Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7

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Abstract

AKT, also referred to as protein kinase B (PKB or RAC), plays a critical role in controlling cell survival and apoptosis. To gain insights into the mechanisms regulating sperm survival after ejaculation, the role of AKT was investigated in stallion spermatozoa using a specific inhibitor and a phosphoflow approach. Stallion spermatozoa were washed and incubated in Biggers–Whitten–Whittingham medium, supplemented with 1% polyvinyl alcohol (PVA) in the presence of 0 (vehicle), 10, 20 or 30 μ M SH5, an AKT inhibitor. SH5 treatment reduced the percentage of sperm displaying AKT phosphorylation, with inhibition reaching a maximum after 1 h of incubation. This decrease in phosphorylation was attributable to either dephosphorylation or suppression of the active phosphorylation pathway. Stallion spermatozoa spontaneously dephosphorylated during *in vitro* incubation, resulting in a lack of a difference in AKT phosphorylation between the SH5-treated sperm and the control after 4 h of incubation. AKT inhibition decreased the proportion of motile spermatozoa (total and progressive) and the sperm velocity. Similarly, AKT inhibition reduced membrane integrity, leading to increased membrane permeability and reduced the mitochondrial membrane potential concomitantly with activation of caspases 3 and 7. However, the percentage of spermatozoa exhibiting oxidative stress, the production of mitochondrial superoxide radicals, DNA oxidation and DNA fragmentation were not affected by AKT inhibition. It is concluded that AKT maintains the membrane integrity of ejaculated stallion spermatozoa, presumably by inhibiting caspases 3 and 7, which prevents the progression of spermatozoa to an incomplete form of apoptosis.

Free Spanish abstract

A Spanish translation of this abstract is freely available at <http://www.reproduction-online.org/content/148/2/221/suppl/DC1>.

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Introduction

Artificial insemination with chilled transported semen is widely used in the equine industry, with most breeding registries now accepting this reproductive technology (Peña *et al.* 2012). However, the viability and fertilisation capacity of cooled stallion spermatozoa are typically maintained for only a few days or, in some cases, only several hours (Macías García *et al.* 2012). Apoptosis is a form of programmed cell death that is executed by caspases, a family of cysteine proteases that are activated through two major pathways. Caspases are intrinsically activated by cellular stress through the liberation of mitochondrial pro-apoptotic proteins that activate caspase 9. Caspases are also extrinsically activated by ligand-activated death receptors that

induce cleavage of caspase 8 (Aitken *et al.* 2011). The occurrence of apoptosis in ejaculated spermatozoa remains under debate. The theory of abortive apoptosis considers spermatozoal apoptosis as a remnant from spermatogenesis (Sakkas *et al.* 2002) on the basis that spermatozoa are terminal, transcriptionally silent cells. This theory is contradicted by findings 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.* 2009a,b, Said *et al.* 2010, Aitken *et al.* 2012). Thus, the apoptosis phenomenon can be triggered in spermatozoa after ejaculation through the intrinsic apoptotic pathway (Peña *et al.* 2003a). Determining whether apoptosis occurs in ejaculated spermatozoa may have substantial

implications for human and animal-assistive reproductive biotechnologies where sperm are manipulated for extended periods of time (Peña *et al.* 2006, Ortega-Ferrusola *et al.* 2009c).

AKT, also referred to as protein kinase B (PKB or RAC), plays a critical role in controlling survival and apoptosis in somatic cells (Hers *et al.* 2011). Growing evidence suggests that AKT 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, phosphatidylinositol 3-kinase (PIK3CA (PI3K)), has been the subject of several studies in sperm biology, AKT has received insufficient attention despite recent research identifying it as a key factor for sperm survival after ejaculation. AKT suppresses sperm capacitation and prevents spermatozoa from entering the apoptotic cascade that leads to sperm senescence and death (Pujianto *et al.* 2010). We recently demonstrated that after cryopreservation (Ortega-Ferrusola *et al.* 2008, 2010) and chilling (Macías García *et al.* 2012), stallion sperm death occurs through apoptosis-related phenomena. In view of these findings and to determine the role of AKT in regulating the survival of ejaculated stallion spermatozoa, we investigated stallion sperm survival using a specific inhibitor and a phosphoflow cytometry (flow cytometry-based approaches to measure changes in the phosphorylation status of key intracellular signalling molecules, at a single-cell level) approach involving antibodies against phosphorylated forms of a particular protein (Tazzari *et al.* 2002, Krutzik *et al.* 2004, Schulz *et al.* 2007, 2012). Our results indicate that survival of ejaculated spermatozoa is regulated by AKT through the inhibition of an apoptosis-like mechanism. This mechanism, previously proposed in humans (Aitken *et al.* 2011), may result in the silent removal of defective spermatozoa from the female genitalia (Lessig *et al.* 2007). If a similar mechanism is conserved in horses, it may be possible to use equine spermatozoa to model human spermatozoa, particularly in areas that are less explored in humans, such as sperm defects and preservation. Moreover, we demonstrate the utility of the phosphoflow approach for studying sperm biology.

Material and methods

Reagents and media

Ethidium homodimer (Eth), 5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl benzimidazolyl carbocyanine iodine (JC-1), Mitrotracker Deep Red, YO-PRO-1, CellEvent caspases 3 and 7 detection reagent, CellROX Deep Red, MitoSox Red, Sytox Green and the TUNEL assay were purchased from Molecular Probes (Leiden, The Netherlands). The AKT inhibitor SH5 and the OxyDNA Assay Kit were purchased from Calbiochem (San Diego, CA, USA). The AKT1/2 kinase inhibitors carbenxolone and probenecid were purchased from Sigma. Anti-pannexin-1 was purchased from Abcam (Cambridge, UK). Anti-phospho-

AKT (pSer⁴⁷³) and anti-phospho-AKT (pThr³⁰⁸) Alexa Fluor 488- and Alexa Fluor 647-conjugated MABs and IgG XP Isotype Controls (Alexa Fluor 488 and Alexa Fluor 647 conjugates) were purchased from Cell Signalling Technology (Danvers, MA, USA).

Semen collection and processing

Semen was obtained from seven Pure Spanish horses (PRE; three ejaculates each) that were individually housed at the Veterinary Teaching Hospital of the University of Extremadura (Cáceres, Spain). Stallions were maintained according to institutional and European regulations. Ejaculates were collected on a regular basis (two collections per week) during the 2012 breeding season, using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. The semen samples were immediately transported to the laboratory for evaluation and processing. The ejaculate samples were diluted 1:2 in INRA-96 (IMV, L'Aigle, France) centrifuged (600 g, 10 min) and re-suspended in Biggers–Whitten–Whittingham (BWW; Macías García *et al.* 2011a,b) medium supplemented with 1% polyvinyl alcohol (PVA) to obtain a concentration of 50×10^6 spermatozoa/ml. All experiments followed a split sample design with every ejaculate sample divided between the control and the treatment groups.

To study the role of AKT in the maintenance of stallion sperm motility and viability, split samples of stallion spermatozoa were incubated in BWW medium in the 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 30 μ M in DMSO) for up to 6 h at 37 °C in BWW medium supplemented with 1% PVA. The final concentration of DMSO in all samples was 0.01%. SH5 is a cell permeable, reversible, substrate-competitive phosphatidylinositol analogue that inhibits AKT activation and the activation of select downstream substrates without decreasing phosphorylation of PDK-1 or other kinases downstream of RAS, such as MAPK. SH5 has been widely used as specific inhibitor of AKT in many cell lines (Lan *et al.* 2011, Busch *et al.* 2012, Qiao *et al.* 2013, Yang *et al.* 2013), acting as a potent inducer of apoptosis that selectively kills various types of cancer cells that contain high levels of active AKT. We used a second inhibitor, a AKT1/2 inhibitor, in additional experiments (Gilot *et al.* 2010). The AKT phosphorylation status was monitored by flow cytometry. All experiments were repeated using three different ejaculates from each of the seven stallions (21 replicates in total).

