

# Activated caspases are present in frozen–thawed canine sperm and may be related to post thaw sperm quality

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## Summary

The identification of early changes in the sperm plasmalemma is currently a factor in the improvement of freezing protocols. We analysed the presence of active caspases in freeze–thawed (FT) dog spermatozoa, and evaluated straws from eight dogs using flow cytometry and fluorescence microscopy with fluorescein isothiocyanate–Val–Ala–Asp–fluoromethylketone (FITC–VAD–fmk) combined with ethidium homodimers. Apoptotic-like changes were evaluated using the YO–PRO-1/ethidium homodimer combination, and changes in mitochondrial membrane potential were monitored with JC-1. Sperm motility post-thaw was evaluated using a CASA system. FITC–VAD–fmk stained sperm cells *in situ* and the subcellular labelling pattern was consistent with known localization of caspases. On average, a high proportion of FT canine sperm showed caspase activity, ranging from 30.2 to 70.7% of the live sperm compared with 7.3 to 24.0% in dead spermatozoa. This observed differentiation between caspase activity in dead and live spermatozoa may be a simple method to disclose subtle differences in sperm quality, since this staining allowed us to find statistically significant differences among dogs. Notably, the sperm sample with overall better results in all sperm parameters studied after thawing had a lower percentage of active caspases in both dead and live spermatozoa.

Keywords: Apoptosis, Canine, Caspases, Flow cytometry, Freezing, Mitochondria, Sperm

## Introduction

In comparison with other domestic species, the development of new tests to improve the spermiogramme in canines have been scarce (Peña *et al.*, 2006). These studies have focused on the evaluation of sperm membranes, acrosomes or capacitation status; IVF tests have also been successfully used (Peña *et al.*, 2004). Recent studies have examined the sperm DNA (Núñez Martínez *et al.*, 2005) and the sperm subpopulation structure (Núñez Martínez *et al.*, 2005a,b, 2006). The importance of the so-called, ‘apoptotic-like’ phenomena is receiving increasing interest both in human and veterinary andrology. Apoptotic-like phenomena are demonstrated to be

involved in human infertility (Said *et al.*, 2004) and in the damage induced by cryopreservation procedures both in humans (Paasch *et al.*, 2004) and animals (Peña *et al.*, 2003, Martin *et al.*, 2005)

Apoptosis is a complex phenomenon regulating cellular proliferation that can be divided into three phases: induction, execution and degradation. After induction of apoptosis, mitochondrial pores are opened, leading to a decreased mitochondrial membrane potential ( $\Delta\Psi_m$ ). Opening of mitochondrial pores causes the release of pro-apoptotic factors in the cytoplasm where they are activated, leading to the degradation phase. During degradation, the sperm membrane permeability increases, and phosphatidylserine (which triggers a non-inflammatory recognition of the apoptotic cells by phagocytes) is externalized (Desagher & Martinou, 2000). Mitochondria play a major role in the control of apoptosis. In addition, mitochondria seem to be the subcellular structures that are more sensitive to the stress of cryopreservation (Peña *et al.*, 2004; Ortega Ferrusola *et al.*, 2008). Improvement of cryo-preservation protocols requires

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an in-depth knowledge of the subcellular and molecular mechanisms involved in cryo-damage, and the development of new techniques able to detect these injuries. Flow cytometry is a highly accurate, and repeatable, technique suitable to detect these subcellular and molecular injuries allowing a rapid clinical application of this technique in assessing cryopreservation (Peña *et al.*, 2006)

The aim of the present study was to investigate the presence of active caspases in frozen thawed canine sperm using a cell permeable fluorescent derivative of the inhibitor peptide VAD-FMK (FITC-VAD-FMK) and assess the correlation of the presence of active caspases with other apoptosis markers,  $\Delta\Psi_m$  and early changes on membrane permeability. Additionally, we investigated the possible correlation of these parameters with sperm motility (CASA).

## Material and methods

### Animals

Eight privately owned dogs (one German shepherd, three golden retrievers, and four Labrador retrievers) were used in this study. All dogs were of known fertility (all had sired at least one litter). The range of the dogs' weight was from 25 to 35 kg, with ages ranging from 2 to 6 years.

### Semen collection and processing

Semen was manually collected (three ejaculates per dog) in a prewarmed graduated test tubes. After collection, sperm samples were kept at 37°C in a water bath for less than 10 min. An aliquot was removed for sperm concentration measurement and evaluation of motility using computer-assisted sperm analysis (CASA) and morphology analysis (phase contrast, microscopy of wet smears using a  $\times 100$  objective). Only samples with at least 70% progressive motility and 80% morphologically normal spermatozoa were included in the study.

### Cryopreservation procedure

Semen was processed using the Uppsala method (Peña & Linde Forsberg, 2000) modified by Nuñez Martinez *et al.* (2006). In brief, collected semen was extended 1:1 in Tris-glucose Extender I (Tris 200 mM, glucose 70 mM, citric acid 63 mM, BSA 3%, penicillin 1000 UI/ml, dihydrostreptomycin 1 mg/ml) and centrifuged for 8 min at 700 g. The seminal plasma was then removed and the sperm pellet resuspended in Extender II at room temperature (Tris 200 mM, 70 mM glucose, 63 mM citric acid, glycerol 3% v/v, egg yolk 20% v/v, penicillin 1000 UI/ml, dihydrostreptomycin 1 mg/ml), and cooled to 5°C over a period of 1 h,

resulting in a concentration of 300–400  $\times 10^6$  sperm per ml. After equilibration, an equal volume of Extender III (Tris 200 mM, 70 mM glucose, 63 mM citric acid 7% v/v, glycerol 20% v/v, egg yolk 1% v/v, Equex STM paste [Nova Chemical Sales Inc. Sciutate] penicillin dihydrostreptomycin) was added at 5°C to a final sperm concentration of 150–200  $\times 10^6$  spermatozoa per ml. The sperm samples were then loaded into 0.5 ml straws and frozen horizontally in racks by placement of samples 4 cm above the surface of liquid N<sub>2</sub> in a closed Styrofoam box for 10 min and then plunging directly in liquid N<sub>2</sub>. After 4 weeks of storage, the straws were thawed in a 70°C water bath for 8 s.

