

Mohammad Shekarriz

D.M. DeWire

Anthony J Thomas, Jr.

Ashok Agarwal

Andrology Laboratory,
Department of Urology,
The Cleveland Clinic Foundation,
Cleveland, Ohio, USA

Key Words

Spermatozoa

Infertility

ROS

Centrifugation

Sperm washing

A Method of Human Semen Centrifugation to Minimize the Iatrogenic Sperm Injuries Caused by Reactive Oxygen Species

Abstract

Current techniques of sperm preparation for in vitro fertilization or intrauterine insemination require centrifugation of human semen to separate spermatozoa from the seminal plasma. Centrifugation increases reactive oxygen species (ROS) formation in semen. Moreover, high levels of ROS are associated with sperm membrane injury through spontaneous lipid peroxidation, which may alter sperm function. We investigated the relationship between centrifugation variables (time and g force) and ROS production to establish an optimal centrifugation protocol for sperm preparation techniques. Semen from 38 men (24 patients and 14 normal volunteers) was evaluated for the formation of ROS before centrifugation and after centrifugation at 200 g for 2 or 10 min and after 500 g for 2 or 10 min. The absence of white blood cells in semen which can also produce ROS was determined with the myeloperoxidase technique (Endtz test). All specimens were negative ($< 1 \times 10^6/\text{ml}$) by the Endtz test. The formation of ROS was measured by chemiluminescence. ROS formation was regarded as high (positive) when the chemiluminescence response was at least 10×10^4 counted photons/min (cpm). The sperm concentration in each sample was adjusted to $15\text{-}20 \times 10^6$ cells/ml before analysis. Eight specimens (7 patients and 1 donor) exhibited high levels of ROS before centrifugation. All 8 showed further, significant increases in ROS formation regardless of g-force or time. The increase in ROS was significantly less when semen was centrifuged for 2 as compared to 10 min ($p < 0.001$). Six specimens previously ROS-negative became ROS-positive after centrifugation for 10 min at 200 and 500 g. We conclude that the time of centrifugation is more important than g-force for inducing ROS formation in semen. Based on these results, we recommend a shorter centrifugation period in the preparation of sperm for assisted reproductive techniques.

Ashok Agarwal, Ph.D.
Department of Urology, A100
The Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland, OH 44195 (USA)

© 1995 S. Karger AG, Basel
0302-2838/95/0281-0031

Introduction

Centrifugation of human semen is the most effective and widely used method for separating seminal plasma from the cellular components [1, 2]. Separation of human spermatozoa from seminal plasma to improve their fertilizing capacity is an essential part of sperm preparation for assisted reproductive techniques, such as intrauterine insemination or in vitro fertilization (IVF) [3]. Moreover, centrifugation of human spermatozoa is also required after cryopreservation because cryopreserved specimens contain various extenders, such as egg yolk, that need to be removed before insemination [4].

The conventional technique for preparing human spermatozoa involves repeated centrifugation and resuspension in a fresh medium before selecting highly motile spermatozoa. The 'washed pellet' method is successful in preparing spermatozoa for IVF in cases where no male factor is present [3]. However, as the indications for IVF are expanded to those with male factor infertility and idiopathic infertility, there is a noted increase in failure [5-7].

Repeated centrifugation induces a significant increase in reactive oxygen species (ROS) formation in a pellet of unselected cells of human semen [1]. Excessive ROS in human semen has recently been associated with impaired function and fertilizing capacity of human spermatozoa [8-10]. The deleterious effect of ROS on spermatozoa is caused by the lipid peroxidation of the sperm membrane, as well as by depletion of mitochondrial ATP in the presence of these free radicals [11, 12].

ROS can be generated by spermatozoa as well as leukocytes. Although the spermatozoal origin of ROS remains unclear, some evidence indicates that sperm damage is caused by ROS as well as by centrifugation [3, 11, 13].

The purpose of this study was two-fold; to investigate the relationship between centrifugation variables, speed and time, and the ROS level in whole semen, and to determine an optimal speed and time of centrifugation that may minimize ROS

generation during sperm preparation.

