

# Lipid peroxidation, assessed with BODIPY-C<sub>11</sub>, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes

C Ortega Ferrusola<sup>1</sup>, L González Fernández<sup>2</sup>, J M Morrell<sup>3</sup>, C Salazar Sandoval<sup>1</sup>, B Macías García<sup>1</sup>, H Rodríguez-Martínez<sup>3</sup>, J A Tapia<sup>2</sup> and F J Peña<sup>1</sup>

<sup>1</sup>Section of Reproduction and Obstetrics, Laboratory of Spermatology, Department of Herd Health and Medicine, Faculty of Veterinary Medicine, Veterinary Teaching Hospital and <sup>2</sup>Department of Physiology, Faculty of Veterinary Medicine, University of Extremadura, Avda de la Universidad s/n, 10071 Cáceres, Spain and <sup>3</sup>Division of Reproduction, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden

Correspondence should be addressed to F J Peña; Email: fjuanpvega@unex.es

## Abstract

Lipid peroxidation (LPO) of stallion spermatozoa was assessed in fresh semen and in samples of the same ejaculates after freezing and thawing. Particular attention was paid to individual differences in the susceptibility to LPO and its possible relationship with freezability. Innate levels of LPO were very low in fresh spermatozoa but increased after thawing, a change that was largely stallion-dependent. The level of LPO in fresh spermatozoa was not correlated with that of the thawed spermatozoa. Negative correlations existed between LPO and intact membranes post-thaw ( $r = -0.789$ ,  $P < 0.001$ ), and also between LPO and spermatozoa with high mitochondrial membrane potential ( $\Delta\psi_m$ ) post-thaw ( $r = -0.689$ ,  $P < 0.001$ ). LPO was also highly and significantly correlated with caspase activity. The correlation between caspase activity in ethidium positive cells and LPO was  $r = 0.772$ ,  $P < 0.001$ . This LPO is unlikely to represent, *per se*, a sign of cryopreservation-induced injury, but it is apparently capable of triggering 'apoptotic-like changes' that could result in the sub-lethal cryodamage often seen among surviving spermatozoa.

*Reproduction* (2009) **138** 55–63

## Introduction

Cryopreservation causes differential damage to the spermatozoa of all mammalian species, of either lethal or sub-lethal nature (Watson 2000). Obviously, sperm death is the major factor explaining the lower longevity of thawed spermatozoa, but non-lethal modifications of the surviving cells also account for their reduced life-span, thus narrowing the window which spermatozoa have for successful fertilization after artificial insemination (AI; Alvarez & Storey 1992, Peña *et al.* 2005). In many species including horses, peroxidation of plasma membrane lipids (lipid peroxidation, LPO) has been claimed to be a major factor involved in this sub-lethal cryodamage (Alvarez & Storey 1992, Agarwall & Said 2005, Almeida & Ball 2005, Aitken *et al.* 2007). The particular susceptibility of the sperm plasma membrane to peroxidative damage is due to a high cellular content of polyunsaturated fatty acids. Attacks by free radicals on those fatty acids generate peroxy ( $\text{ROO}^\cdot$ ) and alkoxyl ( $\text{RO}^\cdot$ ) radicals that – in order to become stable as molecules – subtract a hydrogen atom from an adjacent

carbon atom in a neighboring lipid, thus generating the corresponding acid or alcohol. The subtraction of a hydrogen atom from an adjacent lipid creates a carbon-centered radical that combines with dioxygen to create another lipid peroxide, thus perpetuating the propagation of peroxidative damage throughout various cell membranes (Bileski *et al.* 1983).

Recently, sperm-freezing technology has become an area of increasing interest for the equine industry (Samper *et al.* 2007). One of the major problems of equine sperm cryobiology is the large inter-individual variability in sperm survival during the freezing and thawing procedures. Such variability is often ascribed to the fact that most stallions have been selected by performance and phenotype, and not for sperm quality, either directly or indirectly. However, the physiological and biochemical reasons behind this variability remain unexplained. Sperm-LPO has been studied using many methods, including the use of fluorescent probes. Among these, the recently developed probe BODIPY-C<sub>11</sub> is considered most reliable and easy to assess LPO in

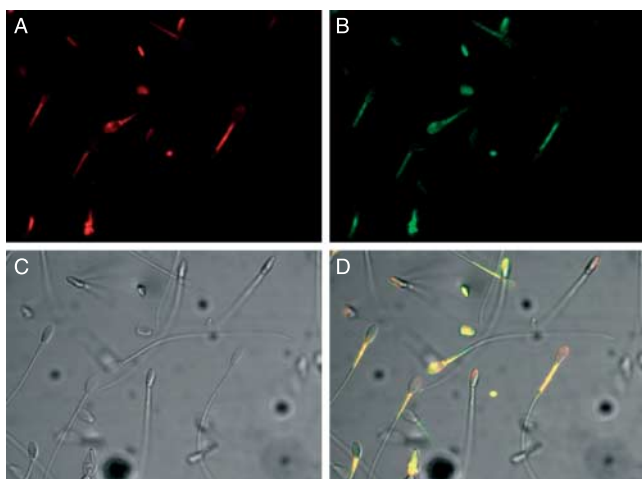
spermatozoa (Pap *et al.* 1999, Brouwers & Gadella 2003, Brouwers *et al.* 2005, Aitken *et al.* 2007). BODIPY-C<sub>11</sub> has, therefore, been used to assess the susceptibility of stallion sperm to this insult after different oxidative challenges (Baumber *et al.* 2000, Ball & Vo 2002, Neild *et al.* 2005). However, these studies did not investigate individual variation among stallions or their ejaculates in their susceptibility to cryopreservation-induced LPO. Therefore, the aim of the present study was to assess the individual responses of spermatozoa from seven stallions to freezing and thawing, including their susceptibility to LPO, as well as to study the relationship between LPO and cell damage.

## Results

### Lipid peroxidation

The proportions of ejaculated spermatozoa showing LPO was low, ranging on average from 0.25 to 1.9%. The peroxidative damage was conspicuous in the mid-piece but less evident in the sperm head or the rest of the sperm tail (Fig. 1).

After thawing, there was an increase in LPO, compared with the ejaculate, fresh spermatozoa ( $P < 0.01$ ). The percentage of sperm depicting peroxidative damage after thawing ranged, on average, between 3.1 and 29.7%. These increases post-thaw were largely stallion-dependent (Table 1) and were not statistically related to the levels present in fresh spermatozoa (ns). The localization of peroxidative damage was the same as that observed in fresh semen, as depicted by confocal laser microscopy.