### Motility analysis

Motility was measured after cryopreservation using a CASA system (ISAS® Proiser, Valencia, Spain). Analysis was based on the examination of 25 consecutive, digitalized images obtained from a single field using a  $\times 10$  negative phase-contrast objective. Images were taken at a time lapse of 1 s and the image capture speed was, therefore, once every 40 ms. The number of objects incorrectly identified as spermatozoa were minimized on the monitor by the playback function. With respect to the setting parameters for the programme, sperm with a mean velocity (VAP)  $< 10 \mu\text{m/s}$  were considered immotile, while sperm with a velocity  $> 15 \mu\text{m/s}$  were considered motile. Spermatozoa deviating  $< 10\%$  from a straight line were designated as linear motile. Sperm motion kinematics measured by CASA included the following:

- curvilinear velocity (VCL) ( $\mu\text{m/s}$ ): measures the sequential progression along the true trajectory;
- linear velocity (VSL) ( $\mu\text{m/s}$ ): measures the straight trajectory of the spermatozoa per unit time;
- mean velocity (VAP) ( $\mu\text{m/s}$ ): measures the mean trajectory of the spermatozoa per unit time;
- linearity coefficient (LIN) (%):  $\text{VSL}/\text{VCL} \times 100$ ;
- straightness coefficient (STR) (%):  $\text{VSL}/\text{VAP} \times 100$ ;
- wobble coefficient (WOB) (%):  $\text{VAP}/\text{VCL} \times 100$ ;
- mean lateral head displacement (ALH) ( $\mu\text{m}$ ): measures the mean head displacement along the curvilinear trajectory;
- BCF (beat cross frequency) (Hz): number of times the sperm head crosses the mean path/s.

### Staining for cytofluorometric assessment of activated caspases

The caspase FITC-VAD-FMK *in situ* marker (Molecular Probes) was used to detect active caspases. This cell permeable caspase inhibitor peptide conjugated to FITC covalently binds activated caspases, thus serving as an *in situ* marker for apoptosis. Spermatozoa ( $1 \times 10^6$ ) were diluted in 1 ml of PBS, 1  $\mu\text{l}$  of

FITC–VAD–FMK was added, and samples were incubated at room temperature in the dark for 20 min. The spermatozoa were then washed in PBS and resuspended to the initial cell concentration, 1  $\mu$ l of ethidium homodimer (1.167 mM) (Molecular Probes) was added, and samples were assessed by flow cytometry and fluorescence microscopy within 10 min.

#### Staining for evaluation of mitochondrial membrane potential ( $\Delta\Psi_m$ )

The lipophilic cationic compound, 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl carbocyanine iodine (JC-1), possesses the unique ability to differentially label mitochondria with low and high membrane potentials. In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength (590 nm) when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers, emitting in the green wavelength (525 to 530 nm) when excited at 488 nm. A 3 mM stock solution of JC-1 (Molecular Probes) in DMSO was used for staining, and a 500  $\mu$ l aliquot of each semen sample was stained with 0.5  $\mu$ l of the JC-1 stock solution. The samples were incubated at 38°C in the dark for 40 min before flow cytometric and fluorescence microscopy analysis.

#### Staining for assessment of subtle membrane changes and viability

Early membrane changes and sperm viability were determined as described by Peña *et al.* (2005) with modifications for adaptation to canine semen. In brief, the following stock solutions in DMSO were prepared: YO–PRO-1 (25  $\mu$ M) and ethidium homodimer-1 (1.167 mM) (Molecular Probes). A total of 500  $\mu$ l of a sperm suspension containing  $5 \times 10^6$  spermatozoa/ml in PBS was stained with 3  $\mu$ l of YO–PRO-1 and 1  $\mu$ l 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. The first subpopulation of unstained spermatozoa are considered live and in the absence of any membrane alteration. A second subpopulation is the YO–PRO-1-positive cells emitting green fluorescence. In early stages of apoptosis, the membrane permeability is known to be modified, selectively allowing the entry of several non-permeable DNA-binding molecules (Ormerod *et al.*, 1993; Wronsky *et al.*, 2002). This subpopulation comprises the spermatozoa that may show early damage or a shift to another physiological state. Membranes become slightly permeable during the first steps of cryoinjury, enabling YO–PRO-1 but not ethidium homodimer to penetrate the plasma membrane (Izdiorek *et al.*, 1995). Neither of the probes enters intact cells. Finally,

two subpopulations of cryoinjury-induced necrotic spermatozoa were easily detected by staining: early necrotic spermatozoa stained with both YO–PRO-1 and ethidium homodimer (emitting both green and red fluorescence), and late necrotic spermatozoa stained only with ethidium homodimer (emitting red fluorescence). The staining patterns were visually checked under combined phase-contrast fluorescence microscopy (Nikon-E80).

#### Flow cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc.) flow cytometer equipped with standard optics. An argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm and the EXPO 2000 software was included. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Non-sperm events (debris) were gated out based on the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to sperm cells but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10 000 events per sample; samples were measured at flow rate of approximately 300 cells/s. Green fluorescence was detected in FL1, red fluorescence was detected in FL3, and orange fluorescence in FL2.

