Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa

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The reactive oxygen species, hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}), were generated with a xanthine–xanthine oxidase system and their effect on human sperm function was studied. The action of reactive oxygen species on selected human spermatozoa resulted in a decreased capacity for ionophore-induced acrosome reaction, a decrease in sperm motility, an increase in the concentration of lipid hydroperoxides and a loss of membrane polyunsaturated fatty acids. H₂O₂ was the key intermediate of the deleterious effects exerted by the xanthine and xanthine oxidase. Among these parameters, the acrosome reaction appeared most susceptible to the reactive oxygen species generated by the xanthine–xanthine oxidase system, and was decreased without sperm motility being affected. Treatment with H₂O₂ was shown to inactivate several enzymatic activities involved in the antioxidant defence of spermatozoa: glutathione peroxidase, superoxide dismutase and glucose-6-phosphate dehydrogenase. H₂O₂ and O₂^{•-} were shown to be involved in the lipid alterations triggered by the xanthine–xanthine oxidase system. Singlet oxygen is proposed to intervene in the lipoperoxidation process. The inefficacy of mannitol in protecting spermatozoa suggests that hydroxyl radicals were not produced in the extracellular medium.

Introduction

The role of reactive oxygen species in the pathophysiology of human sperm function has been emphasized in recent years (Jones *et al.*, 1979; Alvarez *et al.*, 1987; Aitken *et al.*, 1989, 1991, 1993a, b; De Lamirande and Gagnon, 1992a, b). Their production in semen has been associated with loss of motility, decreased capacity for sperm–oocyte fusion and loss of fertility (Aitken *et al.*, 1989, 1991).

In semen preparations, there are two major sources of reactive oxygen species: leucocytes (Aitken and West, 1990; Aitken *et al.*, 1992a; Kessopoulou *et al.*, 1992) and spermatozoa (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Aitken *et al.*, 1992b; Iwasaki and Gagnon, 1992; Zini *et al.*, 1993). Aitken and Clarkson (1987) proposed that production by spermatozoa was dependent upon a membrane-bound NADPH-oxidase. Hydrogen peroxide (H₂O₂) produced by the dismutation of superoxide anion has been recognized as the most toxic oxidizing species for human spermatozoa (De Lamirande and Gagnon, 1992a; Aitken *et al.*, 1993a). Owing to their high content of polyunsaturated fatty acids, it has been proposed that lipid peroxidation of the sperm plasma membrane is largely responsible for defective sperm function (Jones *et al.*, 1979). Reactive oxygen species also affect the sperm axoneme as a result of ATP depletion (De Lamirande and Gagnon, 1992b), inhibit mitochondrial functions, and synthesis of DNA, RNA and

proteins (Comporti, 1989), produce cytoskeletal modifications (Hindshaw *et al.*, 1986) and inhibit sperm–oocyte fusion (Aitken *et al.*, 1993a).

Human spermatozoa possess enzymatic defence systems such as superoxide dismutase (SOD), glutathione peroxidase/reductase (GPX/GRD) and catalase (Nissen and Kreysel, 1983; Alvarez *et al.*, 1987; Alvarez and Storey, 1989; Jeulin *et al.*, 1989) to counteract the toxic effects induced by reactive oxygen species. Although correlations have been reported between the effectiveness of reactive oxygen species and the duration of sperm motility (Alvarez *et al.*, 1987; Alvarez and Storey, 1989), the importance of their action has not been fully elucidated.

This study was designed to investigate the direct effects of reactive oxygen species on membrane phospholipids, enzymatic defence systems, ionophore-induced acrosome reaction and motility. Reactive oxygen species were produced using a combination of xanthine and xanthine oxidase, which catalyses the reduction of ground state oxygen to generate O₂^{•-} and H₂O₂ by spontaneous dismutation of superoxide.

Materials and Methods

Media and reagents

B₂ medium was obtained from Bio-Mérieux (Api system; Montalieu), Percoll from Pharmacia (Uppsala), Ham's F10

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medium from Flow Laboratories (Puteaux), GB₂₄ from Theramex (Monaco) and rhodamine B from Prolabo (Paris). Methanol, chloroform, acetic acid, heptane, dioxane, sulfuric acid and thin layer silica gel plates were obtained from Merck (Darmstadt). All other media and reagents were purchased from Sigma Chemical Company (St Louis, MO).

Sperm preparation and induction of oxidative stress

Semen samples were collected by masturbation from healthy donors or from men attending the fertility laboratory after a sexual abstinence of 2–3 days. Only spermatozoa exhibiting normal semen characteristics ($> 20 \times 10^6$ spermatozoa ml^{-1} , $> 50\%$ motility and $> 50\%$ normal forms) were used. After 15 min liquefaction at 37°C , spermatozoa were fractionated on a four-step discontinuous Percoll gradient (46–69–83–92%). Percoll was made isotonic by adding 0.8 volumes of 0.4 mol Hepes-buffered Ham's F10 l^{-1} (pH 7.4) to 9.2 volumes of Percoll. The resulting solution was further diluted with 0.04 mol Hepes-buffered Ham's F10 l^{-1} to give 46, 69 and 83% Percoll solutions. Semen was layered on the top of the gradient and centrifuged at 500 *g* for 20 min at room temperature. Spermatozoa from the 92% Percoll fractions were resuspended in 5 ml of B₂ medium and centrifuged at 500 *g* for 5 min at room temperature. The supernatant was discarded and spermatozoa were resuspended at a concentration of 20×10^6 spermatozoa ml^{-1} in B₂ medium.