**Figure 1** Confocal laser microscopy images of stallion spermatozoa loaded with BODIPY-C<sub>11</sub>. (A) Red fluorescence associated with the widespread incorporation of the non-oxidized probe in the spermatozoa. (B) Green fluorescence associated with the oxidized probe. (C) Transmission images. (D) Overlaid images in which areas of lipid peroxidation appear yellow. All images were obtained with a Bio-Rad MRC confocal microscope. Magnification, 60 $\times$ .

**Table 1** Individual variation in the percentage of lipid peroxidation (BODIPY-C<sub>11</sub> + cells) after freezing and thawing (means  $\pm$  s.d.;  $n = 7$  stallions, four ejaculates each).

Stallion	BODIPY-C <sub>11</sub> + cells (%)	
	Freshly ejaculated spermatozoa	Frozen-thawed spermatozoa
1	0.9 $\pm$ 0.74 <sup>a,A</sup>	3.8 $\pm$ 0.80 <sup>a,B</sup>
2	0.2 $\pm$ 0.19 <sup>a,A</sup>	2.7 $\pm$ 0.62 <sup>a,B</sup>
3	0.4 $\pm$ 0.15 <sup>a,A</sup>	4.3 $\pm$ 1.94 <sup>a,B</sup>
4	0.6 $\pm$ 0.49 <sup>a,A</sup>	13.6 $\pm$ 5.30 <sup>a,b,B</sup>
5	0.6 $\pm$ 0.29 <sup>a,A</sup>	8.6 $\pm$ 9.78 <sup>a,B</sup>
6	1.9 $\pm$ 1.72 <sup>b,A</sup>	29.7 $\pm$ 16.25 <sup>b,B</sup>
7	0.5 $\pm$ 0.05 <sup>a,A</sup>	3.1 $\pm$ 5.14 <sup>a,B</sup>

Values with different superscript between rows within a column (<sup>a-b</sup>) or between columns within a row (<sup>A-B</sup>), differ significantly ( $P < 0.05$ ).

### Membrane integrity and mitochondrial membrane potential

As expected, freezing and thawing caused major damage to sperm membrane integrity and mitochondrial membrane potential, compared with the status in the ejaculated spermatozoa (Tables 2 and 3). Once again this effect was stallion-dependent.

### Expression of active caspases

Active caspases were present in fresh ejaculated spermatozoa, varying largely among stallions ( $P < 0.001$ ). Most of these spermatozoa depicting caspase activity were ethidium negative (live spermatozoa). After thawing, caspase activity was present especially in ethidium positive spermatozoa (dead-spermatozoa), and the individual variation diminished (Table 4).

### Correlations between proportions of sperm-LPO and sperm quality variables

Significant correlations were found between LPO and sperm quality post-thaw, expressed as sperm membrane integrity and mitochondrial membrane potential (Table 5, Figs 2 and 3). High negative correlations were particularly seen between the proportions of spermatozoa having LPO and intact membranes post-thaw ( $r = -0.789$ ,  $P < 0.001$ ), and with spermatozoa depicting mitochondria with high  $\Delta\psi_m$  ( $r = -0.689$ ,  $P < 0.001$ ). LPO was also highly and significantly correlated with caspase activity. The correlation between caspase activity in the ethidium positive cells and LPO was  $r = 0.772$  ( $P < 0.001$ ; Fig. 4).

### Superoxide dismutase activity

Superoxide dismutase (SOD) activity ranged from 37 U/mg protein in stallion number 1 to 134.16 U/mg protein in stallion number 6 (Fig. 5). However, these

**Table 2** Changes in sperm membrane integrity (YO-PRO-1 and ethidium homodimer) before (freshly ejaculated spermatozoa, FE) and after cryopreservation (frozen–thawed spermatozoa, FT;  $n=7$  stallions, four ejaculates each, means  $\pm$  s.d.)

Stallion	YO-PRO–/Eth–		YO-PRO+/Eth–		YO-PRO+/Eth+		YO-PRO–/Eth+	
	FE	FT	FE	FT	FE	FT	FE	FT
1	74.0 $\pm$ 14.12 <sup>a,A</sup>	35.3 $\pm$ 14.79 <sup>a,B</sup>	1.0 $\pm$ 0.99 <sup>A</sup>	15.9 $\pm$ 7.03 <sup>a,B</sup>	3.3 $\pm$ 3.12 <sup>A</sup>	26.5 $\pm$ 14.28 <sup>B</sup>	21.6 $\pm$ 10.29 <sup>a</sup>	22.3 $\pm$ 7.18 <sup>a</sup>
2	77.0 $\pm$ 7.76 <sup>a,A</sup>	31.7 $\pm$ 7.21 <sup>a,B</sup>	0.6 $\pm$ 0.60 <sup>A</sup>	18.1 $\pm$ 6.20 <sup>a,B</sup>	1.9 $\pm$ 2.72 <sup>A</sup>	22.8 $\pm$ 8.56 <sup>B</sup>	27.3 $\pm$ 5.60 <sup>a</sup>	27.3 $\pm$ 5.60 <sup>a</sup>
3	58.5 $\pm$ 7.28 <sup>b,A</sup>	33.4 $\pm$ 5.31 <sup>a,B</sup>	0.4 $\pm$ 0.20 <sup>A</sup>	6.3 $\pm$ 3.48 <sup>b,B</sup>	1.8 $\pm$ 0.17 <sup>A</sup>	19.3 $\pm$ 13.09 <sup>B</sup>	39.4 $\pm$ 7.44 <sup>b</sup>	40.9 $\pm$ 11.04 <sup>b</sup>
4	53.9 $\pm$ 3.45 <sup>b,A</sup>	27.5 $\pm$ 4.87 <sup>b,B</sup>	0.7 $\pm$ 1.18 <sup>A</sup>	17.5 $\pm$ 4.74 <sup>a,B</sup>	4.7 $\pm$ 7.13 <sup>A</sup>	19.57 $\pm$ 4.21 <sup>B</sup>	40.1 $\pm$ 11.05 <sup>b</sup>	35.1 $\pm$ 9.75 <sup>b</sup>
5	70.4 $\pm$ 4.93 <sup>a,A</sup>	28.5 $\pm$ 11.76 <sup>b,B</sup>	0.4 $\pm$ 0.28 <sup>A</sup>	14.0 $\pm$ 2.53 <sup>a,B</sup>	1.0 $\pm$ 0.54 <sup>A</sup>	31.5 $\pm$ 8.74 <sup>B</sup>	28.1 $\pm$ 4.28 <sup>a</sup>	25.9 $\pm$ 5.19 <sup>a</sup>
6	59.1 $\pm$ 9.86 <sup>b,A</sup>	25.3 $\pm$ 9.59 <sup>b,B</sup>	1.0 $\pm$ 0.66 <sup>A</sup>	9.4 $\pm$ 3.23 <sup>b,B</sup>	4.3 $\pm$ 1.13 <sup>A</sup>	26.1 $\pm$ 14.14 <sup>B</sup>	35.5 $\pm$ 10.28 <sup>b</sup>	39.2 $\pm$ 7.67 <sup>b</sup>
7	71.7 $\pm$ 6.46 <sup>a,A</sup>	34.8 $\pm$ 9.90 <sup>a,B</sup>	0.2 $\pm$ 0.32 <sup>A</sup>	19.4 $\pm$ 5.95 <sup>a,B</sup>	1.6 $\pm$ 1.27 <sup>A</sup>	23.3 $\pm$ 7.06 <sup>B</sup>	26.6 $\pm$ 5.36 <sup>a</sup>	22.4 $\pm$ 4.94 <sup>a</sup>

