Title: Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity

Running title: Antioxidant supplementation in bull sperm

Key words: bull, sperm chromatin, catalase, SCSA, oxidative stress

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Abstract

The potential protective effect of catalase supplementation during in vitro culture of frozen/thawed bull spermatozoa was investigated. Frozen/thawed semen collected from three fighting bulls was diluted in PBS and incubated at 37 °C under different experimental conditions: Control, Catalase (CAT) (200 U/mL), Oxidant (OXI) (100 μM Fe^{2+}/1 mM ascorbate), and Catalase+Oxidant (CAT/OXI). We assessed sperm motility, acrosomal integrity, viability and chromatin status (SCSA®) at 0 h, 2 h and 6 h of incubation. Our results showed that catalase abolished the effect of the oxidant, protecting spermatozoa against ROS, and improving both sperm motility and chromatin status during incubation. The OXI treatment significantly reduced the percentage of motile sperm after six hours of incubation. The statistical model also showed that there were differences in sperm motility between CAT/OXI (20.8 ± 2.9%) and OXI (11.6 ± 7.6%) ($P < 0.001$). There were no significant effects of OXI on sperm viability, acrosomal status or proportion of abnormal tails. %DFI (spermatozoa with moderate or high DNA Fragmentation Index) was significantly higher on OXI ($P < 0.001$). Catalase prevented DNA fragmentation even in the presence of the oxidant (%DFI: 30.3 ± 0.8% OXI vs. 17.4 ± 0.7% CAT/OXI). We conclude that catalase supplementation after thawing could protect bull spermatozoa against oxidative stress, and it could improve media used for processing thawed spermatozoa.

Introduction

The processes of sperm cooling, freezing and thawing generate physical and chemical stresses on the spermatozoa, thereby reducing their viability and fertility. One of the deleterious effects of cryopreservation is the generation of reactive oxygen species (ROS), as demonstrated in many species (Hinshaw et al., 1986; Aitken et al., 1989; Lopes et al., 1998; Chatterjee & Gagnon, 2001). Freezing and thawing increase the generation of ROS (Chatterjee & Gagnon, 2001), resulting in DNA damage (Lopes et al., 1998), cytoskeletal alterations [3], inhibition of sperm-oocyte fusion (Hinshaw et al., 1986), and effects on the sperm axoneme that lead to loss of motility (De Lamirade & Gagnon, 1989). One manner to overcome the effects of ROS after thawing consists in the supplementation of the extender with antioxidants before freezing (Pena et al., 2003, 2004; Roca et al., 2004, 2005; Fernández-Santos et al., 2007), or just after thawing (Berlinguer et al., 2003; Gadea et al., 2005a,b), thus preventing oxidative stress. Catalase catalizes the decomposition of H_2O_2 into water and oxygen, thus removing an initiator of chain reactions leading to lipid peroxidation and to the formation of other reactive radicals (Aitken, 1995). Supplementation of the extender with catalase has resulted in the improvement of sperm parameters in several species (Roca et al., 2005; Fernández-Santos et al., 2007; Michael et al., 2007). However, there are not previous studies considering the protection of catalase in bull thawed semen. Moreover, the addition of antioxidants to the thawing extender seems to improve the sperm ability to penetrate oocytes in vitro (Gadea et al., 2005b). Therefore, antioxidant supplementation after thawing could be of great interest in techniques such as IVF or ICSI, since an excess of ROS in the culture media may impact post-fertilization development (cleavage rate, yield and quality of blastocysts) and ultimately affecting embryo quality and viability (Agarwal et al., 2005).

In this study we have used semen from fighting bulls. This cattle has a great value in Spain, thus the interest in preserving the germplasm of this breed. However, since males are routinely sacrificed in bullfighting, the management to conserve their genetics is complicated. Electroejaculation of selected animals and semen cryopreservation could help to overcome these problems. The nature of these samples makes necessary to obtain the maximum output from them, and using artificial reproductive techniques, such as IVF. As far as we know, currently only three figthing bulls have semen cryopreserved, so it is necessary to obtain the most output from them. Thus, our objective was to test if the supplementation of incubation media with catalase would protect thawed bull spermatozoa against oxidative stress, considering the possible use of these samples for in vitro techniques.

