

INCIDENCE AND LEVEL OF SEMINAL REACTIVE OXYGEN SPECIES IN NORMAL MEN

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ABSTRACT—Objectives. Excessive formation of reactive oxygen species (ROS) in human semen has been associated with impaired sperm function and fertility potential. The presence of ROS in semen specimens from normal fertile men emphasizes the importance of defining a normal range of ROS formation. The purpose of this study was to establish a normal range of ROS generation and to investigate the effect of sperm concentration on the ROS level.

Methods. ROS was determined in 15 healthy donors and 20 men with suspected infertility. After the sperm concentration in normal donors was adjusted to $20 \times 10^6/\text{mL}$, ROS was measured by chemiluminescence using luminol in a Berthold luminometer. A specimen was regarded as positive (abnormal) when the value was at least 10×10^4 counts per minute (cpm). ROS was also evaluated at 4 sperm concentrations (60, 30, 15, and $7.5 \times 10^6/\text{mL}$) from samples obtained from the patients with suspected infertility. In addition, ROS was measured in 7 ROS-positive specimens at a sperm concentration of $15 \times 10^6/\text{mL}$ and $60 \times 10^6/\text{mL}$.

Results. Results showed that ROS formation was negative in all 15 healthy donors (median, 0.9×10^4 cpm; interquartile range, 0 to 1.48×10^4 cpm). The ROS formation value among all the donors was less than 5.5×10^4 cpm. ROS formation was positive in 8 (40%) of the suspected infertile patients. ROS levels were significantly lower at sperm concentrations of $15 \times 10^6/\text{mL}$ or $7.5 \times 10^6/\text{mL}$ compared with $30 \times 10^6/\text{mL}$ or $60 \times 10^6/\text{mL}$ ($P=0.05$). The ROS level increased after centrifugation for 10 minutes at 500 g in all 7 specimens at both 15 and $60 \times 10^6/\text{mL}$. However, the increase in ROS formation at $60 \times 10^6/\text{mL}$ was significantly greater than that at $15 \times 10^6/\text{mL}$ ($P<0.001$).

Conclusions. A range of ROS formation of 0 to 5.5×10^4 cpm at a sperm concentration of $20 \times 10^6/\text{mL}$ may be considered as normal for healthy donor semen. The positive relationship between ROS formation and sperm concentration at the time of measurement emphasizes the importance of concentration adjustment before analysis when comparing ROS levels between different specimens.

Reactive oxygen species (ROS) are free radicals, such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and the hydroxyl radical (OH^\cdot). Recent investigations suggest an association between excessive ROS formation and impaired sperm function and fertilizing potential in some infertile men.¹ Although functionally abnormal

spermatozoa have been considered to be the main source of ROS, studies with spermatozoa obtained from different layers after density gradient centrifugation have shown that ROS generation is not limited to gradients with poor sperm quality.² Nevertheless, a normal level of ROS formation has not been defined.

The use of different methods and the lack of a standardized protocol to assess ROS in humans is partly responsible for this problem. Sperm washing

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procedures using repeated centrifugation or washing with colloidal suspensions (Percoll) have been shown to increase ROS formation in semen.³ Cell (spermatozoa or leukocyte) concentration at the time of ROS analysis is another important variable. The use of buffers to dilute semen before ROS measurement indicates the importance of sperm concentration adjustment at the time of analysis.

Most commonly, ROS is assessed by chemiluminescence. The purpose of this study was to establish the normal level of ROS formation in semen from a group of normal, fertile men and to investigate the effect of sperm concentration on the level of ROS formation in semen from a group of infertile men. Centrifugation of human semen is known to induce a significant increase in ROS formation in a pellet of unselected sperm cells.³ Therefore, ROS was assessed in the basal (uncentrifuged) state and after a one-step centrifugation. The semen specimens from infertile patients were used as positive controls for this study.

