

Kinetics of hydrogen peroxide generated from live and dead ram spermatozoa and the effects of catalase and oxidase substrates addition

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ABSTRACT: The generation of hydrogen peroxide (H_2O_2) by ram spermatozoa (spz) was measured using a flurometric assay with 10-acetyl-3,7-dihydroxyphenoxazine agent as a probe for H_2O_2 detection. The kinetics of H_2O_2 production from both live and dead spz at 1×10^6 , 3×10^6 , and 6×10^6 spz/well concentrations were assessed in the tyrode albumin lactate (TAL) medium every 15 min for 120 min. An increase in H_2O_2 production from both live and dead spz was noted with a significant difference ($P < 0.05$) between the 1×10^6 and 6×10^6 spz/well concentrations. Although dead sperm generated higher amounts of H_2O_2 than live ones, no significant differences ($P > 0.05$) were observed between the two types of sperm for the three different concentrations. The generation of H_2O_2 by ram spz was also compared in the presence and absence of nicotinamide adenine dinucleotide phosphate (NADPH) and phenylalanine, substrates of the two specific oxidases. The supplementation with these substrates significantly ($P < 0.05$) increased the amounts of H_2O_2 generated from both live and dead spz, but for the two substrates, the increase was higher with dead than with live spz especially when phenylalanine was added. Addition of the antioxidant catalase significantly ($P < 0.05$) decreased the generation of H_2O_2 by live and dead spz with no significant differences ($P > 0.05$) between the two types of sperm before or after the antioxidant addition. This study showed the ability of live and dead ram spz to generate H_2O_2 in TAL medium. This ability was significantly influenced by the addition of NADPH and phenylalanine and also by the supplementation of the antioxidant catalase.

Keywords: reactive oxygen species; ram; antioxidant

List of abbreviations: spz = mammalian spermatozoa, ROS = reactive oxygen species, H_2O_2 = hydrogen peroxide, NADPH = nicotinamide adenine dinucleotide phosphate, NOS = nitric oxide synthase, AAO = aromatic amino acid oxidase, SOD = superoxide dismutase, GSH = glutathione, GPx = glutathione peroxidase, CAT = catalase

INTRODUCTION

Mammalian spermatozoa (spz), like all cells living under aerobic conditions, constantly face oxygen that is required to support life. Its metabolites, such as reactive oxygen species (ROS), can stimulate cell functions and endanger cell survival or both (Sanocka and Kurpisz 2004). ROS family includes species such as superoxide anion (O_2^-), hydroxyl radical ($HO\cdot$), and hydrogen peroxide (H_2O_2), which are generally derived from the incomplete reduction of molecular oxygen (Thannickal and Fanburg

2000). Mammalian sperm cells may generate ROS by different ways including nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase system in sperm plasma membrane (Aitken and Buckingham 1992; Rivlin et al. 2004), nitric oxide synthase (NOS) in the acrosome and sperm tail (Rodriguez et al. 2005), and NADH-dependent oxido-reductase diaphorase in the mitochondria (Gavella and Lipovac 1992). Moreover, a specific aromatic amino acid oxidase (AAO) has been identified as the origin of ROS formation from dead bovine and ram sperm (Shannon and Curson 1982a, b; Upreti et al. 1998).

Mammalian ejaculate contains adequate levels of antioxidants given that superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx), and catalase (CAT) are present in the seminal plasma and in the spermatozoa (Bilodeau et al. 2000; Marti et al. 2007). In this respect, the presence of CAT enzyme in sperm has been demonstrated in bull (Bilodeau et al. 2001) and ram species (Upreti et al. 1998). Fernandez-Santos et al. (2009) reported that catalase supplementation after thawing could protect spermatozoa against oxidative stress, and it could improve media used for processing thawed spermatozoa.

When produced in excessive amounts, ROS have toxic effects on both sperm function and quality (Alvarez et al. 1987). These reactive agents can be responsible for reduced sperm motility (De Lamirande and Gagnon 1992), sperm lipid peroxidation (Aitken et al. 1993), damages of the sperm nuclear DNA (Lopes et al. 1998), and decreased capacity of sperm-oocyte fusion (Aitken and Clarkson 1987). Many studies have shown that controlled amounts of ROS are involved in signal transduction pathways (Thannickal and Fanburg 2000). For example, hydrogen peroxide and superoxide anion play a role in the regulation of sperm capacitation by inducing protein tyrosine phosphorylation in human (O'Flaherty et al. 2006; De Lamirande and Lamothe 2009), equine (Baumber et al. 2003), and bull (Rivlin et al. 2004).

