

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Antioxidant level in the Seminal Plasma of Human subjects with different Fertility Potential

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ABSTRACT

In recent years, it is evident from different studies that oxidative stress has a definite role in inducing male infertility. Antioxidants help to fight against the oxidative stress. To estimate the levels of antioxidants viz. ascorbic acid (vitamin C), alpha-tocopherol (vitamin E) and reduced glutathione (GSH) in the seminal plasma of human subjects with different fertility potential. The present study was done on four groups. Four groups were group 1- control (n=10) normozoospermic fertile, group2: Normozoospermics (n=20) infertile, group 3- oligoasthenoteratozoospermics (n=30) infertile, and group 4- Asthenoteratozoospermics (n=20) infertile. Their semen analysis was done and levels of the antioxidant Vitamin C, Vitamin E, and the reduced glutathione was measured. ANOVA test and Bonferroni's post test. Coefficient of correlation (r- value) was found to find relationship between different parameters. Ascorbic acid, α -tocopherol and reduced glutathione level (GSH) was significantly more in group 1 as compared to the other groups. Ascorbic acid, α -tocopherol and reduced glutathione level levels of seminal plasma were found to be positively correlated with sperm concentration ($r = 0.63, r = 0.73, r = 0.55, r = 0.57$), sperm motility ($r = 0.44, r = 0.51, r = 0.57, r = 0.59$), normal sperm morphology ($r = 0.72, r = 0.63, r = 0.73, r = 0.59$). 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.

Keywords: Ascorbic acid, α -tocopherol, reduced glutathione level

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INTRODUCTION

Over the past years, antioxidants have attracted considerable interest as potential cause for the wide variety of disease states including cancer and other states like atherosclerosis, chronic inflammatory diseases and aging. The role of antioxidants is to detoxify reactive oxygen species (ROS) in the body which are the dangerous byproducts of aerobic metabolisms in the body. [1] Spermatozoa were the first cell type suggested to generate highly reactive oxygen species (ROS) in human body. [2] These reactive oxygen species (ROS) have been implicated as a major contributory factor in male infertility, as 40% of infertile men have detectable amounts of ROS in their semen, where as is no ROS activity in the semen of fertile men. Given that male factor problems make up the largest single cause of infertility the role of antioxidants in male infertility has become very important. [3, 4]

These antioxidants can be defined as "Substances that when present in low concentrations relative to the oxidizable substrate significantly delays or reduces oxidation of the substrate". Our hypothesis behind the study was that decreasing seminal plasma antioxidants levels could have significant role in etiology of impaired sperm function. Hence we endeavored to undertake this study. Although a wide variety of antioxidants contribute to reduce oxidative stress, this research was focused on three antioxidants in the semen of infertile male. These are Vitamin C, Vitamin E, and the reduced glutathione.

Subjects and Methods:

Semen samples were obtained from 80 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 [5] 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 90 subjects.

They were grouped as follows:

1. 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.

2. Group 2: Normozoospermics [20 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.
3. Group 3: Oligoasthenoteratozoospermics [30cases] 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.
4. Group 4: Asthenoteratozoospermics [20 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:

1. Controls were adult healthy male fertile volunteers, in the age group of 21-40yrs having at least one issue.
2. 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 judge to be fertile.

Exclusion Criteria:

1. 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.
2. Patients with azoospermia as the effect on functional parameters cannot be studied.
3. Persons with history of drug addiction, smoking and alcohol intake.
4. 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 [6] and modified by Bolin and Book [7] 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. 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^o 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 nm).

METHOD OF ESTIMATION OF VITAMIN E

We used Emmerie Engel [8] assay modified by Baker and Frank. [9] Three stoppered centrifuge tubes are taken and labeled as Test (T), Standard (S) and Blank (B). The addition were 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 tube were mixed and centrifuged for 15min. In other three clean stoppered tube 0.5ml of each xylene layer was transferred. To this 0.5ml of dipyrindyl reagent was added. 0.5 ml of mixture was pipette into spectrophotometer cuvettes and the absorbance was read at 460 nm (A 460) 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 [10]. 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 10,000 rpm. 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 were done from the graph.

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.

