PÉROXYDATION ET POUVOIR FÉCONDANT DU SPERMATOZOÏDE

A. AGARWAL*, S. HAMAMAH**, M. SHEKARRIZ*

La formation excessive de radicaux hydropéroxydes (ROS) dans le sperme humain est associée chez certains hommes infertiles à une dysfonction spermatique et une diminution du pouvoir fécondant. Les spermatozoïdes altérés ainsi que les leucocytes du sperme sont la source principale de ces ROS. De plus, la préparation du sperme (centrifugation et lavage) entraîne une production de ces radicaux péroxydes. Le but de ce travail est d'étudier l'effet de centrifugation (temps, force en g) sur la production des ROS dans le sperme. Il ressort que la durée de centrifugation est plus importante que la force en g dans la génération de la péroxydation et un temps court est donc préconisé dans la préparation du sperme pour la PMA. La forte corrélation entre la coloration de Endz et la péroxydation implique que cette coloration simple peut être utilisée comme indication de la péroxydation dans l'infertilité masculine. *(Contracept. Fertil. Sex., 1994, 22, 5, 327-330.)*

Reactive oxygen species and fertilizing capacity of spermatozoa

Excessive reactive oxygen species formation in human semen has been associated with impaired sperm function and fertility potential in some men with idiopathic infertility. Subnormal spermatozoa as well as white blood cells (WBCs) are the main source of ROS detected in human semen. Furthermore, sperm processing (centrifugation and washing) results in a burst of ROS generation. The purpose of this study was to investigate the correlation between centrifugation parameters (time and g-force) and ROS production and to study the correlation between ROS formation in semen and the leukocytospermia. We conclude that the time of centrifugation is more important than g-force for inducing ROS formation in semen and recommend short-term centrifugation in the sperm preparation for assisted reproductive techniques. The strong positive correlation of Endtz test with the ROS formation in semen indicate that simple, cost efficient myeloperoxidase staining technique (Endtz test) could be utilized as an indicator of excessive ROS formation in semen that may adversely affect fertilizing capacity of spermatozoa. (Contracept. Fertil. Sex., 1994, 22, 5, 327-330.)

Mots clés : Spermatozoïdes - Infertilité - Péroxydation - Leucospermie - Coloration de Endtz.
Key words: Spermatozoa - Infertility - ROS - Leukocytospermia - Endtz test.

INTRODUCTION

Idiopathic infertility represents the most common cause of male factor infertility (1). Advances in assessment of functional properties of spermatozoa revealed that defective sperm function despite normal routine semen analysis is a common condition in this patient population. Recent investigations on the biochemical basis of these abnormalities suggest an association between excessive generation of reactive oxygen species (ROS) and impaired sperm function as characterized by a failure to exhibit sperm-oocyte fusion (2). ROS are free radicals (viz: hydrogen peroxide, the superoxide anion, and the hydroxyl radicals), which can be produced by the main cellular components (spermatozoa and white blood cells) of semen (3). The deleterious effect of ROS on spermatozoa has been shown to be mediated by lipid peroxidation of the sperm membrane. Peroxide applied exogenously to a suspension of human spermatozoa caused peroxidation of phospholipids present in spermatozoa and act as a potent spermicidal agent (4). High content of polyunsaturated fatty acids is thought to be responsible for the susceptibility of human sperm to lipid peroxidation (2, 4). Recently, depletion of mitochondrial ATP in the presence of these free radicals has also been reported (5, 6).

Seminal plasma and spermatozoa contains scavenger systems such as superoxide dismutase and glutathione peroxidase / reductase (7, 8). These scavengers maintain a balance between the generated and scavenged ROS in semen. A disturbance of this equilibrium has been thought to result in an excessive ROS formation (9). Gagnon et coll. (10) reported that 40 % of 172 unselected men consulting for infertility had detectable and 25 % high levels of ROS in semen. In our own studies the incidence of positive ROS was 22 % in a unselected population of infertile men.