Spermatozoa were then incubated in B₂ medium at 37°C under a 5% CO₂ atmosphere to allow capacitation with a combination of xanthine (X: 100–200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (XO: 1–50 mU ml^{-1}), or with H₂O₂ (500 $\mu\text{mol l}^{-1}$) or mercaptosuccinate (MCS: 200 $\mu\text{mol l}^{-1}$). The reactive oxygen species scavengers: superoxide dismutase (500 U ml^{-1}) catalase (500 U ml^{-1}), mannitol (100 $\mu\text{mol l}^{-1}$) and histidine (10 mmol l^{-1}) were also tested.

Analysis of sperm motility

Sperm motility was analysed using the ATS 40 motility analyser (J. C. Diffusion International, La Ferte Fresnel) in a 20 $\mu\text{mol l}^{-1}$ depth chamber (Fertility Technology Inc., Natick, MA) at 37°C . This system was previously validated for measuring the specific movement parameters of human spermatozoa (Le Lannou *et al.*, 1992). System parameter settings for this analysis were 40 frames s^{-1} recorded, 30 consecutive frames analysed, spermatozoon minimum size 8 pixels, spermatozoon maximum size 20 pixels, 20 $\mu\text{m s}^{-1}$ threshold velocity and 300 $\mu\text{m s}^{-1}$ maximum velocity.

A minimum of 100 trajectories were analysed for each sample. The characteristics of movement recorded were motility (%), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight line velocity (VSL, $\mu\text{m s}^{-1}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %) and percentage of hyperactivated spermatozoa (percentage of cells exhibiting a VCL $> 100 \mu\text{m s}^{-1}$, LIN $< 40\%$ and ALH $> 4.5 \mu\text{m}$; adapted from Mortimer and Mortimer, 1990; Burkman, 1991).

Induction and evaluation of acrosome reaction

Acrosome reaction was evaluated after 6 h of incubation, when it was chemically induced with 10 $\mu\text{mol A23187 l}^{-1}$

(stored at $10^{-3} \text{ mol l}^{-1}$ in ethanol at -20°C before use) in B₂ medium for 1 h at 37°C and in an atmosphere of 5% CO₂ and 95% air. Five minutes before the end of the incubation, the vital stain propidium iodide ($10^{-9} \text{ mol l}^{-1}$ final) was added to the sperm suspension. After washing by centrifugation at 500 *g* for 5 min in PBS with 0.1% BSA, the spermatozoa were resuspended with GB₂₄ antibody (1 μg : 100 μl PBS), a mouse monoclonal antibody that recognizes an inner acrosomal membrane-associated protein (Fenichel *et al.*, 1990). After incubation for 45 min at room temperature, the spermatozoa were washed by centrifugation at 500 *g* for 5 min with PBS–BSA and resuspended with FITC-conjugated anti-mouse immunoglobulin at 1:50 in PBS–BSA for 30 min. Spermatozoa were then washed by centrifugation at 500 *g* for 5 min with PBS–BSA and immediately observed by fluorescence microscopy. Two-hundred spermatozoa were evaluated for each sample. Only spermatozoa devoid of red fluorescence (given by propidium iodide) and exhibiting a green homogeneous fluorescence pattern distributed over the entire acrosome cap were scored as positive (living acrosome-reacted spermatozoa). The results were expressed as percentages of positive spermatozoa.

Assay of reactive oxygen species

The generation of O₂^{•−} by the combination of xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) was monitored at 550 nm by the reduction of ferricytochrome C (Gavella *et al.*, 1991). The specificity of the assay was assessed by adding 500 U superoxide dismutase into the control incubation.

The production of H₂O₂ in the same system was measured at 510 nm using the peroxidase-catalysed oxidation of 4-aminoantipyrine in the presence of phenol (Aitken *et al.*, 1993a). The specificity of the assay was assured by adding 500 U catalase into the control incubation.

Assay of superoxide dismutase-like activity

Superoxide dismutase-like activity was measured by the inhibition of the ferricytochrome C reduction due to the O₂^{•−} generated by 200 μmol xanthine l^{-1} and 10 mU xanthine oxidase (Alvarez and Storey, 1983).

The extraction procedure was that of Kobayashi *et al.* (1991). After 3 h of treatment, excess xanthine–xanthine oxidase, H₂O₂ or mercaptosuccinate was removed by centrifugation (500 *g*, 5 min), and spermatozoa were treated for 10 min with 10 mmol potassium phosphate buffer l^{-1} . Complete disruption of spermatozoa was achieved by sonicating them for 2 min at 4°C in 20 s bursts with 20 s intervals between bursts to avoid heating. The supernatant was then collected by centrifugation for 10 min at 1800 *g* and 4°C , and used for the measurement of superoxide dismutase-like activity. The reaction was initiated by the addition of XO and monitored at 25°C . One unit of activity was defined as the amount of enzyme required to inhibit the change of absorbance at 550 nm by 50%.

