

## Free-radical production after post-thaw incubation of ram spermatozoa is related to decreased *in vivo* fertility

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**Abstract.** The aim of the present study was to evaluate the effect of sperm reactive oxygen species (ROS) production and DNA changes on male fertility. For that purpose, six rams with significantly different pregnancy rates were used; these were classified as having high fertility, i.e. 59.4% average pregnancy rate, or low fertility, i.e. 23.1% average pregnancy rate. Sperm quality was assessed after a two-step process of sample thawing followed by an incubation of 2 h, either in the freezing extender (37°C) or after dilution in synthetic oviductal fluid (SOF; 38°C, 5%CO<sub>2</sub>). Sperm viability (YO-PRO-1), ROS production (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein acetyl ester (CM-H<sub>2</sub>DCFDA)) and undamaged chromatin (sperm chromatin structure assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, chromomycin A3) were evaluated by flow cytometry. Although no significant differences in sperm viability were observed, our results showed increased ROS production during incubation in the freezing extender as well as in SOF medium. Comparison between fertility groups showed significant differences in ROS production after 2 h of incubation for the two treatments. Regarding DNA integrity, our results showed no significant differences either between treatments and incubation times or fertility groups. Linear regression analysis showed that ROS production determined by CM-H<sub>2</sub>DCFDA was a good indicator parameter for *in vivo* male fertility of SOF-incubated samples, yielding a fair correlation between both parameters ( $r = -0.92$ ). These results indicate that detection of ROS production by CM-H<sub>2</sub>DCFDA and flow cytometry after 2 h of incubation in SOF could be a useful procedure for predicting fertility of ram spermatozoa.

**Additional keywords:** artificial insemination, flow cytometry, fluorescein (CM-H<sub>2</sub>DCFDA), frozen semen.

Received 6 February 2014, accepted 27 April 2014, published online xx xxxxx xxxx

### Introduction

Oxidant species have a crucial role on sperm physiology, but they may also impair the fertilising ability of spermatozoa. Oxidative stress may be defined as the outcome of the imbalance between reactive oxygen species (ROS) production and the ability of antioxidants to scavenge them. This oxidative stress is then considered of high concern as high levels of ROS have negative effects on sperm functionality (Gil-Guzman *et al.* 2001).

Furthermore, ROS production is considered as one of the mediators of damage caused by cryopreservation (Alvarez and Storey 1992; O'Flaherty *et al.* 1997; Chatterjee and Gagnon 2001; Fernández-Santos *et al.* 2007) as it may lead to DNA damage (Lopes *et al.* 1998), cytoskeletal alterations (Hinshaw *et al.* 1986), inhibition of spermatozoon–oocyte fusion (Aitken *et al.* 1989) and effects on the sperm axoneme followed by loss of motility (de Lamirande and Gagnon 1992). Thus, it could be feasible that oxidative stress may be related to cell death and loss

of cell function during or following a freeze–thaw cycle (O'Flaherty *et al.* 1997; Bailey *et al.* 2000).

Therefore, the study of oxidative stress of thawed sperm samples and its relationship to fertility is of great interest, as these samples could be used for artificial insemination. Many studies have aimed to elucidate the relationship between sperm-quality parameters and *in vivo* fertility, with different outcomes (Schneider *et al.* 1999; Rodríguez-Martínez 2003; Papadopoulos *et al.* 2005; Petrunina *et al.* 2007).

The mechanisms by which oxidative stress limits the functional competence of mammalian spermatozoa involve the peroxidation of lipids, the induction of oxidative DNA damage and the formation of protein adducts (Aitken *et al.* 2012). Thus, the effect of oxidative stress on spermatozoa can also be monitored by assessing the levels of DNA damage (Lewis and Aitken 2005). The sperm chromatin structure assay (SCSA) technique and the terminal deoxynucleotidyl

transferase-mediated dUTP nick-end labelling (TUNEL) assay have been proposed for the evaluation of sperm DNA fragmentation. By using SCSA, a high DNA fragmentation index (DFI) has been related to reduced fertility, longer times to pregnancy and higher spontaneous miscarriage rates in humans (Virro *et al.* 2004; Evenson and Wixon 2006). It has also shown a correlation with fertility and prolificacy in domestic animals (bull, Waterhouse *et al.* (2006); García-Macias *et al.* (2007); boar, Boe-Hansen *et al.* (2008)). Similarly, the TUNEL assay is considered to be a promising technique in accordance with SCSA and fertility results (Waterhouse *et al.* 2006; Benchaib *et al.* 2007). The results of the chromomycin A3 (CMA3) test, a fluorochrome that detects protamine deficiency in loosely packed chromatin, are correlated with the extent of nicked DNA (Gillan *et al.* 2005). Therefore, the aim of this work was to evaluate the effect of ROS production on post-thaw sperm characteristics, in order to gain knowledge about the underlying mechanisms of differences in ram fertility. To this end, ROS production and DNA fragmentation levels of cryopreserved sperm samples were assessed, and an *in vivo* fertilisation test was conducted. Both the study of oxidative stress and DNA analysis could be useful tools for quality assessment of thawed semen doses from domestic animals, before artificial insemination, and potential fertility determination of given semen samples.

## Materials and methods

### Experimental design

Two straws per male were thawed. Sperm samples were previously analysed to ensure that the two straws had similar characteristics and there were no differences between them. Standardised insemination doses were used. Thawed sperm samples were incubated for 2 h (37°C) without dilution (in the freezing diluent) or after dilution 1 : 25 in synthetic oviductal fluid (SOF) medium, at 5% CO<sub>2</sub> (38°C). SOF medium is an *in vitro* fertilisation medium that tries to mimic the female reproductive tract, commonly used as a test of endurance. Samples were analysed just after thawing and after dilution in SOF. After 2 h of incubation, sperm samples were also analysed.

### Reagents and media

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probe propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes were purchased from Invitrogen (Barcelona, Spain) unless otherwise stated. Stock solutions of the fluorescent probes were: PI, 1.5 mM in Milli-Q water; YO-PRO-1, 50 µM in DMSO (dimethyl sulfoxide); CM-H<sub>2</sub>DFCDA, 0.5 mM in DMSO; CMA3, 0.25 mg mL<sup>-1</sup> in DMSO. All fluorescent stocks were kept at -20°C in the dark until needed. The freezing extender was prepared in our laboratory as described by Fiser *et al.* (1987), using reagent-grade chemicals purchased from Panreac Quimica SA (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA). The fluorochrome acridine orange was of electrophoretic grade and purchased from Polysciences Inc. (Warrington, PA, USA).

SOF was composed of 107 mM NaCl, 7.17 mM KCl, 1.19 mM KH<sub>2</sub>PO<sub>4</sub>, 1.71 mM Ca<sub>2</sub>Cl<sub>2</sub>·2H<sub>2</sub>O, 0.49 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 25.07 mM NaHCO<sub>3</sub>, 3.3 mM Na lactate, 0.3 mM Na pyruvate and 200 mM glutamine. Osmolarity was 270–280 mOsm kg<sup>-1</sup> and pH (room temperature) 7.2–7.3 (Gardner *et al.* 1994).

