken *et al.* 2011), may relate to the

silent removal of defective spermatozoa from the female genitalia (Lessig *et al.* 2007). If proven similar, such a mechanism conserved over species would make the equine spermatozoon a suitable model for human spermatozoa, particularly in relation to studies of defectiveness and preservation, areas less explored in human. Moreover, the utility of a phosphoflow approach in the study of sperm biology is evidenced.

MATERIAL AND METHODS

Reagents and media

Ethidium homodimer, 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyne iodine (JC-1), Mitrotracker deep Red, YO-PRO-1, Caspase 3 and 7 detection reagent CellEvent, Cellrox, MitoxSox Red, Sytox Green and TUNEL assay were from Molecular Probes (Molecular Probes, Leiden The Netherlands), Akt inhibitor SH-5, and OxyDNA assay kit were from Calbiochem (San Diego, CA), Akt 1/2 kinase inhibitor, carbenexolone and probenecid from Sigma (St Louis MO), antin pannexin-1 was from AbCam (Cambridge UK), antiphospho Akt (pSer^{473}), Akt (pThr ^{308}) Alexa Flour® 488 and 647 conjugated monoclonal antibodies and IgG XP® Isotype Controls (Alexa Fluor® 488 and 647 conjugates) were purchased from Cell Signaling Technology (Danvers, MA)

Semen collection and processing

Semen was obtained from 7 Pure Spanish horses (PRE) (three ejaculates each) individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. Stallions were maintained according to institutional and European regulations, and were collected on a regular basis (two collections/week) during the 2012-breeding season. Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. The semen was immediately transported to the laboratory for evaluation and processing. The ejaculate was extended 1:1 in INRA-96 centrifuged (600g x 10 min) and re-suspended in BWW media supplemented with 1% PVA to 50x10^6 spermatozoa/mL. All the experiments followed a split sample design with every ejaculate

divided in controls and treatment groups. Every particular experiment was repeated in three different ejaculates from each of the seven stallions (21 replicates in total).

Detection of phosphorylated Akt (Ser ^{473}) and Akt (Thr^{308}) in stallion spermatozoa

Spermatozoa (1x10^6/mL) were washed with saline-Hepes medium and fixed in 2% paraformaldehyde in phosphate buffer saline (PBS) at room temperature for 15 minutes. After fixation, the cells were washed twice with PBS and once with PBS/1%BSA, permeabilized for 30 minutes using 0.1% saponin in PBS containing 1% BSA, and incubated in the same buffer with 2 µL/mL of Phospho-Akt (Ser ^{473}) –Alexa Fluor 488 conjugate (Cat number 4071, Cell signaling) and 2 µL/mL of Phospho-Akt (Thr308) Alexa fluor 647 conjugate catalogue number (C31E5E) for 30 minutes in the dark at 22ºC. Samples were then washed in PBS, the pellets resuspended in 500 µL of PBS and analyzed using a MACSQuant® Flow cytometer (Miltenyi Biotech, Madrid Spain) and a Bio-Rad MRC1024 confocal laser microscope with a X60 oil-immersion objective. Hoechst 33342 was added to restrict analysis to spermatozoa and gate out debris. The specificity of the antibodies was validated using western blotting, and negative controls consisted of unstained samples and isotype controls, IgG XP® Isotype Controls (Alexa Fluor® 488 and 647 conjugates)

Western Blotting

Stallion semen was centrifuged and washed twice with PBS. After washing, sperm cells were sonicated for 5 sec at 4 ºC in 100 µl of Lysis Buffer consisting in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.4 mM EDTA, a protease inhibitor cocktail (Complete, EDTA-free), and 0.2 mM Na_3VO_4. The homogenates were clarified by centrifugation at 10,000 xg (15 min, 4°C) and the supernatant was used for analysis of protein concentration followed by dilution with 4× SDS sample buffer. Proteins (25 µg/well) from stallion sperm lysates were fractionated by SDS-PAGE using 4-20% polyacrylamide gradient gels and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight at 4 °C with anti-pannexin-1 (1:1000), anti- pAkt Ser 473(1:1000), and anti-pAkt Thr 308 (1:1000). The following day, membranes were washed twice and incubated for 45 min at 25 °C with anti-rabbit IgG -HRP conjugated secondary Ab. Membranes were then washed again,

incubated with enhanced chemiluminescence detection reagents, and, finally, exposed to Hyperfilm ECL films (Amersham). Positive controls for pAkt Ser 473 and Thr 308 were pancreatic acini lysates, and positive controls for Pannexin-1 were brain lysates. The intensity and molecular weight of appearing bands were quantified using the software Scion Image for Windows, version 4.02 (Scion Corp., Frederick, MD), normalized to β actin values.

Sperm motility

Sperm motility and kinematics were assessed using a CASA system (ISAS® Proiser Valencia Spain) (Pena *et al.* 2005a, Nunez-Martinez *et al.* 2007a, Gonzalez-Fernandez *et al.* 2009). Semen was loaded in a 20 □m depth Leja chamber (Leja Amsterdam, The Netherlands) and placed on a warmed (37ºC) stage. The analysis was based on the examination of 25 consecutive digitalized images obtained from each field using x10 negative phase contrast objective. At least three different fields were recorded to assure a minimum of 200 spermatozoa per sample. Images were taken with a time lapse of 1 second - the image capture speed was therefore one every 40 milliseconds. The number of objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. With respect to the setting parameters for the program, spermatozoa with a VAP <15 μm/s were considered immotile, while spermatozoa with a velocity >15μm/s were considered motile. Spermatozoa deviating <45 % from a straight line were designated linearly motile and spermatozoa with a circular velocity (VCL) > 45μm/s were designated rapid sperm. Sperm motion absolute and re-calculated kinematic parameters measured by CASA included the following: Curvilinear Velocity (VCL) μm/s, Measures the sequential progression along the true trajectory. Linear Velocity (VSL) μm/s, Measures the straight trajectory of the spermatozoa per unit time. Mean Velocity (VAP) μm/s, Measures the mean trajectory of the spermatozoa per unit of time.

Simultaneous flow cytometric assessment of subtle membrane changes, viability and oxidative stress

The following stock solutions were prepared in DMSO: Yo-Pro-1 (25 μM), Ethidium Homodimer-1 (1.167 mM); and CellRox (5mM). Hoechst 33342 (1.62 mM in water) was used to identify spermatozoa

and eliminate debris from the analysis. One mL of a sperm suspension containing 5 x 10^6 spermatozoa/mL was stained with 1 μL of Yo-Pro-1, 1μL of CellRox and 0.3μL of Hoechst 33342. After thorough mixing, the sperm suspension was incubated at RT in the dark for 25 minutes, the spermatozoa were then washed in PBS and 0.3 μL of ethidium homodimer added and incubated further 5 minutes before reading in the flow cytometer. This staining is modified after previous protocols and distinguishes four sperm subpopulations and the oxidative stress simultaneously (Pena *et al.* 2005b, Nunez-Martinez *et al.* 2007b, Ortega Ferrusola *et al.* 2009). The first is the subpopulation of only Hoechst 33342 positive spermatozoa, considered alive and without any membrane alteration. Another subpopulation is the Yo-Pro-1 positive cells emitting green fluorescence. This subpopulation are those spermatozoa showing early damage or a shift to another physiological state, since membranes become slightly permeable during the first steps of damage, enabling Yo-Pro-1 but not ethidium homodimer to penetrate the plasma membrane. None of these probes enters intact cells. Finally, two subpopulations of dead spermatozoa are easily detected, either apoptotic (spermatozoa stained both with Yo-Pro-1 and ethidium homodimer, emitting both green and red fluorescence), or necrotic spermatozoa (cells stained only with ethidium homodimer emitting red fluorescence). Spermatozoa suffering oxidative stress emit fluorescence in the far red spectrum, while Hoechst 33342 emit blue fluorescence. Representative cytograms of the assay are depicted in figure 1. Positive controls for oxidative stress were samples supplemented with 800 μM SO_4Fe and 200 μL of H_2O_2 (Sigma) to induce the Fenton reaction.

Simultaneous flow cytometric detection of active caspases 3 and 7 and active mitochondria

CellEvent™ Caspase-3/7 Green Detection Reagent is a fluorogenic substrate for activated caspases 3 and 7. The reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. This cell-permeant substrate is intrinsically non‑fluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 and caspase-7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with an absorption/emission maxima of ~502/530 nm. One important advantage of this assay is that no wash steps are required avoiding cell losses during washing. The following stock solutions; CellEvent (2mM in DMSO), ethidium homodimer (1.167mM in DMSO), mitotracker deep red (0.5μM in DMSO) and Hoechst 33342 (1.62mM in water) were prepared. Spermatozoa (5x10^6/ml) in one ml of PBS, were

stained with 1µL of cell event, 0.3µL of Hoechst 33342 and 0.3µL of Mitotracker deep red and incubated in the dark at r.t. for 25 minutes, after that 0.3µL of Ethidum homidimer were added and samples were incubated further five minutes and samples were immediately run in the flow cytometer. Representative cytograms of the assay are placed in figure 2. Since cryopreservation induces caspase activity in stallion sperm (Ortega-Ferrusola *et al.* 2008) cryopreserved samples were used as positive controls (figure 2) for caspase 3 and 7.

