Research Article

Antioxidant level in the seminal plasma of human subjects with different fertility potential

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Abstract

Background: In recent years, it is evident from different studies that the oxidative stress has a definite role in inducing male infertility. Antioxidants help to fight against the oxidative stress. **Aims and Objective:** To estimate the levels of antioxidants namely ascorbic acid (vitamin C), α-tocopherol (vitamin E), and reduced glutathione (GSH) in the seminal plasma of human subjects with different fertility potential. **Materials and Methods:** This study was done on four groups. Four groups were group 1: control (n = 10) normozoospermic fertile, group 2: normozoospermics (n = 20) infertile, group 3: oligo-asthenoteratozoospermics (n = 30) infertile, and group 4: asthenoteratozoospermics (n = 20) infertile. Their semen analysis was done and the levels of the antioxidants vitamin C, vitamin E, and the reduced GSH were measured using analysis of variance (ANOVA) test and Bonferroni's post test. **Result:** Coefficient of correlation (r value) was calculated to find the relationship between different parameters. Ascorbic acid, α-tocopherol, and reduced GSH level was significantly more in group 1 as compared with the other groups. Ascorbic acid, α-tocopherol, and reduced GSH 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), and normal sperm morphology (r = 0.72, r = 0.63, r = 0.73, r = 0.59). **Conclusion:** Decreasing levels of seminal plasma antioxidants could have a significant role in the etiology of impaired sperm function. The seminal plasma antioxidant levels are closely related to male fertility; and the decreased level of antioxidants in seminal plasma may be one of the causes of male infertility.

KEY WORDS: Ascorbic Acid; α-Tocopherol; Reduced Glutathione Level

Introduction

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.[1] Spermatozoa were the first cell type suggested to generate highly ROS in human body.[2] These 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, whereas no ROS activity is seen in the semen of fertile men. Given that male-factor problems make up the single largest 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 levels of seminal plasma antioxidants could have significant role in the 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 (GSH).

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Materials and Methods

Semen samples were obtained from 80 male patients aged 21–40 years attending the semen analysis laboratory of Department of Physiology, Government Medical College,

Nagpur. Detailed history of the present and past illness as well as the medical and surgical management was taken. Selected male partners underwent thorough surgical examination of genitourinary system to rule out the exclusion criteria. Subjects with normally developed genitourinary organs were included in the study. All the tests were conducted with due permission 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 for sperm concentration, motility, and 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 million/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. Group2: Normozoospermics (20 cases) persons with sperm concentration of 20 million/mL or more, sperm motility of 50% or more (a + b type motility), normal sperm morphology of 30% or more.
3. Group 3: Oligoasthenoteratozoospermics (30 cases) 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 million/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 groups 2, 3, and 4 were those having no issues in spite of at least 1 year of unprotected intercourse.

Inclusion Criteria

1. Controls were adult healthy male fertile volunteers, in the age group of 21–40 years having at least one issue.
2. Infertile males (normozoospermic $\geqslant 20 \times 10^6$ spermatozoa/mL and oligozoospermic $< 20 \times 10^6$ spermatozoa/mL) were those having no issues in spite of at least 1 year of unprotected intercourse in the age group of 21–40 years.

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

Exclusion Criteria

1. Persons with occupation near hot furnace or in chemical industries using the substances such as 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 a history of drug addiction, smoking, and alcohol intake.
4. Persons with a previous history of hydrocele, varicocele, hernia, and operations on genital tract.

After taking permission from the ethical committee of Government Medical College and with due consent of the subjects, their clinical examination was performed.

Estimation of Ascorbic Acid in Seminal Plasma

Ascorbic acid is estimated by a method based on the principles of methods by Roe and Obsterling[6] and modified by Bolin and Book[7] with a little modification to suit semen. A total of 0.5 mL of seminal plasma was taken in a centrifuge tube. To it 1 mL of 10% TCA (trichloroacetic acid) solution was added and kept for 5 min, to this 7 mL of distilled water was added and mixed, then centrifuged for 15 min. Three test tubes were taken and marked A, B, and C and 2 mL of the filtrate was taken in each tube. A drop of 2:6 dichlorophenol-indophenol solution was added to test tubes A and B only and they were mixed till some color would persist. A total of 0.1 ml of thiourea solution was added to all the three test tubes. A drop more to tube C was added. A total of 0.5 mL of 2:4 di-nitrophenyl hydrazin (DNPH) solution was added to tube B and C and preserve the test tube A as blank. All the three tubes were incubated in an incubator at 370°C for exactly 3 h. To all the three tubes 1.5 mL of 95% sulfuric acid was added drop by drop while the tubes were immersed in ice. To the blank tube A 0.5 mL of 2:4 DNPH solution was added. All the tubes were taken out from the ice and kept at room temperature for half an hour. Colorimetric readings were taken using green filter (wavelength 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 was made as follows. Standard was prepared by taking 0.5 mL of standard solution in a test tube, to this 0.5 mL of ethanol and 0.5 mL of xylene was added. Blank was prepared by taking 0.5 mL of distilled water in a test tube, to this 0.5 mL of ethanol and 0.5 mL of xylene was added. Test was prepared by taking 0.5 mL of seminal plasma in a test tube, to this 0.5 mL of ethanol and 0.5 mL of xylene was added. All the three stoppered centrifuge tubes were mixed and centrifuged for 15 min. In other three clean stoppered tubes, 0.5 mL of each xylene layer was transferred, to this 0.5 mL of dipyridyl reagent was added. A total of 0.5 mL of the mixture was pipetted into a spectrophotometer cuvette and the absorbance was read at 460 nm (A 460) of the test (T) and standard (S) against the blank (B). The wavelength of the filter used was 520 nm. Then beginning with blank 0.33 mL $FeCl_3$ solution was added into all the tubes, mixed and kept aside for 1.5 min. The absorbance at 520 nm (A 520) of the test (T) and standard (S) against the blank (B) was read.

