Human sperm chemotaxis depends on critical levels of reactive oxygen species

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Objective: To verify whether chemotaxis is in part an oxidative process mediated by reactive oxygen species (ROS).
Design: In this prospective study, after removal of seminal plasma, the sperm suspension received no treatment (control), ROS formation by stimulation with phorbol 12-myristate 13-acetate (PMA), antioxidant treatment (with catalase), or PMA stimulus in the presence of catalase. At time zero and after 3 hours of incubation, the percentage of capacitated and oriented spermatozoa and the ROS levels were determined.
Setting: Andrology laboratory in a medical research institution.
Patient(s): Normal semen was obtained from eight men.
Intervention(s): The semen samples were evaluated to determine the effect of ROS production by stimulation with PMA and/or antioxidant treatment (with catalase) on the percentage of capacitated and oriented spermatozoa.
Main Outcome Measure(s): The sperm capacitation, chemotaxis and reactive oxygen species were assessed before and after PMA and/or antioxidant treatment.
Result(s): Prolonged exposure to high quantities of ROS decrease the sperm chemotactic response, probably because of oxidative damage of the cell. However, this effect may be reduced by the addition of antioxidants like catalase.
Conclusion(s): Similar to capacitation, chemotaxis seems to depend on the production of ROS, but in the latter process there may be a critical level of ROS necessary for chemotaxis to occur. (Fertil Steril® 2010;93:150–3. ©2010 by American Society for Reproductive Medicine.)

Key Words: Human spermatozoa, ROS, antioxidant, chemotaxis, sperm capacition

Mammalian spermatozoa usually produce reactive oxygen species (ROS) as part of a normal metabolism. However, oxidative stress may result from an imbalance between the production of free radicals (mainly ROS) and the ability of the antioxidants to degrade them. Consequently, significant oxidative damage occurs to many cellular organelles by damaging lipids, proteins, DNA, and carbohydrates, thus leading to cell death. The sperm cell is particularly susceptible to oxidative damage because of the lack of cytoplasm, which is also a source of antioxidants (1, 2). Immature spermatozoa and seminal leukocytes are the major source of ROS in semen. Seminal leukocytes produce H_2O_2, which is the most toxic form of ROS for spermatozoa since it rapidly crosses their membranes. Excessive ROS generation can overwhelm the protective mechanism and initiate lipid peroxidation in sperm plasma membranes. Lipid peroxidation causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased nonspecific permeability to ions. Thus, sperm oxidative stress may affect reproduction and fertility (1). On the other hand, antioxidant molecules could reduce the impact of H_2O_2. For example, catalase catalyzes the decomposition of H_2O_2 into water and oxygen (2).

Nevertheless, spermatozoa need ROS to normally function, and low levels of ROS are essential for capacitation, hyperactivation, motility, and acrosome reaction (3, 4). Sperm capacitation is a process that prepares the spermatozoa to fertilize the egg. It has been reported that ROS induce sperm capacitation in human and animal models by the redox-mediated protein tyrosine phosphorylation (1).

Furthermore, only capacitated spermatozoa are able to orient their movement toward the source of an attractant, which is called chemotaxis (5–8). Since sperm capacitation depends on ROS formation (1, 9) and chemotaxis depends on capacitation (5, 6), the purpose of this work was to verify whether chemotaxis is also influenced by ROS.

MATERIALS AND METHODS

Chemicals and Culture Medium

Chemicals were from Sigma-Aldrich (St. Louis), except dihydroethidine, which is from Molecular Probes (Eugene,

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OR), and the salts, which are from Merck (Darmstadt, Germany). Bovine follicular fluid was obtained and lyophilized as described by Kurosaka et al. (10). The culture medium was Biggers, Whitten, Whittingham medium (BWW) (11), at pH 7.4, supplemented with Hepes (45 mM) and 4% bovine serum albumin (fraction V).