Non-stained cells (YO-PRO-1–/Eth–), corresponding to viable spermatozoa, (YO-PRO+/Eth–) spermatozoa (early apoptotic, spermatozoa depicting an increase in membrane permeability), YO-PRO-1+/Eth+ (late apoptotic) and YO-PRO–/Eth+ spermatozoa (necrotic cells). Values with different superscript between rows within a column (<sup>a–b</sup>) or between columns within a row (<sup>A–B</sup>), differ significantly ( $P<0.05$ ).

differences were not statistically significant. No correlations were detected between the SOD activity and LPO.

### Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity ranged from 1.54 nmol/min per mg protein in stallion number 7 to 3.43 nmol/min per mg protein in stallion number 6 (Fig. 6). The differences were not statistically significant. Once again, no correlations between LPO and GPx activity were detected.

### Discussion

As previously reported, cryopreservation induces LPO in stallion spermatozoa, mainly at their mid-piece (Neild *et al.* 2005). Although the effect of cryopreservation on the induction of LPO has been previously described in many species including horses (Baumber *et al.* 2000, Christova *et al.* 2004, Neild *et al.* 2005, Ball 2008, Cosci *et al.* 2008), this is – to the best of our knowledge – the first time that a significant stallion-to-stallion variation

in susceptibility to LPO of their cryopreserved spermatozoa has been shown. The first practical implication of this finding is that differences in susceptibility to peroxidation may explain the large individual variation in the cryoresistance of the stallion ejaculate. The second fact is that innate LPO, present in the recently ejaculated spermatozoa, is not related to the peroxidation levels found after freezing and thawing, implying that the process of cryopreservation imposes peroxidative changes unrelated to the intrinsic levels of a particular stallion. Apparently, this susceptibility is not related to the antioxidant systems of the semen, since we were unable to find direct correlations among SOD or GPx activity and LPO thus, probably this susceptibility relates to the lipid composition of the sperm plasma membrane.

In our work, we studied the relationship between LPO, sperm membrane integrity, mitochondrial membrane potential ( $\Delta\psi_m$ ), and caspase activity. Probably for the first time, we were able to show a direct mathematical relationship between LPO, sperm membrane integrity  $\Delta\psi_m$ , and caspase activity. To explain these relationships, the factors causing cell death and sub-lethal

**Table 3** Changes in sperm mitochondrial membrane potential (% of either high, low, or intermediate  $\Delta\psi_m$ ) using JC-1 before (freshly ejaculated spermatozoa, FE) and after cryopreservation (frozen–thawed spermatozoa, FT;  $n=7$  stallions, four ejaculates each, means  $\pm$  s.d.)

Stallion	Percentages of JC-1 differentially stained spermatozoa					
	Orange		Orange + green		Green	
	FE	FT	FE	FT	FE	FT
1	20.5 $\pm$ 16.40 <sup>A</sup>	2.6 $\pm$ 2.04 <sup>B</sup>	21.6 $\pm$ 14.82	39.8 $\pm$ 20.26	57.1 $\pm$ 20.38	64.7 $\pm$ 12.62
2	15.1 $\pm$ 6.22 <sup>A</sup>	2.6 $\pm$ 4.32 <sup>B</sup>	32.5 $\pm$ 6.58	34.9 $\pm$ 1.68	52.2 $\pm$ 11.54	62.0 $\pm$ 3.08
3	11.8 $\pm$ 3.38 <sup>A</sup>	4.7 $\pm$ 7.42 <sup>B</sup>	25.1 $\pm$ 22.32	31.0 $\pm$ 2.80	63.1 $\pm$ 18.75	63.8 $\pm$ 10.76
4	19.8 $\pm$ 13.58 <sup>A</sup>	1.5 $\pm$ 0.73 <sup>B</sup>	19.7 $\pm$ 7.61	27.5 $\pm$ 10.56	60.0 $\pm$ 18.38	70.7 $\pm$ 11.18
5	23.8 $\pm$ 11.18 <sup>A</sup>	0.4 $\pm$ 0.25 <sup>B</sup>	34.6 $\pm$ 7.86	15.6 $\pm$ 7.10	40.6 $\pm$ 3.42 <sup>A</sup>	83.7 $\pm$ 7.03 <sup>B</sup>
6	27.2 $\pm$ 16.23 <sup>A</sup>	0.5 $\pm$ 0.93 <sup>B</sup>	39.8 $\pm$ 16.56 <sup>a</sup>	8.20 $\pm$ 10.98 <sup>b</sup>	32.4 $\pm$ 18.58 <sup>A</sup>	89.9 $\pm$ 11.06 <sup>B</sup>
7	17.8 $\pm$ 13.05 <sup>A</sup>	3.7 $\pm$ 5.97 <sup>B</sup>	33.3 $\pm$ 12.12	23.7 $\pm$ 14.68	48.4 $\pm$ 23.92	72.0 $\pm$ 19.69

Orange fluorescence represents spermatozoa depicting high mitochondrial membrane potential. Green fluorescence represents spermatozoa depicting low mitochondrial membrane potential. Orange and green fluorescence simultaneously represent spermatozoa having heterogeneous mitochondria, with high and low mitochondrial membrane potential within the same spermatozoa. Values with different superscript between rows within a column (<sup>a–b</sup>) or between columns within a row (<sup>A–B</sup>), differ significantly ( $P<0.05$ ).