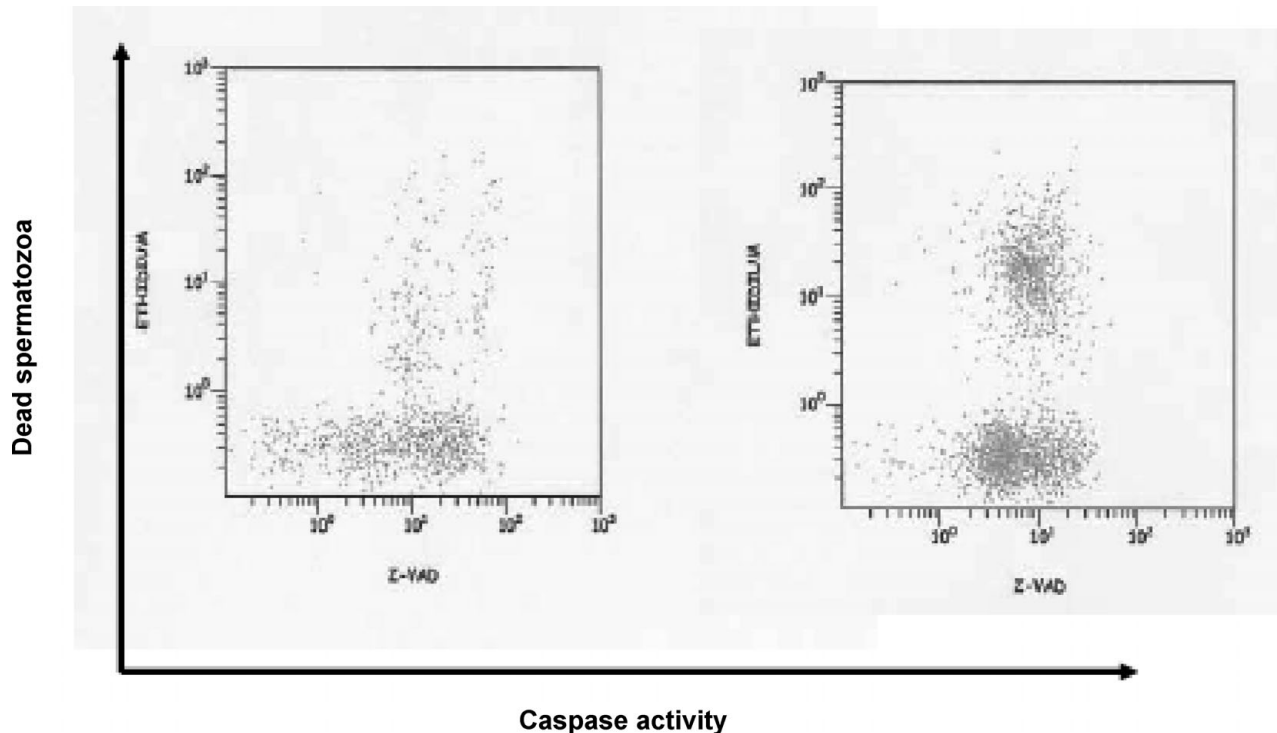
#### Statistical analysis

The data were first examined using the Kolmogorov–Smirnov test to determine their distribution. In view of the non-Gaussian distribution of most of the data gathered, multivariate analysis of variance was performed, and when significant differences were found, the non-parametric Mann–Whitney *U*-test was used to directly compare pairs of values. The Spearman non-parametric test was used to study the correlation between sperm analysis prefreezing and sperm quality post-thaw. All analyses were performed using SPSS ver. 15.0 for Windows (SPSS Inc.). Significance was set at  $p \leq 0.05$ .

## Results

#### Presence of active caspases in frozen–thawed sperm

Typical cytograms of FT canine spermatozoa labelled with FITC–VAD–FMK/Eth are shown in Fig. 1. Four cell patterns were detected by cytometric analysis: live cells without caspase activity, live cells with caspase activity, and two groups of dead cells, dead with caspase activity and dead cells without caspase activity. Staining of spermatozoa for cellular caspase activity



**Figure 1** Flow cytometry analysis of activation of caspase family members in frozen-thawed dog sperm. Representative cytograms in two dogs. Left panel indicates a freezer that worked, while right panel shows results from samples stored in a defective freezer. Events in the lower left region represent intact sperm, and events in the lower right region represent spermatozoa showing caspase activity. Events in the upper right and left regions are ethidium positive, thus dead spermatozoa.

revealed two patterns of caspase localization; caspase activity (green fluorescence) was observed in the mid piece, tail and over the acrosomal region, or observed only in the midpiece and tail (Fig. 2). On average, a high proportion of FT canine sperm showed caspase activity (Table 1), ranging from with 30.2 to 70.7% of live sperm and 7.3 to 24.0% of dead sperm as caspase positive.

#### Mitochondrial membrane potential

The mean per cent of sperm cells showing low  $\Delta\Psi_m$  ranged from 8 to 39.8%, and significant differences among the dogs were not identified. The proportion of mitochondria showing high and low  $\Delta\Psi_m$  varied significantly (Fig. 3).

#### Presence of apoptotic and dead spermatozoa (increase in sperm membrane permeability)

No significant differences were observed among dogs either in the percent of live spermatozoa or in the presence of spermatozoa showing early changes in sperm membrane permeability (Table 2). However, significant differences were observed among dogs in the per cent of ethidium positive spermatozoa.