MATERIAL AND METHODS

SELECTION OF SUBJECTS

Semen samples were obtained from 15 normal volunteers who were selected on the basis of normal semen analysis results (volume at least 2.0 mL, sperm count at least $20 \times 10^6/\text{mL}$, motility at least 50%, and morphology at least 30% normal spermatozoa).⁴ Semen samples were also collected from 20 patients who came to our laboratory because of suspected subfertility. Only specimens with a sperm concentration of at least $60 \times 10^6/\text{mL}$ were included in this group.

SEMEN COLLECTION AND ASSESSMENT OF SEMEN VARIABLES

Semen specimens were collected by masturbation after 2 to 5 days of sexual abstinence and were then liquified at 37°C for 30 minutes. Five μL of specimen were loaded on a 20 μL Microcell chamber (Conception Technologies, San Diego, CA) and analyzed on a Hamilton-Thorn Motility Analyzer (HTM version 10, IVOS model, Hamilton-Thorn Research, Beverly, MA).

QUANTITATION OF WHITE BLOOD CELLS

The presence of leukocytes in semen specimens was assessed by the Endtz test.⁵ A 20 μL volume of liquified specimen was placed in a 2.0 mL cryogenic vial (Corning Costar Corp, Cambridge, MA); 20 μL of phosphate-buffered saline (pH 7.0) and 40 μL of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 minutes.

Peroxidase-positive white blood cells (WBCs) staining dark brown were counted in all 100 squares of the grid in a Makler chamber (Sefi Medical, Haifa, Israel) under the bright-field objective ($\times 20$). The results after correction for dilution were recorded as counts $\times 10^6/\text{mL}$. All specimens were negative (less than $1 \times 10^6/\text{WBC}/\text{mL}$)⁴ by the Endtz test.

ASSESSMENT OF ROS ACTIVITY IN SEMEN FROM NORMAL VOLUNTEERS

The sperm concentration in specimens obtained from normal men was adjusted to $20 \times 10^6/\text{mL}$ before ROS measurement. Modified human tubal fluid (HTF) medium, (Irvine Scientific, Santa Ana, CA) with human serum albumin (5.0 mg/mL) was used to adjust the concentration. Each specimen was then divided in 2 equal aliquots of 0.5 mL and placed in 17 \times 120 mm polystyrene tubes (Falcon, Lincoln Park, NJ).

ROS formation was measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). A 100 mmol/L stock luminol solution was prepared by dissolving 100 mg luminol powder (Bio Orbit, Turku, Finland) in 5.64 mL dimethyl sulfoxide (DMSO). The working solution (5 mmol/L luminol) was prepared by further dilution (1:20) with DMSO before measurement. Twenty μL of working solution was then added to each semen aliquot for the analysis. Chemiluminescence was measured 10 minutes after addition of luminol using a Berthold (Autolumat LB 953, Wallac Inc., Gaithersburg, MD) luminometer in the integration mode at 37°C. ROS production was expressed as counted photons $\times 10^4$ per minute (cpm). One aliquot was used to measure the background luminescence for each specimen before adding luminol. The background readings were subtracted from the actual test value to obtain the true ROS level. The second aliquot was used to determine the ROS level.

The ROS level was considered abnormal (positive) when the luminescence curve peaked (1 to 4 minutes) after luminol was added (Fig. 1). A positive response was always associated with a value of at least 10×10^4 cpm in the integration mode. Therefore, based on our observations, a chemiluminescence response of 10×10^4 cpm was considered as the upper limit of normal.

