Autophagy and Apoptosis Have a Role in the Survival or Death of Stallion Spermatozoa during Conservation in Refrigeration

Juan M. Gallardo Bolaños1, Álvaro Miró Morán2, Carolina M. Balao da Silva1, Antolín Morillo Rodríguez1, María Plaza Dávila1, Inés M. Aparicio2, José A. Tapia2, Cristina Ortega Ferrusola1, Fernando J. Peña1*

1Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura Cáceres, Cáceres, Spain, 2Department of Physiology, Faculty of Veterinary Medicine, University of Extremadura Cáceres, Cáceres, Spain

Abstract

Apoptosis has been recognized as a cause of sperm death during cryopreservation and a cause of infertility in humans, however there is no data on its role in sperm death during conservation in refrigeration; autophagy has not been described to date in mature sperm. We investigated the role of apoptosis and autophagy during cooled storage of stallion spermatozoa. Samples from seven stallions were split; half of the ejaculate was processed by single layer centrifugation, while the other half was extended unprocessed, and stored at 5°C for five days. During the time of storage, sperm motility (CASA, daily) and membrane integrity (flow cytometry, daily) were evaluated. Apoptosis was evaluated on days 1, 3 and 5 (active caspase 3, increase in membrane permeability, phosphatidylserine translocation and mitochondrial membrane potential) using flow cytometry. Furthermore, LC3B processing was investigated by western blotting at the beginning and at the end of the period of storage. The decrease in sperm quality over the period of storage was to a large extent due to apoptosis; single layer centrifugation selected non-apoptotic spermatozoa, but there were no differences in sperm motility between selected and unselected sperm. A high percentage of spermatozoa showed active caspase 3 upon ejaculation, and during the period of storage there was an increase of apoptotic spermatozoa but no changes in the percentage of live sperm, revealed by the SYBR-14/PI assay, were observed. LC3B was differentially processed in sperm after single layer centrifugation compared with native sperm. In processed sperm more LC3B-II was present than in non-processed samples; furthermore, in non-processed sperm there was an increase in LC3B-II after five days of cooled storage. These results indicate that apoptosis plays a major role in the sperm death during storage in refrigeration and that autophagy plays a role in the survival of spermatozoa representing a new pro-survival mechanism in spermatozoa not previously described.

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* E-mail: fjuanpvega@unex.es

Introduction

Artificial insemination is the reproductive technology most widely used in the equine industry since most of breed registries have accepted its use [1]. The objective of cooling stallion sperm is to extend the life expectancy of the spermatozoa, and thus be transported to breed mares located distant from the stallion. However cooled stallion spermatozoa last only for a few days viable and loses rapidly fertilizing ability [2]. Cooling imposes a number of stresses to the spermatozoa, collectively termed “cold shock” [3,4] that finally leads to cell damage and/or death. This cold shock is attributed to lipid re-arrangements during cooling and again at warming. In addition during cooling storage, spermatozoa may suffer other insults such as lipid peroxidation [5,6,7], changes in pH [8], ATP depletion [9] and bacterial contamination [10], that after a variable time of storage finally ends in the death of most, if not all, spermatozoa. However the molecular mechanisms behind such sperm death remains largely unveiled. It is plausible to think that if the molecular mechanisms of sperm survival/death can be revealed [11,12], improved strategies to store sperm during longer periods can be developed [13]. Autophagy is a conserved catabolic process primarily responsible for nonspecific degradation of redundant or faulty cell components. This occurs as part of cell's daily activities in response to metabolic or hypoxic stress and starvation, since to preserve normal cellular function a fast and responsive mechanism to degrade irreversibly damaged proteins is essential. Processed LC3B-II is a cellular readout of autophagy levels [14,15]. LC3 is the commonly used name for microtubule associated protein 1 light chain. The unprocessed form of LC3 (pro LC3) is proteolytically cleaved by Atg4 protease, resulting in the LC3-I form with a carboxyterminal exposed glycine. Upon induction of autophagy the exposed glycine is conjugated by Atg7, Atg3 and by Atg12-Atg5 and Atg16L multimers to phosphatidylethanolamine (PE) moiety to generate LC3-II. To date LC3-II is the only well characterized protein that is specifically localized to autophagic
structures throughout the process from phagophore to lysosomal degradation [15].

Death by apoptosis has been described in the stallion spermatozoa as a result of cryopreservation [16,17], the same mechanism has been implicated in human [18] and bovine [19] sperm cryoinjury. However, there is no data regarding the molecular mechanisms leading to sperm death of stallion spermatozoa maintained in refrigeration. We hypothesize that apoptotic mechanisms may be also involved in the death of stallion spermatozoa during cooled storage. Furthermore we investigated and described for the first time the presence of autophagy in stallion spermatozoa and propose a role of this mechanism in sperm survival.

Materials and Methods

Experimental design

The study evaluated sperm quality, apoptotic markers (motility, membrane integrity, membrane permeability, mitochondrial membrane potential, lipid peroxidation (LPO) phosphatidylserine translocation and caspase 3 and 7 activity) and autophagy in fresh semen maintained in refrigeration at 5°C up to five days. Motility and membrane integrity were evaluated daily, while the rest of sperm parameters were evaluated on days 1, 3 and 5 of storage. Autophagy was investigated on day 1 and on day 5. To have two distinct subpopulations with different sperm quality, two groups were established: extended semen (FE), and extended semen after colloid centrifugation (CC); it has been demonstrated that the subpopulation of spermatozoa obtained after CC has better quality in vitro, survives longer and has better fertility [20,21,22]. All experiments were reviewed and approved by the Ethical committee of the University of Extremadura, Spain, ref AGL201020758, the only manipulation of animals was semen collection using standard procedures.