Evaluation of mitochondrial membrane potential (ΔΨm)

The lipophilic cationic compound 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyne iodine (JC-1) has the unique ability to differentially label mitochondria with low and high membrane potential (Pena *et al.* 2003a, Ortega Ferrusola *et al.* 2009, Ortega-Ferrusola *et al.* 2009). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers, that emit in the green wavelength (525 to 530 nm) when excited at 488 nm. For staining, a 3mM stock solution of JC-1 (Molecular Probes Europe, Leiden, The Netherlands) in dimetylsulfoxide (DMSO) was prepared. From each sperm sample, 1 mL of a sperm suspension in PBS (5 x10^6/mL) was stained with 0.5 µL JC-1 stock solution. The samples incubated at 37 °C in the dark for 40 minutes before flow cytometric analysis.

Determination of DNA oxidation, 8-oxoguanine assay

This assay is based in the direct binding of a fluorescent probe to the DNA adduct 8- oxoguanine (OxyDNA Assay Kit, Calbiochem), a major oxidation product and an important indicator of free radical-induced DNA damage and oxidative stress. The assay was performed according to manufacturer instructions and following previously published protocols(De Iuliis *et al.* 2009, Koppers *et al.* 2011). In brief, spermatozoa (5 x 10^6/mL) were separated from each sample, washed in PBS and fixed in a 2% paraformaldehide solution in 0,1M PBS (pH 7.6) for 15 minutes at room temperature. Cells were then washed twice in PBS and once in PBS 1% BSA. Permeabilization was made by incubation in PBS 1% BSA supplemented 0.1% saponin for 30 minutes. Samples were washed with 1mL of wash solution (1:25

dilution in water of Wash Concentrate provided by the manufacturer). Staining with 100µL of 1X FITC-Conjugate (1:10 dilution of FITC-Conjugate with wash solution) was done for 60 minutes in the dark, at room temperature. Finally, cells were washed twice and resuspended in 1 mL of PBS for flow cytometry analysis. The amount of 8-oxiguanosine formed (excitation at 495 nm and emission at 515 nm), was measured in the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany), as green fluorescence being proportional to the oxidative damage caused to the DNA. Positive controls were made after incubation of additional samples in 800µM Fe2+ and 200µM H_2O_2

Mitosox red assay

Generation of mitochondrial superoxide anion was investigated using Mitosox Red (MSR, Molecular probes) as previously described (Koppers *et al.* 2008, Koppers *et al.* 2011). Stock solutions of Mitosox Red (5mM in DMSO) and Sytox Green (0.125 mM in DMSO) were prepared. Spermatozoa (5×10^6 /mL) were stained with a final concentration of 2 µM MiToSox Red and incubated for 15 min at 37º C, followed by centrifugation for 5 min at 600 × *g* and resuspension in BWW. SYTOX Green (0.05 µM) was then added for a final 15-min incubation. The MSR (red) and SYTOX Green (green) fluorescence was then measured using 530/30 band pass (green) and 585/42 band pass (red) filters. Non sperm-specific events were gated out after staining with Hoechst 33342 (0.1µL of a 16 mM solution), and10,000 cells were examined.

TUNEL Assay Terminal TdT (deoxynucleotidyltransferase)-mediated dUTP nick end labeling assay

DNA cleavage may yield double stranded and single stranded DNA breaks. Both type of breaks can be detected by labeling the free 3'-OH terminals with fluorescent nucleotides (labeled dUTPs) in an enzymatic reaction catalized by TDT(Koppers *et al.* 2011). Samples were washed in PBS and fixed in a 2% paraformaldehide solution in 0,1M PBS (pH 7.6) for 15 minutes at room temperature. Cells were then washed in PBS and maintained in 5mL methanol 70% at -20ºC. For recognition of DNA fragmentation of apoptotic cells, APO-BrdU TUNEL Assay Kit (Invitrogen, Molecular Probes) was used. This Kit allows detection of 3'-hydroxyl ends, serving as starting points for terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction labels the break sites. Once incorporated into the DNA, BrdUTP is detected by an anti-BrdUTP antibody, which is labeled by

an Alexa Fluor 488 dye (Ex 495 nm, Em 519 nm), while total cellular DNA content is labeled by propidium iodide (Ex 535 nm, Em 617 nm). Frozen cells were washed twice in wash buffer and resuspended in the DNA-labeling solution for 60 minutes at 37ºC in a water bath. Suspensions were washed twice in rinse buffer, and the Alexa Fluor 488 dye-labeled anti-BrdU antibody was added to each of them. Incubation was performed for 30 minutes at room temperature in the dark. Finally, 0,5mL of propidium iodide RNase staining buffer was added, incubated for 30 minutes at room temperature in the dark, followed by flow cytometry.

Flow Cytometry

Flow cytometric analyses were carried out with a MACSQuant Analyzer 10 (Miltenyi Biotech) flow cytometer equipped with 3 lasers emitting at 405, 488 and 635 nm and 10 photomultiplier tubes (PTM): V1 (Ex 405 Em 450/50) V2 (Ex 405 filter 525/50), B1 (Ex 488 filter 525/50) B2 (Ex 488 filter 585/40) B3 (Ex 488 filter 655-730 (655LP + split 730)) B4 (Ex 499 filter 750 LP), R1 (Ex 635 filter 655-730 (655LP+split 730) and R2 (Ex 635 filter 750 LP. The system is equipped with the MACSQuantify software. Sperm subpopulations were divided by quadrants, and the frequency of each subpopulation quantified. Forward and sideways light scatter were recorded for a total of 50,000 events per sample. Non-sperm events were eliminated gating the sperm population after Hoechst 33342 staining. The instrument was calibrated daily using specific calibration beads provided by the manufacturer, and compensation overlap performed before each specific experiment.

Statistical analysis

All experiments were repeated at least three times in independent samples (three independent ejaculates from each of the seven stallions) and the results analyzed using ANOVA with the SPSS. 19.0 software for Mac. Differences with a P value < 0.05 were regarded as significant

RESULTS

Identification and subcellular localization of phosphorylated AkTt (Ser ^{473}) and Akt (Thr^{308}) in stallion spermatozoa.

The presence of phosphorylated Akt in stallion spermatozoa was demonstrated using specific antibodies, western blotting, flow cytometry and confocal laser microscopy. Akt was mainly identified in the caudal part of the post-acrosomal region and the mid-piece (Figure 3). This subcellular localization complies with a putative role; inhibiting pro-apoptotic proteins localized in the mitochondrial membrane.

Inhibition of Akt leads to reduced stallion sperm motility and kinematics

To study the role of AKT in the maintenance of stallion sperm motility, split samples of stallion spermatozoa were incubated in BWW media in presence of a specific inhibitor of AKT, [D-3-deoxy-2-O-methyl-myo-inositol-1-[(R)-2- methoxy-3-(octadecyloxy) propyl hydrogen phosphate]] (SH-5)(Sethi *et al.* 2008) (0 (vehicle), 10, 20 and 30μM in DMSO) up to 6 hours at 37ºC in BWW media supplemented with 1% PVA. The final concentration of DMSO in all samples was 0.01‰. This is this is a cell permeable, reversible, and substrate competitive phosphatidylinositol analog that inhibits the activation of Akt and select downstream substrates without decreasing phosphorylation of PDK-1 or other kinases downstream of Ras, such as MAPK. This compound has been widely used as specific inhibitor of AKT in a wide number of cell lines (Lan *et al.* 2011, Busch *et al.* 2012, Qiao *et al.* 2013, Yang *et al.* 2013) acting as as a potent inducer of apoptosis, and selectively kills a variety of cancer cell lines that contain high levels of active Akt. A second inhibitor, Akt 1/2 inhibitor was also used in additional experiment (Gilot *et al.* 2010). The Akt phosphorylation status was monitored using flow cytometry (Fig 4). In stallion spermatozoa Akt appeared phosphorylated at Thr308 and Ser473. Inhibition of Akt phosphorylation was monitored after 1 and four hours of incubation. Time of incubation had a significant effect on Akt phosphorylation, with the percentage of spermatozoa with phosphorylated Akt both at Thr 308 and Ser 473 decreasing significantly after four hours of incubation (Figure 4). After one hour of incubation in presence of SH-5 the percentage of spermatozoa depicting phosphorylated Akt decreased significantly, however after 4 hours of incubation differences among control and treated samples were no longer different (Figure 4), the same effect was observed using Akt 1/2 inhibitor (Fig 9 D). After one, two, four or six hours of incubation, sperm motility and velocities were determined using CASA analysis. After one

hour of incubation, both Akt inhibitors induced, at 30μM, a significant decrease in the percentage of total motile spermatozoa (30% vs 50% in controls), progressive sperm (6% vs 18% in controls), circular (39. 5 vs 63.5 μm/s in controls), average (21.5 vs 36.7 μm/s in controls) and straight line velocities (12.5 vs 21μm/s in controls) ($p<0.05$). After two hours of incubation at 37º C, the inhibitors significantly reduced circular velocity and average velocity ($p<0.05$) (figure 5 and figure 9 A and B). These data suggest that AKT plays a role in the regulation of stallion sperm motility and velocities. After 4 and 6 hours of incubation all parameters of sperm motility and kinematics were reduced ($p<0.01$), independently of treatment.

