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EFFECT OF SPERM WASHING ON LEVELS OF REACTIVE OXYGEN SPECIES IN SEMEN

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The possibility was evaluated that the production of reactive oxygen species (ROS) by human sperm is stimulated by the repeated cycles of centrifugation and resuspension involved in conventional sperm preparation. ROS generation by human sperm was monitored before and after the washing of sperm from 55 men (43 men with suspected subfertility and 12 normal volunteers). The ROS activity of all 55 specimens before washing was inversely correlated with original sperm motility ($r = .278$, $p < .05$). The mean level of ROS activity was significantly higher after washing than before processing ($p < .05$) for the 26 specimens with normal sperm motility, the 20 specimens with normal sperm morphology, and the 12 specimens with both normal motility and normal morphology. In contrast, the mean ROS level was not significantly changed after washing in the 27 specimens with poor sperm motility, the 16 specimens with abnormal sperm morphology, or the 13 specimens with both abnormal motility and abnormal morphology. It would appear that repeated centrifugation, resuspension, and vortexing cause excessive generation of ROS in the motile sperm population of the washed specimen. Washing procedures involving excessive manipulation of sperm may, in fact, cause the most harm to motile sperm, i.e., those that the method is trying to select. Procedures that minimize multiple centrifugation, resuspension, and vortexing steps should therefore be used for the preparation of semen specimens for assisted-reproduction techniques.

Keywords spermatozoa, infertility, ROS, centrifugation, sperm washing

Defective sperm function has been identified as the largest defined cause of human infertility, accounting for attendance at infertility clinics in about 27% of all cases [7]. Despite its high incidence, few effective therapies for this condition are available. This paucity of therapeutic options is largely a consequence of our lack of knowledge concerning the precise biochemical nature of the defects responsible for the sperm's loss of fertilizing potential. Human

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sperm are particularly susceptible to peroxidative damage because they contain an extremely high concentration of polyunsaturated fatty acids, exhibit no capacity for membrane repair, and possess a significant ability to generate reactive oxygen species (ROS), chiefly superoxide anion and hydrogen peroxide [2, 4, 9].

The conventional technique for preparing human sperm involves repeated cycles of centrifugation and resuspension in a simple culture medium. This procedure separates the sperm from the seminal plasma but does not incorporate any element of sperm selection. Cell suspensions prepared by the repeated-centrifugation technique retain leukocytes, squamous epithelial cells, or noncellular debris that may have contaminated the original semen sample as well as a varying number of nonmotile and nonviable sperm. In an investigation of the possibility that free radical-generating activity might be stimulated by the mechanical perturbation of the plasma membrane associated with this particular preparation technique, we monitored ROS generation by human sperm before and after washing.

MATERIALS AND METHODS

Selection of Subjects. Semen samples were obtained from 43 randomly selected men coming to our laboratory because of suspected subfertility. Specimens from 12 volunteers with normal results of semen analysis served as controls.

Collection of Semen and Assessment of Semen Parameters. Semen specimens were collected by masturbation after at least 2 days of sexual abstinence and liquefied at 37°C; 5 μL was loaded on a 20-μ Microcell chamber (Fertility Technologies, Inc., Natick, MA, USA) and analyzed on a Hamilton–Thorn motility analyzer, HTM version 7 (Hamilton–Thorn Research, Beverly, MA, USA). Semen smears prepared from liquefied specimens were stained by Hemacolor (EM Diagnostic, Inc., Gibstown, NJ, USA) for assessment of sperm morphology by standard methods. One hundred cells were scored according to the guidelines established by the World Health Organization [13] for normal forms; head, neck, and tail defects; and undifferentiated round cells at a magnification of 400× (Olympus BH2, Tokyo, Japan).

Quantitation of White Blood Cells. The presence of granulocytes in semen specimens was assessed by the Endtz test [6]. A 20-μL volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial; 20 μL of phosphate-buffered saline and 40 μL of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 min. Peroxidase-positive white blood cells (WBCs) staining dark brown were counted in all 100 squares of the grid in a Makler chamber under the 20× bright-field objective. The results after correction for dilution were recorded as counts × 10^6/mL.