#### Motility and kinematic parameters

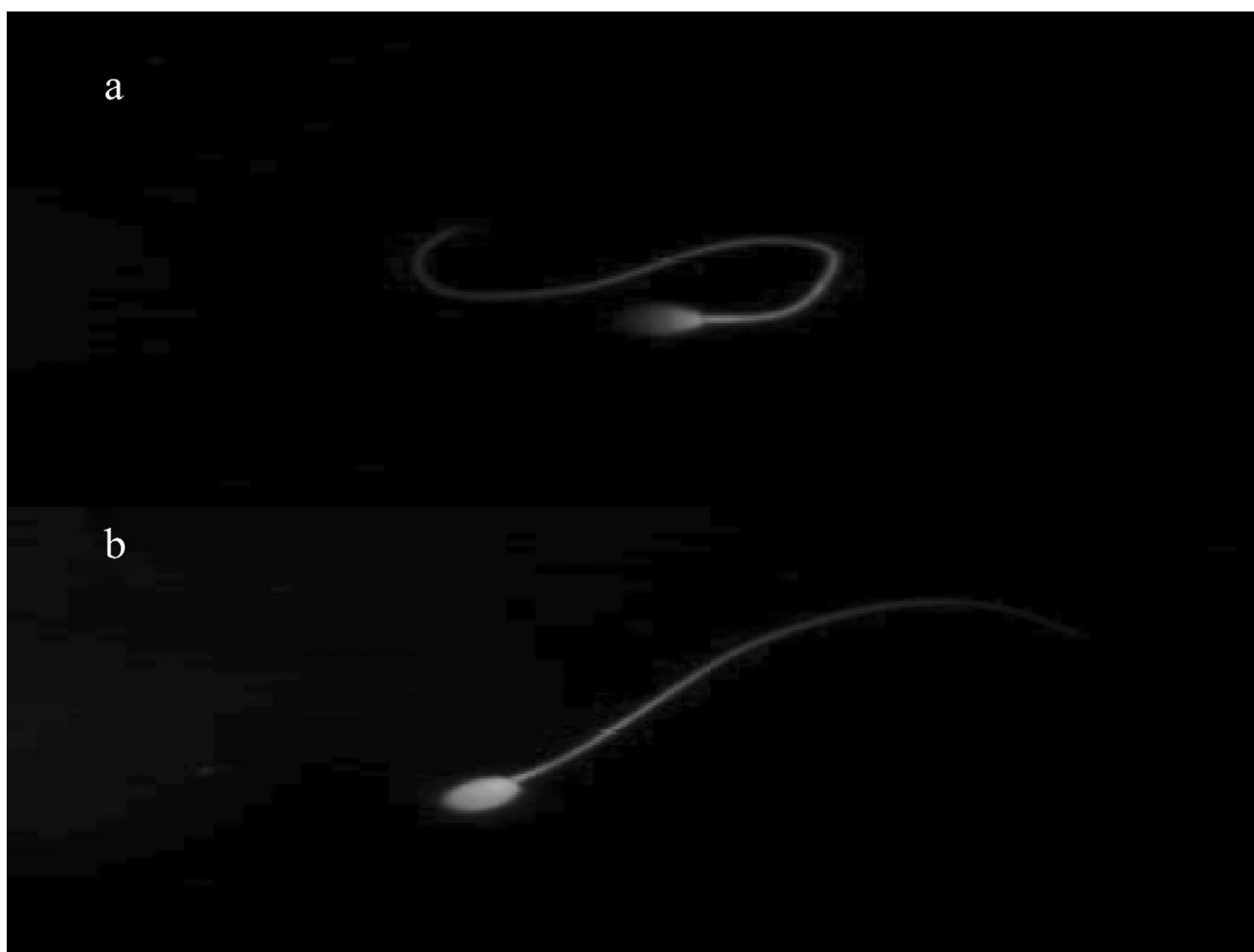
Significant differences were observed in the per cent of linear motile spermatozoa, VCL VAP, BCF and VSL among dogs (Tables 3 and 4).

#### Correlations among caspase activity, $\Delta\Psi_m$ , membrane integrity and motility

Significant negative correlations were observed among sperm showing low  $\Delta\Psi_m$  and live spermatozoa showing caspase activity. Ethidium positivity was negatively correlated with live spermatozoa showing caspase activity. Significant positive correlations were observed among the per cent of sperm showing low  $\Delta\Psi_m$  and dead spermatozoa. The percentage of YO-PRO-1+/ Eth+ sperm was negatively correlated with the percentage of linear motile sperm (Table 5).

#### Discussion

Cryopreservation induces major damage to sperm. During this process, crystallization of intracellular and extracellular water leads to mechanical damage, and chemical damage due to osmotic stress also occurs (Mazur, 1984) Recently, biochemical damage,



**Figure 2** Localization of FITC–VAD–FMK fluorescence in FT dog spermatozoa. Active caspases are present in (a) the midpiece, tail and acrosome or (b) in the midpiece and tail.

such as lipid peroxidation, premature ageing and phase membrane transitions, have been recognized as important factors that contribute to the sublethal damage of the spermatozoa that survive freezing and

thawing (Watson., 2000). The mitochondrion has been recently recognized as the subcellular structure of the spermatozoa most sensitive to this biotechnology (Peña *et al.*, 2003, Ortega Ferrusola *et al.*, 2008).

