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Effect of hydrogen peroxide on thawed ovine sperm motility

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Abstract

Oxidative stress, resulting from excessive levels of ROS in semen, has a negative impact on functional parameters and sperm fertility. In this study we examined the influence of the oxidative stress induced by hydrogen peroxide (H_2O_2) in ovine sperm motility after thawing and catalase (CAT) ability to preserve sperm motility. Semen was incubated at 37°C with 100 µM H_2O_2, 1 U catalase + 100 µM H_2O_2 or no treatment, for 30 min. Immediately after adding treatments, sperm motility was determined by computer-assisted semen analysis (CASA). Incubation with H_2O_2 led to a significant ($P < 0.05$) decrease in motility parameters whereas catalase prevented a decline in motility secondary to oxidative stress. After 30 min of incubation with H_2O_2, total motility (12.0% *vs.* control 73.0%, H_2O_2 +CAT 70.0%), progressive motility (0.0% *vs.* control 19.0%, H_2O_2 +CAT 19.0%) and rapid motility (1.0% *vs.* control 43.0%, H_2O_2 +CAT 40.0%) decreased significantly ($P < 0.05$), whereas percentage of static cells increased (84.0% *vs.* control 18.0%, H_2O_2 +CAT 20.0%). We conclude that H_2O_2 causes damage to ovine sperm motility and that catalase is able to avoid detrimental effect of H_2O_2 on sperm motility.

Keywords: antioxidants, catalase, oxidative stress, ROS.

Introduction

Oxidative stress is determined by the balance between the generation and degradation of reactive oxygen species (ROS) within a tissue. Sperm and seminal plasma posses a number of enzymes and low-molecular weight antioxidants that scavenge ROS in order to prevent possible cellular damage (Aitken, 1995; Halliwell and Gutteridge, 1999).

The action of ROS on sperm function is concentration-dependent. Low concentrations of ROS, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are needed for sperm capacitation, hyperactivation of motility, acrosome reaction and sperm-oocyte fusion (De Lamirande and Gagnon, 1993; Aitken, 1995; Baumber *et al*., 2003; O'Flaherty *et al*., 2003). On the other hand, excessive levels of ROS due to overproduction and/or inadequate antioxidant defenses leads to oxidative stress, resulting in a negative impact on functional parameters and fertility of sperm (Aitken, 1995; Armstrong *et al*., 1999; Peris *et al*., 2007).

The ROS most commonly generated by spermatozoon are: the superoxide anion, hydrogen peroxide and hydroxyl radical (Aitken, 1995). Several studies have shown that H_2O_2 exogenously added or produced by sperm, is toxic to mammalian spermatozoon causing damage to spermatic cell, including inhibition of motility and decline in energy metabolism (Armstrong *et al*., 1999; O'Flaherty *et al*., 1999; Baumber *et al*., 2000, 2002; Bilodeau *et al*., 2001, 2002; Garg *et al*., 2009). Once produced, the H_2O_2 is removed by antioxidant enzymes such as catalase, glutathione peroxidase and peroxiredutases that catalyze their reduction to O_2 and water (Halliwell and Gutteridge, 1999).

Catalase has been used successfully in extenders in order to improve the antioxidant capacity of semen and preserve sperm motility after thawing (Krzyzosiak *et al*., 2000; Baumber *et al*., 2002; Bilodeau *et al*., 2002; Maia *et al*., 2009).

In light of the relationship between ROS generation and spermatic function, we examined the effect of the addition of exogenous H_2O_2 on frozen/thawed ram sperm motility during incubation for 30 min and the catalase ability to preserve sperm motility in semen incubated with H_2O_2.

Materials and Methods

Chemicals were purchased from SIGMA- ALDRICH, Inc. (St Louis, MO, USA) except hydrogen peroxide, which was obtained from Merck Millipore (Darmstadt, Germany) and Orvus® Es Paste (sodium lauryl sulfate), which was purchased from Procter & Gamble (Cincinnati, OH, USA).