Materials and Methods

Selection of Subjects

Semen samples were obtained from 38 patients and donors over a 4 month period. The first group consisted of specimens from 14 normal volunteers who were selected on the basis of normal semen analysis results (volume \geq 2.0 ml, sperm count \geq 20×10^6 /ml, motility \geq 50% and morphology \geq 50% normal spermatozoa). The second group consisted of semen samples collected from 24 patients who came to our laboratory because of suspected subfertility. Only specimens with a sperm concentration of \geq 15×10^6 /ml were included in this study.

Semen Collection and Assessment of Characteristics

Semen specimens were collected by masturbation after at least 2 days of sexual abstinence and liquified at 37 ° C for 30 min. Five microliters of specimen were loaded on a 20-p1 Microcell chamber (Conception Technologies, San Diego, Calif.) and analyzed on a Hamilton-Thorn Motility Analyzer, HTM version 10, model IVOS (Hamilton-Thorn Research, Beverly, Mass.).

Quantitation of White Blood Cells

The presence of granulocytes in semen specimens was assessed by the Endtz test [14]. A 20-g1 volume of liquified specimen was placed in a Corning 2.0-ml cryogenic vial; 20 g1 of phosphate-buffered saline and 40 μ l of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 min. Peroxidase positive white blood cells, 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 $\times 10^6$ /ml. All specimens were negative ($<$ 1×10^6 /ml) by the Endtz test.

Measurement of ROS Activity

Sperm concentration in each sample was adjusted to 15 - 20×10^6 /ml before ROS measurement. Each specimen was then divided into seven equal aliquots of 0.5 ml using 17 x 120 mm polystyrene

tubes (Falcon, Lincoln Park, N.J.). Modified human tubal fluid (Irvine Scientific, Santa Ana, Calif.) medium with human serum albumin (5.0 mg/ml) was used for concentration adjustment.

ROS formation was measured by the chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione).

Luminol is a sensitive chemiluminescent probe that reacts with a variety of free radicals (hydrogen peroxide, hydroxyl radicals, superoxide anions) [1, 8, 15]. A 100 mmol stock solution of luminol was prepared by dissolving 100 mg of luminol powder (Bio Orbit, Turku, Finland) with 5.64 ml of dimethyl sulfoxide (DMSO). The working solution (5 mmol luminol) was prepared by further dilution (1:20) with DMSO before measurement. Twenty microliters of the above solution were then added to each semen aliquot for the analysis.

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 min after the addition of luminol in the integration mode at 37°C. ROS production was expressed as $\times 10^4$ counted photons/min (cpm). One aliquot was used to measure the background luminescence for each specimen before adding luminol. The background readings were subtracted from the test value to give the level of ROS. The second aliquot was used to determine the basal ROS level and was not centrifuged. Four aliquots were used for different centrifugation protocols as described below.

ROS level was considered abnormal (positive) when the luminescence curve (fig. 1) peaked 1-4 min after the addition of luminol. A positive response was associated with a value of at least 10×10^4 cpm in the integration mode. Therefore, all values greater than or equal to 10×10^4 cpm were considered as abnormal or positive.

Centrifugation Procedure

Four out of five remaining aliquots were centrifuged in a tabletop IEC centrifuge (International Equipment, Needham, Mass.) at the

following speeds and times: 200 g 2 min, 200 g 10 min, 500 g 2 min, and 500 g 10 min. The sperm pellet was resuspended in supernatant after centrifugation, and each aliquot was transferred into a 120 x 75 mm polystyrene test tube. ROS was measured immediately after adding 20 μ l of 5 mmol luminol solution as described above.

ROS Activity of Seminal Plasma

The seventh aliquot from each specimen with an ROS-positive response was centrifuged at 1,000 g for 10 min. The seminal plasma was transferred into a polystyrene tube to assess ROS activity in seminal plasma.

Statistical Analysis

The nonparametric Wilcoxon signed-rank test was used because the data were not normally distributed. A p value of less than 0.05 was considered significant. All statistical analyses were performed using the SAS statistical software package.