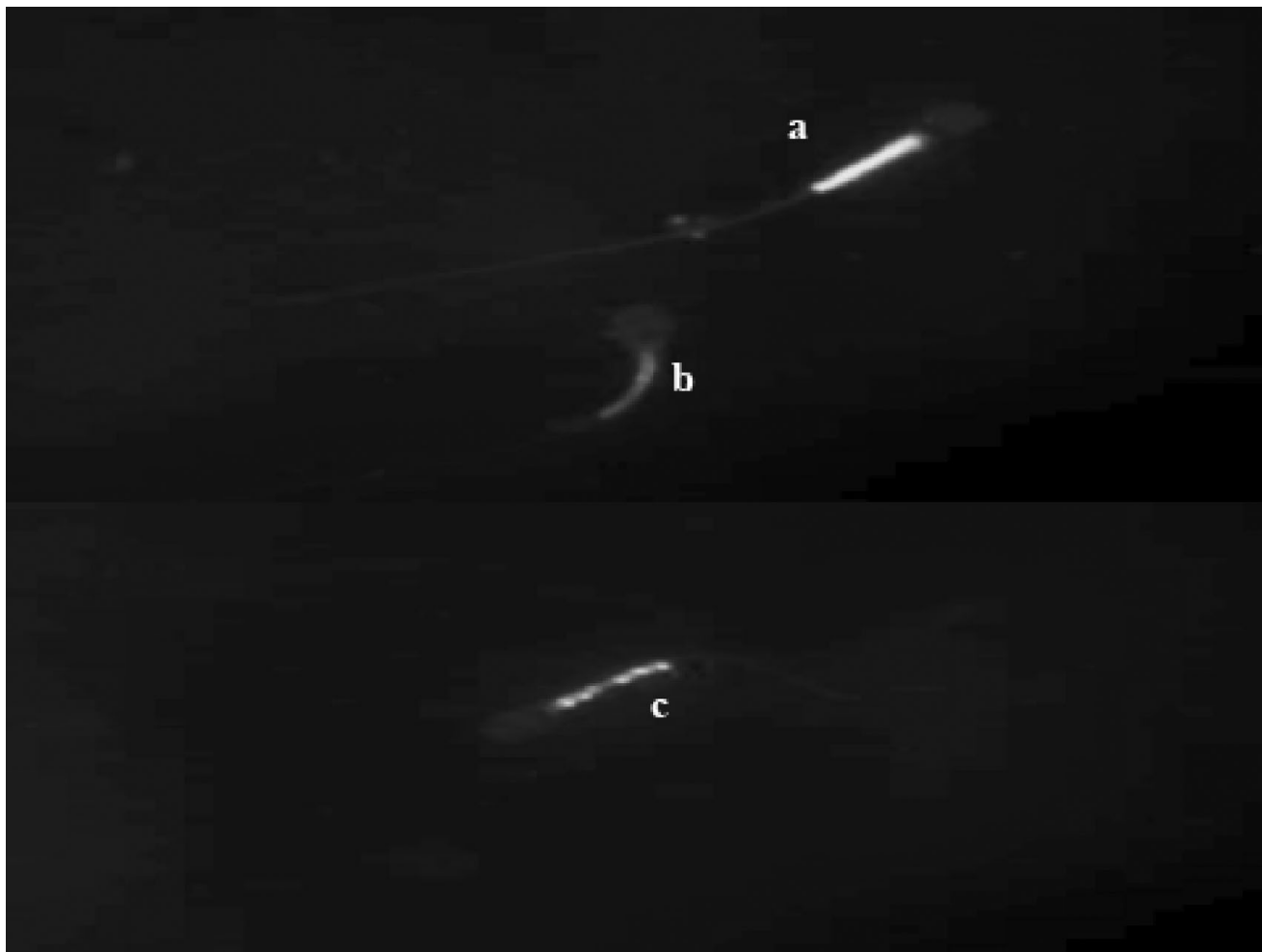
**Table 1** Mean percentages of caspase-positive and -negative live and dead spermatozoa stained with FITC–VAD–FMK and ethidium homodimer.

Dog	ZVAD– Eth– (%)	ZVAD+ Eth– (%)	ZVAD+ Eth+ (%)	ZVAD– Eth+ (%)
1	13.2 ± 10.53	63.7 ± 9.19	22.2 ± 2.12 <sup>a</sup>	0.8 ± 0.77
2	41.1 ± 30.05	30.2 ± 28.49	22.4 ± 9.19 <sup>a</sup>	6.3 ± 7.63
3	16.5 ± 19.94	58.1 ± 10.61	24.0 ± 9.16 <sup>a</sup>	1.3 ± 0.35
4	13.2 ± 6.71	70.7 ± 16.61	11.4 ± 5.37	3.9 ± 5.51
5	43.5 ± 26.94	43.4 ± 30.05	7.3 ± 1.20 <sup>b</sup>	5.6 ± 4.38
6	18.3 ± 13.07	61.5 ± 20.6	18.0 ± 9.01	2.1 ± 1.50
7	15.9 ± 5.52	64.0 ± 6.17	18.4 ± 3.05	1.7 ± 1.05
8	26.4 ± 7.91	56.6 ± 18.54	16.0 ± 6.22	2.2 ± 1.69

ZVAD–/Eth– (non-stained cells corresponding to viable spermatozoa); ZVAD+/Eth– spermatozoa (early apoptotic, confirmed caspase activity); ZVAD+/Eth+ (late apoptotic caspase activity in dead sperm); ZVAD–/Eth+ spermatozoa (necrotic cells).

<sup>a,b</sup>Within a column values with different superscripts differ significantly ( $p < 0.05$ ).





**Figure 3** Patterns of JC-1 fluorescence in FT dog spermatozoa. (a) Spermatozoa with high  $\Delta\Psi_m$ ; and (b) spermatozoa with low  $\Delta\Psi_m$ . (c) Spermatozoa showing an intermediate situation (mitochondria showing low and high  $\Delta\Psi_m$  within the same spermatozoan).

Mitochondria have a major role in apoptosis, and recently the role of apoptosis in the cellular injury induced by the cryopreservation procedure has been characterized (Martin *et al.*, 2003; Grunewald *et al.*,

2005; Ortega Ferrusola *et al.*, 2008). Even under the best conditions, only 50% of the spermatozoa remain motile after thawing and, of these, only 5% are considered completely competent spermatozoa for fertilization

**Table 2** Mean percentages of live and dead spermatozoa with and without early membrane changes in frozen-thawed semen. Flow cytometric evaluation of early membrane changes and changes in membrane integrity.