Semen collection and processing

Semen was collected from sixteen adult (24-32 months of age) Santa Inês rams (*Ovis aries*) with an artificial vagina and the study was carried out using one ejaculate per male. Immediately after collection, ejaculates were evaluated for volume, concentration (Neubauer chamber) and motility (using CASA). Semen was diluted to 400 x 10^6 cells/ml with a Tris- egg yolk extender, containing 250.25 mM Tris-hydroxymethyl-aminomethane, 79.71 mM monohydrated citric acid, 9.99 mM glucose, 20.0% (v/v) egg yolk, 1.0% (v/v)

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Orvus ES Paste, 100 mg/100 ml streptomycin sulfate and 100 000 IU Potassic Penicillin (Fraction A, pH: 6.91; osmolarity: 308 mOsm) and 14.0% glycerol (v/v) in the Fraction B (Maia *et al*., 2009). Dilution was performed at 32°C in a two-step process. The extended semen was filled in 0.25 French straws, sealed with polyvinyl alcohol and then cooled and frozen in an automatic freezer (Tetakon® -TK 3000, TK Tecnologia em Congelação Ltda, Uberaba, MG, Brazil) at a rate of -0.25°C/min from 32 to 5°C and -20°C/min from 5 to -120°C.

Thawing was carried out in a water bath at 42°C for 20 s. After thawing, straws were wiped and their content was poured in a micro tube, and then split in three aliquots for treatments: control; oxidant treatment (H_2O_2) and catalase/oxidant treatment (H_2O_2+CAT). In the control treatment, 60 µl semen (24 x 10^6 cells/ml) was diluted in 440 µl Tris-citric acid buffer solution (pH 7.4, Maia *et al*., 2010); the oxidant treatment constituted of semen sample, 60 µl, 435 µl Tris-citric acid buffer solution and 5 µl of 10 mM H_2O_2 (100 µM; Bilodeau *et al*., 2001); the catalase/ oxidant treatment constituted of semen sample, 60 µl, 415 µl Tris-citric acid buffer solution, 5 µl of 10 mM H_2O_2 (100 µM) and 20 µl of 25 U/ml catalase solution (2 U/ml; Bilodeau *et al*., 2002). The hydrogen peroxide (Perhydrol 30% H_2O_2 PA- Merck) and catalase (Catalase from bovine liver, SIGMA, C-9322) were prepared just prior to being added to the incubation medium.

Samples were then incubated at 37°C for 30 min, in order to induce oxidative stress, and sperm motility was determined immediately after adding of the treatments (time 0) and then at 15 min intervals.

Evaluation of sperm motility using CASA

Motility was assessed using a computer assisted sperm analysis (CASA). A sample of 10 µl of semen was placed in a pre-warmed MAKLER chamber (37°C) and transferred to the CASA analyzer (Hamilton-Thorn Biosciences IVOS 12, Beverly, MA, USA). For each semen sample, three fields were selected at random and various motility parameters including total motility (TM; %), progressive motility (PM; %), sperm with rapid motility (RAPID; %), static cells (STATIC; %), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), amplitude of lateral head displacement (ALH; µm), beat-cross frequency (BCF; Hz), straightness (STR; %) and linearity (LIN; %; Maia *et al*., 2009) were evaluated.

The settings used for ovine spermatozoa were negative phase-contrast optics, at a recording rate of 60 frames/s, minimum contrast 60, minimum cell size five pixels, cell intensity 55, non motile head size 2, non motile head intensity 50, progressive VAP threshold value 75 µm/s, slow cells VAP cutoff 21.9 µm/s, slow cells VSL cutoff 6.0 µm/s, threshold STR 80%. Progressive cells were those exhibiting a VAP of 75 µm/s and an STR of 80%. For each sperm sample, three fields were selected at random and assessed to generate data from at least 450 sperm/sample.

Detection of hydrogen peroxide

The presence of hydrogen peroxide in a sample was assessed using the HRPO-dependent oxidation of phenol red to a yellow derivate which concentration was measured by its absorbance at 610 nm (Maia *et al*., 2010).

Statistical analysis

One ejaculate from each of the 16 rams was used in the 3 treatments (control, H_2O_2 and H_2O_2+CAT) and 3 incubation times (0; 15 and 30 min) totaling n = 144. Analyses of variance were performed employing a randomized block design. Each male was considered a block and treatments and time were random variables blocked within rams. The linear model for ANOVA was:

$$Y_{ijk} = \mu + Trat_i + Time_j + I_{ij} + Male_k + \varepsilon_{ijk}$$

Where Y is a motility parameter; µ the overall mean, Trat is the effect of treatment; Time is the effect of incubation time; I, effect of interaction (Trat x time); Male, effect of ram and ε the error component. Mean values that were different at $P < 0.05$ were tested using Duncan's test. The values for sperm motility parameters were expressed as the mean ± SEM.