MEASUREMENT OF ROS ACTIVITY AT DIFFERENT SPERM CONCENTRATIONS

ROS formation was measured at various sperm concentrations (60, 30, 15, and $7.5 \times 10^6/\text{mL}$) in

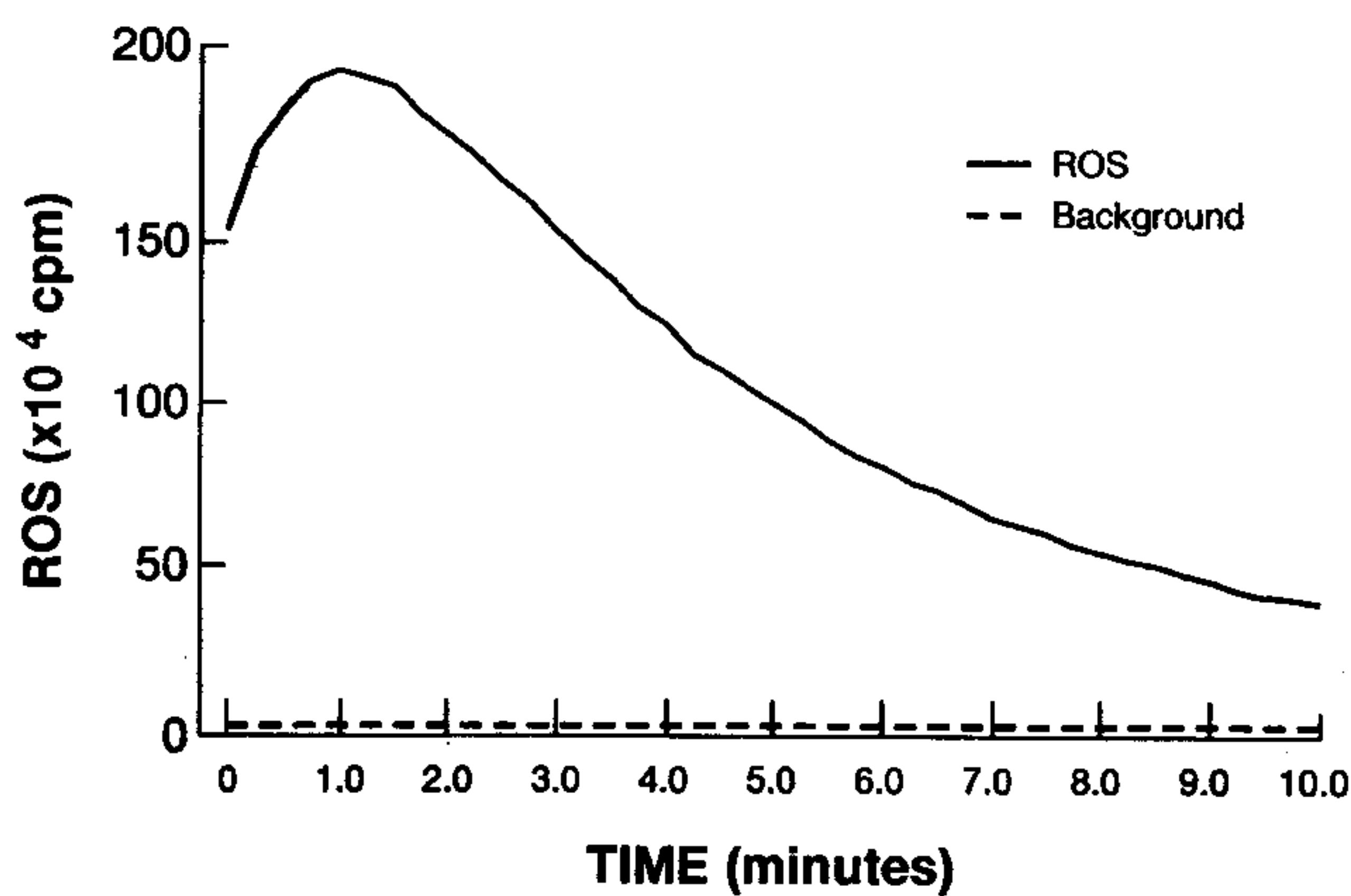


FIGURE 1. A positive reactive oxygen species curve. The chemiluminescence peaks 1 to 2 minutes after luminol is added. The baseline curve shows the background luminescence.

the 20 specimens obtained from patients consulting for infertility. Each specimen was divided into five to seven aliquots. HTF solution was used to adjust the sperm concentration of each aliquot. ROS measurement was conducted with 0.5 mL diluted sperm aliquot.

