Effects of Hydrogen Peroxide on Human Spermatozoa*

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Purpose: Reactive oxygen species (ROS) have been reported widely to cause deleterious effects on sperm viability and function due to peroxidation of membrane lipids. However, their action appears more selective at low concentrations; recent evidence indicates that the superoxide anion can promote capacitation and induce hyperactivated motility (HA) in human spermatozoa and that hydrogen peroxide (H2O2) may participate in capacitation of hamster spermatozoa. The objective of these studies was to investigate the direct effects of H2O2 on functions crucial to fertilization in human spermatozoa.

Methods: In these prospective studies, we examined the dose- and time-dependent effects of H2O2 on sperm membrane-mediated events (binding to the zona pellucida and changes in intracellular calcium concentration [Ca2+]i, motility patterns, and acrosome reaction). Sperm from fertile donors were used in the experiments under capacitating conditions after separation of the motile fraction by wash-swim-up. [Ca2+]i was measured by the fluorescent fura-2 indicator, and sperm-zona pellucida binding was assessed with the hemizona assay (HZA). Hyperactivated motility was evaluated by computerized analysis, and the percentage of acrosome reacted sperm was detected by FITC-Pisum sativum lectin and indirect immuno- fluorescence.

Results: In the HZA, H2O2 did not influence sperm-zona pellucida binding at low concentrations (0.05 mM and 0.1 mM), but significantly reduced binding at 0.2 mM (P < 0.004 vs controls). H2O2 significantly decreased HA in a dose-dependent manner (P < 0.0001) and had a significant effect (P < 0.01) on acrosome reaction (stimulatory effect at 0.01 mM). H2O2 did not affect basal [Ca2+]i; however, H2O2 (0.1 mM through 10 mM) decreased the initial phase of progesterone-induced (P4: 1 μM) enhancement of [Ca2+]i in a dose- and time-dependent fashion. Preincubation of sperm with catalase (20 μg/ml) potentiated the P4-induced increase of [Ca2+]i. H2O2 did not significantly modify [Ca2+]i increase in response to inomycin (10 μM).

Conclusions: These experiments show that H2O2 directly affects sperm functions crucial to fertilization in a dose- and time-dependent fashion. Low concentrations maintain capacitation, whereas higher concentrations have deleterious effects, as determined by the end points of the capacitation process. The latter effects are probably dependent on modifications of plasma membrane and intracellular homeostasis by the oxidative process.

KEY WORDS: hydrogen peroxide; reactive oxygen species; human sperm.

INTRODUCTION

Reactive oxygen species (ROS) have been widely reported to cause deleterious effects on sperm viability and function due to peroxidation of membrane lipids. Reactive oxygen species such as the superoxide anion, hydrogen peroxide (H2O2), and the hydroxyl radical can be produced by spermatozoa (1,2). Additionally, phagocytic leukocytes present in semen have also been shown to produce ROS (3,4). Due to their high content of polyunsaturated fatty acids, human spermatozoa are especially sensitive to ROS-induced damage and H2O2 appears to be the most toxic ROS under in vitro conditions (5–9). Investigations have shown that damaged or
defective spermatozoa produce high levels of ROS, whereas sperm from fertile men (donors) do not (1,2,6,9).

To counteract the effects of ROS, spermatozoa and seminal plasma possess systems to scavenge ROS and prevent intracellular damage, including the presence of superoxide dismutase, the glutathione peroxidase/reductase system, catalase, proteins (such as albumin) and other molecules (vitamin E and C, taurine, and hypotaurine) (5,8,10-13). Recently, it was demonstrated that the superoxide anion, generated by the combination of xanthine plus xanthine oxidase (in the presence of catalase), induced hyperactivated motility (HA) and capacitation in human spermatozoa (14). Furthermore, H₂O₂ was also shown to be involved in the capacitation process of hamster spermatozoa (15).

Because of the dual effects observed with H₂O₂ (deleterious effects at high concentrations, and more selective, positive effects on capacitation at low concentrations), we designed these studies to examine dose- and time-dependent effects of H₂O₂ on functions crucial to fertilization in human spermatozoa. These functions included sperm–zona pellucida binding, HA, acrosome reaction and its prerequisite, an increase in the intracellular levels of calcium [Ca²⁺].

MATERIALS AND METHODS

Semen Donors and Sperm Preparation

Semen samples were obtained from four normozoospermic men (donors) by masturbation after 2 to 4 days of sexual abstinence. These men were active participants of the donor insemination program of our Institute, had fathered a pregnancy within the last year, and had negative bacteriologic semen cultures. All semen samples were studied after liquefaction was complete (<30 min) and within 1 h of collection. Following liquefaction, the semen samples were subjected to a basic evaluation of sperm parameters (concentration, progressive motility, and morphology as previously published) followed by a swim-up separation of the sperm motile fraction (16). All ejaculates used in the experiments had an original sperm concentration ≥60 x 10⁶/ml, progressive motility ≥50%, normal morphology >14% (strict criteria), and <0.1 x 10⁶/ml leukocytes (by peroxidase staining). For the swim-up separation, 1 ml of semen was diluted with 1 ml of medium consisting of Ham’s F-10 (Gibco Lab., Grand Island, NY) supplemented with 0.3% human serum albumin (HSA) (Irvine Sci., Santa Anna, CA) and centrifuged gently at 290 g for 8 min. The sperm pellet was washed by centrifugation for 5 min. Finally, 1 ml of medium was gently layered over the undisturbed pellets, and specimens were incubated for 1 h at 37°C in 5% CO₂ in air. The supernatant was removed and adjusted to appropriate concentrations depending on experiments.

Hyperactivated Motility

Motility characteristics were objectively measured with the HTM-2030 motility analyzer (Hamilton-Thorn Research, Danvers, MA) using fixed parameter settings previously established (17). Each sample (a 5-µl aliquot of swim-up adjusted to 20 x 10⁶ motile sperm/ml) was loaded by capillary action into a microcell counting chamber (20 µm deep) and the tube transferred to the HTM-2030 where it was maintained at 37°C for 1 min prior to the start of data acquisition. Data collection was completed on randomly selected fields until at least 100 motile spermatozoa were analyzed. Motility patterns consistent with HA were identified as previously described (17,18).

Hemizona Assay

Tight binding of sperm to the human zona pellucida was assessed using the HZA as previously detailed (19,20). Briefly, salt-stored human oocytes were microbisected into matching hemizonae using Narishige micromanipulators (Narishige, Tokyo, Japan) mounted on a phase-contrast inverted microscope (Nikon Diaphot, Garden City, NY). Control and test sperm droplets (100 µl of a final dilution of 0.5 x 10⁵ motile sperm/ml) were separately incubated under oil with the matching hemizonae for 4 h at 37°C in 5% CO₂ in air. After the coincubation period, the hemizonae were washed to remove loosely attached sperm using a finely drawn glass pipette and the number of sperm tightly bound to the outer zona surface was counted under phase-contrast microscopy (x200).

Acrosomal Status

The proportion of acrosome-reacted sperm was determined using the fluorescent probe fluorescein isothiocyanate (FITC)-labeled Pisum sativum lectin.