Materials and methods

Experimental design

All chemicals were at least of Reagent grade, and acquired from Sigma (Madrid, Spain). We collected and cryopreserved samples from three bulls. After thawing, sperm was diluted in PBS and split between a Control (CTL), an antioxidant treatment (200 U/mL of catalase; CAT), oxidant treatment (100 µM Fe^{2+}/1 mM ascorbate; OXI) and a mixed treatment (catalase/oxidant; CAT/OXI). Fe^{2+} oxidizes to Fe^{3+}, which is recycled by the ascorbate, producing the highly reactive hydroxyl radical (•HO). Samples were incubated at 37 °C, in order to induce oxidative stress, and were evaluated at 0, 2 and 6 hours. We assessed sperm motility, acrosomal integrity, abnormal tails, sperm viability and sperm chromatin integrity.

Animals, electroejaculation and semen cryopreservation

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609. The bulls were kept isolated in individual enclosures. They were healthy and reproductively mature at the time of the collection of the study. Previously to electroejaculation, bulls were anesthetized using Zylazine (0.1 mg/kg Rompun 2%, Bayer, Germany) and Mepivacaine (0.16 mg/kg Mepivacaine-HCl 2%, Braun, Germany) (Alvarez et al., 2006).

Semen was collected by electroejaculation using a sine-wave stimulator (P. T. Electronics, Boring, OR, USA). The stimulator was capable of monitoring voltage and amperage, producing a maximum of 55 V and 1.5 A. The stimulating voltage was delivered using rectal probes with three longitudinal surface-mounted electrodes. Probe diameter, probe length, and electrode length were 4.5, 37.5 and 8.5 cm, respectively. For the electroejaculation procedure, we lubricated the probe and gently inserted it into the rectum, with the electrodes positioned ventrally. The penis was prolapsed beyond the prepuce before starting. The electroejaculation regime was based on that employed previously for ungulates (Howard et al., 1981; Roth et al., 1998) with various modifications. It consisted of consecutive series of 5-s pulses of similar voltage, each separated by a 5-s break. Each series consisted of a total of 4 pulses (Garde et al., 2003). Semen was collected using a 30-mL sterile plastic container, which was kept warm by covering it with the hand. After the collection, it was placed at 30 ºC in a water bath until processed.

For freezing, we supplemented Biladyl (Fraction A and B; IMV, L'Aigle, France) with 20% egg yolk. Semen was diluted in glass tubes to 100 x 10^6 cells/mL with Biladyl Fraction A at 30 ºC. The tubes were put into beakers with 100 mL of water at the same

temperature, which were placed into a walk-in fridge at 5 ºC (slow cooling). When water temperature reached 5 ºC, the same volume of Biladyl Fraction B (12% glycerol) was added to the tubes (final concentration of glycerol: 6%). The samples were left to equilibrate for 4 h, and then packed in 0.5-mL straws (50×10^6 cells/straw). Freezing was carried out in liquid nitrogen vapors (4.5 cm above liquid nitrogen level), and straws were stored in liquid nitrogen for a year.

Thawing and sample processing

Thawing was carried out by dropping the straws into a water bath at 37 ºC. Two straws of each male were thawed, making, two replicates of each male. After 30 s, straws were thoroughly wiped and its content were poured in a tube and kept at 37 ºC. Semen was diluted in PBS (NaCl 8 g/L, KCl 0.2 g/L, $MgCl_2\times6H_2O$ 0.1 g/L, $CaCl_2$ 0.1 g/L, HNa_2PO_4 1.0 g/L, NaH_2PO_4 0.15 g/L, KH_2PO_4 0.2 g/L, BSA 5 g/L) to 10 x 10^6 cells/mL, and split in 1-mL aliquots for each treatment. We added catalase to the CAT and CAT/OXI aliquots, to a final concentration of 200 U/mL (stock solutions of catalase 2×10^5 U/mL in PBS (Roca et al., 2005)). We added 10 µL of a solution of 10 mM $FeSO_4$ and 100 mM sodium ascorbate in water to the OXI and CAT/OXI tubes, for a final concentration of 100 µM Fe^{2+} and 1 mM ascorbate. Samples were then incubated in a water bath at 37 ºC.

Spermatozoa evaluation

Motility

We calculated a sperm motility index (SMI) as indicated by Comizzoli et al. (2001). A 5 mL drop was put on a prewarmed Makler counting chamber and immediately assessed. The sample (at least five fields) was examined with a phase contrast microscope (Nikon Eclipse 80i; negative contrast optics), with a warming stage at 37 ºC. We assessed the percentage of motile sperm and the quality of movement (0–5

scale) was subjectively assessed by using phase contrast microscopy. Then, we calculated the sperm motility index using the formula SMI = (% individual motility + quality of motility × 20) × 0.5.