Reagents and media

Ethidium homodimer, 5’,6’,6’-tetrachloro-1’,3’,3’ tetraethylbenzimidazolyl carbocyanine iodine (JC-1), YO-PRO-1, BOD-IPY 581/591C11, Caspase 3 and 7 detection kit and Alexa 488-benzimidazolyl carbocianyne iodine (JC-1), YO-PRO-1, BOD-IPY 581/591C11, Caspase 3 and 7 activity) and autophagy in fresh semen maintained in refrigeration at 5°C up to five days. Motility and membrane integrity were evaluated daily, while the rest of sperm parameters were evaluated on days 1, 3 and 5 of storage. Autophagy was investigated on day 1 and on day 5. To have two distinct subpopulations with different sperm quality, two groups were established: extended semen (FE), and extended semen after colloid centrifugation (CC); it has been demonstrated that the subpopulation of spermatozoa obtained after CC has better quality in vitro, survives longer and has better fertility [20,21,22]. All experiments were reviewed and approved by the Ethical committee of the University of Extremadura, Spain, ref AGL201020758, the only manipulation of animals was semen collection using standard procedures.

Semen collection and processing

Semen (four ejaculates per stallion) was obtained from 7 Andalusian horses 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 2011 breeding season. Ejaculates were collected using a Missouri model artificial vagina with an inline filter to eliminate the gel fraction, lubricated and warmed to 45–50°C. The semen was immediately transported to the laboratory for evaluation and processing. Half of the ejaculate was extended 1:1 in INRA-96 centrifuged (600g x 10 min) and re-suspended in INRA 96 to a final concentration of 50x10⁶ sperm/ml. The other half was processed by single layer colloid centrifugation as previously described [21,23] and extended in INRA-96 to 50x10⁶ sperm/ml.

Sperm motility

Sperm kinematics was assessed using a CASA system (ISAS® Proser) Valencia Spain). The analysis was based on the examination of 25 consecutive, digitalized images obtained from a single field using x10 negative phase contrast objective and a warmed (37°C) stage. Semen was loaded in a 20 μm depth Leja chamber (Leja Amsterdam, The Netherlands). Images were taken with a time lapse of 1 sec - the image capture speed was therefore one every 40 msec. 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 >15 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were designated linearly motile and spermatozoa with a circular velocity (VCL) >45 μm/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 time. Average lateral head displacement (ALH) μm, Measures the mean head displacement along the curvilinear trajectory. BCF (Hz), Number of times the sperm head crosses the mean path/second.

Staining for evaluation of sperm membrane integrity

The LIVE/DEAD® Sperm Viability Kit [Molecular Probes, Leiden The Netherlands] was used following the indications of the manufacturer. In brief, 1 to 5x10⁶ stallion spermatozoa were re-suspended in a final volume of 1 ml of HEPES buffered saline solution (10mM HEPES, 150mM NaCl, 10% BSA, pH 7.4). Sperm was then stained with 5 μL SYBR-14 (100nM) and incubated at 37°C for 10 minutes in the dark and then samples were stained with 5 μL Propidium iodide (PI) (12 μM) and incubated for additional 10 minutes before reading in the flow cytometer. SYBR-14 fluorescence was detected in FL1 and PI fluorescence was detected in FL3.

Assessment of subtle membrane changes and viability

The following stock solutions in DMSO were prepared: Yo-Pro-1, (25 μM), and Ethidium Homodimer-1 (1.167 mM) (Molecular Probes Europe, Leiden, The Netherlands), then one mL of a sperm suspension containing 5x10⁶ spermatozoa/mL was stained with 3 μL of Yo-Pro-1 and one μL of ethidium homodimer. After thorough mixing, the sperm suspension was incubated at 37°C in the dark for 16 min. This staining distinguishes four sperm subpopulations [17,24,25]. The first is the subpopulation of unstained spermatozoa. These spermatozoa are considered alive and without any membrane alteration. Another subpopulation is the Yo-Pro-1 positive cells emitting green fluorescence. It has been demonstrated that in early stages of apoptosis, there is a modification of membrane permeability that selectively allows entry of some non permeable DNA-binding molecules. This subpopulation are the spermatozoa showing early damage or a shift to another physiological state, since membranes become
slightly permeable during the first steps of cryoinjury, enabling Yo-
Pro-1 but not ethidium homodimer to penetrate the plasma 
membrane. None of these probes enters intact cells. Finally two 
subpopulations of cryoinjury-induced necrotic spermatozoa were 
easily detected, early necrotic, spermatozoa stained both with Yo-
Pro-1 and ethidium homodimer (emitting both green and red 
fluorescence), and late necrotic spermatozoa, cells stained only 
with ethidium homodimer (emitting red fluorescence).

## Staining for detection of phosphatidylserine 
translocation Annexin-V assay

Phosphatidylserine (PS) translocation was detected with the use 
of Alexa Fluor 488 annexin V (Dead Cell Apoptosis Kit, 
Molecular Probes, Leiden The Netherlands), which detects the 
translocation of PS from the inner to the outer leaflet of the 
plasma membrane, and Propidium Iodide (PI) nucleic acids stain, 
a probe able to detect dead cells, and therefore exclude these cells 
from the analysis [26]. To 100 µL of semen, 500 µL of 5X 
annexin binding-buffer (4 parts of nanopure water and 1 part of 
commercial binding buffer: 50mM HEPES, 700mM NaCl, 
12.5mM CaCl2, pH 7.4) was added. To this sample, 5 
µL of Alexa Fluor 488 annexin V (solution in 25mM HEPES, 140mM 
NaCl, 1mM EDTA, pH 7.4, 0.1% BSA) and 10 µL of Propidium 
iodide (1mg/mL in deionized water) were added. After 
15 minutes of incubation in the dark at room temperature, 
400 µL of 5X annexin binding-buffer were added. Cytometry 
analysis was then performed. Apoptotic cells show green 
fluorescence (530nm), dead cells show red fluorescence (620nm) 
and live cells have no fluorescence, after excited with Argon-laser 
ion of 488nm.

