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Absence of beneficial effects on rabbit sperm cell cryopreservation by several antioxidant agents

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Summary

The generation of reactive oxygen species associated with cryopreservation could be responsible for mammalian sperm damage and the limitable value of stored semen in artificial insemination. The aim of this study was to assess several antioxidant agents supplemented in a commercial freezing extender (Gent B®) in order to improve post-thaw rabbit sperm quality. Ejaculates of 26 New Zealand White rabbit bucks were collected, evaluated and frozen using a conventional protocol. Antioxidant agents were tested at different concentrations: bovine serum albumin (BSA; 5, 30 or 60 mg/ml), retinol (RO; 50, 100 or 200 μM) and retinyl (RI; 0.282 or 2.82 μg/ml). Per cent viability, morphological abnormalities and intact acrosomes were determined using eosin–nigrosin staining. Motility and progressivity were analyzed by computer-assisted sperm analysis (CASA). In general, all sperm quality parameters were negatively affected by the cryopreservation process, the largest effect seen was for total motility. The addition of antioxidant agents did not improve thaw sperm quality. Furthermore, for RI groups a significant decrease in sperm quality parameters was recorded. In conclusion, rabbit sperm quality is negatively affected by the cryopreservation process. To our knowledge this report is the first using these antioxidants to supplement rabbit freezing extender. BSA and RO at concentrations used in the study did not improve sperm quality parameters after thawing, whereas RI supplementation appeared to be toxic. More studies are required to find the appropriate antioxidants necessary and their most effective concentrations to improve rabbit post-thaw sperm quality.

Keywords: Bovine serum albumin, Rabbit, Retinol, Retinyl, Spermatozoa

Introduction

Artificial insemination (AI) in rabbits is used extensively in Mediterranean countries, performed mainly by cooled semen due to the low fertility and prolificacy rates achieved with cryopreserved sperm cells (Viudes-de-Castro *et al.*, 1999; Roca *et al.*, 2000; Lopez-Gatius *et al.*, 2005).

During cryopreservation, mammalian sperm cells can suffer physical and chemical stress due to low temperatures, cryoprotectants, ice formation and changes in osmolarity (Watson, 2000). In fact, less than 50% of the sperm cells that survive keep their fertilizing ability (Holt, 2000; Watson, 2000; Prathalingam *et al.*, 2006; Waterhouse *et al.*, 2006). The generation of reactive oxygen species (ROS) induced by the cryopreservation process (Griveau & Le Lannou, 1997; Calamera *et al.*, 2001; Neild *et al.*, 2003) and the reduction in antioxidant enzyme activities in semen after a cycle of freezing–thawing (Bilodeau *et al.*, 2000; Chatterjee & Gagnon, 2001; Bucak *et al.*, 2008; Martí *et al.*, 2008) could be responsible for mammalian sperm damage (Griveau & Le Lannou, 1997; Calamera *et al.*, 2001; Neild *et al.*, 2003) and limitable value of stored semen in AI

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(La Falci *et al.*, 2011). The oxidative stress derived from the overproduction of ROS induces sublethal damage that results in a reduction of spermatozoa life span, loss of motility, viability, antioxidant enzyme activity, *in vivo* fertilizing ability, alteration on acrosome and plasma membrane integrity, and DNA damage (Aitken *et al.*, 1998; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002; Sanocka & Kurpisz, 2004). However, the negative effects produced on sperm cells will vary between species depending on the initial sperm plasma membrane composition (Holt, 2000) as the plasma membrane is the primary site of damage induced by cryopreservation (Hammerstedt *et al.*, 1990; Parks & Graham, 1992; Holt & North, 1994; Watson, 1995).