Measurement of ROS Activity. The formation of ROS was measured with a computer-driven Luminometer (model 1251, LKB-Wallac, Turku, Finland). Luminescence was recorded after the addition of 20 μL of 4 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Bio-Orbit, Turku, Finland) to 1.0 mL of washed sperm in human tubal fluid (HTF) medium (Fertility Technologies, Inc., Natick, MA, USA). Measurement of ROS activity vas started 3 min after the addition of luminol and continued for 30 cycles. The readings were taken in the integration mode, with constant mixing of the analyzed sample, for 10 s. HTF medium alone acted as a control for each specimen analyzed for ROS activity. ROS activity was expressed (mV/s/10^9 sperm) as the mean ± SE for 30 cycles. The difference between experimental and control readings was considered significant if the comparison yielded a p value of $<.05$.

Sperm Washing Procedure. The semen was mixed with HTF medium in a 1:4 ratio and centrifuged for 10 min at 500*g* (1600 rpm) in an IEC centrifuge (International Equipment, Needham, MA, USA). The supernatant was discarded, and pelleted spermatozoa were resuspended in 3.0 mL of fresh medium and centrifuged under the same conditions. After the second spin, the supernatant was discarded and the final pellet was resuspended in 0.5 mL of HTF medium and used for analysis of ROS formation.

Statistical Analysis. Statistical analysis included comparison by Wilcoxon's signed rank test of the mean ROS values before and after washing of specimens. A p value of $<.05$ was considered significant. If the p value was $>.05$ and $<.1$, the result was considered marginally significant. Correlation of ROS generation with semen parameters was evaluated by least-squares linear regression with use of the InStat program (GraphPAD Software, Inc., San Diego, CA, USA) in an IBM PS/2 computer.

RESULTS

A total of 55 specimens were tested for ROS activity. Of the specimens from patients, 35 were Endtz negative ($<1 \times 10^6$ WBCs/mL) and 8 were Endtz positive. Of the 12 specimens from donors with normal fertility, 10 were Endtz negative and 2 were Endtz positive.

ROS and Sperm Motility

The mean level of ROS generation for all 55 semen specimens before washing was inversely correlated with sperm motility in the unprocessed raw semen ($r = .287$, $p < .05$). The 26 specimens with normal sperm motility had a significantly higher mean ROS level after sperm washing than before processing ($p < .05$). In contrast, no significant difference was seen before and after washing in the 27 specimens with abnormal initial sperm motility (Table 1).

The mean ROS value increased significantly ($p < .05$) after sperm washing for the 19 Endtz-negative specimens with normal sperm motility (51.81 ± 25.26 mV/s/10^9 sperm) over the mean value for raw specimens (22.61 ± 20.33 mV/s/10^9 sperm). However, no significant difference in mean ROS level before and after sperm washing was found for the 27 Endtz-negative specimens with abnormal sperm motility. Before washing, the mean level of ROS generation was significantly lower ($p < .0001$) for specimens with normal motility than for

TABLE 1 Comparison of ROS Generation in 55 Semen Specimens (43 from Men with Suspected Subfertility and 12 from Donors with Normal Fertility) Before and After Sperm Washing

Sperm Characteristics (Number of Specimens)	ROS (Mean ± SE, mV/s/10^9 Sperm		Probability
	Unprocessed Specimen	Washed Specimen	
Normal motility (26)	11.93 ± 11.18	29.48 ± 10.01	.0039
Abnormal motility (27)	16.63 ± 6.26	64.22 ± 51.79	NS
Normal morphology (20)	3.48 ± 1.46	19.09 ± 7.69	.0025
Abnormal morphology (16)	6.95 ± 1.88	16.57 ± 7.86	NS
Normal motility and morphology (12)	0.40 ± 0.09	20.23 ± 11.77	.0005
Abnormal motility and morphology (13)	8.40 ± 2.12	10.50 ± 3.83	NS

Note. NS, not significant.

those with abnormal motility. No such difference between these two groups was seen after washing (Figure 1).

ROS and Sperm Morphology

The mean ROS level increased significantly ($p < .05$) after sperm washing for the 20 specimens with initially normal sperm morphology, but not for the 16 specimens with initially abnormal sperm morphology (Table 1). The mean level of ROS generation increased significantly ($p < .05$) after sperm washing for the 13 Endtz-negative semen specimens) with normal morphology (27.25 ± 11.29 mV/s/10^9 sperm) over the mean value before washing (5.10 ± 2.14 mV/s/10^9 sperm). No such difference was found for the 14 Endtz-negative specimens with abnormal morphology ($n = 14$). Before washing, the mean level of ROS generation was significantly lower ($p < .05$) for specimens with normal morphology than for those with abnormal morphology. There was difference between these two groups after washing (Figure 1).