## Staining for detection of active caspases 3 and 7

The caspase FITC-DEVD-FMK (caspase 3 and 7) in situ marker 
(Molecular Probes, Leiden, The Netherlands) was used to detect 
avtive caspases. This cell-permeable specific caspase inhibitor 
peptide is conjugated to fluorescein isothiocyanate (FITC) and 
binds covalently to active caspases 3 and 7 serving as in situ marker 
for apoptosis [17,27]. A sample of 5x10⁶ spermatozoa were 
suspended in 1mL of PBS, and -after adding one µL of FITC 
DEVD-FMK (5mM) the suspension was incubated at room 
temperature (22-25°C) in the dark for 20 minutes. After 
incubation, the spermatozoa were washed with PBS, followed by 
the addition of 1 µL of ethidium homodimer (1.167 mM) 
(Molecular Probes Europe, Leiden, The Netherlands) to detect 
membrane damage. Flow cytometry was conducted within 
10 minutes.

## Staining for detection of lipid peroxidation

Lipid peroxidation was measured using the probe BODIPY 
581/591 C₁₁ (Molecular Probes) as previously described in our 
laboratory [17,28]. BODIPY 581/591 C₁₁ is a fatty acid that is 
a sensitive fluorescent reporter for LPO, undergoing a shift from 
red to green fluorescence when in presence of this process, due 
to the oxidation of the phenylbutadiene segment of the 
fluoresophore. This permits fluorescent ratio emission of live cells. 
A suspension of 2x10⁶ spermatozoa in PBS was loaded with the 
probe at a final concentration of 2 µM. Spermatozoa were then 
incubated at 37°C for 30 minutes, washed by centrifugation at 
1000 rpm during 5 minutes to remove the unbound probe, and 
analyzed using a flow cytometer. Positive controls were obtained 
after the addition of 80 µM ferrous sulfate to additional sperm 
suspensions.

## Evaluation of mitochondrial membrane potential 
(ΔΨm)

The lipophilic cationic compound 5,5',6,6'-tetrachloro-
1,1',3,3' tetraethylbenzimidazolyl carbocyanine iodine (JC-1) 
has the unique ability to differentially label mitochondria with 
low and high membrane potential [29,30]. 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, these monomers 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 dimethylsulfoxide (DMSO) was prepared. From 
each sperm sample, 1 mL of a sperm solution in PBS containing 
5x10⁶ cells/ mL was stained with 0.5 µL JC-1 stock solution. The 
samples incubated at 38°C in the dark for 40 minutes before flow 
cytometric analysis.

## Flow Cytometry

Flow cytometric analyses were carried out with a Coulter 
EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow 
cytometer equipped with standard optics. The standard four color 
set up uses 525, 575, 620 and 675 nm band pass filters and 488, 
550, 600 and 645nm dichroic long pass filters. The system has an 
argon-ion laser (Cytonics, Coherent, Santa Clara, CA, USA) 
performing 15 mW at 488 nm and EXPO 2000 software. 
Subpopulations were divided by quadrants, and the frequency of 
each subpopulation was quantified. Forward and sideways light 
scatter were recorded for a total of 10,000 events per sample. Non 
sperm events were calculated and eliminated as previously 
described [31]. Samples were measured at a flow rate of 200– 
300 cells/sec. Green fluorescence was detected in FL1(525 nm 
band pass filter) red fluorescence was detected in FL3 (620 nm 
band pass filter), and orange fluorescence in FL2 (575 nm band 
pass filter).

## Western blotting

During autophagy, LC3B-I is processed and converted to 
LC3B-II, thus the amount of LC3B-II is correlated with the 
number of autophagosomes [14].Western blotting was performed 
as previously described [32,33]. Briefly, stallion semen 
was centrifuged and washed twice with PBS. After washing, sperm 
cells were sonicated for 5 sec at 4°C 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 Na₃VO₄. The homogenates were clarified by centrifugation 
at 10,000 x g (15 min, 4°C) and the supernatant was used 
for analysis of protein concentration followed by dilution with 4x 
SDS sample buffer. Proteins (25 µg/well) from stallion sperm 
lysates were fractionated by SDS-PAGE using 4-20% polyacryl-
amide gradient gels and transferred to nitrocellulose membranes. 
After blocking, membranes were incubated overnight at 4°C with 
anti-LC3B (1:2000). 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 (Amer-
sham). 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.
Immunocytochemistry

Spermatozoa were washed and suspended in PBS adjusting the cell concentration to 1x10^6 cells per ml. Fifteen µl of the sperm suspension were spread on poly-l-lysine coated slides and allowed to attach for 10 min. Cells were then fixed with 3% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Slides were washed three times for 10 min with PBS and incubated in PBS supplemented with 5% BSA (w/v) for 90 min to block non-specific sites. After blocking, slides were incubated overnight at 4°C with anti-TNF-R1 (1:100), anti-TNF-R2 (1:100) or anti-TNF-alpha (1:100) antibodies diluted in PBS containing 5% BSA (w/v). Next day samples were extensively washed with PBS and further incubated with a goat anti-rabbit Alexa 488-conjugated antibody for 45 min at room temperature. Finally, slides were washed with PBS and examined with a Bio-Rad MRC1024 confocal microscope with a X60 objective in oil immersion. Samples were excited at 488 nm with an argon laser and emission was recorded using a 515-nm longpass filter set. Processing the samples without primary antibody assessed the absence of non-specific staining.

Statistical analysis

Data were first examined using the Kolmogorov-Smirnov test to determine their distribution, a multivariate analysis of variance was performed (ANOVA) and when significant differences were found, the non-parametric Mann-Whitney U-test was used to compare pairs of values directly if data did not adjust to a normal distribution. All analyses were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). The Spearman non-parametric test was used to study the correlations among apoptosis and autophagy and the results of the sperm analysis. Significance was set at P<0.05.