Differences in fatty acid composition and sterol levels have been associated with tolerance to cold shock and cryopreservation (Parks & Lynch, 1992; White, 1993). In general, mammalian sperm cells can be divided in two groups in accordance with their susceptibility to cold temperatures. Sperm cell membranes composed of a high content of poly-unsaturated fatty acids (PUFA) and low cholesterol levels (i.e. bull, boar or ram) make membranes susceptible to peroxidative damage, compared with those with low PUFA and high cholesterol levels (i.e. dog, human), making these species more resistant to cold shock (Bailey *et al.*, 2000; La Falci *et al.*, 2011). Rabbit sperm plasma membrane presents a high cholesterol:phospholipid ratio and a low ratio of PUFA:saturated fatty acid in phospholipids (Darin-Bennet & White, 1977; Castellini *et al.*, 2006), giving the membrane structure of intermediate fluidity. This composition makes rabbit sperm cells quite resistant to cold shock (Darin-Bennet & White, 1977). The high concentrations of PUFA within the lipid structure require efficient antioxidant systems to defend against peroxidative damage produced by ROS (Alvarez & Storey, 1989; Aitken & Fisher, 1994; La Falci *et al.*, 2011). However, the protective antioxidant systems in spermatozoa are primarily of cytoplasmic origin, which is mostly discarded during the terminal stages of differentiation (Bucak *et al.*, 2010), and it is also reduced by the storage protocol (Lasso *et al.*, 1994; Bilodeau *et al.*, 2000) being insufficient in preventing lipid peroxidation (LPO) during the freeze–thawing process (Aurich *et al.*, 1997; Storey, 1997). Consequently, sperm cells are unable to resynthesize their membrane components (Michael *et al.*, 2007), which leads to structural damage (Sinha *et al.*, 1996) and subsequent sperm dysfunction (Alvarez & Storey, 1989; Aitken & Fisher, 1994; Storey, 1997).

Antioxidant molecules could reduce the impact of oxidative stress, and thus improve semen quality after thawing. In fact, it has been stated that supplementation of antioxidants (i.e. glutathione, cysteine, lipid-soluble vitamins) improved sperm quality (even post-thawing) in boar (Funahashi & Sano, 2005; Szczesniak-Fabianczyk *et al.*, 2006), bull (Bilodeau *et al.*, 2001; Bucak *et al.*, 2010; Tuncer *et al.*, 2010), ram (Maxwell & Stojanov, 1996; Bucak *et al.*, 2008; Anghel *et al.*, 2009), stallion (Denniston *et al.*, 2000; Baumber *et al.*, 2005), dog (Neagu *et al.*, 2010) and rabbit (Castellini *et al.*, 2000; Yousef *et al.*, 2003) sperm cells. However, antioxidants were not beneficial in other studies (Denniston *et al.*, 2000; Marco-Jiménez *et al.*, 2006). Antioxidants play an important role in scavenging ROS (Baumber *et al.*, 2000) and there are many varieties of antioxidant agents that could be used in this matter, without eliminating completely ROS, because oxidative mechanisms play an important role in the physiological control of mammalian sperm functions as well (sperm capacitation or sperm–egg fusion; Aitken & Fisher, 1994; Griveau & Le Lannou, 1997; Saleh & Agarwal, 2002; Aitken *et al.*, 2004; Ford, 2004; Bennetts & Aitken, 2005; Agarwal *et al.*, 2006).

Although rabbit cryopreserved sperm is not used for commercial purposes at present, rabbit is a valuable laboratory animal, and there is a need for reliable methods of rabbit sperm resource banking (Foote & Carney, 2000). Furthermore, sperm cryopreservation can be an alternative to the transport of animals between countries (Liu *et al.*, 2007) or to the preservation of endangered rabbit breeds (Bolet *et al.*, 2000). However, the methods of freezing semen require further improvements.

The aim of this study was to test bovine serum albumin (BSA), retinol and retinyl as antioxidant agents supplemented at different concentrations in a commercial freezing extender in order to improve rabbit post-thawing sperm quality.

Materials and methods

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Experimental design

The extender used in the study was the commercial Gent B® (Control group; Minitüb, Tiefenbach, Germany) that contains egg yolk and glycerol. The effect of antioxidant agent addition to the commercial extender sperm quality after freezing–thawing was assessed in three different experiments:

- Gent B with BSA supplemented at three different concentrations: 5 mg/ml (BSA 1), 30 mg/ml (BSA 2) and 60 mg/ml (BSA 3).

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- Gent B with retinol (RO) at three different concentrations: 50 μM (RO 1), 100 μM (RO 2) and 200 μM (RO 3).
- Gent B with retinyl (RI) at two different concentrations: 0.282 μg/ml (RI 1) and 2.82 μg/ml (RI 2).