Dog	YO-PRO-/Eth- (%)	YO-PRO+/Eth- (%)	YO-PRO+/Eth+ (%)	YO-PRO-/Eth+ (%)
1	69.9 ± 16.12	11.6 ± 8.41	15.0 ± 6.78	3.4 ± 0.91 <sup>a</sup>
2	59.2 ± 4.17	23.3 ± 0.98	12.6 ± 1.13	4.8 ± 2.05 <sup>a</sup>
3	45.4 ± 11.73	25.6 ± 7.99	19.9 ± 5.65	9.0 ± 1.90 <sup>b</sup>
4	71.6 ± 25.24	19.4 ± 23.33	6.6 ± 3.25	2.3 ± 1.34 <sup>a</sup>
5	58.0 ± 5.72	20.8 ± 4.87	13.9 ± 1.27	7.2 ± 0.56
6	57.4 ± 15.87	25.8 ± 13.79	13.2 ± 6.70	3.5 ± 2.70
7	53.2 ± 19.48	25.9 ± 10.45	14.7 ± 9.54	6.1 ± 0.55
8	64.2 ± 11.10	18.3 ± 3.39	12.9 ± 3.81	4.5 ± 3.88

YO-PRO-1-/Eth- (non-stained cells corresponding to viable spermatozoa); YO-PRO+/Eth- (early apoptotic, spermatozoa with increased membrane permeability); YO-PRO-1+/Eth+ (late apoptotic); and YO-PRO-/Eth+ spermatozoa (necrotic cells).

<sup>a,b</sup>Within a column values with different superscript differ significantly ( $p < 0.05$ ).

**Table 3** Mean percentages of sperm motility and velocity after thawing.

Dog	LM (%)	NLM (%)	VCL ( $\mu\text{m/s}$ )	VSL ( $\mu\text{m/s}$ )	VAP ( $\mu\text{m/s}$ )
1	3.8 $\pm$ 1.97 <sup>a</sup>	36.5 $\pm$ 4.38	35.5 $\pm$ 1.90	17.25 $\pm$ 3.60	21.6 $\pm$ 9.44
2	18.0 $\pm$ 10.04 <sup>b</sup>	36.9 $\pm$ 11.31	54.5 $\pm$ 14.28	28.2 $\pm$ 11.95 <sup>a</sup>	33.5 $\pm$ 11.80 <sup>a</sup>
3	4.4 $\pm$ 5.72 <sup>a</sup>	17.1 $\pm$ 7.84	42.8 $\pm$ 25.25	16.8 $\pm$ 9.05	22.0 $\pm$ 12.09
4	36.1 $\pm$ 41.1 <sup>b</sup>	30.7 $\pm$ 34.13	31.5 $\pm$ 5.44	14.1 $\pm$ 5.79	20.2 $\pm$ 3.88
5	21.2 $\pm$ 17.46 <sup>b</sup>	36.8 $\pm$ 8.13	60.5 $\pm$ 27.36 <sup>a</sup>	29.5 $\pm$ 12.86 <sup>a</sup>	35.6 $\pm$ 14.56 <sup>a</sup>
6	1.7 $\pm$ 1.44 <sup>a</sup>	35.6 $\pm$ 16.31	27.3 $\pm$ 2.56 <sup>b</sup>	11.4 $\pm$ 5.79 <sup>b</sup>	15.0 $\pm$ 0.95 <sup>b</sup>
7	17.0 $\pm$ 12.82	31.6 $\pm$ 9.82	52.3 $\pm$ 15.27	27.5 $\pm$ 9.64 <sup>a</sup>	33.0 $\pm$ 10.53 <sup>a</sup>
8	18.1 $\pm$ 0.84	45.2 $\pm$ 1.13	52.5 $\pm$ 6.29	27.6 $\pm$ 9.64	32.8 $\pm$ 1.34 <sup>a</sup>

LM, linear motile sperm; NLM, non-linear motile sperm; VCL, circular velocity; VSL, straight line velocity; VAP, average velocity.

<sup>a,b</sup>Within a column values with different superscript differ significantly ( $p < 0.05$ ).

**Table 4** Mean kinematic parameters after thawing.

Dog	LIN (%)	STR (%)	WOB (%)	ALH ( $\mu\text{m}$ )	BCF (Hz)
1	48.4 $\pm$ 7.49	79.4 $\pm$ 6.01	60.7 $\pm$ 4.87	3.3 $\pm$ 0.21	9.6 $\pm$ 0.01 <sup>a</sup>
2	50.7 $\pm$ 8.69	83.1 $\pm$ 6.43	60.8 $\pm$ 5.65	4.1 $\pm$ 0.28	10.3 $\pm$ 0.35
3	39.9 $\pm$ 2.61	76.3 $\pm$ 0.70	52.3 $\pm$ 2.90	3.2 $\pm$ 2.19	5.3 $\pm$ 4.94 <sup>a</sup>
4	46.7 $\pm$ 6.64	72.9 $\pm$ 8.48	65.3 $\pm$ 0.28	2.8 $\pm$ 1.69	8.4 $\pm$ 3.89 <sup>a</sup>
5	48.9 $\pm$ 0.98	82.5 $\pm$ 2.26	59.3 $\pm$ 2.75	4.5 $\pm$ 0.28	35.7 $\pm$ 36.2 <sup>b</sup>
6	42.2 $\pm$ 5.72	76.3 $\pm$ 3.17	55.2 $\pm$ 5.48	2.7 $\pm$ 0.87	7.4 $\pm$ 3.06 <sup>a</sup>
7	52.2 $\pm$ 9.55	82.7 $\pm$ 4.19	62.9 $\pm$ 8.29	4.3 $\pm$ 0.75	10.3 $\pm$ 0.60
8	51.9 $\pm$ 11.38	81.9 $\pm$ 4.87	63.1 $\pm$ 10.18	3.9 $\pm$ 1.20	10.8 $\pm$ 1.34

LIN, linearity coefficient ( $\text{VSL}/\text{VCL} \times 100$ ); STR, straightness coefficient ( $\text{VSL}/\text{VAP} \times 100$ ); WOB, wobble coefficient ( $\text{VAP}/\text{VCL} \times 100$ ); ALH, mean lateral head displacement; BCF (beat cross frequency).

