

Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility

Tamer M. Said, M.D.,^a Ashok Agarwal, Ph.D., H.C.L.D.,^a Rakesh K. Sharma, Ph.D.,^a Edward Mascha, M.S.,^b Suresh C. Sikka, Ph.D., H.C.L.D.,^c and Anthony J. Thomas, Jr., M.D.^d

Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics and Gynecology, The Cleveland Clinic Foundation, Cleveland, Ohio

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Reprint requests: Dr. Ashok Agarwal, Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195 (FAX: 216-445-6049; E-mail: agarwaa@ccf.org)

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

^b Department of Biostatistics and Epidemiology, The Cleveland Clinic Foundation.

^c Department of Urology, Tulane University Health Sciences Center, New Orleans, Louisiana.

^d Glickman Urological Institute, The Cleveland Clinic Foundation.

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Objective: To measure levels of reactive oxygen species (ROS) including H₂O₂ and O₂^{•-} generation in infertile men and determine whether sperm quality is correlated with levels of ROS triggered by the exogenous reduced form of β nicotinamide adenine dinucleotide phosphate (NADPH).

Design: Prospective study.

Setting: Male infertility clinic at a tertiary healthcare center.

Patient(s): Eleven infertile men and six healthy donors.

Intervention(s): Chemiluminescence assay using luminol and lucigenin as probes before and after incubating sperm samples with 5 mM and 10 mM of NADPH.

Main Outcome Measure(s): The ROS generation (10⁶ counted photons per minute/10⁶ sperm).

Result(s): Baseline levels of O₂^{•-} generation were significantly higher in the infertile patients than in the healthy donors ($r = 0.73$, 95% confidence interval [median (25th, 75th percentiles): 0.73 (0.5, 5.5) vs. 0.2 (0.0, 0.5)] when lucigenin was used as the probe. Compared with basal levels, O₂^{•-} generation was significantly higher after coinubation with NADPH (5 mM and 10 mM) in the entire combined study population, and patients only but not donors. The O₂^{•-} generation was negatively correlated with sperm concentration ($r = -0.75$, 95% CI 0.38–1), motility ($r = -0.69$, 95% CI 0.28–1), and percentage of normal morphology ($r = -0.78$, 95% CI 0.36–1).

Conclusion(s): Spermatozoa from infertile men produce higher levels of O₂^{•-} in the presence of exogenous NADPH compared to healthy donors. The ability of spermatozoa to generate O₂^{•-} increases as the semen quality declines. (Fertil Steril® 2004;82:871–7. ©2004 by American Society for Reproductive Medicine.)

Key Words: Chemiluminescence, male infertility, NADPH, ROS, superoxide anion, luminol, lucigenin

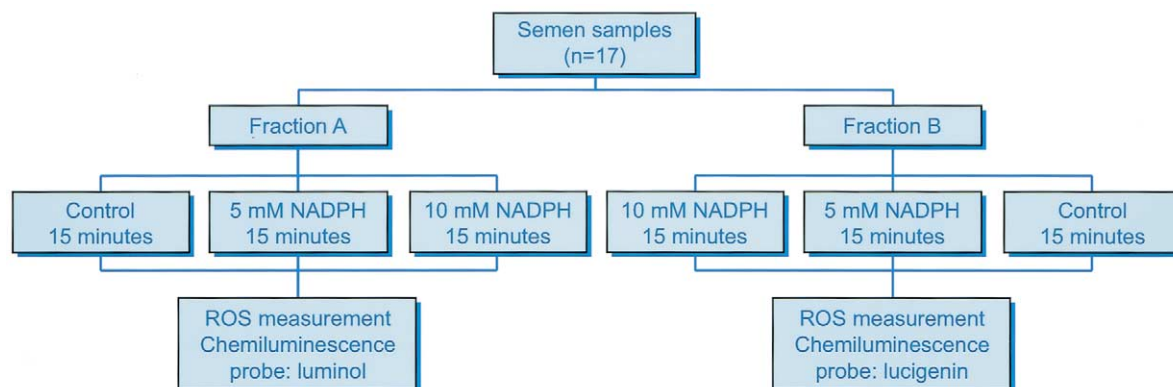
Mammalian spermatozoa generate a variety of reactive oxygen species (ROS), which are thought to play a physiological role during sperm capacitation, acrosome reaction, and oocyte fusion (1, 2). However, oxidative stress (OS) occurs if the generation of ROS overwhelms the limited antioxidant defenses. Seminal OS precipitates a wide range of pathologies that may afflict the reproductive function of the spermatozoa (3–5). The primary product of the spermatozoon's free radical generating system appears to be O₂^{•-}, which secondarily dismutates to H₂O₂ through the catalytic action of superoxide dismutase (SOD) (6). The combination of O₂^{•-} and H₂O₂ is potentially harmful, and in the presence of tran-