ROS and Sperm Motility and Morphology

The mean ROS level increased significantly after washing for the 12 specimens with normal sperm motility and morphology ($p < .001$) but not for those with initially abnormal sperm motility and morphology (Table 1). Before washing, the mean level of ROS generation was significantly lower ($p < .01$) for specimens with normal motility and morphology than for

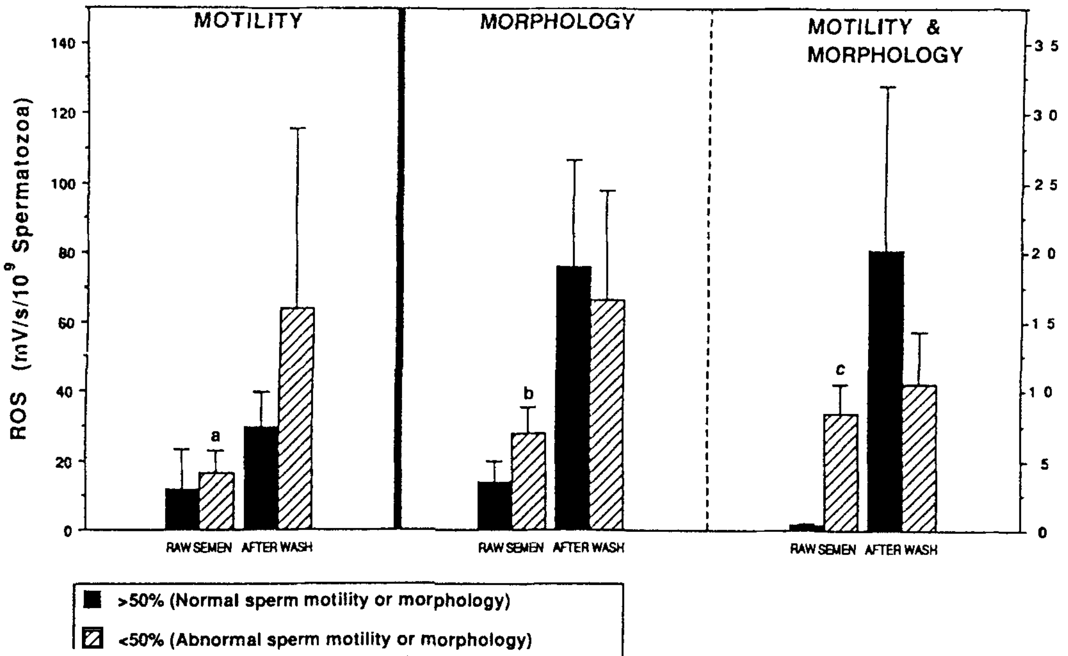

FIGURE 1 Mean level of formation of reactive oxygen species (ROS) in different semen parameter groups. Before sperm washing, ROS levels in specimens with >50% motility and/or morphology are always significantly lower than those in specimens with <50% motility and/or morphology. a, $p < .0001$; b, $p = .0435$; c, $p = .0017$.

those with abnormal motility and morphology, whereas no such difference between these two groups was seen after washing (Figure 1).

DISCUSSION

Growing interest in oxygen toxicity and free-radical reactions in biology and medicine has led to the hypothesis that cell damage can be produced by increased generation of free radicals (OH^- and O^-) [5]. In seminal plasma the presence of trace amounts of transition elements such as iron [10] may lead to the initiation of ROS synthesis, causing lipid peroxidation by hydroxyl radicals in the sperm plasma membrane [1]. Accumulation of lipid hydroperoxides in a membrane disrupts its structure and can cause it to collapse. In addition, lipid peroxides can decompose to yield a range of highly cytotoxic products. Lipid peroxidation, in turn, leads to a decrease in membrane fluidity [12], as a consequence of which the capacity of the sperm to participate in the membrane-fusion events associated with fertilization is diminished [8, 11].