Assay of glutathione peroxidase and glutathione reductase activities and glutathione content

The activities of glutathione peroxidase and glutathione reductase and glutathione content were determined in hypo-osmotically treated spermatozoa after 3 h of incubation with xanthine–xanthine oxidase, H_2O_2 or mercaptosuccinate and based on the coupled oxidation of NADPH, and reduction of oxidized glutathione (GSSG) by glutathione reductase (GRD) (Alvarez *et al.*, 1987). In this series of experiments, enzymatic activities and glutathione content were determined in the whole hypotonic sperm suspension. The change in absorbance due to the oxidation of NADPH was monitored at 365 nm.

Assay of glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured using a kit (Sigma Diagnostics, St Louis, MO) after 3 h of incubation with xanthine ($200 \mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}). The increase in absorbance due to the formation of NADPH (proportional to the G6PDH activity) was recorded using a spectrophotometer at 340 nm. The reduction of NADP^+ was linear for up to 20 min.

Extraction and analysis of sperm phospholipid

Semen from 2–3 donors was pooled to obtain sufficient phospholipids, and spermatozoa were selected on Percoll gradient and treated with xanthine ($200 \mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}). After incubation for 6 h, the lipids were extracted with chloroform/methanol (2/1, v/v) according to the method of Folch *et al.* (1957). The mixture of lipids was fractionated by thin layer chromatography (TLC) using the solvent system hexane/diethylether/acetic acid (90/10/0.5, v/v/v) for 45 min at room temperature. The different spots of lipids were visualized in ultraviolet after reaction with rhodamine and identified by comparison with lipid standards. The silica gel was scraped and the fatty acids transesterified according to the method of Hagenfeld (1966). Fatty acid methyl esters were then analysed by gas liquid chromatography (GLC) on a Chrompack CP 9001 apparatus equipped with a BPX70 capillary column (SGE, villeneuve-Saint Georges), $25 \text{ m} \times 0.32 \text{ mm i.d.}$, film thickness $0.22 \mu\text{mol l}^{-1}$ and a flame ionization detector. The vector gas was nitrogen. The analysis of fatty acids was carried out with an internal standard, nonadecanoic acid (19:0). The results obtained are given as percentages of the total fatty acids.

Lipid hydroperoxide determination

Amounts of lipid hydroperoxides were spectrophotometrically determined after incubation for 3 h according to the method developed by Hicks and Gebicki (1979) and based on the liberation of iodine after the 1:1 stoichiometric reaction between hydroperoxides and potassium iodide. Briefly, total lipid extracts were prepared from spermatozoa as described above and stored under liquid nitrogen. Twenty-five microlitres of the extract were added to 1.8 ml deoxygenated methanol/acetic acid (1/1, v/v) and 1.9 ml of a 20%

(w/v) potassium iodide solution in methanol. The change in absorbance was recorded at 360 nm until any further change was small and linear with time. Amounts of lipid hydroperoxides were calculated using the extinction coefficient of $2.80 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Singlet oxygen determination

Singlet oxygen formation was investigated by the use of 1,3-diphenylisobenzofuran (DPBF), which forms 2,2-dibenzoylbenzene (DBB) when it reacts with $^1\text{O}_2$ (Pederson and Aust, 1973). DPBF (10 mmol l^{-1}) was first dissolved in chloroform, and the solvent was removed under a stream of N_2 and the deposit was emulsified in PBS by sonication. The reaction was initiated by addition of xanthine ($200 \mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}). The reaction mixture was incubated for 1 h at 37°C and extracted with 0.5 ml chloroform. One-hundred microlitres of each extract was applied to thin layer plate silica gel and developed by heptane/dioxane (3/1, v/v). The plate was then sprayed with 0.5% (w/v) of 2,4-dinitrophenylhydrazine in 2 mol HCl l^{-1} .

Statistical analysis

Unless stated otherwise, the statistical significance of the results was assessed using the paired Student's *t* test using the STATWORKS statistical package (Heyden and Sons Inc., Philadelphia, PA). Differences with values of $P < 0.05$ were considered to be statistically significant.

Results

Production of reactive oxygen species and effects on sperm motility and acrosome reaction

A combination of xanthine ($200 \mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) resulted in the production of $40.0 \pm 0.7 \mu\text{mol O}_2^{\circ-} \text{ l}^{-1}$ and $79.0 \pm 3.2 \mu\text{mol H}_2\text{O}_2 \text{ l}^{-1}$ (from the spontaneous dismutation of $\text{O}_2^{\circ-}$) over 4 and 6 min, respectively (Fig. 1). The specificity of the assays was assured by superoxide dismutase and catalase which respectively scavenged $\text{O}_2^{\circ-}$ and H_2O_2 . Within 5 min, $\text{O}_2^{\circ-}$ could not be detected in the medium, whereas H_2O_2 decreased only slightly for several hours. However, in the presence of spermatozoa ($20 \times 10^6 \text{ spermatozoa ml}^{-1}$), the amount of H_2O_2 decreased very rapidly and was insignificant after 1 h of incubation (data not shown).

