

Role of Measurement of Reactive Oxygen Species in Semen Sample of Patients with Male Factor Infertility and Treatment with Antioxidants in Patients with High ROS Levels

Satyabrato Chatterjee¹, Pankaj Talwar²

ABSTRACT

Introduction: It is a known fact that human sperms, produce reactive oxygen species in small amounts, which is useful for normal functioning of the sperm mainly sperm oocyte interaction acrosome reaction and capacitation. Recent research has shown that this ROS in increased amounts is a cause of male infertility.

Material and methods: It is a prospective control trial to determine the abnormal pattern of reactive oxygen species in semen sample of patients with male factor infertility and to determine the effects of antioxidants in semen sample of patients with high ROS in semen sample. 150 patients were selected who were undergoing IVF treatment. Patients were divided into three groups according to the severity of abnormality in the semen analysis. The ROS levels of all the patients were measured and compared with 50 normal fertile donors. The patients whose ROS levels was found to be high were then given antioxidants for 2 months and their ROS levels in semen measured for improvement.

Results: The ROS levels were significantly high in all the three groups compared to fertile donors. The patients with high ROS levels were then treated with antioxidants and it was seen that all the three groups had significant improvement in their ROS levels.

Conclusion: So, to conclude it is hence proved by my study that ROS level measurement is useful for patients with male factor infertility and the use of antioxidants significantly helps in reducing ROS levels in semen.

Keywords: Male Factor Infertility, ROS Levels, Antioxidants

INTRODUCTION

There has been significant advancement in the understanding of male factor infertility. Earlier the primary focus of Infertility investigation and management used to be that of the female counterpart. It is now well recognised fact that male factor as a sole cause of infertility is 5% and as a contributory factor in about 20%- 40% of reproductive failure.¹ Correct and early detection and goal directed therapy can help infertile men to achieve natural conception.

Recently the cause of male infertility has shifted from simple semen analysis and culture to more complex array of tests like the ROS, Sperm function tests, DFI and many more.

ROS is normally produced by sperm and it has some beneficial effects.² It helps in capacitation and sperm acrosome reaction.^{3,4} It also helps in the sperm oocyte interaction.⁵ The mechanism involved in it is the Phospholipase A₂ activity demonstrated in the sperm membrane.⁶ The concentration of ROS required for these functions is minimal and generally

not detected by the current detection kits in the semen of fertile men.⁷ Idiopathic infertility has been seen in patients having high levels of ROS in seminal fluid.

The major source of ROS in semen is the sperm membrane and the mitochondrial system. The mechanism involved in production of the ROS is the NADPH oxidase system found in sperm membrane and the NADPH dependent oxidoreductase found in the mitochondria.⁸ Huge amount of ROS is found in morphologically abnormal sperm were increased amount of NADPH acts as a substrate to Superoxide Dismutase which converts NADPH to H₂O₂. The mitochondrial NADPH oxidoreductase is the major source of ROS in abnormal sperms.⁹

ROS affects the different parameters of sperm which include morphology, motility and concentration.^{10,11,12} The factor most affected by increased ROS production is sperm motility which is affected by more than 60% compared to normal motile sperm where ROS is not detected.¹³

There are a number of techniques available to measure ROS levels in semen and each one of them has its inherent limitations. The techniques available are measurement of ROS at the cell membrane surface intra and extracellular measurement of ROS.¹⁴

The 1st technique utilizes Tetrazolium Nitroblue for measuring Superoxide but the sperm count should be adequate for measurement and this method lacks sensitivity.¹⁵ In the second technique fluorescent based methods are utilized and it measures both intra and extracellular ROS levels utilizing electron spin method and is more sensitive than others. The ROS is measured using Luminol based chemiluminescence methods. The intracellular ROS present in the sperm causes deoxygenation of Luminol. The signal produced by Luminol is then measured using peroxidase inhibitors and azide insensitive peroxidases. Some more specific methods use

¹Department of ART, Army Hospital (R&R), New Delhi, ²Professor (HOD ART), Department of ART, Army Hospital (R&R), New Delhi, India

Corresponding author: Dr Pankaj Talwar, Dr Satyabrato Chatterjee, Assistant Professor, Department of Assisted Reproductive Techniques

How to cite this article: Satyabrato Chatterjee, Pankaj Talwar. Role of measurement of reactive oxygen species in semen sample of patients with male factor infertility and treatment with antioxidants in patients with high ROS levels. International Journal of Contemporary Medical Research 2019;6(2):B1-B5.