In this study, the effect of sperm preparation techniques on the level of ROS was investigated. The conventional technique for preparing human spermatozoa involves repeated centrifugation and resuspension in a fresh medium before selecting highly motile spermatozoa (11). Repeated centrifugation induces a significant increase in (ROS) formation in a pellet of unselected cells of human semen (12). The purpose of this study was two-fold, first to investigate the correlation of centrifugation parameters, speed and time with the ROS level in whole semen. Secondly, to determine an optimal speed and time of centrifugation that may minimize ROS generation during sperm preparation.

The second part of this study addresses the correlation of leukocytospermia with the formation of ROS as determined by a myelope-

roxidase staining technique (13). Although, the significance of leukocytospermia in semen remains controversial, there is now evidence that white blood cells (WBCs) could adversely affect sperm function and act as a potential cofactor in male infertility (14-16). The mechanism by which leukocytes could alter sperm function in vivo is currently unknown. Recent investigations suggest that reactive oxygen species (ROS) generated by the polymorphonuclear (PMN) granulocytes could adversely affect sperm function (17).

MATERIALS AND METHODS

Selection of subjects

Semen samples were obtained from 46 men who came to our laboratory because of suspected subfertility and 16 normal volunteers who were selected on the basis of normal semen analysis results (volume ≥ 1.5 mL, sperm count ≥ 20 x 10^6 / ml, motility ≥ 50 % and morphology ≥ 50 % normal spermatozoa). Only specimens with a sperm concentrations of ≥ 15 x 10^6 / mL were included in this study.

Semen collection and assessment of semen parameter

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

Quantitation of White Blood Cells

The presence of granulocytes in semen specimens was assessed by the Endtz test (13). A 20-µL volume of liquified 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 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 20 X bright-field objective. The results after correction for dilution were recorded as counts X 10^6 / mL.. All specimens were negative (< 1 X 10^6 / mL) by the Endtz test.

Measurement of ROS activity

Sperm concentration in all Endtz negative specimens was adjusted to 15-20 X 10^6 / mL before ROS measurement. Modified HTF (human tubal fluid, Irvine Scientific, Santa Ana, CA) medium with human serum albumin (5.0 mg / mL) was used for concentration adjustment. Two aliquot of 0.5 mL from each specimens was then used for measurement of background luminescence before adding luminol and basal (uncentrifuged) ROS formation in all specimens by chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). The background readings were substracted from the test values to give the level of ROS.

A 100-mM stock solution luminol was prepared by dissolving 100 mg powder of luminol (Bio Orbit, Turku, Finland) with 5.64 mL of dimethyl sulfoxide (DMSO). The working solution (5 mM luminol) was prepared by further dilution (1:20) with DMSO prior to measurement. Twenty µL of the above working solution was then added to the semen aliquot for the analysis. Luminol is a sensitive chemiluminescent probe which has been shown to react with a variety of free radicals (hydrogen peroxide, hydroxyl radicals, superoxide anions). The reaction of luminol with free radicals results in light emission which is proportional to the ROS level in the sample. Chemiluminescence was measured using a Berthold (Autolumat LB 953, Wallac Incorporated, Gaithersburg, MD) luminometer 10 minutes after the addition of luminol in the integration mode at 37 °C. ROS production was expressed as X10^4 counted photons per minutes (cpm).

ROS level was considered abnormal (positive) when the luminescence curve peaked 1-4 minutes after addition of luminol (figure 1). A positive response was associated with a value of at least ≥ 10 X 10^4 cpm in the integration mode. All values greater than or equal to 10 X 10^4 cpm were considered as abnormal or positive.