Inhibition of Akt increases sperm membrane permeability and leads to sperm death

In order to determine whether Akt relates to early changes in membrane integrity and permeability, stallion spermatozoa were analyzed using flow cytometry after incubation in presence or absence of the Akt inhibitor in BWW media up to 6 hours at 37ºC. After one hour of incubation at 37ºC, the percentage of spermatozoa with intact membranes decreased significantly ($p<0.05$) from 50% in controls to 38% in presence of 30μM Akt inhibitor. Simultaneously, the percentage of spermatozoa with increased membrane permeability, apoptotic and of necrotic spermatozoa increased ($P<0.05$)(figure 6). However, the effect of the inhibitor disappeared after longer incubation periods, although the percentage of dead spermatozoa was higher after 6 hours of incubation at 37 ºC in presence of 30μM Akt inhibitor.

Pannexin-1 channel is present in stallion spermatozoa, and YoPro-1 uses this channel to penetrate the sperm membrane

In somatic cells YoPro-1 detects early apoptotic cells, since this probe uses specific channels (Pannexin-1) to penetrate the cell membrane; these channels open in the very early stages of apoptosis (Elliott *et al.* 2009, Chekeni *et al.* 2010, Sandilos *et al.* 2012). In order to confirm the presence of this channel in sperm and that YoPro-1 uses the pannexin-1 channel to penetrate the sperm membrane a specific experiment was conducted. . First the presence of Pannexin-1 channels was investigated using Western Blotting and specific antibodies, and secondly the channel was blocked using two unrelated inhibitors (probenecid 1mM an carbenexolone 100μm) and sperm was stained with YoPro-1. Pannexin-1 was present in stallion spermatozoa, moreover Probenecid and carbenexolone significantly reduced the percentage of YoPro-1+

(Figure 7) spermatozoa supporting that YoPro-1 uptake in this population of spermatozoa is due to the opening of pannexin-1 channels. This may also indicate that these spermatozoa are in a early stage of an apoptotic phenomena similar to the situation described in somatic cells (Chekeni *et al.* 2010) .

Inhibition of Akt phosphorylation (Ser ^{473}) and (Thr^{308}) increases caspase 3 and 7 activity in stallion spermatozoa

One possible mechanism to explain our findings is that the phosphorylation status of Akt participates in the regulation of an apoptotic-like mechanism. To test this hypothesis, we evaluated caspase 3 and 7 activation in stallion spermatozoa after incubation in presence of the Akt inhibitor. If Akt is inhibiting an apoptotic mechanism the inhibition of its activity shall lead to an increase in caspase 3 and 7 activities. After one hour of incubation, all concentrations tested reduced the percentage of caspase-negative, live spermatozoa in a positive dose-dependent manner ($p<0.01$), (figure 8). The percentage of caspase 3 positive spermatozoa also increased in a dose dependent manner, being this effect especially evident after four hours of incubation at 37º C (figure 8 C). These results indicate that Akt phosphorylation at Ser 473 and Thr308 inhibits caspase 3and 7 activation in ejaculated stallion spermatozoa. In order to provide further evidences supporting that these effects were due to Akt inhibition a further experiment was conducted using simultaneously two different Akt inhibitors (SH-5 and Akt 1-2 Kinase inhibitor 0 and 30μM) and addressing its effects on Akt phosphorylation, sperm motility and velocity and caspase 3 and 7 activation after 1 hour of incubation at 37ºC. Both inhibitors showed similar effects, with a reduction in sperm motilities and velocities, increases in caspase 3 and 7 activities and in dephosphorylation of Akt (figure 9).

Inhibition of Akt reduces mitochondrial membrane potential in stallion spermatozoa

To study the effect of the inhibition of Akt phosphorylation on sperm mitochondria two different assays were performed. Mitochondrial membrane potential was studied after JC-1 and Mitotracker deep red staining. Inhibition of Akt phosphorylation affected, in a dose and time dependent manner, mitochondrial membrane potential of stallion spermatozoa. After one hour of incubation, 30μM of Akt inhibitor significantly reduced mitochondrial membrane potential, evaluated with two probes (figures 10 and 11).

After two hours of incubation, 20 and 30 μM of Akt inhibitor decreased mitochondrial membrane potential when determined with mitotracker deep red (figure 10), but not with JC-1. For the latter, mitochondrial membrane potential was only significantly decreased at 30 μM (figure 11).

Inhibition of Akt does not increase the production of reactive oxygen species (ROS), mitochondrial superoxide production and DNA oxidation, and does not increase DNA fragmentation

Since disruption of mitochondria may lead to increased ROS-production and oxidative stress (Koppers *et al.* 2008), the effect of an Akt inhibitor was tested. However, the differences observed in the production of ROS were not statistically significant (figure 12). Neither was there an increase in mitochondrial superoxide production when Akt phosphorylation was inhibited (data not shown). Moreover, inhibition of Akt phosphorylation did not lead to significant increases in DNA oxidation on stallion spermatozoa (data not shown). Since DNA fragmentation is a landmark of apoptosis, a TUNEL assay was used, but inhibition of Akt had no significant effect on the percentage of fragmented DNA (data not shown).

DISCUSSION

The presence and role of Akt in stallion spermatozoa was investigated using specific inhibitors. The overall results indicated that Akt maintains sperm survival after ejaculation through the inhibition of caspase 3 and 7. The PI3 kinase/Akt pathway has been previously studied in spermatozoa using specific inhibitors such as Wortmannin and LY294002, but to the authors´ knowledge this is the first time that a specific inhibitors of Akt are used to study the Akt pathway in mammalian spermatozoa, and reveals a direct effect of Akt inhibiting caspase 3 and 7, since Akt inhibition rapidly lead to caspase 3 and 7 activation. The data thus suggest that inhibition of an apoptotic like mechanism, probably sperm specific, plays a role in the survival of ejaculated stallion spermatozoa, and confirm similar recent findings in humans (Koppers *et al.* 2011) supporting the idea that post ejaculatory survival of stallion and human spermatozoa is tightly regulated through the inhibition of an truncated apoptotic cascade. This inhibition is lost over the incubation time, since spontaneous dephosphorylation of Akt occurs, suggesting that this is a mechanism to remove redundant spermatozoa in the female genital tract (Aitken *et al.* 2011). Our study suggest that Akt is necessary to maintain sperm motility, viability and mitochondrial membrane

potential through an inhibition of the activation of caspases 3 and 7. This was hereby demonstrated by the rapid increase in caspase 3 and 7 activity upon inhibition of Akt. Inhibition of Akt was accompanied by decreases in the percentage of sperm motility and velocity, increases in sperm membrane permeability and decreases of mitochondrial membrane potential, all suggesting a role for Akt in maintaining sperm viability. Although we show evidence for each point, the link between them remains to be determined. These findings strongly suggest that stallion sperm death during storage is largely an apoptotic phenomena, as has been previously proposed (Ball 2008, Ortega-Ferrusola *et al.* 2008, da Silva *et al.* 2011, Macias Garcia *et al.* 2012). Other forms of sperm dead during storage are also possible, and after 6 hours of storage we evidenced and increase in the percentage of necrotic sperm incubated in presence of 30 μM SH-5, however most of these population are also caspase +, suggesting an apoptotic phenomena behind, moreover when a different Akt inhibitor was used the same effect was reproduced further suggesting that Akt inhibition leads to a truncated apoptotic pathway leading to cessation of sperm motility and death. Although we present strong evidences of the existence of this pathway remains to be fully described; for example recent evidences suggest that a extrinsic, mediated by death receptors, pathway may also be present in spermatozoa (Macias Garcia *et al.* 2012, Mendoza *et al.* 2013)

Moreover we demonstrated for the first time the presence of Pannexin-1 channel in stallion spermatozoa, and a putative role in early stages of membrane destabilization is proposed as evidenced in somatic cells (Elliott *et al.* 2009, Chekeni *et al.* 2010); in addition, since YoPro-1 uptake occurs through pannexin-1 channels, the value of the YoPro-1 assay in the detection of this early stages of apoptosis was evidenced. Recently we demonstrated a high and negative correlation between YoPro+ spermatozoa and ATP content in stallion spermatozoa (Balao da Silva *et al.* 2013), further supporting this theory, since ATP depletion in early apoptotic cells occur through pannexin-1 channels as well (Elliott *et al.* 2009)

Apoptotic mechanisms are involved in cryodamage in mammalian spermatozoa (Ortega Ferrusola *et al.* 2010, Said *et al.* 2010) and recently anti-apoptotic proteins have been successfully used to improve sperm survival after thawing (Shimokawa *et al.* 2012). In our experiment, Akt inhibition was not accompanied with increased ROS production and DNA fragmentation, contrary to findings in humans where inhibition of PI3K was accompanied with increased mitochondrial production of reactive oxygen species and oxidative DNA damage, but without changes in mitochondrial membrane potential (Koppers *et al.* 2011).