### Animals and sperm collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. Adult males belonged to the Regional Center for Animal Selection and Reproduction in Valdepeñas (CERSYRA). A total of six males of Manchega sheep breed (age >3 years) were used. Semen collection was performed using an artificial vagina. Volume, concentration, wave motion (0 no movement to 5 strong wave movement) and sperm motility were assessed shortly after collection. Only ejaculates with wave-motion values of 4 or 5 and sperm motility higher than 80% were frozen.

### Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. Initially, before freezing, the ejaculates were diluted to a final concentration of 200 × 10<sup>6</sup> spermatozoa mL<sup>-1</sup>. First, sperm samples were diluted in fraction 1 of the diluent to a concentration of 400 × 10<sup>6</sup> spermatozoa mL<sup>-1</sup> and slowly cooled from 30 to 5°C in 2 h. Then, sperm samples were further diluted (2 : 1) with fraction 2 (5°C) and held for equilibration at 5°C for 2 h (total refrigeration time at 5°C was 4 h). At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen. The straws were frozen in a programmable biofreezer (IceCube 14S-Ver. 1.30; SY-Laboratory Geräte GmbH, Minitüb®, Tiefenbach, Germany) at -20°C min<sup>-1</sup> to -100°C, and at -10°C min<sup>-1</sup> from -100°C to -140°C and then plunged into liquid nitrogen. Two straws per male were thawed for 30 s at 37°C in a warm bath and aliquots were used to assess sperm quality and concentration.

### Artificial insemination trials

Thawed sperm samples from the six males were used to inseminate 351 ewes in eight farms. Sperm samples from each male were used to inseminate between 27 and 147 females. The number of inseminated ewes per male was 49, 29, 27, 147, 42 and 57 for Males 1, 2, 3, 4, 5 and 6, respectively. The insemination dose was 100 × 10<sup>6</sup> spermatozoa and contained more than 55% motile spermatozoa. Only one ejaculate per male was used in this study. Sperm samples sourced from selected rams of a reproductive selection centre. All samples had a minimum quality that includes low morphological abnormalities.

The ewes were synchronised using progestagen pessaries (30 mg fluorogestone acetate, (FGA); Chronogest, Intervet, Booxmer, The Netherlands) for 13 days followed by 500 IU equine chorionic gonadotrophin (eCG) at pessary removal. Ewes were inseminated intrauterine by laparoscopy at 55–58 h after pessary removal. Two technicians carried out all intrauterine inseminations on different dates.

We considered that a male scored a successful fertilisation when the female lambled. The fertility rate for each male was calculated as follows: number of lambled ewes/number of ewes inseminated  $\times$  100. This rate was called male fertility.

The males were classified according to fertility in two groups: high fertility, those with fertility above mean (male fertility 55%) and, low fertility, those with fertility below mean (male fertility  $<$ 55%).

#### Flow cytometric analysis

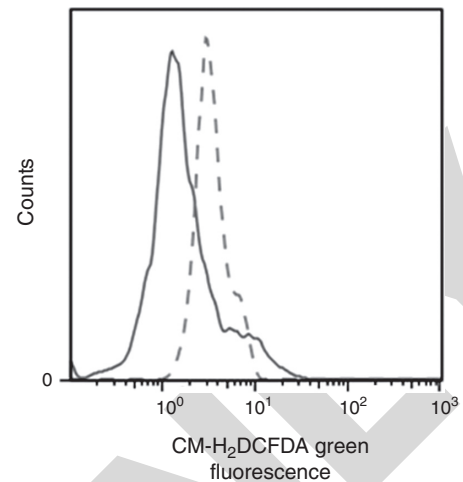
After appropriate treatment with fluorescent probes (see below), sperm suspensions preincubated with or without SOF were analysed on a Cytomics FC500 flow cytometer (Beckman Coulter) controlled by WEASEL software Ver. 3 (WEHI, Melbourne, Vic., Australia). Cells were passed through the instrument at 150–300 cells  $s^{-1}$ , and data were collected for 10 000 cells. The cells were excited at 488 nm using an argon laser. Fluorescein isothiocyanate (FITC), CM-H<sub>2</sub>DCFDA, CMA3 and acridine orange (AO) green fluorescence was detected with a 530/28 band-pass filter (FL-1) while propidium iodide and AO red fluorescence was detected with a 620/40 band-pass filter (FL-3), both on logarithmic scales. The control settings were adjusted for measuring the fluorescence in FL-1 and FL-3 channels essentially as described in the Supplementary Material available in the online version of this paper.

#### Sperm viability

Sperm viability was checked by means of propidium iodide staining (PI, 15  $\mu$ M; stock, 50  $\mu$ M in DMSO) and YO-PRO-1 (50 nM; stock, 7.5 mM in milli-Q water) in combination with flow cytometry as described by Martínez-Pastor *et al.* (2008). The staining solution was prepared using SOF-HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) ( $\sim$ 1–2  $\times$  10<sup>6</sup> spermatozoa  $mL^{-1}$ ). After 15 min of incubation in the dark, sperm samples were analysed by flow cytometry. Propidium iodide binds to DNA in membrane-defective spermatozoa and allows the identification of viability. YO-PRO-1 is a probe capable of staining early apoptotic cells (with intact plasma-membrane, not stained by PI, but showing increased permeability; Martínez-Pastor *et al.* 2009). The control settings were adjusted for measuring the fluorescence in FL-1 and FL-3 channels essentially as described in the Supplementary Material. The percentage of YO-PRO-1-positive non-DNA particles ranged from 15.7% (freezing extender) to 17.9% (SOF).

#### Production of reactive oxygen species

Reactive oxygen species production was recorded using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein acetyl ester (CM-H<sub>2</sub>DCFDA) as described by Domínguez-Rebolledo *et al.* (2010). CM-H<sub>2</sub>DCFDA penetrates the plasma membrane and is retained after intracellular esterases cleave the acetate groups and emits green fluorescent (504 nm) upon oxidation. The intensity of fluorescence of CM-H<sub>2</sub>DCFDA increases as ROS production increases. Sperm suspensions (1  $\times$  10<sup>8</sup> cells  $mL^{-1}$ ) were loaded at 37°C, 20 min with CM-H<sub>2</sub>DCFDA in SOF-HEPES (final concentration 0.5  $\mu$ M; stock solution, 500  $\mu$ M). Main population at 0 h was set under