Western blotting

Stallion semen was centrifuged and washed twice with PBS. After washing, the sperm cells were sonicated for 5 s at 4 °C in 100 μ l of lysis buffer consisting of 50 mM Tris/HCl, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.4 mM EDTA, a protease inhibitor cocktail (Complete, EDTA-free, cat no. 11836170001, Roche Diagnostics GmbH) and 0.2 mM Na₃VO₄, pH 7.5. The homogenates were clarified by centrifugation at 10 000 g (15 min, 4 °C), and the

supernatants were used for protein analysis after dilution with 4×SDS sample buffer. Total protein samples (25 µg/well) from stallion sperm were fractionated by SDS–PAGE using 4–20% polyacrylamide gradient gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4 °C with anti-pannexin-1 (1:1000), anti-pAKT Ser⁴⁷³ (1:1000) and anti-pAKT Thr³⁰⁸ (1:1000). On the following day, the membranes were washed twice and incubated for 45 min at 25 °C with anti-rabbit IgG HRP-conjugated secondary antibody. The membranes were then washed again, incubated with ECL detection reagents and finally were used to expose Hyperfilm ECL Films (Amersham). The positive controls for pAKT Ser⁴⁷³ and Thr³⁰⁸ were pancreatic acini lysates. The positive controls for pannexin-1 were brain lysates. The intensity and molecular weight of the bands were quantified using Scion Image Software for Windows, version 4.02 (Scion Corp., Frederick, MD, USA) and normalised against the values for β-actin.

Immunocytochemistry

Spermatozoa were washed and suspended in PBS, with an adjustment of the cell concentration to 1×10^6 cells/ml. Fifteen microlitres of the sperm suspension were spread on poly-L-lysine-coated slides and allowed to attach for 10 min. The cells were then fixed with 3% formaldehyde in PBS for 15 min and permeabilised with 0.2 Triton X-100 in PBS for 5 min. Slides were washed three times for 10 min each with PBS and incubated in PBS supplemented with 5% BSA (w/v) for 90 min to block non-specific sites. After blocking, the slides were incubated overnight at 4 °C with anti-phospho-AKT Ser⁴⁷³ (1:50) diluted in PBS containing 5% BSA (w/v). On the next day, samples were extensively washed with PBS and further incubated with an Alexa 488-conjugated goat anti-rabbit antibody for 45 min at room temperature (RT). Finally, the slides were washed with PBS and examined with a Bio-Rad MRC1024 confocal microscope with a 60× objective in oil immersion. The samples were excited at 488 nm with an argon laser and emission was recorded using a 515 nm long-pass filter set. Samples without any primary antibody were assessed to confirm the absence of non-specific staining.

Sperm motility

Sperm motility and kinematics were assessed using a CASA system (ISAS Proiser, Valencia, Spain; Peña *et al.* 2005a, Nuñez-Martínez *et al.* 2007a, Gonzalez-Fernandez *et al.* 2009). Semen was loaded in a Leja chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a warmed (37 °C) stage. The analysis was based on an examination of 60 consecutive digitalised images obtained from each field using a 10× negative phase-contrast objective. At least three different fields were recorded to ensure that at least 200 spermatozoa were analysed per sample. Images were taken at 1-s intervals. Objects incorrectly identified as spermatozoa were excluded by observation using the playback function. Spermatozoa with a VAP of <15 µm/s were considered immotile, whereas spermatozoa with a VAP >15 µm/s were considered motile. Spermatozoa deviating

<45% from a straight line were designated as linearly motile and spermatozoa with a circular velocity (VCL) >45 µm/s were designated as rapid sperm. The absolute and re-calculated kinematic parameters measured by CASA included the following (Martínez-Pastor *et al.* 2011): curvilinear velocity, the time-average velocity of the sperm head along its actual trajectory (VCL (µm/s)); straight line velocity, the time average velocity of the sperm head along a straight line from its first-detected position to its last-detected position (VSL (µm/s)) and average path velocity, the time average velocity of the sperm head along its average trajectory. The average trajectory is computed by smoothing the average trajectory (VAP (µm/s)).

Flow cytometry

Flow cytometric analyses were conducted using a MACSQuant Analyser 10 (Miltenyi Biotech, Madrid, Spain) flow cytometer, equipped with three lasers emitting at 405, 488 and 635 nm and ten photomultiplier tubes (V1 (excitation 405 nm and emission 450/50 nm), V2 (excitation 405 nm and emission 525/50 nm), B1 (excitation 488 nm and emission 525/50 nm), B2 (excitation 488 nm and emission 585/40 nm), B3 (excitation 488 nm and emission 655–730 nm (655LP+split 730)), B4 (excitation 488 nm and emission 750 LP), R1 (excitation 635 nm and emission 655–730 nm (655LP+split 730)) and R2 (excitation 635 nm and emission filter 750 LP)). The system was controlled using MACSQuantify Software. Sperm subpopulations were divided by quadrants to quantify the frequency of each subpopulation. Forward and sideways light scatter were recorded for a total of 50 000 events/sample. Non-sperm events were eliminated by gating the sperm population after Hoechst 33342 staining. All positive events for Hoechst 33342 within a certain range of the side scatter (SCC) distribution were considered spermatozoa. The instrument was calibrated daily using specific calibration beads provided by the manufacturer, and compensation of spectral overlap was performed before each experiment. Compensation was performed before each particular experiment using negative (unstained) and positive (single stained) controls for each single-stained compensation control sample following published recommendations for multicolour flow cytometry (Baumgarth & Roederer 2000, Roederer 2001) and the instructions in the MacsQuant Instrument Software guide (Miltenyi Biotech). Also thresholds for each quadrant were determined using unstained, isotype controls and single-stained control samples for each of the probes used in each individual experiment.

Phosphoflow detection of phosphorylated AKT (Ser⁴⁷³) and AKT (Thr³⁰⁸) in stallion spermatozoa

The spermatozoa (1×10^6 /ml) were washed with saline–HEPES medium and fixed in 2% paraformaldehyde in PBS at RT for 15 min. After fixation, the cells were washed twice with PBS and once with PBS/1% BSA, permeabilised for 30 min using 0.1% saponin in PBS containing 1% BSA and incubated in the same buffer with 2 µl/ml phospho-AKT (Ser⁴⁷³) Alexa Fluor 488 conjugate (cat no. 4071, Cell Signalling Technology) and 2 µl/ml phospho-AKT (Thr³⁰⁸) Alexa Fluor 647 conjugate

(cat no. C31E5E) for 30 min in the dark at 22 °C. The samples were then washed in PBS. The pellets were resuspended in 500 µl of PBS and analysed using a MACSQuant flow cytometer (Miltenyi Biotech, Madrid, Spain). Hoechst 33342 was added to the restrict analysis to spermatozoa and to exclude debris. The specificity of the antibodies was validated using western blotting. Negative controls consisted of unstained samples and IgG XP isotype controls (Alexa Fluor 488 and 647 conjugates).

