

Correlation of Sperm Function Test with Antioxidant Levels in Seminal Plasma

Shrikant Shete¹, Sandip Meghnad Hulke², Avinash Thakare², Prashant Patil²

¹ Jhalawar Medical college, NH12, Kota Road, Jhalawar

² Department of Physiology, Laxminarayan Medical College, Bhopal

Correspondence to:

Sandip M Hulke
(smh555@rediffmail.com)

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ABSTRACT

Background: Sperm function testing is used to determine if the sperm have the biologic capacity to perform the tasks necessary to reach and fertilize ova and ultimately result in live births. There is growing evidence that damage to spermatozoa by reactive oxygen species may affect sperm function and Antioxidant help to fight against such reactive oxidant species.

Aims & Objective: To find the correlation between antioxidants and sperm function tests.

Materials and Methods: Cross section study done on four group with different fertility potential. Four groups were group 1- control(n=10) normozoospermic fertile, group2:- Normozoospermics (n=18) infertile, group 3- oligoasthenoteratozoospermics (n=27) infertile, and group 4- Asthenoteratozoospermics (n=23) infertile. Their semen analysis was done and levels of the antioxidant Vitamin C, Vitamin E, and the reduced glutathione was measured. Correlation was found between sperm function test and antioxidant level in seminal plasma. Statistical analysis was done using ANOVA test and Bonferroni's post test. Coefficient of correlation (r- value) was found to find relationship between different parameters. Graphpad prism 5 Software was used.

Results: Sperm function test and antioxidant level in seminal plasma was significantly more in group 1 as compared to the other groups. Significant correlation was seen between sperm function test and antioxidant level.

Conclusion: Decreasing seminal plasma antioxidants levels could have significant role in etiology of impaired sperm function. Seminal plasma antioxidants levels is closely related to male fertility, and the decreased level of antioxidants levels in seminal plasma may be one of the causes of male infertility.

KEY WORDS: Ascorbic Acid; α -tocopherol; Reduced Glutathione Level; Sperm Function Test

INTRODUCTION

Semen analysis as an integral part of infertility investigations is taken as a surrogate measure for male fecundity in clinical andrology, male fertility, and pregnancy risk assessments. Clearly, laboratory seminology is still very much in its infancy. The routine seminal analysis lacks the specificity and sensitivity, as they (parameters) are only indirect indicators of quality of semen. In the 5th edition of the World Health Organization (WHO) manual, sperm function test had been recommended.^[1] Sperm function testing is used to determine if the sperm have the biologic capacity to perform the tasks necessary to reach and fertilize ova and ultimately result in live births. Vasan SS et al^[2] had recommended that if sperm abnormalities are observed in the *basic* semen analysis or if the couple is diagnosed as *unexplained* infertility, the workup should proceed to the analysis of sperm functional tests. Thus sperm function test had emerged as important lab investigation in the diagnosis of infertility.

The role of antioxidants is to detoxify reactive oxygen species (ROS) in the body which are the dangerous by products of aerobic metabolisms in the body. Spermatozoa were the first cell type suggested to generate highly reactive oxygen species (ROS) in human body.^[3,4,5] Antioxidants are present in low concentrations relative to the oxidizable substrate and they significantly delays or reduces oxidation of the substrate. In the past many studies had been performed where correlation was seen between antioxidant level with routine sperm analysis.^[6,7] In the past very few studies had been performed, where relation is seen between sperm function test and antioxidant level in seminal plasma.^[8,9] In the present study, we had performed the sperm function tests viz. Hypo-osmotic swelling test (HOS), Acrosome intactness test (AI), Sperm mitochondrial activity index (SMAI), and Nuclear chromatin decondensation test (NCDT) and we had elucidated the relation between antioxidants and sperm function tests.