DOI: <http://dx.doi.org/10.21276/ijcmr.2019.6.2.30>

oxidized components having longer half-life examples are Thiobarbitivue Reacted Malondialdehyde which is an index of Lipid Peroxidation.¹⁵

Strategies to reduce oxidative stress requires proper insight into the generation of ROS in our human body. This will help evaluate the strategy to reduce oxidative stress in spermatozoa.

Study aimed at measurement of reactive oxygen species in semen sample of patients with male factor infertility and treatment with antioxidants in patients with high ROS levels.

MATERIAL AND METHODS

Patients attending the infertility clinic of Army Hospital (Research and Referral) during the time period 1 Jan 2018-30 Jun 2018 provided the semen sample during conducting usual seminal test which is the initial step for evaluating infertility for routine semen analysis.

The population to study includes a total of 200 patients with 50 healthy donors and 150 patients with infertility due to male factor. The patients with (MFI) will be divided into 3 equal groups of 50 patients with Group 1 having one unusual sperm parameter, Group 2 with two abnormal sperm functions, Group 3 with more than two to all abnormal parameters. Semen analysis will be done at the 1st visit to hospital. It will be repeated after 15 days with proper instructions for 3 days of abstinence. The ROS levels will be measured during the 2nd semen analysis and patient divided into one of the 3 groups of MFIs. Semen analysis and ROS levels of healthy donors will be done at 1st visit with proper instructions for 3 days of abstinence. ROS levels will be tabulated and correlated with sperm parameters. Patients with increased levels of ROS will be given treatment with antioxidants for 2 months and ROS levels again measured along with semen analysis after intervention. Improvement in the levels of ROS will be noted.

Study population

The population to study includes normal healthy donors (50) and MFI patients (150). Male factor infertility will be defined as when couple does not give birth to child after practising unprotected coitus with her co-partner or wife who has no abnormal history of reproduction and a normal ovulatory cycle that has been diagnosed with TVS, progesterone level post ovulatory biopsy of endometrium. With normal fallopian tube with bilateral spillage in HSG or DHL.

Inclusion criteria

Normal healthy donors 50.

Male factor infertility consists of 3 groups.

Each group consisting of 50 patients

Group 1 consists of patients with one abnormal sperm parameter according to WHO criteria.

Group 2 consists of patients with two abnormal sperm parameters

Group 3 consists of couples with more than two to all abnormal sperm parameters

Exclusion criteria

Patients with Azoospermia

Patients and donors with positive leukocytospermia (white blood cell counts $1 \times 10^6/\text{mL}$ ejaculate)

SEMEN ANALYSIS

The samples of semen will be collected by masturbating after 2-3 days of abstinence. seminal fluid will be collected into sterile plastic containers keeping liquefaction time of 20mins at 37°C. The sperm parameter will be assessed according to WHO criteria.

Lower Reference values for Semen Analysis (WHO 2010)

S.No	Parameters	Lower Reference Limits
1.	Volume	1.5 (1,4-1,7) ml
2.	Sperm Concentration	15 (12-16) million/ml
3.	Total Sperm Number	39 (33-46) million/ejaculate
4.	Total motility	40 (38-42)%
5.	Progressive Motility	32 (31-34)%
6.	Normal Morphology	4 (3-4)%
7.	Vitality	58(55-63)%

Measurement of seminal ROS

Place the melted gel tube containing Nitroblue Tetrazolium at 37° C for 5 mins.

Add 200ul of semen sample to melted agarose gel tube and mix gently to avoid bubble formation and stress to sperm cells.

Incubate the tube for 55 mins at 37°C.

After incubating the tube observe the colour change immediately and compare the colour code immediately to determine the level of oxidative stress.

- The outcomes analysed from this study
- Patients divided into 4 groups
- Study Group 1 consists of patients with one unusual sperm parameter according to criteria of WHO.
- Study Group 2 consists of infertile couples with one abnormal sperm function.
- Study Group 3 consists of patients with all abnormal sperm function
- Comparisons ROS levels of fertile donors and infertile men
- Comparison of ROS of infertile men with high ROS after antioxidant treatment for 2 months with ROS levels before treatment.

RESULTS

ROS levels were measured in semen sample using chemiluminescence method. It was found that in Group 3 patients who had more than 2 abnormalities in semen had maximum number of ROS positive semen sample and in Group 1 patients who had no semen abnormalities had the minimum number of ROS positive semen sample.