sition metals, it can precipitate the generation of hydroxyl radicals (7).

The process by which spermatozoa produce ROS is not fully understood. Human spermatozoa may contain a reduced form of β nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is similar to that found in phagocytic leukocytes (8, 9). This theory is based mainly on two observations. First, adding pharmacological doses of NADPH to purified sperm suspensions increases O₂^{•-} production, which is associated with reduced sperm function (10–12). Second, this increased generation of O₂^{•-} can be inhibited by SOD, which protects spermatozoa against the toxic effects of NADPH (10, 13, 14).

FIGURE 1

Experimental flow diagram showing reactive oxygen species (ROS) measurements in different semen aliquots. NADPH = exogenous reduced form of nicotinamide adenine dinucleotide phosphate.



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The cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PD) controls the rate of glucose flux and intracellular availability of NADPH through the hexose monophosphate shunt. This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS through NADPH oxidase (NOX 5) (2, 10). However, Armstrong et al. (9) recently demonstrated that NOX 5 activity is more pronounced in white blood cells than in human spermatozoa. The role of NADPH oxidase in ROS production by spermatozoa is not clear, although ROS production has been strongly correlated with defective immature sperm characterized by incomplete extrusion of cytoplasm (15–19). Abnormal spermatozoa from infertile patients are more likely to produce ROS than those from fertile patients (20). How these abnormal spermatozoa respond to exogenous NADPH, which plays a role in ROS production, is not yet known.

Our study has three main objectives: [1] to assess the levels of $O_2^{\cdot-}$ and H_2O_2 generation in a group of infertile men and healthy donors using luminol and lucigenin as probes; [2] to evaluate the role of NADPH in ROS generation by human spermatozoa; and [3] to evaluate whether levels of ROS generation triggered by exogenous NADPH correlate with semen quality.

MATERIALS AND METHODS

Subject Selection

The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation and all patients granted their written informed consent. Semen samples were collected from normal healthy donors (n = 6) and patients undergoing infertility evaluation (n = 11).

Semen Collection and Assessment of Semen Variables

Semen specimens were collected by masturbation after 48–72 hours of abstinence. After liquefaction at 37°C for 20 minutes, 5 μ L of each specimen was loaded on a 20- μ L Microcell chamber (Conception Technologies, San Diego, CA) and analyzed for sperm concentration and motility. For morphological evaluation, seminal smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL) and assessed by the one individual (T.S.) according to World Health Organization (WHO) guidelines (21).

Quantitation of White Blood Cells

The presence of white blood cells (WBCs) in all specimens was assessed using myeloperoxidase staining (Endtz test). A 20- μ L volume of liquefied specimen was placed in a 2.0-mL cryogenic vial (Corning Costar Corp., Cambridge, MA), diluted 1:1 in phosphate buffered saline (PBS; pH 7.0) and mixed with 40 μ L of benzidine solution. The mixture was allowed to sit at room temperature for 5 minutes. An aliquot (5 μ L) was counted for the peroxidase positive WBCs (brown cells) in all 100 squares in a Makler chamber (Sefi Medical, Haifa, Israel). Specimens with WBCs greater than 1×10^6 /mL were excluded from the study because our purpose was to analyze only those semen samples without any evidence of leukocytospermia that may lead to abnormal ROS production.