MATERIALS AND METHODS

Semen samples were obtained from 78 male patients of 21-40 yrs of age attending the semen analysis laboratory of Department of Physiology, Government Medical College, Nagpur. Detailed history of present and past illness as well as medical and surgical management was taken. Selected male partners were underwent thorough surgical examination of genito-urinary system to rule out the exclusion criteria. Subjects with normally developed genito-urinary organs were included in the study. All the tests were conducted with due per-mission of the ethical committee of the Institute and with written consent from the subjects. Specimens of semen were collected by masturbation after 3 days of sexual abstinence. After complete liquefaction, samples were analyzed by SQA II B sperm quality analyzer (M.E.S. Ltd., Israel) for sperm concentration, motility, morphology according to WHO guideline^[1] and grouped into four categories with following criteria:

The present study was conducted in the semen analysis laboratory of Department of Physiology, Government Medical College, Nagpur. The study included 78 subjects.

They were grouped as follows:

Group 1 [Control (Normozoospermics - 10 cases)]: Persons with sperm concentration of 20 millions/mL or more, sperm motility of 50% or more (a+b type motility), normal sperm morphology of 30% or more. They were having at least one issue and served as control.

Group 2 [Normozoospermics - 18 cases]: Persons with sperm concentration of 20 millions/mL or more, sperm motility of 50% or more (a+b type motility), normal sperm morphology of 30% or more.

Group 3 [Oligoasthenoteratozoospermics - 27cases]: Persons with sperm concentration less than 20 million/mL, sperm motility below 50% (a+b type motility), normal sperm morphology in less than 30 % of sperms.

Group 4 [Asthenoteratozoospermics - 23 cases]: Persons with sperm concentration of 20 millions/mL or more, sperm motility below 50% (a+b type motility), normal sperm morphology in less than 30 % of sperms.

The subjects belonging to the group 2, 3 and 4 were those having no issues in spite of at least one year of unprotected inter-course.

Inclusion Criteria:

- Controls were adult healthy male fertile volunteers, in the age group of 21-40yrs having at least one issue.
- Infertile males (normozoospermic $\geq 20 \times 10^6$ spermatozoa/ml and oligozoospermic $< 20 \times 10^6$ spermatozoa/ml) were those having no issues in spite of at least one year of unprotected inter-course in the age group of 21-40yrs.

In all cases, the sexual partner had completed a full gynecological work-up, and all were judged to be fertile.

Exclusion Criteria:

- Persons with occupation near hot furnace or in chemical industries using the substances like benzene or aniline dyes, which are known to produce alterations in spermatogenesis.
- Patients with azoospermia as the effect on functional parameters cannot be studied.
- Persons with history of drug addiction, smoking and alcohol intake.
- Persons with previous history of hydrocele, varicocele, hernia and operations on genital tract. After taking permission from the ethical committee of Govt. Medical College and due consent of the subjects, their clinical examination was performed.

Estimation of Ascorbic Acid in Seminal Plasma:

Ascorbic acid is estimated by methods based on principles of methods of Roe and Oesterling^[10] and modified by Bolin and Book^[11] with a little modification to suit semen. 0.5ml of seminal plasma was taken in centrifuge tube. To it 1 ml of

10% TCA Solution (Trichloroacetic acid) was added and waited for 5 minutes. To this 7 ml of distilled water was added and mixed then centrifuged for 15 minutes. Three test tubes were taken and marked A, B, and C and 2 ml of the filtrate in each tube was taken. A drop of 2:6 di-chlorophenol-indophenol solution was added to test tubes A and B only and mixed till some colour should persist. 0.1 ml of thiourea solution was added to all the three test tubes. A drop more to tube 'C' added. 0.5 ml of 2:4 DNPH Solution was added to tube 'B' and 'C' preserved the test tube 'A' as blank. All the three tubes were incubated in an incubator at 37°C for exactly 3 hours. To all the three tubes 1.5 ml of 95% sulphuric acid was added drop by drop while tubes were immersed in ice. To the blank tube 'A' was added 0.5 ml. of 2:4 DNPH Solution. All the tubes were taken out from ice and kept at room temperature for half an hour. Colorimetric readings were taken using green filter (Wave length 540).