Comparing the three groups with control it was found that, the p value was significant all the three groups. Group 1 (.023), Group 2 and 3 (.0001). While comparing the patient groups (Group 1 Vs Group 2) and (Group 1 Vs Group 3) p value is significant .0005 and .0001 respectively. While comparing 2 Vs Group 3 p value is 0.280.

Patients with high ROS were treated with antioxidants and ROS levels compared for improvement. All the three groups

Variables	Patients with High ROS Levels	Percentage	Patients with Low ROS Levels	Percentage
Controls, N=50	8	16%	42	84%
Group 1, N=50	18	36%	32	64%
Group 2, N=50	32	64%	18	36%
Group 3, N=50	37	74%	13	26%

Table-1: ROS levels in fertile donors and infertile men

Variables	Controls, N=50	Group 1, N=50	Group 2, N=50	Group 3, N=50
Patients with High ROS Levels	8	18	32	37
Patients with Low ROS Levels	42	32	18	13
Controls Vs Group 1	0.023			
Controls Vs Group 2	0.0001			
Controls Vs Group 3	0.0001			
Group 1 Vs Group 2	0.005			
Group 1 Vs Group 3	0.0001			
Group 2 Vs Group 3	0.280			

Table-2: Comparison of ros levels between different groups

Variables	High ROS Before Treatment	Normal ROS After Treatment	%	p value
Group 1	18 (36%)	15	83.3%	.001
Group 2	32 (64%)	20	62.5%	.001
Group 3	37 (74%)	21	56.8%	.001

Table-3: ROS levels after treatment with antioxidant

Assay	Probe	End product	Extracellular / Intracellular
Indirect measurement Lipid peroxidation levels	TBA	MDA	Oxidized end products are measured
Antioxidants Trace elements and vitamin	HPLC	Alphatocopherol	Serum and seminal plasma
Antioxidant-pro-oxidant status	Total antioxidant	TEAC	Low chain breaking level
Chemokines	ELISA	Interleukin-6 Interleukin- 8	Seminal plasma
Direct measurement Terazolium Nitroblue Chemiluminescence	Ferricytochrome C Luminol Xanthine Oxidase system DNDH/HRP	$O_2 \cdot H_2O_2, O_2 \cdot \cdot OH$ SOD $H_2O_2, \cdot OH$	Extracellular Both Extracellular Both

Table-4:

had significant improvement in ROS levels after treatment with antioxidants.

DISCUSSION

Our study included a total of 200 patients who presented to us during time period of 1st Jan 2018 – 30 Jun 2018 and provided the semen sample during conducting usual seminal test which is the initial step for evaluating infertility in males. There was a total of 50 healthy patients and 150 patients who were divided into 3 groups of 50 patients each depending on the number of parameters that were deranged according to WHO criteria (2010). In our study system semen analysis was done in 1st visit of the patient at our clinic. The test was again repeated after 15 days of abstinence. Patient were divided into one of the three groups of male infertility after that. The ROS levels were then measured during the second semen analysis.

Patients with leukocytospermia were not included in the test since it is a marker of infection of the genital tract.¹⁶ There are a number of controversies regarding leukocytospermia and its association with male infertility.¹⁷ Some studies

quote that genital tract infection and leukocytospermia has no correlation with infertility. Whereas some studies say infection is associated with damage to sperm function and decrease in fertility potential.¹⁸ The WBCs are itself a source of generation of ROS in the seminal fluid. In WBCs the granulocytes have largely been held responsible for the damage of sperm function and it is present in the secretions from the seminal vesicle and prostrate.^{19,20} Since we are discussing ROS generation in sperm, we also have to keep in mind that seminal fluid capable enough to generate ROS which could impair sperm function so the factor was kept away from our study protocol.

In our study raw semen sample after liquefaction was used for ROS measurement. In other studies, raw and processed semen sample after swim up was used for measurement of ROS. Both has its advantages and disadvantages. Processing the semen sample removes the pathogens and WBCs which are a source of ROS, but here we have already excluded semen sample with leukocytospermia. Using raw semen sample reduces the time taken for the test and makes the

procedure simple and shows no significant difference in ROS levels between processed and raw semen sample.²⁶ There are studies which show that sperm washing techniques can itself cause ROS generation and impair sperm function.²¹ It has been found samples which are negative for ROS has been found positive after sperm washing. Samples with low sperm count and motility become negative for ROS after sperm washing but samples with good count and motility become positive after washing.²² Among the various washing techniques, the samples become positive for ROS more in cases of double density wash compared to simple swim up technique. Both centrifugation speed and time length are directly related to ROS generation, but centrifugation time is more important than speed in generation of ROS.²³ Looking at the various factors it was considered to use raw sample to measure ROS so that it will give first-hand information regarding ROS status of the patient.