Results

The effect of H_2O_2 on sperm motility during incubation is shown in Table 1. Significant differences were observed in motility parameters after exposure to H_2O_2. During incubation, catalase prevented the decrease in all motility parameters.

Total motility (TM) in H_2O_2 group was significantly lower ($P < 0.05$) than in control from the beginning of incubation and after 15 min incubation compared to the H_2O_2/ CAT group. At 15 min incubation a significant reduction in total motility ($P < 0.05$) in H_2O_2/ CAT group was observed compared to control, whereas at 30 min no difference was found between the two groups.

Progressive motility was the parameter most affected by exposure to H_2O_2. After 15 min incubation a significant reduction in progressive motility ($P < 0.05$) was observed in H_2O_2 group compared to the other groups. After 30 min of incubation, no sperm with progressive motility was observed in this group.

After 15 min incubation, the percentage of sperm with rapid motility was significantly lower ($P < 0.05$) in H_2O_2 than in the control and H_2O_2+CAT group.

Exposure to H_2O_2 rapidly led to a significant increase in the percentage of static cells when compared with control and H_2O_2+CAT treatment. At 30 min incubation the percentage of static sperm in control, H_2O_2

and H_2O_2+CAT groups were 18 ± 2.6%, 84 ± 2.6% and 20 ± 2.6% respectively. The other motility parameters (VAP, VSL, VCL, ALH, BCF, STR and LIN) decreased significantly after 15 min of incubation in H_2O_2 group compared to the other groups.

After incubation of the sample for 30 min, the concentration of H_2O_2 detected in control was 7.4 ± 2.8 nmol/ml, in H_2O_2 group 19.6 ± 5.7 nmol/ml and in H_2O_2+CAT group was 6.6 ± 2.6 nmol/ml. The concentration of H_2O_2 was greater ($P < 0.05$) in H_2O_2 group than in samples from control or H_2O_2+CAT, that were similar.

Table 1. Effect of hydrogen peroxide (H_2O_2) on frozen-thawed sperm motility during incubation at 37°C for 30 min.

