

Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index

Nabil Aziz, M.D.,^a Ramadan A. Saleh, M.D.,^{b,c} Rakesh K. Sharma, Ph.D.,^b
Iwan Lewis-Jones, M.D.,^a Navid Esfandiari, D.V.M., Ph.D.,^{b,d}
Anthony J. Thomas, Jr., M.D.,^b and Ashok Agarwal, Ph.D., H.C.L.D.^b

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Reprint requests: Ashok Agarwal, Ph.D., H.C.L.D., Director, Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, 9500 Euclid Avenue, Desk A19.1, Cleveland, Ohio 44195 (FAX: 216-445-6049; E-mail: agarwaa@ccf.org).

^a Reproduction Medicine Unit, Liverpool Women's Hospital, University of Liverpool.

^b Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic Foundation.

^c Department of Dermatology, Venereology, and Andrology, Faculty of Medicine, Sohag, Egypt.

^d IVF/Andrology Laboratory, Toronto Center for Assisted Reproductive Technology, Toronto, Ontario, Canada.

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Reproduction Medicine Unit, Liverpool Women's Hospital, University of Liverpool, Liverpool, United Kingdom; and Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic Foundation, Cleveland, Ohio

Objective: To examine the relationship between sperm reactive oxygen species (ROS) production and sperm morphology in a group of infertile men and healthy fertile donors.

Design: A prospective clinical study.

Setting: Male infertility clinic, Glickman Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, and the Reproductive Medicine Unit, Liverpool Women's Hospital, United Kingdom

Patient(s): Thirty-nine infertile men and 13 healthy fertile donors (control).

Intervention(s): Standard semen analysis, seminal leukocyte concentration, assessment of sperm morphology, and measurement of sperm ROS production.

Main Outcome Measure(s): Levels of sperm ROS production, percentages of different sperm morphological abnormalities, and the sperm deformity index (SDI) scores.

Result(s): A significant negative correlation was observed between sperm ROS production and the proportion of sperm with normal morphology and borderline morphology. Reactive oxygen species production was positively correlated with the proportion of sperm with amorphous heads, damaged acrosomes, midpiece defects, cytoplasmic droplets, tail defects, and SDI scores. Logistic regression analysis identified a two-variable model including SDI and percentage sperm motility, which correctly identified 84% of individuals with high seminal ROS and 85% of individuals with low seminal ROS. The model had an overall accuracy of 85%.

Conclusion(s): The standard semen analysis to assess sperm motility, sperm morphology, and the SDI scores is a useful tool in identifying infertile men with high seminal ROS in infertility clinics where facilities for measuring levels of seminal ROS are not available. (Fertil Steril® 2004;2:349–54. ©2004 by American Society for Reproductive Medicine.)

Key Words: Spermatozoa, reactive oxygen species, morphology, sperm deformity index, male infertility

It is now recognized that low, controlled levels of extracellular reactive oxygen species (ROS) produced by spermatozoa are involved in sperm capacitation and acrosome reaction (1, 2). The mechanism by which ROS regulates these processes is unclear, but may involve tyrosine phosphorylation of sperm proteins (3). High ROS levels in seminal plasma have been associated with the inhibition of sperm function and viability due to the peroxidation of membrane polyunsaturated fatty acids (4). This

leads to the loss of sperm membrane fluidity, which is required for sperm adhesion and oocyte fusion. It is reported that up to 40% of infertile men have high seminal ROS levels (5, 6). Moreover, high ROS production has been found to be inversely correlated with the outcome of in vitro fertilization (7, 8). Infertile males who produce high levels of ROS have a fivefold less chance of initiating a pregnancy than infertile males who produce low levels of ROS (7).