Method of Estimation of Vitamin E:

We used Emmerie Engel^[12] assay modified by Baker and Frank.^[13] Three stoppered centrifuge tubes are taken and labeled as Test (T), Standard (S) and Blank (B). The addition was made as follows. Standard was prepared by taking 0.5ml of standard solution in a test tube, to this 0.5ml of ethanol and 0.5ml of xylene was added. Blank was prepared by taking 0.5ml of distilled water in a test tube, to this 0.5ml of ethanol and 0.5ml of xylene was added. Test was prepared by taking 0.5ml of seminal plasma in a test tube, to this 0.5ml of ethanol and 0.5ml of xylene was added. All the three stoppered centrifuge tubes were mixed and centrifuged for 15min. In other three clean stoppered tubes 0.5ml of each xylene layer was transferred. To this 0.5ml of dipyrindyl reagent was added. 0.5 ml of mixture was pipetted into spectrophotometer cuvettes and the absorbance was read at 460 nm (A₄₆₀) of Test (T) and Standard (S) against the Blank (B). The wavelength was 520 nm. Then beginning with Blank 0.33 ml FeCl₃ solution was added, mixed and waited for 1.5min. The absorbance at 520nm (A₅₂₀) of the Test (T) and standard (S) against the blank (B) was read.

Method of Estimation of Reduced Glutathione (GSH):

Reduced glutathione (GSH) is estimated by methods based on principles of methods of Moron et al.^[14] 0.5 ml of seminal plasma was taken in a test tube and 2ml of distilled water was added, mixed well. Then centrifuged for 5 min. at 5000 rpm. 0.5 ml of supernatant was taken; to which 0.5ml of TCA (5%) was added and then centrifuged for 10 min. at 10,000rpm. 0.5 ml of supernatant was taken to which 2.5ml of phosphate buffer (pH 8) was added. To this 1ml DTNB was added. This solution was inverted for 3 times to mix. The absorbance was read on spectrophotometer at 412nm within 4 min. of preparing the mixtures. Standard graph of reduced glutathione GSH concentrations was plotted. Determination of reduced glutathione GSH concentration in seminal plasma was done from the graph.

Hypo-osmotic Swelling Test (HOS)^[15]:

The test evaluates the functional integrity of sperm plasma membrane. Hypo-osmotic solution is prepared by adding 1.351 gm of fructose along with 0.735 gm of sodium citrate in 100 ml of distilled water. An initial count of sperms with curled tail is made. One ml of hypo-osmotic solution is placed at 37° C for 10 minutes, to which 0.1 ml of semen sample is added. It is incubated at 37° C for half an hour. A drop of above solution is placed on pre-cleaned glass slide and observed under microscope (400 X magnification) after putting the coverslip. The percentage of sperms showing swollen tail and "curling" was noted. To get the final hypo-osmotic swelling test score the initial percentage of sperms with coiled tail was subtracted from the later. The observation was duplicated to take the mean. If more than 60% of spermatozoa, shows curling then it is considered as normal.

Acrosomal Intactness Test (AI)^[16]:

Acrosomal intactness test is one of the several tests analyzing the quality of enzymes in the acrosome. Forty microlitre of warm (500 C), 5% of aqueous solution of gelatin was spread on each

cleaned glass slide. The slides were then placed horizontally in refrigerator at 40 C for 24 hours. Slides were removed and fixed in the PBS (Phosphate buffered saline) - glutaraldehyde solution for 2 minutes, were washed twice with distilled water and stored again at 40 C in vertical position in refrigerator. In this way gelatin slide is prepared. Semen was diluted 20 fold with PBS-D-Glucose and incubated at 37° C for thirty minutes. In the meantime, the gelatin-coated slide was allowed to come at room temperature. A drop of diluted semen was spread over gelatin slide. The moisture was allowed to evaporate from slide by keeping at room temperature for 5-10 minutes. The slide is then transferred to petri dish containing moist filter paper and incubated at 37° C for two hours. The slides were examined under the phase-contrast microscope using 40 X objective. The percentage of sperms having halos around their heads was counted.