Despite the extensive knowledge on ROS production from mammalian sperm and the molecular targets and the mechanisms by which ROS act on spermatozoa, the kinetics of H_2O_2 generation from dead and live ram sperm was not previously indicated. Therefore, the objective of this study was to examine the production of H_2O_2 by live and dead ram spz, and to test the possibility to induce H_2O_2 production by adding different oxidase substrates. Also, the effect of catalase on live and dead ram spz H_2O_2 production was assessed.

MATERIAL AND METHODS

Animals and semen processing. This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km SE of Damascus. Semen was obtained from three sexually-experienced Awassi rams, aged between 3 and 4 years and weighing (mean \pm SD) 92.3 ± 8.8 kg. Semen samples were collected with the aid of an electro-ejaculator (Elec-

troJac 5; Neogen Corp., Ideal Instruments, Lansing, USA) administering a series of 32 cycles of short electrical stimuli with each cycle delivering a slightly higher intensity. All used animals were under veterinary care supervision after semen collection and animals' welfare was highly respected. Upon collection, the semen was immediately evaluated for its general appearance and volume. Sperm concentration was estimated using a hemacytometer. An initial analysis of sperm motility was performed using CASA system (Hamilton Thorne Biosciences, Beverly, USA). Sperm samples with a motility score $\geq 75\%$ of motile sperm and a concentration of $\geq 1 \times 10^9$ spermatozoa/ml were employed. Prior to H_2O_2 measurement, sperm were washed by mixing 500 μ l of the mixed semen with 2 ml of TAL solution (tyrode, albumin, lactate medium without pyruvate) containing 25mM bicarbonate, 22mM Na-lactate, 6 mg/ml fatty acid free bovine serum albumin (BSA) and centrifuged at 300 g for 10 min, then 2 ml of the supernatant was discarded.

Experimental design. The kinetics of H_2O_2 production from both live and dead sperm was defined in four experiments using 39 ejaculates. To diminish the effect of individual variation between the animals and for each assay, a mixture of spz from the three rams was used. Each experiment was repeated at least thrice.

Experiment 1: measurement of H_2O_2 generated by live and dead ram spermatozoa. Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) (Invitrogen, Molecular Probes, Eugene, USA) was used to monitor the H_2O_2 production. In the presence of horseradish peroxidase, amplex red reacts with H_2O_2 in a 1 : 1 stoichiometric reaction to produce resorufin, a highly fluorescent end product. A stock of amplex red was prepared in DMSO (10mM), while horseradish peroxidase was prepared in phosphate buffer (450 unit/ml, pH 7.5). Both stocks were stored at -20°C until the assay. In each assay, sperm concentration was calculated using a hemocytometer (Thoma cell counting chamber; Neubauer Improved, Marienfeld, Germany), and the sperm was diluted in TAL to a final concentration of 1×10^6 spz/well, 3×10^6 spz/well, and 6×10^6 spz/well. These concentrations were equivalent concentrations of 10, 30, and 60×10^6 spermatozoa/ml, respectively. 20 μ l of the sperm suspensions were added to 96-well plates (Nunc, Roskilde, Denmark). The remaining spz were killed by repeated freezing and thawing for at least three consecutive

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cycles and 20 μl of dead sperm suspension were added to the 96-well plates. In each assay, H_2O_2 standards (100, 50, 25, 12.5, 6.25, and 3.12 μM) were prepared extemporaneously in TAL medium. 80 μl of TAL containing 100 μM amplex red and 1 unit of horseradish peroxidase/ml were added to each well. Sperm samples, standard solutions, and blanks were assayed in duplicate. Microplates were incubated at 38°C, and the generation of H_2O_2 was measured using a fluorimeter (excitation wavelength 530 nm; emission wavelength 590 nm) (Fluoroskan Ascent FL; Labsystems, Thermo Bio-Analysis Company, Helsinki, Finland). Fluorescence was recorded at 15-minute intervals for 120 min. The concentration of H_2O_2 was determined from the standard curve based on the blank-corrected fluorescence for each measured time point and expressed in μM .

Experiment 2: effect of NADPH addition on H_2O_2 generation. Using the same method as for the above experiment, H_2O_2 formation from live and dead spz was determined with and without the addition of 1mM NADPH. The sperm was diluted in TAL solution to a final concentration of 3×10^6 spz/well, which is considered as one of the commonly used concentrations in the *in vitro* fertilization. The natural fluorescence of NADPH was taken into account when calculating the H_2O_2 production.