Sperm Preparation

Human semen samples were obtained by masturbation from eight healthy donors after 3–5 days of sexual abstinence. The donors were informed verbally and in writing about the aims of the investigation and signed the consent for the use of their sperm. This study was approved by the Institutional Review Board of the Faculty of Medicine at the Universidad de La Frontera. Only those samples showing normal sperm density, motility, and morphology according to World Health Organization guidelines were included in the study. The semen samples were allowed to liquefy for 30 minutes at 37°C, and then spermatozoa were separated from the seminal plasma by the migration-sedimentation technique to prevent damage produced by centrifugation (12) in an atmosphere of 5% CO_2 in air at 37°C. The highly motile sperm population was recovered and adjusted to 4.5×10^6 cells/mL. The cells were immediately divided into four treatments as follows: spermatozoa in BWW (as a control), spermatozoa incubated with 0.1 mM phorbol 12-myristate 13-acetate (PMA) to induce ROS production (13), spermatozoa with 2600 U/mL catalase as an antioxidant, spermatozoa with both PMA and catalase as indicated above. In each treatment, the spermatozoa were evaluated for capacitation, chemotaxis, and ROS production at time zero (defined as the end of the sperm separation from the seminal plasma) and after 3 hours of incubation under the capacitating conditions mentioned above.

Capacitation

The criterion to evaluate the level of capacitated spermatozoa was the percentage of spermatozoa that underwent the acrosome reaction upon pharmacological stimulation. As previously recommended by the Andrology Special Interest Group of the European Society for Human Reproduction and Embryology, we used the calcium ionophore A23187 to stimulate the acrosome reaction (14) only in capacitated spermatozoa (15, 16).

The level of capacitated sperm was determined as described elsewhere (6) from the difference between the percentage of acrosome-reacted spermatozoa before (spontaneous acrosome reaction) and after calcium ionophore stimulation. The acrosome-reacted spermatozoa were visualized by the acrosome marker *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate.

Chemotaxis Assay

Chemotaxis assays were performed at 37°C in a modified Zigmond chemotaxis chamber consisting of two wells separated by a wall and closed with a coverslip sealed with paraffin as described elsewhere (6). Briefly, one of the wells contained spermatozoa in BWW and the other one contained the attractant or BWW as a negative control. Bovine follicular fluid (10^{-4} in BWW) was used as the attractant since it has been shown to chemoattract human spermatozoa (7) and because large quantities of it can be easily obtained. This construction allowed the formation of a stable, one-dimensional concentration gradient of the chemoattractant between both compartments. The movement of sperm cells on top of the partition wall (in the middle of the field between the two wells) was videotaped at 63× for 5 minutes. The sperm tracks were subsequently analyzed using the Sperm Track 2.9 software (Center for Cellular and Molecular Biology, Universidad Nacional de Córdoba, Argentina), and the percentage of oriented cells in each treatment was determined. The occurrence of chemotaxis was calculated by discounting the percentage of oriented spermatozoa observed in the absence of attractant (culture medium alone) from the value obtained in the presence of the attractant (follicular fluid).

ROS Determination

ROS production was measured by means of 20 μM dihydroethide, a permeant fluorescence oxidative marker that changes into 2-hidroxyethide when it is oxidated by the superoxide anion; it is then trapped inside the nucleus and generates a red fluorescence (17). Spermatozoa were incubated with dihydroethide for 15 minutes at 37°C. The cells were subsequently centrifuged at $300 \times g$ for 10 minutes. The samples were smeared into a slide, and the percentage of red fluorescence–labeled cells (ROS-producing cells) was determined under an epifluorescence microscope (Nikon, Tokyo, Japan) at ×1000.

Statistical Analysis

Eight experiments were performed, analyzing a total of 1600 cells per treatment for induced acrosome reaction, 1200 for chemotaxis, and 1600 for ROS production. Data were analyzed with a nonparametric analysis of variance and Tukey tests using the Prisma 3.0 GraphPad software (GraphPad Software, Inc., La Jolla, CA). $P \leq .05$ was considered statistically significant.

RESULTS

Capacitation

At time zero, there were no significant differences between treatments in the level of induced acrosome reacted spermatozoa (Fig. 1A). However, after 3 hours of incubation, there was a significant decrease in the level of induced acrosome reacted spermatozoa in the samples treated with PMA in comparison with time zero and in the samples subjected to other treatments after 3 hours of sperm incubation ($P<.001$). The capacitated cells under catalase treatment did not significantly differ from those observed in the control group.