Sperm Mitochondrial Activity Index (SMAI)^[16,17]:

This test evaluates the ability of mitochondria to provide the energy for the motility of spermatozoa by estimating the level of respiratory enzymes present in the mitochondria. A drop of semen and a drop of NBT (Nitro blue tetrazolium) solution were placed in cavity slide with a coverslip over it and was transferred to a moist petri dish and incubated at 37 C for thirty minutes. After this coverslip was removed and contents were well mixed in cavity itself and a smear was prepared on clean glass slide. The smear was allowed to dry at room temperature for ten minutes. The smear was fixed in neutral formalin for 2 hours, washed twice with distilled water and allowed to dry. The smear was examined under phase-contrast microscope (100 X objective). The intensity and distribution of formazan reaction was graded as below:

- Standard: Bluish-black precipitate along length of mid-piece.
- Sub-standard: Irregular precipitate on mid-piece and post acrosomal region.
- Low: Occasional formation of the precipitate in mid-piece.
- Residual: Precipitate all along the tail.

Hundred sperms were counted and categorized as above. Numbers of sperm of each category were classified and by using a multiplication factor, SMAI score was obtained. e.q.

Category	Number	Multiplication factor	Product
Standard	70	1.0	70
Sub-standard	20	0.7	14
Low	5	0.3	1.5
Residual	3	0.1	0.3
Nil	2	0.0	00
Total score			85.8

Nuclear Chromatin Decondensation Test (NCDT)^[16,18]:

The test detects the ability of the spermatozoal nuclear material to decondense in vitro. The semen sample is centrifuged (400 g for 15 minutes) to separate the plasma. The pellets were washed in 0.05 M borate buffer twice. Nine volumes of 6 mM EDTA and 1% SDS mixture are added to one volume of sample. The equal volume of 2.5% glutaraldehyde in 0.05 M borate buffer is added. It is incubated at 37° C for 60 minutes. A drop of this mixture is transferred to pre-cleaned glass slide and covered with coverslip to observe under phase optics (400 X). The numbers of condensed and decondensed heads are counted and their percentage is calculated.

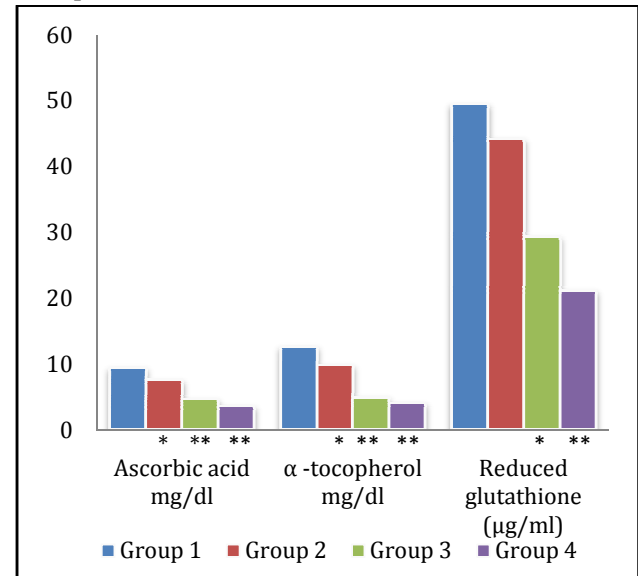
Statistical analysis was done using ANOVA test and Bonferroni's post test and P values < 0.05 were taken as significant. (p < 0.05 was considered statistically significant). The relationship between different parameters was tested by calculating coefficient of correlation (r-value). All the calculations were done by using Graph pad prism 5 software. Approval for the above study was taken from institutional ethics committee.

RESULTS

Semen samples were obtained from 78 male patients of 21-40 yrs of age attending the semen analysis laboratory of Department of Physiology, Government Medical College, Nagpur. Ascorbic acid, α -tocopherol and reduced glutathione level (GSH) was significantly more in group 1 as

compared to the other groups [figure1]. Similar finding was also observed in sperm function test score [figure2]. r value between sperm function test and antioxidant level in seminal plasma in different group is shown in table 1, 2, 3 and 4. Significant correlation was seen between sperm function test and antioxidant level.

