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WATER SOLUBLE VITAMIN AS AN ANTI-OXIDANT TO PROTECT AGAINST SEMINAL OXIDATIVE STRESS IN MALE INFERTILITY

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ABSTRACT

Oxidative stress can lead to sperm damage, deformity and eventually male infertility. This study was aimed to evaluate the relationship between malondialdehyde (MDA) & vitamin C levels in seminal plasma with sperm parameters. Thirty fertile men and thirty eight infertile men were recruited in this study. Seminal fluid MDA and Vitamin C levels were measured by spectrophotometric method and correlated with semen parameters including; volume, sperm count, sperm motility and morphology of sperm (3.15 ± 0.6 ml, 30.74 ± 8.11 millions/ml, 33.56 ± 6.68 %, 19.64 ± 4.8 % respectively) were significantly lower ($p<0.005$) in infertile men than control (3.58 ± 0.53 ml, 78.94 ± 13.08 millions/ml, 74.58 ± 7.83%, 67.25 ± 7.73% respectively). The seminal level of MDA was significantly higher ($p<0.03$) in infertile men (2.27 ± 0.39 nmol/ml) than control (1.40 ± 2.27 nmol/ml). Vitamin C level was significantly lower ($p<0.0001$) in infertile men (9.40 ± 4.13 mg/dl) than control (16.99 ± 4.67 mg/dl). Present study concludes the role of vitamin C to protect sperm against oxidative damage & suggests therapeutic use of vitamin C in male infertility although series of clinical trials are needed to investigate the prospect.

KEY WORDS

Oxidative stress (OS), Reactive oxygen species (ROS), malondialdehyde (MDA), Vitamin C, and Male infertility.

INTRODUCTION:

The World Health Organization defines infertility as the inability of a couple to achieve conception or bring a pregnancy to term after 1 year or more of regular, unprotected sexual intercourse[1]. Infertility is a major clinical concern, affecting 15% of all reproductive-aged couples, and male factors, including decreased semen quality, are responsible for 25% of these cases[2]. Currently, the etiology of suboptimal semen quality is poorly understood, and many physiological, environmental, and genetic factors, including oxidative stress have been implicated[2–5].

Oxidative stress is the state of imbalance between pro-oxidant and antioxidant forces in any given system. Pro-oxidants are the substances capable of generating Reactive Oxygen Species (ROS) or free radicals causing cellular damage at different level leading to a number of morbidity involving various organs.[6] ROS has recently been proposed as one of the major cause of OS and directly damaging the spermatozoa[7-11]. Increased levels of ROS have been correlated with decreased sperm motility[12-14]. Increased sperm DNA damage[15–17], sperm cellular membrane lipid peroxidation[18-20] and decreased efficacy of oocyte-sperm fusion[21].

The human sperm cell membrane is particularly susceptible to oxidation due to the existence of high concentration of polyunsaturated fatty acids (PUFA). Seminal plasma malondialdehyde which is the stable lipid per-oxidation product is a simple method to evaluate the effect of lipid peroxidation on sperm. The presence of considerable amounts of antioxidants, e.g. vitamin C (ascorbic acid), vitamin E (tocopherol) as well as the enzymes superoxide dismutase, glutathione peroxidase and catalase have been known[22].

Ascorbate also has the ability to scavenge free radicals independent of vitamin E[23]. Apparently, minimal levels of ascorbic acid are required to protect the formation, maturation and delivery of spermatozoa from endogenous reactive oxidants, being the main extracellular water-soluble antioxidant[24]. Necrospermic as well as azoospermic semen samples apparently contain less content of ascorbic acid than normozoospermic samples[25, 26]. Also, seminal plasma ascorbic acid was shown to reduce nonspecific sperm-agglutinin and ensures the molecular coating of the sperm heads[27]. Its other role is in the epithelium of the germinal layer and cauda epididymidis where sperm maturation occurs[28].

The aim of the study is to evaluate the levels of malondialdehyde as an indicator of lipid peroxidation, ascorbic acid as an water soluble anti oxidant vitamin in seminal plasma of fertile and infertile men and the significance of any difference, if any, detected between both groups.