**Table 4** Changes in the proportions of stallion spermatozoa depicting expression of active caspases before (freshly ejaculated spermatozoa, FE) and after cryopreservation (frozen–thawed spermatozoa, FT;  $n=7$  stallions, four ejaculates each, means  $\pm$  s.d.).

Stallion	Caspase +		Caspase + /Eth +	
	Fresh	FT	Fresh	FT
1	70.4 $\pm$ 17.25 <sup>a,A</sup>	25.4 $\pm$ 4.41 <sup>a,B</sup>	22.3 $\pm$ 17.73 <sup>a,A</sup>	57.5 $\pm$ 6.76 <sup>B</sup>
2	60.2 $\pm$ 38.00 <sup>A</sup>	25.8 $\pm$ 12.60 <sup>B</sup>	13.7 $\pm$ 8.82 <sup>a,A</sup>	52.5 $\pm$ 5.75 <sup>B</sup>
3	37.9 $\pm$ 11.63 <sup>b,A</sup>	12.2 $\pm$ 2.41 <sup>b,B</sup>	43.7 $\pm$ 8.92 <sup>b,A</sup>	54.2 $\pm$ 12.99 <sup>B</sup>
4	32.5 $\pm$ 12.62 <sup>b,A</sup>	20.8 $\pm$ 11.38 <sup>B</sup>	43.8 $\pm$ 4.68 <sup>b,A</sup>	59.6 $\pm$ 9.46 <sup>B</sup>
5	68.1 $\pm$ 3.90 <sup>a,A</sup>	21.7 $\pm$ 8.91 <sup>B</sup>	27.9 $\pm$ 4.11 <sup>A</sup>	64.9 $\pm$ 9.81 <sup>B</sup>
6	51.9 $\pm$ 18.47 <sup>A</sup>	18.5 $\pm$ 10.91 <sup>b,B</sup>	35.2 $\pm$ 11.08 <sup>b,A</sup>	55.4 $\pm$ 6.37 <sup>B</sup>
7	37.9 $\pm$ 18.22 <sup>A</sup>	33.6 $\pm$ 4.64 <sup>a,B</sup>	33.3 $\pm$ 12.07 <sup>A</sup>	53.5 $\pm$ 6.57 <sup>B</sup>

Values with different superscript between rows within a column (<sup>a–b</sup>) or between columns within a row (<sup>A–B</sup>), differ significantly ( $P < 0.05$ ).

cellular damage during freezing and thawing should be considered. Previously, the two injury-factors theory (the presence of intracellular ice crystals, acting as an agricultural plow, tearing apart membranes on one hand and dehydration of the cells causing osmotic stress) was largely accepted. However, recent research indicates that at the freezing rates currently used in animal sperm cryobiology, the formation of intracellular ice crystals is unlikely to occur, with most damage being mainly osmotic in nature, occurring both during freezing and at thawing (Morris *et al.* 2007).

In addition, a large percentage of the surviving spermatozoa experience changes that reduce their lifespan within the female reproductive tract. In the past, these changes were termed as ‘cryocapacitation’ (Watson 2000). Today, these changes, although resembling capacitation as studied using the Chlortetracycline test, appear in a different light and are called ‘capacitation-like’ (Peña *et al.* 2004, Bravo *et al.* 2005, Saravia *et al.* 2007). Cryopreserved spermatozoa experience premature aging, involving mechanisms such as LPO and an apoptosis-like mechanism, at least in some species, including the horse (Kemal Duru *et al.* 2001, Anzar *et al.* 2002, Peña *et al.* 2003, Martin *et al.* 2004, 2007, Paash *et al.* 2004, Neild *et al.* 2005, Brum *et al.* 2008, Ortega Ferrusola *et al.* 2008). The sperm mitochondria are the main source of reactive oxygen species in the spermatozoon (Koppers *et al.* 2008), mainly generated at the complexes I and III of the respiratory chain.

Although the sperm mitochondria have been largely ignored for years, recent research indicates a major role of these structures in many physiological and pathological processes in the spermatozoa (Gallon *et al.* 2006, Gur & Breitbart 2006, Rasola & Bernardi 2007, Peña *et al.* 2009). A possible explanation for the results presented in the current study may be the following:

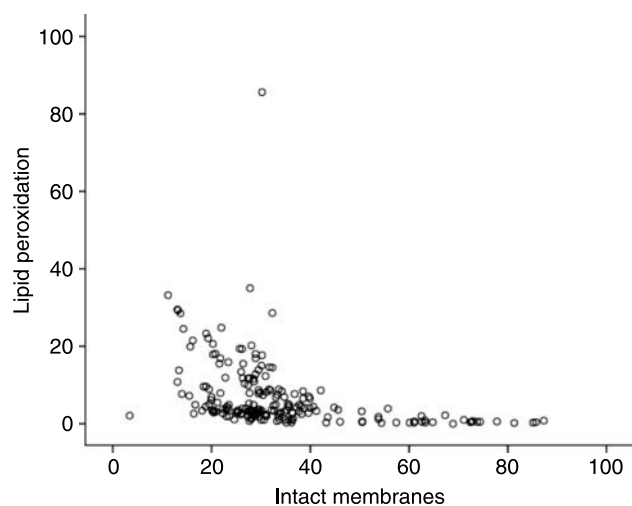
freezing and thawing increases ROS production in the sperm mitochondria while an osmotic mechanism may increase mitochondrial membrane permeability allowing ROS to diffuse rapidly, activating cellular machinery similar to the intrinsic pathway of apoptosis. In fact, oxidative stress is a well-documented inductor of apoptosis (Desagher & Martinou 2000, Lachaud *et al.* 2004, Ott *et al.* 2007). Data from our laboratory support this theory, since active caspases 3 and 7 (major proteins involved in the activation of apoptosis), and caspase 9 (an inductor protein involved in the activation of the mitochondrial pathway of apoptosis) have been described in fresh and frozen–thawed equine spermatozoa (Ortega Ferrusola *et al.* 2008). In addition, and supporting the concept that apoptotic-like phenomena may occur in ejaculated stallion spermatozoa, there is a report where ejaculated spermatozoa were capable of triggering a nuclear matrix associated topoisomerase IIB that concomitantly with a nuclease can cleave the entire sperm DNA into small fragments (Shaman *et al.* 2006). The theory of an apoptotic-like process being induced by LPO to explain the concept of ‘premature aging’ of cryopreserved spermatozoa, is also supported by the mathematical relationship found in our study, between LPO, membrane integrity, and  $\Delta\psi_m$ . LPO may activate an ‘apoptotic-like’ phenomenon leading to premature aging ending in a reduced lifespan of the processed spermatozoa.