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), Eth-1 (1.167 mM) and CellRox (5 mM). Hoechst 33342 (1.62 mM in water) was used to identify spermatozoa and eliminate debris from the analysis. A sperm suspension (1 ml) containing 5×10^6 spermatozoa/ml was stained with 1 µl of Yo-Pro-1, 1 µl of CellRox and 0.3 µl of Hoechst 33342. After thorough mixing, the sperm suspension was incubated at RT in the dark for 25 min. The spermatozoa were then washed in PBS and 0.3 µl of Eth was added and incubated for 5 min before reading in the flow cytometer. This staining protocol was a modified version of previous protocols and distinguishes four sperm subpopulations while simultaneously measuring oxidative stress (Peña *et al.* 2005b, Nuñez-Martínez *et al.* 2007b, Ortega Ferrusola *et al.* 2009b). The first subpopulation, positive for only Hoechst 33342, was considered to be alive and without any membrane alterations. Another subpopulation, the Yo-Pro-1-positive cells emitting green fluorescence, showed signs of early damage and a shift to another physiological state, as membranes become slightly

permeable during the first steps of damage, thus enabling Yo-Pro-1 but not Eth to cross the plasma membrane. Neither probe enters intact cells. Finally, two subpopulations of dead spermatozoa were easily detected. These were either apoptotic (spermatozoa stained both with Yo-Pro-1 and Eth, emitting both green and red fluorescence) or necrotic (cells stained only with Eth, emitting red fluorescence). Spermatozoa exhibiting oxidative stress emit fluorescence in the far red spectrum. Representative cytograms of the assay are depicted in Fig. 1. The positive controls for oxidative stress were samples supplemented with 800 µM SO_4Fe and 200 µl of H_2O_2 (Sigma) to produce 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 absorption/emission maxima of ~502/530 nm. One important advantage of this assay is that no wash steps are required, thus preventing cell loss during washing. Stock solutions of CellEvent (2 mM in DMSO), Eth (1.167 mM in DMSO), Mitotracker Deep Red (0.5 µM in DMSO) and Hoechst 33342 (1.62 mM in water) were prepared. Spermatozoa (5×10^6 /ml) in 1 ml of PBS were stained with 1 µl of CellEvent, 0.3 µl of Hoechst 33342 and 0.3 µl of Mitotracker

Figure 1 Representative cytograms for simultaneous flow cytometric assessment of subtle membrane changes, viability and reactive oxygen species (ROS). Samples were stained with Hoechst 33342, Yo-Pro-1, ethidium homodimer (Eth) and CellROX Deep Red, as described in the 'Materials and methods' section. Hierarchical gating was applied to exclude debris from the analysis and simultaneously measure viability, apoptosis, necrosis and ROS. (A) Hoechst 33342 fluorescence was detected using the V1 channel (Ex 405 bandpass filter 450/50 nm), and a gate was applied to positive (DNA-containing particles) events to gate out debris. The gated region was analysed. (B) Yo-Pro-1 was detected using the B1 channel (Ex 488 bandpass Filter 525/50 nm), Eth was detected using the B3 channel (Ex 488 bandpass filter 655–730 nm) and (D) CellROX Deep Red was detected using the R1 channel (Ex 635 nm bandpass filter 655–730 nm). (C) Positive control for oxidative stress (800 µM FeSO_4 plus 200 µl of H_2O_2), $n=7$ stallions.

Figure 2 Representative cytograms for simultaneous flow cytometric assessment of caspases 3 and 7 and mitochondrial activity in stallion spermatozoa. Samples were stained using CellEvent, ethidium homodimer (Eth), Mitotracker Deep Red and Hoechst 33342, as described in the 'Materials and methods' section. Hierarchical gating was applied to exclude debris from the analysis and simultaneously measure viability, caspase activity, necrosis and mitochondrial activity. (A) Hoechst 33342 fluorescence was detected using the V1 channel (Ex 405 bandPass filter 450/50 nm), and a gate was applied to positive (DNA-containing particles) events to gate out debris. The gated region was analysed. (B) Mitotracker Deep Red was detected using the R1 channel (Ex 635 nm bandpass filter 655–730 nm). (C) Caspases 3- and 7-positive controls (frozen thawed sperm) and (D) Caspases 3 and 7 were detected using the B1 channel (Ex 488 bandpass Filter 525/50 nm), Eth was detected using the B3 channel (Ex 488 bandpass filter 655–730 nm), $n=7$ stallions.

Deep Red, and incubated in the dark at RT for 25 min. Next, 0.3 μ l of Eth was added to each sample. After incubation for 5 min, the samples were immediately run on the flow cytometer. Representative cytograms of the assay are shown in Fig. 2. Because cryopreservation induces caspase activity in stallion sperm (Ortega-Ferrusola *et al.* 2008), cryopreserved samples were used as positive controls (Fig. 2) for caspases 3 and 7.

Evaluation of mitochondrial membrane potential ($\Delta\Psi_m$)

The lipophilic cationic compound JC-1 has the unique ability to differentially label mitochondria with low and high membrane potential (Peña *et al.* 2003b, Ortega Ferrusola *et al.* 2009a,b, Ortega-Ferrusola *et al.* c). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange region (590 nm) when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers that emit in the green region (525–530 nm) when excited at 488 nm. For mitochondrial membrane potential staining, a 3 mM stock solution of JC-1 (Molecular Probes Europe) in DMSO was prepared. For each sperm sample, 1 ml of sperm suspension in PBS (5×10^6 /ml) was stained with 0.5 μ l of JC-1 stock solution. The samples were incubated at 37 °C in the dark for 40 min before flow cytometric analysis.

Determination of DNA oxidation using the 8-oxoguanine assay

This assay is based on the direct binding of a fluorescent probe to the DNA adduct 8-oxoguanine (OxyDNA Assay Kit,

Calbiochem), which is a major oxidation product and an important indicator of free radical-induced DNA damage and oxidative stress. The assay was carried out according to the manufacturer's instructions following previously published protocols (De Iuliis *et al.* 2009, Koppers *et al.* 2011). In brief, spermatozoa (5×10^6 /ml) were separated from each sample, washed in PBS and fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.6) for 15 min at RT. The cells were then washed twice in PBS and once in PBS containing 1% BSA. Permeabilisation was performed for 30 min in PBS containing 1% BSA supplemented with 0.1% saponin. Samples were washed with 1 ml of wash solution (1:25 dilution in water of the wash concentrate provided by the manufacturer). Staining with 100 μ l of 1 $\times$ FITC-conjugate (1:10 dilution of FITC-conjugate in the wash solution) was carried out for 60 min in the dark at RT. Finally, the cells were washed twice and resuspended in 1 ml of PBS for flow cytometric analysis. The amount of 8-oxoguanosine (excitation at 495 nm and emission at 515 nm) was measured in a MACSQuant Analyser 10 (Miltenyi Biotech, Bergisch Gladbach, Germany) as green fluorescence proportional to the oxidative damage to the DNA. Positive controls were produced by incubation of additional samples in 800 μ M Fe^{2+} and 200 μ M H_2O_2 .

MitoSox Red assay

The generation of mitochondrial superoxide anions was investigated using MitoSox Red (MSR, Molecular Probes) as previously described (Koppers *et al.* 2008, 2011). Stock solutions of MitoSox Red (5 mM 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 μ M MitoSox Red

and incubated for 15 min at 37 °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. MSR (red) and SYTOX Green (green) fluorescence were detected using 530/30 nm bandpass (green) and 585/42 nm bandpass (red) filters. Non-sperm-specific events were gated out after staining with Hoechst 33342 (0.1 µl of a 16 mM solution). In total, 10 000 cells were examined.

