ROLE OF ASCORBIC ACID IN HUMAN SEMINAL PLASMA AGAINST THE OXIDATIVE DAMAGE TO THE SPERMS

Piyali Das, A R Choudhari, A Dhawan and Ramji Singh

Reproductive Biology Unit, Department of Physiology, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha- 442102.

ABSTRACT

This study evaluated the extent of oxidative stress by measuring malondialdehyde and ascorbic acid in the seminal plasma of human subjects with different fertility potential. Semen samples from 148 subjects were evaluated (48 normozoospermics, 34 oligoasthenoteratozoospermics, 34 asthenoteratozoospermics and 32 azoospermics). malondialdehyde level was found to be significantly higher in the abnormal groups (oligoasthenoterato and asthenoterato-zoospermics) than normozoospermics ($P < 0.01$). Negative correlation was also found between malondialdehyde level, sperm concentration, sperm motility and sperm morphology. Level of ascorbic acid was found to be significantly higher in normozoospermics than other abnormal groups ($P < 0.01$). It was found to be correlated positively with all seminogram parameters and negatively with malondialdehyde level. The study revealed that, excess lipid peroxidation reflected by high malondialdehyde level with reduced ascorbic acid in human seminal plasma is associated with poor semen quality where as ascorbic acid content has positive correlation with fertility potential.

KEY WORDS

Ascorbic acid, Infertility, Oxidative stress, Human Seminal Plasma.

INTRODUCTION

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 (1). On the other hand antioxidants are the compounds disposing, scavenging, suppressing the formation or opposing the action of toxic oxidants (2). In context to human reproduction, excessive generation of pro-oxidants that exceeds critical level of physiological requirement can overwhelm the antioxidant defense strategies of spermatozoa as well as seminal plasma. The resultant condition is "oxidative stress" which potentially targets all the cellular components

including lipids, proteins, nucleic acids, sugars etc. According to a recent report, high levels of ROS were detected in semen samples of 25-40% of infertile men (3). Different life style behavior like smoking, alcohol abuse; environmental factors like radiation, pollutants, chemical fertilizers and pesticides, different infective and inflammatory conditions of the reproductive tract including accessory glands may lead to overproduction of ROS and thus the oxidative stress (4). Human spermatozoa because of abundant content of poly unsaturated fatty acid in their cell membrane and very scanty antioxidant support from the cytoplasm are very much susceptible to oxidative stress. Excess amount of lipid peroxidation producing high levels of spermicidal cytotoxic end products like malondialdehyde (MDA) may cause defective changes in sperm and diminish fertility (5). The potential method that can evaluate Oxidative Stress is to measure the oxidized end product (Thiobarbituric acid reactive substance, MDA) in body fluids (i.e. seminal plasma). However, there are lot of controversies about the seminal MDA activity and the sperm quality. Suleiman et al (6) demonstrated that the MDA concentration in the seminal plasma was not correlated with sperm concentration and motility which contradicts the findings of a number of previous studies (7, 5).

Address for Correspondence :

Dr. Ramji Singh,
Department of Physiology,
Mahatma Gandhi Institute of Medical Sciences
Sevagram, Wardha- 442102.
E-mail : sramji57@gmail.com
Ph: 09423421794

Ascorbic acid or Vitamin C is a major water soluble antioxidant acting as a scavenger for a wide range of ROS. It is present at approximately ten fold higher concentration in seminal plasma as compared to blood and is mostly secreted from seminal vesicles (8). Ascorbic acid acts as a powerful electron donor that reacts with superoxide, peroxide and hydroxyl radicals to form dehydroascorbic acid. It also recycles Vit E. It protects sperms against DNA damage induced by H_2O_2 radical & also reduces nitrite (4). But Vitamin C can also have a paradoxical effect as it can produce some ROS by its action on transition metal ions (9). A few studies have also addressed a possible relationship between ascorbic acid and DNA fragmentation in infertile men (10). Thus still now, there is gross discrepancy regarding the role as well as level of ascorbic acid in human seminal plasma.