Results

Sperm motility and kinematics

As expected all parameters of sperm motility and kinematics decreased over the incubation period (Table 1). The percentages of total motile sperm and progressive sperm decreased in the second day of incubation (p<0.01), however on day 3 the percentage of total motile sperm was significantly different from day 2. The percentage of progressive motile sperm also decreased the second and third day of incubation (p<0.01), but the difference between the forth and fifth day was not statistically significant. Sperm velocities and the percentage of rapid sperm also decreased over the incubation period. VCL significantly decreased on the second day (p<0.01), but VCL on the second day was not different from VCL on the third day. On the other hand, VLS and VAP also decreased on the third day of storage (p<0.01). ALH and BCF only changed significantly (p<0.01) at the end of the period of storage; in addition ALH was significantly influenced by colloidal centrifugation that significantly reduced this parameter (p<0.01).

Sperm membrane integrity

There was not a significant decrease in the percentage of live sperm over the period of cooled storage. The percentage of live sperm at the end of the storage period was above 70% both in fresh extended sperm and in sperm subjected to CC. The overall percentage of live sperm was higher in CC selected sperm (p<0.01) (table 2).

Membrane intactness and subtle changes in membrane permeability

Colloidal centrifugation increased the percentage of spermatozoa showing intact membranes (p<0.01). The percentage of intact membranes decreased on day 3 of storage, but only in the CC group, while in the FE group the percentage on intact spermatozoa decreased after 5 days of storage (table 3). The change in the percentage of intact spermatozoa was parallel to change in the percentage of apoptotic spermatozoa that significantly increased in both groups at the end of the period of storage (p<0.01).

Active caspase 3 and 7

High caspase 3 and 7 activity was found in stallion spermatozoa, ranging from 19 to 30%. There was no effect of CC or days of storage in the percentage of spermatozoa showing high caspase 3 and 7 activity (table 4).

Detection of phosphatidylserine (PS) translocation/Annexin assay

The percentage of intact sperm (A-/PI-) decreased at day 3 of storage (p<0.01), but did not decrease further on day 5. The percentage of live sperm was higher in CC selected sperm (p<0.01) (table 2).

Table 1. Sperm motility and kinematics after computer assisted sperm analysis (CASA) of stallion spermatozoa stored during five days (day 1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation.

|        | FE  | CC  |
|--------|-----|-----|
| day | TM% | LM% | RS% | VCL/s | VSL/s | VAPμm/s | ALHμm | BCF Hz |
| 1     | 84.2±11.1  | 87.7±8.5  | 65.4±14.1  | 72.9±18.6  | 60.9±16.5  | 57.8±23.5  | 48.6±17.3  | 11.1±2.1  |
| 2     | 55.7±11.9  | 66.0±12.5  | 38.1±15.9  | 49.2±22.5  | 29.9±11.3  | 29.1±21.5  | 20.1±11.9  | 9.0±1.1  |
| 3     | 34.9±15.1  | 44.9±19.7  | 14.2±8.7   | 22.7±15.0  | 10.9±8.0   | 13.5±14.8  | 6.5±4.0   | 8.1±0.8  |
| 4     | 83.2±18.4  | 90.8±19.5  | 62.3±15.2  | 68.8±22.1  | 58.4±15.0  | 57.2±17.4  | 53.7±13.4  | 18.1±5.7  |
| 5     | 34.1±9.0   | 44.9±11.4  | 25.5±10.2  | 32.8±12.5  | 21.2±6.9   | 22.1±10.4  | 18.1±5.7   | 15.0±3.9  |

Table 1: The percentage of intact sperm (A-/PI-) decreased at day 3 of storage (p<0.01), but did not decrease further on day 5. The percentage of necrotic sperm (A-/PI+) followed the opposite trend.
increasing on day 3 but no further on day 5 (table 5). The percentage of annexin+ sperm did not vary over the time of storage while the percentage of A+/PI+ spermatozoa increased on the 5th day (p<0.01). CC had no effect on the percentage of spermatozoa showing PS translocation.

Mitochondrial membrane potential and percentage of Lipid peroxidation (LPO)

There were no changes on the mitochondrial membrane potential related either to the storage period or the sperm processing (table 6). In a similar way LPO was not affected either by the period of storage or the sperm processing.

LC3B processing to measure autophagy

Measuring processing of endogenous LC3B by western blot is the most common approach to measure autophagy in cells [14]. In native (non filtrated, fresh extended sperm) storage induced a significant increase in LC3B processing at day 5 indicating that autophagy was already activated at the beginning of the period of storage (figure 1), and did not change over the time.

Identification and subcellular distribution of TNF, TNFR1 and TNFR2 in stallion spermatozoa

The subcellular distribution of TNF, TNFR1 and TNFR2 was investigated in fixed and permeabilized stallion sperm using specific monoclonal antibodies. TNF and both receptors / TNFR1 and TNFR2 were present in stallion spermatozoa (figure 2). TNF was localized in the mid piece and rest of the tail, TNFR1, was present in the acrosomal region and mid piece, while TNFR2 was present in the post acrosomal region mid piece and rest of the tail.

Correlations among apoptotic markers and the sperm analysis

Positive as well as negative correlations were found among sperm quality and different apoptotic markers. The percentage of motile sperm was positively correlated with the percentage of intact sperm (YoPro-/Eth-) (r = 0.602, p<0.01), live sperm (SYBR-14+/PI-) (r = 0.549, p<0.01) and non apoptotic sperm (A-/PI-) (r = 0.713 p<0.01). Necrotic and apoptotic markers negatively correlated with motility; (YoPro+/Eth+) (r = −0.445, p<0.01), A+/PI+ (r = −0.280 p<0.01), YoPro+/Eth+ (r = 0.291 p<0.01) However low caspase 3 and 7 activity was correlated positively with motility (r = 0.314 p<0.01). High caspase 3 and 7 activity was correlated with apoptotic sperm (YoPro+, r = 0.312 p<0.01; A+ r = 0.287 p<0.01) and negatively correlated with necrotic sperm (Eth+ r = −0.362 p<0.01) and LPO (r = −0.436 p<0.01). Low caspase activity correlated positively with annexin negative sperm (r = 0.345 p<0.01).