The response of spermatozoa to this profile of reactive oxygen species production was a progressive and significant decrease with time in percentage motility ($P < 0.01$) and every parameter of sperm movement (Table 1) except for ALH, which was even significantly increased ($P < 0.05$) after incubation for 3 h.

The influence of reactive oxygen species on the capacity of human spermatozoa to undergo the acrosome reaction after capacitation for 6 h was assessed with a concentration of xanthine at which the percentage of motile spermatozoa was unaltered. Exposure to $100 \mu\text{mol xanthine l}^{-1}$ and 50 mU

Fig. 1. Time course of reactive oxygen species production induced by 200 $\mu\text{mol l}^{-1}$ xanthine and 50 mU xanthine oxidase ml^{-1} . The generation of $\text{O}_2^{\bullet-}$ was monitored by the reduction of ferricytochrome C, and the generation of H_2O_2 was monitored by the peroxidase-catalysed oxidation of 4-aminoantipyrine in the presence of phenol. Values are means $\pm$ SEM, $n = 3$. $\square$: H_2O_2 ; $\blacksquare$: $\text{O}_2^{\bullet-}$.

xanthine oxidase ml^{-1} was compatible with the maintenance of the percentage of motility for up to 6 h ($85.0 \pm 4.0\%$ for control versus $77.8 \pm 4.7\%$ for xanthine–xanthine oxidase). Despite an unchanged sperm motility, the percentage of the ionophore-induced acrosome reaction was significantly diminished ($26.2 \pm 3.1\%$ for control versus $4.5 \pm 1.5\%$ for xanthine–xanthine oxidase, $P < 0.001$).

A series of experiments was performed in which the concentration of xanthine was fixed at 100 $\mu\text{mol l}^{-1}$ but that of xanthine oxidase varied from 1 to 50 mU ml^{-1} (Fig. 2) to determine whether the speed at which reactive oxygen species were generated had any influence on the ionophore-induced acrosome reaction. As the concentration of enzyme increased, the effects on the ionophore-induced acrosome reaction were more pronounced. With the lowest concentration of xanthine oxidase (1 mU ml^{-1}), the response to A23187 was unchanged, whereas at 5 mU ml^{-1} or above, treated spermatozoa exhibited a dose-dependent diminished response to induction of the acrosome reaction.

Influence of the xanthine oxidase system on sperm phospholipids

An oxidation of the membrane fatty acids was observed after exposure of the human spermatozoa to xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}). GLC analysis revealed that the polyunsaturated fatty acid (PUFA) concentrations were preferentially affected (Fig. 3a) and dropped by 20%, whereas saturated and mono-unsaturated fatty acid concentrations remained stable.

A more detailed analysis of the behaviour of the major phospholipid PUFAs revealed that they were differentially affected (Fig. 3b). Linoleic acid (18:2n-6) showed a high resistance to reactive oxygen species exposure and remained stable at $98.6 \pm 3.2\%$ of its initial value. The two other major PUFAs: arachidonic acid (20:4n-6) and docosaenoic acid (22:6n-3) were more affected with, respectively, $75.0 \pm 12.9\%$ and $72.3 \pm 5.6\%$ of their initial concentrations remaining after incubation for 6 h.

Effects of reactive oxygen species scavengers

A series of specific scavengers was tested on spermatozoa exposed to xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) to determine which reactive oxygen species was responsible for the decreases in motility, acrosome reaction and PUFA content.

Among the scavengers tested, only catalase completely reversed the toxic effects induced by the xanthine–xanthine oxidase system on all the parameters considered (Table 2).

Superoxide dismutase prevented loss of PUFA content, with $96.5 \pm 4.9\%$ of initial concentrations remaining after 6 h of incubation (arachidonic and docosaenoic acids were equally protected: 96.8 ± 1.3 and $96.0 \pm 1.4\%$ of native spermatozoa, respectively) but exhibited no capacity to preserve sperm motility and ionophore-induced acrosome reaction.

The hydroxyl radical scavenger, mannitol (100 mmol l^{-1}), did not modify any of the cytotoxic effects of the reactive oxygen species.

Reactive oxygen species, enzymatic defence systems and lipid hydroperoxides

Treatment of spermatozoa with the combination of xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) caused a

Table 1. Influence of the combination of xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) on the movement characteristics of human spermatozoa after 3 and 6 h of incubation

Movement characteristics	Time			
	Control	3 h X-XO	Control	6 h X-XO
Motility (%)	87.7 ± 2.3	$63.7 \pm 8.8^*$	85.7 ± 4.1	$54.2 \pm 2.9^*$
VCL ($\mu\text{m s}^{-1}$)	92.0 ± 2.3	$75.4 \pm 2.1^{***}$	90.8 ± 2.6	$66.6 \pm 2.9^{***}$
VSL ($\mu\text{m s}^{-1}$)	69.7 ± 5.6	$47.7 \pm 2.6^{**}$	70.1 ± 3.1	$43.8 \pm 2.8^{***}$
LIN (%)	75.2 ± 4.4	$62.9 \pm 2.5^*$	76.5 ± 1.7	$64.3 \pm 2.3^{**}$
ALH ($\mu\text{m s}^{-1}$)	3.2 ± 0.3	$4.0 \pm 0.2^*$	3.2 ± 0.2	3.3 ± 0.2
Hyperactivated (%)	10.2 ± 3.1	$1.6 \pm 1.0^{***}$	3.7 ± 2.3	1.6 ± 1.1

Values are means $\pm$ SEM, $n = 10$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control values.