Animals and samples collection

The animals used in this study came from the Caldes line (Gómez *et al.*, 2002), which is selected for growth rate during the fattening period. Twenty-six New Zealand White rabbit bucks (180 days old and from different litters) individually housed in commercial wire cages were submitted to 17°C as mean temperature, 60% of relative humidity and cycles of 16 h day–light. An ejaculate per male was collected manually using the gloved-hand method and analyzed to guarantee the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was diluted (1:2; v/v) in the commercial extender Galap liquid diluent (IMV Technologies). Semen samples with the presence of calcium carbonate deposits and urine were discarded. The ejaculates underwent standard analysis for volume and sperm concentration. Part of the diluted semen was sent at room temperature to the laboratory within 30 min. Once in the laboratory, the samples were kept in an incubator at 37°C until sperm quality analysis were done.

Sperm cell cryopreservation

Semen was centrifuged at 2000 rpm for 10 min at 25°C in a programmable refrigerated centrifuge (Hermle Z300K). Afterwards supernatants were discarded and each pellet was re-extended with the different freezing extenders achieving a final concentration of 100×10^6 spermatozoa/ml. Diluted sperm samples were then packaged into 0.25 ml straws and immediately cooled to 4°C for 2 h. Subsequently, straws were exposed 10 min to liquid nitrogen (N_2) vapours and then plunged into liquid N_2 (–196°C) for storage. Frozen samples were stored in liquid N_2 for at least 2 months. The thawing process was performed then by an immediate immersion of the straws in a water bath at 37°C for 30 s. Straws were carefully wiped and opened, and samples were evaluated to determine post-thaw sperm quality parameters on a warmed platina.

Sperm cell quality parameters

Percentages of viability, morphological abnormalities and intact acrosomes were determined using eosin–nigrosin staining. This technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a pinkish stain was indicative of non-viable sperm cells (Bamba, 1988). All determinations were performed after analyzing a minimum of 200 spermatozoa/samples through an optical microscope (magnification: ×1000). Motility and progressivity were analyzed by computer-assisted sperm analysis (CASA; Integrated Sperm Analysis System, V.1.2., Proiser S.L., Valencia, Spain). Five-microlitre aliquots of pre-warmed samples were placed on a warmed (37°C) slide and covered with a 22 mm^2 coverslip. The analysis was based upon the study of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of ×100 on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 ms. Four to five separate fields were taken for each sample. The motility descriptors obtained after CASA analyses were:

- Curvilinear velocity (VCL): the mean path velocity of the sperm head along its actual trajectory (units: μm/s).
- Linear velocity (VSL): the mean path velocity of the sperm head along a straight line from its first to its last position (units: μm/s).
- Mean velocity (VAP): the mean velocity of the sperm head along its average trajectory (units: μm/s).
- Linearity coefficient (LIN): (VSL/VCL) ×100 (units: %).
- Straightness coefficient (STR): (VSL/VAP) ×100 (units: %).
- Wobble coefficient (WOB): (VAP/VCL) ×100 (units: %).
- Mean amplitude of lateral head displacement (ALH): the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: μm).
- Frequency of head displacement (BCF): the frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).
- Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s, and progressivity as the percentage of spermatozoa which showed a VAP above 50 μm/s and 70% of STR.

Statistical analysis

Data were processed using the Statistical Analysis Systems Package (SAS. SAS/STAC Software. Cary, NC, USA: SAS Inst. Inc. 2000). Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. A PROC GLM procedure was applied to test for significant differences in sperm parameters ($P < 0.05$) among the samples subjected to different groups of

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Table 1 Means ± standard error of the mean (SEM) of the sperm quality analysis from fresh and thawed ejaculates