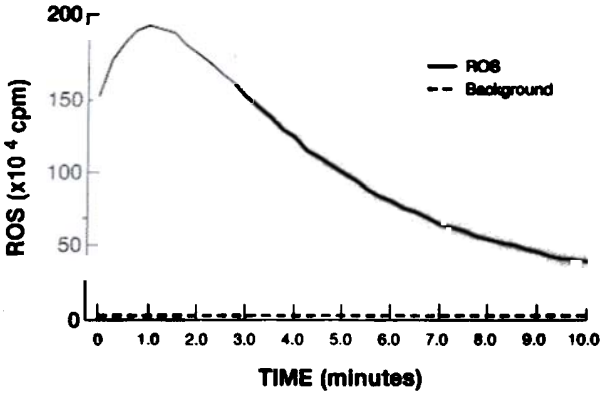

Figure 1 : A positive ROS curve in a Endtz-positive specimen. The chemiluminescence peaks 1-2 minutes after adding luminol. The baseline curve demonstrate the background luminescence.

Centrifugation procedure

Specimens from 38 men (24 patients and 14 normal donors) were used for this study. After adjustment of sperm concentration as explained above. Each specimen was divided into six equal aliquots of 0.5 mL using 17 X 120 mm polystyrene tubes (Falcon, Lincoln park, NJ) for centrifugation. Two aliquots were ten used for determination of basal ROS. The four remaining aliquots were centrifuged in a table top IEC centrifuge (International Equipment, Needham, MA) and centrifuged at the following speed or time intervals: 200 Xg 2 min, 200 Xg 10 min, 500 Xg 2 min and 500 Xg 10 min. The sperm pellet was resuspended in supernatant after centrifugation and each aliquot was transferred into a 12 X 75 mm polystyrene test tube. ROS was measured immediately after adding 20 µL of 5 mM luminol solution as described above.

Statistical analysis

The non-parametric Wilcoxon signed rank test was used as data was found to be not normally distributed for comparing ROS formation in the basal state and after centrifugation between different groups. A Fisher's Exact test was used to compare the incidence of leukocytospermia and ROS formation between patients and

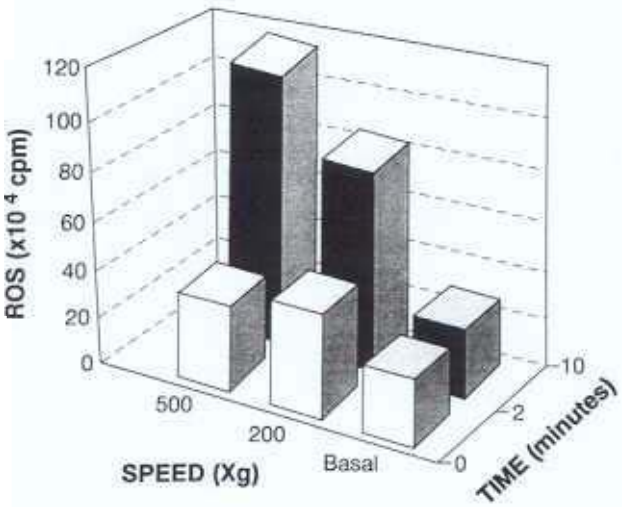

Figure 2 : ROS levels after centrifugation were compared with those in basal stage. A short-term centrifugation for 2-minute could significantly increase the ROS formation ($p < 0.001$). The differences between 200 Xg and 500 Xg were not significant. The increase in centrifugation time to 10 minutes at both 200 Xg or 500 Xg induced a further significant increase in ROS formation ($p < 0.001$) . However, the level of ROS after centrifugation at 500 Xg for 10-min was significantly higher than at 200 Xg ($p < 0.001$).

donors as well as the association between Endtz test results and positive ROS formation. A student's t-test was used to compare ROS values between Endtz positive and Endtz negative as well as patients and donors specimens. A P value of < 0.05 was considered significant. All statistical analysis were performed using the SAS statistical software package.