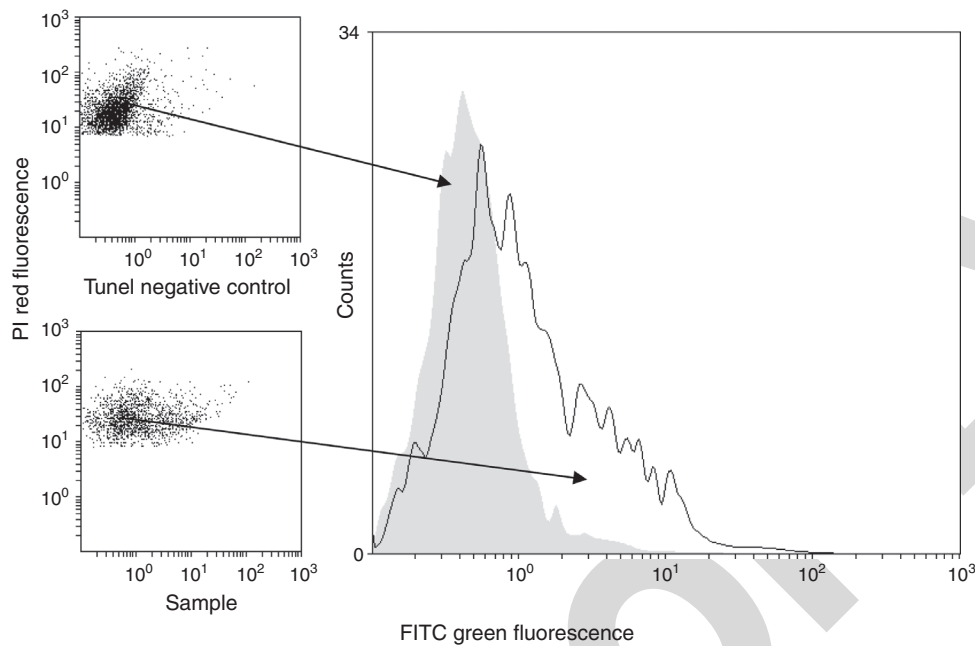
**Fig. 1.** Free-radical production assessed by CM-H<sub>2</sub>DCFDA histogram for incubated sperm samples in SOF. Continuous line indicates free-radical production at 0 h. Semi-continuous line indicates free-radical production at 2 h of incubation at 37°C. Displacement of the mean was analysed to assess the increase in radical production.

the first decade of the logarithmic scale of the 530/28 BP photodetector as reference value in a histogram. With these settings, samples were analysed at 0 h and 2 h of incubation and the mean value was noted (Fig. 1).

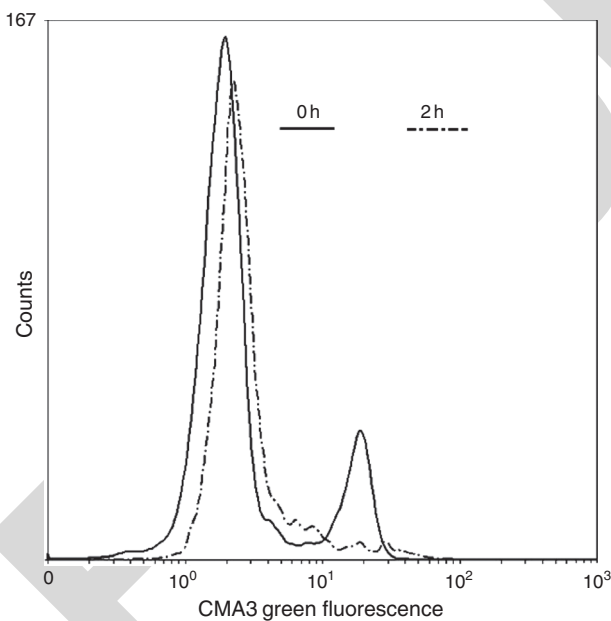
#### Sperm chromatin structure assay (SCSA)

Chromatin stability was assessed following the sperm chromatin structure assay (SCSA), based on the susceptibility of sperm DNA to acid-induced denaturation *in situ* and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson *et al.* 2002). Acridine orange (AO) fluorescence shifts from green (double strand (dsDNA)) to red (single strand (ssDNA)). Samples were diluted in TNE buffer (0.01 M TRIS-HCl, 0.15 M NaCl, 1 mM EDTA; pH 7.4) to a final sperm concentration of 2  $\times$  10<sup>6</sup> cells  $mL^{-1}$ . Samples were frozen ( $-80^{\circ}C$ ) until needed. For analysis, the samples were thawed in crushed ice. Acid-induced denaturation of DNA *in situ* was achieved by adding 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl; pH 1.4) to 200  $\mu$ L of sample. After 30 s, the cells were stained by adding 1.2 mL of an acridine orange solution (0.1 M citric acid, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 0.15 M NaCl, 6  $\mu$ g  $mL^{-1}$  acridine orange; pH 6.0). The stained samples were analysed by flow cytometry exactly 3 min after adding the acridine orange solution.

A tube with 0.4 mL of acid-detergent solution and 1.2 mL of acridine orange solution was run through the system before running any samples and between samples. At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0–1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence versus total intensity of the fluorescence (red/[red+green]  $\times$  100), called the DNA fragmentation index (DFI; formerly called  $\alpha$ ) for each spermatozoon, representing the shift from



**Fig. 2.** Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. PI is used to discriminate fixed cells from debris. Grey histogram represents basal fluorescence (negative control) from no-antibody FITC-conjugated treated cells. White histogram represents sample fluorescence. Sample cells exhibiting a greater green fluorescence than the basal level are considered TUNEL positive and are expressed as a percentage (%).



**Fig. 3.** Sperm histone re-emplacment measured by CMA3. Dotted line indicates chromatin compaction at 2 h of incubation. Changes after 2 h and displacement of the mean were compared.

green to red fluorescence. High values of DFI indicate chromatin abnormalities. Flow cytometry data was processed to obtain %DFI (percentage of spermatozoa with DFI >25) and high DNA stainability (HDS; percentage of spermatozoa with green fluorescence higher than channel 600 of 1024 channels).

*TUNEL assay*

DNA damage was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) assay, following the manufacturer's instructions (ApoTarget APO-BRDU Kit; Life Technologies). Briefly, spermatozoa diluted in phosphate-buffered saline (PBS;  $10^6$  cells  $\text{mL}^{-1}$ ) were fixed for 1 h in 2% paraformaldehyde. The cells were washed twice with PBS and resuspended in 70% ethanol at 0°C. The samples were left at -20°C overnight. Then, the cells were washed twice using the wash buffer provided with the kit, adding the DNA labelling mixture after removing the wash buffer. After 60 min at 37°C (with agitation), the cells were washed twice using the rinse buffer. Finally, the cells were resuspended in the antibody solution (FITC-anti-BrdUTP mAb) and incubated for 30 min at room temperature in the dark. Samples were resuspended in a PI-RNase A solution and analysed by flow cytometry within 2 h. Positive and negative controls (incubation of fixed cells with DNase A and substituting water for the DNA labelling mixture, respectively) were used to standardise the assay. DNA damage was measured using FL1 for the detection of the emission of FITC in the green spectrum and the FL3 photodetector for the emission of PI in the red spectrum. Non-red events (PI-) were discarded as debris. Setting non-DNA-labelled spermatozoa as control their basal green fluorescence was established as a threshold; all events above that limit were considered TUNEL+ (Fig. 2).