With this background, in the present study attempt was made to assess the oxidative stress by measuring MDA and vitamin C in the seminal plasma of human subjects with different fertility potential and to correlate the level of MDA with that of Vitamin C as well as different seminogram parameters. Correlation of vitamin C with different seminogram parameters was also tested statistically.

MATERIALS AND METHODS

Semen samples were obtained from 148 male patients of 24-47 yrs of age attending the Reproductive Biology Unit, MGIMS, Sevagram, with complaints of infertility (both primary and secondary infertility cases). Detailed history of present and past illness as well as medical and surgical management was taken. Selected male partners underwent thorough surgical examination of genito-urinary system to rule out the exclusion criteria. Only subjects with normally developed genito-urinary organs were included in the study. All the tests were done 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 by SQA II B sperm quality analyzer (M.E.S. Ltd., Israel) for sperm concentration, motility, morphology according to WHO guideline (11) and grouped into four categories with following criteria -

1. **Normozoospermics-(Normo)-** (48 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. **Oligoasthenoteratozoospermics-(OAT)-** (34 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.

3. **Asthenoteratozoospermics-(AT)-** (34 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.
4. **Azoospermics-(Azoo)-** (32 cases) – Total absence of Spermatozoa in semen (even after centrifugation).

Except for the normozoospermics, all other subjects with abnormal seminogram profile were asked to come for repeat semen analysis after 1 month. If the second report was also abnormal then only they were included in the abnormal groups and the semen samples were utilized for further physiochemical assessment.

Exclusion criteria: Subjects with varicocoele, hydrocoele, undescended testes or any other structural abnormality or any history of surgical intervention in the genitourinary tract which may interfere with male fertility were excluded from the study. Subjects with any acute febrile ($>38^\circ C$ body temperature) illness or a history of similar episode in last six month or treatment history with drugs like cancer chemotherapy, nitrofurantoin, niridazole, colchicine or any hormonal preparation which may directly suppress the spermatogenesis were also excluded from the study. Azoospermics with normal testicular volume (total testicular volume ≥ 30 ml) were subjected to testicular biopsy to rule out obstructive azoospermia cases which were excluded from the study. According to the semen analysis report, those who had <5 millions sperm /ml of semen 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 (5). 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 was added and then heated for 1 hour in a boiling water bath. After cooling the tube was centrifuged for 10 minutes at 4000 rpm and the supernatant absorbance was read on a spectrophotometer at 534 nm.

Estimation of Ascorbic acid by 2, 4 Dinitrophenyl – hydrazine (2,4 DNPH) method (12): 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 which is measured at 520 nm.

Statistics: The differences of mean values of MDA and

ascorbic acid in different groups were evaluated statistically using Student's 't' test. (p- Value < 0.05 was considered significant). The relationships between different parameters were tested by calculating coefficient of correlation (r- value). All the calculations were done by using STATA 10 software.

RESULTS

The lipid peroxidation as expressed by MDA level (nmol/ml) in seminal plasma was found to be significantly higher in abnormal groups (Oligoasthenoterato, Asthenoterato) than in normal group (Normozoospermics). In Table 1, Mean MDA level in abnormal groups were found to be 2.39 ± 0.81 nmol/ml in Oligoasthenoterato, 2.18 ± 0.61 in Asthenoterato; where as the level in normal group was 1.46 ± 0.52 ('P' value < 0.01). MDA level in Azoospermics was found to be 1.54 ± 0.41 . MDA level of seminal plasma was found to have a negative correlation with sperm concentration ($r = -0.58$), sperm motility ($r = -0.57$) and normal sperm morphology ($r = -0.59$).