VCL: curvilinear velocity; VSL: straight line velocity; LIN: linearity; ALH: lateral head displacement.

Fig. 2. Effects of various concentrations of xanthine oxidase on the capacity of human spermatozoa to undergo the acrosome reaction after exposure to 100 μmol xanthine l^{-1} . Acrosome reaction was induced after 6 h of capacitation by 10 μmol calcium ionophore A23187 l^{-1} . Values are means $\pm$ SEM, $n = 5$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control values. : 50 mU ml^{-1} ; .: 20 mU ml^{-1} ; ■: 5 mU ml^{-1} ; □: 1 mU ml^{-1} .

significant decrease in superoxide dismutase-like and glutathione peroxidase activities (Table 3). In contrast, glutathione reductase activity was not affected by this treatment. A concomitant oxidation of reduced glutathione (GSH) to oxidised glutathione (GSSG) and a rise in the concentration of lipid hydroperoxides was observed.

Catalase provided protection against inhibition of both superoxide dismutase-like and glutathione peroxidase activities. Oxidation of GSH and an increase in lipid hydroperoxides were also prevented.

Although superoxide dismutase offered a partial protection against the rise in concentration of lipid hydroperoxides, its presence in the medium of incubation failed to protect glutathione peroxidase activity.

Treatment with 500 μmol H_2O_2 l^{-1} led to the same decrease in superoxide dismutase-like and glutathione peroxidase activities, GSH oxidation and rise in concentration of lipid hydroperoxides as that observed with the xanthine–xanthine oxidase system.

The direct inactivation of glutathione peroxidase by 200 μmol mercaptosuccinate l^{-1} (a specific inhibitor of glutathione peroxidase) emphasized the important role played by this enzymatic system in protection against oxidative stress. Although superoxide dismutase-like activity was maintained and a complete conversion of GSSG to GSH observed, concentrations of lipid hydroperoxides were significantly ($P < 0.01$) increased.

The accumulation of GSSG, despite normal glutathione reductase activity, led us to investigate the production of NADPH (which allows the recycling of GSSG to GSH by glutathione reductase) by G6PDH, an enzyme of the pentose phosphate cycle (the main metabolic pathway leading to the

production of NADPH in the cell). In agreement with our hypothesis, treatment of spermatozoa with the combination of xanthine (200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (50 mU ml^{-1}) resulted in a significant decrease ($P < 0.005$) in the production of NADPH by the G6PDH (0.98 ± 0.02 versus 0.74 ± 0.05 $\mu\text{mol NADPH formed min}^{-1} 10^{-8}$ spermatozoa, $n = 6$).

Effects of singlet oxygen

In the study reported here, we have shown that external superoxide dismutase protected the spermatozoa PUFAs against oxidation after incubation with the xanthine–xanthine oxidase system. Some previous studies suggest that the dismutation of lipid peroxyl radicals (Nakano *et al.*, 1976) and the spontaneous dismutation of $\text{O}_2^{\bullet-}$ in aqueous solution (Corey *et al.*, 1987) produce $^1\text{O}_2$. It has also already been shown that $^1\text{O}_2$ may be produced by xanthine oxidase (Kellogg and Fridovich, 1975, 1977).

The generation of $^1\text{O}_2$ in the pro-oxidant system used in this study was verified by investigating the formation of *o*-dibenzoylbenzene, by reaction of $^1\text{O}_2$ with 1,3 diphenylisobenzofuran by thin layer chromatography. The aerobic incubation of 200 μmol xanthine l^{-1} and 50 mU xanthine oxidase ml^{-1} converted 1,3 diphenylisobenzofuran to *o*-dibenzoylbenzene (data not shown). This reaction was inhibited by superoxide dismutase.

The role played by $^1\text{O}_2$ in the alterations induced by the xanthine–xanthine oxidase system was studied with histidine, a $^1\text{O}_2$ scavenger. It was first verified that histidine did not interfere with the production of reactive oxygen species by xanthine and xanthine oxidase. Histidine partially prevented

Fig. 3. Gas liquid chromatography analysis of the phospholipid fatty acid content after 6 h exposure to 200 μmol xanthine l⁻¹ and 50 mU xanthine oxidase ml⁻¹. (a) Relative modifications of the proportions of the different classes of fatty acids. Results are expressed as percentages of intact fatty acids remaining compared with the control, considered to be 100% for each class. Values are means ± SEM, *n* = 5. **P* < 0.01 compared with the control. (b) Analysis of the main phospholipid polyunsaturated fatty acids, linoleic acid (18:2 n-6), arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3). Results are expressed as percentages of intact polyunsaturated fatty acid (PUFA) compared with the control. Values are means ± SEM, *n* = 5. **P* < 0.001 compared with the control.

the increase in concentration of lipid hydroperoxides and the loss of PUFA content in sperm plasma phospholipids (Table 4).