Isolation of Pure Sperm Population by Gradient Separation

An aliquot (1 mL) of the liquefied semen was loaded onto a 47% and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA) and centrifuged at $500 \times g$ for 20 minutes at room temperature. The resulting pellet was

TABLE 1

Sperm parameters in the donors and patients.

Variable	Donors (n = 6)	Patients (n = 11)	P ^a
Concentration (10 ⁶ /mL)	62.01 (39.75, 147.87)	19.90 (2.4, 80)	.07
Motility (%)	69 (50, 88.5)	32 (10, 9)	.04
% Normal morphology (WHO)	34 (28, 43)	25 (3, 44)	.03

Note: Values are median and interquartile range (25th and 75th percentile).

^a Wilcoxon rank-sum test (comparing donor and patient groups); $P < .05$ was considered significant.

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washed with Biggers–Whitten–Whittingham medium (BWW) by centrifugation at $500 \times g$ for 7 minutes. The supernatant was aspirated, and the pellet was resuspended in 5 mL of PBS to provide a concentration of ≥ 2 million sperm/mL.

Sample Preparation and Chemiluminescence Measurement

The sperm suspension was equally divided into two fractions for measuring ROS production using luminol (fraction A) and lucigenin (fraction B) as the probes. Both fractions were further divided into three equal parts: [1] basal ROS production, [2] incubation with 5 mM of NADPH, and [3] incubation with 10 mM of NADPH (Fig. 1). A fresh stock solution of NADPH (200 mM) was prepared in PBS. Sperm suspensions containing ≥ 2 million sperm/mL were incubated with 5 mM or 10 mM of NADPH at 37°C for 15 minutes.

Levels of ROS were measured in fraction A using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma, St Louis, MO). Luminol was prepared as 5 mM stock in dimethyl sulfoxide (DMSO) (22); 10 μ L of the stock was added to 400 μ L of the sperm suspension. In fraction B, ROS levels were measured using 25 mM lucigenin (*bis-N*-methylacridinium nitrate; Sigma) (23). A 4- μ L aliquot of lucigenin stock was added to 400 μ L of sperm suspension. Negative controls were prepared by adding equal amount (10 μ L and 4 μ L) of luminol and lucigenin to 400 μ L of PBS.

Levels of ROS were determined by measuring chemiluminescence with a Berthold luminometer (model: LKB 953, Wallace Inc., Gaithersburg, MD) for 15 minutes (22). Results were expressed as 10⁶ counted photons per minute (cpm) per 20 million sperm.

Statistical Analysis

All study groups were evaluated using Wilcoxon rank-sum tests. All hypothesis testing was two-tailed, with a significance level of .05. Within-group changes were assessed with the Wilcoxon signed-rank test. A Bonferroni correction was used for multiple comparisons within a hypothesis, so that when two comparisons were made for a variable, a significance criterion .025 ($P < .025$) was used (.05/2). When three comparisons were made, a sig-

nificance criterion of .017 was used. Correlation between variables was assessed using Spearman's correlation coefficients (r) and corresponding 95% confidence intervals (CI). Summary statistics are presented as median and quartiles (25th and 75th percentile). Data were analyzed using a SAS statistical software package (version 8.1; SAS Institute Inc., Cary, NC).

RESULTS

The sperm parameters in the donors and patients are listed in Table 1. The basal levels of ROS were significantly higher in patients with male factor infertility than in donors ($r = [0.73 (0.5, 5.5)$ vs. $0.20, (0.0, 0.5)$; $P = 0.03$) when lucigenin was used as a probe and the sperm were not exposed to exogenous NADPH. On the other hand, use of luminol did not show any significant difference between male infertility patients and donors [$0.64 (0.2, 0.9)$ vs. $0.20 (0.0, 0.6)$; $P = 0.13$].

Compared with basal levels, O₂⁻ generation was significantly higher after incubation with NADPH (5 mM and 10

TABLE 2

Effect of exogenous NADPH (5 mM or 10 mM) on levels of lucigenin detectable superoxide or luminol detectable hydrogen peroxide ROS generation by human spermatozoa in patients and donors.