Results

Basal Level of ROS in Patients and Donors

Thirty specimens (17 patients and 13 donors) had a negative basal response (no increase in chemiluminescence curve, $< 10 \times 10^4$ cpm). The ROS levels (median and interquartile range) in specimens with a negative response was 0.5 (0-1.4) $\times 10^4$ cpm in patients and 0.9 (0-1.5) $\times 10^4$ cpm in donors. In the ROS-negative specimens, basal ROS levels did not differ significantly between patient and donor groups. Seven of 24 (29%) patients and 1 of 14 (7%) normal volunteers had abnormally high ($\geq 10 \times 10^4$ cpm) ROS levels in the uncentrifuged basal condition.

Relationship between ROS Formation and Semen Parameter

Out of 7 ROS-positive patients 2 were oligospermic and one of these patients had abnormal sperm morphology (1 % normal spermatozoa) according to Kruger's criteria. Three patients were asthenospermic with normal sperm count. The remaining 2 patients had normal routine semen analysis.

Relationship between ROS Formation and Clinical Diagnosis

The relationship between clinical diagnosis and ROS formation was investigated in 7 ROS-positive patients. In 2 patients out of 7, one was asthenospermic and the other oligospermic, both presented with a varicocele. One patient with normal semen analysis had a history of vasectomy reversal. The remaining 4 patients had idiopathic infertility.

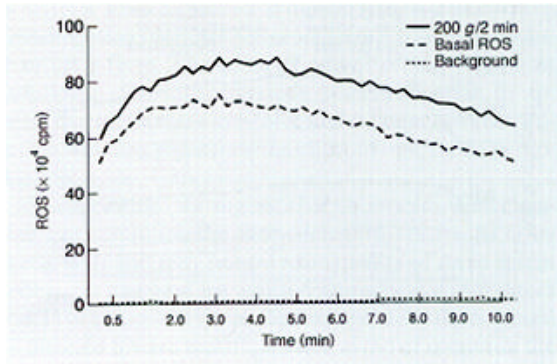


Fig. 1. A positive chemiluminescence response. Background was defined as chemiluminescence of the specimen before adding luminol. Chemiluminescence was expressed as $\times 10^4$ cpm in the integration mode over 10 min measurement time. Centrifugation for 2 min at 200 g significantly increased ROS formation ($n = 1$).

Comparison of ROS Levels before and after Centrifugation

The basal ROS level (median and interquartile range) in ROS-positive specimens before centrifugation was $25.2 (18.8-67.8) \times 10^4$ cpm. ROS formation after 2 min of centrifugation at 200 g for 2 min was $68.6 (55.9-110.8) \times 10^4$ Cpm ($p < 0.001$) as compared to $69.8 (55.9-146.6) \times 10^4$ cpm after 500 g for 2 min ($p = 0.02$). Similarly, ROS formation after 10 min of centrifugation at 200 g was $194.9 (103.8-231.9) \times 10^4$ cpm as compared to $346 (199.6-663.1) \times 10^4$ cpm at 500 g ($p < 0.001$) (fig. 2, 3). ROS formation was not detected in the seminal plasma of semen specimens initially tested positive for ROS.

Effect of Centrifugation on ROS-Negative Specimens

Although most specimens ($n = 30$) with an initial low level of ROS ($< 10 \times 10^4$ cpm) did not show a significant increase in ROS formation after

centrifugation, 6 specimens (4 patients and 2 donors) with initially low (negative) ROS formation had a late positive response after 10 min of centrifugation at 200 or 500 g. The basal ROS levels in this group were $2.86 (1.2-5.4) \times 10^4$ cpm as compared to $48.6 (38.2-68.2) \times 10^4$ cpm after centrifugation at 500 g for 10 min ($p < 0.001$) (fig. 4).

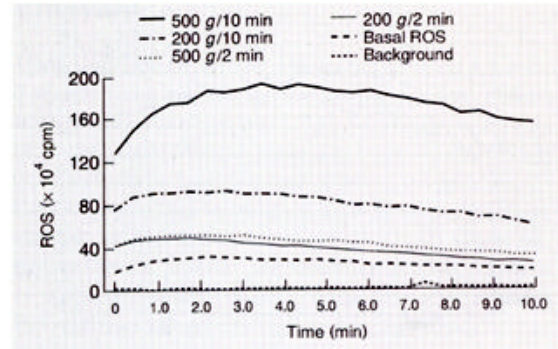


Fig. 2. Centrifugation response of a ROS-positive specimen ($n = 1$). Centrifugation was done at two different speeds (200 and 500 g) and time intervals (2 and 10 min). The highest level of ROS was seen after centrifugation at 500 g for 10 min.