Discussion

The xanthine–xanthine oxidase system is known to produce reactive oxygen species that are involved in cellular degradation in several cell types, including spermatozoa. The present

study shows that exposure of selected human spermatozoa to the reactive oxygen species generated by a xanthine–xanthine oxidase system leads to an increase of lipid hydroperoxide content, loss of membrane PUFAs, diminished capacity to undergo the acrosome reaction and loss of motility. The ionophore-induced acrosome reaction was a more sensitive parameter than motility in relation to oxidative stress, and both the rate of production and the amount of reactive oxygen species generated by the xanthine–xanthine oxidase system influenced the inhibition of the acrosome reaction. The use of specific scavengers (catalase, superoxide dismutase) allowed us to show that H₂O₂ and O₂^{•-} play a role in the alterations induced by xanthine and xanthine oxidase. The presence of catalase completely reversed all the cytotoxic effects observed, whereas superoxide dismutase inhibited only the increase of lipid hydroperoxide content and the degradation of PUFAs, but did not have any protective effect on the ionophore-induced acrosome reaction and motility. In fact, all the effects of the xanthine–xanthine oxidase system on the enzymatic antioxidant defence systems, glutathione and lipid hydroperoxide concentrations can be mimicked by the direct addition of H₂O₂, and can be inhibited by the presence of catalase. These results are in agreement with previous studies in which H₂O₂ was proposed as the most toxic reactive oxygen species for human spermatozoa (Alvarez and Storey, 1989; De Lamirande and Gagnon, 1992a; Aitken *et al.*, 1993a).

The accumulation of GSSG after an incubation of spermatozoa with H₂O₂ was described by Alvarez and Storey (1989) who concluded that this accumulation was responsible for a decrease in glutathione peroxidase activity. The results presented here show that glutathione peroxidase is inhibited *per se* and not by a lack of its substrate GSH after exposure to H₂O₂ or the xanthine–xanthine oxidase system. They also show that GSSG accumulates in spermatozoa in spite of a decreased glutathione peroxidase activity and an unchanged glutathione reductase activity. The efficacy of the protection against lipoperoxidation afforded by glutathione peroxidase and GSH is dependent upon the recycling of oxidized glutathione (GSSG) into its reduced form (GSH). This recycling is done by glutathione reductase and needs NADPH as a reducing co-factor. The accumulation of GSSG despite normal glutathione reductase activity may be linked to a lack of NADPH in the spermatozoa. The main metabolic pathway leading to the production of NADPH in cells is the pentose phosphate cycle in which G6PDH transforms glucose-6-phosphate into phospho-6-glucono-lactone with the production of NADPH.

Table 2. Effects of reactive oxygen species scavengers on motility, ionophore-induced acrosome reaction and membrane polyunsaturated fatty acid (PUFA) content after 6 h of incubation with the combination of xanthine (X) (200 μmol l⁻¹) and xanthine oxidase (XO) (50 mU ml⁻¹)

Parameter	Control	X-XO	X-XO + catalase	X-XO + superoxide dismutase	X-XO + mannitol
Motility (%)	87.6 ± 3.0	66.4 ± 6.1*	83.2 ± 3.0	69.5 ± 11.0*	58.4 ± 7.1**
Acrosome reaction (%)	22.3 ± 1.2	9.3 ± 0.7***	19.3 ± 1.8	8.7 ± 1.8***	12.0 ± 1.2***
% PUFA/control	100	79.1 ± 5.8*	99.0 ± 0.9	96.5 ± 4.9	81.5 ± 6.3*

Values are means ± SEM, *n* = 5.

P* < 0.01; *P* < 0.005; ****P* < 0.001 compared with control values.

Table 3. Influence of the combination of xanthine (X; 200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (XO; 50 mU ml^{-1}), H_2O_2 and mercaptosuccinate (MCS) on the superoxide dismutase (SOD)-like and glutathione peroxidase/reductase (GPX/GRD) activities, oxidized glutathione/reduced glutathione (GSSG/GSH) content, and hydroperoxide concentrations after 3 h of incubation

Treatment	Activity (U 10^{-8} spermatozoa) SOD	Activity (nmol min^{-1} 10^{-8} spermatozoa)		Glutathione content (nmol 10^{-8} spermatozoa)			Hydroperoxides (nmol 10^{-8} spermatozoa)
		GPX	GRD	Total	GSSG	GSH	
Control	6.5 $\pm$ 0.9	20.8 $\pm$ 1.9	45.5 $\pm$ 2.6	6.2 $\pm$ 0.6	2.8 $\pm$ 0.6	3.4 $\pm$ 1.2	4.8 $\pm$ 0.2
X-XO	1.2 $\pm$ 0.6**	8.1 $\pm$ 0.8***	47.6 $\pm$ 5.6	6.2 $\pm$ 0.5	5.2 $\pm$ 1.5*	1.0 $\pm$ 2.0*	10.7 $\pm$ 0.9**
X-XO + catalase	6.3 $\pm$ 0.8	16.5 $\pm$ 2.0	46.2 $\pm$ 2.9	6.3 $\pm$ 1.0	3.1 $\pm$ 0.8	3.2 $\pm$ 1.8	5.7 $\pm$ 0.7
X-XO + SOD	—	7.8 $\pm$ 1.5***	45.8 $\pm$ 4.0	6.2 $\pm$ 0.7	4.7 $\pm$ 1.0*	1.5 $\pm$ 1.7*	6.5 $\pm$ 0.4
H_2O_2	3.6 $\pm$ 0.8*	8.5 $\pm$ 1.3***	46.0 $\pm$ 3.0	6.2 $\pm$ 0.6	4.1 $\pm$ 1.1*	2.1 $\pm$ 1.7*	10.2 $\pm$ 0.8**
MCS	5.7 $\pm$ 0.5	3.8 $\pm$ 1.0***	46.2 $\pm$ 3.1	6.4 $\pm$ 0.3	0.2 $\pm$ 0.1 [†]	6.2 $\pm$ 0.4 [†]	12.9 $\pm$ 0.9**