This discrepancy could be attributed to species differences and/or to the differential effect of the inhibitor used in the PI3K/AKT pathway, since we inhibited specifically Akt, that is located downstream of PI3K. Inhibition of PI3K may also affect other downstream targets. In relation to this, inhibition of PI3K results in an increase in cAMP levels and in the tyrosine phosphorylation of PKA-anchoring protein AKAP3 in human spermatozoa, suggesting a negative role for PI3-K in the regulation of motility (Luconi *et al.* 2001, du Plessis *et al.* 2004, Luconi *et al.* 2005). Moreover, studies in pig spermatozoa showed no effect of PI3K on the percentage of motile or progressive motile spermatozoa, but evidenced an increase in sperm velocity (Aparicio *et al.* 2005). In addition, both Wortmannin and LY294002 lack specificity and provoke different responses on spermatozoa (Nauc *et al.* 2004). A recent study(Pujianto *et al.* 2010) showed more intense changes in caspase activation and motility decreases after incubation of human spermatozoa in presence of an Akt inhibitor than in presence of the PI3K inhibitor Wortmannin. In contrast to human sperm, Akt inhibition in stallion spermatozoa decreased mitochondrial membrane potential but did not lead to oxidative stress, thus supporting the existence of species-specific features in the apoptotic mechanisms in sperm senescence. Previous reports from our laboratory indicate that the mitochondrial permeability transition pore has a role in the apoptotic mechanism associated to sperm senescence induced by cryopreservation (Ortega Ferrusola *et al.* 2010); agreeing with the role of Akt preventing translocation of Bax from the cytosol to the mitochondria.

Inhibition of Akt did not result in significant increases of DNA fragmentation, suggesting that this apoptotic mechanism does not lead to DNA damage, as has previously been suggested in human spermatozoa (Aitken & Koppers 2011, Koppers *et al.* 2011, Aitken *et al.* 2012). This has been attributed to the special architecture of the spermatozoa that prevents endonucleases activated during the apoptotic process at the mid-piece, to reach the sperm nucleus (Koppers *et al.* 2011). Another possible explanation arises due to the relatively high resistance of stallion spermatozoa to oxidative insults when compared to those in human (Ortega Ferrusola *et al.* 2009), a factor that probably relates to the different lipid composition of the sperm membranes between species (Garcia *et al.* 2011, Macias Garcia *et al.* 2011). Multicolor flow cytometry for the study of sperm biology is worth mentioning, alongside the development of new assays for the simultaneous evaluation of several sperm characteristics and to improve the discrimination from debris in the analysis, a major concern in many flow cytometric sperm assays (Petrunkina & Harrison 2010, Petrunkina & Harrison 2011b, Petrunkina & Harrison 2011a). In

short, inhibition of a sperm-specific apoptotic mechanism is involved in the survival of stallion spermatozoa after ejaculation; this special form of apoptosis in spermatozoa could well be termed *spermptosis*. Regulation of sperm survival after ejaculation emerges as an interesting area of research with relevant implications for the study of infertility in humans, particularly for the lack of studies dealing with intrinsic sperm dysfunction, and developments of reproductive technologies in animals. In addition, stallion spermatozoa experiences an apoptosis phenomenon leading to cellular senescence after ejaculation sharing biochemical and molecular characteristics with human sperm, thus emerging as a suitable model for the study of apoptotic mechanisms involved in male factor human infertility

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FIGURES

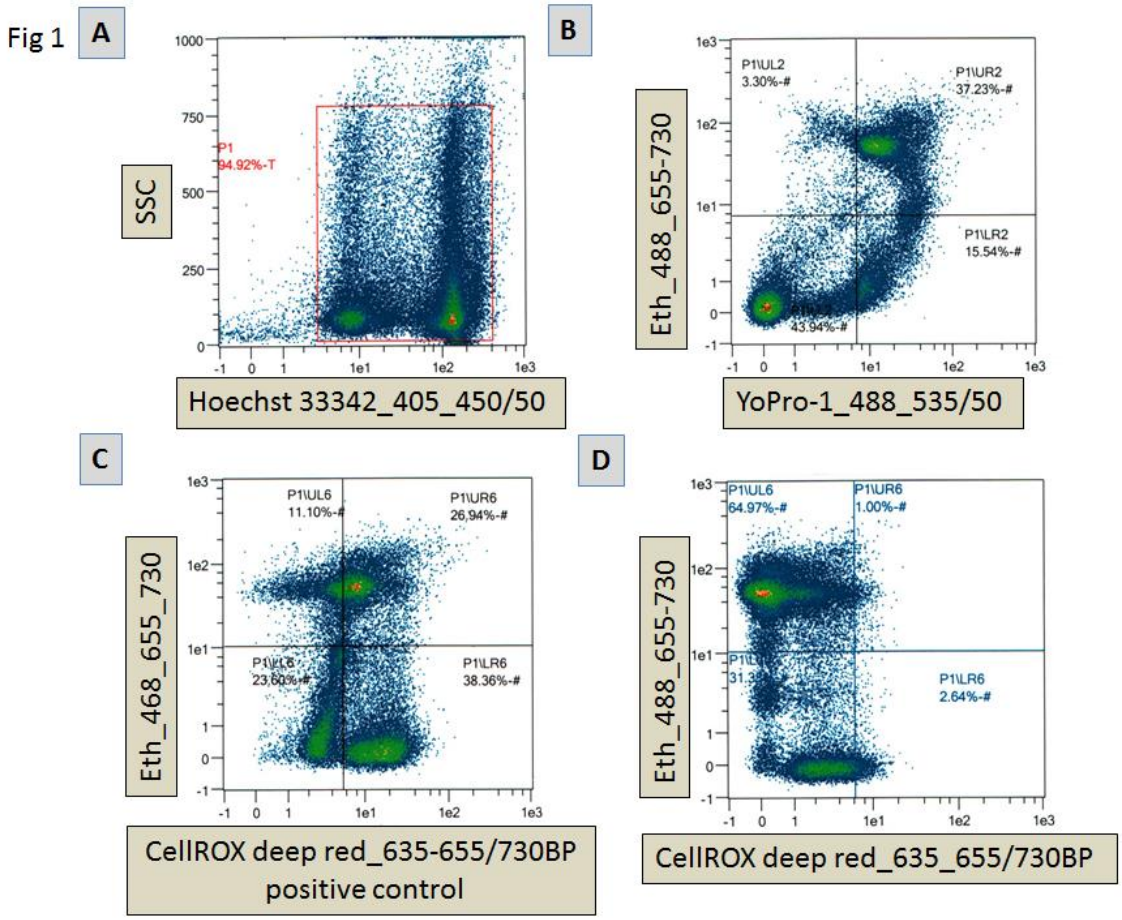

FIGURE 1. Representative cytograms of the simultaneous flow cytometric assessment of subtle membrane changes, viability and reactive oxygen species (ROS). Samples were stained with Hoechst 33342, YoPro-1, Ethidum Homodimer and CellROX as described in material and methods. Hierarchical gating was applied to exclude debris from the analysis and simultaneously measure viability, apoptosis, necrosis and ROS. (A), Hoescht 33342 fluorescence was detected at V1 channel (Ex 405 Band Pass filter 450/50 nm), and a gate was applied to positive (DNA containing particles) events to gate out debris. Then, the gated region was analyzed (B) YoPro-1 was detected on B1 channel (Ex 488 Band Pass Filter 525/50), ethidium homodimer was detected in B3 (Ex 488 Band pass filter 655-730nm) and (C) CellROX was detected in R1 (Ex 635 nm Band pass filter 655-730 nm). (D) Positive controls for oxidative stress (800µM plus 200µL H_20_2)