Discussion

In the present study spermatozoa were investigated for the presence of LC3B in order to determine if the molecular machinery related to autophagy was present; for the first time we found that both LC3B-I and LC3B-II were present in ejaculated stallion spermatozoa. In somatic cells the primary role of autophagy is to protect cells under stress conditions such as starvation. Thus, can be considered that a major function of autophagy is lifespan extension [34,35] and consequently a

### Table 2. Sperm membrane integrity (SYBR-14/PI) of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation.

|       | FE  | CC  | FE  | CC  | FE  | CC  | FE  | CC  | FE  | CC  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Live% | 78.7±13.1<sup>a</sup> | 84.7±11.3<sup>a</sup> | 82.9±7.7<sup>b</sup> | 88.8±6.7<sup>b</sup> | 73.8±13.1<sup>a</sup> | 81.6±5.3<sup>b</sup> | 74.0±9.1<sup>b</sup> | 80.3±11.0<sup>b</sup> | 72.1±7.7<sup>b</sup> | 74.4±14.0<sup>b</sup> |
| Dead% | 16.3±10.3<sup>a</sup> | 10.0±8.1<sup>a</sup> | 12.5±7.3<sup>b</sup> | 6.6±7.0<sup>b</sup> | 17.9±11.9<sup>a</sup> | 11.5±6.1<sup>b</sup> | 17.5±7.2<sup>b</sup> | 12.2±9.5<sup>b</sup> | 19.0±8.8<sup>b</sup> | 17.9±13.1<sup>b</sup> |
| Damaged% | 5.0±5.9<sup>a</sup> | 5.2±9.5<sup>a</sup> | 4.5±2.8<sup>b</sup> | 3.9±5.6<sup>b</sup> | 8.2±7.7<sup>b</sup> | 6.9±6.7<sup>b</sup> | 8.5±8.9<sup>b</sup> | 7.4±8.9<sup>b</sup> | 8.8±7.7<sup>b</sup> | 7.6±6.5<sup>b</sup> |

### Table 3. Membrane intactness and subtle changes in sperm membrane integrity of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation.

|       | FE  | CC  | FE  | CC  | FE  | CC  | FE  | CC  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| Intact% | 58.1±12.9<sup>a</sup> | 72.4±13.2<sup>a</sup> | 51.3±9.9<sup>a</sup> | 60.4±9.1<sup>a</sup> | 45.8±11.7<sup>b</sup> | 49.9±11.0<sup>b</sup> |
| YoPro+ % | 12.7±6.7<sup>a</sup> | 8.6±2.9<sup>a</sup> | 9.4±2.7<sup>a</sup> | 8.4±3.3<sup>a</sup> | 20.7±18.9<sup>b</sup> | 18.5±10.8<sup>b</sup> |
| YoPro+/Eth+ % | 21.6±12.9 | 12.3±11.1 | 28.2±9.9 | 18.5±3.7 | 22.9±13.1 | 25.9±9.9 |
| Eth+ % | 7.5±6.2<sup>a</sup> | 6.6±7.0<sup>a</sup> | 11.0±8.6<sup>a</sup> | 12.8±6.2<sup>a</sup> | 10.5±10.3 | 5.5±4.6<sup>a</sup> |

Intact spermatozoa are those not stained and thus represent spermatozoa with completely intact membranes. YoPro+ are early apoptotic sperm depicting an increase in sperm membrane permeability, YoPro+/Eth+ are late apoptotic and Eth+ are necrotic spermatozoa. Within a row with values different superscript differ statistically a-c p<0.01. (means ± SD) Results are derived from 28 identical experiments (7 stallions, 4 ejaculates per stallion).

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strategy for survival in mammalian cells. Both situations -stress and extension of the lifespan of the spermatozoa- are hallmarks of sperm conservation. This is to the authors’ knowledge the first report indicating that the mammalian spermatozoa have the molecular machinery necessary for autophagy. It is true that the presence of LC3B may simply represent a remnant from spermatogenesis, in a similar way as has been proposed in the theory of abortive apoptosis[36], for apoptotic markers present in mature spermatozoa. However, autophagy appears to be differentially activated in the spermatozoa, with a subpopulation of sperm that have higher relation of LC3B-II/LC3B-I (Figure 1) on day 1. This subpopulation is also characterized by higher percentage of intact sperm on day 1 but not in day 5, however on day 5 both subpopulations of spermatozoa have similar relation of LC3B-II/LC3B-I intensity. These findings indicate that an “autophagy like” phenomena may have in sperm distinct functions as has been proposed for somatic cells[37]. On one side this autophagy-like mechanism can be related to sperm survival since the spermatozoa selected through gradient centrifugation had higher percentages of live and intact sperm and also had higher levels of processed LC3B. In the unselected subpopulation there was an increase of processed LC3B after 5 days of storage. This may represent a response of the spermatozoa to nutrient deprivation along the storage period, but taking in account that in the selected population there was not a change in LC3 processing another role for autophagy in sperm can not be completely ruled out. Although autophagy may also lead to programmed cell death in somatic cells[38,39] it is believed that autophagy is mainly a strategy for survival and the fact that the subpopulation of more viable spermatozoa at the same time had