	Viability (%)	Total motility (%)	Progressivity (%)	Altered acrosomes (%)	Morphological abnormalities (%)
Fresh	77.0 ± 2.2^{a}	74.1 ± 3.9^{a}	30.1 ± 2.8^{a}	27.4 ± 3.1^{a}	24.2 ± 2.4^{a}
Gent B	$36.5 \pm 2.6^{b,d}$	4.9 ± 0.6^{b}	2.7 ± 0.4^{b}	67.7 ± 2.3^{b}	18.9 ± 1.7^{a}
BSA 1	$39.6 \pm 4.3^{b,d}$	6.3 ± 0.8^{b}	4.0 ± 0.6^{b}	68.7 ± 3.1^{b}	19.8 ± 2.0^{a}
BSA 2	$42.3 \pm 5.1^{b,d}$	$4.5 \pm 0.9^{b,c}$	$3.0 \pm 0.7^{b,c}$	64.8 ± 3.3^{b}	20.5 ± 2.5^{a}
BSA 3	45.4 ± 4.6^{b}	6.5 ± 1.3^{b}	4.8 ± 1.2^{b}	68.4 ± 4.1^{b}	21.6 ± 4.6^{a}
RO 1	$34.7 \pm 4.0^{b,d}$	$4.1 \pm 1.0^{b,c}$	$1.1 \pm 0.4^{b,c}$	62.4 ± 2.8^{b}	15.2 ± 2.7^{a}
RO 2	$34.6 \pm 5.2^{b,d}$	$2.7 \pm 1.0^{b,c}$	$1.1 \pm 0.4^{b,c}$	66.1 ± 4.6^{b}	14.6 ± 3.3^{a}
RO 3	$36.6 \pm 3.7^{b,d}$	$1.6 \pm 0.5^{b,c}$	$0.8 \pm 0.3^{b,c}$	66.3 ± 3.9^{b}	15.0 ± 1.7^{a}
RI 1	27.0 ± 2.3^{d}	$2.3 \pm 0.5^{b,c}$	$1.6 \pm 0.4^{b,c}$	72.8 ± 3.9^{b}	20.7 ± 1.9^{a}
RI 2	9.5 ± 2.0^{c}	0.2 ± 0.1^{c}	0.1 ± 0.1^{c}	92.5 ± 2.6^{c}	20.8 ± 2.9^{a}

[a–d]Different superscripts in a column indicate significant differences.

Table 2 Means ± standard error of the mean (SEM) of the specific motility parameters from fresh and thawed ejaculates

	VCL (μm/s)	VSL (μm/s)	VAP (%)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Fresh	109.6 ± 0.5^{a}	44.3 ± 0.3^{a}	76.7 ± 0.4^{a}	43.9 ± 0.3^{a}	59.7 ± 0.3^{a}	70.9 ± 0.2^{a}	$3.70 \pm 0.02^{a,b}$	9.4 ± 0.1^{a}
Gent B	84.7 ± 1.7^{b}	$29.7 \pm 0.8^{b,c}$	$44.7 \pm 0.9^{b,c}$	37.8 ± 0.9^{b}	$68.5 \pm 1.1^{b,c}$	53.7 ± 0.6^{b}	$3.71 \pm 0.08^{a,b}$	$9.7 \pm 0.2^{a,b,c}$
BSA 1	$92.8 \pm 2.9^{b,c}$	34.1 ± 1.4^{b}	49.8 ± 1.6^{b}	$38.9 \pm 1.4^{a,b}$	$69.4 \pm 1.8^{b,c}$	54.5 ± 0.9^{b}	$4.04 \pm 0.14^{a,b,c}$	$9.6 \pm 0.4^{a,b,c}$
BSA 2	$90.5 \pm 3.5^{b,c}$	$33.4 \pm 1.7^{b,c}$	$47.7 \pm 1.8^{b,c}$	$40.1 \pm 1.8^{a,b,c}$	$71.7 \pm 2.4^{b,c}$	53.7 ± 1.2^{b}	$3.89 \pm 0.18^{a,b,c}$	$10.5 \pm 0.4^{a,b,c}$
BSA 3	$86.5 \pm 3.1^{b,c}$	$32.9 \pm 1.4^{b,c}$	$44.5 \pm 1.7^{b,c}$	$40.6 \pm 1.4^{a,b}$	76.0 ± 1.7^{c}	52.4 ± 1.1^{b}	$3.68 \pm 0.15^{a,b}$	10.5 ± 0.4^{b}
RO 1	$89.7 \pm 4.5^{b,c}$	24.0 ± 1.6^{c}	$46.2 \pm 2.4^{b,c}$	$30.1 \pm 1.5^{c,d}$	56.0 ± 2.7^{a}	52.7 ± 1.1^{b}	$4.16 \pm 0.20^{b,c}$	7.8 ± 0.4^{c}
RO 2	$96.6 \pm 5.9^{a,c}$	$28.7 \pm 2.4^{b,c}$	$47.8 \pm 3.1^{b,c}$	$33.3 \pm 2.2^{b,c,d}$	$62.8 \pm 2.9^{a,b}$	50.8 ± 1.6^{b}	4.43 ± 2.73^{c}	$8.9 \pm 0.6^{a,c}$
RO 3	$103.6 \pm 6.0^{a,b,c}$	$35.2 \pm 3.3^{a,b,c}$	56.9 ± 3.3^{b}	$36.3 \pm 3.1^{a,b,c}$	$63.1 \pm 4.5^{a,b,c}$	56.1 ± 1.9^{b}	4.73 ± 0.32^{c}	$9.5 \pm 0.7^{a,b,c}$
RI 1	69.4 ± 3.5^{e}	$28.3 \pm 1.7^{b,c}$	$36.4 \pm 1.9^{c,d}$	$40.6 \pm 1.7^{a,b}$	$75.2 \pm 1.9^{b,c}$	51.9 ± 1.5^{b}	2.97 ± 0.14^{e}	$9.8 \pm 0.5^{a,b}$
RI 2	28.1 ± 8.4^{d}	$13.1 \pm 4.1^{b,c}$	17.4 ± 5.4^{d}	22.6 ± 7.2^{d}	34.8 ± 10.1^{d}	29.7 ± 9.1^{c}	1.11 ± 0.35^{d}	4.7 ± 1.6^{d}