From our study we can infer that susceptibility to LPO explains differences in cryoresistance among stallions and ejaculates within the same stallion. However, LPO appears to be overestimated as a major factor involved in cryodamage. Innate LPO levels were low in fresh samples of ejaculated spermatozoa, and for some stallions cryopreservation only increased LPO by 2–3% after thawing. However, despite LPO not being a major factor in sperm cryodamage, it may trigger

**Table 5** Non parametric correlations (Spearman  $\rho$ ) between the percentage of sperm lipid peroxidation, membrane, and mitochondrial status.

	YO-PRO–/Eth–	YO-PRO+	YO-PRO+/Eth+	Orange	Green	Caspase +	Caspase +/Eth +
BODIPY-C <sub>11</sub>	–0.789 <sup>†</sup>	0.681*	0.728*	–0.689*	0.537 <sup>†</sup>	–0.562*	0.772 <sup>†</sup>

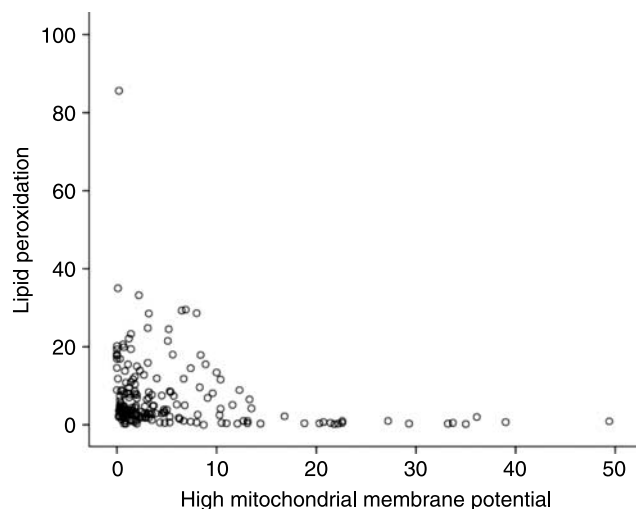
\* $P < 0.01$ , <sup>†</sup> $P < 0.001$ .



**Figure 2** Correlation analysis of the relationship between LPO and intact membranes in equine spermatozoa ( $r = -0.789$ ,  $P < 0.001$ ).

mechanisms indirectly leading to sperm damage, and as a more practical and important aspect, LPO is highly correlated to cryoresistance.

The present study also confirms previous findings from our laboratory (Ortega Ferrusola *et al.* 2008) and others (Brum *et al.* 2008), indicating that the equine ejaculate is characterized by a high percentage of spermatozoa depicting caspase activity, and that the expression of caspases increases as a result of cryopreservation. It is unlikely that the presence of active caspases in ejaculated spermatozoa represents only a pathological phenomenon. The stallions involved in the present study were of known fertility and used as donor sires for AI in our institution. Although apoptosis is a characteristic form of cell death that usually serves to remove unwanted cells, there are some examples where



**Figure 3** Correlation analysis of the relationship between LPO and spermatozoa depicting high mitochondrial membrane potential ( $r = -0.689$ ,  $P < 0.01$ ).

apoptosis-like events do not lead to death but rather are involved in the terminal differentiation of certain cell types (Said *et al.* 2004, Marchiani *et al.* 2007). On the other hand, since the overall survival time of the spermatozoa (both fresh and frozen-thawed) could not be prolonged by inhibitors of caspases (Weil *et al.* 1998, Peter & Linde-Forsberg 2003, Heninger *et al.* 2004) the mere presence of active caspases does not seem to be a crucial lethal factor in ejaculated spermatozoa. We can, therefore, speculate that active caspases potentially display additional physiological roles in mature spermatozoa rather than being involved only in detrimental changes.

In summary, LPO levels are low in freshly ejaculated stallion spermatozoa, but increase after cryopreservation in a stallion-dependent manner. This LPO is unlikely to represent a sign of cryopreservation-induced injury *per se*, but it is apparently capable of triggering apoptotic-like changes that could result in the sub-lethal cryo-damage often seen among surviving spermatozoa.

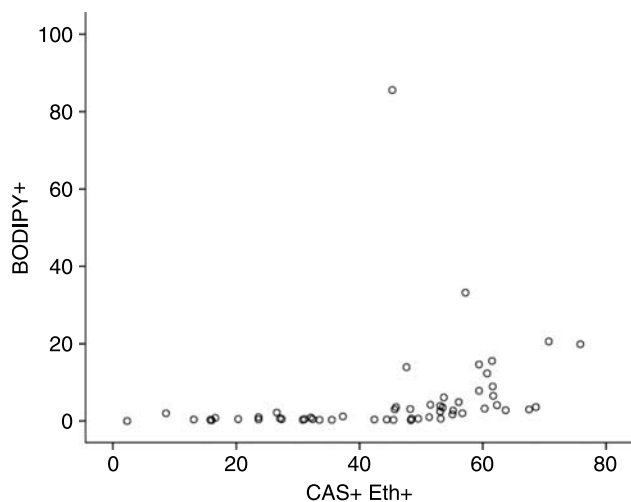
## Materials and Methods

### Study design

The study design comprised evaluation of sperm quality (sperm motility, membrane integrity, mitochondrial membrane potential, and caspase activity) and of LPO in freshly ejaculated spermatozoa, which were subsequently subjected to cryopreservation using a routine procedure. Spermatozoa were also studied for the same variables when thawed after at least 4 weeks of storage in liquid  $N_2$  (LN<sub>2</sub>).