Apoptosis is a major cause of sperm damage during cryopreservation in the stallion spermatozoa [17,29,40,41], and in the present study we investigated whether apoptosis also plays a role in sperm aging during cooled storage. Although apoptosis in mature spermatozoa has been under debate due to the fact that are highly differentiated terminal cells, recently new evidences suggest that apoptosis in mature sperm, has a role in the removal of these cells from the male and female reproductive tracts once viability has been lost [42]. Most of the ejaculated spermatozoa die in the female reproductive tract after insemination. Active caspases 3 and 7 were present in fresh sperm, indicating that upon ejaculation an important percentage of spermatozoa are already activated to die through apoptosis. This finding confirms previous reports from our laboratory [16]. Caspase 3 is considered the most important effector caspase, with its activation marking a “point of no return” in apoptosis [43]. In fact, lose of sperm quality seems to be an apoptotic phenomenon [44,45]; this is further demonstrated in our study by the fact that high caspase 3 and 7 activity was correlated with apoptotic sperm (YoPro\(^+\)), but at the same time negatively correlated with necrotic sperm (Ethidium\(^+\)). Another fact is that there were no changes in the percentage of live sperm thorough the storage period, while was evidenced a significant increase in the percentage of spermatozoa with increased membrane permeability. Increased membrane permeability is a sign of early apoptosis both in somatic cells [46] and spermatozoa [16,47]. This increase in membrane permeability is related to the fact that apoptotic cells release ‘find-me’ signals at the earliest stages of death to recruit phagocytes. The nucleotides ATP and UTP represent one class of “find-me” signals that are released by mechanisms that imply increases in membrane permeability [46,48], using the same channels to be released from the cell as

| Table 4. Active caspases 3 and 7 of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation. |
|----------------------------------|------------------|------------------|------------------|
|                                | D1   | D3   | D5   |
|                                | FE   | CC   | FE   | CC   | FE   | CC   |
| High activity%                 | 27.9±31.8 | 28.3±32.6 | 18.9±15.9 | 18.2±25.3 | 30.2±28.9 | 28.3±22.9 |
| Low activity%                  | 43.7±27.7 | 48.6±35.3 | 37.9±15.0 | 42.6±9.7 | 34.6±30.7 | 34.6±24.4 |
| Dead sperm%                    | 28.6±17.7 | 23.3±19.7 | 43.5±4.5 | 40.8±8.8 | 35.4±18.8 | 37.5±10.6 |

Table 5. Annexin-V assay of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation.

| Table 5. Annexin-V assay of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation. |
|----------------------------------|------------------|------------------|------------------|
|                                | D1   | D3   | D5   |
|                                | FE   | CC   | FE   | CC   | FE   | CC   |
| Live (%A-P)                     | 71.8±15.7 | 69.5±18.2 | 47.9±10.6 | 53.6±12.6 | 40.0±15.4 | 48.9±10.2 |
| A+ (%)                         | 6.1±4.3 | 14.2±15.7 | 8.5±10.0 | 8.5±10.0 | 15.8±15.8 | 10.3±9.0 |
| A+P (%)                       | 1.0±1.5 | 0.8±1.1 | 2.2±2.0 | 2.8±2.3 | 2.9±3.9 | 2.9±3.3 |
| A+P+ (%)                      | 20.9±14.8 | 15.6±11.3 | 41.4±9.2 | 31.2±15.9 | 41.2±12.9 | 37.9±12.8 |

Live % percentage of live sperm, A+, annexin positive sperm, spermatozoa depicting translocation of PS, A+P+PI dead spermatozoa depicting PS translocation, A+P+ necrotic spermatozoa. Within a row values with different superscript differ statistically a-b p<0.01. (Means ± SD) Results are derived from 28 identical experiments (7 stallions, 4 ejaculates per stallion).
YoPro-1 uses to enter the cell. This fact has also implications for other areas of sperm reproductive technology; since more classical approaches to measure sperm quality such as the combination SYBR-14/PI does not allow detecting spermatozoa that have already initiated a way to cell death. Assays capable to detect apoptotic spermatozoa can identify defective spermatozoa at an early stage of damage and thus can be better assays of sperm quality [26,49].

The use of YoPro-1 is considered a simple, quick, inexpensive and repeatable method to detect apoptosis in somatic cells [50] and spermatozoa [51] and is routinely used in our laboratory to detect early sperm damage [24,25,29,52]. Our findings support the superior value of this technique for sperm analysis confirming previous reports [51,53]. Another marker of apoptosis that also revealed differences along the incubation period was the monitoring of PS translocation. During more advanced stages of apoptosis dying cells display in the outer membrane PS that is recognized by phagocytes to remove these cells without an inflammatory response, being an “eat-me” signal [18,54,55]. At the end of the storage period there was an increase in sperm depicting PS translocation further supporting the existence of apoptotic sperm death during cooled storage.

Sperm motility is routinely used as the main indicator of sperm quality under clinical settings [56,57]. In our study we found that loss of motility correlated with the percentage of necrotic and also apoptotic sperm; this fact supports the assumption that stallion spermatozoa dye both through necrotic and apoptotic mechanisms. Interestingly loss of motility correlated better with intact spermatozoa than with live sperm, adding value to assays able to detect apoptotic spermatozoa.

During cryopreservation stallion spermatozoa experiences apoptosis involving the intrinsic pathway [16], however in our experiment there were not changes in mitochondrial membrane potential, or changes in LPO, suggesting either that the intrinsic pathway does not play a role in sperm death during cooled storage or that this pathway is already activated at ejaculation. The extrinsic pathway of apoptosis is activated by tumor necrosis factor family of receptors; activation of these receptors results in

**Table 6.** Mitochondrial membrane potential (Δψm) and lipid peroxidation (LPO) of stallion spermatozoa stored during five days (day 1 D1 to day 5 D5) at 5°C FE fresh extended sperm, CC sperm processed through colloidal centrifugation.

|            | D1 FE | D1 CC | D3 FE | D3 CC | D5 FE | D5 CC |
|------------|-------|-------|-------|-------|-------|-------|
| High %     | 4.4±.8| 8.3±12.2 | 4.4±7.4| 5.6±6.2 | 2.2±4.4 | 6.2±5.8 |
| Low %      | 27.3±16.3 | 23.2±23.6 | 23.6±17.3 | 30.4±22.2 | 31.4±13.5 | 10.9±16.8 |
| LPO%       | 68.1±22.5 | 67.8±33.3 | 71.9±22.5 | 63.9±26.9 | 62.2±17.7 | 82.9±18.4 |

(Mean±SD) Results are derived from 28 identical experiments (7 stallions, 4 ejaculates per stallion). High spermatozoa depicting high Δψm, High and Low spermatozoa depicting simultaneously mitochondria with low and high Δψm, Low spermatozoa with low Δψm, LPO spermatozoa showing peroxidation of the lipids of their membranes.

