Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients

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Objective: To investigate whether there is an impact of different sources of reactive oxygen species (ROS) on sperm functions.
Design: Prospective study.
Setting: Patients at the Center for Dermatology and Andrology, Giessen, Germany.
Patient(s): Semen collected from 63 randomly collected patients attending the IVF unit of the University of Giessen, Germany.
Intervention(s): Only patients with nonleukocytospermia were included in this study.
Main Outcome Measure(s): Sperm count and motility before and after sperm separation by swim-up, morphology, DNA fragmentation, and extrinsic (by leukocytes) and intrinsic ROS production (by spermatozoa) were evaluated.
Result(s): Leukocytes correlated significantly with extrinsic ROS production ($r = 0.576$), but markedly less with intrinsic ROS production ($r = 0.296$). Sperm count, morphology, and motility in the ejaculate were markedly more affected by extrinsic than by intrinsic ROS. The DNA fragmentation was strongly positively correlated with intrinsic ROS production, whereas this correlation was weaker for extrinsic ROS production. No correlation was found between DNA fragmentation and the number of leukocytes, whereas the correlations with motility in the ejaculate and the motile sperm count after swim-up were highly significant. Moreover, significant differences were observed for extrinsic and intrinsic ROS production between groups of patients having a high ($\geq 1 \times 10^6$/mL) and a low number ($< 1 \times 10^6$/mL) of leukocytes in the ejaculate.
Conclusion(s): The origin of ROS seems to have an influence on the site of the damage. Because leukocyte counts $< 1 \times 10^6$/mL caused a significant decrease of motility and DNA integrity, the threshold given by the World Health Organization (WHO) should be re-evaluated. (Fertil Steril® 2005;83:635–42. ©2005 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, sperm functions, DNA fragmentation, leukocytospermia, human spermatozoa

Fertilization and pregnancy are dependent on a series of functional sperm parameters, which are reportedly affected by reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), or hydroxyl radical ($\cdot OH$) (1–5). These highly reactive substances, which exhibit half-life times in the nanosecond ($\cdot OH$) to the millisecond range (O_2^-), are very strong oxidants and physiologically produced in any living cell during respiration.

Because of the extraordinary high content of polyunsaturated fatty acids in the plasma membrane, spermatozoa are highly susceptible to oxidative stress (1), which has repeatedly been shown to be a cause of impaired sperm function and thus male infertility (6–8). This is probably due to the very low content of protective systems and the absence of catalase. On the other hand, it was shown that ROS also have a key position in the control of sperm function by redox regulation of tyrosine phosphorylation (9–11).

In the male reproductive system, ROS can derive from leukocytes or from the sperm cells themselves. In the ejaculate, spermatozoa have been repeatedly shown to be only a minor source of ROS production, whereas infiltrating or contaminating leukocytes (peroxidase-positive cells) are the predominant source of these oxidants (7, 12, 13). Leukocytes are present in almost any ejaculate (14) and produce at least 1,000 times more ROS than spermatozoa (15) within the scope of their normal physiological function, immunosurveillance, and thus immunologic defense with elimination of pathogenic germs.

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TABLE 1

Summary statistics of the 63 non-leukocytospermic patients analyzed in the study.

Parameter	Mean ± SD	Median
Age of patients (y)	30.04 ± 4.96	36.11
Sperm count in the ejaculate (10^6/mL)	63.72 ± 75.19	36.00
Motility in the ejaculate (%)	49.49 ± 21.17	55.00
Progressive motility (%)	39.33 ± 18.01	40.00
Normal morphology (%)	24.17 ± 12.27	30.00
Sperm count after swim-up (10^6/mL)	10.46 ± 10.11	7.00
Motility after swim-up (%)	79.73 ± 17.43	84.44
Number of peroxidase-positive cells (10^6/mL)	0.17 ± 0.21	0.10
ROS production in the ejaculate (counts/10^7 live sperm)	296,188.92 ± 1,043,603.95	4440.00
Percentage of ROS-producing sperm (%)	47.16 ± 15.14	47.62
Percentage of TUNEL-positive sperm (%)	17.25 ± 9.03	15.64

Henkel. Intrinsic and extrinsic ROS. Fertil Steril 2005.