TUNEL assay

DNA cleavage may yield double- and single-stranded DNA breaks. Both types of break can be detected by labelling the free 3'-OH terminals with fluorescent nucleotides (labelled dUTPs) in an enzymatic reaction catalysed by TdT (Koppers *et al.* 2011). The samples were washed in PBS and fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.6) for 15 min at RT. The cells were then washed in PBS and maintained in 5 ml of 70% methanol at -20 °C. For recognition of DNA fragmentation in apoptotic cells, an APO-BrdU TUNEL Assay Kit (Invitrogen, Molecular Probes) was used. This kit allows detection of 3'-hydroxyl ends that serve as starting points for TdT, which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analogue BrdUTP to the TdT reaction labels the break sites. Once incorporated into the DNA, BrdUTP is detected by an anti-BrdUTP antibody, which is labelled by Alexa Fluor 488 (495 nm excitation and 519 nm emission). The total cellular DNA content is measured using propidium iodide (excitation 535 nm and emission 617 nm). The frozen cells were washed twice in wash buffer and resuspended in the DNA-labelling solution for 60 min at 37 °C in a water bath. The suspensions were washed twice in rinse buffer, and the Alexa Fluor 488-labelled anti-BrdU antibody was added to each suspension. The samples were incubated for 30 min at RT in the dark. Finally, 0.5 ml of propidium iodide RNase staining buffer was added. After 30 additional minutes of incubation in the dark at RT, flow cytometry was carried out.

Statistical analysis

Three ejaculates from seven individual stallions were collected. All experiments were repeated at least three times with independent samples (three independent ejaculates from each of the seven stallions). The normality of the data was previously assessed using the Kolmogorov-Smirnov test, in view of the normality of the distribution the results were analysed by ANOVA and the Tukey's *post hoc* test to perform pair wise comparisons using SPSS 19.0 Software for Mac. $P < 0.05$ was regarded as significant, with $*P < 0.05$ and $**P < 0.01$. Results are given as means $\pm$ s.d.

Results

Identification and subcellular localisation of phosphorylated AKT (Ser⁴⁷³) and AKT (Thr³⁰⁸) in stallion spermatozoa

Phosphorylated AKT in stallion spermatozoa was detected using specific antibodies, western blotting, flow cytometry and confocal laser microscopy. Three labelling patterns were present in equine sperm, i.e. non-labelled cells (not shown), cells labelled in the mid piece (Fig. 3) and cells labelled in the post acrosomal region (not shown). This subcellular localisation is consistent with the putative role of inhibiting pro-apoptotic proteins localised in mitochondrial membranes. Not all spermatozoa expressed phosphorylated AKT, and the staining pattern was fairly constant.

Effect of SH5 on AKT phosphorylation in stallion spermatozoa

In stallion spermatozoa, AKT was phosphorylated at Thr³⁰⁸ and Ser⁴⁷³. Inhibition of AKT phosphorylation was monitored at the beginning and after 1 and 4 h

Figure 3 Subcellular localisation of phospho-AKT in stallion spermatozoa. Ejaculated stallion spermatozoa were washed and stained using specific anti-phospho-AKT antibodies and visualised using confocal laser microscopy, as described in the 'Materials and methods' section. Three labelling patterns of phosphorylated AKT were present in equine sperm, i.e. non-labelled cells (not shown), cells labelled in the mid piece (shown in this figure) and cells labelled in the post acrosomal region (not shown). (A) Transmission image, (B) fluorescence recorded using the 540/30 nm emission filter, (C) merged images, (D, E and F) negative controls, stained only with the secondary antibody, $n = 7$ stallions.

Figure 4 Effect of the AKT inhibitor SH5 on AKT phosphorylation at Ser⁴⁷³ and Thr³⁰⁸ in stallion spermatozoa. Stallion spermatozoa were washed and incubated in the presence of AKT inhibitor (0 or 30 μM) for up to 4 h. They were then stained with specific anti-phospho-AKT antibodies (phospho-AKT (Ser⁴⁷³) Alexa Fluor 488 conjugate and phospho-AKT (Thr³⁰⁸) Alexa Fluor 647 conjugate, as described in the 'Materials and methods' section. Samples were analysed by flow cytometry and representative cytograms are given. Events in the lower right quadrant represent spermatozoa positive for pAKT Ser⁴⁷³. Events in the upper right quadrant represent spermatozoa positive for pAKT at both Ser⁴⁷³ and Thr³⁰⁸. Events in the upper left quadrant represent spermatozoa positive for pAKT Thr³⁰⁸. The specificity of the antibodies was validated using western blotting analysis (upper left corner), as described in the 'Materials and methods' section. Lanes one and two represent sperm lysates from two different stallions and lane three represents the positive control (pancreatic acini). In the three lower panels, the quantification of the phosphorylation status of AKT is given at three different times. Figures represent percentages of sperm in the quadrants of interest; isotype controls were used to set the quadrants as described in 'Materials and methods' section. Results are given as mean ± s.d. Comparisons are made between each sample and the control (** $P < 0.01$ and * $P < 0.05$), $n = 7$ stallions.

of incubation. After 1 h of incubation in presence of SH5, phosphorylated AKT decreased significantly; however, after 4 h of incubation, differences among control and treated samples were no longer significant (Fig. 4). The same effect was observed for the AKT1/2 inhibitor (Fig. 9D).

Inhibition of AKT leads to reduced stallion sperm motility and kinematics

After 1, 2, 4 or 6 h of incubation, sperm motility and velocity were determined using a CASA analysis. After 1 h of incubation, both AKT inhibitors (used at 30 μM) significantly decreased the percentage of total motile spermatozoa (30 vs 50% in controls); the percentage of progressive sperm (6 vs 18% in controls); and the circular velocity (39.5 vs 63.5 μm/s in controls), average velocity (21.5 vs 36.7 μm/s in controls) and straight-line velocity (12.5 vs 21 μm/s in controls) ($P < 0.05$). After 2 h of incubation at 37 °C, the inhibitors significantly reduced the circular velocity and average velocity of the sperm cells ($P < 0.05$; Figs 5 and 9A and B). These are average values of all motile sperm. These data suggest that AKT plays a role in the regulation of stallion sperm motility and velocity. After 4 and 6 h of incubation, all parameters for sperm motility and kinematics decreased ($P < 0.01$), regardless of the treatment.

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

To determine whether AKT regulates early changes in membrane integrity and permeability, stallion spermatozoa were analysed using flow cytometry after incubation in the presence or absence of the AKT inhibitor in BWW medium for up to 6 h at 37 °C. After 1 h of incubation at 37 °C, the percentage of spermatozoa with intact membranes decreased significantly ($P < 0.05$) from 50% in controls to 38% in the presence of 30 μM AKT inhibitor. Simultaneously, the percentage of spermatozoa with increased membrane permeability and the percentages of necrotic spermatozoa increased ($P < 0.05$) (Fig. 6). The effect of the inhibitor disappeared after longer incubation periods, although the percentage of dead spermatozoa was higher after 6 h of incubation at 37 °C in the presence of 30 μM AKT inhibitor than in the control.

Yo-Pro-1 enters stallion spermatozoa through pannexin-1 channels

In somatic cells, Yo-Pro-1 detects early apoptotic cells by using specific channels (pannexin-1) that are open in the very early stages of apoptosis to enter cells (Elliott *et al.* 2009, Chekeni *et al.* 2010, Sandilos *et al.* 2012).

Figure 5 Effect of inhibition of AKT phosphorylation on stallion sperm motility and kinematics. Stallion spermatozoa were washed and resuspended in BWB supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h or (D) 6 h of incubation at 37 °C. TM%, percentage of total motile spermatozoa; PM%, percentage of progressive motile spermatozoa; VCL ($\mu\text{m/s}$), circular velocity; VAP ($\mu\text{m/s}$), average velocity; VSL ($\mu\text{m/s}$), straight line velocity. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control (** $P < 0.01$ and * $P < 0.05$), $n = 7$ stallions.