*Chromomycin A3 (CMA3) determination*

The CMA3 penetrability test was used to assess the extent of sperm nuclear protamination and as an indicator of chromatin

condensation. CMA3 labelling and analysis by flow cytometry were performed as described previously by Zubkova *et al.* (2005). CMA3 has affinity for DNA but is unable to access it when histones have been replaced by protamines or when the disulfide bridges are intact. Sperm samples were stained for 20 min in McIlvaine's buffer with CMA3 antibiotic at a concentration  $0.25 \text{ mg mL}^{-1}$ . CMA3 emits green fluorescence when bound to DNA. The mean of the green fluorescence was taken for each sample, once adjusted with a control (green emission at 0 h incubation was adjusted at first decade of the logarithmic scale). Variations between 0 h and 2 h were compared (Fig. 3).

#### Statistical analysis

Three statistical analyses were performed in this study. First, a multiple regression analysis was carried out to determine which variables significantly affected *in vivo* fertility. Four explicative variables were considered: date of insemination, farm and male as the factors of interest, with the number of ewes inseminated included as a weighing factor. Second, the effect of incubation times and extenders was compared with ANOVA, carrying out a pairwise comparison (Bonferroni's correction) when any of the effects was significant.

**Table 1. GLM (generalised linear model) of male fertility on number of inseminated ewes, insemination date, farm and male (model:  $R^2 = 0.95$ ;  $P = 0.02$ )**

Dependent variable	Independent variable	<i>P</i> value
Male fertility	Number of inseminated ewes	0.441
	Insemination date	0.100
	Farm	0.560
	Male	0.006

Finally, the two fertility groups were compared for all the sperm parameters assessed on thawed samples using a *t*-test. This allowed us to examine if sperm characteristics differed between the two groups, and therefore could be responsible for the fertility differences. The relationship between ROS production and *in vivo* fertility was examined by using linear regression analysis.

All statistical analyses were performed using SPSS for Windows version 20.0 (IBM SPSS Statistics Inc., Chicago, IL, USA). Differences were considered to be statistically significant at  $P < 0.05$ .

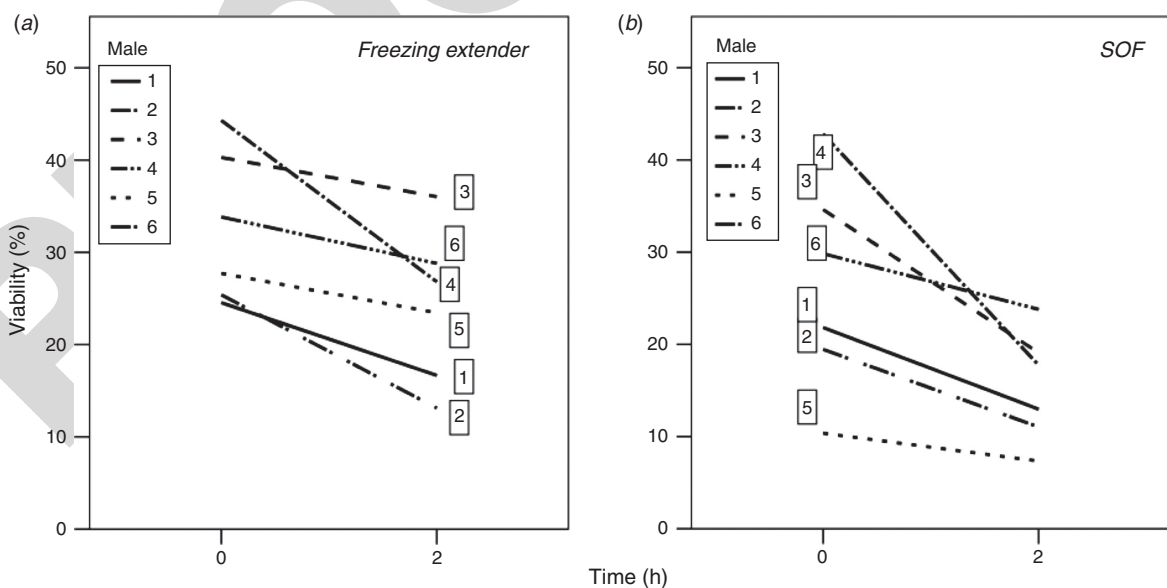
#### Results

Male fertility rates ranged from 22% to 64% with a mean value of 40.5%. Differences in fertility rates among males were significant ( $P = 0.006$ ). Of all effects we considered to affect the fertility success, only male showed a significant effect. Thus, the insemination date, the farm in which insemination took place and the number of inseminated ewes per male did not affect male fertility in a significant way (Table 1).

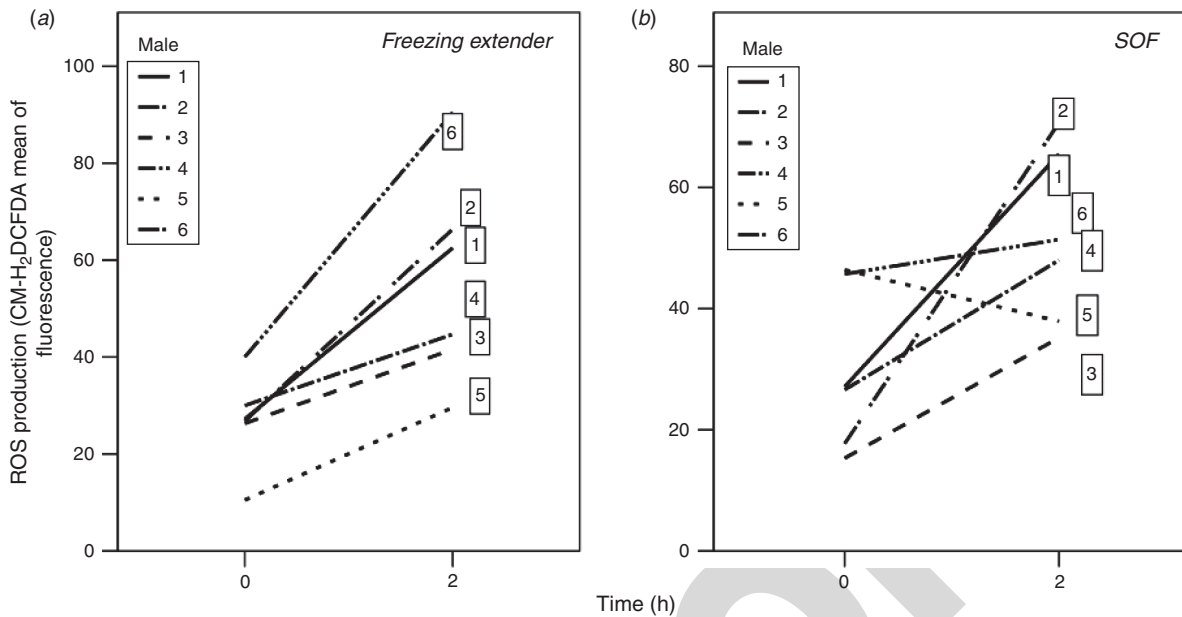
Sperm viability results of each male and for the two treatments are shown in Fig. 4. There were no significant differences in viability between the two treatments (freezing extender vs SOF) either after thawing ( $P = 0.30$ ) or after 2 h of incubation ( $P = 0.06$ ).