High levels of seminal ROS have also been correlated with poor sperm morphology, as indicated by low proportions of sperm with normal morphology in semen. Literature review identified only one study that correlated a specific morphological defect of human sperm with sperm ROS production (9). In this study, a complex methodology for quantifying the residual cytoplasm present in the midpiece of human spermatozoa revealed a significant correlation between excess residual cytoplasm in the midpiece and the enhanced generation of ROS. The relationship between other sperm morphological defects and sperm ROS production has not been studied yet.

The sophistication brought into sperm morphological examination by the inclusion of the strict morphological criteria (10, 11) and sperm deformity index (SDI) has enhanced this parameter's predictive power and reproducibility (12). The SDI is the average number of deformities per spermatozoa scored. It is a novel quantitative expression of sperm morphological quality, and is a more powerful predictor of sperm function and the outcome of oocyte fertilization *in vitro* than either the normal morphology or multiple anomalies index. It has been hypothesized that the SDI is an indirect measure of the functional integrity of morphologically normal spermatozoa (12).

Clinically, semen analysis including the assessment of sperm morphology remains an essential investigative tool of male infertility. The paucity of our knowledge regarding the relationship between sperm morphological defects and seminal ROS levels is addressed in this study by examining the possible correlation between seminal ROS production and sperm morphological attributes, including the SDI. The results of this study may aid in predicting the cause of male infertility where dedicated and expensive laboratory facilities for measuring seminal ROS levels are not available.

MATERIALS AND METHODS

Semen Samples

Once the Institutional Review Board (IRB) at the Cleveland Clinic granted approval, semen samples were collected from males undergoing infertility screening ($n = 39$) and from normozoospermic healthy donors ($n = 13$) of proven fertility (initiated a successful pregnancy within the last 12 months) as controls. Samples with a sperm concentration $< 1 \times 10^6/\text{mL}$ were excluded from this study. All specimens were collected by masturbation at the clinical andrology laboratory after an abstinence period of 48–72 hours. After liquefaction, routine semen analysis was performed to measure sperm concentration and percentage motility.

Myeloperoxidase-Staining Test

The presence of peroxidase positive leukocytes (neutrophils and macrophages) in semen was assessed by a myeloperoxidase-staining test (13). A 20- μL volume of liquefied semen specimen was placed in a Corning 2.0-mL

cryogenic vial (Corning Costar Corp., Cambridge, MA) with 20 μL of phosphate-buffered saline (PBS) (pH 7.0) and 40 μL of benzidine solution. The solutions were mixed and allowed to sit at room temperature for 5 minutes. Peroxidase-positive leukocytes that stained brown were counted by a Makler's counting chamber (Sefi Medical, Haifa, Israel) under the bright-field objective (magnification, $\times 20$). The results after correction for dilution were recorded as $\times 10^6$ peroxidase-positive leukocytes/mL of semen. A seminal leukocyte concentration of $\leq 1 \times 10^6/\text{mL}$ (14) was considered normal.

Measurement of Sperm ROS Production

Levels of ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as a probe (15). Liquefied semen was centrifuged at 300 g for 7 minutes, and the seminal plasma was separated. The pellet was washed with PBS and resuspended in the same media at a concentration of 20×10^6 sperm/mL, and finally divided into 400 μL aliquots for assessment of basal ROS levels. Eight microliters of horseradish peroxidase (HRP) (12.4 U of HRP Type VI, 310 U/mg; Sigma Chemical Co., St. Louis, MO) were added to sensitize the assay for measurement of extracellular H_2O_2 . Ten microliters of luminol, prepared as a 5-mM stock in dimethyl sulfoxide (DMSO), were added to the mixture. A negative control was prepared by adding 10 μL of 5-mM luminol to 400 μL of PBS.