MATERIALS AND METHODS

After obtaining due permission of the ethical committee of the Institute and with written consent from the subjects, semen samples were collected from 68 married men aged 19-51 years by masturbation only after confirming 3 days of sexual abstinence. After complete liquefaction, samples were analyzed microscopically for sperm concentration, motility, and morphology according to WHO guideline[29] and grouped into two categories with following criteria;

1. Normozoospermics- (30 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.
2. Asthenoteratozoospermics- (38 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.

These subjects had complaints of infertility (both primary and secondary infertility cases) even after one year of regular, unprotected intercourse. Wives of the infertile subjects included had no obvious causes of infertility like tubal blockage or ovulation disorders.

Exclusion criteria:

Subjects with those complaints that may interfere with male fertility like varicocoele, hydrocoele, undescended testes or any other structural abnormality or any history of surgical intervention in the genitourinary tract or any acute febrile ($>38^0$ C body temperature) illness or a history of similar episode in last six month or treatment history with drugs like cancer chemo-therapy, nitrofurantoin, niridazole, colchicine or any hormonal preparation which may directly suppress the spermatogenesis were also excluded from the study.

Assessment of oxidative stress by measuring MDA levels of seminal plasma:

MDA levels were measured as per Thiobarbituric Acid (TBA) method described by Yao-Yuan Hsieh et al[30]. Seminal plasma was separated at 5000 rpm for 10 minutes at room temperature after complete liquefaction. Then 0.1 ml of seminal plasma was added to 0.9 ml of distilled water in a glass tube, to it 0.5 ml of TBA reagent (0.67 gm of Thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 gm of NaOH and 100 ml of glacial acetic acid) was added and heated for 1 hour in a boiling water bath. After cooling the tube was centrifuged for 10 minutes at 4000 rpm and the absorbance of supernatant was read on a spectrophotometer at 534 nm.

Estimation of Ascorbic acid by 2, 4 Dinitrophenylhydrazine (2, 4 DNPH) method:

Ascorbic acid in seminal plasma is oxidized by cupric (Cu^{+2}) ion to form dehydro ascorbic acid which reacts with acidic 2, 4 – dinitrophenyl – hydrazine to form a red bis–hydrazone[31]. Briefly, ascorbic acid in the

sample was converted to dehydroascorbate in the presence of thiourea and copper sulphate. 0.5 ml samples were mixed with 0.4 ml DTCs (containing thiourea, copper sulphate and DNPT, DNPH, 12M) and incubated for 3 hours at 37°C. Dehydroascorbate was then coupled with 2, 4-dinitrophenylhydrazine forming its bis derivative. Upon treatment with 2 ml, 12 mol sulphuric acid, the derivative yields a stable red colour, which was measured spectrophotometrically at 520 nm[32].

Statistical analysis:

It was performed between group 1 (normozoospermics) and group 2 (asthenozoospermics) by using MS office Excel 2007 and the software 'GraphPad Quick Cals t-test calculator'. Results were expressed as mean (M), standard deviation (S.D.) and considered significant when the p value less than 0.05 determined by the unpaired student's t-test. Pearson's correlation (r value) was determined to find positive and negative correlations among parameters.

RESULTS

Results were expressed as Mean ± SD for each parameter. Statistically significant differences among normozoospermic (fertile) and asthenozoospermic (infertile) men are indicated in **Table 1** along with their significant values. The various parameters studied were; seminal volume, sperm count, sperm motility (both actively motile and sluggish, viz. a+b,) and morphology of normal sperm cells (3.15 ± 0.6 ml, 30.74 ± 8.11 millions/ml, 33.56 ± 6.68 %, 19.64 ± 4.8 % respectively) were significantly lower (P<0.005) in infertile men than control fertile men (3.58 ± 0.53 ml, 78.94 ± 13.08 millions/ml, 74.58 ± 7.83%, 67.25 ± 7.73% respectively). The seminal level of MDA is significantly higher (p<0.03) in infertile men (2.27 ± 0.39 nmol/l) than fertile men (1.40 ± 2.27 nmol/l). But the seminal level of Vitamin C was significantly lower (p<0.0001) in infertile men (9.40 ± 4.13 mg/dl) compared to their counterpart fertile men (16.99 ± 4.67 mg/dl).