### Semen collection and processing

Semen (four ejaculates per stallion) was obtained from seven Andalusian stallions individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, with semen being collected on a regular basis (two collections per week) during the 2008 breeding season. Ejaculates were collected using a Missouri-model artificial vagina, lubricated and pre-warmed to 45–50 °C, fitted with an in-line filter to separate the gel fraction. The collected ejaculate was immediately transported to the laboratory for evaluation and processing. The filtered ejaculate was extended 1:1 (v/v) with INRA 96 (IMV, L'Aigle, France), and centrifuged at 600 *g* for 10 min. The resulting sperm pellet was re-extended in the freezing medium (Ghent, Minitüb Ibérica, Spain) to a final concentration of  $100 \times 10^6$  spermatozoa/ml. The spermatozoa were slowly cooled to 4 °C within 1 h, loaded in 0.5 ml plastic straws and frozen horizontally in racks placed 4 cm above the surface of LN<sub>2</sub> for 10 min, after which they were directly plunged in LN<sub>2</sub> for storage. After at least 4 weeks of storage, the straws were thawed in a water bath at 37 °C for 30 s for analyzes.



**Figure 4** Correlation analysis of the relationship between LPO and caspase activity in dying (ethidium+) equine spermatozoa. ( $r=0.772$ ,  $P<0.001$ ).

### Cytofluorometric assessment of activated caspases

The caspase FITC-VAD-FMK *in situ* marker (Molecular Probes, Leiden, The Netherlands) was used to detect active caspases in the spermatozoa. This cell-permeable caspase inhibitor peptide is conjugated to FITC and binds covalently to active caspases serving as an *in situ* marker for apoptosis (Martin *et al.* 2004). A sample of  $5 \times 10^6$  freshly ejaculated or post-thawed spermatozoa was suspended in 1 ml PBS, and – after adding 1  $\mu$ l FITC-VAD-FMK (5 mM) – the suspension was incubated at room temperature (22–25 °C) in darkness for 20 min. After incubation, the spermatozoa were washed with PBS, followed by the addition of 1  $\mu$ l ethidium homodimer (1.167 mM; Molecular Probes Europe) to detect membrane damage. Flow cytometry and fluorescence microscopy were conducted within 10 min.

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

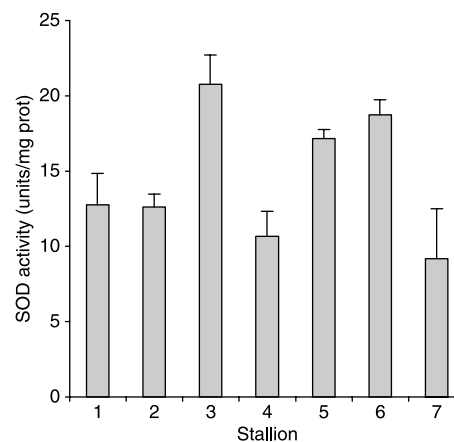
The lipophilic cationic compound 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl carbocyanine iodine (JC-1, Molecular Probes Europe) has the unique ability to label differentially mitochondria with low and high membrane potential. In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. However, at the same excitement wavelength (488 nm) in mitochondria with low membrane potential, the JC-1 forms monomers that emit in the green wavelength (525–530 nm). For staining, a 3 mM stock solution of JC-1 was prepared in DMSO. From each sperm suspension,  $5 \times 10^6$  freshly ejaculated or post-thawed spermatozoa were placed in 1 ml PBS and stained with 0.5  $\mu$ l JC-1 stock solution. The samples were incubated at 38 °C in darkness for 40 min before flow cytometric analysis.

### Assessment of subtle sperm membrane changes and viability

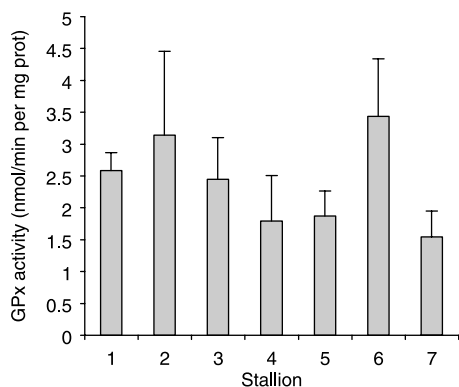
Early sperm membrane changes and viability were determined as described in Peña *et al.* (2005), with modifications for adaptation to the equine species (Ortega Ferrusola *et al.* 2008). In brief, 1 ml sperm suspension ( $5 \times 10^6$  spermatozoa/ml) from freshly ejaculated or post-thawed spermatozoa was loaded with 3  $\mu$ l YO-PRO-1 (25  $\mu$ M) and 1  $\mu$ l ethidium homodimer-1 (1.167 mM; Molecular Probes Europe, Leiden, The Netherlands). After a thorough mixing the suspension was incubated at 37 °C in darkness for 16 min. This staining distinguishes four sperm subpopulations. The first is the subpopulation of unstained spermatozoa. These spermatozoa are considered alive and without any membrane alteration. Another sperm subpopulation consists of YO-PRO-1 positive cells emitting green fluorescence. It has been demonstrated that in the early stages of apoptosis there is a modification of membrane permeability that selectively allows entry of some non-permeable DNA-binding molecules (Ormerod *et al.* 1993, Wronsky *et al.* 2002). This subpopulation contains spermatozoa which may show 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 (Idziorek *et al.* 1995, Wronsky *et al.* 2002). Neither of these probes enters intact cells. Finally, two subpopulations of cryo-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; Fig. 7).

### Staining for detection of LPO

LPO was measured using the probe BODIPY<sup>581/591</sup>-C<sub>11</sub> (Molecular Probes). A suspension of  $2 \times 10^6$ /ml spermatozoa (freshly ejaculated or post-thawed) was loaded with the probe at a final concentration of 2  $\mu$ M. Since egg yolk was found to



**Figure 5** Superoxide dismutase (SOD) activity expressed as arbitrary units of absorbance normalized to mg of protein in seven stallions. The differences observed were not statistically significant.



**Figure 6** Glutathione peroxidase (GPx) activity normalized to mg of protein nmol/min per mg protein in seven stallions. The differences observed were not statistically significant.

bind the lipophilic BODIPY<sup>581/591</sup>-C<sub>11</sub>, the egg yolk containing freezing extender (Ghent) was first removed by centrifugation through a 35% density gradient (Pure Sperm Nidacon, Gothenburg, Sweden). The spermatozoa were then incubated at 37 °C for 30 min, washed by centrifugation to remove the unbound probe, and analyzed using a flow cytometer and a confocal laser microscope. Positive controls were obtained after addition of 80 μM ferrous sulfate to additional sperm suspensions (Aitken *et al.* 2007).