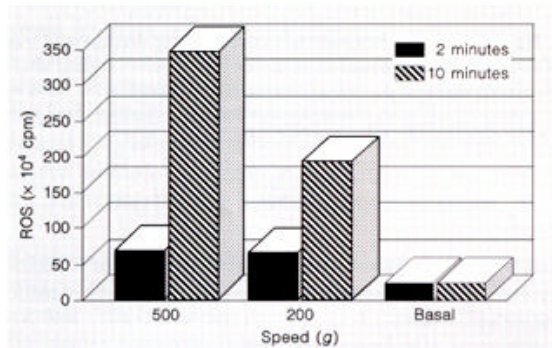


Fig. 3. ROS levels after centrifugation were compared with those in basal stage ($n = 8$). A short-term centrifugation for 2 min could significantly increase the ROS formation ($p < 0.001$). The differences between 200 and 500 g were not significant. The increase in centrifugation time to 10 min at both 200 or 500 g induced a further significant increase in ROS formation ($p < 0.001$). However, the level of ROS after centrifugation at 500 g for 10 min was significantly higher than at 200 g ($p < 0.001$).

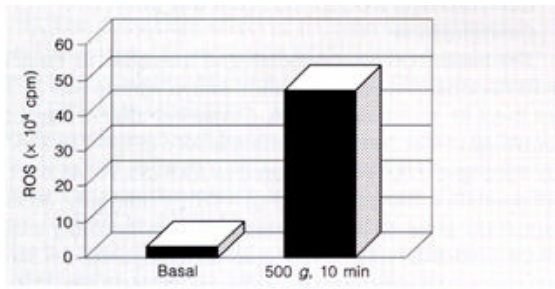


Fig. 4. Six specimens with negative ROS levels in the basal state cpm showed a ROS formation after 10 min centrifugation at 500 g ($p < 0.001$).

Discussion

ROS increased significantly, after even a short centrifugation interval, in all specimens with abnormally high basal ROS levels. Five out of 7 patients with high levels of ROS presented with some abnormal semen parameters and 2 had a varicocele. This is in agreement with the previous findings [10]. The formation of ROS in patients with normal semen analysis results may explain the possible role of ROS in idiopathic infertility [8, 9]. Larger numbers of patients are necessary to study the generation of ROS in subgroups of infertile men.

The mechanism stimulating ROS formation by centrifugation as described by Aitken and Clarkson [1] is thought to be through the mechanical damage of the sperm plasma membrane associated with centrifugation. However, WBCs in human semen are also capable of ROS generation [15, 16]. To minimize the contribution of leukocytes, the Endtz test [14] was used to assess the leukocyte concentration in semen. Only specimens with a negative (Endtz test $< 1 \times 10^6$ WBCs/ml) were used for this study. None of the ROS-positive specimens showed any ROS activity in seminal plasma indicating the cellular origin of measured ROS. This is in agreement with the previous findings [10]. The centrifugation used to separate seminal plasma from the cellular component of semen before ROS assessment can also change the ROS level in some specimens; therefore, we used the entire ejaculate in our experiment. Moreover,

to evaluate the significance of ROS in human semen and their impact on the impairment of sperm function clinically, the measurement needs to be performed in whole semen as natural antioxidants (free-radical scavengers) of seminal plasma such as: superoxide dismutase and catalase maintain the equilibrium of ROS level in human semen [17, 18]. Centrifugation damage to the human spermatozoa seems to be sublethal. The plasma membrane of human spermatozoa has a high content of polyunsaturated fatty acids [17, 19, 20]. Lipid peroxidation of these fatty acids is thought to be responsible for the deleterious effect of ROS on spermatozoa [20, 21]. Depletion of cellular ATP and the consequent axonemal damage in the presence of the ROS have also been found recently in a demembrated spermatozoal model; this damage was associated with decreased sperm motility [11, 12].