Table 1: Level of different antioxidants in seminal plasma

Group	Ascorbic acid (mg/dl)	α-Tocopherol (μg/mL)	Reduced glutathione (μg/mL)
Group 1 (n = 10)	9.36 ± 2.38 (8.45–10.27)	12.59 ± 4.41 (10.92–14.27)	49.49 ± 12.6 (38.12–60.12)
Group 2 (n = 20)	7.65 ± 1.90 (6.9–8.37)*	9.79 ± 4.5 (8.05–11.53)*	39.59 ± 17.7 (25.12–53.23)*
Group 3 (n = 30)	4.65 ± 1.83 (3.77–5.5)**, *****	4.9 ± 2.5 (3.75–6.04)**, *****	20.53 ± 10.30 (10.36–33.35)**, *****
Group 4 (n = 20)	3.65 ± 1.33 (3.2–5.66)***, ****	3.9 ± 2.15 (3.445–5.94)***, ****	19.53 ± 10.21 (10.116–31.15)***, ****

95% confidence intervals are given in parentheses. Values are mean ± standard deviation (SD).
*$p < 0.05$ significant: comparison between group 1 and group 2, **$p < 0.001$ significant: comparison between group 1 and group 3, ***$p < 0.001$ 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.

Method of Estimation of Reduced Glutathione (GSH)

Reduced GSH is estimated by a method based on the principles of methods by Moron et al.[10] A total of 0.5 mL of seminal plasma was taken in a test tube and 2 mL of distilled water was added and mixed well, then it was centrifuged for 5 min at 5,000 rpm. From the supernatant 0.5 mL was taken, to which 0.5 mL of TCA (5%) was added and then centrifuged for 10 min at 10,000 rpm. Again from the supernatant 0.5 mL was taken to which 2.5 mL of phosphate buffer (pH 8) was added and to this 1 mL 5, 5'-Dithiobis (2-nitrobenzoic acid) was added. This solution was inverted three times to mix. The absorbance was read on the spectrophotometer at 412 nm within 4 min of preparing the mixtures. Standard graph of the reduced GSH concentrations was plotted. Determination of the reduced GSH concentration in seminal plasma was done from the graph.

Statistical analysis was done using the analysis of variance (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 GraphPad Prism 5 Software.

Result

Semen samples were obtained from 90 male patients aged 21–40 years attending the semen analysis laboratory of Department of Physiology, Government Medical College, Nagpur. Ascorbic acid, α-tocopherol, and reduced GSH level were significantly more in group 1 as compared with the other groups. Ascorbic acid, α-tocopherol, and reduced GSH 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), and normal sperm morphology (r = 0.72, r = 0.63, r = 0.73, r = 0.59).

The findings are summarized in Table 1.

Discussion

Male gametes are highly specialized and differentiated cells designed for the fertilization of the oocyte. The cellular generation of ROS was first observed in mammalian spermatozoa in the late 1940s and subsequently their importance in damaging mammalian spermatozoa was first reported by Aitken et al.[1] Oxidative stress is a condition associated with an increased rate of cellular damage. Oxidative stress arises as a consequence of excessive ROS production and/or impaired antioxidant defense 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, the seminal plasma is well endowed with an array of antioxidant defense mechanism to protect spermatozoa against the oxidants. In this study, we investigated different antioxidant levels in the seminal plasma. Ascorbic acid, α-tocopherol, and reduced GSH level were significantly more in normozoospermics. Also a positive correlation was seen between antioxidant level and sperm concentration, sperm motility, and normal sperm morphology. Various authors had also found similar result comparable to us.[12–16] Ochsendorf et al.,[17] however, observed that there was no statistical difference between the reduced GSH level of seminal plasma of patients and different fertility potential, nor there was any association found between the parameters of seminogram and reduced GSH content of seminal plasma.

Mahfouz et al.[13] 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 et al.[18] 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 a consequence of increased oxidative stress.[12,16] Recently, it has been found that high molecular weight antioxidants (superoxide dismutase and catalase) were less effective than the low molecular weight antioxidants such as vitamin C (ascorbic acid), reduced GSH, and the lipid soluble antioxidant vitamin E (α-tocopherol) in seminal plasma. To make a more complete assertion about the antioxidative capacity of seminal plasma, it was considered necessary to investigate antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), and reduced 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 GSH. Decreased levels of these antioxidants reduce the capacity of recycling and increases susceptibility of sperms to ROS damage.[15] Judging by the higher concentrations present in a 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 cells in the blood plasma.[21] Ascorbic acid was found to be a major antioxidant whereas α-tocopherol and reduced GSH were found to be mainly contributory antioxidants in the seminal plasma.[15] These findings lead to the hypothesis that a lack of antioxidative protection may play a major role in male infertility. 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. vitamin C, vitamin E, and reduced GSH) in normozoospermic and oligozoospermic infertile males may improve the number of healthy spermatozoa and may improve the chances of fertilization. Similarly, in in vitro fertilization to counter the effects of depleted antioxidative defense in the seminal plasma of normozoospermic infertile patients, supplementation of these antioxidants during sperm preparation for assisted conception techniques should be considered.

Limitations

Less sample size. Not all antioxidants were studied.

Conclusion

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

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