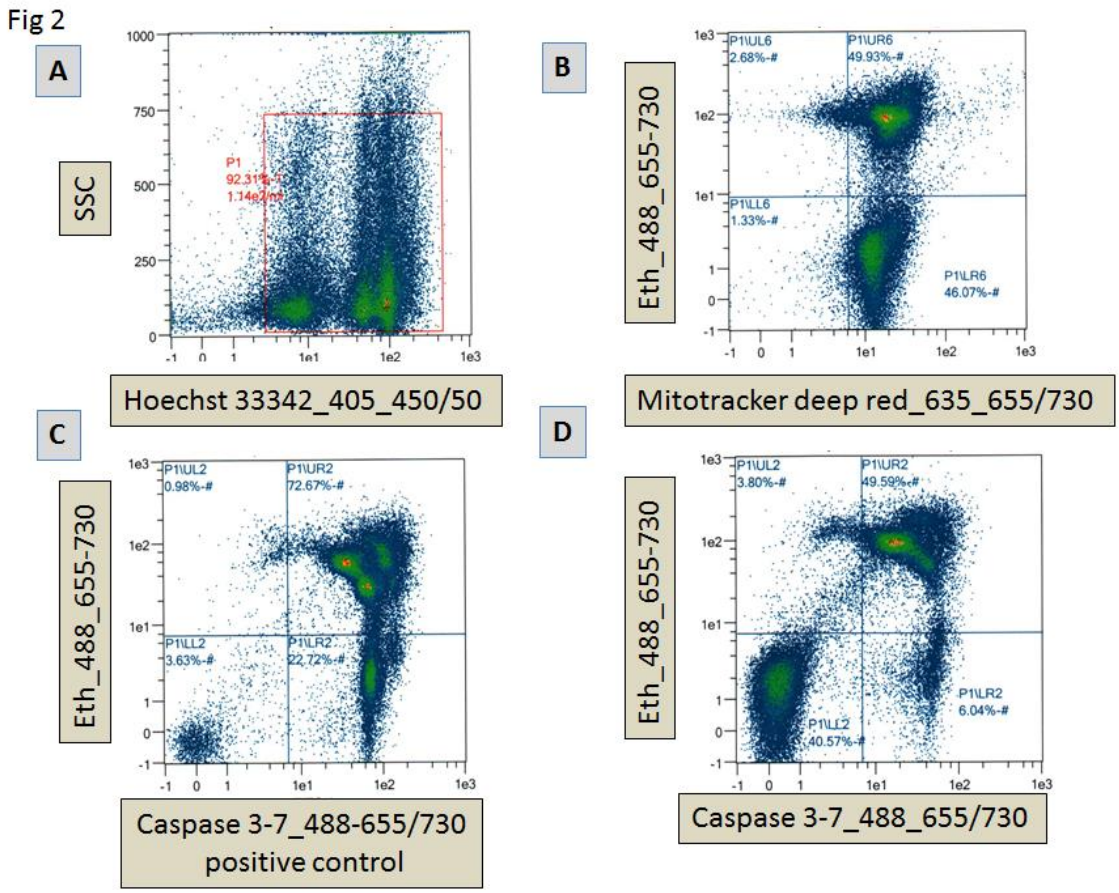

FIGURE 2. Representative cytograms of the simultaneous flow cytometric assessment of caspase 3 and 7 and mitochondrial activity in stallion spermatozoa. Samples were stained with CellEvent ,ethidium homodimer, mitotracker deep red and Hoechst 33342 as described in material and methods. Hierarchical gating was applied to exclude debris from the analysis and simultaneously measure viability, caspase activity, necrosis and mitochondrial activity. (A), Hoescht 33342 fluorescence was detected at V1 channel (Ex 405 Band Pass filter 450/50 nm), and a gate was applied to positive (DNA containing particles) events to gate out debris. Then, the gated region was analyzed (B) Caspase 3 and 7 were detected on B1 channel (Ex 488 Band Pass Filter 525/50), ethidium homodimer was detected in B3 (Ex 488 Band pass filter 655-730nm) and (C) Mitotracker deep red was detected in R1 (Ex 635 nm Band pass filter 655-730 nm). (D) Caspase 3 and 7 positive controls (frozen thawed sperm).

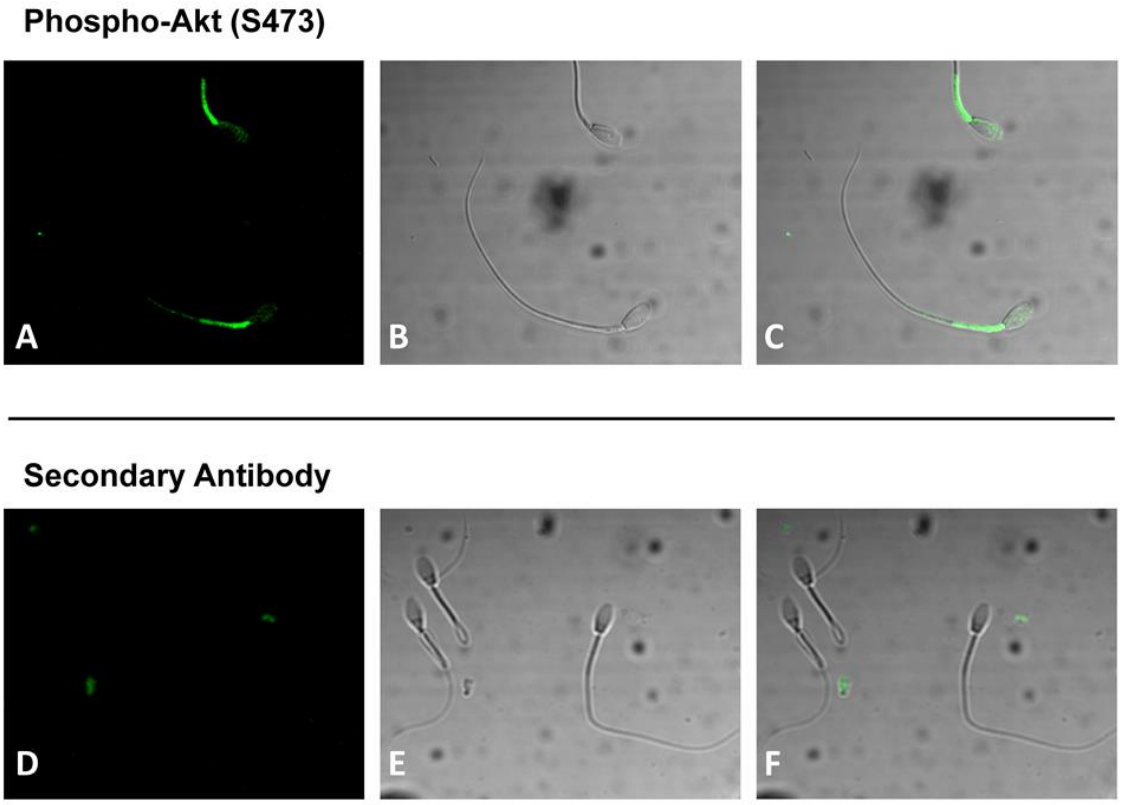

FIGURE 3. Subcellular localization of Phospo Akt in stallion spermatozoa. Ejaculated stallion spermatozoa were washed and stained using specific anti phospho Akt antibodies and visualized under confocal laser microscopy as described in material and methods. Phosporylated Akt was present in the post acrosomal region and mid piece. A Transmission image, B Florescence recorded at 540/30 emission filter, C merged images.

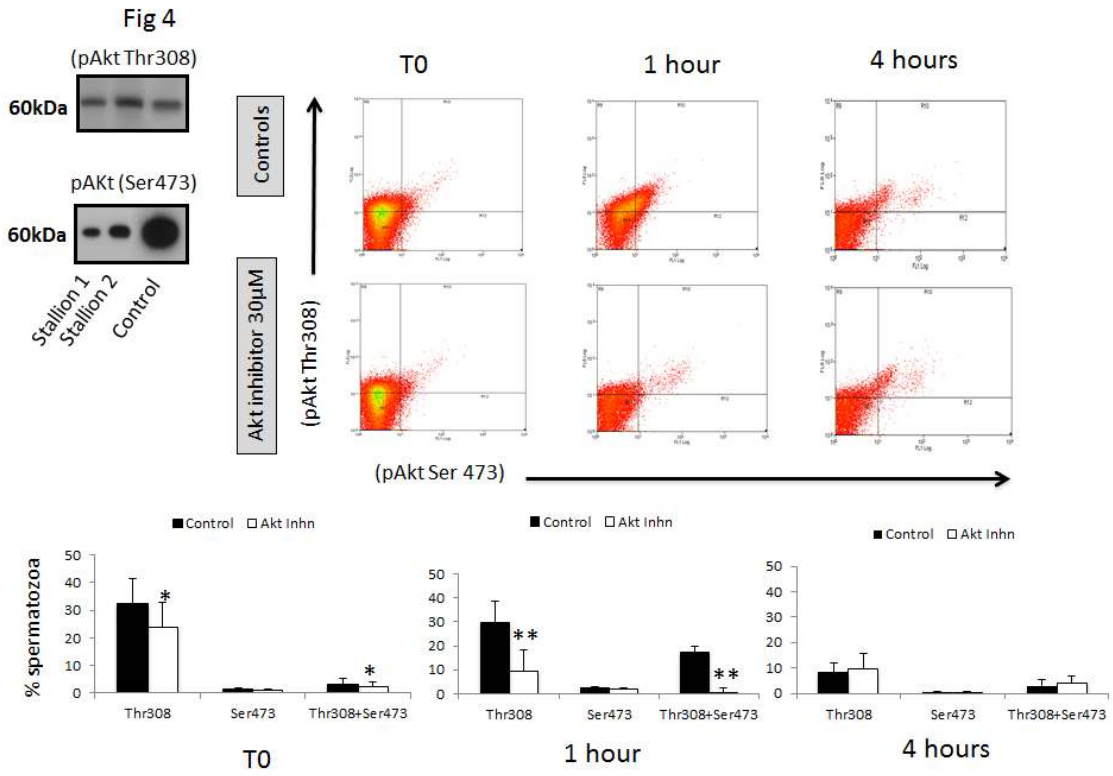

FIGURE 4. Effect of the Akt inhibitor SH5 on Akt phosphorylation at Ser473 and Thr308 of stallion spermatozoa. Stallion spermatozoa were washed and incubated in presence of Akt inhibitor (0 and 30 µM), up to four hours, then were stained with specific antiphospho Akt antibodies (Phospho-Akt (Ser 473) –Alexa Fluor 488 conjugate and Phospho-Akt (Thr308) Alexa fluor 647 conjugate as described in material and methods. Samples were analyzed flow cytometrically. Events in the lower right quadrant represent spermatozoa positive for pAkt Ser 473, events in upper right quadrant represent spermatozoa positive for pAkt Ser 473 and Thr308 simultaneously and event in upper left quadrant represent spermatozoa positive for pAkt Thr308. The specificity of the antibodies was validated using western blotting (upper left corner) as described in material and methods, lanes 1 and 2 represent sperm lysates from two different stallions, lane 3 positive controls (pancreatic acini)