To confirm Yo-Pro-1 entry through pannexin-1 channels, we first investigated pannexin-1 levels using western blotting with specific antibodies. We then blocked pannexin-1 by incubating the spermatozoa for 1 h at 37 °C in presence of two unrelated inhibitors (1 mM probenecid and 100 μM carbenexolone) and subsequently stained only with Yo-Pro-1 in presence

of Hoechst 33342 to gate out debris. Pannexin-1 was present in stallion spermatozoa, and probenecid and carbenexolone reduced the percentage of Yo-Pro-1 + spermatozoa (Fig. 7), supporting the hypothesis that Yo-Pro-1 uptake in this population of spermatozoa occurs through opening of pannexin-1 channels. Moreover, this experiment may also indicate that the stallion

Figure 6 Effect of inhibition of AKT on stallion sperm membrane permeability and integrity. Stallion spermatozoa were washed and resuspended in BWB supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μM for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h and (D) 6 h of incubation at 37 °C. Intact %, percentage of spermatozoa with completely intact membranes; Yo-Pro+, percentage of spermatozoa with increased permeability in their membranes; Yo-Pro+/Eth+, percentage of apoptotic spermatozoa; Eth+, percentage of dead spermatozoa. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control (* $P < 0.05$), $n = 7$ stallions.

Figure 7 Identification and function of the pannexin-1 channel in stallion spermatozoa. (Left panel) Western blot analysis showing the presence of pannexin-1 in stallion sperm lysates. Lane 1 is the control and lanes 2–4 are sperm lysates from three different stallions. (Right panel) Whisker and box plots showing the effect of two different pannexin-1 channel blockers on the uptake of Yo-Pro-1 by stallion spermatozoa. The uptake of Yo-Pro-1 was significantly inhibited by both inhibitors. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control ($*P < 0.05$), $n = 7$ stallions.

spermatozoa are in the early stages of apoptotic phenomena, similar to the situation described in somatic cells (Chekeni *et al.* 2010).

Inhibition of AKT (Ser⁴⁷³) and (Thr³⁰⁸) phosphorylation increases caspases 3 and 7 activity in stallion spermatozoa

One possible mechanism that explains our findings is that the phosphorylation of AKT participates in the regulation of an apoptotic-like mechanism. To test this hypothesis, we evaluated caspases 3 and 7

activation in stallion spermatozoa after incubation in presence of the AKT inhibitor. If AKT inhibits apoptosis, its inhibition should increase caspases 3 and 7 activity. After 1 h of incubation, all concentrations tested reduced the percentage of caspase-negative, live spermatozoa in a positive concentration-dependent manner ($P < 0.01$; Fig. 8). The percentage of caspase 3-positive spermatozoa also increased in a concentration-dependent manner and was especially evident after 4 h of incubation at 37 °C (Fig. 8C). These results indicate that AKT phosphorylation at Ser⁴⁷³ and Thr³⁰⁸ inhibits activation of caspases 3 and 7 in ejaculated stallion spermatozoa. To provide further evidence supporting the role of AKT inhibition, an additional experiment was conducted using two different AKT inhibitors (SH-5 and AKT1/2 kinase inhibitors at either 0 or 30 μ M for 1 h of incubation at 37 °C) to address the effects of AKT phosphorylation on sperm motility and velocity in addition to the activation of caspases 3 and 7. Both inhibitors induced similar effects, causing reduced sperm motility and velocity, increase in caspases 3 and 7 activity and dephosphorylation of AKT (Fig. 9).

Inhibition of AKT reduces the mitochondrial membrane potential in stallion spermatozoa

To study the effect of inhibiting AKT phosphorylation on sperm mitochondria, two different assays were carried out. The mitochondrial membrane potential was studied after JC-1 and Mitotracker Deep Red staining. Inhibition of AKT phosphorylation affected the mitochondrial membrane potential of stallion

Figure 8 Effect of inhibition of AKT on caspases 3 and 7 activation in stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μ M for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h and (D) 6 h of incubation at 37 °C. 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. Results are given as means $\pm$ s.d. Comparisons are made between each sample and the control ($**P < 0.01$ and $*P < 0.05$), $n = 7$ stallions.

Figure 9 Effect of two different inhibitors of AKT (AKT1/2 kinase inhibitor and SH5) on motility, velocity, caspases 3 and 7 activation and AKT phosphorylation in stallion spermatozoa. The samples were washed and resuspended in BWV media and incubated for 1 h at 37 °C in the presence of 0 or 30 μM AKT1/2 kinase inhibitor and 30 μM SH5. (A) Effects on stallion sperm motility. TM %, percentage of total motile sperm; PM %, percentage of progressive motile sperm. (B) Effects on stallion sperm velocity. VCL (μm/s), circular velocity; VSL (μm/s), straight line velocity; VAP (μm/s), average velocity. (C) Effects on caspases 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 Thr³⁰⁸; Ser⁴⁷³, percentage of spermatozoa with AKT phosphorylated at Ser⁴⁷³; Thr³⁰⁸+Ser⁴⁷³, percentage of spermatozoa with AKT phosphorylated simultaneously at Thr³⁰⁸ and Ser⁴⁷³. Results are given as mean ± s.d. Comparisons are made between each sample and the control (* $P < 0.05$ and ** $P < 0.01$), $n = 7$ stallions.

spermatozoa in a concentration- and time-dependent manner. After 1 h of incubation, the AKT inhibitor at 30 μM significantly reduced the mitochondrial membrane potential, as determined by both probes (Figs 10 and 11). After 2 h of incubation, the AKT inhibitor at 20 and 30 μM decreased the mitochondrial membrane potential, as determined by Mitotracker Deep Red (Fig. 10). However, determined using JC-1, the mitochondrial membrane potential only decreased significantly at 30 μM (Fig. 11).

Inhibition of AKT does not increase the production of reactive oxygen species or mitochondrial superoxide, DNA oxidation or DNA fragmentation

Because the disruption of mitochondria may lead to increased reactive oxygen species (ROS) production and oxidative stress (Koppers *et al.* 2008), the effect of the AKT inhibitor on apoptosis was tested. However, the differences in production of ROS were not statistically significant (Fig. 12). Furthermore, no increase in mitochondrial superoxide production was observed when AKT phosphorylation was inhibited (data not shown). Moreover, inhibition of AKT phosphorylation did not lead to significant increases in DNA oxidation (data not shown). Because DNA fragmentation is a hallmark of apoptosis, a TUNEL assay was used to evaluate apoptosis. However, inhibition of AKT did not increase the fraction of fragmented DNA (data not shown).

Discussion

The presence and the role of AKT in stallion spermatozoa were investigated using specific inhibitors. Overall, the results indicate that AKT maintains sperm survival after ejaculation through inhibition of caspases 3 and 7. The PI3 kinase/AKT pathway has been previously studied in spermatozoa using specific inhibitors such as Wortmannin and LY294002 (du Plessis *et al.* 2004, Aparicio *et al.* 2005). However, to our knowledge, this is the first study to report the effects of specific inhibitors of AKT on mammalian spermatozoa. The data reveal a direct inhibitory effect of AKT on caspases 3 and 7, as AKT inhibition led to rapid activation of caspases 3 and 7. Thus, inhibition of an apoptosis-like mechanism (most likely being sperm-specific mechanism) plays a role in the survival of ejaculated stallion spermatozoa, which confirms similar recent findings in humans (Koppers *et al.* 2011) and supports the idea that post-ejaculatory survival of stallion and human spermatozoa is tightly regulated through the inhibition of a truncated apoptotic cascade. This inhibition is lost over time through spontaneous dephosphorylation of AKT, which suggests its function to remove redundant spermatozoa in the female genital tract (Aitken *et al.* 2011). However the fact that the inhibitor reduced the percentage of phospho-AKT Thr³⁰⁸ at the beginning of the incubation period suggests inhibition of active phosphorylation rather than dephosphorylation of this protein.