[a–e]Different superscripts in a column indicate significant differences.

study. The LSMEANS (least squares means) procedure was used to identify significant differences. The total number of spermatozoa analyzed following this protocol was 9521 (4195, 2031 and 3295 from BSA, RO and RI groups, respectively).

Results

Effect of cryopreservation on post-thawing sperm cell quality

In accordance with the sperm quality parameters, significant differences were found between fresh ejaculates and post-thawed sperm cells (Table 1). In general, all the quality parameters were affected negatively by the cryopreservation process. The total motility was the most affected parameter by the freezing–thawing protocol (74.07 vs. 4.86%, for fresh and sperm cells frozen with Gent B respectively). Alterations in total motility and progressivity were also associated with significant changes in specific motility parameters (Table 2). Thus, specific motility parameters were decreased in general after the cryopreservation process except STR and ALH. Only normalcy, those sperm cells without any morphological abnormality (i.e. head abnormality, coiled-tail, tailless), was not affected by the process.

Effect of BSA on thawed quality parameters

In general, the addition of BSA to the freezing extender was not more beneficial than the use of Gent B alone. However, in terms of viability, the percentage increased as the BSA concentration increases, although without significant differences (Figure 1). In terms of altered acrosomes and normalcy, no differences were found between groups of study. Hence, similar sperm quality was obtained by using or not BSA as supplementation.

Taking into account total motility and progressivity, the best results were found when BSA at 5 and 30 mg/ml concentrations were present in the freezing extender. Nevertheless, significant differences were not detected once more. Hence, similar results in sperm motility are expected by both using BSA or not. In accordance, changes in specific motility parameters after cryopreservation varied according to

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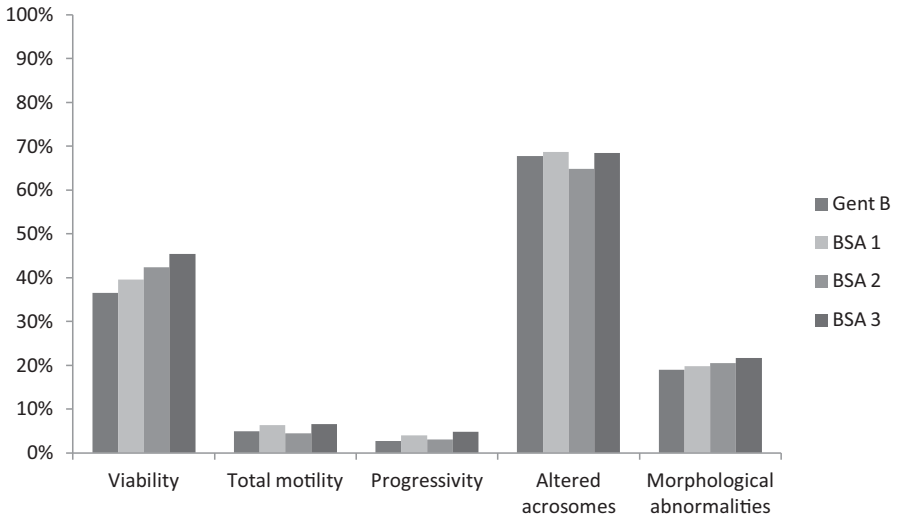

Figure 1 Semen quality parameters with the addition of bovine serum albumin (BSA) to the freezing extender compared with no supplemented samples.