Values for the sperm ROS production after thawing and after 2 h of incubation in the two different media are shown in Fig. 5. ROS production increased significantly after incubation both in the freezing extender ( $P = 0.02$ ) and in the SOF medium ( $P = 0.01$ ).

As can be observed in Table 2, there were no significant differences in viability between high-fertility males and low-fertility ones, at any time of evaluation, either in the freezing extender or in SOF. After 2 h of incubation, males classified as



**Fig. 4.** These plots show the effect of the individual males (1–6) on sperm viability (YO-PRO-1–PI–; live spermatozoa with intact plasmalemma) after 2 h of incubation in (a) freezing extender or (b) SOF medium (5%  $\text{CO}_2$ ,  $38^\circ\text{C}$ ).



**Fig. 5.** These plots show the effect of the individual males (1–6) on ROS production assessed by CM-H<sub>2</sub>DCFDA (mean of the fluorescence) after 2 h of incubation in (a) freezing extender or (b) SOF medium (5% CO<sub>2</sub>, 38°C).

**Table 2.** Values (least squares mean ± s.e.m.) for the sperm parameters in the groups of high and low fertility

FE, freezing extender; SOF, synthetic oviductal fluid; viability, percentage of YO-PRO-1-/PI- spermatozoa (live with intact plasmalemma); ROS production, mean fluorescence intensity for CM-H<sub>2</sub>DCFDA; tDFI, DNA fragmentation index (SCSA; % of DNA fragmentation); TUNEL, percentage of TUNEL+ spermatozoa; CMA3, chromatin compaction status (mean fluorescence intensity for CMA3). \**P* < 0.05

Time of evaluation	Fertility group	Viability (%)	ROS production (mean)	tDFI (%)	TUNEL (%)	CMA3 (mean)
After thawing	High	29.2 ± 7.2	29.4 ± 8.6	0.6 ± 0.04	7.1 ± 0.9	32.4 ± 7.4
	Low	23.7 ± 7.2	30.1 ± 8.6	0.7 ± 0.04	8.5 ± 0.9	42.3 ± 7.4
After dilution in SOF	High	37.4 ± 4.1	22.2 ± 5.2	0.3 ± 0.04	7.4 ± 2.7	46.6 ± 10.0
	Low	27.9 ± 4.1	31.3 ± 5.2	0.4 ± 0.04	9.7 ± 2.7	55.3 ± 10.0
2 h incubation in FE	High	15.9 ± 3.8	40.3 ± 6.9	0.8 ± 0.15	12.1 ± 2.1	32.3 ± 2.1
	Low	14.7 ± 3.8	62.5 ± 4.9*	0.7 ± 0.15	9.8 ± 2.1	37.9 ± 2.1
2 h incubation in SOF	High	28.7 ± 4.2	38.6 ± 6.9	0.3 ± 0.03	20.7 ± 5.4	30.2 ± 2.4
	Low	19.5 ± 4.2	73.1 ± 6.9*	0.5 ± 0.03	11.7 ± 5.4	36.2 ± 2.4

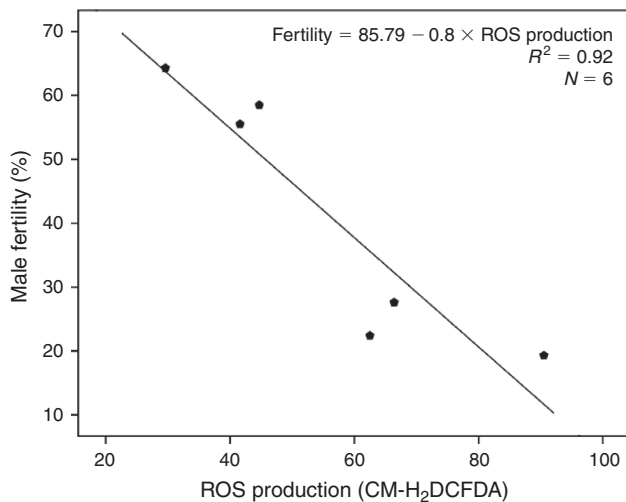
of high fertility had significantly lower ROS production than those of low fertility, both in the freezing extender (FE;  $40.36 \pm 6.9$  vs  $62.53 \pm 6.9$ ;  $P = 0.03$ ) and SOF ( $38.63 \pm 6.9$  vs  $73.13 \pm 6.9$ ;  $P = 0.02$ ). Once differences in ROS production between fertility groups were identified, we decided to investigate whether DNA sperm parameters were also modified between both groups. Nevertheless, there were no significant differences for DNA fragmentation either after thawing or after incubation, as detected by both the SCSA and TUNEL techniques. Furthermore, we did not find any significant difference for packaging quality as assessed by CMA3 (Table 2).

Finally, we examined the relationship between ROS production levels and *in vivo* male fertility. Although initially we did not find a relationship between ROS production and *in vivo* male fertility, after 2 h of incubation in SOF, ROS production levels showed a negative correlation ( $r = -0.92$ ;  $P < 0.01$ ) with male fertility (Fig. 6). However, this correlation was not found after 2 h of incubation in the freezing extender.

## Discussion

In the present study, despite finding no significant differences in sperm viability, our results showed increasing ROS production during incubation both for freezing extender and SOF medium. Comparison between fertility groups showed significant differences in ROS production after 2 h of incubation for the two treatments. After 2 h of incubation in SOF, ROS production levels were negatively correlated with *in vivo* male fertility.

Oxidative stress has an important role in sperm physiology. In this context, oxidative stress is increasingly recognised as a major cause in the aetiology of human male infertility (Aitken and Krausz 2001; Tremellen 2008). Moreover, cryopreservation not only reduces sperm quality, but also induces oxidative stress and decreases the antioxidants in semen (Aisen *et al.* 2005; Peris *et al.* 2007). The mechanisms of oxidative stress that limit the functional competence of mammalian spermatozoa involve the peroxidation of lipids, the induction of oxidative DNA



**Fig. 6.** Relationship between pregnancy rate and ROS production assessed by CM-H<sub>2</sub>DCFDA after 2 h of incubation in SOF medium (5% CO<sub>2</sub>, 38°C).

damage and the formation of protein adducts (Aitken *et al.* 2012). It is clear that ROS are important contributors to the regulation of sperm function from a positive and a negative point of view. The beneficial and detrimental effects of ROS probably represent a continuum (Aitken 2011). Thus, following insemination, these cells generate low levels of ROS in order to promote capacitation and the functional evolution of sperm behaviours needed for fertilisation, including hyperactivation and the presentation of zona-recognition molecules on their surface (Aitken *et al.* 1989; de Lamirande and Gagnon 1993). If fertilisation does not occur, as is usually the case for an individual spermatozoon, then the continued ROS generation overwhelms the meagre intrinsic antioxidant defences of these cells and activates the intrinsic apoptotic cascade. In light of the foregoing, an excessive production of ROS could be related to early development of capacitation and hyperactivation processes, preventing spermatozoa from developing properly. Thus, as our results show, incubation in IVF media may also result in an overproduction of ROS, which impairs sperm function and fertility. The increase of ROS could not result in negative effects by itself, but could indicate a subjacent cause (mitochondrial failure, pathway deregulation.etc), which produces both increased ROS and lower fertility. Thus, the increased ROS would not be the cause of the lower fertility, but a signal that something is not being regulated correctly in the spermatozoa.