Centrifugation per se has also been shown to damage human spermatozoa. The percentage of motile sperm diminished after centrifugation as a function of time. Deterioration of sperm motility was believed to be caused by a direct mechanical effect of centrifugation [2]. Both the direct effect of centrifugation through mechanical membrane damage as well as the indirect adverse effect caused by excessive ROS formation is responsible for the deterioration of sperm function [1, 3].

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 min of centrifugation in 6 cases (2 donors, 4 patients). Two explanations are possible for this finding. Centrifugation can activate seminal leukocytes (in concentrations $< 1 \times 10^6$) and can disturb the balance between the ROS formation and the scavengers present in semen. The increase in ROS formation may also result from mechanical perturbation of sperm plasma membrane. Theoretically, a longer period of centrifugation may then induce ROS formation in a subpopulation of spermatozoa with suboptimal membrane function. This explanation is supported by an earlier finding that cells from the Percoll gradient with poor sperm quality were the major source of ROS formation [1].

Our results indicate that even a single-step centrifugation for a short period (2 min in our study) can significantly increase ROS formation in human semen. The effect of centrifugation time on ROS generation is more significant than the g-force. The effect of g-force seems to be most significant after longer periods of centrifugation. It appears that an increase in g-force under prolonged centrifugation will then potentiate the effect of time on ROS production.

In conclusion, we suggest that short-term centrifugation can minimize the formation of excessive ROS by sperm cells and may improve the quality of some sperm specimens prepared for

assisted reproductive techniques (ART) or cryopreservation. Future research efforts should be directed toward techniques that avoid or minimize the use of centrifugation for sperm preparation for ART.

Acknowledgement

The authors wish to thank Jar-Chi Lee and Marlene Goormastic for their help in the statistical analysis of results; Christine Schneider for her help with computer graphics; Debbie Garlak for her help selecting patient specimens for this study; and Tracy McAlpine for her secretarial assistance.

References

- 1 Aitken RJ, Clarkson JS: Significance of reactive oxygen species and antioxidants in defining the efficiency of sperm preparation techniques. *J Androl* 1989;9:367.
- 2 Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero PP, 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.
- 3 Mortimer D: Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum Reprod* 1991;6:173.
- 4 McLaughlin RH, Graham JK, Nolan JP: Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J Reprod Fertil* 1992;95:527.
- 5 Mahadevan M, Trounson AO, Leeton JF: The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to success of in vitro fertilization. *Fertil Steril* 1983;40:755.
- 6 Overstreet JW, Yanagimachi R, Katz DF, Hayashi K, Hanson FW: Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: Study of fertile donors and infertile patients. *Fertil Steril* 1980; 33:534.
- 7 Trounson AO, Leeton JF, Wood C, Webb J, Kovacs G: The investigation of idiopathic infertility by the in vitro fertilization. *Fertil Steril* 1980;34:431.
- 8 Aitken RJ, Clarkson JS: Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species. *J Reprod Fertil* 1987;81:459.
- 9 Gagnon C, Iwasaki A, de Lamirande E, Kovalski N: Reactive oxygen species and human spermatozoa. *Ann NY Acad Sci* 1992;637:436.
- 10 Iwasaki A, Gagnon C: Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;57:409.
- 11 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.
- 12 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.
- 13 Kovalski NN, 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.
- 14 Endtz AW: A rapid staining method for differentiating granulocytes from 'germinal cells' in Papanicolao-stained semen. *Acta Cytol* 1974; 18:2.
- 15 Aitken RJ, West KM: 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.
- 16 Kessopoulou E, Tomlinson MJ, Barratt CLR, Bolton AE, Cooke ID: Origin of reactive oxygen species in human semen: Spermatozoa or leucocytes. *J Reprod Fertil* 1992;94:463.
- 17 Alvarez JG, Touchstone JC, Blasco L, Storey BT: 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.
- 18 Alvarez JG, Storey BT: Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res* 1989;23:77.
- 19 Jones R, Mann T, FRS, Sherins RJ: Adverse effects of peroxidized lipid on human spermatozoa. *Proc R Soc Lond* 1978;201:413.
- 20 Jones R, Mann T, Sherins RJ: Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 1979;5:531.
- 21 Aitken RJ, Clarkson JS, Fishel S: Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989;40: 183-197.