CENTRIFUGATION PROCEDURE

Seven ROS-positive specimens were used for this part of the experiment. Two aliquots of 0.5 mL each at a sperm concentration of $60 \times 10^6/\text{mL}$ and $15 \times 10^6/\text{mL}$ were centrifuged in a table-top centrifuge (International Equipment Company, Needham, MA) at 500 g for 10 minutes. The sperm pellet was resuspended in supernatant after centrifugation, and each aliquot was transferred to a 120×75 mm polystyrene test tube. The ROS was measured immediately after the addition of 20 μL of 5 mmol/L luminol solution as described above. In addition, a third aliquot (0.5 mL) at a concentration of 60×10^6 mL from each specimen was centrifuged at 500 g for 10 minutes. The seminal plasma was transferred to a polystyrene tube to assess the ROS activity in seminal plasma.

STATISTICAL ANALYSIS

To analyze postcentrifugation semen variables between groups, the Wilcoxon signed rank test was used to compare groups because data were not normally distributed. The results were expressed as median and interquartile range. The interquartile range is defined as the range in which the measurements fall between the lower quartile (25th percentile) and upper (75th percentile) quartile. A *P* value of less than 0.05 was considered significant. The statistical analysis was performed using the Statistical Analysis System (SAS Institute Inc., 1992).