Acrosomal Integrity and tail abnormalities

Sperm samples were fixed in 2% glutaraldehyde (0.165 M cacodylate/HCl buffer at pH 7.3). We evaluated the fixed cells by phase contrast microscopy (×400; 200 cells/sample), noting the percentage of spermatozoa with normal acrosomal ridges (NAR), considered as having an intact acrosome, and the percentage of sperm with coiled or bent tails (TA).

Sperm viability

Assessment of sperm viability was evaluated by using the nigrosine-eosine sperm viability staining (Tamuli, 1994). We mixed 5 µl of sample with 10 µl of nigrosine/eosine stain on a pre-warmed slide. After 30 s at 37 ºC on a warming plate, the drop was smeared and left to dry on the plate. The samples were evaluated using bright field microscopy at ×400. In this assay, live spermatozoa remain unstained, whereas dead cells are pink stained. We recorded the percentage of live spermatozoa for each sample (viability).

Assessment of sperm chromatin stability

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique. This technique is based in the susceptibility of the sperm DNA to acid-induced denaturation in situ and in the metachromatic staining Acridine Orange (AO). Acridine orange shifts from green (dsDNA) to red (ssDNA) fluorescence depending on the degree of DNA denaturation (Evenson & Jost, 200). Samples were diluted with TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) in

cryotubes, at a final sperm concentration of approximately 2×10^6 cells/mL. The samples were flash frozen by dropping the cryotubes in LN_2 and stored at -80 ºC. For the analysis, samples were thawed and kept on crushed ice. Acid-induced denaturation of DNA in situ was attained by mixing 0.2 mL of sample with 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl; pH 1.2) in a cytometry tube. After 30 s, cells were stained by adding 1.2 mL of a solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1mM EDTA, 0.15 M NaCl; pH 6.0) containing 6 μg/mL AO. After 3 min, we ran the sample through a LSR-I flow cytometer (BD Biosciences, San José, CA, USA). We excited the acridine orange using an argon-ion laser providing 488 nm light. The red fluorescence was detected using the FL-3 photodetector (670LP filter) and the green one using the FL-2 photodetector (530/28BP filter). Fluorescence was amplified using linear scales. Flow velocity was adjusted to 200 cels/s before starting. Sample acquisition was carried out with the CellQuest v. 3 software.

Flow cytometry files were converted to tabbed text using the WinMDI software (Scripps Research Institute, La Jolla, USA). Data was processed to obtain the DFI (DNA fragmentation index; formerly called αt) for each spermatozoa, that express the shift from green to red fluorescence, and is expressed as the ratio of red fluorescence to total intensity of the fluorescence (red/[red+green]×100). High values of DFI indicate chromatin abnormalities. We calculated the standard deviation of DFI (SDDFI), the percentage of spermatozoa with DFI>25 (%DFI), and the percentage of spermatozoa with green fluorescence higher than cytometer channel 600 (of 1024 channels), termed HDS (High DNA Stainability). DNA parameters are explained in Figure 1.

Statistical analysis

Statistical analyses were carried out using the R software (http://www.r-project.org). Data was fitted to linear mixed-effect models by maximizing the restricted log-

likelihood (RML) (Pinheiro & Bates, 2000). Male was always included as random effect, and time (covariate) and treatment (factor with four levels: Control, Antioxidant, Oxidant and Antioxidant+Oxidant) were included as fixed effects. The tested model was:

$$Y_{ijk}=\mu + Time_i + Time_i \times Treatment_j + Male_k + \varepsilon_{ijk}$$

For comparing treatments at a given time or between 0 h and sampling times, we used the contrasts provided by the analysis (adjusting P values by Holm's correction for multiple comparisons).

Results

The model used for studying the effect of the different treatments on thawed bull sperm indicated that incubation time decreased SMI ($P < 0.001$) and increased TA ($P = 0.048$). The rest of the parameters analyzed by microcopy did not seem to be affected by the incubation at 37 °C. SMI decreased from 45.8 ± 14.2% to 27.5 ± 8.8%, while TA increased only from 10.0 ± 2.0% to 15.0 ± 4.6% (Table 1). Treatments did not affect the increase of TA significantly, but OXI accelerated the loss of SMI ($P = 0.01$) while the media supplemented with catalase (CAT and CAT/OXI) maintained a similar rate than the control ($P > 0.05$). Treatments reflected in a significant difference between OXI at 6 h and the other treatments, as showed in Table 1. Although a positive effect of catalase compared to control might be expected, since ROS are overproduced after cryopreservation, there were no significant differences between C and CAT .