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 BWB supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μ M for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h and (D) 6 h of incubation at 37 °C. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control (* P <0.05), n =7 stallions.

Our study suggests that AKT is necessary to maintain sperm motility, viability and mitochondrial membrane potential by inhibiting activation of caspases 3 and 7, as demonstrated by the rapid increase in caspases 3 and 7 activities upon inhibition of AKT. Inhibition of AKT was accompanied by decreases in the sperm motility and

velocity, increases in sperm membrane permeability and decreases in mitochondrial membrane potential, all suggesting a role for AKT in the maintenance of sperm viability. Although we show evidence for each of these effects, the link between them remains to be determined. These findings strongly suggest that stallion

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 BWB supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μ M for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h and (D) 6 h of incubation at 37 °C. High %, percentage of spermatozoa with high mitochondrial membrane potential; intermediate %, percentage of spermatozoa with mitochondria with both high and low potential; low %, percentage of spermatozoa showing low mitochondrial potential. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control (** P <0.01 and * P <0.05), n =7 stallions.

Figure 12 Effect of inhibition of AKT on the percentage of stallion spermatozoa showing oxidative stress. Samples were washed and resuspended in BWB supplemented with 1% PVA in the presence of the AKT inhibitor SH5 at 0, 10, 20 and 30 μ M for up to 6 h. (A) After 1 h, (B) 2 h, (C) 4 h and (D) 6 h of incubation at 37 °C. Results are given as mean $\pm$ s.d. Comparisons are made between each sample and the control, $n=7$ stallions.

sperm death during storage is largely an apoptotic phenomenon, as has been previously proposed (Ball 2008, Ortega-Ferrusola *et al.* 2008, da Silva *et al.* 2011, Macías García *et al.* 2012). Other forms of sperm death during storage are also possible, as after 6 h of storage we observed an increase in the percentage of necrotic sperm incubated in the presence of 30 μ M SH5. However, most of the necrotic population was also caspase positive, which suggests an underlying apoptotic phenomenon. Moreover, when a different AKT inhibitor was used, the same effect was reproduced, which further suggests that AKT inhibition results in a truncated apoptotic pathway that causes cessation of sperm motility and death. Although we present strong evidence for the existence of this pathway, it remains to be fully described. For example, recent studies have suggested that an extrinsic pathway mediated by death receptors may also be present in spermatozoa (Macías García *et al.* 2012, Mendoza *et al.* 2013).

We also demonstrated for the first time that pannexin-1 is present in stallion spermatozoa and that it has a putative role in the early stages of membrane destabilisation, as proposed for somatic cells (Elliott *et al.* 2009, Chekeni *et al.* 2010). In addition, because Yo-Pro-1 uptake occurs through pannexin-1 channels, we demonstrated the value of the Yo-Pro-1 assay in the detection of the early stages of apoptosis. Recently, we have demonstrated a strong negative correlation between Yo-Pro-positive spermatozoa and ATP content in stallion spermatozoa (Balao da Silva *et al.* 2013). This finding further supports our theory, as ATP depletion

in early apoptotic cells occurs through pannexin-1 channels (Elliott *et al.* 2009).

Apoptotic mechanisms are involved in cryodamage in mammalian spermatozoa (Ortega Ferrusola *et al.* 2010, Said *et al.* 2010). Recently, anti-apoptotic proteins have been used to improve sperm survival after thawing (Shimokawa *et al.* 2012). In our experiment, AKT inhibition was not accompanied by increased ROS production and DNA fragmentation, contrary to findings in humans where inhibition of PI3CA was accompanied by increased mitochondrial production of ROS and oxidative DNA damage 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 on the PI3CA/AKT pathway (we specifically induced inhibition of AKT downstream of PI3CA). The inhibition of PI3CA may also affect other downstream targets. The inhibition of PI3CA increases cAMP levels and tyrosine phosphorylation of the PKA-anchoring protein AKAP3 in human spermatozoa, suggesting a negative role for PI3K in the regulation of motility (Luconi *et al.* 2001, du Plessis *et al.* 2004, Luconi *et al.* 2005). Moreover, studies using pig spermatozoa showed no effect of PI3CA on the percentage of motile or progressive motile spermatozoa, but showed an increase in sperm velocity (Aparicio *et al.* 2005). In addition, both Wortmannin and LY294002 lack specificity and provoke different responses from spermatozoa (Nauc *et al.* 2004). A recent study (Pujianto *et al.* 2010) has shown more intense changes in caspase activation and loss of motility

of human spermatozoa with an AKT inhibitor than with the PIK3CA inhibitor Wortmannin. In contrast to human sperm, stallion spermatozoa showed a decreased mitochondrial membrane potential but did not show oxidative stress upon AKT inhibition. This finding supports the existence of species-specific features in the apoptotic mechanisms of sperm senescence. Previous reports from our laboratory indicate that the mitochondrial permeability transition pore plays a role in the apoptotic mechanism associated with sperm senescence induced by cryopreservation (Ortega Ferrusola *et al.* 2010), which is consistent with AKT preventing translocation of Bax from the cytosol to the mitochondria.

The inhibition of AKT did not result in significant increases in DNA fragmentation, suggesting that this apoptotic mechanism does not cause DNA damage, as has previously been suggested in human spermatozoa (Aitken & Koppers 2011, Koppers *et al.* 2011, Aitken *et al.* 2012). This lack of DNA damage can be attributed to the special architecture of spermatozoa that prevents endonucleases activated at the mid-piece during the apoptotic process from reaching the nucleus (Koppers *et al.* 2011). Another possible explanation arises from the relatively high resistance of stallion spermatozoa to oxidative insults relative to human sperm (Ortega Ferrusola *et al.* 2009a,b), a factor that most likely relates to the different lipid compositions of the sperm membranes between the species (Garcia *et al.* 2011, Macías Garcia *et al.* 2011a,b).