Levels of ROS were assessed by measuring the luminol-dependent chemiluminescence with a luminometer (model LKB 953; Wallac Inc., Gaithersburg, MD) in the integrated mode for 15 minutes. The results were expressed as $\times 10^6$ counted photons per minute (cpm) per 20×10^6 sperm. In order to study the relationship between sperm morphology and sperm ROS production, infertile patients were categorized into two groups: low ROS level group ($\leq 1 \times 10^6$ cpm per 20×10^6 sperm) or high ROS level group ($> 1 \times 10^6$ cpm per 20×10^6 sperm). This criteria was based on our previous report, which demonstrated that semen specimens from normal healthy controls have ROS values of $< 1 \times 10^6$ cpm per 20×10^6 sperm (16).

Assessment of Sperm Morphology

Thin smears of the well-mixed ejaculated semen were prepared in duplicate by placing 2–5 μL (depending on the sperm concentration) on clean poly-L-lysine-coated slides. Thin semen smears facilitated sperm morphology assessment by avoiding sperm cell overlap and ensuring that the sperm were scattered at the same focal depth. After drying in air, the slides were stained with Papanicolaou. Slides were coded (andrology laboratories, Cleveland Clinic Foundation) and randomly evaluated by the investigator (N. Aziz, Liverpool Womens Hospital, UK). A total of 100 spermatozoa were scored per slide using bright field illumination and an oil immersion objective with a total magnification of $\times 2000$. At least ten high-power fields selected at random from dif-

TABLE 1

Sperm ROS production and sperm abnormalities in seminal ejaculates of fertile donors and infertile men with low and high ROS levels. Results are presented as medians (25th, 75th percentiles).

Variables	Donors (n = 13)	Infertile			P value ^b		
		Low ROS ≤1 × 10 ⁶ cpm/20 × 10 ⁶ sperm/mL) (n = 26)	High ROS >1 × 10 ⁶ cpm/20 × 10 ⁶ sperm/mL) (n = 13)	P value ^a	A	B	C
Sperm concentration	64 (40, 93)	50 (36, 77)	20 (12, 26)	<.0001	.3	<.0001	.0004
Sperm motility (%)	69 (59, 76)	59 (36, 65)	38 (31, 53)	<.0001	.001	<.0001	.01
ROS level (× 10 ⁶ cpm)	.15 (0.1, 0.9)	.3 (.07, .05)	4.6 (3, 64)	<.0001	.9	<.0001	<.0001
Seminal leukocytes (× 10 ⁶ /mL)	.1 (0.0, 0.2)	.1 (0, .2)	1 (0.2, 2.8)	.0003	.99	.0005	.0004
Normal morphology (%)	10 (8, 18)	5 (0, 12)	2 (0, 8)	.001	.001	.0001	.39
Borderline morphology (%)	16 (10, 21)	12 (6, 20)	5 (2, 9)	.0001	.07	<.0001	.003
Amorphous (%)	38 (33, 45)	42 (29, 54)	47 (42, 68)	.02	.45	.01	.07
Acrosomal damage (%)	19 (14, 29)	21 (20, 31)	33 (24, 52)	.004	.25	.001	.03
Nuclear abnormalities (%)	2 (1, 6)	7 (4, 12)	8 (5, 12)	.005	.017	.001	.8
Cytoplasmic droplet (%)	4 (0, 7)	8 (4, 10)	8 (4, 20)	.0004	.15	<.0001	.006
Midpiece abnormalities (%)	19 (9, 22)	16 (12, 28)	26 (21, 31)	.001	.1	.0002	.02
Tail defects (%)	4 (2, 6)	6 (4, 12)	12 (7, 29)	.0007	.1	<.0001	.02
Sperm deformity index	1.5 (1.4, 1.6)	1.6 (1.5, 1.9)	2 (1.7, 2.27)	.0004	.15	<.0001	.006

Note: A = fertile donors vs. infertile patients with low ROS levels; B = donors vs. patients with high ROS levels; C = infertile patients with low vs. high ROS levels. *P* < .05 was considered significant.

^a Univariate comparison of variables among groups was performed with the Kruskal–Wallis test.

^b Simultaneous pairwise comparisons among groups were performed with the Convar–Inman test.