Mean ascorbic acid level of seminal plasma (in mg / dl) was found to be highest in Normozoospermics (20.53 ± 13.81) followed by Asthenoterato (4.66 ± 3.12), Oligoasthenoterato (4.35 ± 5.38) and Azoospermics (2.86 ± 0.41). Levels in each abnormal group were significantly different from that of Normozoospermics ($P < 0.05$). Seminogram parameters (sperm concentration, motility, normal sperm morphology) were found to be positively correlated with seminal ascorbic acid ('r' value: 0.62, 0.58 and 0.62 respectively). Ascorbic acid was also found to have a negative correlation with seminal MDA ('r' value: - 0.63).

DISCUSSION

It is now evident from different research works that free radical induced injury to the sperm plays a major role in the pathogenesis of male infertility. The amount of lipid peroxide content of the semen reflects the extent of oxidative stress

that the spermatozoa have suffered during their life history. But there is lot of controversies regarding the levels of MDA in different group of subjects. Kobayashi et al in (13) demonstrated that MDA concentration was significantly related to the number of immotile spermatozoa. Elevated MDA level was also noted by Fraczek et al (14) in Oligoasthenoteratozoospermics. Conversely, Suleiman et al (6) demonstrated that the MDA concentration in the seminal plasma was not correlated with sperm concentration and motility. In our present study, we found significantly higher level of MDA in the seminal plasma of the abnormal groups (including oligoasthenoterato and asthenoteratozoospermics). The finding may be supportive of the fact that major source of MDA in seminal plasma are abnormal spermatozoa as well as infiltrating leukocytes that are preponderant in abnormal ejaculates (4). A negative correlation was observed between sperm concentration, motility, normal morphology and seminal MDA level which were in agreement with the findings of Yao-Yuan Hsieh et al (5). So it was shown from our study that, high lipid peroxidation is associated with over all poor semen quality. However, MDA levels in azoospermics were found to be slightly higher than the normozoospermic group though not statistically significant which may be due to presence of inflammatory cells or epithelial castings within the semen.

In present study we found the highest level of ascorbic acid in the seminal plasma of normozoospermics (20.53 ± 13.81 mg/dl) followed by asthenoterato (4.66 ± 3.12 mg/dl), oligoasthenoterato (4.35 ± 5.38 mg/dl) and azoospermics (2.86 ± 0.41 mg/dl). Findings are similar to that observed by Blumenkrantz et al (15).

The observations of Koets and Michelson (16) were thoroughly different from that of us. They found 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 (17), 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 (8). Simultaneous increase in the level of MDA in abnormal ejaculates further supports the possibility of excess ROS generation and thereby enhanced lipid peroxidation process which becomes uncontrollable with the available antioxidant resource of seminal plasma. In case of normal ejaculates, as the production of ROS is within controllable limit, it gets efficiently scavenged by the existing antioxidant store of

Table 1: Levels of MDA (nmol/ml) and ascorbic acid (mg/dl) in different study Groups

Groups (n)	MDA (nmol/ml)	Ascorbic acid (mg/dl)
Normozoospermics (48)	1.46 ± 0.52	20.53 ± 13.81
Oligoasthenoteratozoospermics (34)	$2.39 \pm 0.81^*$	$4.35 \pm 5.38^*$
Asthenoteratozoospermics (34)	$2.18 \pm 0.61^*$	$4.66 \pm 3.12^*$
Azoospermics (32)	1.54 ± 0.41	$2.86 \pm 0.41^*$

* P < 0.01

seminal plasma. A significant negative correlation between seminal ascorbic acid and MDA as shown from our study indicates towards the defensive role of ascorbic acid against the lipid peroxidation process. We also found a positive correlation between seminal ascorbic acid and concentration, motility, normal morphology of spermatozoa which specifically supports the findings of Thiele et al (10). The findings may lead to the possibility of improving quality of semen by therapeutic supplementation of ascorbic acid to the patients of impaired fertility potential. However, Rolf et al (18), reported in their double blinded study that oral treatment with high dose of vitamin C and vitamin E did not improve semen parameters in asthenozoospermic men, though there are much other studies showing the role of supplemented ascorbic acid in prevention of sperm DNA damage and other oxidative injury (19, 20).