Parameter	Treatment	Incubation time (min)		
		0	15	30
TM (%)	Control	71 ± 2.5^{Aa}	75 ± 2.5^{Aa}	73 ± 2.5^{Aa}
	H_2O_2	59 ± 2.5^{Bb}	24 ± 2.5^{Cc}	12 ± 2.5^{Bd}
	H_2O_2 + CAT	64 ± 2.5^{Aa}	66 ± 2.5^{Ba}	70 ± 2.5^{Aa}
PM (%)	Control	22 ± 1.0^{Aa}	23 ± 1.0^{Aa}	19 ± 1.0^{Ab}
	H_2O_2	20 ± 1.0^{Ab}	1.0 ± 0.6^{Bc}	0.4 ± 0.1^{Bc}
	H_2O_2 + CAT	20 ± 1.0^{Ab}	20 ± 1.0^{Ab}	19 ± 1.0^{Ab}
RAPID (%)	Control	39 ± 2.2^{Aa}	46 ± 2.2^{Aa}	43 ± 2.2^{Aa}
	H_2O_2	32 ± 2.2^{Ab}	3.0 ± 2.2^{Bc}	0.9 ± 0.2^{Bc}
	H_2O_2 + CAT	38 ± 2.2^{Ab}	39 ± 2.2^{Ab}	40 ± 2.2^{Ab}
STATIC (%)	Control	17 ± 2.6^{Ba}	16 ± 2.6^{Ca}	18 ± 2.6^{Ba}
	H_2O_2	31 ± 2.6^{Ac}	70 ± 2.6^{Ab}	84 ± 2.6^{Aa}
	H_2O_2 + CAT	27 ± 2.6^{Ac}	24 ± 2.6^{Bc}	20 ± 2.6^{Bd}
VAP (μm/s)	Controle	84.5 ± 1.6^{ABa}	87.0 ± 1.6^{Aa}	84.1 ± 1.6^{Aa}
	H2O2	80.2 ± 1.6^{Bb}	46.0 ± 1.6^{Bc}	45.0 ± 1.6^{Bc}
	H2O2 + CAT	87.0 ± 1.6^{Aa}	83.3 ± 1.6^{Aa}	81.5 ± 1.6^{Aa}
VSL (μm/s)	Controle	62.0 ± 1.3^{ABb}	62.5 ± 1.3^{Ab}	59.3 ± 1.3^{Ab}
	H2O2	60.4 ± 1.3^{Bb}	32.0 ± 1.3^{Bc}	25.2 ± 1.3^{Bd}
	H2O2 + CAT	65.0 ± 1.3^{Aa}	62.0 ± 1.3^{Aab}	59.0 ± 1.3^{Ab}
VCL (μm/s)	Controle	161.0 ± 3.1^{Aa}	160.1 ± 3.1^{Aa}	159.0 ± 3.1^{Aa}
	H2O2	151.4 ± 3.1^{Aa}	112.0 ± 3.1^{Bb}	101.0 ± 3.1^{Bc}
	H2O2 + CAT	161.4 ± 3.1^{Aa}	154.0 ± 3.1^{Aa}	152.4 ± 3.1^{Aa}
ALH (μm)	Controle	8.1 ± 0.5^{Aa}	7.7 ± 0.5^{Aa}	7.7 ± 0.5^{Aa}
	H2O2	7.5 ± 0.5^{Aa}	4.4 ± 0.5^{Bb}	3.1 ± 0.5^{Bb}
	H2O2 + CAT	7.6 ± 0.5^{Aa}	7.4 ± 0.5^{Aa}	7.2 ± 0.5^{Aa}
BCF (Hz)	Controle	38.0 ± 0.7^{Aa}	36.0 ± 0.7^{Aa}	36.0 ± 0.7^{Bb}
	H2O2	37.0 ± 0.7^{Ab}	31.4 ± 0.7^{Cc}	31.5 ± 0.7^{Cc}
	H2O2 + CAT	37.4 ± 0.7^{Ab}	37.0 ± 0.7^{Ba}	36.5 ± 0.7^{Ba}
STR (%)	Controle	69 ± 1.3^{Aa}	70 ± 1.3^{Aa}	68 ± 1.3^{Aa}
	H2O2	71 ± 1.3^{Aa}	68 ± 1.3^{Bb}	55 ± 1.3^{Bc}
	H2O2 + CAT	72 ± 1.3^{Aa}	72 ± 1.3^{Aa}	70 ± 1.3^{Aa}
LIN (%)	Controle	38 ± 1.2^{Aa}	39 ± 1.2^{Aa}	38 ± 1.2^{Aa}
	H2O2	40 ± 1.2^{Aa}	30 ± 1.2^{Bb}	27 ± 1.2^{Bb}
	H2O2 + CAT	40 ± 1.2^{Aa}	40 ± 1.2^{Aa}	39 ± 1.2^{Aa}

Values with different superscript letters in the same row (lower case letters) or column (capital letters) differ significantly at $P < 0.05$. Data is expressed as mean ± S.E of 16 replicates (one ejaculate from each of the sixteen rams).

Discussion

Our findings support the evidences that hydrogen peroxide is toxic to mammalian spermatozoon. The exposure to exogenous H_2O_2 affected adversely all sperm motility parameters, like what was observed in previous reports with various species (O'Flaherty *et al*., 1999; Bilodeau *et al*., 2001; 2002; Peris *et al*., 2007; Garg *et al*., 2009; Du Plessis *et al*., 2010). This study also confirmed the beneficial effect of catalase on sperm survival during incubation under oxidative stress (Aitken *et al*., 1993; Armstrong *et al*. 1999; Baumber *et al*., 2000; Bilodeau *et al*., 2002; Fernández-Santos *et al*., 2008) indicating that H_2O_2 exerts a direct cytotoxic effect on spermatozoa.

Previous reports showed that catalase on concentration ranging from 1 - 4000 U/ml prevents the decrease of sperm motility in semen under oxidative stress, induced by H_2O_2 exogenous or by ROS generate systems (Aitken *et al*., 1993; Armstrong *et al*., 1999; Baumber *et al*., 2000; Bilodeau *et al*., 2002; Fernández-Santos *et al*., 2008). In our study, the addition of only 2 U/ml of catalase to the incubation medium was sufficient to prevent the loss of sperm motility caused by H_2O_2. This could be partly explained because during the catalytic reaction to eliminate two molecules of H_2O_2, free catalase is regenerated and molecular oxygen is released (Chance *et al*., 1979; Bartosz, 2005). Thus the enzyme may continue to react and a single molecule of catalase is capable of decomposing thousands of molecules of H_2O_2. According to Bartosz (2005) the number of peroxide molecules decomposed per CAT molecule per second is 3.5×10^6. Moreover, the catalase is most effective when the concentration of H_2O_2 is highly elevated which may have favored the catalase activity in our study. The catalytic reaction in which H_2O_2 react with catalase and Compound I does not obey Michaelis-Menten Kinetics; the rate of substrate decomposition increases linearly with hydrogen peroxide concentration over a wide concentration range (Bartosz, 2005). According to Chance *et al*. (1979) catalase is especially effective as a "safety valve" for dealing with much H_2O_2. The catalase activity like H_2O_2 scavenger was observed in our study, in sperm treated with H_2O_2 and catalase, the H_2O_2 levels remain similar to untreated sperm.