RESULTS

Comparison of ROS levels before and after centrifugation

The basal ROS level before centrifugation was 28.64 ± 125 X 10^4 cpm. ROS formation after two minutes of centrifugation at 200 Xg for 2 min was 43.88 ± 147 X 10^4 cpm ($p < 0.01$) as compared to 37.39 ± 122.4 X 10^4 cpm after 500 Xg for 2 min ($p = 0.02$). The differences were not statistically significant. Similarly, ROS formation after 10 min of centrifugation at 200 Xg was 85.35 ± 258 X 10^4 cpm as compared to 117.45 ± 341.7 X 10^4 cpm at 500 Xg ($p < 0.001$) (figure 2).

Effect of centrifugation on ROS negative specimens

Although, most specimens (n = 24) with a low level of ROS (< 10 X 10^4 cpm) did not show a significant increase in ROS formation after centrifugation, six specimens (four patients and two donor) with initially low (negative) ROS formation demonstrated a late response after 10 min of centrifugation at 200 Xg or 500 Xg with an increase in chemiluminescence curve. ROS levels in this group in the basal state was 3.78 ± 3.4 X 10^4 cpm as compared to 67.9 ± 60 X 10^4 cpm after centrifugation at 500 Xg for 10 min ($P < 0.001$) (figure 3).

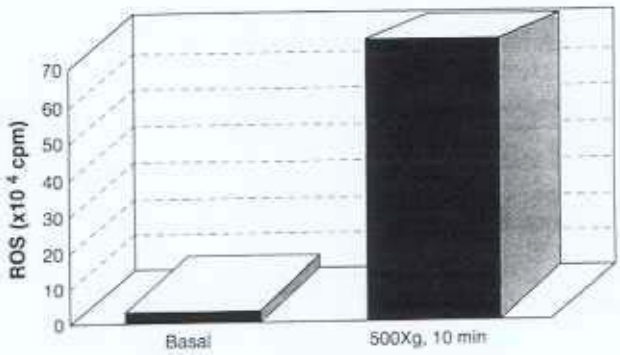

Figure 3 : Six specimens with negative ROS levels in the basal state cpm showed a positive ROS formation after ten minutes centrifugation at 500 Xg ($p < 0.001$).

Leukocytospermia in patients and donors

The incidence of significant leukocytospermia (≥ 1 X 10^6 / mL sperm) as determined by Endtz test in patients was 21.7.2 % (n = 10) as compared to 6.2 % (n = 1) in donors). The differences were not significant.

Incidence of ROS formation in Endtz-negative and Endtz-positive specimens

Of the 36 Endtz negative patients eight (22.2 %) were found to be positive for ROS formation. Out of the 15 Endtz negative donors two (13.3 %) were positive for ROS formation. All specimens with a positive Endtz test (n = 11) were also positive for ROS formation (≥ 10 X 10^4 cpm). Comparison of ROS positive specimens between Endtz positive and Endtz negative specimens in donor and patients showed a strong correlation between a positive Endtz test result and positive ROS formation ($p < 0.001$; table 1).

Table 1 : Comparison of ROS positive-Endtz negative with the ROS positive-Endtz specimens. The correlation of a positive Endtz test with positive ROS formation was highly significant ($p < 0.001$).

Specimen	Endtz(-) subjects*	Endtz(+) subjects	Total
ROS (-)	41 (80.4 %)	0 (0.0 %)	41
ROS (+)	10 (19.6 %)	11 (100.0 %)	21
Total	51	11	62

ROS levels in ROS-positive and ROS-negative specimens

Comparison of ROS levels between Endtz positive-ROS positive specimens and Endtz negative-ROS positive specimens showed no significant difference (161.49 ± 258.1 vs 305.95 ± 720 ; ROS level in specimens defined as negative (< 10 x 10^4 cpm) was 0.93 ± 1.01 in patients as compared to 0.92 ± 1.95 in donors. These differences were not statistically significant.

Correlation between WBCs concentration and ROS levels

Although, a very high ROS formation was seen in some specimens with high leukocyte concentration, a correlation between the leuko-

cyte concentration and the level of ROS at the time of measurement was not seen.