Table 1: The Mean Values Of Sperm Count, Sperm Motility, Sperm Morphology, Seminal MDA, & Vitamin C In Normozoospermic & Asthenozoospermic Men.

Parameters	Normozoospermic (n=30)	Asthenozoospermic (n= 38)
Seminal Volume (ml)	3.58 ± 0.53	3.15 ± 0.6**
Sperm count (millions/ml)	78.94 ± 13.08	30.74 ± 8.11***
Sperm motility[#] (both a+b)%	74.58 ± 7.83	33.56 ± 6.68***
Normal sperm mopphology %	67.25 ± 7.73	19.64 ± 4.8***
MDA(nmol/ml)	1.40 ± 2.27	2.27 ± 0.39*
Vitamin C (mg/dl)	16.99 ± 4.67	9.40 ± 4.13***

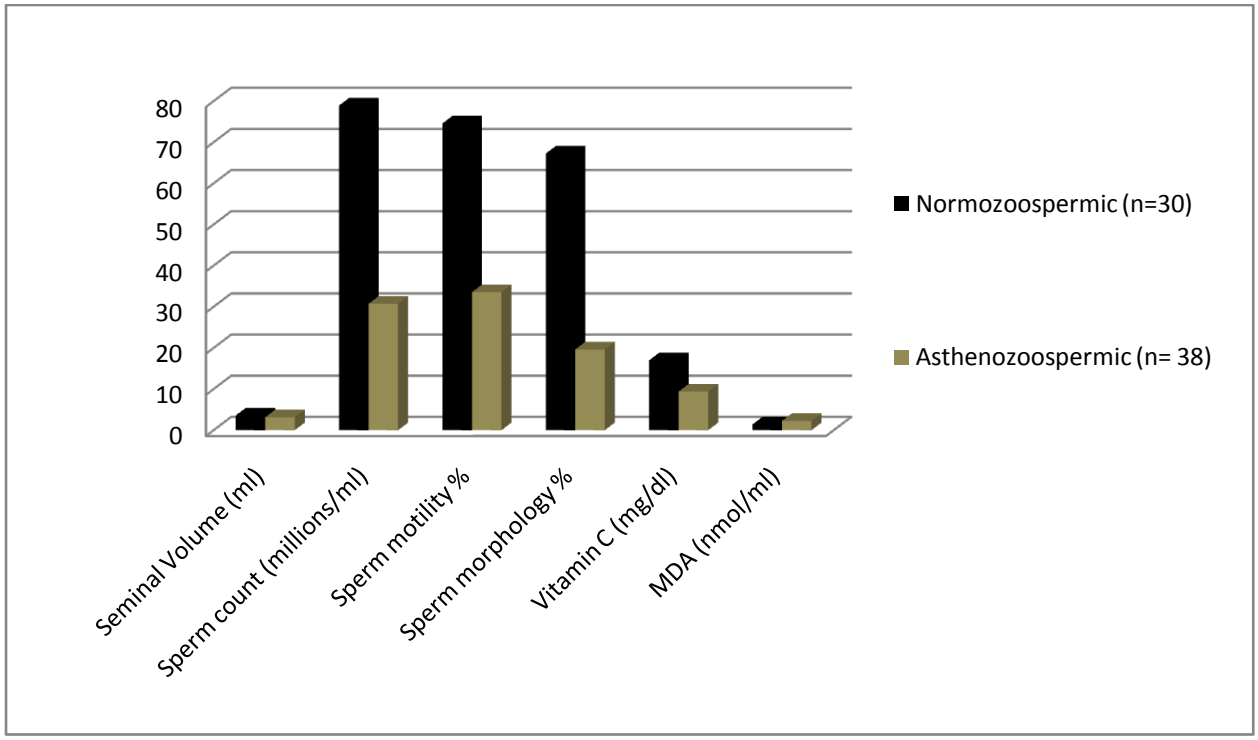
(Mean ± S.D., Comparison with control: *p<0.03 statistically significant, * *p<0.005 statistically highly significant, and ***p<0.0001 statistically extremely significant.), # Sperm motility a= actively motile, b= sluggish.