Experiment 3: effect of phenylalanine addition on H_2O_2 generation. H_2O_2 formation from live and dead spz was determined with and without the addition of 100 μM phenylalanine. The sperm was diluted in TAL solution to a final concentration

of 3×10^6 spz/well. Amplex red assay was then performed as above, and was replicated thrice.

Experiment 4: effect of catalase addition on H_2O_2 generation. Inhibition of H_2O_2 generation by ram sperm was examined by using catalase as scavenger for H_2O_2 . Catalase (derived from *Aspergillus niger*) was stored as a stock solution (2000 U/ml in TAL) for up to 3 weeks at 5°C. The sperm was diluted in TAL solution to a final concentration of 3×10^6 spz/well and 1000 U/ml of catalase was added.

Statistical analysis. Statistical analysis was conducted with the Minitab Statistical Software (Version 13.31, 2000). The normality of values distribution was first tested with the Shapiro-Wilk test. Data regarding H_2O_2 were subjected to a factorial analysis of variance for the three concentrations at each time point (ANOVA 2, General Linear Model procedure) including sperm concentration as fixed factor and replication as random factor, followed by multiple pairwise comparisons using a post-hoc Tukey's test. The slopes of the curves obtained by linear regression were compared using ANOVA (GLM) including the presence or the absence of additives (NADPH or phenylalanine or catalase), sperm status (live or dead) as fixed factors, and replication as random factor. The threshold of signification was set at $P < 0.05$.

RESULTS

Measurement of H_2O_2 generation by live and dead ram spermatozoa. H_2O_2 from live and dead

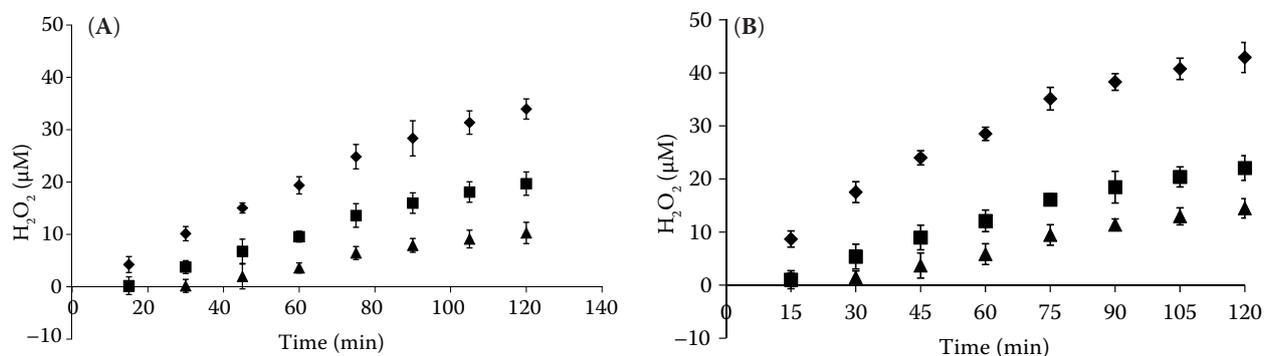


Figure 1. Generation of hydrogen peroxide (mean \pm SEM) by live (A) and dead (B) spermatozoa during a 120-minute incubation in tyrode albumin lactate medium

ram spermatozoa were incubated in a 96-well microplate at the concentration of 1×10^6 (▲), 3×10^6 (■), and 6×10^6 (◆) spermatozoa/well

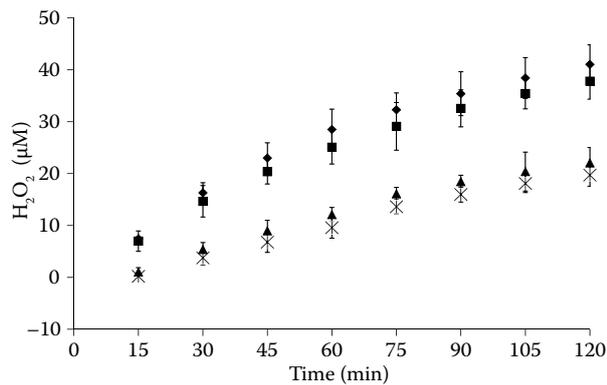


Figure 2. Effect of NADPH addition on the generation of hydrogen peroxide (mean \pm SEM) by live spermatozoa with (■) and without (⋈) the addition of NADPH and dead spermatozoa with (◆) and without (▲) the addition of NADPH during a 120-minute incubation in tyrode albumin lactate medium

ram spermatozoa were incubated in a 96-well microplate at the concentration of 3×10^6 spermatozoa/well

spz was generated in TAL medium during 120 min of *in vitro* incubation (Figure 1). Amounts of H_2O_2 increased significantly ($P < 0.05$) with time (for the three concentrations the averages ranged from 0.09, 0.2, 4.3 μM at 15 min to 10.3, 19.7, 33.9 μM at 120 min for live spz and from 1.3, 1.02, 8.7 μM at 15 min to 14.5, 22.1, 42.9 μM at 120 min for dead spz, respectively). Clear significant differences ($P = 0.002$; $P = 0.0013$) were observed between the