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Figure 1. Changes in LC3B processing in stallion spermatozoa stored under refrigeration (5°C) for five days, after single layer centrifugation (Filtrated) or unprocessed (native sperm). In native sperm storage induced a significant increase in LC3B processing at day 5 indicating that autophagy was activated during the period of storage. On the other hand, filtration of sperm selected a subpopulation of spermatozoa in which autophagy was already activated at the beginning of the period of storage and did not change over the time. Results are representative of 28 identical experiments (seven stallions, four ejaculates per stallion) * p<0.01.

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TNF was localized in the mid piece and rest of the tail, TNFR1, was present in the acrosomal region and mid piece, while TNFR2 was present in the fixed and permeabilized stallion spermatozoa was assessed by immunocytochemistry with specific antibodies as described in material and methods.

References

1. Rigby SL, Brinko SP, Cochran M, Blanchard TL, Love CC, et al. (2001) Advances in cooled semen technologies: seminal plasma and semen extenders. Anim Reprod Sci 68: 171–180.

2. Moran DM, Jasko DJ, Squires EL, Amann RP (1993) Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 38: 999–1012.

3. Chandler E, Abraham-Peskir JV, Little S, McCann C, Medenwaldt R (2000) Effect of cooling on the mobility and function of human spermatozoa. Cryobiology 41: 125–134.

4. Drobinski EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, et al. (1993) Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J Exp Zool 265: 432–437.

5. White IG (1993) Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod Fertil Dev 5: 639–658.

6. Windsor DP, White IG, Selley ML, Swan MA (1993) Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J Exp Zool 265: 432–437.

7. White IG (1993) Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod Fertil Dev 5: 639–658.

8. Purdy PH, Tharp N, Stewart T, Spiller SF, Blackburn HD (2010) Implications of cryopreservation of stallion spermatozoa. Anim Reprod Sci 68: 171–180.

9. Halangk W, Bohnensack R, Kunz W (1980) Lipid peroxidation mechanisms. J Pathol 221: 117–124.

10. Hulka WC, Bohnensack R, Kunz W (1980) [Relation between intactness and adenine nucleotide pattern of ejaculated bull spermatozoa]. Acta biologica et medica Germanica 39: 791–808.

11. Ortega-Ferrusola C, Gonzalez-Fernandez L, Muriel A, Macias-Garcia B, et al. (2009) Lipid peroxidation, assessed with BODIPY-C11, modifies the sperm subpopulation structure of frozen-thawed stallion semen. Anim Reprod Sci 68: 171–180.

12. Halangk W, Bohnensack R, Kunz W (1980) [Relation between intactness and adenine nucleotide pattern of ejaculated bull spermatozoa]. Acta biologica et medica Germanica 39: 791–808.

13. Holt WW (2011) Does apoptosis hold the key to long-term sperm storage mechanisms in vivo? Mol Reprod Dev 78: 464–465.

14. Barth S, Glick D, Macled CF (2010) Autophagy: assays and artifacts. J Pathol 221: 117–124.

15. Glick D, Barth S, Macled CF (2010) Autophagy: cellular and molecular mechanisms. J Pathol 221: 3–12.

16. Ortega-Ferrusola C, Solitro-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, et al. (2008) Detection of “apoptosis-like” changes during the cryopreservation process in equine sperm. J Androl 29: 213–219.

17. Ortega-Ferrusola C, Gonzalez Fernandez L, Morrell JM, Salazar Sandoval C, Macias Garcia B, et al. (2009) Lipid peroxidation, assessed with BODIPY-C11, modifies the sperm subpopulation structure of frozen-thawed stallion semen. Anim Reprod Sci 108: 53–63.

18. Said TM, Gagliani A, Agarwal A (2010) Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online 21: 456–462.

19. Martin G, Sabido O, Durand P, Levy R (2004) Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 71: 20–37.

20. Johannisson A, Morrell JM, Thoren J, Jonsson M, Dalin AM, et al. (2009) Colloidal centrifugation with Androcoll-E prolongs stallion sperm motility, viability and chromatin integrity. Anim Reprod Sci 116: 119–128.

21. Macias Garcia B, Gonzalez Fernandez L, Morrell JM, Ortega Ferrusola C, Tapia JA, et al. (2009) Single-layer centrifugation through colloidal positively modifies the sperm subpopulation structure of frozen-thawed stallion sperma-tozoa. Reprod Domest Anim 44: 325–329.

22. Morrell JM, Rodrigez-Martinez H, Johannisson A (2010) Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. Equine Veterinary Journal 42: 379–385.

23. Macias Garcia B, Morrell JM, Ortega-Ferrusola C, Gonzalez-Fernandez L, Tapia JA, et al. (2009) Centrifugation on a single layer of colloidal selects improved quality spermatozoa from frozen-thawed stallion semen. Anim Reprod Sci 114: 193–202.

To summarize, major findings of our study are: (i) discovery that LC3B is differentially activated during cooled storage of stallion spermatozoa, (ii) that during cooled storage stallion spermatozoa experiences apoptotic damage (iii) demonstrates the major value of the YoPro-1 assay for the early detection of defective spermatozoa (iv) opens clues to disclose, whether other forms of programmed cell death (apoptosis) occur in the spermatozoa, and more importantly (v) may open new strategies of sperm conservation based in the modulation of cellular pathways leading to sperm death, or enhancing mechanisms of sperm survival.