Table 2: Correlation Coefficient Of Various Parameters Studied In Infertile Men.

Parameters	MDA	Vitamin C
MDA	--	-0.67
Vitamin C	-0.67	--
Sperm Count	-0.13	0.36
Sperm Motility (a+b)	-0.16	0.4
Sperm Morphology	0.07	-0.01

Correlation coefficient of various parameters as indicated in **Table 2** MDA. There was negative correlation of malondialdehyde (MDA) with Vitamin C in infertile men. The Seminal plasma MDA had a negative correlation with sperm count, and motility but positively correlated with sperm morphology. Similarly, vitamin C levels were positively correlated with sperm count & motility but it was negatively correlated with sperm morphology in infertile men.

Figure 1: Comparison of Seminal Parameters between Normozoospermic and Asthenozoospermic Group



DISCUSSION

Recently the over-production of ROS in the male reproductive tract has become a potential cause of male infertility. Though it has been shown that small amounts of ROS are essential for regulation of normal sperm functions like sperm capacitation, acrosome reaction and oocyte fusion but at high levels they have potential toxic effects on sperm quality and function. Sperm plasma membrane has a high concentration of PUFA which makes it susceptible to lipid peroxidation by ROS, this can leads to loss of membrane fluidity and integrity, as a result of this the spermatozoa lose their competence to participate in the membrane fusion events associated with fertilization. Also they can attack DNA, induced strand breaks and oxidative stress damage in spermatozoa[33].

In present study, we found high levels of MDA in infertile men as compared to fertile and it was negatively correlated with sperm count and sperm motility. Our result of MDA was in accordance with studies by Nabil H. et al[34], Hsieh YY et al[35] and Fraczek et al[36]. Nabil H. et al[34] reported elevated seminal MDA concentration in patients with oligozoospermic and azoospermic groups. Several studies have reported that spermatozoa from oligozoospermic or asthenozoospermic men showed a greater production of oxidative stress[33]. Our results were in contrast with Suleiman et al[37] who demonstrated that MDA concentration in the seminal plasma was not related with the sperm concentration and motility.

The highest level of ascorbic acid seen in the seminal plasma of normozoospermics was similar to that observed by Blumenkrantz et al[38] & Piyali Das,et al[39]. Although some different results were observed by some researchers, The Observation of Koets and Michelson[40] shows a significantly higher value of ascorbic acid (13.7 mg/dl) in the semen of impaired quality than that of normal sample (7.2 mg/dl). Our study also contradicts the findings of Chinoy et al[41] who found highest amount of free ascorbic acid in the semen of azoospermics. Lower levels of ascorbic acid in abnormal ejaculates as found in our study suggests the excess generation of ROS over there by different pathophysiological processes which leads to excess consumption of the antioxidant molecules like ascorbic acid[42].

The negative correlation between seminal Vitamin C and MDA obtained from our study indicates the defensive role of ascorbic acid against the lipid peroxidation process. We also found a positive correlation between seminal ascorbic acid concentration and motility but negative correlation with normal morphology of spermatozoa which specifically supports the findings of Thiele et al[43] & Zunzarrao G. Badade et al[33] although they had found positive correlation between seminal plasma Vitamin C & normal morphology of spermatozoa.

Rolf et al[44], in their double blinded study reported that oral treatment with high dose of vitamin C and vitamin E did not improve semen parameters in asthenozoospermic men, though there are various other studies showing the role of supplemented ascorbic acid in prevention of sperm DNA damage and oxidative injury[45, 46].

Our study suggests that increased seminal plasma MDA and decreased Vitamin C level might have significant role in the etiology of sperm abnormality. Negative correlation of sperm parameters with MDA, and positive correlation with Vitamin C indicates, oxidative stress adversely affects in male infertility by interfering with sperm count & motility regardless of sperm morphology. Hence evaluation of MDA and Vitamin C can be used for diagnosis and prognosis of male infertility. Therapeutic usage of the water soluble antioxidant like Vitamin C in the treatment of male infertility should be studied extensively. A series of clinical trials are needed to investigate the prospect.

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