Aziz. *Sperm abnormalities and ROS. Fertil Steril* 2004.

ferent areas of the slide were examined. A calibrated microscope on the eyepiece of the light microscope was used to measure sperm dimensions when there was doubt about sperm classification.

All slides were assessed using a morphological classification based on a modification of the method of Eliasson (17) and applying the strict criteria for normal sperm morphology (10, 11). A multiple entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed. The SDI was calculated by dividing the total number of deformities observed by the number of sperm randomly selected and evaluated, irrespective of their morphological normality. Borderline forms were considered abnormal and included:

1. Spermatozoa with slightly elongated head with loss of its oval shape.
2. Those with rounded heads and intact acrosome.
3. Those with normal heads and a thickened midpiece.

Quality control of sperm morphology assessment revealed no significant difference in repeated estimation of different sperm morphological forms.

Statistical Analysis

Data were analyzed using inbuilt functions within the Statistical Package for Social Science (SPSS UK Ltd., Chertsey, Surrey, UK). Summary statistics are presented as me-

dian and interquartile ranges (25th and 75th percentiles). Univariate comparison of continuous variables among the groups was performed with the Kruskal–Wallis test. Simultaneous multiple pairwise comparisons among groups were performed with the Conover–Inman test, which is simply Fisher’s least significance difference method performed on ranks. Spearman’s rank correlation test was used to provide a distribution-free test of independence between sperm ROS production and sperm attributes. All hypothesis testing was two-tailed; *P* < .05 was considered statistically significant. Forward stepwise logistic regression analysis was used to identify a suitable model predicting high sperm ROS production.

RESULTS

Sperm ROS Production

Semen specimens from 52 individuals were studied: 39 were infertile men and 13 were fertile healthy donors. Of the 39 infertile patients, 14 were placed into the low ROS group (≤1 × 10⁶ cpm per 20 × 10⁶ sperm) and 25 into the high ROS group (>1 × 10⁶ cpm per 20 × 10⁶ sperm). No significant differences were observed in sperm ROS production between the healthy donors and the low ROS group of infertile patients (Table 1). The leukocyte count in semen between the healthy donors was comparable with that of the low ROS infertile group; however, the seminal leukocytic

count was significantly higher in infertile men with high ROS than in the other two groups (Table 1).

ROS and Sperm Concentration and Motility

The median and interquartile values (25th, 75th percentiles) of sperm concentration and motility in the three study groups are listed in Table 1. The healthy donors and patients with low ROS had significantly higher sperm concentrations and a higher percentage of sperm motility than the infertile patients with high ROS. No significant difference was observed in sperm concentrations between healthy donors and the group of infertile patients with low ROS; however, percentage sperm motility was different between these two groups (Table 1). When the 52 individuals were considered collectively, there was a significant negative correlation between sperm ROS production and the sperm concentrations ($r = -.6$, 95% CI = $-.75$ to $-.38$; $P < .0001$) and percentage sperm motility ($r = -.5$, 95% CI = $-.67$ to $-.26$; $P < .0001$).

Sperm Morphology and Sperm ROS Production

The median and interquartile values (25th, 75th percentiles) of different sperm morphological forms are listed in Table 1. Compared with the healthy donors, infertile patients with low ROS had a significantly lower proportion of sperm with normal morphology and a significantly higher proportion of sperm with nuclear deformities. Other sperm morphological abnormalities and SDI scores were comparable between these two groups. Compared with the healthy donors and the low ROS infertile patients, the high ROS infertile patients had significantly lower proportions of sperm with borderline sperm morphology, significantly higher proportions of sperm with acrosomal damage, cytoplasmic droplets, midpiece defects, and tail defects, as well as significantly higher SDI scores (Table 1).