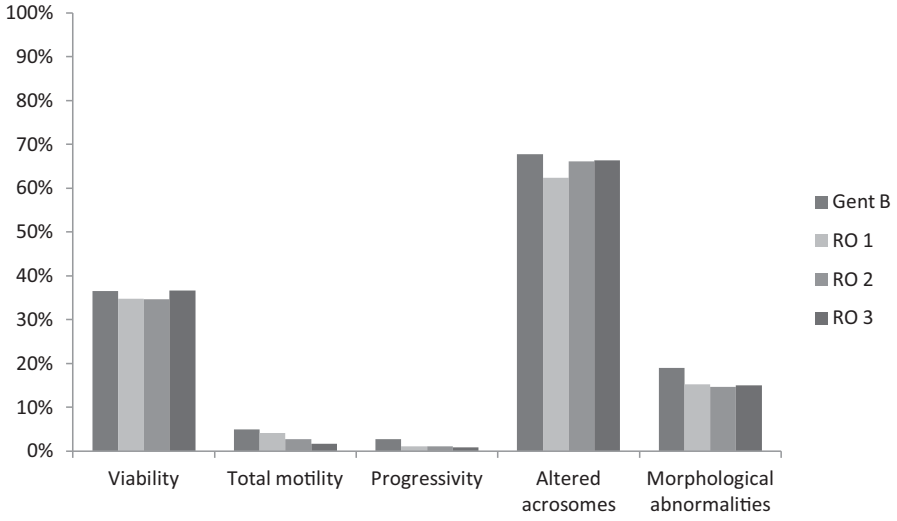

Figure 2 Semen quality parameters with the addition of retinol (RO) to the freezing extender compared with no supplemented samples.

BSA supplementation (Table 2). In general, all BSA concentrations showed higher parameters of sperm velocity (as represented by VCL, VSL, VAP), LIN and STR and also BCF. However, no significant differences were detected.

Effect of RO on thawed quality parameters

RO supplementation did not show a beneficial effect in comparison with the use of Gent B without antioxidant addition. Significant differences were not found in terms of viability, acrosome integrity or normalcy (Figure 2).

However, based on sperm movements, the supplementation of RO decreased sperm total motility and progressivity as its concentration increased (Figure 2), although the findings were not significant. Therefore, improvements are not expected by using or not RO in the extender. According to these results the changes in specific motility parameters after cryopreservation varied also according to supplementation (Table 2). RO concentrations showed higher values of sperm velocity, although the best results were detected using RO at the 200 μM concentration (higher values of VCL, VSL, VAP, WOB and ALH).

Effect of RI on thawed quality parameters

The supplementation of RI to the freezing extender resulted in no significant differences in sperm parameters when RI at the 0.282 μg/ml concentration was used. However, the use of RI at 2.82 μg/ml

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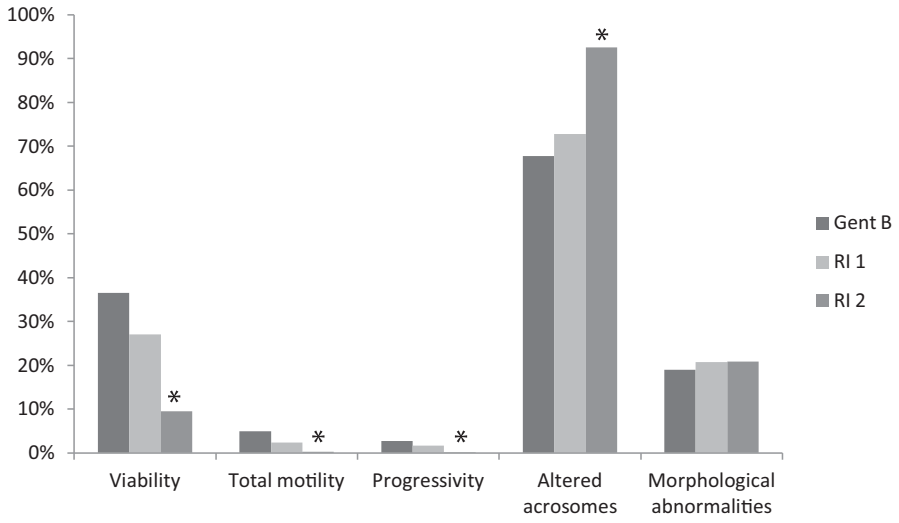