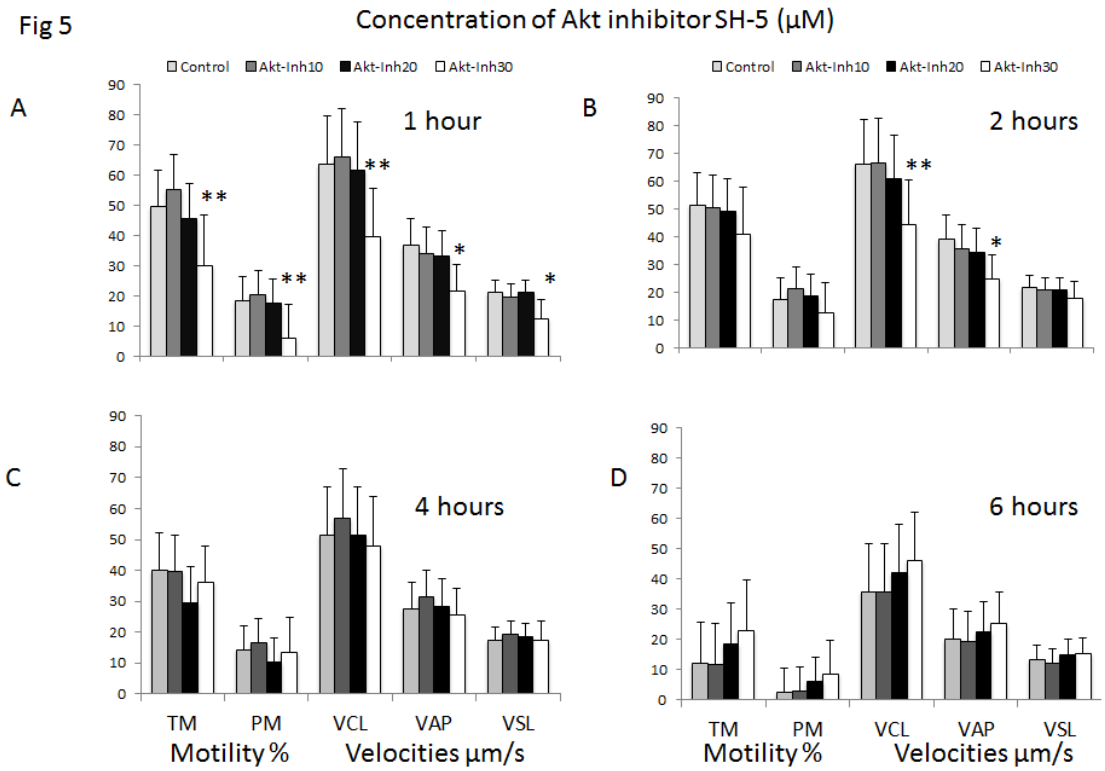

FIGURE 5. Effect of inhibition of Akt phosphorylation on stallion sperm motility and kinematics. Stallion spermatozoa were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 µM for up to 6 hours. **A** after 1 hour of incubation at 37ºC, **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC. TM %percentage of total motile spermatozoa, PM % percentage of progressive motile spermatozoa, VCL (µm/s) circular velocity, VAP (µm/s) average velocity VSL (µm/s) straight line velocity. ** $p<0.01$, * $p<0.05$

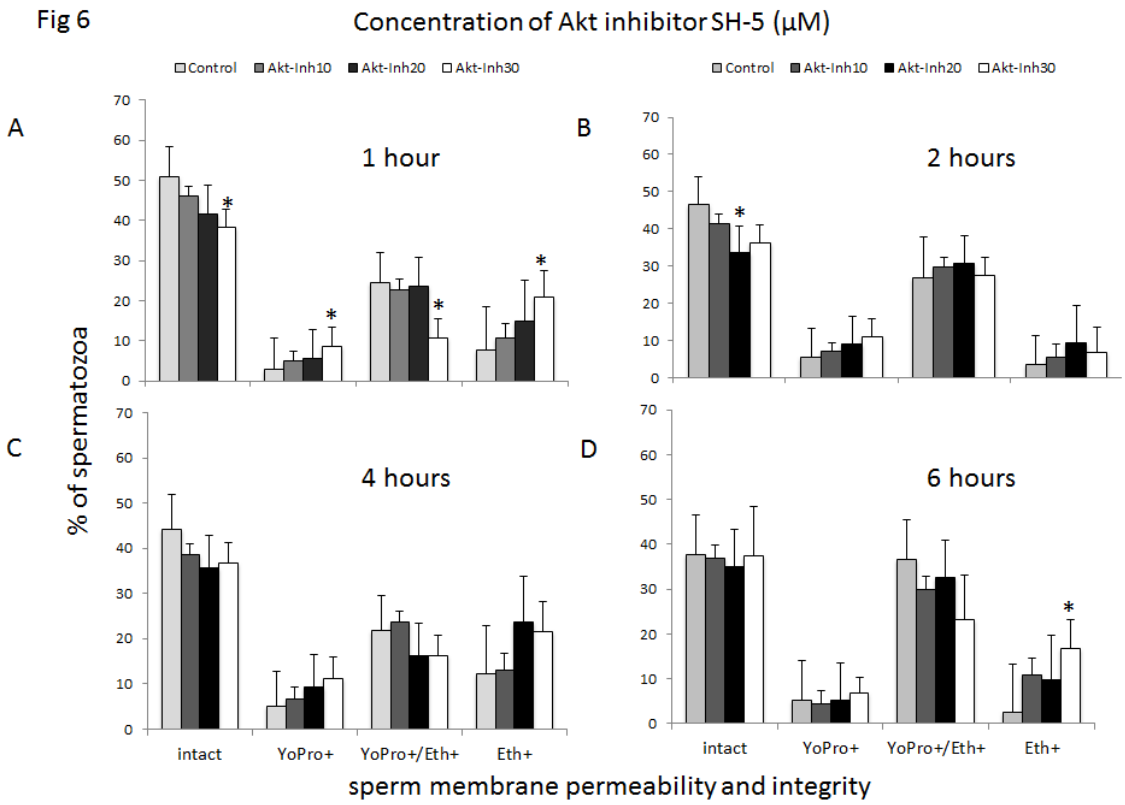

FIGURE 6. Effect of inhibition of Akt on stallion sperm membrane permeability and integrity. Stallion spermatozoa were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 hours. **A** after 1 hour of incubation at 37ºC, **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC. Intact % percentage of spermatozoa with completely intact membranes YoPro+ percentage of spermatozoa with increased permeability in their membranes, YoPro+/Eth+ percentage of apoptotic spermatozoa Eth+ percentage of dead spermatozoa. * $p<0.05$

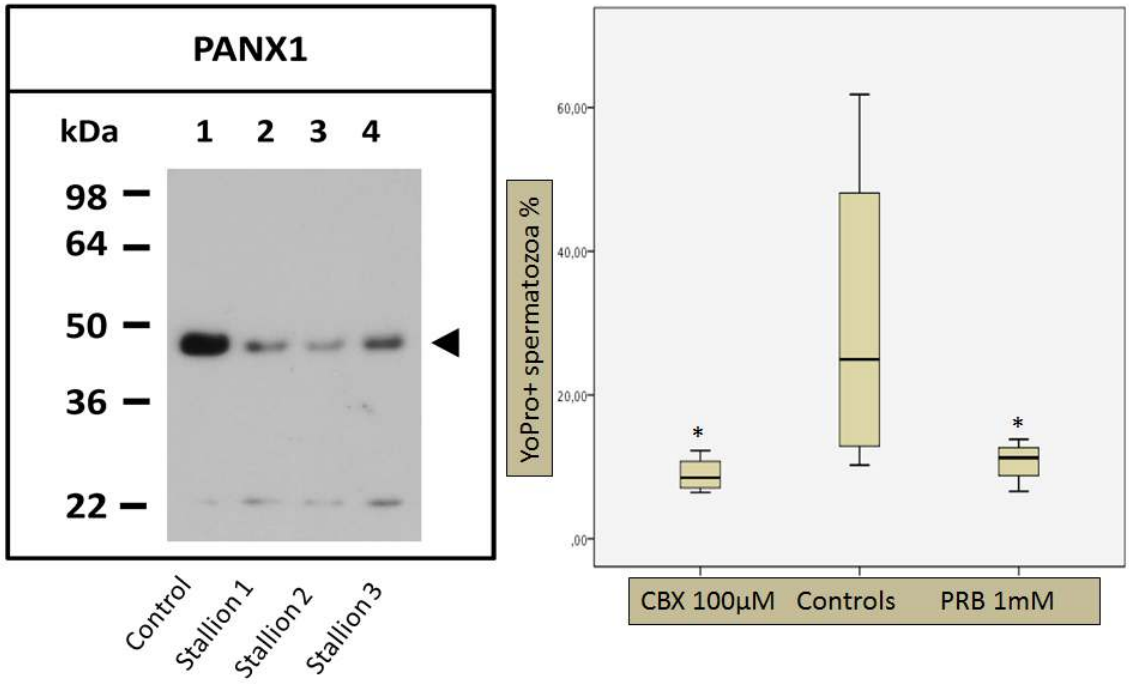

FIGURE 7. Identification and function of the Pannexin-1 channel in stallion spermatozoa. Left panel western blotting showing the presence of pannexin-1 in stallion sperm lysates, lane -1 control, lanes 2-4 sperm lysates from three different stallions. Right panel whisker and box graphics showing the effect of two different blockers of the pannexin-1 channel in the uptake of YoPro-1 by stallion spermatozoa. The uptake of YoPro-1 was significantly inhibited by both inhibitors (* $p<0.05$)