The technique used for measuring ROS in semen sample in our study was by chemiluminescence assay using NBT. It is a qualitative way of measuring the ROS levels where after adding the dye after liquefaction of the semen sample we observed the colour change immediately in the semen sample and measured it with the colour code to determine level of oxidative stress.

There are a number of test available presently which has been tabulated and put in the chart below.

In a study by it quotes that NBT test is easily performed and can be used as a routine andrology lab procedure to assess seminal OS without need of expensive luminometer. It has also been proposed by (Evenson et al, 1999) that no single laboratory test can accurately and precisely assess male infertility.²⁴

It was further proposed by (Aitken et al, 1995) that one single diagnostic test is impossible in case of male infertility because male infertility is not a single entity but a collection of many causes and other pathological conditions which manifest as male infertility.²⁵ Therefore, till the different causes of male infertility are understood and the threshold at which it will cause male infertility with certainty, no single test will, be able to accurately judge the problem.

In our study when fertile donors were tested for ROS and 7 out of 50 fertile donors tested positive for seminal ROS. While in Group 1, 2 and 3 was that of 18, 32 and 37 respectively. When P value was compared with that of control it was significant in all the three group patients (Group1- .023, Group 2 and 3 being .0001). In a similar study by (R. Saleh and A. Agarwal 2002) when ROS levels were compared using ROS- TAC score in donors and infertility patients P value was found to be significant for donors versus infertile men with high ROS and infertile men with low ROS versus infertile men with high ROS.²⁶

Studies by (Ollero et al and Gilm Guzman et al 2001) quotes that when we compare ROS levels with sperm morphology on WHO criteria a negative correlation was found between the two.²⁷

Similarly considering other sperm parameters like sperm motility (Agarwal et al 1994 and Armstrong et al 1999)

quotes that there is a reduction in sperm mobility because of increased ROS that can be attributed to phosphorylation of protein present in axoneme of the sperm which affects the fluidity sperm membrane and fertilization potential of the sperm.^{28,29} It was found that as the level of severity increased in semen analysis the percentage of patients with increased ROS levels also increased (Group 1- 36%, Group 2- 64% and Group 3- 74%) and the values were significant comparing Group 1 with Group 2 and Group 2 with Group 3 (P values .005 and .0001) respectively but comparison of Group 2 Vs Group 3 was not significant (P value .280). It suggests that ROS levels are increased in patients with deranged semen parameters and percentage of positive patients increases as the severity increases.

In our study patients who tested positive for ROS were treated with antioxidant carnitine 1000mg once daily and Vit E 500mg twice daily for 2 months and ROS levels measured again. The treatment showed that there was significant improvement in ROS levels in all the three groups. Our study results were consistent with other study results (Kessopoulou et al 1995) says significant improvement was found in semen parameters after oral administration of Vit E but he also commented that patient should undergo IVF rather than waiting for natural conception.³⁰

CONCLUSION

ROS in seminal fluid in physiological or normal concentrations helps on sperm function and maturation while in abnormal concentrations imperil sperm capacity and function.

Significantly high amount of ROS has been found in infertile patients with deranged semen parameters in comparison with fertile donors with normal semen parameters.

The measurement of ROS in seminal fluid can help choose subgroups among infertility patients in whom stress is a significant factor.

The prevention of oxidative stress by using antioxidant supplements for increasing fertility is strongly recommended. Presently it seems to suggest that no single drug or adjuvant have the capacity to improve sperm function in infertile men with abnormal ROS levels and a blend of conceivable techniques that are not harmful at doses given would be a possible approach.