The damaging effect caused by 100 µM H_2O_2 (100 nmol/ml) on sperm motility during incubation in the diluents without catalase was very fast, 15 min after its addition to the extender; the same was observed by Bilodeau *et al*. (2001, 2002) and Garg *et al*. (2009). Possibly, this occurred due to the amount of H_2O_2 added to the system, which was much greater than the concentrations generated spontaneously by ram sperm, after cryopreservation (7.39 nmol/ml). However, after 30 min of incubation, the amount of hydrogen peroxide detected in the H_2O_2 group was only about 20% of original concentration added to the system (100 nmol/ml), possibly reflecting a scavenging effect of spermatozoa and seminal plasma. Nevertheless, this concentration was sufficient to affect sperm motility. It has been established that the decline in sperm motility, after treatment with H_2O_2, occurs in a dose-dependent manner (Armstrong *et al*., 1999; Peris *et al*., 2007). Thus, the amount of H_2O_2 added to the system was enough to cause a severe oxidative stress to sperm, resulting in a rapid decline (after 15 min) in all motility parameters.

The parameter most affected by H_2O_2 was the progressive motility, such as that detected in human sperm by Calamera *et al*. (2001) and du Plessis *et al*. (2010). Moreover, Calamera *et al*. (2001) observed that although progressively immovable, the spermatozoa were alive and showing no signs of major membrane damage. Thus, the mechanism by which H_2O_2 affects sperm motility is still unclear. Some studies (Armstrong *et al*., 1999; Bilodeau *et al*., 2002) attributed the loss of sperm motility to the fact that the H_2O_2 inhibits sperm ATP production by different ways. However, Calamera *et al*. (2001) reported that the loss of motility was accompanied by a parallel increase in sperm ATP concentration. Therefore, depletion of ATP may not be the cause of the decrease of sperm movement, instead, the decrease in the use or consumption by progressively immotile spermatozoa.

Furthermore, the inactivation of the intracellular scavenger systems may also be involved in decline in sperm motility, after treatment with H_2O_2. According to Krzyzosiak *et al*. (2000) the effect of H_2O_2 on sperm motility during incubation without catalase could be related to the inactivation of glutathione peroxidase/reductase system. This system is present in ovine semen (Bucak *et al*., 2008; Marti *et al*., 2008). Since biological membranes are highly permeable to H_2O_2, and glutathione peroxidase is present in the mitochondria (Chance *et al*., 1979), probably, when the intracellular concentration of H_2O_2 increased, intracellular glutathione (GSH) began to go down as well as the sperm motility. Therefore, the loss of sperm motility caused by H_2O_2 could be because no antioxidant enzyme that reduces H_2O_2 was present in the media.

On the other hand, in the semen incubated with H_2O_2 and catalase there was a time lag before the effect of catalase on motility was detected. Possibly, this occurred because the catalase reacted with H_2O_2 only after most GSH had been consumed, since glutathione peroxidase has a higher affinity for H_2O_2 (Bartosz, 2005) than does catalase. In erythrocyte Mendiratta *et al*. (1998) observed that with increasing fluxes of H_2O_2 coming into the cells, intracellular GSH decreased as the amount of H_2O_2 increased, reaching the catalase and that GSH need not be substantially depleted before H_2O_2 reaches catalase. In this way, the increase in sperm motility, arising from the effect of catalase, compared to the H_2O_2 group was only observed after 15 min of incubation.

In conclusion, the deleterious effect of H_2O_2 on sperm motility can be counteracted by adding catalase to the extender. The finding that H_2O_2 is cytotoxic to ovine spermatozoon suggests the importance of the addition of systems for H_2O_2 scavenge, like catalase, in the semen cryopreservation extender.

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