### Confocal microscopy

Sperm samples were loaded with the membrane probe BODIPY<sup>581/591</sup>-C<sup>11</sup> and observed under a spectral inverted confocal microscope (Bio-Rad MRC 1024). The localization of the peroxidated probe emission was assessed at a wavelength range of 495–545 nm, whereas intact probe emission was selected at the wavelength range of 580–620 nm. Samples were excited with an argon ion laser emitting at 488 nm.

### Flow cytometry

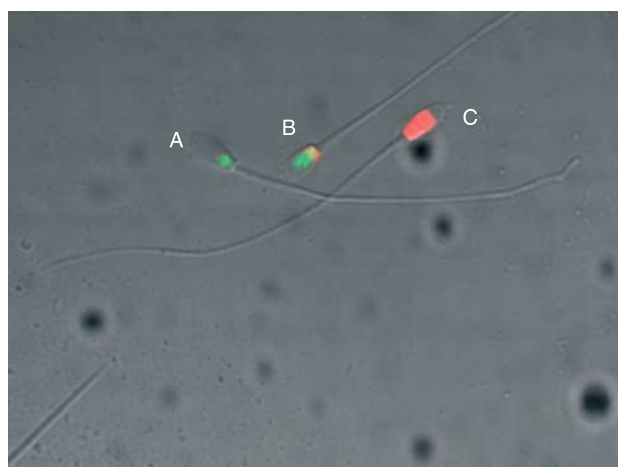
Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow cytometer equipped with standard optics, an argon-ion laser (Cyomics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm, and an EXPO 2000 software. Subpopulations were divided into 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-plots by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to spermatozoa 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 (YO-PRO-1, caspases, and BODIPY C<sub>11</sub>), or 30 000 events for JC-1. Samples were measured at flow rate of 200–300 cells/s. Green fluorescence was detected in FL1, red fluorescence was detected in FL3, and orange fluorescence in FL2.

### SOD determination

SOD activity was estimated by using a SOD determination kit following the directions provided by the manufacturer (Sigma-Aldrich). Briefly, SOD activity was indirectly estimated by a colorimetric method based on the reduction of WST-1, a highly water-soluble tetrazolium salt which renders a water-soluble formazan dye upon its reduction with a superoxide anion. Since the absorbance of WST-1 formazan at 440 nm is proportional to the amount of superoxide anion, the SOD activity was indirectly estimated by measuring the decrease in the color development at 440 nm. Frozen/thawed seminal plasma was diluted in a ratio of 1:10 in deionized water and assayed by triplicate. Each assay consisted in 20 μl diluted sample, 200 μl WST working solution, and 20 μl a solution containing active xanthine oxidase in the appropriate buffer. After the incubation for 20 min at 37 °C the color development was measured at 440 nm. SOD activity was expressed as arbitrary units of absorbance normalized to mg of protein.

### GPx determination

GPx activity was determined by using a GPx assay kit following the directions provided by the manufacturer (Calbiochem, La Jolla, CA, USA). Briefly, the GPx activity was indirectly estimated by the monitorization of the oxidation of NADPH to NADP<sup>+</sup>, which is accompanied by the decrease of the absorbance at 340 nm. Frozen/thawed seminal plasma (20 μl) was diluted with assay buffer (100 μl), mixed with 50 μl of a mixture containing NADPH, glutathione and glutathione reductase, and assayed in triplicate. In addition to samples, non-enzymatic samples (background) as well as samples containing bovine erythrocyte GPx (positive controls) were also assayed. The decrease in the absorbance at 340 nm was



**Figure 7** Confocal laser microscopy images of stallion spermatozoa loaded with YO-PRO-1/ethidium staining. (A) Green fluorescence, represents spermatozoa with an initial increase in membrane permeability (YO-PRO-1 fluorescence). (B) Green and red fluorescence represents an advanced stage of membrane damage, both YO-PRO-1 and ethidium homodimer stains the sperm nucleus. (C) Red fluorescence, ethidium homodimer, this is the advanced degree of membrane damage. All images were obtained with a Bio-Rad MRC confocal microscope. Magnification, 60×.

measured each min during 6 min at 25 °C. GPx activity was normalized to mg of protein and expressed as nmol/min per mg protein.

### Statistical analysis

The data were first examined using the Kolmogorov–Smirnov test to determine their distribution. In view of the non-Gaussian distribution of some of the data gathered, multivariate ANOVA was performed, and when significant differences were found, the non-parametric Mann–Whitney *U*-test was used to compare pairs of values. The Spearman non-parametric test was used to study the correlation between sperm analysis pre-freezing and sperm quality post-thaw. All analyses were performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was set at  $P < 0.05$ .

### 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.

### Funding

The authors received financial support from Ministerio de Educación y Ciencia- FEDER Madrid, Spain (Grants AGL 2007-60598 (GAN), BFU-2007-62423 (BFI) and INIA RZ2008-00018-00-00) and the Swedish Foundation for Equine Research (SSH), Stockholm, Sweden.

### Acknowledgements

This research is dedicated to the memory of Circe (1995–2009).