REFERENCES

1. Lipschultz LY, and Howards SS: Evaluation of the sub fertile man, *Infertility in the Male*. New York: Churchill Livingstone, 1983, pp 187-192.
2. Schreck R, Rieber P, and Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-kappa p transcription factor and HIV-1. *EMBO J* 1991;10:2247-2258.
3. Bennet PJ, Moatti JP, Mansat A, Ribbes H, Cayrac JC, Pontonnier F, Chap H, and Douste-Blazy L: Evidence for the activation of phospholipase during acrosome reaction of human sperm elicited by calcium ionophore A23187. *Biochim Biophys Acta* 1987; 919: 255-265.
4. Burkman LJ: Hyperactivated motility of human

- spermatozoa during in vitro capacitation and implications for fertility, in Gagnon C (Ed): Controls of Sperm Motility. Boca Raton, CRC Press, 1990, pp 303-329.
5. DeLamirande E, Eiley D, and Gagnon C: Inverse relationship between the induction of human sperm capacitation and spontaneous acrosome reaction by various biological fluids and the superoxide scavenging capacity of these fluids. *Int J Androl* 1993;16: 258-266.
 6. Goldman R, Ferber E, and Zort U: Reactive oxygen species are involved in the activation of cellular phospholipase A₂. *FEBS Lett* 1992;309: 190-192.
 7. Iwasaki A, and Gagnon C: Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992; 57:409-416.
 8. Holland MK, Alvarez JG, and Storey BT: Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol Reprod* 1982; 27: 1109-1118.
 9. Plante M, de Lamirande E, and Gagnon C: Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertil Steril* 1994; 62:387-393.
 10. Agarwal A, Ikemoto I, and Loughlin KR: Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. *J Urol* 1994; 152:107-110.
 11. D'Agata R, Vicari E, Moncada ML, Sidoti G, Calogero AE, Fornito MC, Minacapilli G, Mongioi A, and Polosa P: Generation of reactive oxygen species in subgroups of infertile men. *Int J Androl* 1990;13: 344-351.
 12. Aitken RJ, and Clarkson JS: Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl* 1988; 9: 367-376.
 13. Iwasaki A, and Gagnon C: Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992; 57:409-416.
 14. Wymann MP, von Tscherner V, Deranleau DA, and Baggiolini M: The onset of the respiratory burst in human neutrophils. Real time studies of H₂O₂ formation reveal a rapid agonist-induced transduction process. *J Biol Chem* 1987; 22:12048- 12053.
 15. AlvarezJG, Touchstone JC, Blasco L and 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: 338-348.
 16. Aitken RJ, West K, and Buckingham D: Leukocyte infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *J Androl* 1994; 15: 343-352.
 17. Eschenbach DA, Buchanan TM, Pollock HM, Forsyth PS, Alexander ER, Lin JS, Wang SP, Wentworth BB, MacCormack WM, and Holmes KK: Polymicrobial aetiology of acute pelvic inflammatory disease. *N Engl J Med* 1975; 293: 166-169.
 18. Hanssen L, and Mard PA: In vitro tests of the adherence of Chlamydia trachomatis to human spermatozoa. *Fertil Steril* 1984;42:102-107.
 19. Allen RC: Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis. *Methods Enzymol* 1986;133:449-493.
 20. Ryan TC, Weil GJ, Newburger PE, Haugland R, and Simons ER: Measurement of superoxide release in the phago vacuoles of immune complex-stimulated human neutrophils. *J Immunol Methods* 1990; 130:223-233.
 21. Iwasaki A, and Gagnon C: Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;57:409-416.
 22. Agarwal A, Ikemoto I, and Loughlin KR: Effect of sperm washing on levels of reactive oxygen species in semen. *Archiv Andro* 1994;133: 157- 162.
 23. Shekarriz M, DeWire DM, Thomas AJ Jr, and Agarwal A: A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Uro* 1995;128:31-35.
 24. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, Angelis PD, Claussen OP: Utility of sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod.* 1999; 14:1039-1049
 25. Aitken RJ. Free radicals, lipid peroxidation, sperm function. *Reprod Fertil Dev.* 1995; 7:659-668.
 26. Ramadan A. Saleh and Ashok Agarwal: Oxidative Stress and Male Infertility: From Research Bench to Clinical Practice, *Journal of Andrology* 2002; Vol. 23, No. 6, November/December.
 27. Ollero M, Gil-Guzman E, Lopez MC, et al. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod.* 2001; 16.
 28. Agarwal A, Ikemoto I, Loughlin KR. Relationship of sperm parameters to levels of reactive oxygen species in semen specimens. *J Urol.* 1994a; 152:107-110.
 29. Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med.* 1999; 26:869-880.
 30. Kessopoulou E, Tomlinson MJ, Banat CLR, Bolton AE, Cooke ID. Origin of reactive oxygen species in human semen-spermatozoa or leukocytes. *J Reprod Fertil.* 1992; 94:463-470.

Source of Support: Nil; **Conflict of Interest:** None

Submitted: 02-01-2019; **Accepted:** 14-02-2019; **Published:** 2702-2019