<sup>a,b</sup>Within a column values with different superscript differ significantly ( $p < 0.05$ ).

(Holt & Van Lock, 2004). This observation implies that the identification of subtle changes in the spermatozoa is of utmost importance to the identification of competent spermatozoa and the improvement of current cryopreservation protocols.

We identified, for the first time, active caspases in frozen–thawed canine sperm. Active caspases have been previously identified in frozen–thawed bovine sperm (Martín *et al.*, 2004), human sperm, mainly in infertile patients (Marchetti *et al.*, 2004; Hu *et al.*, 2006), rodents (Cisternas & Moreno, 2006), rams (Martí *et al.*, 2006) and horses (Ortega Ferrusola *et al.*, 2008).

**Table 5** Correlation among caspase activity and other sperm characteristics in frozen–thawed sperm.

	Live caspase	Dead	Linear motile
Low mitochondrial membrane potential	−0.494*	0.490*	−
YOPRO−/Eth+	−0.482*	−	−0.479
YOPRO+/Eth+	−	−	−

\* $p < 0.05$ .

The mitochondrial apoptotic pathway seems to be involved in caspase activation in spermatozoa. Caspase activation has been induced with betulinic acid (Grunewald *et al.*, 2005) and bovine spermatozoa have been demonstrated to contain the apoptotic machinery, especially the one related to mitochondrial pathway.

Under physiological situations, pro-apoptotic factors remain inactivated within the mitochondria. Caspases are unrelated to sperm survival or capacitation and the acrosome reaction (Wundrich *et al.*, 2006). Cytochrome *c* and the apoptosis inducing factor (AIF) have been detected in bovine spermatozoa (Martín *et al.*, 2007), and in addition, pro-caspase 9 and activated caspase 9, involved in the mitochondrial pathway, were detected in bovine (Martín *et al.*, 2007) and human sperm (Wundrich *et al.*, 2006). Cryopreservation induces an increase in mitochondrial permeability leading to the release of proapoptotic factors. Also, apoptosis plays an important role in spermatogenesis (Konrad *et al.*, 2006; Zheng *et al.*, 2006) reflecting the important role of this mechanism in the male reproductive sphere.

Among the interesting findings of our study were the identification of various degrees of membrane injury and the detection of active caspases both in live and dead sperm. Two patterns of caspase activity in dead

spermatozoa were observed in our analyses: (1) caspase activity in the midpiece tail; and (2) in the midpiece tail and acrosomal region. In live (ethidium-negative) spermatozoa, caspase activity was detected only in the midpiece tail region. In previous reports, caspase activity was detected only in the midpiece and no differentiation was made between live and dead sperm showing caspase activity (Martín *et al.*, 2005).

The presence of caspase activity in the acrosomal region is not easy to interpret. One possibility is that the staining is simply an artefact, while the presence of caspase activity in the midpiece supports the theory of the mitochondrial pathway of apoptosis (Cyali *et al.*, 2004). Also the presence of live (ethidium-negative) caspase-positive spermatozoa supports the theory of an apoptotic-like mechanism involved in sublethal damage after freeze-thawing.

From a practical point of view the differentiation between caspase activity in dead and live spermatozoa may be a simple method to disclose subtle differences in sperm quality. This staining allowed us to find statistically significant differences in the sperm characteristics among dogs. In fact, the sample that showed overall better results in all the sperm parameters studied after thawing was the one with a lower percentage of active caspases, both in dead and live spermatozoa. Also, the human study that found significant correlations between the per cent of sperm with active caspases and the outcome of IVF (Marchetti *et al.*, 2004) strengthen this assay.

JC-1 staining showed high variations among and within dogs. This fact was related to numerous intermediate situations (different percentages of low and high  $\Delta\Psi_m$  within the same spermatozoa) that made it difficult to properly gate the different subpopulations in the flow cytometric dot plots. Experiments are in progress in our laboratory to improve the identification of these intermediate situations. Analysis of correlations among different parameters of sperm quality provided important information. The percentage of live sperm that showed caspase activity was negatively correlated with sperm that showed low  $\Delta\Psi_m$ . This fact seems contradictory, however it can be interpreted when taking into account that low  $\Delta\Psi_m$  was positively correlated with the percentage of dead sperm. Bearing that in mind, we distinguished two categories of caspase positive spermatozoa. Live, caspase-positive spermatozoa may represent those with a very early stage of cell injury; the negative correlation of YO-PRO-1/Eth+ (necrotic sperm) with live sperm that showed caspase activity supports this theory.

On the other hand, it is noteworthy that apoptotic sperm were negatively correlated with the per cent of linear motile spermatozoa. This subpopulation may represent spermatozoa with subtle damage, leading in

this case to a reduction or compromised linear pattern of movement.

In short, we successfully used flow cytometric analysis to detect active caspases in frozen-thawed canine sperm. This may represent a novel and simple test to assess the quality of a semen sample, as this technique allows the differentiation among semen samples of similar quality with conventional analysis.