DISCUSSION

Our results indicate that even a single-step centrifugation for a short period (2 minutes in our study) can significantly increase ROS formation in some human semen. Although, most of the specimens with a low initial ROS level did not show any increase after centrifugation, ROS formation was a late response after 10 minutes centrifugation in six (two donor, four patient) cases. This data emphasize the importance of ROS assessment in the whole semen for its clinical significance, since aside from the elimination of the protective effect of seminal plasma, centrifugation used to separate seminal plasma from the cellular component of semen before ROS assessment can also change the ROS level in some specimens. The effect of centrifugation time on ROS generation is significantly more than the speed. However, after longer periods of centrifugation, the increase in centrifugation speed will potentiate the effect of time on ROS production.

The mechanism stimulating ROS formation by centrifugation as described by Aitken and Clarkson (12) is thought to be through the mechanical damage of the sperm plasma membrane associated with centrifugation. Theoretically, a longer period of centrifugation may then induce ROS formation by a subpopulation of spermatozoa with a suboptimal membrane function. Alternatively, centrifugation can activate seminal leukocytes (in concentration < 1 X 10^6) and can disturb the balance between the ROS formation and the scavengers present in semen (18). Nevertheless, centrifugation *per se* has also been shown to damage human spermatozoa. The percentage of motile sperm diminished after centrifugation as a function of time (19). Deterioration of sperm motility was believed to be caused by a direct mechanical effect of centrifugation (19). Both the direct effect of centrifugation through mechanical damage as well as the indirect adverse effect of excessive ROS formation may cause deterioration of sperm function.

A high correlation was seen in our study between a positive Endtz test and a positive chemiluminescence response for ROS in whole semen. The diagnosis of leukocytospermia has been reported with cytochemical, immunohistochemical and morphological techniques (20, 21). The Endtz test is based on the peroxidase activity of polymorphonuclear leukocytes (PMN) and has been recommended by the World Health Organization (WHO) (22) for determination of WBCs in semen. The advantage of this method is its simplicity and low cost as compared to more complicated and expensive methods such as the use of monoclonal antibodies. For clinical purposes, an ideal test must be easy to perform, fast, and inexpensive. The limitation of this technique is the lack of lymphocyte detection in semen. However, peroxidase positive leukocytes (neutrophils and macrophages) are the main leukocytes present in semen (20), which also represent the source of ROS formation by phagocytosis. This make the use of the Endtz test for the screening of high level of ROS in semen suitable.

In conclusion, we suggest a positive Endtz test is an indicator of positive chemiluminescence for ROS in unprocessed human semen regardless of WBCs activity or presence of seminal plasma. This test is cost-efficient and easy to perform and its results can be utilized for prediction of the possible peroxidative damage to spermatozoa which may adversely affect their fertilizing potential.

Future research efforts should be directed toward techniques that avoid or minimize the use of centrifugation for sperm preparation for ART. Further investigations of factors which may influence ROS formation in semen would improve our understanding of the role of ROS in idiopathic infertility.

Acknolewdgments

We wish to thank Dr Cl. Barthélémy for her advice and consultation in the preparation of this paper.