Table 2 summarizes SCSA results. Incubation time did not significantly affect SDDFI ($P = 0.183$), but caused an increase of %DFI ($P = 0.024$). However, despite the lack of signification of the time effect, both CAT and CAT/OXI showed a significant negative effect on SDDFI at 6 h ($P < 0.01$). In the case of %DFI, the model indicated

that CAT significantly counteracted the effect of incubation time ($P = 0.017$), while OXI increased it considerably ($P < 0.001$). Both at 2 h and 6 h, the %DFI of OXI was significantly higher than for the other treatments ($P < 0.05$ at 2 h and $P < 0.001$ at 6 h). Besides, our model showed that HDS, a measure of DNA stainability by acridine orange, was negatively affected by incubation time ($P < 0.001$). The treatments did not significantly affect this parameter.

Discussion

In this study we have showed that adding catalase just after thawing can prevent the deleterious effects of oxidative stress, at least in part. This protection is very important, since free radicals can be generated from many sources after thawing: sperm metabolism, transition metals, leucocytes, etc. (Agarwal et al., 2005), decreasing sperm viability. Moreover, when using the spermatozoa for IVF, the oocytes and cells from the cumulus may generate ROS (Bedaiwy et al., 2004). The generation of oxidative stress in in-vitro culture media may have detrimental effects on post-fertilization development and assisted reproduction outcomes (Comporti, 1989; Aitken et al., 1993).

Our results showed that catalase supplementation after thawing prevented both the decrease of sperm motility and the increase of DNA damage under oxidant stress. In our experiment we used the pair Fe^{2+}/ascorbate, which generates hydroxyl radicals (•OH) from H_2O_2. Since catalase activates the decomposition of H_2O_2 into water and oxygen, the generation of radicals by Fe^{2+} is less efficient in the presence of this enzyme. The block of this pathway reduces the negative effects of H_2O_2 and other radicals, which are known to decrease sperm motility (Alvarez & Storey, 1982; O'Flaherty et al., 1997; Bilodeau et al., 2001).

The use of catalase for protecting spermatozoa has been previously tested with varying results (Maxwell & Stojanov, 1996; Roca et al., 2005; Fernández-Santos et al., 2007; Michael et al., 2007). Bilodeau et al. (2002) indicated that the addition of antioxidants to the extender was beneficial in terms on the motility of frozen-thawed spermatozoa, even in absence of an external source of oxidative stress. Maxwell and Stojanov (1996) found that the addition of catalase to the extender improved survival of liquid-stored ram spermatozoa, and Roca et al. (2005) have reported improvements in sperm motility when catalase was added to the freezing extender used for boar spermatozoa. Our group (Fernández-Santos et al., 2007) reported that catalase supplementation to the freezing extender improved the post-thawing motility of red deer spermatozoa. Nevertheless, all these studied tested the supplementation with antioxidants either for refrigerated storage or for cryopreservation, but not after thawing.

Even more important is that, in our experiment, catalase maintained chromatin stability along the incubation under oxidative stress. Although some studies (Lopes et al., 1998) indicated that catalase was ineffective in preventing human sperm DNA damage and suggested that H_2O_2 has little effect on sperm chromatin, most research has shown otherwise. Moreover, excessive generation of ROS in the semen is associated with lower fertilization rates in conventional IVF (Krausz et al., 1992; Suckcharoen et al., 1996). Smith et al. (2006) found a lower level of sperm chromatin condensation when the antioxidant was added to the medium after thawing, and Baumber et al. (2005), working in the cryopreservation of equine spermatozoa, found that catalase had protective effects on the DNA.

The addition of antioxidant after thawing could be of great interest especially for in vitro techniques, such as IVF or ICSI, where spermatozoa may be submitted to

oxidative stress. Agarwal et al. (2005) have proposed that culture media (for IVF or ICSI) should be supplemented with antioxidants, in order to protect spermatozoa against DNA damage, improve embryo development and decrease apoptosis. In fact, Gadea et al. (2005b) showed that the pre-treatment of sperm with antioxidant might improve sperm decondensation and male pronuclear formation after fertilization. Nevertheless, the use of antioxidants in IVF was advantageous only under very specific conditions. We consider that in vitro techniques could have an important role for breeds such as the fighting bull, because of their problematic genetic management. Techniques such as IVF or ICSI may improve the utilization of semen doses from very valuable individuals, and, therefore, efforts should be directed towards any improvement of the yield of these techniques. Thus, catalase supplementation, preventing oxidative stress, may contribute greatly to these efforts.