Probe	Subjects (n = 17)	P ^a
Luminol		
NADPH (5 mM)	-0.01 (-0.17, 0.48)	.78
NADPH (10 mM)	-0.01 (-0.29, 0.49)	.64
Lucigenin		
NADPH (5 mM)	13.70 (-35.09, -6.45)	<.001
NADPH (10 mM)	-18.61 (-52.80, -8.99)	<.001

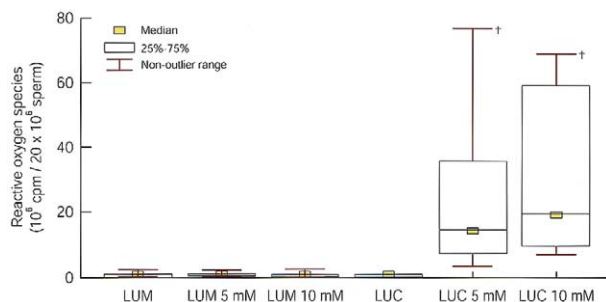
Note: Results are expressed as median (25th and 75th percentile); ROS levels are expressed as $\times 10^6$ counted photons per minute (cpm)/20 $\times 10^6$ sperm and represent the within-subject differences between postincubation and preincubation values.

^a Wilcoxon sign-rank test (comparing the values in absence and in presence of NADPH); $P < .025$ was considered significant.

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FIGURE 2

The reactive oxygen species levels detected by chemiluminescence in human spermatozoa from donors and patients (n = 17) using luminol and lucigenin as probes. LUM = luminol; LUC = lucigenin; 5 mM = incubation with exogenous reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) 5 mM; 10 mM = incubation with NADPH 10 mM. †LUC 5 mM and LUC 10 mM are significantly higher than (P<.001 Wilcoxon rank-sum test).



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mM) in the entire study population (P<.001 and P<.001). The ROS levels pre- and post-NADPH incubation are summarized in Table 2. The overall increase was detectable only when using lucigenin but not luminol by both doses of NADPH (Fig. 2).

Table 3 shows the effect of NADPH on levels of ROS detected by luminol and lucigenin. The within-subject differences, which are summarized in the Table, represent the difference between postincubation and preincubation values. The negative results reflect higher ROS values after incubation with NADPH. In addition, significant changes were detected in the patient group between basal O₂⁻ levels and those after incubation with 5 mM of NADPH [-26.3 (-52.7, -6.5); P=.001]. Similarly, levels of O₂⁻ were higher than the basal values when the sperm were incubated

with 10 mM of NADPH [-22.4 (-53.5, -8.8); P=.002]. NADPH (5 mM or 10 mM) did not increase the levels of O₂⁻ in the normal donor group.

Changes in O₂⁻ levels on incubation with 5 mM or 10 mM of NADPH demonstrated a positive correlation with sperm concentration, motility, and percentage of normal forms (Fig. 3). However, the degree of correlation did not increase in accordance with the NADPH concentration.

DISCUSSION

Human spermatozoa can produce ROS, which may be responsible for defective sperm function (2, 5, 6, 9, 24-26). Although many radicals constitute ROS, it appears that O₂⁻ is the main inducer of lipid peroxidation in spermatozoa (27, 28). Regardless of the clinical etiology, patients diagnosed with male factor infertility exhibit elevated levels of ROS (2, 5, 20, 29). Using lucigenin as the chemiluminescence probe, we were able to demonstrate that the elevated ROS levels were mainly composed of O₂⁻.

The chemiluminescence assay is one of the most commonly used methods to detect oxidized end products (2, 5, 13, 22, 26, 30). Lucigenin yields a chemiluminescence that is more specific for extracellular O₂⁻. In contrast, luminol is not able to differentiate between intracellular and extracellular ROS generation. Luminol reacts with a variety of reactive oxygen species, especially in samples with leukocytospermia where H₂O₂ appears to be the predominant form of ROS (13, 31-33).