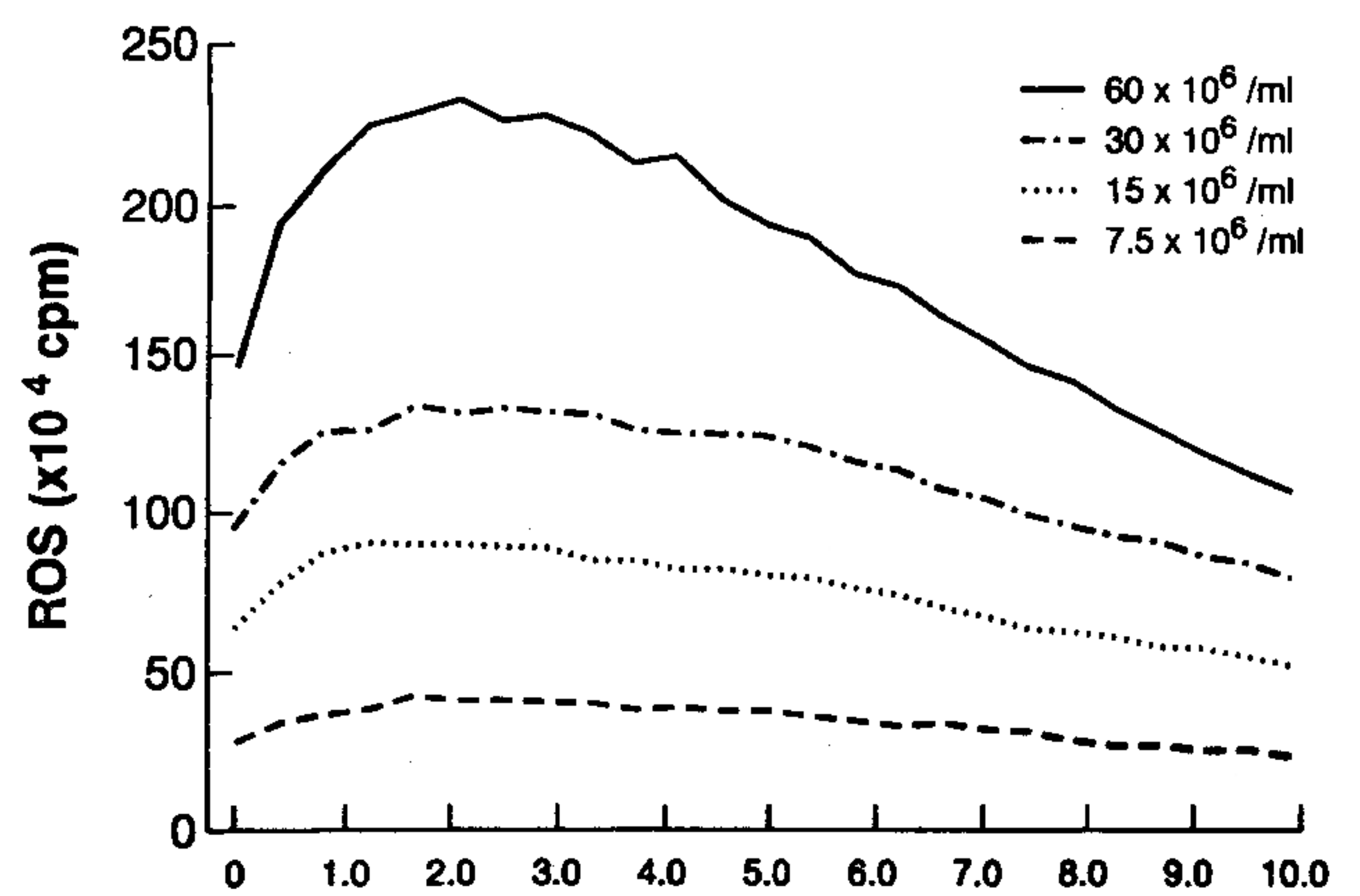


FIGURE 2. An example of the changes in chemiluminescence response at various sperm concentrations from the same specimen. A decrease in ROS formation was seen at the lower sperm concentration.

RESULTS

ROS FORMATION IN NORMAL MEN

All 15 donors were negative for ROS formation ($<10 \times 10^4$ cpm). The ROS formation value among all the donors was $<5.5 \times 10^4$ cpm. The median value of ROS in donors was 0.9×10^4 cpm with an interquartile range of 0 to 1.48×10^4 cpm at a sperm concentration of $20 \times 10^6/\text{mL}$.

EFFECT OF SPERM CONCENTRATION ON THE ROS LEVELS IN WHOLE (RAW) SEMEN

Eight of the 20 patients consulting for infertility showed ROS formation. Although the ROS level decreased in all specimens at lower sperm concentrations, the differences were significant ($P < 0.05$) in those specimens with an initial positive response (Table I; Fig. 2). Two initially positive specimens did not show a positive response at a sperm count of $7.5 \times 10^6/\text{mL}$.

ROS ACTIVITY AFTER CENTRIFUGATION AT VARIOUS SPERM CONCENTRATIONS

The ROS level increased after centrifugation in all seven specimens at both 15 and $60 \times 10^6/\text{mL}$ ($P < 0.003$). However, the median ROS level of 1282 (interquartile range 132.6 to 1799.4×10^4 cpm) differed significantly in specimens with a sperm count of $60 \times 10^6/\text{mL}$ compared with $15 \times 10^6/\text{mL}$, median 257.3 (interquartile range 16 to 663.9×10^4 cpm) ($P < 0.001$; Fig. 3).

ROS ACTIVITY OF SEMINAL PLASMA

None of the seven specimens with initially positive ROS formation showed ROS activity in seminal plasma (ie, without sperm).

COMMENT

Normal men in this study showed ROS levels between 0 and 5.5×10^4 cpm, a range that can

TABLE I. Effect of sperm concentration on reactive oxygen species level (ROS) in patients with suspected subfertility

Sperm Concentration ($\times 10^6/\text{mL}$)	ROS Level ($\times 10^4$ cpm)	
	All Patients (n = 20) Median* (Interquartile Range)	ROS-Positive Patients (n = 8) Median (Interquartile Range)
60	3.62 (0 to 197.5)	297.3 (38.1 to 491.1)
30	2.05 (0.39 to 126.5)	165.6 (13.7 to 237.4)
15	1.23 (0 to 77.6)	77.6 (9.9 to 139.2)
7.5	0.12 (0 to 28.4)	37.0 (21.3 to 46.4)