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## References

- Cisternas, P. & Moreno, R.D. (2006). Comparative analysis of apoptotic pathways in rat mouse and hamster spermatozoa. *Mol. Reprod. Develop.* **73**, 1318–25.
- Cyali, S., Sakkas, D., Vigue, L., Demir, L. & Huszar, G. (2004). Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and BCL-XL levels in mature and diminished maturity sperm. *Mol. Hum. Reprod.* **10**, 365–72.
- Desagher, S. & Martinou, J.C. (2000). Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* **10**, 369–77.
- Grunewald, S., Paasch, U., Said, T.M., Sharma, R.K., Glandner, H.J. & Agarwal, A. (2005). Caspase activation in human spermatozoa in response to physiological and pathological stimuli. *Fertil. Steril. Suppl.* **1**, 1106–12.
- Holt, W.V. & Van Look, K.J.W. (2004). Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. *Reproduction* **127**, 527–535.
- Hu, X.Y., Xu, Y.M., Qiao, Y., Wu, D.L., Sa, Y.L., Fu, Q., Yu, J.J., Zhang, X.R., Zhang, J., Gu, B.J., Chen, R. & Xie, H. (2006). Reduced semen quality in chronic prostatitis patients that induce the release of apoptotic protein Omi/HtrA2 from spermatozoa. Prostate cancer and prostatic diseases. doi:10.1038/sj.pcan.4500919.
- Idziorek, T., Estaquier, J., de Bels, F. & Ameisen, J.C. (1995). YO-PRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immunol. Meth.* **185**, 249–85.
- Konrad, L., Keilani, M.M., Laible, L., Nottelmann, U. & Hofmann, R. (2006). Effects of TGF betas and a specific antagonist on apoptosis of immature rat male germ cells in vitro. *Apoptosis* **11**, 739–48.
- Marchetti, C., Gallego, M.A., Defossez, A., Formstecher, P. & Marchetti, P. (2004). Staining of human sperm with fluorochrome labelled inhibitor of caspases: correlation with apoptosis and sperm parameters. *Hum. Reprod.* **19**, 1127–34.
- Marti, E., Perez Pe, R., Muiño Blanco, T. & Cebrian Perez, J.A. (2006). Comparative study of four different sperm washing methods using apoptotic makers in ram spermatozoa. *J. Androl.* **27**, 746–53.



- Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P. & Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum. Reprod.* **22**, 380–8.
- Martin, G., Sabido, O., Durand, Ph. & Levy, R. (2004). Cryopreservation induces an apoptosis like mechanism in bull sperm. *Biol. Reprod.* **71**, 28–37.
- Martin, G., Sabido, O., Durand, P. & Levy, R. (2005). Phosphatidylserine externalization in human semen induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. *Biol. Reprod.* **20**, 3459–68.
- Mazur, P. (1984). Freezing of living cells: mechanism and implications. *Am. J. Physiol.* **16**, C125–42.
- Núñez Martínez, I., Morán, J.M. & Peña, F.J. (2007). Sperm indexes obtained using computer assisted morphometry provide a forecast of the freezeability of canine sperm. *Int. J. Androl.* **30**, 182–9.
- Núñez Martínez, I., Moran, J.M. & Peña, F.J. (2005). Do computer assisted derived morphometric sperm characteristics reflect DNA status in canine spermatozoa? *Reprod. Dom. Anim.* **40**, 37–43.
- Núñez Martínez, I., Moran, J.M. & Peña, F.J. (2006). A three step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. *Reprod. Dom. Anim.* **41**, 408–15.
- Núñez-Martínez, I., Morán, J.M. & Peña, F.J. (2006). Two step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. *J. Androl.* **27**, 596–603.
- Ormerod, M.G., Sun, X.M., Snowden, R.T., Davies, R., Fearnhead, H. & Cohen, G.M. (1993). Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. *Cytometry* **14**, 595–602.
- Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J.M., González-Fernández, L., Tapia, J.A. & Peña, F.J. (2008). Detection of apoptosis like changes during the cryopreservation process in equine sperm. *J. Androl.* **29**, 213–21.
- Paasch, U., Sharma, R.K., Gupta, A.K., Grunewald, S., Mascha, E.J., Thomas, A.J., Glander, H.J. & Agarwal, A. (2004). Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol. Reprod.* **71**, 1828–37.
- Peña, A. & Linde-Forsberg, C. (2000). Effect of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology* **54**, 703–18.
- Peña, A.I. (2004). Canine fresh and cryopreserved evaluation. *Anim. Reprod. Sci.* **82–3**, 209–24.
- Peña, F.J., Johannisson, A., Wallgren, M. & Rodriguez Martinez, 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**, 85–8.
- Peña, F.J., Johannisson, A., Wallgren, M. & Rodriguez Martinez, H. (2003). Assessment of fresh and frozen thawed boar semen using an annexin assay: a new method of evaluating sperm membrane integrity. *Theriogenology* **60**, 677–89.
- Peña, F.J., Johannisson, A., Wallgren, M. & Rodriguez Martinez, H. (2004). Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. *Zygote* **12**, 117–27.
- Peña, F.J., Johannisson, A., Wallgren, M. & Rodriguez Martinez, H. (2005). A new and simple method to evaluate early membrane changes in frozen–thawed boar spermatozoa. *Int. J. Androl.* **28**, 107–14.
- Peña, F.J., Núñez Martínez, I. & Morán, J.M. (2006). Semen technologies in dog breeding: an update. *Reprod. Dom. Anim.* **41(S2)**, 21–9.
- Said, T.M., Paasch, U., Glander, H.J. & Agarwal, A. (2004). Role of caspases in male infertility. *Hum. Reprod. Update* **10**, 39–51.
- Watson, P.F. (2000). The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* **60–1**, 481–92.
- Wronski, R., Golob, N., Grygar, E. & Winsdish, M. (2002). Two color, fluorescence-based microplate assay for apoptosis detection. *Biotechniques* **32**, 666–68.
- Wundrich, K., Paasch, U., Leicht, M. & Glander, H.J. (2006). Activation of caspases in human spermatozoa during cryopreservation: an immunoblot study. *Cell Tissue Bank* **7**, 81–90.
- Zheng, S., Turner, T.T. & Lysiak, J.J. (2006). Caspase 2 activity contributes to the initial wave of germ cell apoptosis during the first round of spermatogenesis. *Biol. Reprod.* **74**, 1026–33.