Indeed, it seems that the information provided by the CM-H<sub>2</sub>DCFDA test combined with SOF incubation is useful for improving sperm-quality assessment, and both methods could be considered as a standard tool for sperm-quality determination. In fact, other results indicate that spermatozoa with higher levels of ROS, measured by CM-H<sub>2</sub>DCFDA, produced embryos that could not progress beyond the first division (Silva *et al.* 2007).

The assessment of the fertility potential of a semen sample has been the paramount objective of semen analysis. Thus, our objective is to develop laboratory techniques that allow us, in a quick and effective manner, to evaluate the potential fertility of a

given sperm sample. However, the predictive power of these tests is generally low and very variable among different authors (Rodríguez-Martínez 2007). The objective assessment of sperm function could increase the chances of predicting the fertilising capacity of a frozen-thawed semen sample or diagnosing infertility problems. Taking into account the importance of ROS production in thawed sperm samples, we have addressed this issue evaluating its effect on fertility. It is remarkable that García-Álvarez *et al.* (2009) showed that heterologous *in vitro* fertilisation was a good procedure to predict the fertility of ram semen. According to our results, differences in ROS production could be responsible for different *in vitro* fertilisation results. However, methods based on IVF are costly and time-consuming. There are many *in vitro* techniques that have attempted to correlate with *in vivo* fertility (Schneider *et al.* 1999; O'Meara *et al.* 2008; García-Álvarez *et al.* 2009). In this context, significant positive correlation between different velocity parameters and fertilisation percentage has been reported (Amann *et al.* 2000; Kathiravan *et al.* 2008; Fernández-Santos *et al.* 2011). Recently, Del Olmo *et al.* (2013) showed that evaluation of kinematic ram sperm motility parameters by computer assisted sperm analyser (CASA) might be useful to identify samples with poor fertility. Specifically, O'Meara *et al.* (2008) did not find any relationship between *in vitro* sperm functional test results and *in vivo* fertility of rams following artificial insemination of ewes with frozen-thawed semen. In this context, it is remarkable that designing a method that could predict *in vivo* fertility is of great interest as assessing individual male fertility by artificial insemination (AI) is an expensive and laborious procedure. Hence, there is a clear need for alternative techniques capable of evaluating *in vitro* sperm characteristics with good correlation to *in vivo* fertility.

Knowing that an excessive accumulation of ROS could cause damage not only to the plasma membrane but also the sperm DNA (Bennetts and Aitken 2005), we decided to evaluate DNA integrity and its relationship to *in vivo* fertility. DNA damage in spermatozoa has been linked to reduced rates of fertilisation, impaired preimplantation development, miscarriage and morbidity in the offspring (Zini and Sigman 2009; Aitken and De Iulii 2010; Avendaño and Oehninger 2011). In general, significant DNA damage is rarely found in a proven fertile male, and the incidence of DNA damage is higher in infertile males. Although most researchers would intuitively agree that DNA damage is probably detrimental, the clinical impact of DNA damage has been more challenging to prove, perhaps because the correct functional end points have not been identified. Some studies suggest that sperm DNA damage levels can predict success using assisted reproductive techniques (ART) (Bungum *et al.* 2007) and they may also predict the likelihood of recurrent pregnancy loss (Carrell *et al.* 2003). Zini *et al.* (2005) suggested that sperm DNA damage may provide a useful correction biomarker for detrimental fertility impairing conditions. Since our results showed that free-radical production after post-thawing incubation of ram spermatozoa is related to decreased *in vivo* fertility, it is possible that DNA damage could be related to oxidation processes. In this regard, Thomson *et al.* (2011) have shown that 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative DNA damage, is a marker with high predictive values of

human semen fertility after artificial insemination. Although some authors have found a negative relationship of DNA damage with fertility (Evenson *et al.* 2002) we could not find this relationship in our study either analysing DNA with SCSA or with TUNEL assay and CMA3. We had no fragmentation, and thus we could say that our fertility results have not been affected by the chromatin status of samples. This effect is more commonly observed for human spermatozoa than for ruminants, maybe due to the high degree of DNA packaging of ruminants (Januskauskas *et al.* 2001). Thus, our results showed that the possible mechanism responsible for fertility differences is not due to DNA damage.

Having discovered a significant connection between ROS production and field fertility, we suggest that the addition of antioxidant to the freezing extender could have positive effects on the freezability of ram spermatozoa and therefore on the results of fertility after artificial insemination with thawed ram sperm samples. In this regard, many researchers have demonstrated the benefits of adding antioxidants to the freezing extender (Breininger *et al.* 2005; Roca *et al.* 2005; Fernández-Santos *et al.* 2007) or after thawing (Fernández-Santos *et al.* 2009; Bucak *et al.* 2010; Domínguez-Rebolledo *et al.* 2010). However, it should be determined which antioxidants are suitable for the preservation of ram sperm samples, because depending on the origin of samples and conservation, antioxidants may be beneficial or not (Foote *et al.* 2002; Fernández-Santos *et al.* 2007; Fernández-Santos *et al.* 2009; Domínguez-Rebolledo *et al.* 2010). In any case, benefits of antioxidant addition on *in vivo* fertility in sheep have thus far not been shown.

In conclusion, we have found that the information provided by the CM-H<sub>2</sub>DCFDA test combined with the SOF incubation is useful and could be considered a standard method for improving sperm-quality assessment; however, the limited number of males used in this study suggests a cautious interpretation. This combination seems to be a good procedure to identify the signal that something is not being regulated correctly in the spermatozoa and that directly affects fertility. Nevertheless, the experimental approach in the present study evaluating the semen samples after a 2-h incubation at 38.0°C (5% CO<sub>2</sub>) in the SOF medium merits further research, and it could be the basis of protocols for predicting the *in vivo* fertility of frozen-thawed ram sperm samples.

### Acknowledgements

This research was supported by Education and Science Council (JCCM, Spain) grant (PAI09-0006-3806). Enrique del Olmo is supported by a grant for training of technical personnel associated with PTA2008-0858-P project (Spanish ministry of Science and Innovation, MICINN). Manuel Ramón is supported by the DOC-INIA program.