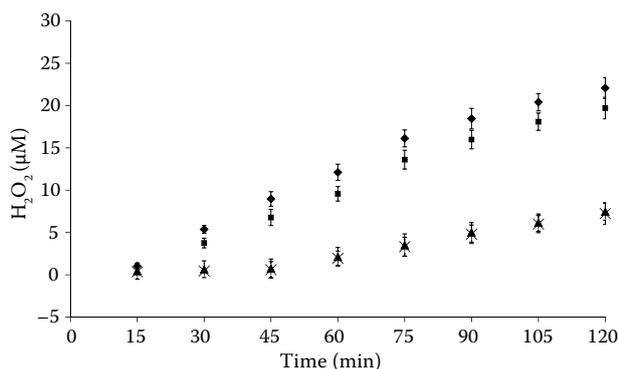


Figure 4. Effect of catalase addition on the generation of hydrogen peroxide (mean \pm SEM) by live spermatozoa with (⋈) and without (■) the addition of catalase and dead spermatozoa with (▲) and without (◆) the addition of catalase during a 120-minute incubation in tyrode albumin lactate medium

ram spermatozoa were incubated in a 96-well microplate at the concentration of 3×10^6 spermatozoa/well

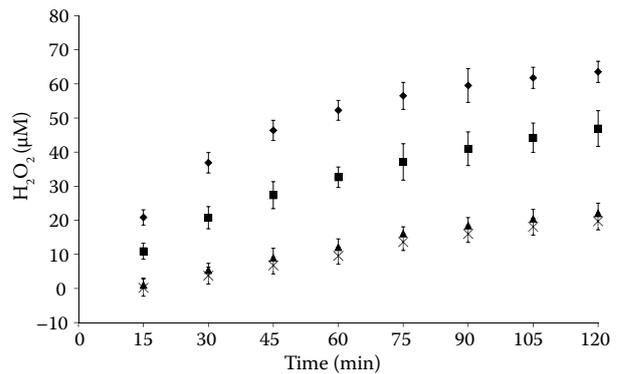


Figure 3. Effect of phenylalanine addition on the generation of hydrogen peroxide (mean \pm SEM) by live spermatozoa with (■) and without (⋈) the addition of phenylalanine and dead spermatozoa with (◆) and without (▲) the addition of phenylalanine during a 120-minute incubation in tyrode albumin lactate medium

ram spermatozoa were incubated in a 96-well microplate at the concentration of 3×10^6 spermatozoa/well

1 and 6×10^6 spz/well concentrations for the live and dead spz. Although the production of H_2O_2 was higher from the dead sperm concentrations than from the live ones, no significant differences ($P > 0.05$) were noted between the two types of sperm for the three concentrations.

Effect of NADPH on H_2O_2 generation. NADPH addition significantly ($P < 0.05$) increased the amount of H_2O_2 generated by live and dead sperm (Figure 2). The increase was higher with dead than with live sperm (on average 41.1 μM at 120 min for the dead sperm vs 37.8 μM for the live ones). However, no significant differences ($P > 0.05$) were noted between the live and the dead sperm before or after the NADPH addition.

Effect of phenylalanine on H_2O_2 generation. The addition of phenylalanine significantly ($P < 0.05$) increased the amount of H_2O_2 generated by live and dead sperm (Figure 3). The rate of H_2O_2 production was more important with dead sperm than with live sperm (on average 63.6 μM for dead sperm vs 46.9 μM for live sperm at 120 min) with a significant difference ($P < 0.05$) between the two types of sperm after the substrate addition.

Inhibition of H_2O_2 generation by the addition of catalase. The addition of catalase significantly ($P < 0.05$) decreased the amount of H_2O_2 generated by live and dead sperm (Figure 4). No significant differences ($P > 0.05$) were noted between live and dead sperm before or after the catalase addition.