The World Health Organization (WHO) has defined seminal leukocyte counts of $>10^6$ leukocytes/mL as leukocytospermia (16). A large number of patients (about 10%–20% of infertile men) (14) show, according to the WHO criteria, an increased number of leukocytes in the ejaculate. Although the determination of leukocytes in the ejaculate is one of the corner stones in andrological diagnosis the clinical relevance of leukocytospermia has been questioned repeatedly. Although some investigators found that ROS levels and seminal leukocyte counts appear to have only little or even no prognostic value for reproduction (17, 18), other researchers attributed even favorable effects on sperm morphology or on some sperm functions (19, 20). On the other hand, there are also reports showing its importance for the deterioration of semen quality and male factor infertility (21, 22) and it was suggested recently that the cutoff value of 10^6 leukocytes/mL for leukocytospermia might be too high (23, 24).

In this context, the question raises whether the influence of ROS produced by leukocytes or the sperm cells themselves affect sperm functions in non-leukocytospermic patients. Moreover, it is of clinical interest for assisted reproduction programs to know whether or not these two sources of ROS act differently on male germ cells. Therefore, this study aimed at investigating the impact of extrinsic ROS produced in the ejaculate by leukocytes and defective spermatozoa, and intrinsic ROS produced by the spermatozoa themselves on motility and DNA fragmentation.

MATERIALS AND METHODS

To investigate the influence of intrinsic ROS, produced by the sperm cells themselves, and extrinsic ROS, mainly produced by leukocytes in the ejaculate, on sperm functions, ejaculates from 63 randomly selected nonleukocytospermic (leukocyte count $<1 \times 10^6$/mL semen) patients attending the Centre for IVF at the University of Giessen, Germany, were examined. Sperm count, sperm motility, and the number of peroxidase-positive cells (leukocytes) were analyzed according to WHO guidelines (16), whereas normal sperm morphology was performed after Shorr stain according to the Düsseldorf classification and is correlated with the results obtained with "strict criteria" (25, 26). In addition, motile sperm count before and after conventional swim-up procedure was determined. In brief, semen aliquots were diluted 1:5 with human tubular fluid medium according to Quinn et al. (27) containing 1% serum albumin (Centeon Pharma, Marburg, Germany) (HTF-HSA), centrifuged for 10 minutes at $400 \times g$. The supernatant was discarded and the remaining pellet was overlaid with 1 mL of fresh HTF-HSA and incubated at 37°C for 1 hour. Afterwards, the medium containing the motile spermatozoa was collected.

For the determination of sperm DNA fragmentation, a detection kit (Apoptosis Detection System Fluorescein; Promega, Mannheim, Germany) was used and the test was performed as described previously (28). After the final rinses, excess water was drained off, a drop of Anti-Fade solution (Molecular Probes, Eugene, OR) was added, coverslip applied, and 300 randomly selected spermatozoa were immediately analyzed with an epifluorescence microscope (Zeiss, Oberkochen, Germany) at a ×1,000 magnification. The percentage of sperm showing green fluorescence (TUNEL-positive) was determined. Negative controls without the TdT enzyme were prepared for each batch of analyzed slides.

To determine the intrinsic ROS production within the spermatozoa, 100 μL of native ejaculate were diluted 1:2 with phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) and centrifuged at $300 \times g$ for 10 minutes. The supernatant was discarded and the remaining pellet resuspended to a final sperm count of $20–30 \times 10^6$/mL. Twenty microliters of a 20 μM dihydroethidine solution (Molecular Probes) was added to 180 μL of the cell suspension and incubated for 15 minutes at 37°C. This uncharged, cell-permeant compound,

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