### References

Aisen, E., Quintana, M., Medina, V., Morello, H., and Venturino, A. (2005). Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology* **50**, 239–249. doi:10.1016/J.CRYOBIOL.2005.02.002

- Aitken, R. J. (2011). The capacitation–apoptosis highway: oxysterols and mammalian sperm function. *Biol. Reprod.* **85**, 9–12. doi:10.1095/BIOLREPROD.111.092528
- Aitken, R. J., and De Iulius, G. N. (2010). On the possible origins of DNA damage in human spermatozoa. *Mol. Hum. Reprod.* **16**, 3–13. doi:10.1093/MOLEHR/GAP059
- Aitken, R. J., and Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122**, 497–506. doi:10.1530/REP.0.1220497
- Aitken, R. J., Clarkson, J. S., and Fishel, S. (1989). Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.* **41**, 183–197. doi:10.1095/BIOLREPROD41.1.183
- Aitken, R. J., Jones, K. T., and Robertson, S. A. (2012). Reactive oxygen species and sperm function – in sickness and in health. *J. Androl.* **33**, 1096–1106. doi:10.2164/JANDROL.112.016535
- Alvarez, J. G., and Storey, B. T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl.* **13**, 232–241.
- Amann, R. P., and Seidel, G. E., Jr, and Mortimer, R. G. (2000). Fertilizing potential *in vitro* of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology* **54**, 1499–1515.
- Avendaño, C., and Oehninger, S. (2011). DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era? *J. Androl.* **32**, 356–363. doi:10.2164/JANDROL.110.012005
- Bailey, J. L., Bilodeau, J. F., and Cormier, N. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J. Androl.* **21**, 1–7.
- Benchaib, M., Lormage, J., Mazoyer, C., Lejeune, H., Salle, B., and François Guerin, J. (2007). Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertil. Steril.* **87**, 93–100. doi:10.1016/J.FERTNSTERT.2006.05.057
- Bennetts, L. E., and Aitken, R. J. (2005). A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol. Reprod. Dev.* **71**, 77–87. doi:10.1002/MRD.20285
- Boe-Hansen, G. B., Christensen, P., Vibjerg, D., Nielsen, M. B. F., and Hedeboe, A. M. (2008). Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology* **69**, 728–736. doi:10.1016/J.THERIOGENOLOGY.2007.12.004
- Breininger, E., Beorlegui, N. B., O’Flaherty, C. M., and Beconi, M. T. (2005). Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology* **63**, 2126–2135. doi:10.1016/J.THERIOGENOLOGY.2004.08.016
- Bucak, M. N., Tuncer, P. B., Sariözkan, S., Başpınar, N., Taşpınar, M., Cayan, K., Bilgili, A., Akalın, P. P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroğlu, A., and Oztuna, D. (2010). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology* **61**, 248–253. doi:10.1016/J.CRYOBIOL.2010.09.001
- Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., and Giwercman, A. (2007). Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum. Reprod.* **22**, 174–179. doi:10.1093/HUMREP/DEL326
- Carrell, D. T., Liu, L., Peterson, C. M., Jones, K. P., Hatasaka, H. H., Erickson, L., and Campbell, B. (2003). Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch. Androl.* **49**, 49–55. doi:10.1080/01485010290099390
- Chatterjee, S., and Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol. Reprod. Dev.* **59**, 451–458. doi:10.1002/MRD.1052