When the 52 individuals were considered collectively, a significant negative correlation existed between the sperm ROS production and the proportion of sperm with normal morphology ($r = -0.44$, 95% CI = $-.63$ to $-.2$; $P = .001$) and borderline morphology ($r = -.55$, 95% CI = $-.7$ to $-.33$; $P < .0001$). A significant positive correlation also existed between sperm ROS production and the proportion of sperm with amorphous heads ($r = .34$, 95% CI = $.08$ to $.56$; $P = .014$), damaged acrosomes ($r = .52$, 95% CI = $.29$ to $.7$; $P = .0001$), midpiece defects ($r = .45$, 95% CI = $.2$ to $.65$; $P = .0006$), cytoplasmic droplets ($r = .28$, 95% CI = $.008$ to $.51$; $P = .04$), tail defects ($r = .47$, 95% CI = $.23$ to $.66$; $P = .0005$), and the SDI scores ($r = .51$, 95% CI = $.3$ to 0.7 ; $P = .0001$).

No significant correlation was observed between levels of seminal ROS and the proportions of spermatozoa with large, small, pyriform, or tapering heads or spermatozoa with nuclear defects.

Sperm Morphology as a Predictor of High Sperm ROS Production

Sperm count, motility, and morphology variables as well as seminal leukocyte concentrations were subjected to forward stepwise logistic regression analysis to identify a suitable model for predicting high seminal ROS. A two-variable model, including SDI and percentage sperm motility, correctly identified 84% (21/25) of individuals with high seminal ROS and 85% (23/27) of individuals with low seminal ROS. The model had an overall accuracy of 85%.

DISCUSSION

This is the first study, to our knowledge, to assess the relationship between detailed sperm morphology (including the SDI scores applying the strict criteria) and sperm ROS production. Our study demonstrated that sperm ROS production was negatively correlated with percentage normal and borderline morphology and positively correlated with the percentage of sperm with amorphous heads, acrosomal damage, cytoplasmic droplets, midpiece, and tail defects as well as the SDI scores.

Our study reveals a significant association between high ROS production and reduced sperm concentration and percentage motile sperm in semen. This is in agreement with other independent reports demonstrating an association between high seminal ROS levels and reduced sperm count (18–19) and motility (18, 20–22). Reactive oxygen species produced by polymorphonuclear leukocytes have been shown to decrease sperm motility (23–25). In one report, however, seminal oxidative stress in patients presenting with prostatitis was not associated with a reduction in sperm concentration, percentage of motility, and morphology compared with healthy controls (19).

Low sperm count is perhaps due to prolonged exposure of the seminiferous epithelium to high levels of ROS-producing spermatozoa, which could damage the seminiferous tubules, lead to testicular atrophy and reduce total sperm production (26). This is consistent with reports demonstrating that varicocele is associated with both high levels of ROS production (27) and progressive testicular atrophy (28–30).

Compared with healthy donors, patients with both low and high ROS had lower percentages of sperm with normal and borderline morphology. The lack of a significant difference in the proportion of spermatozoa with normal sperm morphology between the low and high ROS groups of patients substantiates the argument for careful assessment of the morphology of the remaining sperm population in a semen sample. In contrast, a significant negative correlation was observed between borderline sperm morphology and sperm ROS production. This may suggest that a subtle deviation in sperm head shape is not associated with excessive ROS production.

The significant correlation between sperm ROS production and the proportions of spermatozoa with cytoplasmic

droplet demonstrated in this study is in agreement with the reported excessive production of ROS by spermatozoa associated with the retention of residual cytoplasm in the sperm midpiece following spermiation (9, 26). Spermatozoa with cytoplasmic droplet, frequently referred to as immature spermatozoa, appear more frequently in human semen compared with other animals. This has been attributed to inefficient human spermiogenesis that involves fewer steps leading to less rigorous quality control (31).