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DISCUSSION

The data presented in this study confirm that live and dead ram sperm are capable of generating H_2O_2 . Moreover, H_2O_2 concentrations generated by ram sperm increased when the numbers of spermatozoa increased. Similarly, a detectable accumulation of H_2O_2 was recorded after 30–60 min of incubation in a suspension of live and dead equine and bull spz (Ball et al. 2001; Alomar and Donnay 2006). Despite the difficulties encountered when comparing the different studies, higher rate of H_2O_2 appears to be generated from rams' spz than from equine and human (Aitken and Buckingham 1992; Ball et al. 2001). In contrast to our results, an approximately 5-fold increase in the amount of H_2O_2 was generated by dead equine sperm compared with the amount generated by live ones (Ball et al. 2001). Thus, the source or the amplitude of ROS generation from dead spz could differ among different species.

In our study, we attempted to determine the source of ROS generated from live and dead ram sperm by adding specific substrates of the NADPH-oxidase and the aromatic amino acid oxidase. NADPH supplementation increased the amount of H_2O_2 generated by the two types of sperm (live and dead). Thus, ram spz may possess a NADPH oxidase in their plasma membrane similar to that described in human and equine semen (Aitken 1997; Ball et al. 2001). It is well known that NADPH serves as a source of electrons for the generation of ROS via a proposed NADPH-oxidase reaction in spermatozoa (Aitken 1997). Moreover, this substrate appears to be implicated in sperm capacitation by inducing tyrosine phosphorylation of several different proteins regulated by cAMP-dependent PKA signalling pathway (Baumber et al. 2003; O'Flaherty et al. 2006). Considerable rates of H_2O_2 were generated from dead ram sperm supplemented with NADPH. After sperm death, the stimulation of NADPH-oxidase appears to be possible as in the case of live sperm. This could be due to an increased access of NADPH, but also to a decrease in antioxidant mechanisms after sperm death.

Phenylalanine was also able to increase H_2O_2 generation from the two sperm populations. This amino acid is considered one of the major substrates of the AAAO enzyme. The generation of H_2O_2 after phenylalanine addition in our work

supports previous data showing AAAO activity in bovine sperm (Shannon and Curson 1982b) and ram sperm (Upreti et al. 1998). This oxidase could be located in the tail of bovine spz, and its activity was detected only after sperm death (Shannon and Curson 1982a, b). Moreover, this enzyme can dehydrogenate several amino acids including phenylalanine to produce H_2O_2 (Shannon and Curson 1982b). Lapointe and Sirard (1998) pointed out that phenylalanine had significant negative influence on bovine sperm motility and velocity and this was related to H_2O_2 effect. It must be noted that ram spermatozoa contained higher levels of AAAO activity than bull spermatozoa, although the physico-chemical properties of the enzyme were generally similar in both species (Upreti et al. 1998). The increase in H_2O_2 amounts generated by live ram sperm after the addition of phenylalanine might be related to the progressive sperm death occurring during the 120 min of the incubation period. However, the potential physiological role of such enzyme in dead or in live sperm remains an open question.

Contradictory reports exist concerning the effect of catalase supplementation to mammalian semen. In this respect, the addition of this enzyme to semen extender contributed to the reduction of sperm membrane lipid peroxidation by ROS during semen cryopreservation (El-Sisy et al. 2008). In contrast, Sicherle et al. (2010) reported that the addition of catalase to post thawed ram semen at specific concentrations failed to remove ROS produced during the freezing and thawing process. Our results clearly showed the positive effect of catalase supplementation in inhibiting H_2O_2 generation in *in vitro* conditions. Anyhow, it must be awarded that the antioxidant treatments should not lead to complete ROS elimination, since the oxidative mechanisms play a crucial role during the physiological control of sperm functions.

Tyrode Albumin Lactate Pyruvate (TALP) medium is one of the most important fertilization media used in *in vitro* experiments (Farrell et al. 1996). Pyruvate was removed from the TALP medium throughout the present study. Previous studies showed that alpha-keto carboxylic acids, such as pyruvate, can be decarboxylated in the presence of H_2O_2 to produce acetate carbon dioxide and water (Morales et al. 1999). In addition, the presence of catalase was beneficial to sperm

motility when pyruvate was eliminated (Upreti et al. 1998). However, it has been reported that either catalase or pyruvate could be used as an antioxidant in diluents for spermatozoal storage (Upreti et al. 1998; Bilodeau et al. 2002).

CONCLUSION

Live and dead ram spermatozoa generate H_2O_2 possibly via both NADPH-oxidase and aromatic amino acid oxidase systems. Dead ram sperm is an important source of H_2O_2 , which is interesting to know due to the high rates of sperm mortality observed during sperm cryopreservation and also during the process of *in vitro* fertilization. Catalase is clearly a beneficial antioxidant for the two types of ram sperm. This in turn raises the possibility for potential applications of this antioxidant in the fields of sperm cryopreservation and also for ram *in vitro* fertility.

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