- de Lamirande, D. E., and Gagnon, C. (1992). Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J. Androl.* **13**, 368–378.
- de Lamirande, D. E., and Gagnon, C. (1993). Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic. Biol. Med.* **14**, 157–166. doi:10.1016/0891-5849(93)90006-G
- Del Olmo, E., Bisbal, A., Maroto-Morales, A., García-Alvarez, O., Ramon, M., Jimenez-Rabadan, P., Martínez-Pastor, F., Soler, A. J., Garde, J. J., and Fernandez-Santos, M. R. (2013). Fertility of cryopreserved ovine semen is determined by sperm velocity. *Anim. Reprod. Sci.* **138**, 102–109. doi:10.1016/J.ANIREPROSCI.2013.02.007
- Domínguez-Rebolledo, A. E., Fernández-Santos, M. R., Bisbal, A., Ros-Santaella, J. L., Ramón, M., Carmona, M., Martínez-Pastor, F., and Garde, J. J. (2010). Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. *Reprod. Fertil. Dev.* **22**, 856–870. doi:10.1071/RD09197
- Evenson, D. P., and Wixon, R. (2006). Predictive value of the sperm chromatin assay in different populations. *Fertil. Steril.* **85**, 810–811. doi:10.1016/J.FERTNSTERT.2005.11.021
- Evenson, D. P., Larson, K. L., and Jost, L. K. (2002). Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **23**, 25–43.
- Fernández-Santos, M. R., Martínez-Pastor, F., García-Macias, V., Esteso, M. C., Soler, A. J., Paz, P., Anel, L., and Garde, J. J. (2007). Sperm characteristics and DNA integrity of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa frozen in the presence of enzymatic and non-enzymatic antioxidants. *J. Androl.* **28**, 294–305. doi:10.2164/JANDROL.106.000935
- Fernández-Santos, M. R., Domínguez-Rebolledo, A. E., Esteso, M. C., Garde, J. J., and Martínez-Pastor, F. (2009). Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity. *Int. J. Androl.* **32**, 353–359. doi:10.1111/J.1365-2605.2008.00871.X
- Fernández-Santos, M. R., Soler, A. J., Ramón, M., Ros-Santaella, J. L., Maroto-Morales, A., García-Álvarez, O., Bisbal, A., Garde, J. J., Coloma, M. A., and Santiago-Moreno, J. (2011). Effect of post-mortem time on post-thaw characteristics of Spanish ibex (*Capra pyrenaica*) spermatozoa. *Anim. Reprod. Sci.* **129**, 56–66.
- Fiser, P. S., Ainsworth, L., and Fairfull, R. W. (1987). Evaluation of a new diluent and different processing procedures for cryopreservation of ram semen. *Theriogenology* **28**, 599–607. doi:10.1016/0093-691X(87)90276-7
- Foote, R. H., Brockett, C. C., and Kaproth, M. T. (2002). Motility and fertility of bull sperm in whole-milk extender containing antioxidants. *Anim. Reprod. Sci.* **71**, 13–23. doi:10.1016/S0378-4320(02)00018-0
- García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Fernández-Santos, M. R., Esteso, M. C., Pérez-Guzmán, M. D., and Soler, A. J. (2009). Heterologous *in vitro* fertilisation is a good procedure to assess the fertility of thawed ram spermatozoa. *Theriogenology* **71**, 643–650. doi:10.1016/J.THERIOGENOLOGY.2008.09.036
- García-Macias, V., de Paz, P., Martínez-pastor, F., Alvarez, M., Gomes-Alves, S., Bernardo, J., Anel, E., and Anel, L. (2007). DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halamax (bright-field microscopy and fluorescence microscopy) in bull sperm. *Int. J. Androl.* **30**, 88–98. doi:10.1111/J.1365-2605.2006.00723.X
- Gardner, D. K., Lane, M., Spitzer, A., and Batt, P. A. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins and culturing embryos in groups stimulate development. *Biol. Reprod.* **50**, 390–400. doi:10.1095/BIOLREPROD50.2.390
- Gil-Guzman, E., Ollero, M., Lopez, M. C., Sharma, R. K., Alvarez, J. G., Thomas, A. J., Jr, and Agarwal, A. (2001). Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum. Reprod.* **16**, 1922–1930. doi:10.1093/HUMREP/16.9.1922
- Gillan, L., Evans, G., and Maxwell, W. M. C. (2005). Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* **63**, 445–457. doi:10.1016/J.THERIOGENOLOGY.2004.09.024
- Hinshaw, D. B., Sklar, L. A., Bohl, B., Schraufstatter, I. U., Hyslop, P. A., Rossi, M. W., Spragg, R. G., and Cochrane, C. G. (1986). Cytoskeletal and morphologic impact of cellular oxidant injury. *Am. J. Pathol.* **123**, 454–464.
- Januskauskas, A., Johannisson, A., and Rodríguez-Martínez, H. (2001). Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology* **55**, 947–961. doi:10.1016/S0093-691X(01)00456-3
- Kathiravan, P., Kalatharan, J., Edwin, M. J., and Veerapandian, C. (2008). Computer automated motion analysis of crossbred bull spermatozoa and its relationship with *in vitro* fertility in zona-free hamster oocytes. *Anim. Reprod. Sci.* **104**, 9–17.
- Lewis, S. E., and Aitken, R. J. (2005). DNA damage to spermatozoa has impacts on fertilisation and pregnancy. *Cell Tissue Res.* **322**, 33–41. doi:10.1007/S00441-005-1097-5
- Lopes, S., Jurisicova, A., Sun, J. G., and Casper, R. F. (1998). Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod.* **13**, 896–900. doi:10.1093/HUMREP/13.4.896
- Martínez-Pastor, F., Fernández-Santos, M. R., del Olmo, E., Domínguez-Rebolledo, A. E., Esteso, M. C., Montoro, V., and Garde, J. J. (2008). Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. *Reprod. Fertil. Dev.* **20**, 547–556. doi:10.1071/RD08002
- Martínez-Pastor, F., Aisen, E., Fernández-Santos, M. R., Esteso, M. C., Maroto-Morales, A., García-Álvarez, O., and Garde, J. J. (2009). Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction* **137**, 225–235. doi:10.1530/REP-08-0357
- O’Flaherty, C., Beconi, M., and Beorlegui, N. (1997). Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. *Andrologia* **29**, 269–275. doi:10.1111/J.1439-0272.1997.TB00481.X
- O’Meara, C. M., Hanrahan, J. P., Prathalingam, N. S., Owen, J. S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A. C., and Lonergan, P. (2008). Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. *Theriogenology* **69**, 513–522. doi:10.1016/J.THERIOGENOLOGY.2007.12.003
- Papadopoulos, S., Hanrahan, J. P., Donovan, A., Duffy, P., Boland, M. P., and Lonergan, P. (2005). *In vitro* fertilisation as a predictor of fertility from cervical insemination of sheep. *Theriogenology* **63**, 150–159. doi:10.1016/J.THERIOGENOLOGY.2004.04.015
- Peris, S. I., Bilodeau, J. F., Dufour, M., and Bailey, J. L. (2007). Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation and functional parameters in ram sperm. *Mol. Reprod. Dev.* **74**, 878–892. doi:10.1002/MRD.20686
- Petrunkina, A. M., Waberski, D., Günzel-Apel, A. R., and Töpfer-Petersen, E. (2007). Determinants of sperm quality and fertility in domestic species. *Reproduction* **134**, 3–17. doi:10.1530/REP-07-0046
- Roca, J., Rodríguez, M. J., Gil, M. A., Carvajal, G., Garcia, E. M., Cuello, C., Vazquez, J. M., and Martinez, E. A. (2005). Survival and *in vitro* fertility

- of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J. Androl.* **26**, 15–24.
- Rodríguez-Martínez, H. (2003). Laboratory semen assessment and prediction of fertility: still utopia? *Reprod. Domest. Anim.* **38**, 312–318. doi:10.1046/J.1439-0531.2003.00436.X
- 5 Rodríguez-Martínez, H. (2007). State of the art in farm-animal sperm evaluation. *Reprod. Fertil. Dev.* **19**, 91–101. doi:10.1071/RD06104
- Schneider, C. S., Ellington, J. E., and Wright, R. W., Jr (1999). Relationship between bull field fertility and *in vitro* embryo production using sperm preparation methods with and without somatic cell co-culture. *Theriogenology* **51**, 1085–1098. doi:10.1016/S0093-691X(99)80013-2
- 10 Silva, P. F., Gadella, B. M., Colenbrander, B., Roelen, B. A., Peris, S. I., Morrier, A., and Dufour, M. (2007). Exposure of bovine sperm to pro-oxidants impairs the developmental competence of the embryo after the first cleavage. *Theriogenology* **67**, 609–619. doi:10.1016/J.THERIOGENOLOGY.2006.09.032
- 15 Thomson, L. K., Zieschang, J. A., and Clark, A. M. (2011). Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. *Fertil. Steril.* **96**, 843–847. doi:10.1016/J.FERTNSTERT.2011.07.356
- 20 Tremellen, K. (2008). Oxidative stress and male infertility – a clinical perspective. *Hum. Reprod. Update* **14**, 243–258. doi:10.1093/HUMUPD/DMN004
- Virro, M. R., Larson-Cook, K. L., and Evenson, D. P. (2004). Sperm chromatin structure assay (SCSA) parameters are related to fertilisation, blastocyst development and ongoing pregnancy in *in vitro* fertilisation and intracytoplasmic sperm injection cycles. *Fertil. Steril.* **81**, 1289–1295. doi:10.1016/J.FERTNSTERT.2003.09.063
- 5 Waterhouse, K. E., Haugan, T., Kommisrud, E., Tverdal, A., Flatberg, G., Farstad, W., Evenson, D. P., and De Angelis, P. M. (2006). Sperm DNA damage is related to field fertility of semen from young Norwegian red bulls. *Reprod. Fertil. Dev.* **18**, 781–788. doi:10.1071/RD06029
- 10 Zini, A., and Sigman, M. (2009). Are tests of sperm DNA damage clinically useful? Pros and cons. *J. Androl.* **30**, 219–229. doi:10.2164/JANDROL.108.006908
- Zini, A., Blumenfeld, A., Libman, J., and Willis, J. (2005). Beneficial effect of microsurgical varicocelectomy on human sperm DNA integrity. *Hum. Reprod.* **20**, 1018–1021. doi:10.1093/HUMREP/DEH701
- 15 Zubkova, E. V., Wade, M., and Robaire, B. (2005). Changes in spermatozoal chromatin packaging and susceptibility to oxidative challenge during ageing. *Fertil. Steril.* **84**, 1191–1198. doi:10.1016/J.FERTNSTERT.2005.04.044