It is worth mentioning that multicolour flow cytometry, alongside the development of new assays for the simultaneous evaluation of several sperm characteristics and improved discrimination of sperm from debris during analysis, offers a major improvement for flow cytometric sperm assays (Petrunikina & Harrison 2011). In conclusion, inhibition of a sperm-specific apoptotic mechanism is involved in the survival of stallion spermatozoa after ejaculation. This special form of apoptosis in spermatozoa can be termed spermtosis. Regulation of sperm survival after ejaculation is an interesting area of research with relevance to infertility in humans, particularly with respect to intrinsic sperm dysfunction and the development of reproductive technologies. In addition, stallion spermatozoa experience an apoptosis phenomenon leading to cellular senescence after ejaculation that shares biochemical and molecular characteristics with the process observed in human sperm. Thus, stallion sperm may be suitable models for studying the apoptotic mechanisms involved in male factor human infertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Aitken RJ & Koppers AJ 2011 Apoptosis and DNA damage in human spermatozoa. *Asian Journal of Andrology* **13** 36–42. (doi:10.1038/aja.2010.68)
- Aitken RJ, Findlay JK, Hutt KJ & Kerr JB 2011 Apoptosis in the germ line. *Reproduction* **141** 139–150. (doi:10.1530/REP-10-0232)
- Aitken RJ, De Iuliis GN, Gibb Z & Baker MA 2012 The simmet lecture: new horizons on an old landscape – oxidative stress, DNA damage and apoptosis in the male germ line. *Reproduction in Domestic Animals* **47** (Suppl 4) 7–14. (doi:10.1111/j.1439-0531.2012.02049.x)
- Aparicio IM, Gil MC, Garcia-Herreros M, Pena FJ & Garcia-Marin LJ 2005 Inhibition of phosphatidylinositol 3-kinase modifies boar sperm motion parameters. *Reproduction* **129** 283–289. (doi:10.1530/rep.1.00447)
- Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S & Ando S 2007 Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Human Reproduction* **22** 2594–2605. (doi:10.1093/humrep/dem243)
- Balao da Silva CM, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolanos JM, Plaza Davila M, Morrell JM, Rodriguez Martinez H, Tapia JA, Aparicio IM & Pena FJ 2013 Sex sorting increases the permeability of the membrane of stallion spermatozoa. *Animal Reproduction Science* **138** 241–251. (doi:10.1016/j.anireprosci.2013.02.021)
- Ball BA 2008 Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal Reproduction Science* **107** 257–267. (doi:10.1016/j.anireprosci.2008.04.014)
- Baumgarth N & Roederer M 2000 A practical approach to multicolor flow cytometry for immunophenotyping. *Journal of Immunological Methods* **243** 77–79. (doi:10.1016/S0022-1759(00)00229-5)
- Busch F, Mobasheri A, Shayan P, Lueders C, Stahlmann R & Shakibaei M 2012 Resveratrol modulates interleukin-1 β -induced phosphatidylinositol 3-kinase and nuclear factor κ B signaling pathways in human tenocytes. *Journal of Biological Chemistry* **287** 38050–38063. (doi:10.1074/jbc.M112.377028)
- Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS *et al.* 2010 Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* **467** 863–867. (doi:10.1038/nature09413)
- De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B & Aitken RJ 2009 DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biology of Reproduction* **81** 517–524. (doi:10.1095/biolreprod.109.076836)
- Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P *et al.* 2009 Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* **461** 282–286. (doi:10.1038/nature08296)
- Garcia BM, Fernandez LG, Ferrusola CO, Salazar-Sandoval C, Rodriguez AM, Martinez HR, Tapia JA, Morcuende D & Pena FJ 2011 Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. *Reproduction in Domestic Animals* **46** 141–148. (doi:10.1111/j.1439-0531.2010.01609.x)

- Gilot D, Giudicelli F, Lagadic-Gossmann D & Fardel O 2010 AKT1-2, an allosteric inhibitor of AKT 1 and 2, efficiently inhibits CaMKI α activity and aryl hydrocarbon receptor pathway. *Chemico-Biological Interactions* **188** 546–552. (doi:10.1016/j.cbi.2010.08.011)
- Gonzalez-Fernandez L, Ortega-Ferrusola C, Macías-García B, Salido GM, Peña FJ & Tapia JA 2009 Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. *Biology of Reproduction* **80** 1239–1252. (doi:10.1095/biolreprod.108.073486)
- Hers I, Vincent EE & Tavaré JM 2011 AKT signalling in health and disease. *Cellular Signalling* **23** 1515–1527. (doi:10.1016/j.cellsig.2011.05.004)
- Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA & Aitken RJ 2008 Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **93** 3199–3207. (doi:10.1210/jc.2007-2616)
- Koppers AJ, Mitchell LA, Wang P, Lin M & Aitken RJ 2011 Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. *Biochemical Journal* **436** 687–698. (doi:10.1042/BJ20110114)
- Krutzik PO, Irish JM, Nolan GP & Perez OD 2004 Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clinical Immunology* **110** 206–221. (doi:10.1016/j.clim.2003.11.009)
- Lan TH, Xu ZW, Wang Z, Wu YL, Wu WK & Tan HM 2011 Ginsenoside Rb1 prevents homocysteine-induced endothelial dysfunction via PI3K/AKT activation and PKC inhibition. *Biochemical Pharmacology* **82** 148–155. (doi:10.1016/j.bcp.2011.04.001)
- Lessig J, Spalteholz H, Reibetanz U, Salavei P, Fischlechner M, Glander HJ & Arnhold J 2007 Myeloperoxidase binds to non-vital spermatozoa on phosphatidylserine epitopes. *Apoptosis* **12** 1803–1812. (doi:10.1007/s10495-007-0113-5)
- Luconi M, Marra F, Gandini L, Filimberti E, Lenzi A, Forti G & Baldi E 2001 Phosphatidylinositol 3-kinase inhibition enhances human sperm motility. *Human Reproduction* **16** 1931–1937. (doi:10.1093/humrep/16.9.1931)
- Luconi M, Torcia S, Grillo D, Fiorenza MT, Forti G, Mangia F & Baldi E 2005 Enhancement of mouse sperm motility by the PI3-kinase inhibitor LY294002 does not result in toxic effects on preimplantation embryo development. *Human Reproduction* **20** 3500–3504. (doi:10.1093/humrep/dei248)
- Macías García B, Gonzalez Fernandez L, Ortega Ferrusola C, Morillo Rodríguez A, Gallardo Bolanos JM, Rodriguez Martinez H, Tapia JA, Morcuende D & Pena FJ 2011a Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. *Theriogenology* **75** 811–818. (doi:10.1016/j.theriogenology.2010.10.021)
- Macías García B, Miro Moran A, Gonzalez Fernandez L, Ortega Ferrusola C, Morillo Rodríguez A, Gallardo Bolaños JM, Balao da Silva CM, Rodriguez Martinez H & Peña FJ 2011b The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic induced stress: role of the c Jun terminal kinase (JMK) pathway. *Journal of Andrology* **33** 105–113. (doi:10.2164/jandrol.110.011957)
- Macías García B, Ortega Ferrusola C, Aparicio IM, Miro-Moran A, Morillo Rodríguez A, Gallardo Bolanos JM, Gonzalez Fernandez L, Balao da Silva CM, Rodriguez Martinez H, Tapia JA *et al.* 2012 Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. *Theriogenology* **77** 1280–1289. (doi:10.1016/j.theriogenology.2011.10.033)
- Martínez-Pastor F, Jorge Tizado E, Garde JJ, Anel L & de Paz P 2011 Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* **75** 783–795. (doi:10.1016/j.theriogenology.2010.11.034)
- Mendoza N, Casao A, Perez-Pe R, Cebrian-Perez JA & Muino-Blanco T 2013 New insights into the mechanisms of ram sperm protection by seminal plasma proteins. *Biology of Reproduction* **88** 149. (doi:10.1095/biolreprod.112.105650)
- Moran JM, Madejon L, Ortega Ferrusola C & Peña FJ 2008 Nitric oxide induces caspase activity in boar spermatozoa. *Theriogenology* **70** 91–96. (doi:10.1016/j.theriogenology.2008.02.010)
- Nauc V, De Lamirande E, Leclerc P & Gagnon C 2004 Inhibitors of phosphoinositide 3-kinase, LY294002 and Wortmannin, affect sperm capacitation and associated phosphorylation of proteins differently: Ca²⁺-dependent divergences. *Journal of Andrology* **25** 573–585. (doi:10.1002/j.1939-4640.2004.tb02828.x)
- Núñez-Martínez I, Moran JM & Peña FJ 2007a Identification of sperm morphometric subpopulations in the canine ejaculate: do they reflect different subpopulations in sperm chromatin integrity? *Zygote* **15** 257–266. (doi:10.1017/S0967199407004248)
- Núñez-Martínez I, Moran JM & Peña FJ 2007b Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. *International Journal of Andrology* **30** 182–189. (doi:10.1111/j.1365-2605.2007.00743.x)
- Ortega-Ferrusola C, Sotillo-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, Gonzalez-Fernandez L, Tapia JA & Peña FJ 2008 Detection of “apoptosis-like” changes during the cryopreservation process in equine sperm. *Journal of Andrology* **29** 213–221. (doi:10.2164/jandrol.107.003640)
- Ortega Ferrusola C, Gonzalez Fernandez L, Macías García B, Salazar-Sandoval C, Morillo Rodríguez A, Rodriguez Martinez H, Tapia JA & Peña FJ 2009a Effect of cryopreservation on nitric oxide production by stallion spermatozoa. *Biology of Reproduction* **81** 1106–1111. (doi:10.1095/biolreprod.109.078220)
- Ortega Ferrusola C, García BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, Tapia JA & Peña FJ 2009b Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* **138** 55–63. (doi:10.1530/REP-08-0484)
- Ortega-Ferrusola C, García BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, Tapia JA & Peña FJ 2009c Apoptotic markers can be used to forecast the freezability of stallion spermatozoa. *Animal Reproduction Science* **114** 393–403. (doi:10.1016/j.anireprosci.2008.10.005)
- Ortega Ferrusola C, Gonzalez Fernandez L, Salazar Sandoval C, Macías García B, Rodriguez Martinez H, Tapia JA & Peña FJ 2010 Inhibition of the mitochondrial permeability transition pore reduces “apoptosis like” changes during cryopreservation of stallion spermatozoa. *Theriogenology* **74** 458–465. (doi:10.1016/j.theriogenology.2010.02.029)
- Parte PP, Rao P, Redij S, Lobo V, D’Souza SJ, Gajbhiye R & Kulkarni V 2012 Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC–MS(E)) reveals altered proteomic signatures in asthenozoospermia. *Journal of Proteomics* **75** 5861–5871. (doi:10.1016/j.jpro.2012.07.003)
- Peña FJ, Johannisson A, Wallgren M & Rodriguez-Martinez H 2003a Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. *Theriogenology* **60** 677–689. (doi:10.1016/S0093-691X(03)00081-5)
- Peña FJ, Johannisson A, Wallgren M & Rodriguez-Martinez H 2003b Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science* **78** 85–98. (doi:10.1016/S0378-4320(03)00049-6)
- Peña FJ, Saravia F, Garcia-Herreros M, Núñez-Martínez I, Tapia JA, Johannisson A, Wallgren M & Rodriguez-Martinez H 2005a Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. *Journal of Andrology* **26** 716–723. (doi:10.2164/jandrol.05030)
- Peña FJ, Saravia F, Johannisson A, Walgren M & Rodriguez-Martinez H 2005b A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. *International Journal of Andrology* **28** 107–114. (doi:10.1111/j.1365-2605.2005.00512.x)
- Peña FJ, Núñez-Martínez I & Moran JM 2006 Semen technologies in dog breeding: an update. *Reproduction in Domestic Animals* **41** (Suppl 2) 21–29. (doi:10.1111/j.1439-0531.2006.00766.x)
- Peña FJ, Ortega-Ferrusola C, Tapia JA & Aparicio IM 2012 How stallion sperm age *in vitro*? Scenario for preservation technologies. *Journal of Equine Veterinary Science* **32** 451–454. (doi:10.1016/j.jevs.2012.05.074)
- Petrunkina AM & Harrison RA 2011 Mathematical analysis of mis-estimation of cell subsets in flow cytometry: viability staining revisited. *Journal of Immunological Methods* **368** 71–79. (doi:10.1016/j.jim.2011.02.009)