Author Contributions

Conceived and designed the experiments: FJP. Performed the experiments: JMGB AMM CAMBS AMR MPD COF IMA. Analyzed the data: IMA JAT FJP. Wrote the paper: FJP.
24. Nunez-Martinez I, Moran JM, Peña FJ (2007) Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. Int J Androl 30: 182–189.
25. Peña FJ, Saravia F, Johansson A, Walgren M, Rodriguez-Martinez H (2005) A new method of evaluating sperm membrane integrity. Theriogenology 69: 677–689.
26. Peña FJ, Johansson A, Walgren M, Rodriguez-Martinez H (2003) Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Int J Androl 26: 107–114.
27. Grunewald S, Sharma R, Pasch U, Glander HJ, Agawal A (2009) Impact of caspase activation in human spermatozoa. Micro Res Tech 72: 478–488.
28. Balbo da Silva CM, Macias-Garcia B, Miro-Moran A, Gonzalez-Fernandez L, Morillo-Cordovan G, et al. (2011) Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. J Pineal Res 51: 172–179.
29. Ortega-Ferrusola C, Gonzalez Fernandez L, Macias Garcia B, Salazar-Sandoval C, Morillo Rodriguez A, et al. (2009) Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol Reprod 81: 1106–1111.
30. Peña FJ, Johansson A, Walgren M, Rodriguez-Martin H (2003) Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 78: 85–98.
31. Petrunkina AM, Waberski D, Bollwein H, Sieme H (2010) Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. Theriogenology 73: 995–1000.
32. Gonzalez-Fernandez L, Ortega-Ferrusola C, Macias-Garcia B, Salido GM, Peña FJ, et al. (2009) Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. Biol Reprod 80: 1239–1252.
33. Ortega-Ferrusola C, Gonzalez Fernandez L, Macias Garcia B, Salazar-Sandoval C, Morillo Rodriguez A, et al. (2008) Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol Reprod 81: 1106–1111.
34. Chen Y, Kliomsky DJ (2011) The regulation of autophagy - unanswered questions. J Cell Sci 124: 161–170.
35. Yang Z, Kliomsky DJ (2010) Eaten alive: a history of macroautophagy. Nature cell biology 12: 814–822.
36. Sakkas D, Selig E, Bizzaro D, Tarozzi N, Manicardi GC (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. Reprod Biomed Online 6: 426–432.
37. Yu L, Strandberg L, Lenardo MJ (2008) The selectivity of autophagy and its role in cell death and survival. Autophagy 4: 567–573.
38. Yu L, Alca A, Su H, Dutt P, Freundt E, et al. (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304: 1300–1302.
39. Yu L, Lenardo MJ, Bachrache EH (2004) Autophagy and caspases: a new cell death program. Cell cycle 3: 1124–1129.
40. Ortega-Ferrusola C, Garcia BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, et al. (2009) Apoptotic markers can be used to forecast the freezability of stallion spermatozoa. Anim Reprod Sci 114: 393–403.
41. Brum AM, Sabeur K, Ball BA (2008) Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology 69: 1041–1053.
42. Aiiken RJ, Findlay JK, Hunt KJ, Kerr JB (2011) Apoptosis in the germ line: Reproduction 141: 139–150.
43. Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Bioch 68: 383–424.
44. Peña FJ, Rodriguez-Martinez H, Tapia JA, Ortega-Ferrusola C, Gonzalez Fernandez L, et al. (2009) Mitochondria in mammalian sperm physiology and pathology: a review. Reprod Dom Anim 44: 345–349.
45. Peña FJ, Nunez-Martinez I, Moran JM (2006) Semen technologies in dog breeding: an update. Reprod Dom Anim 41(Suppl 2): 21–29.
46. Chekera FB, Elliot MR, Sandilands JK, Walk SF, Kuncl JM, et al. (2010) Pannexin 1 channels mediate ‘find-me’ signal release and membrane permeability during apoptosis. Nature 467: 863–867.
47. Peña FJ, Saravia F, Johansson A, Walgren M, Rodriguez-Martinez H (2003) A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. Int J Androl 26: 107–114.
48. Elliott MR, Chekera FB, Trampont PC, Lazarowski ER, Kauf A, et al. (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461: 282–286.
49. Anzar M, He L, Buhr MM, Kroetz SC, Pauls KP (2002) Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 66: 354–360.
50. Idziorek T, Estaquier J, De Bel F, Ameisen JC (1993) YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J Immunol Meth 185: 249–258.
51. Martin G, Sabido O, Durand P, Levy R (2004) Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 71: 29–37.
52. Peña FJ, Saravia F, Johansson A, Wallgren M, Rodriguez-Martinez H (2007) Detection of early changes in sperm membrane integrity pre-freezing can estimate post-thaw quality of boar spermatozoa. Anim Reprod Sci 97: 74–83.
53. Martin G, Cagnoni N, Sabido O, Sohn B, Grizard G, et al. (2007) Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum Reprod 22: 380–383.
54. Aiiken RJ, Koppers AJ (2011) Apoptosis and DNA damage in human spermatozoa. Asian J Androl 13: 56–42.
55. Li MO, Sarkisian MR, Mehal WZ, Rakic P, Flavell RA (2003) Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302: 1560–1563.
56. Martinez IN, Moran JM, Peña FJ (2006) Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J Androl 27: 396–403.
57. Ortega-Ferrusola C, Macias Garcia B, Suarez Rama V, Gallardo-Bolanos JM, Gonzalez-Fernandez L, et al. (2009) Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. Reprod Dom Anim 44: 419–423.
58. Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, et al. (2004) Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 71: 1826–1837.
59. Paasch U, Grunewald S, Agarwal A, Glander HJ (2004) Activation pattern of caspases in human spermatozoa. Fertil Steril 81(Suppl 1): 802–809.
60. Zhang DW, Shao J, Lin J, Zhang N, Liu BJ, et al. (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325: 332–336.
61. Peter AT, Linde-Forsberg C (2003) Efficacy of the anticaspase agent zVAD-fmk on post-thaw viability of canine spermatozoa. Theriogenology 59: 1525–1532.

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