As we have not explored the exact etiology of azoospermic cases, the lower levels of ascorbic acid in them can not be explained from our study. This may be due to genetic defects or hormonal discrepancies which may lead to subnormal activity of accessory reproductive organs like seminal vesicles in azoospermics.

Therefore, our study findings support the possible role of ascorbic acid in the prevention of lipid peroxidation and thereby protecting the sperms against the oxidative injury and thus contributing to improve the over all semen quality.

Present study concluded that high lipid peroxidation may have negative impact on human semen quality where as ascorbic acid seems to play defensive role in the seminal plasma to protect the sperms against oxidative damage. Therapeutic usefulness of ascorbic acid in the treatment of male infertility however merits a series of future clinical trials.

REFERENCES

- Halliwell B, Cutleridge JMC. Free radicals, ageing, and disease. In: Free radicals in Biology and Medicine. 2nd ed. Oxford University Press. 1989: 123-419.
- Sikka Suresh C. Oxidative Stress and role of Antioxidants in Normal and Abnormal Sperm Function. *Frontiers in Biosciences* 1996; 1: 78-86.
- WHO manual for the Standardized investigations, diagnosis and management of infertile couple. Cambridge University Press. Cambridge. 2000; 4th Ed: 8-61.
- Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Ind J Exp Biol* 2005; 43: 963-74.
- Yao-Yuan Hsieh, Chi-Chen Chang, Chich- Sheng Lin. Seminal malonaldehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration & motility. *Int J Biol-Sci* 2006; 2: 23-9.
- Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid Peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996; 17: 530.
- Geva E, Lessing JB, Bartoov B, Lerner- Geva L, Zabludovski N, Amit A. The effect of antioxidant treatment on human spermatozoa fertilization rate in an invitro fertilization programme. *Fertil Steril* 1996; 66: 430-4.
- Song GJ, Norkus EP, Lewis V. Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. *Int J androl* 2006; 29: 569-75.
- Lutsenko EA, Carcamo JM, Golde DW. Vitamin C prevent DNA Mutations Induced by Oxidative Stress. *J Biol Chem* 2002; 277(19): 16895-99.
- Thiele JJ, Friesleben HJ, Fuchs J, Ochsendorf FR. Ascorbic Acid & Urate in Human Seminal Plasma: Determination & Interrelationship with Chemiluminescence In washed semen. *Human Reproduction* 1995; 10: 110.
- WHO Laboratory manual for the examination of human semen and sperm cervical mucus interaction. 3rd ed. Cambridge University Press. Cambridge. 1992: 1-107.
- McCormick DB, Green HL. Methods for the determination of ascorbic acid. In: Text Book of Clinical Chemistry, 2nd Edn. edited by NW Teitz, WB Saunders Company. 1994; 1313-14.
- Kobayashi T, Jinno M, Sugimura K, Nozaya S, Sugiyama T, Lida E. Sperm morphological assessment based on strict criteria and in vitro fertilization outcome. *Hum Reprod* 1991; 6: 983-6.
- Fraczek M, Szkutnik D, Sanocka D, Kurpisz M. Peroxidation components of sperm lipid membranes in male infertility. *Ginek Pol* 2001; 72: 73.
- Blumenkrantz N, Calamera JC, Lavieri JC, Mancini RE. Ascorbic acid and metabolically related substances in normal and pathological human semen. *Fertil Steril* 1967; 18(6): 801-06.
- Koets P, Michelson L. Relation between ascorbic acid content and quality of human sperm. *Fertil Steril* 1956; 7: 15.
- Chinoy NJ, Buch RP. Metabolic Significance of Ascorbic acid in Human Semen. *Ind J Exp Biol* 1977; 15: 921- 22.
- Rolf C, Copper TG, Yeung CH, Nieschlag E. Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo- controlled, double blind study. *Hum Reprod* 1999; 14: 1028-33.
- Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertil Steril* 1992; 58: 1034- 39.
- Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant. *J Androl* 2005; 26: 349-53.