The production of O₂⁻ by spermatozoa is usually rapid and transient (34) and may be stimulated by NADPH (7). We have detected higher chemiluminescence signals using lucigenin after exposure to 5 mM and 10 mM of NADPH. Our findings support the hypothesis that exogenous NADPH enhances ROS generation by human spermatozoa (7, 23, 35, 36). Because the overall detection was possible using lucigenin only, it appears that the impact of NADPH on the levels

TABLE 3

Effect of exogenous NADPH on levels of ROS detected by luminol and lucigenin showing the within-subject differences.

NADPH concentration	Donors (n = 6)	P ^a	Patients (n = 11)	P ^a	P ^b
Probe: Luminol					
NADPH 5 mM	-0.01 (-0.1-0.6)	>.999	-0.02 (-0.2-0.4)	.85	.87
NADPH 10 mM	0.24 (-0.1-0.9)	.63	-0.07 (-0.3-0.4)	>.999	.45
Probe: Lucigenin					
NADPH 5 mM	-11.6 (-13.7--3.9)	.03	-26.3 (-52.7--6.5)	.001	.12
NADPH 10 mM	-18.2 (-26.8--13.6)	.13	-22.4 (-53.5--8.8)	.002	.84

Note: Results are expressed as median (25th and 75th percentile).

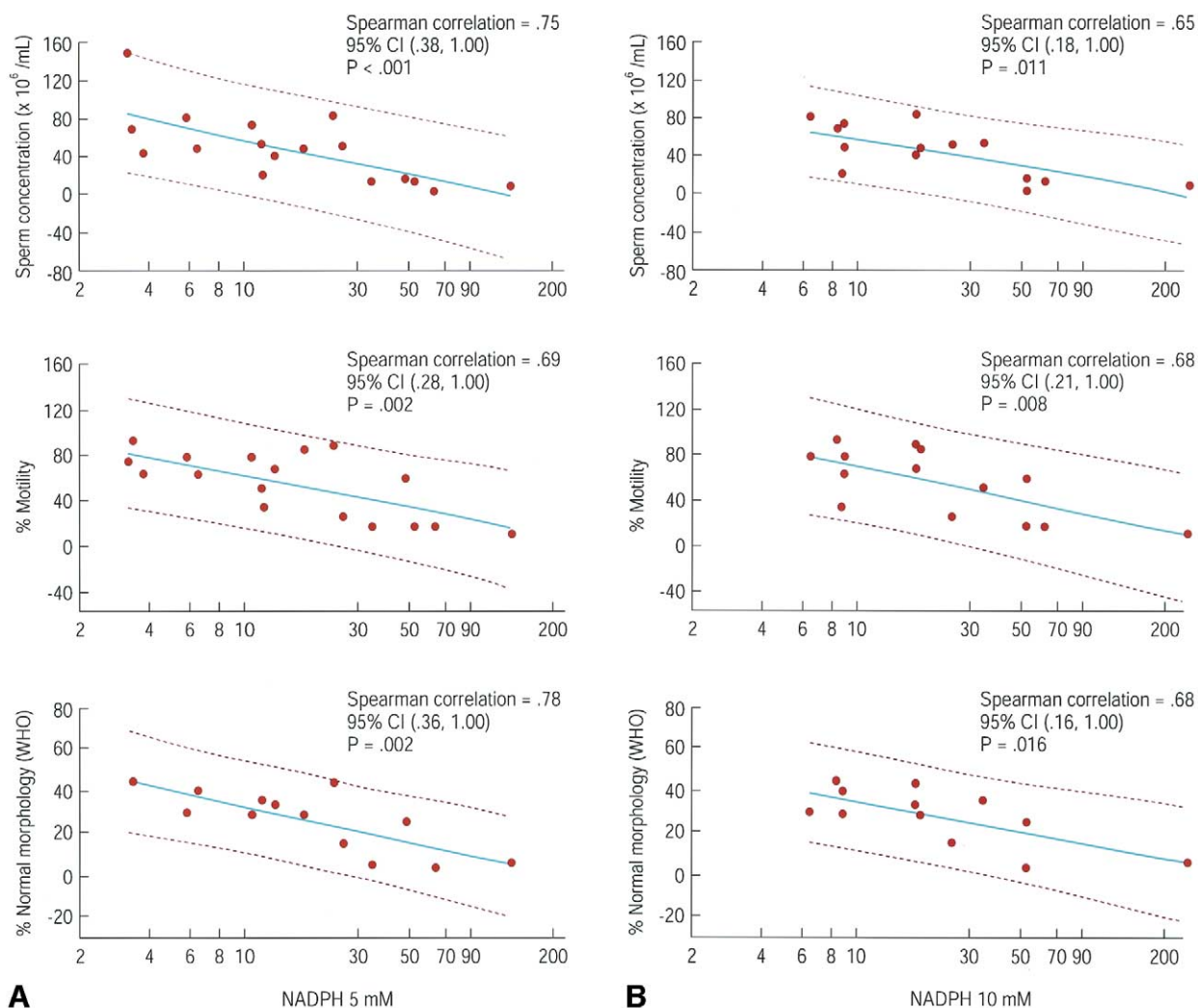
^a Wilcoxon sign-rank test (testing difference within group - the within-subject differences between postincubation and preincubation values); P<.025 was considered statistically significant.