Figure 3 Semen quality parameters with the addition of retinyl (RI) to the freezing extender compared with no supplemented samples.
Note: Significant differences ($P < 0.05$) between RI 2 and commercial extender (*).

concentration significantly decreased sperm quality comparing it with the use of the commercial extender without additives.

In general, supplementation of RI had a negative effect on all sperm quality parameters except normalcy (Figure 3), although only RI 2 showed significant differences. Low values were found in terms of viability and acrosome integrity (9.50 vs. 36.49%; 7.48 vs. 32.3%, respectively) between RI 2 and Gent B groups.

In terms of total motility and progressivity, the lowest values obtained were also detected in those samples frozen in the presence of RI at 2.82 μg/ml concentration (Table 1). In accordance, changes in specific motility parameters after cryopreservation were found (Table 2) and, in general, low values were found using both concentrations in comparison with the control samples, the worst values were always found in the RI 2 group.

Discussion

Our results clearly demonstrated the harmful effects of the cryopreservation process on rabbit sperm quality, especially on sperm motility and progressivity. However, the use of the antioxidant agents in an attempt to improve thaw sperm quality was not sufficient to achieve significant results, at least at the concentrations used in this study.

The ROS derived from the freezing process affects sperm membrane integrity leading to a decrease of sperm quality and consequently their fertilizing ability. The high content of unsaturated fatty acids on sperm membranes (White, 1993; Buhr *et al.*, 1994) makes the spermatozoa particularly sensitive to oxidative stress by ROS. As a result, lipid peroxidation increases resulting in loss of viability (Aitken *et al.*, 1998; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002), reduced sperm motility (de Lamirande & Gagnon, 1992; Baumber *et al.*, 2000; Guthrie & Welch, 2006), loss of acrosomal and plasma membrane integrity (Aitken *et al.*, 1998; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002) and leads to DNA fragmentation (Aitken *et al.*, 1998; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002; Bennetts & Aitken, 2005). Rabbit sperm membranes have lower levels of PUFA (Darin-Bennet & White, 1977; Castellini *et al.*, 2006) making these cells less sensitive to oxidative damage, although reduction of sperm quality is still significant as shown in the present study, with a dramatic decrease in total sperm motility and progressivity. Our results are in accordance with those of Moce & Vicente (2009) who reviewed rabbit cryopreservation studies remarking that the freezing–thawing cycle causes decreases in the percentages of motile and live sperm cells and sperm with intact acrosomes.

Although cryopreserved sperm is not used for commercial purposes at present, there is a need for reliable methods of rabbit sperm resource banking. This approach could facilitate animal genetic diffusion between countries (Liu *et al.*, 2007), and it can be also useful for the preservation of endangered rabbit breeds (Bolet *et al.*, 2000). The values for sperm quality after cryopreservation observed in our study are significantly lower than those found in other species. The reason for this difference is that there are many extenders and protocols developed for rabbit sperm

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cryopreservation as reviewed by Moce & Vicente (2009). There is no consensus about the best extender and protocol for rabbit sperm cell cryopreservation. Finally, each research group uses their own protocol and extender, in our case a commercial one that may not have an appropriate composition for rabbit sperm cryopreservation. This situation may explain why, if rabbit sperm is more resistant to cold shock than other species (Darin-Bennet & White, 1977; Castellini *et al.*, 2006), the sperm quality parameters in our study are so critically affected.