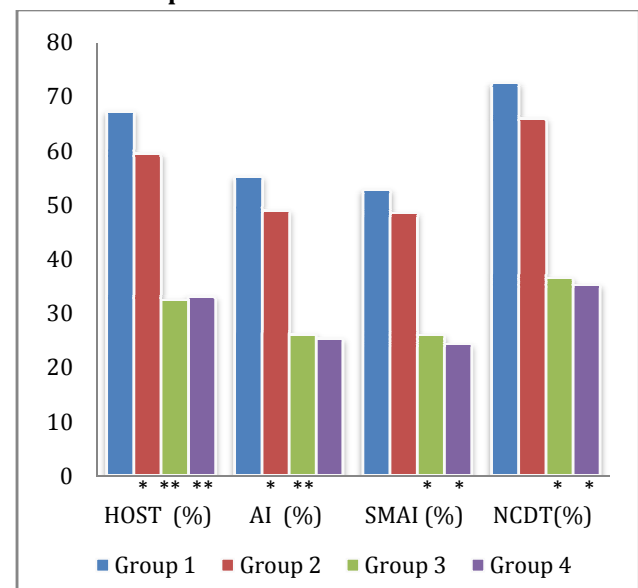
Figure-1: Comparison of Ascorbic Acid, α-tocopherol and Reduced Glutathione in Four Groups



* p < 0.05 significant: comparison with group 1

** p < 0.01 very significant: comparison with group 1

Figure-2: Comparison of Hypo-osmotic Swelling Test (HOST), Acrosomal Intactness Test (AI), Sperm Mitochondrial Activity Index (SMAI) and Nuclear Chromatin Decondensation Test (NCDT) in Four Groups



* p < 0.05 significant: comparison with group 1

** p < 0.01 very significant: comparison with group 1

Table-1: Correlation (r Value) Between Sperm Function Test and Antioxidant Level in Seminal Plasma in the Group 1

Group 1	Ascorbic Acid	α -tocopherol	Reduced Glutathione
HOST	0.95**	0.99**	0.88**
AI	0.48*	0.54*	0.63*
SMAI	0.55*	0.66*	0.76*
NCDT	0.53*	0.62*	0.62*

* p < 0.05 significant; ** p < 0.01 very significant

Table-2: Correlation (r Value) Between Sperm Function Test and Antioxidant Level in Seminal Plasma in the Group 2

Group 2	Ascorbic Acid	α -tocopherol	Reduced Glutathione
HOST	0.54*	0.54*	0.51*
AI	0.54*	0.51*	0.49*
SMAI	0.47*	0.46*	0.42*
NCDT	0.56*	0.54*	0.57*

* p < 0.05 significant

Table-3: Correlation (r Value) Between Sperm Function Test and Antioxidant Level in Seminal Plasma in the Group 3

Group 3	Ascorbic Acid	α -tocopherol	Reduced Glutathione
HOST	0.56*	0.54*	0.66*
AI	0.89**	0.92**	0.81**
SMAI	0.60*	0.60*	0.69*
NCDT	0.58*	0.59*	0.71*

* p < 0.05 significant; ** p < 0.01 very significant

Table-4: Correlation (r Value) Between Sperm Function Test and Antioxidant Level in Seminal Plasma in the Group 4

Group 4	Ascorbic Acid	α -tocopherol	Reduced Glutathione
HOST	0.46*	0.51*	0.56*
AI	0.49*	0.44*	0.51*
SMAI	0.45*	0.51	0.49
NCDT	0.48*	0.51*	0.61*

* p < 0.05 significant

DISCUSSION

The male factor is considered a major contributory factor to infertility. Apart from the conventional causes for male infertility such as varicocele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma and tumors, a new and important cause has been identified: oxidative stress.^[3] Oxidative Stress is a condition associated with an increase rate of cellular damage. Oxidative stress arises as a consequence of excessive ROS production and/or