The possibility that peroxidative damage to the sperm plasma membrane is involved in those cases of infertility characterized by a failure to exhibit sperm–oocyte fusion was suggested by studies indicating an association between the appearance of such defects and the hyperactive production of ROS by the sperm [2, 3]. It is postulated that, under such conditions, the main defense mechanisms of human sperm—superoxide dismutase, glutathione reductase, and a-tocopherol (vitamin E) [4, 11]—are overwhelmed, and the resulting combination of hydrogen peroxide and excess superoxide anion favors the production of hydroxyl radicals that result in peroxidative damage to human sperm.

The generation of ROS in unprocessed semen specimens in our study was found to be a property exhibited by a subpopulation of sperm characterized by poor motility and morphology. The significant increase we observed in ROS formation in specimens with normal sperm motility and/or morphology after sperm washing may have been due to damage inflicted on the normal sperm-cell membrane by repeated centrifugation and resuspension. However, in specimens with abnormal sperm parameters, which had a higher ROS level before sperm washing than specimens with normal parameters, the lack of significant increase in the ROS levels after washing may be due to the fact that already damaged sperm may have lost the capacity to generate ROS. ROS generated by the sperm in response to centrifugation should be able to initiate lipid peroxidation and, as a consequence of the resulting loss of membrane fluidity and integrity, bring about the observed decline in sperm function [2, 9].

Since oxygen radicals are rapidly exported from the germ cell, they can disrupt the plasma membranes of normal functional cells in the immediate vicinity [3]. It is therefore essential that this radical-generating subpopulation of sperm be removed from the sperm suspension before any attempt is made to centrifuge the cells. This task can be accomplished by the use of the swim-up, L4 filter membrane, albumin column, or Percoll gradient technique. The highly motile cells isolated by such procedures generate low levels of ROS.

The present experiment clearly establishes that sperm are the prime source of ROS generation in Endtz-negative semen specimens (i.e., those without WBCs). Clearly, human sperm alone can produce high levels of ROS that adversely affect their fertilizing ability. Identification of the most effective means of isolating human sperm has practical implications for such therapeutic techniques as in vitro fertilization and intrauterine insemination. It would appear that repeated centrifugation, resuspension, and vortexing cause excessive generation of ROS

by the most motile sperm in washed specimens. Washing procedures involving excessive manipulation of sperm may, in fact, cause more harm to the motile and morphologically normal sperm, i.e., those that the method is trying to select. Thus the procedure chosen should minimize centrifugation and vortex steps.

REFERENCES

1. Aitken RJ (1989): The role of free oxygen radicals and sperm function. Int J Androl 12:95–97.
2. Aitken RJ, Clarkson JS (1987): Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 81:459–469.
3. Aitken RJ, Clarkson JS (1988): Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 9:367–376.
4. Alvarez JG, Touchstone JC, Blasco L, Storey BT (1987): Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. J Androl 8:338–348.
5. Bellavite AW (1974): The superoxide-forming enzymatic system of phagocytes. Free Radic Biol Med 4:225–261.
6. Endtz AW (1974): A rapid staining method for differentiating granulocytes from "germinal cells" in Papanicolaou-stained semen. Acta Cytol 18:2–7.
7. Hull MGR (1986): Infertility: nature and extent of the problem. In Human Embryo Research: Yes or No, Book G, O'Connor M (eds). Ciba Foundation, London: Tavistock, pp 24–38.
8. Iwasaki A, Gagnon C (1992): Formation of reactive oxygen species in infertile patients. Fertil Steril, 57:409–416.
9. Jones R, Mann T, and Skerins R (1979): Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 31:531–537.
10. Kwenang A, Kroos MJ, Kosler JF, Van Eijk HG (1987): Iron, ferritin and copper in seminal plasma. Hum Reprod 2:387–388.
11. Mennela MRF, Jones R (1980): Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal ion catalyzed lipid peroxidation reactions in semen. Biochem J 191:289–297.
12. Ohyashiki T, Ohtsaka T, Mohr T (1988): Increase of the molecular rigidity of the protein characteristics and sperm penetration into cervical mucus in vitro. J Reprod Fertil 78:93–102.
13. World Health Organization (1987): WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, 2d ed. Cambridge, UK: The Press Syndicate of the University of Cambridge, p. 10.