In conclusion, catalase supplementation after thawing might prevent the loss of sperm motility and also prevent DNA fragmentation associated to oxidative stress in bull sperm. The main application of our findings may be the improvement of media used for IVF and ICSI, considering the importance of the breeding we used and the eventual importance of these techniques in their reproductive management. Nevertheless, our conclusions must be considered having into account the limited number of samples available for this study.

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Legends to figures

FIGURE 1. (A) Schematic DFI histogram (red/red + green fluorescence). Cells out of the main population are included in DFIt (fragmented DNA, termed %DFI in this study). (B) Cytographs of native DNA stainability (green fluorescence; FL1) vs. fragmented DNA (red fluorescence; FL3). Cells with green fluorescence above 600 belong to HDS (immature DNA).

Tables

Table 1

Effects of catalase oxidant treatments on sperm quality parameters, in the 0, 2 h, and 6 h of incubation. Data are expressed as mean±S.E.M.

		Sperm parameters			
Time (h)	Treatment	SMI	NAR	VIABILITY	TA
0	Control	45.8 ± 14.2	64.0 ± 18.2	53.6 ± 21.8	10.0 ± 2.0
2	C	24.2 ± 8.0	49.6 ± 18.6	55.0 ± 21.1	19.6 ± 6.7
	CAT	30.8 ± 9.5	50.6 ± 16.2	54.6 ± 18.1	12.6 ± 4.0
	OXI	24.2 ± 5.8	44.6 ± 20.3	44.6 ± 16.1	15.3 ± 4.6
	CAT/OXI	20.8 ± 7.5	51.3 ± 21.1	55.0 ± 21.5	14.3 ± 6.4
6	C	27.5 ± 8.8 [a]	49.0 ± 8.5	45.7 ± 15.5	15.0 ± 1.4
	CAT	28.3 ± 5.2 [a]	41.0 ± 11.4	59.0 ± 23.9	14.0 ± 6.6
	OXI	11.6 ± 7.6 [b]	34.6 ± 18.2	50.5 ± 22.3	16.0 ± 2.6
	CAT/OXI	20.8 ± 2.9 [a]	41.3 ± 16.8	47.3 ± 25.6	19.6 ± 2.5

SMI: sperm motility index (%); NAR: % of spermatozoa with normal acrosome ridge; VIABILITY: % of viable spermatozoa: TA: % of spermatozoa with tail abnormalities.

[a,b] values with different superscripts indicate significant differences ($P<0.05$).

Table 2

Effects of catalase and oxidant treatments on SCSA results, in the 0, 2 h, and 6 h of incubation. Data are expressed as mean±S.E.M.

		DNA sperm parameters		
Time (h)	Treatment	SD-DFI	%DFI	HDS
0	Control	8.4 ±0.2	11.7 ± 0.5	7.9 ± 4.5
2	C	9.4 ± 0.3	17.0 ± 0.8 [a]	1.2 ± 0.5
	CAT	9.0 ± 0.2	15.5 ± 0.4 [a]	2.1 ± 0.7
	OXI	9.3 ± 0.2	30.3 ± 0.8 [b]	1.5 ± 0.5
	CAT/OXI	9.3 ± 0.2	17.4 ± 0.7 [a]	2.0 ± 0.9
6	C	9.4 ± 0.3 [ab]	19.1 ± 0.7 [a]	0.7 ± 0.7
	CAT	8.1 ± 0.3 [a]	14.7 ± 0.7 [a]	1.2 ± 0.8
	OXI	9.5 ± 0.4 [b]	51.2 ± 2.9 [b]	0.3 ± 0.7
	CAT/OXI	8.0 ± 0.1 [a]	12.8 ± 0.8 [a]	0.5 ± 0.7

SD-DFI: Standard deviation of DNA fragmentation index (DFI); %DFI: % of spermatozoa with DFI>25%. HDS: % of spermatozoa with high DNA stainability (green fluorescence higher than channel 600).

[a,b] values with different superscripts indicate significant differences ($P<0.05$).

Figures

Figure 1