BIBLIOGRAPHY

1 - AITKEN R. J., CLARKSON J. S. - Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species. *J. Rep. Fertil.*, 1987, 81, 459.
2 - AITKEN R. J., CLARKSON J. S., FISHEL S. - Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol. Reprod.*, 1989, 40, 183.
3 - KESSOPOULOU E., TOMLINSON M. J., BARRAT C. L. R., BOLTON A. E., COOKE I. D. - Origin of reactive oxygen species in human semen: spermatozoa or leukocytes. *J. Reprod. Fert.*, 1992, 94, 463.
4 - JONES R., MANN T., SHERINS R. - Peroxidative breakdown of phopholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil. Steril.*, 1979, 5, 531.
5 - DE LAMIRANDE E., GAGNON C. - Reactive oxygen species and human spermatozoa I. Effect on the motility of intact spermatozoa and on sperm axonemes. *J. Androl.*, 1992, 13, 368.
6 - DE LAMIRANDE E., GAGNON C. - Reactive oxygen species and human spermatozoa II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J. Androl.*, 1992, 13, 379.
7 - ALVAREZ J. G., TOUCHSTONE J. C., BLASCO L., STOREY B. T. - Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa-superoxide dismutase as major enzyme protectant against oxygen toxicity. *J. Androl.*, 1987, 8, 338.
8 - ALVAREZ J. G., STOREY B. T. - Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.*, 1989, 23, 77.
9 - GAGNON C., IWASAKI A., DE LAMIRANDE E., KOVALSKI N. - Reactive oxygen species and human spermatozoa. *Ann. NY Acad. Sci.*, 1992, 637, 436-444.
10 - IWASAKI A., GAGNON C. - Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil. Steril.*, 1992, 57, 409.
11 - MORTIMER D. - Sperm preparation techniques and iatrogenic failures of in vitro fertilization. *Hum. Reprod.*, 1991, 6, 173.
12 - AITKEN R. J., CLARKSON J. S. - Significance of reactive oxygen species and antioxidants in defining the efficiency of sperm preparation techniques. *J. Androl.*, 1989, 9, 367.
13 - ENDTZ A. W. - A rapid staining method for differentiating granulocytes from « germinal cells » in papanicolaou-stained semen. *Acta. Cytol.*, 1974, 18, 2.
14 - WOLFF H., POLITCH J. A., MARTINEZ A., HAIMOVICI F., HILL J. A., ANDERSON D. J. - Leukocytospermia is associated with poor sperm quality. *Fertil. Steril.*, 1990, 53, 528.
15 - EL-DEMIRY, YOUNG H., ELTON R. A., HARGREWAVE T. B., JAMES K., CHISHLM G. D. - Leucocytes in ejaculate from fertile and infertile men. *Br. J. Urol.*, 1986, 58, 715.
16 - TOMLINSON M. J., BARRATT C. L., COOKE J. D. - Prospective study of leukocytes and leukocyte subpopulations in semen suggests that are not a cause of male infertility. *Fertil. Steril.*, 1993, 60, 1069.
17 - KOVALSKI N. N., DE LAMIRANDE E., GAGNON C. - Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effect of seminal plasma and scavengers. *Fertil. Steril.*, 1992, 58, 809.
18 - AITKEN R. J., WEST K. M. - Analysis of the relationship between reactive oxygen species production and leukocyte infiltration in fractions of human semen separated on percoll gradients. *Int. J. Androl.*, 1990, 13, 433.
19 - ALVAREZ J. G., LASSO J. L., BLASCO L., NUNEZ R. C., HEYNER S., CABALLERO P. P., STOREY B. - Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Fertil. Steril.*, 1993, 57, 409.
20 - WOLFF H., PANHANS A., ZEBHAUSER M., MEURER M. - Comparison of three methods to detect white blood cells in semen: leukocyte esterase dipstick test, granulocyte elastase enzyme immunoassay, and peroxidase cytochemistry. *Fertil. Steril.*, 1992, 58, 1260.
21 - JOCHUM M., PAPST W., SCHILL W. B. - Granulocyte elastase as a sensitive diagnostic parameter of silent male genital inflammation. *Andrologia*, 1986, 18, 413.
22 - World Health Organization. WHO Laboratory manual for the examination of human semen and semen-cervical mucus interaction. *2nd ed. Cambridge: The Press Syndicate of the University of Cambridge*, 1987, 29.

* Department of Urology, A 100, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA
** Unité de biologie de la reproduction, Département de gynéco-obs. reproduction et médecine foetale, CHU Bretonneau, 37044 Tours, France