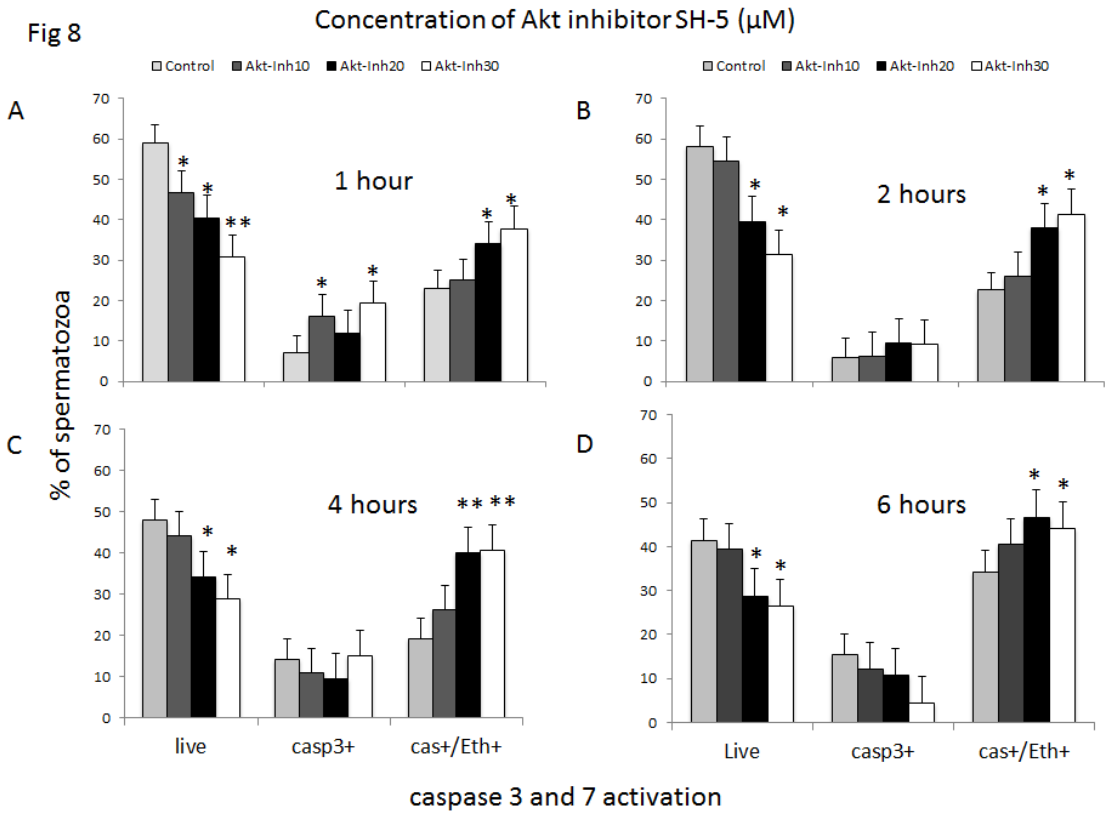

FIGURE 8. Effect of inhibition of Akt on caspase 3 and 7 activation in stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 hours. **A** after 1 hour of incubation at 37ºC, **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC. Live % percentage of live spermatozoa, casp3+ % percentage of live spermatozoa with activated caspases 3 and 7, cas+/Eth+% percentage of dead spermatozoa with activated caspases 3 and 7. ** $p<0.01$, * $p<0.05$

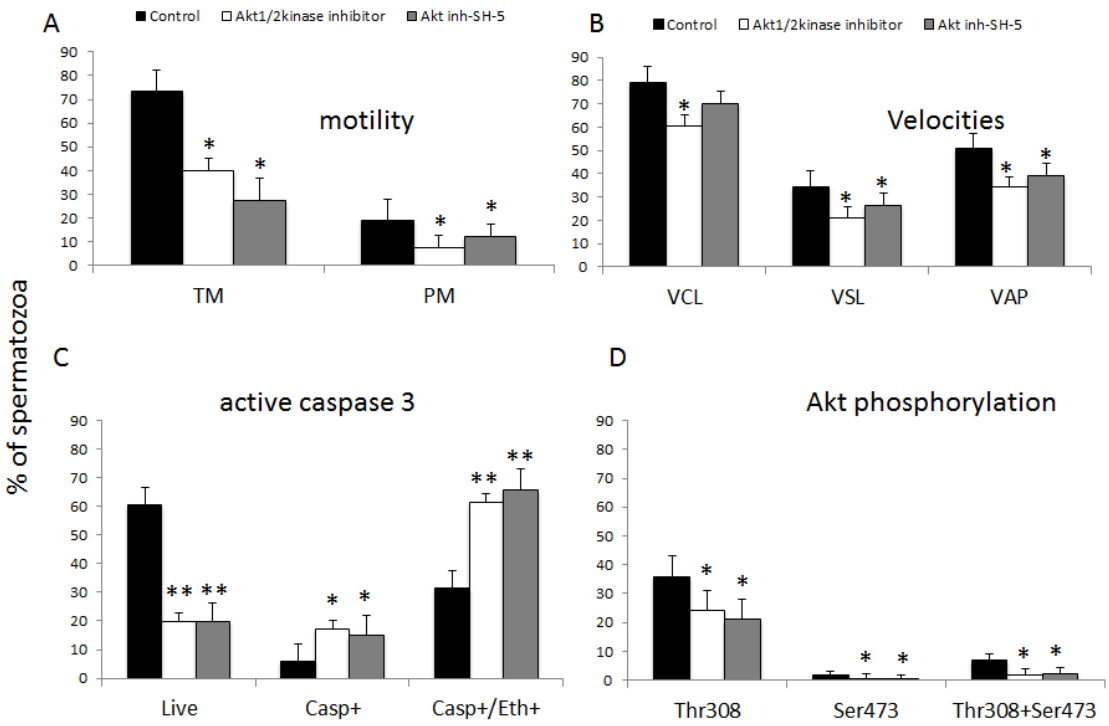

FIGURE 9. Effect of two different inhibitors of Akt (Akt ½ kinase inhibitor and SH-5) on motility, velocity, caspase 3 and 7 activation and Akt phosphorylation on stallion spermatozoa. Samples were washed and resuspended in BWW media, incubated for 1 hour at 37ºC in presence of 0 or 30 µM of Akt 1/2 kinase inhibitor and 30 µM of SH-5. A.- Effects in stallion sperm motility, TM % percentage of total motile sperm, PM % percentage of progressive motile sperm. B.- Effects of stallion sperm velocities, VCL (µm/s) circular velocity, VSL (µm/s) straight line velocity, VAP (µm/s) average velocity. C.- Effects on caspase 3 and 7 activation, live percentage of live sperm, Casp+ percentage of live sperm with active caspases, Casp+/Eth+ percentage of dead sperm with active caspases. D.- Effect on Akt phosphorylation Thr308 percentage of spermatozoa with Akt phosphorylated at threonine 308, Ser473 percentage of spermatozoa with Akt phosphorylated at serine473, The308+ Ser 473 percentage of spermatozoa with Akt phosphorylated simultaneously at threonine 308 and serine 473. * $p<0.05$, ** $p<0.01$.