- du Plessis SS, Franken DR, Baldi E & Luconi M 2004 Phosphatidylinositol 3-kinase inhibition enhances human sperm motility and sperm-zona pellucida binding. *International Journal of Andrology* **27** 19–26. (doi:10.1111/j.1365-2605.2004.00440.x)
- Pujianto DA, Curry BJ & Aitken RJ 2010 Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of AKT phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* **151** 1269–1279. (doi:10.1210/en.2009-0964)
- Qiao Q, Jiang Y & Li G 2013 Inhibition of the PI3K/AKT-NF- κ B pathway with curcumin enhanced radiation-induced apoptosis in human Burkitt's lymphoma. *Journal of Pharmacological Sciences* **121** 247–256. (doi:10.1254/jphs.12149FP)
- Roederer M 2001 Spectral compensation for flow cytometry: visualization artifacts, limitations and caveats. *Cytometry* **45** 194–205. (doi:10.1002/1097-0320(20011101)45:3<194::AID-CYTO1163>3.0.CO;2-C)
- Said TM, Gaglani A & Agarwal A 2010 Implication of apoptosis in sperm cryoinjury. *Reproductive Biomedicine Online* **21** 456–462. (doi:10.1016/j.rbmo.2010.05.011)
- Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N & Bizzaro D 2002 Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biology of Reproduction* **66** 1061–1067. (doi:10.1095/biolreprod66.4.1061)
- Sandilos JK, Chiu YH, Cheleni FB, Armstrong AJ, Walk SF, Ravichandran KS & Bayliss DA 2012 Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal autoinhibitory region. *Journal of Biological Chemistry* **287** 11303–11311. (doi:10.1074/jbc.M111.323378)
- Schulz KR, Danna EA, Krutzik PO & Nolan GP 2007 Single-cell phosphoprotein analysis by flow cytometry. In *Current Protocols in Immunology*, ch 8, unit 8 17. Ed John E Coligan et al. Wiley Blackwell, NJ, USA
- Schulz KR, Danna EA, Krutzik PO & Nolan GP 2012 Single-cell phosphoprotein analysis by flow cytometry. In *Current Protocols in Immunology*, ch 8, unit 8 17, pp 11–20. Ed John E Coligan et al. Wiley Blackwell, NJ, USA
- Sethi G, Ahn KS, Sung B, Kunnumakkara AB, Chaturvedi MM & Aggarwal BB 2008 SH-5, an AKT inhibitor potentiates apoptosis and inhibits invasion through the suppression of anti-apoptotic, proliferative and metastatic gene products regulated by I κ B α kinase activation. *Biochemical Pharmacology* **76** 1404–1416. (doi:10.1016/j.bcp.2008.05.023)
- Shimokawa K, Oshiro R, Yamanaka K, Ashizawa K, Ohta S & Tatemoto H 2012 Improvement of the post-thaw qualities of Okinawan native Agu pig sperm frozen in an extender supplemented with antiapoptotic PTD-FNK protein. *Theriogenology* **78** 1446–1455. (doi:10.1016/j.theriogenology.2012.06.005)
- da Silva CM, Macias-Garcia B, Miro-Moran A, Gonzalez-Fernandez L, Morillo-Rodriguez A, Ortega-Ferrusola C, Gallardo-Bolanos JM, Stilwell G, Tapia JA & Pena FJ 2011 Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. *Journal of Pineal Research* **51** 172–179. (doi:10.1111/j.1600-079X.2011.00873.x)
- Tazzari PL, Cappellini A, Bortul R, Ricci F, Billi AM, Tabellini G, Conte R & Martelli AM 2002 Flow cytometric detection of total and serine 473 phosphorylated AKT. *Journal of Cellular Biochemistry* **86** 704–715. (doi:10.1002/jcb.10262)
- Yang CM, Lee IT, Lin CC, Wang CH, Cherng WJ & Hsiao LD 2013 c-Src-dependent MAPKs/AP-1 activation is involved in TNF- α -induced matrix metalloproteinase-9 expression in rat heart-derived H9c2 cells. *Biochemical Pharmacology* **85** 1115–1123. (doi:10.1016/j.bcp.2013.01.013)

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