*The differences between all sperm concentration groups were significant ($P \leq 0.005$)

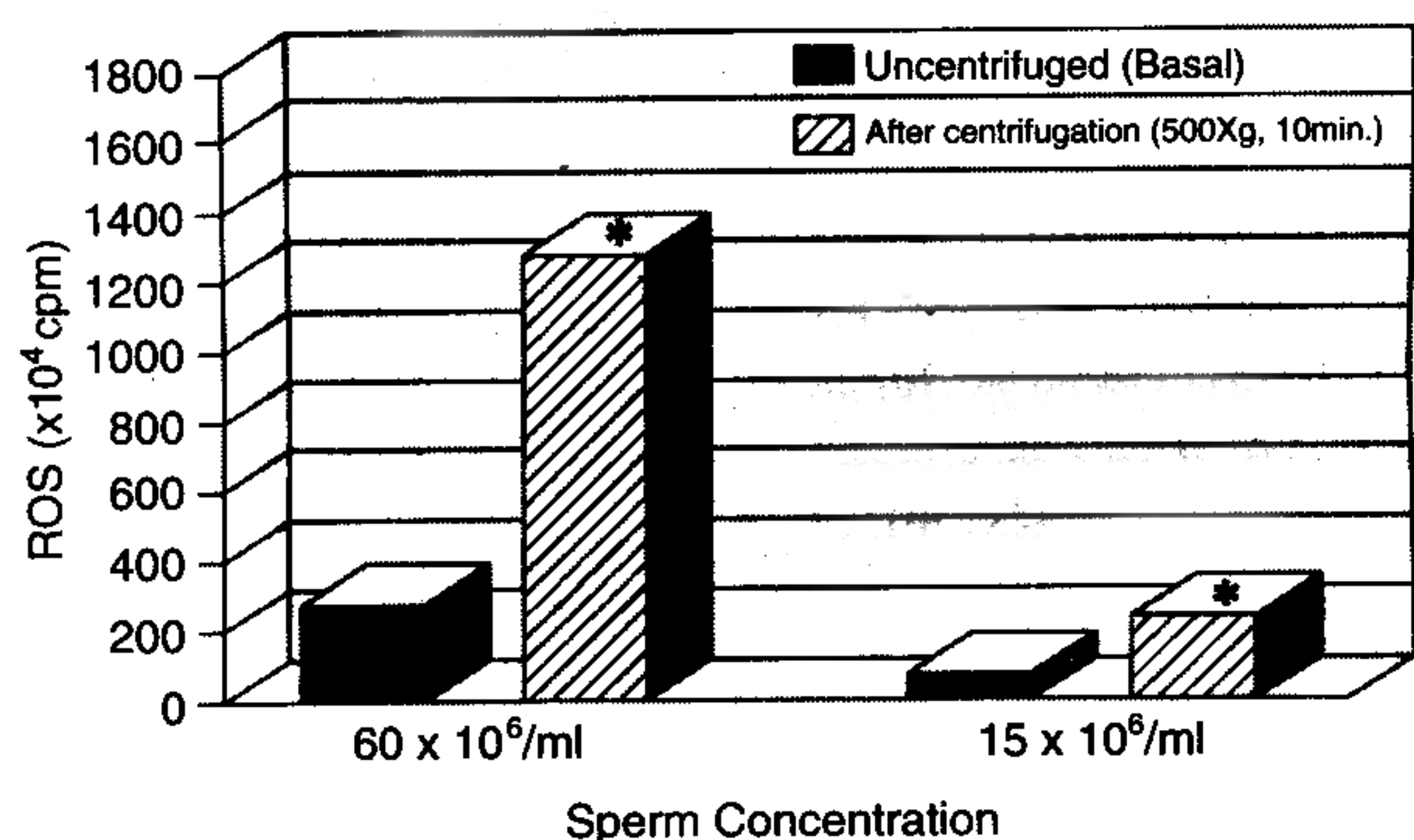


FIGURE 3. ROS levels after centrifugation at 500 g for 10 minutes at 2 different sperm concentrations. The ROS levels at sperm counts of $60 \times 10^6/\text{mL}$ and $15 \times 10^6/\text{mL}$ after centrifugation differ significantly from levels obtained from uncentrifuged sperm (* $P < 0.003$).