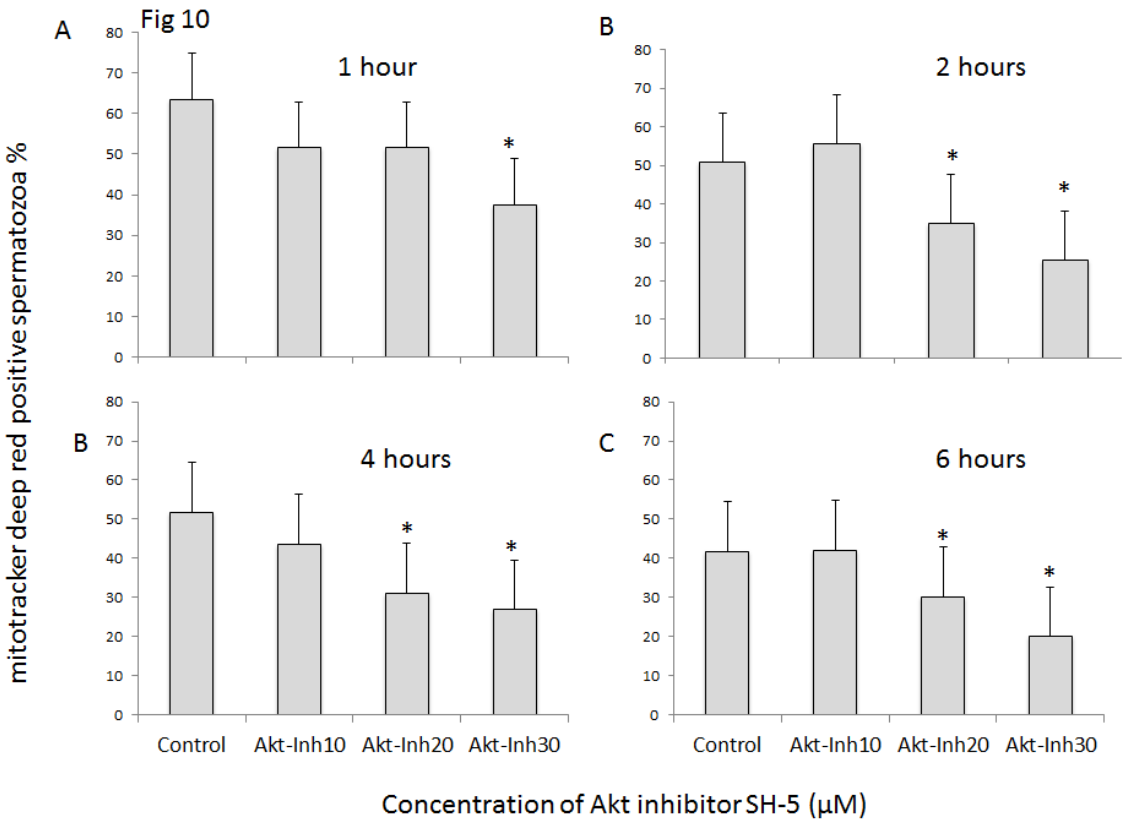

FIGURE 10. Effect of inhibition of Akt on the percentage of active mitochondria in stallion spermatozoa determined after mitotracker staining. Samples were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 hours **A** after 1 hour of incubation at 37ºC **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC. * $p<0.05$

Fig 11

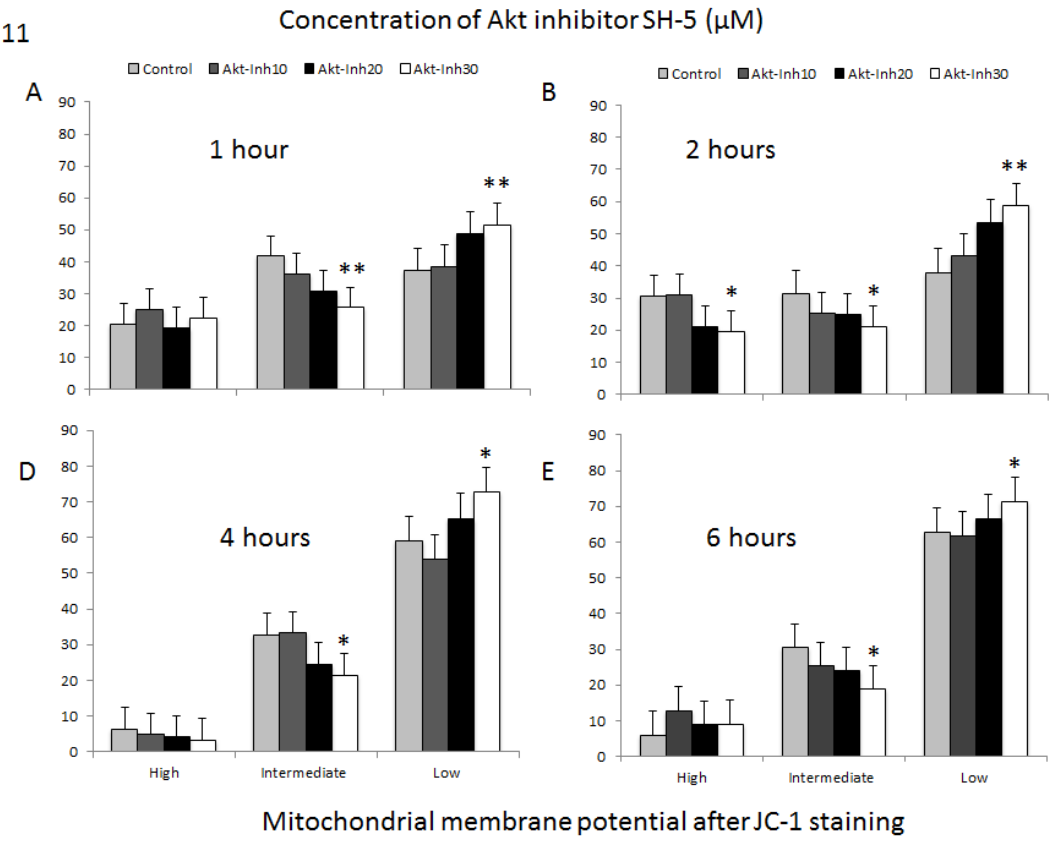

FIGURE 11. Effect of inhibition of Akt on the percentage of active mitochondria in stallion spermatozoa determined after JC-1 staining. Samples were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 hours. **A** after 1 hour of incubation at 37ºC **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC. High % of spermatozoa with high mitochondrial membrane potential, Intermediate % percentage of spermatozoa depicting simultaneously mitochondria with high and low potential, Low % percentage of spermatozoa showing low potential mitochondria. ** $p<0.01$, * $p<0.05$

Fig 12

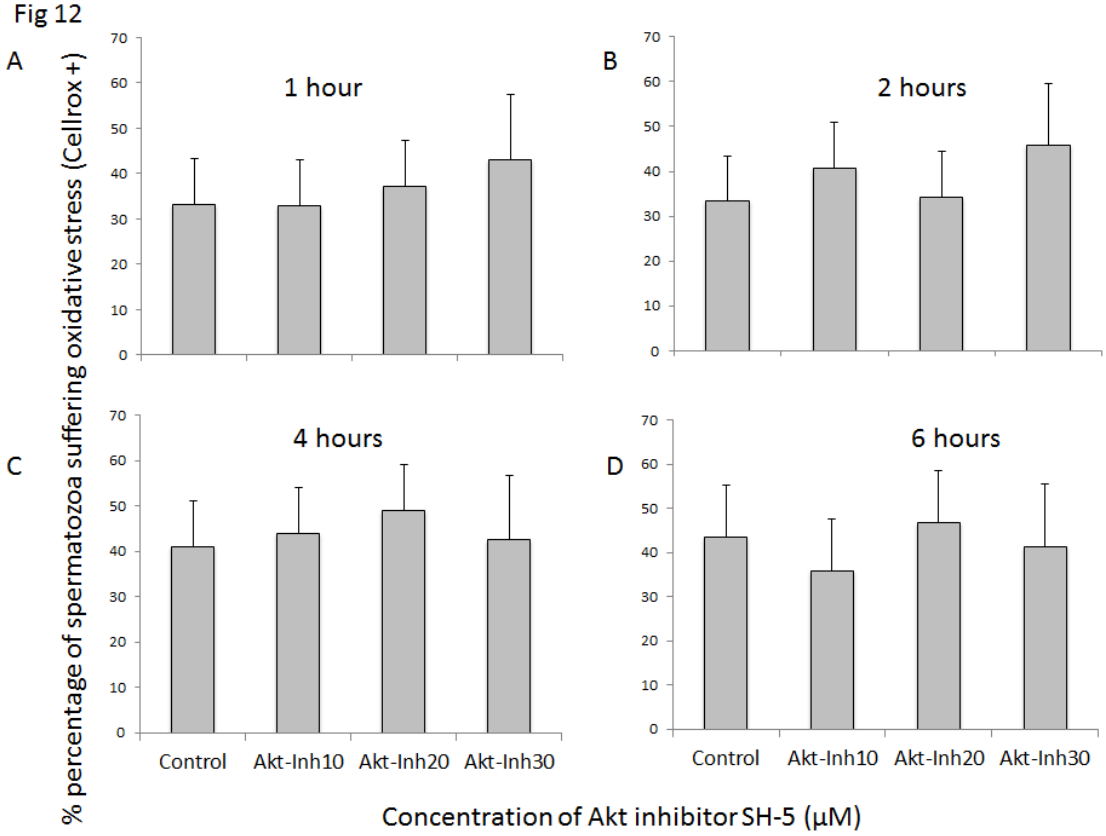

FIGURE 12. Effect of inhibition of Akt on the percentage of stallion spermatozoa showing oxidative stress. Samples were washed and resuspended in BWW supplemented with 1% PVA in presence of Akt inhibitor SH5 at 0, 10, 20 and 30 µM for up to 6 hours **A** after 1 hour of incubation at 37ºC **B** after 2 hours, **C** after 4 hours and **D** after 6 hours of incubation at 37ºC.