In an attempt to minimise the negative effects of all these stressors, research efforts have been focused on the optimization of cryopreservation methods, trying to find the best freezing extender composition. As spermatozoa and seminal plasma have a limited antioxidant capacity (Aurich *et al.*, 1997; Storey, 1997), the effort to decrease ROS overproduction due to freezing process by the use of antioxidant agents as quenchers of ROS has been studied in a wide range of species [(i) human: Sinclair, 2000; (ii) ram: Maxwell & Stojanov, 1996; Upreti *et al.*, 1998; Baumber *et al.*, 2005; Uysal & Bucak, 2007; Anghel *et al.*, 2009; Maia *et al.*, 2010; (iii) bull: Beconi *et al.*, 1991, 1993; Chen *et al.*, 1993; Foote *et al.*, 1993; Bilodeau *et al.*, 2001; Bucack *et al.*, 2010; Tuncer *et al.*, 2010; (iv) boar: Funahashi & Sano, 2005; Szczesniak-Fabianczyk *et al.*, 2006; (v) dog: Michael *et al.*, 2007; Eulenberger *et al.*, 2009; Neagu *et al.*, 2010; (vi) buffalo: El-Kon, 2011; and (vii) stallion: Aurich *et al.*, 1997; Baumber *et al.*, 2000]. The aim of using antioxidant treatments should not be complete ROS elimination as oxidative mechanisms play an important role in the physiological control of mammalian sperm functions (Aitken & Fisher, 1994; Griveau & Le Lannou, 1997; Saleh & Agarwal, 2002; Aitken *et al.*, 2004; Ford, 2004; Bennetts & Aitken, 2005; Agarwal *et al.*, 2006). There are many varieties of antioxidants that could be used in this matter. Unfortunately controversial results have been described. It is therefore necessary to adjust the extender composition, cryoprotectants and cryoprotectant concentrations, as well as the cryopreservation protocol for each single species or even breeds. In rabbits, antioxidants have been especially used to improve semen quality by adding these to the diet (especially vitamins E and C; Castellini et al, 2000; Yousef *et al.*, 2003). Hence, to our knowledge, there is no literature on the addition of antioxidant agents on freezing extenders trying to improve rabbit post-thaw quality.

Bovine serum albumin has been used in some studies as an antioxidant agent to improve sperm quality. It is known that its function is to eliminate free radicals generated by oxidative stress (Lewis *et al.*, 1997), and the protection of membrane integrity of sperm cells from heat shock during freezing–thawing of canine semen (Uysal *et al.*, 2005). It has also been used to enhance motility and viability of ram sperm cells following the cryopreservation process (Matsuoka *et al.*, 2006), 20 mg/ml being the more beneficial concentration detected (Uysal & Bucak, 2007). This reported concentration is similar to those used in our investigation. However, our results did not show significant findings, and we found only quantitative records in terms of viability. As reported by Uysal & Bucak (2007), lower concentrations did not protect sperm cells from lipid peroxidation, although in our study a higher concentration (60 mg/ml) was insufficient to improve sperm quality. Higher concentrations may be needed for rabbit sperm as progressive improvements were found in viability as BSA concentration increases. Further investigations on this topic are needed to elucidate the best BSA concentration for rabbit sperm supplementation in freezing extender.

To our knowledge this study is the first to use retinol and retinyl as supplementation for freezing extenders in order to improve thawed sperm quality. Retinol and retinyl have been used to improve the quality of oocytes and embryos with significantly good results (Lima *et al.*, 2004; Livingston *et al.*, 2004; Hajializadeh *et al.*, 2008; Vahedi *et al.*, 2009; Rajesh *et al.*, 2010). Some precursors of retinol and retinyl, such as β-carotene and lycopene, are known to be important components of antioxidant defence against lipid peroxidation in living cells (Agarwal *et al.*, 2005) and protecting plasma membrane against lipid peroxidation (Di Mascio *et al.*, 1989). However, it should be pointed out that the concentrations used in our study were not sufficient to alleviate the harmful effects of cryopreservation, and even retinyl may be toxic for sperm cells, as a drastic decrease in sperm quality has been recorded as concentration increases. So, further investigations in the use of retinol are needed to find the best concentration, mainly using lower concentrations as the sperm quality decreases as retinol presence in the extender increases. Although, the use of retinyl should be avoided as our results demonstrate.

In conclusion, rabbit sperm quality is negatively affected by the cryopreservation process and could be improved by the use of antioxidant agents supplemented in the freezing extender. To our knowledge this report is the first using these antioxidants as supplementation in rabbit freezing extender. BSA and retinol did not improve sperm quality parameters after thawing at concentrations used in our study. However, retinyl supplementation seems to be toxic for rabbit sperm (and is not recommended). More studies are needed to find the appropriate antioxidants and to define the most effective concentrations that will improve post-thaw quality.

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