^b Wilcoxon rank-sum test (comparing donors and patients); P<.025 was considered statistically significant.

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FIGURE 3

Scatter plot showing correlation between semen quality parameters and reactive oxygen species generation using lucigenin as a probe in response to (A) exogenous 5 mM reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and (B) exogenous 10 mM NADPH. $P < .05$ was considered significant. In all cases, the correlations between semen parameters and NADPH at both 5 and 10 mM were significantly different from zero. CI = confidence interval.



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of free radical generation by the spermatozoa is solely due to $O_2^{\cdot-}$. Other radicals, such as H_2O_2 , do not seem to be affected in this study, mainly because we did not include subjects with leukocytospermia.

There is a lack of consensus on the exact concentration of NADPH required to cause increased ROS levels in spermatozoa that is capable of affecting its function. Exogenous NADPH concentrations of >5 mM in our study produced significantly high levels of ROS. Because higher chemiluminescent signals were detected in the infertile patient population compared with the donors, it is possible that poor

quality spermatozoa from patients with male factor infertility have deranged redox metabolic activity and greater ability to produce $O_2^{\cdot-}$. Our findings are in agreement with earlier studies (14–16, 37, 38) finding that NADPH, which is present in the residual sperm cytoplasm of the midpiece, plays an important role in $O_2^{\cdot-}$ production and can result in the production of increased levels of such toxic oxygen radicals.

Our study documented for the first time a strong inverse correlation between levels of $O_2^{\cdot-}$ produced in response to exogenous NADPH and sperm concentration, motility, and

morphology. Defective spermatozoa, characterized by poor motility and poor morphology are known to generate higher level of ROS than functionally normal cells. However, the reason for this correlation is not clear. One rational explanation is that poor quality spermatozoa possess defective leaky plasma membranes that are relatively permeable to NADPH.

This allows the nucleotide present in high concentrations in cytoplasmic droplets to penetrate intracellular sites, especially mitochondria, where ROS generation can be initiated (10, 19, 39). However, the correlation of ROS levels with decreased sperm concentration cannot be clearly explained and may require further evaluation on ROS production at the testicular level. Although our study is limited by its small sample size, our objective was to detect and clarify the role of $O_2^{\cdot-}$ production in patients with male factor infertility. Significant results were obtained even in this small sample size.

In conclusion, it appears that NADPH in human spermatozoa mediates ROS production, specifically the superoxide anion. Chemiluminescence coupled with the lucigenin probe may serve as a reliable method for detecting high levels of $O_2^{\cdot-}$ generation, which occurs more frequently in semen samples characterized with poor sperm motility, morphology, and concentration. Thus, the assessment of this superoxide ion should constitute an essential component for reporting the levels of oxidative stress in a semen sample of infertile patients.

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