RESULTS

Semen samples were obtained from 90 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. Ascorbic acid, α -tocopherol and reduced glutathione level levels of seminal plasma were found to be positively correlated with sperm concentration ($r = 0.63$, $r = 0.73$, $r = 0.55$, $r = 0.57$), sperm motility ($r = 0.44$, $r = 0.51$, $r = 0.57$, $r = 0.59$), normal sperm morphology ($r = 0.72$, $r = 0.63$, $r = 0.73$, $r = 0.59$). The findings are summarized in Table I.

Group	Ascorbic acid Mg/dl	α -tocopherol μ g/ml	Reduced glutathione (μ g/ml)
Group 1 (n=10)	9.36 \pm 2.38 (8.45-10.27)	12.59 \pm 4.41 (10.92-14.27)	49.49 \pm 12.6 (38.12-60.12)
Group 2 (n=20)	7.65 \pm 1.90 (6.9-8.37)*	9.79 \pm 4.5 (8.05-11.53)*	39.59 \pm 17.7 (25.12-53.23)*
Group 3 (n=30)	4.65 \pm 1.83 (3.77-5.5)**@	4.9 \pm 2.5 (3.75-6.04)**@	20.53 \pm 10.30 (10.36-33.35)**@
Group 4 (n=20)	3.65 \pm 1.33 (3.2-5.66) ^c +	3.9 \pm 2.15 (3.445-5.94) ^c +	19.53 \pm 10.21 (10.116-31.15) ^c +

95% confidence intervals are given in parentheses. Values are means + SD.

*:- P<0.05 significant : comparison between group 1 and group 2

**:-p<0.001 significant: comparison between group 1 and group 3.

^c:-p<0.001 significant: comparison between group 1 and group 4.

+:- P<0.05 significant : comparison between group 2 and group 4

@: P<0.05 significant : comparison between group 2 and group 3

No significant difference between group 3 and group 4.

DISCUSSION

Male gametes are highly specialized and differentiated cells designed for the fertilization of the oocyte. The cellular generation of reactive oxygen species (ROS) was first observed in mammalian spermatozoa in late 1940s and subsequently their importance in damaging mammalian spermatozoa was first reported by Aitkin. [1] 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. [2, 11] 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 the present study, we investigated different antioxidants level in seminal plasma. Ascorbic acid, α -tocopherol and reduced glutathione level (GSH) was significantly more in Normozoospermics. Also the positive correlation was seen between antioxidant level with sperm concentration, sperm motility, and normal sperm morphology. Various authors had also

found similar result comparable to us. [12-16] Oschendorf et al [17], however observed that there was no statistical difference between the reduced glutathione level of seminal plasma of patients with different fertility potential, nor there was any association found between the parameters of seminogram and reduced glutathione content of seminal plasma.

Mahfouz R [13] 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. Sierens J [18] 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. [12, 16]

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. To make a more complete assertion about the antioxidative capacity of seminal plasma, it was considered necessary to investigate antioxidants like vitamin C (ascorbic acid), vitamin E (alpha-tocopherol) and reduced glutathione (GSH) in seminal plasma. [15, 19, 20]

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 [15].

Judging by higher concentrations present in normal seminal plasma ascorbic acid appears to be a key chain breaking antioxidant protecting sperm from oxidative assault in the same way as it protects somatic cell in blood plasma [21] Ascorbic acid was found to be major antioxidant while alpha-tocopherol and reduced glutathione were found to be mainly contributory antioxidants in seminal plasma. [15] These findings lead to the hypothesis that in normozoospermic and oligozoospermic infertile patients a lack of antioxidative protection may play a major role. If the lack of antioxidative protection was a major cause for male infertility the question arises whether or not supplementation of these antioxidants would be of therapeutic use. Supplementation of these antioxidants (viz. : vit. C, vit E and reduced glutathione) in normozoospermic and oligozoospermic infertile males may improve number of healthy spermatozoa and improve chances of fertilization. Similarly in in-vitro fertilization to counter the effects of depleted antioxidative defence in the seminal plasma of normozoospermic infertile patients, supplementation of these antioxidants during sperm preparation for assisted conception techniques should be considered.

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.

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