The increased presence of residual cytoplasm in infertile males suggests the control of spermiogenesis is even less efficient than that observed under normal conditions. This results in the release of significantly higher numbers of immature spermatozoa with cytoplasmic retention into the seminiferous tubules. Independent reports have demonstrated that biochemical markers of the cytoplasmic space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (9, 32). The reason why cells with an excess of residual cytoplasm exhibit high rates of ROS generation may be related to the enhanced presence of glucose-6-phosphate dehydrogenase. This enzyme fuels the generation of NADPH that, in turn, stimulates the production of ROS (1, 3, 33).

One of the interesting findings of our study was the correlation between increased proportion of sperm with tail defects and the significant increase in sperm ROS production. In standard IVF treatment, men who failed to fertilize had a significantly higher mean proportion of spermatozoa with tail defects in semen than those who were successful fertilizers (12). In another report, the percentage of sperm with tail defects correlated negatively with fertilization rates in vitro both before and after sperm preparation techniques (34).

Multiple tail abnormalities have been associated with chromosomal abnormalities including aneuploidy of the sex chromosomes (35, 36). Further work is needed to elucidate the mechanism(s) by which spermatozoa with tail defects increase production of ROS. One hypothesis is that these spermatozoa are probably genetically abnormal and may be producing excessive amounts of ROS. Spermatozoa with abnormal tails may be associated with increased cytoplasm in the tail region (as may be the case in coiled tails) with a consequent increase in sperm ROS production. The elevated seminal ROS levels may lead, in turn, to a derangement in function of apparently morphologically normal spermatozoa (3, 26). Another relevant issue is the observation that the percentage tail abnormalities are not reduced by a sperm preparation method such as density gradient separation (34, 37). This finding is of significance when reporting levels of ROS in neat semen and following sperm preparation.

The multiple-entry technique, presently used in our study, avoids the current confusion in classifying those spermatozoa displaying more than one deformity and ensures that deformities of different parts of the sperm are accounted for

equally. This enables us to evaluate the influence of specific sperm structural deformities, alone or in combinations, on sperm function. It also allows the calculation of the SDI. When the results of this study were subjected to logistic regression analysis, the score and percentage sperm motility was included in a two-model to predict high sperm ROS production. The model's total accuracy of 85% indicates that standard semen analysis (including SDI) is a useful tool in identifying infertile men with high seminal ROS levels in infertility clinics where dedicated, expensive laboratory facilities for applying labor-intensive techniques are not available.

In light of our findings of significant positive correlation between sperm morphological abnormalities and ROS production, and given the fact that spermatozoa are highly packed in both the seminiferous tubules and in the epididymis, it is conceivable that the coexistence of ROS-producing deformed spermatozoa with mature spermatozoa during migration from the seminiferous tubules to the epididymis could result in oxidative damage of mature spermatozoa. If that were the case, this could result in loss of motility and DNA damage of mature spermatozoa. This is also consistent with the observation that centrifugation of semen samples containing high levels of ROS-producing spermatozoa results in significant cross-damage of mature spermatozoa (38).

In summary, this is the first study to assess the relationship between detailed sperm morphology, including the SDI scores, the strict criteria and sperm ROS production. Reactive oxygen species levels correlated negatively with percentage normal and borderline morphology and positively with the percentage of sperm with amorphous head, acrosomal damage, cytoplasmic droplets, midpiece, and tail defects as well as SDI scores. The significant negative correlation of ROS with borderline sperm morphology may suggest that a subtle deviation in sperm head shape may not interfere with sperm metabolism. The association between increased sperm ROS production and tail defects may be attributed to increased cytoplasmic retention. Specific staining of sperm cytoplasm is needed, however, to examine this possibility.

This study lends further evidence to support the theory that the possible mechanism by which the function of morphologically normal sperm is compromised is caused by their coexistence with morphologically abnormal sperm, thus producing excessive amounts of ROS. The standard semen analysis to assess sperm motility, sperm morphology, and the SDI scores is a useful tool in identifying infertile men with high seminal ROS levels in infertility clinics where facilities to measure seminal ROS levels are not available.

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