impaired antioxidants defence mechanisms.^[3,4,5] Owing to their deleterious effects on human spermatozoa, excessive ROS must be continuously inactivated. Sperm cytoplasmic volume is very low and its cytoplasm contains only low concentrations of free radical scavenging enzymes. In contrast, seminal plasma is well endowed with an array of antioxidant defense mechanism to protect spermatozoa against oxidants. In this research work, we tried to evaluate the functional competence of four important spermatozoal organelles and these are Sperm membrane, Mitochondria, Acrosome, Nuclear chromatin. These function tests evaluate the overall structure of the spermatozoa. The HOS test detects the integrity of sperm membrane. The AI test detects acrosome intactness of spermatozoa. The SMAI test detects mitochondrial index and NCDT test detects DNA integrity of the spermatozoa. So after determining these seminal plasma antioxidants and we performed vis-a-vis sperm function tests (HOS, AI, SMAI, NCDT)^[16,17,18] to elucidate their effect on the fertilizing potential of the spermatozoa. Significant correlation was observed between sperm function test and antioxidant level in all groups. In the past various studies had been performed where correlation was seen between antioxidant level with routine sperm analysis and they had found the positive correlation.^[6,7]

We had gone one step further and found out the correlation between sperm function test and antioxidant level. Sierens J^[19] et al found significant positive effects of ascorbic acid levels in seminal plasma on DNA integrity of spermatozoa. Reduced antioxidant activity may also cause the disruption in the membrane integrity of spermatozoa as consequence of increased oxidative stress.^[20,21] Spermatozoa utilizes the reduced glutathione present in seminal plasma to maintain its motility, viability, mitochondrial status, oocyte binding capacity and fertilizing capability.^[18,22] Dandekar SP et al^[9] found that reduced glutathione dependent enzymes decrease (Water test) score, which is like HOS test. Thus sperm function is definitely affected by the levels of the antioxidant and

decreasing seminal plasma antioxidants levels could have significant role in etiology of impaired sperm function however in one study there was no significant relationship between sperm DNA damage and total antioxidant capacity, suggesting other mechanisms for sperm dysfunction.^[23]

It is important to measure these three antioxidants as they show the chain reaction, where the vitamin E gets recycled by vitamin C and vitamin C itself gets reduced by reduced glutathione. Decreased levels of these antioxidants reduce capacity of recycling and increases susceptibility of sperms to ROS damage.^[24] Also Recently, it has been found that high molecular weight antioxidants [superoxide dismutase (SOD) and catalase] were less effective than the low molecular weight antioxidants like vitamin C (ascorbic acid), reduced glutathione (GSH) and lipid soluble antioxidant vitamin E (alpha-tocopherol) in seminal plasma.^[24,25,26] Ascorbic acid was found to be major antioxidant while alpha-tocopherol and reduced glutathione were found to be mainly contributory antioxidants in seminal plasma.^[24,27] However in the present study we found significant correlation with all the three antioxidant so according to us all these low molecular weight antioxidant are equally important for sperm function. Thus the supplementation of these antioxidants would be of therapeutic use. Because oxidative stress is a major cause of sperm DNA damage, then antioxidants should have an important therapeutic role to play in the clinical management of male infertility.^[28] Study of aspects of oxidative stress will help us to design better antioxidant trials in future, with emphasis on identifying appropriate doses and optimal durations.^[3,29]

The evaluation of oxidative status and antioxidant defenses may be taken as an important tool for diagnosis and treatment of male infertility.^[30] The measurement of the antioxidative and oxidative agents could serve to evaluate human infertility in those cases where the result of the spermatoanalysis appears normal.^[31] Although consensus is growing about the clinical utility of seminal oxidative stress

testing in infertility clinics, standardization of protocols to measure is crucial before introducing these tests into routine clinical practice.^[32] Mahfouz RI^[6] et al had concluded that total antioxidant capacity of the seminal plasma as measured by the colorimetric assay is a reliable and simple test for the diagnosis and management of male infertility.

To summarize, decreasing seminal plasma antioxidants levels could have significant role in etiology of impaired sperm function. Seminal plasma antioxidants levels is closely related to male fertility, and the decreased level of antioxidants levels in seminal plasma may be one of the causes of male infertility. Supplementation of these antioxidants would be of therapeutic use in improving the chance of fertilization.

CONCLUSION

Decreasing seminal plasma antioxidants levels could have significant role in etiology of impaired sperm function. Seminal plasma antioxidants levels is closely related to male fertility, and the decreased level of antioxidants levels in seminal plasma may be one of the causes of male infertility.

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