### References

- Agarwall A & Said TM 2005 Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU International* **95** 503–507.
- Aitken RJ, Wingate JK, De Iulius GN & McLaughlin EA 2007 Analysis of lipid peroxidation in human spermatozoa using BODIPY C<sub>11</sub>. *Molecular Human Reproduction* **13** 203–211.
- Almeida J & Ball BA 2005 Effect of alpha tocopherol succinate on lipid peroxidation in equine spermatozoa. *Animal Reproduction Science* **87** 321–337.
- Alvarez JG & Storey B 1992 Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *Journal of Andrology* **13** 232–241.
- Anzar M, He L, Buhr MM, Kroetsch G & Pauls KP 2002 Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction* **66** 354–360.
- Ball BA 2008 Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal Reproduction Science* **107** 257–267.
- Ball BA & Vo A 2002 Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C<sub>11</sub> BODIPY<sup>581/591</sup>. *Journal of Andrology* **23** 259–269.
- Bamber J, Ball BA, Gravance CG, Medina V & Davies Morel MCG 2000 The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *Journal of Andrology* **21** 895–902.
- Bileski BHJ, Arudi RL & Sutherland MW 1983 A study of the reactivity of HO<sub>2</sub>/O<sub>2</sub> with unsaturated fatty acids. *Journal of Biological Chemistry* **254** 4759–4761.
- Bravo MM, Aparicio IM, Garcia-Herreros M, Gil MC, Peña FJ & Garcia-Marin LJ 2005 Changes in tyrosine phosphorylation associated with true capacitation and capacitation like state in boar spermatozoa. *Molecular Reproduction and Development* **71** 88–96.
- Brouwers JF & Gadella BM 2003 *In situ* detection and localization of lipid peroxidation in individual bovine sperm cells. *Free Radical Biology and Medicine* **35** 1382–1391.
- Brouwers JF, Silva PF & Gadella BM 2005 New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze thawing. *Theriogenology* **15** 458–469.
- Brum AM, Sabuer K & Ball BA 2008 Apoptotic like changes in equine spermatozoa separated by density gradient centrifugation. *Theriogenology* **69** 1041–1055.
- Christova Y, James PS & Jones R 2004 Lipid diffusion in sperm plasma membranes exposed to peroxidative injury from oxygen free radicals. *Molecular Reproduction and Development* **68** 365–372.
- Cosci I, Moretti E & Collodel G 2008 Lipid peroxidation in human spermatozoa from men with genitourinary infections. *Systems Biology in Reproductive Medicine* **54** 75–83.
- Desagher S & Martinou JC 2000 Mitochondria as the central control point of apoptosis. *Trends in Cell Biology* **10** 369–377.
- Gallon F, Marchetti C, Jouy N & Marchetti P 2006 The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. *Fertility and Sterility* **86** 1526–1530.
- Gur Y & Breitbart H 2006 Mammalian sperm translate nuclear encoded proteins by mitochondrial-type ribosomes. *Genes and Development* **20** 411–416.
- Henninger NL, Staub C & Blanchard TL 2004 Germ cell apoptosis in the testes of normal stallions. *Theriogenology* **62** 283–297.
- Idziorek T, Estaquier J, de Bels F & Ameisen JC 1995 YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *Journal of Immunological Methods* **185** 249–285.
- Kemal Duru N, Morshendi M, Schuffer A & Oehninger S 2001 Cryopreservation–thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine. *Fertility and Sterility* **75** 263–268.
- Koppers AJ, De Iulius 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.
- Lachaud C, Tesarik J, Cañadas ML & Mendoza C 2004 Apoptosis and necrosis in human ejaculated spermatozoa. *Human Reproduction* **19** 607–610.
- Marchiani S, Tamburrino L, Maoggi A, Vannelli GB, Forti G, Baldi E & Muratori M 2007 Characterization of M450 bodies in human semen: evidence that they are apoptotic bodies. *Molecular Human Reproduction* **13** 621–631.
- Martin G, Sabido O, Durand P & Levy R 2004 Cryopreservation induces an apoptosis like mechanism in bull sperm. *Biology of Reproduction* **71** 28–37.
- 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. *Human Reproduction* **22** 380–388.
- Morris GJ, Faszler K, Green JE, Draper D, Grout BWW & Fonseca F 2007 Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. *Theriogenology* **68** 804–812.
- Neild DM, Brouwers JFHM, Colenbrander B, Agüero A & Gadella BM 2005 Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. *Molecular Reproduction and Development* **72** 230–238.



- Ormerod MG, Sun XM, Snowden RT, Davies R, Fearnhead H & Cohen GM** 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 JM, González Fernández L, Tapia JA & Peña FJ** 2008 Detection of apoptosis like changes during the cryopreservation process in equine sperm. *Journal of Andrology* **29** 213–221.
- Ott M, Gogvadze V, Orrenius S & Zhivotovsky B** 2007 Mitochondria, oxidative stress and cell death. *Apoptosis* **12** 913–922.
- Paash U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ, Glander HJ & Agarwal A** 2004 Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biology of Reproduction* **71** 1828–1837.
- Pap EHW, Drummen GPC, Winter VJ, Kooij TWA, Rijken P, Wirtz KWA, Op den Kamp JAF, Hage WJ & Post JA** 1999 Ratio fluorescence microscopy of lipid oxidation in living cells using C<sub>11</sub>-BODIPY<sup>581/591</sup>. *FEBS Letters* **453** 278–282.
- Peña FJ, Johannisson A, Wallgren M & Rodríguez Martínez H** 2003 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.
- Peña FJ, Johannisson A, Wallgren M & Rodríguez Martínez H** 2004 Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. *Zygote* **12** 117–124.
- Peña FJ, Saravia F, Johannisson A, Wallgren M & Rodríguez Martínez H** 2005 A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. *International Journal of Andrology* **28** 107–114.
- Peña FJ, Rodríguez Martínez H, Tapia JA, Ortega Ferrusola C, González Fernández L & Macías García B** 2009 Mitochondria in mammalian sperm physiology and pathology: a review. *Reproduction in Domestic Animals* **44** 345–349.
- Peter AT & Linde Forsber C** 2003 Efficacy of the anticaspase agent z VAD-fmk on post thaw viability of canine spermatozoa. *Theriogenology* **59** 1525–1532.
- Rasola A & Bernardi P** 2007 The mitochondrial membrane transition pore and its involvement in cell death and disease pathogenesis. *Apoptosis* **12** 815–833.
- Said TM, Paasch U & Glander HJ** 2004 Role of caspases in male infertility. *Human Reproduction Update* **10** 39–511.
- Samper JC, Estrada AJ & McKinnon AO** 2007 Insemination with frozen semen. In *Current Therapy in Equine Reproduction*, pp 285–289. Eds JC Samper, JF Pycock & AO MacKinnon. St Louis, MO: Saunders Elsevier.
- Saravia F, Hernández M, Wallgren MK, Johannisson A & Rodríguez-Martínez H** 2007 Cooling during semen cryopreservation does not induce capacitation of boar spermatozoa. *International Journal of Andrology* **30** 485–499.
- Shaman JA, Prisztoka R & Ward WS** 2006 Topoisomerase IIB and extracellular nuclease interact to digest sperm DNA in an apoptotic like manner. *Biology of Reproduction* **75** 741–748.
- Watson PF** 2000 The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science* **60–61** 481–492.
- Weil M, Jacobson MD & Raff MC** 1998 Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes *Journal of Cell Science* **111** 2707–2715.
- Wronsky R, Golob N, Grygar E & Winsdish M** 2002 Two color, fluorescence – based microplate assay for apoptosis detection. *BioTechniques* **32** 666–668.

---

Received 20 November 2008

First decision 23 December 2008

Revised manuscript received 16 March 2009

Accepted 20 April 2009