be regarded as normal. A standard sperm concentration for analysis ($20 \times 10^6/\text{mL}$ in this study) allows ROS levels to be compared in normal men. However, a positive chemiluminescence response in our study was consistent with a value equal to or greater than 10×10^4 cpm. The ROS values from 5.5 to 10×10^4 cpm, which were seen in some of our patients being evaluated for infertility (unpublished data), may be regarded as an intermediate range. The presence of a poor-quality sperm subpopulation in semen from normal donors may explain the variation in ROS formation in normal men. Our results support previous findings that the incidence of ROS formation in a population of normal, fertile men is lower than in men with suspected infertility.⁶

Chemiluminescence is based on the reaction of luminol with free radicals, which results in light emission. The level of ROS in a specimen is proportional to the light emission from the sample. At neutral pH, luminol permeates the spermatozoa membrane. The generated ROS, therefore, has been considered to be of intra- as well as extracellular origin.⁷ Increased intracellular ROS gen-

eration indicates a functional defect of the spermatozoal membrane, whereas the extracellular ROS in seminal plasma damages the normal spermatozoa by lipid peroxidation.⁸

Luminescence is not specific to the source of ROS formation. The presence of WBCs in semen may also result in a positive response. There have been conflicting data regarding the relationship between WBC concentration in semen and the level of ROS formation.⁹⁻¹¹ The use of different techniques to determine the WBC count explains this controversy. We have found that the association between the positive Endtz test (more than 1×10^6 WBC/mL) and positive ROS formation in semen is highly significant (unpublished data). Therefore, by using the Endtz test to quantitate leukocytes in our study, we were able to determine the leukocytic contribution to positive responses. Nevertheless, the variability in normal ROS levels in the normal group may also be partially a result of subclinical leukocyte concentration. The higher incidence of leukocytospermia in men consulting for infertility may also contribute to the higher incidence of ROS formation.¹²

Assessment of ROS in human semen has been performed using two principal protocols: measurement in a washed suspension of spermatozoa after removal of seminal plasma, and measurement in whole semen. In initial studies of washed spermatozoa, a burst of ROS formation was seen in response to the divalent cation ionophore A23187.⁷ However, repeated centrifugation was used to separate spermatozoa from the cellular components. A single-step centrifugation in this study significantly increased the ROS level in all positive specimens. The increase in ROS formation was significantly greater in specimens with a higher sperm concentration. Furthermore, removal of seminal plasma per se may increase the ROS level regardless of sperm processing. This idea is supported by the finding that application of seminal plasma to suspension of spermatozoa or leukocytes can

decrease ROS activity.^{6,13} The presence of several natural antioxidants, such as superoxide dismutase, in seminal plasma may explain this finding.¹⁴ Therefore, measurement of ROS in unprocessed semen is more accurate for clinical purposes.

The relationship between ROS level and sperm concentration may be explained by the lack of ROS activity in seminal plasma. The ROS level changed significantly at various sperm concentrations taken from the same specimen in ROS-positive specimens and may not be detectable at very low sperm concentrations.

In conclusion, using the chemiluminescence method, ROS formation of 0.0 to 5.5×10^4 cpm at a sperm concentration of $20 \times 10^6/\text{mL}$ may be considered as normal for healthy donor semen. The positive relationship between ROS formation and the sperm concentration at the time of measurement emphasizes the importance of concentration as a variable when comparing ROS levels between repeated measurements or between different positive specimens. However, evaluating the effect of ROS on semen quality requires an initial measurement in whole semen in patients consulting for infertility.

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