Values are means $\pm$ SEM, $n = 7$.

GSH was calculated as the difference between the total glutathione content and GSSG.

Statistical analyses were performed using the unpaired Student's *t*-test.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control values, [†] $P < 0.01$ compared with all other groups.

Table 4. Protective effects of histidine on hydroperoxides and membrane polyunsaturated fatty acid (PUFA) content after treatment by the xanthine (X; 200 $\mu\text{mol l}^{-1}$) and xanthine oxidase (XO; 50 mU ml^{-1})

Treatment	Hydroperoxides (nmol 10^{-8} spermatozoa)	% PUFA/control
Control	5.5 $\pm$ 0.5	100
X-XO	10.2 $\pm$ 0.8***	79.1 $\pm$ 5.8**
X-XO + histidine	7.6 $\pm$ 0.5*	92.5 $\pm$ 1.4*

Values are means $\pm$ SEM, $n = 5$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ compared with control values.

G6PDH is known to be very susceptible to oxidative stress (Oliver *et al.*, 1987a), and its inactivation may lead to the blockade of the pentose phosphate pathway, and a resulting decrease in NADPH concentration in the cell, leading to a decrease in the antioxidative protection exerted by the glutathione-dependent system. This has been shown to occur in other biological models (Starke-Reed and Oliver, 1989) and also in physiological processes like ageing (Noy *et al.*, 1985; Oliver *et al.*, 1987b) and the hypothesis can now be extended to spermatozoa. The activity of G6PDH is partially impaired by the xanthine-xanthine oxidase system. This significant decrease of the G6PDH activity may be one factor leading to a decrease of NADPH in spermatozoa and the inhibition of the recycling of GSSG into GSH despite the unaltered enzymatic activity of the glutathione reductase. In recent studies, De Lamirande and Gagnon (1992a, b) showed that reactive oxygen species produced by xanthine and xanthine oxidase, or by H_2O_2 interfere with the energy production metabolism in human spermatozoa leading to a drop in ATP concentrations. They hypothesized that these effects may be due to the inhibition of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the glycolytic pathway. The results presented in this study show that reactive oxygen species can effectively inhibit a metabolic pathway involved in energy production

(NADPH). They also suggest that such an inhibition can have important consequences for the antioxidant protection of spermatozoa, and point out a possible link between these two phenomena.

The decrease of the superoxide dismutase-like and glutathione peroxidase activities after addition of H_2O_2 suggests that the mechanism by which H_2O_2 mediates its effects on spermatozoa may be linked to the inhibition of the activities of these two protective enzymes. However, the activity of glutathione reductase remained unchanged pointing out that the observed reduction of superoxide dismutase-like and glutathione peroxidase activities were not due to leakage of the enzymes from peroxidized cells. It has been shown that the decrease of glutathione peroxidase activity induced by the xanthine-xanthine oxidase system or by H_2O_2 was linked to a direct inhibition of glutathione peroxidase. Such an inhibition by hydroperoxides has been described in mammalian cells and shown to be associated to an oxidative modification of the protein (Ochi, 1990). The selenium atoms present at the catalytic site of the enzyme undergo a redox cycle during the enzymatic detoxification of hydroperoxides by glutathione peroxidase, between a reduced (selenol) and an oxidized form (selenic acid) (Flohe, 1982 cited in Ochi, 1990). It was hypothesized that the increase of the oxidation level of the cell slows down the return to the reduced state of selenium, leading to a reversible inhibition of the enzyme. Thus, if the observed oxidation of GSH to GSSG does not account directly for the decreased glutathione peroxidase activity in our model, the depletion of reducing substances in the spermatozoa after xanthine-xanthine oxidase treatment may be responsible for the presence of the inactive form of the enzyme.

H_2O_2 -induced impairment of superoxide dismutase activity has been previously reported (Salo *et al.*, 1990; Zini *et al.*, 1993) but it is the first time, to our knowledge, that it is shown to occur in human spermatozoa after reactive oxygen species treatment. Superoxide dismutase plays a key role in protecting human spermatozoa since its activity is well correlated with time of motility loss (Alvarez *et al.*, 1987). The inhibition of superoxide dismutase-like activity by H_2O_2 and the

Fig. 4. Schematic representation for the hypothesis of the events triggered by the xanthine–xanthine oxidase system on some enzyme activities of human spermatozoa. Lipid peroxidation is shown as the result of the hydrogen peroxide-induced inhibition of the antioxidant enzymatic defence systems of the spermatozoa. By decreasing the superoxide dismutase, glutathione peroxidase and glucose-6-phosphate dehydrogenase activities, hydrogen peroxide would permit, in synergy with the endogenous production of reactive oxygen species, the accumulation of toxic lipid peroxides and the development of lipid peroxidation. SOD: superoxide dismutase; GPX: glutathione peroxidase; GRD: glutathione reductase; G6PDH: glucose-6-phosphate dehydrogenase; GSSG: oxidized glutathione; GSH: reduced glutathione.

consequences of decreased glutathione peroxidase activity on the functional properties of human spermatozoa emphasize the importance of these two enzymes in the antioxidant defence system of spermatozoa, and the importance of their cooperation in exerting optimal protection of the cells. The role of glutathione peroxidase in the antioxidant protection of the lipid constituents of spermatozoa is further shown by its direct inactivation by mercaptosuccinate, which provokes a rise in the concentration of lipid hydroperoxides.

Lipid peroxidation was induced by the xanthine–xanthine oxidase system, as shown by the increase in the lipid hydroperoxide content and the degradation of membrane PUFAs. H_2O_2 and $\text{O}_2^{\bullet-}$ were both involved in the initiation of peroxidation since catalase and superoxide dismutase can prevent the degradation of the PUFAs. It is known that H_2O_2 and $\text{O}_2^{\bullet-}$ can produce $^{\circ}\text{OH}$ in the presence of transition metals (Fenton reaction), but as Aitken *et al.* (1993a) reported, mannitol was ineffective in protecting spermatozoa against xanthine–xanthine oxidase-induced lipoperoxidation. However, the involvement of $^{\circ}\text{OH}$ radicals cannot be ruled out. Mannitol is a polar molecule restricted to the extracellular medium, whereas H_2O_2 can diffuse freely across biological membranes. An intracellular Fenton reaction is possible with $^{\circ}\text{OH}$ scavengers and iron chelators capable of penetrating cells. This possibility is emphasized by results showing that

dimethyl sulfoxide, a lipophilic $^{\circ}\text{OH}$ scavenger, delays the immobilization of spermatozoa induced by a xanthine–xanthine oxidase system (De Lamirande and Gagnon, 1992a). Moreover, the cellular functions inhibited by H_2O_2 , the acrosome reaction and motility are dependent on intracellular structures such as acrosomes, mitochondria and axonemes. As H_2O_2 is produced in the extracellular medium by the xanthine–xanthine oxidase system, this may imply that H_2O_2 must cross the plasma membrane to exert its toxic effects. Singlet oxygen reacts strongly with unsaturated fatty acids to form hydroperoxides (Rawls and Van Santen, 1970). Histidine is known to be an $^1\text{O}_2$ scavenger (Lynch and Fridovich, 1978) but can also react with $^{\circ}\text{OH}$. The inefficacy of mannitol to protect the spermatozoa, the conversion of 1,3 diphenylisobenzofuran to *o*-dibenzoylbenzene in the presence of the xanthine–xanthine oxidase system and the partial protection afforded to the PUFAs by histidine suggest that $^1\text{O}_2$ oxidation of fatty acids significantly contributes to the lipid alterations of spermatozoa induced by the xanthine–xanthine oxidase system.

The results presented in this study lead us to propose the following sequence of events triggered by the xanthine–xanthine oxidase system against human spermatozoa (Fig. 4). First, the xanthine–xanthine oxidase system produces $\text{O}_2^{\bullet-}$ which dismutates spontaneously into H_2O_2 and possibly $^1\text{O}_2$.

Hydrogen peroxide diffuses across the membranes into the cells and inhibits the activities of some enzymes: G6PDH, leading to a decrease in the production of NADPH and a concomitant accumulation of GSSG, glutathione peroxidase and superoxide dismutase, leading to a decrease in the antioxidant defences of the spermatozoa. The disorganization of the antioxidant defences then leads to the peroxidation of the membrane phospholipids by reactive oxygen species. As a consequence of the changes in membrane fluidity and integrity induced by the accumulation of lipid peroxides, the ionophore-induced acrosome reaction and sperm movement are disrupted.

Much remains to be directly studied, but this hypothesis takes into account the results obtained in the many studies on reactive oxygen species and human spermatozoa. H_2O_2 appears to be the most toxic species because of its ability to cross membranes freely and to inhibit enzyme activities and cellular functions. The inhibition of energy metabolism by H_2O_2 may then explain the reversible immobilization of spermatozoa. Overall membrane degradation, as measured by the phospholipid fatty acids degradation, appears to occur later, possibly as a consequence of the inhibition of G6PDH, glutathione peroxidase and superoxide dismutase activities. A global lipid peroxidation may indicate a switch between reversible alterations due to the early inhibition of energy metabolism and irreversible structural degradations. H_2O_2 produced by xanthine and xanthine oxidase is responsible for the inhibition of the antioxidant defence system of spermatozoa, but the production of reactive oxygen species by this pro-oxidant system stops rapidly. This suggests that the reactive oxygen species responsible for the lipid alterations may be produced by the spermatozoa themselves. It also suggests that reactive oxygen species induce chain reactions independent of the reactive oxygen species themselves, the effects of which are observed only hours later.

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