EFFECT OF ANTIOXIDANTS AND ANTIBIOTICS ON LEVELS OF SEMINAL OXIDATIVE STRESS IN LEUKOCYTOSPERMIC INFERTILE MEN

Sunil B.Yadav¹, Adinath N. Suryakar², Anil D. Huddedar³ and Pramod S. Shukla¹

¹ Department of Biochemistry, ³ Department of Urosurgery, Krishna Institute of Medical Sciences, Karad (MS), India

² Department of Biochemistry, Dr. V. M. Medical College, Solapur (MS), India

ABSTRACT

Defective sperm function is the most common cause of infertility. A prospective study was carried out to correlate the concentration of nitrite (the stable metabolite of nitric oxide) in seminal plasma with leukocytospermia, and sperm membrane integrity. Total Fifty-seven normozoospermic subjects with and without leukocytospermia visiting the Infertility clinic at KH and MRC, Karad, were included in the present study. Semen samples were checked for sperm concentration, total sperm count, sperm motility, seminal leukocyte concentration and sperm membrane integrity as Hypoosmotic Swelling Test. Similarly the concentration of nitrite in seminal plasma was measured by Griess reaction and total antioxidant power measured as ferric reducing ability of plasma. The concentration of nitrite in seminal plasma was found to be raised with significantly increased leukocyte concentration in semen. Also significantly lowered levels of total antioxidant power along with defective sperm function was observed. Our results suggest that supplementary treatment of antioxidants with antibiotic for leukocytospermic infertile male patients may improve the sperm membrane integrity.

KEY WORDS

Leukocytes and Total antioxidant power, Nitric oxide (as a nitrite), Sperm membrane integrity.

INTRODUCTION

Reproductive health is a state of complete physical, mental and social well-being in all aspects relating to the reproductive system and to its functions and processes (1). Defective sperm function is the most common cause of infertility because many etiological agents like Klinefelter syndrome, varicoceles, hypogonadotropic state and many diseased conditions like mumps, tuberculosis, syphilis, pancreatitis etc. are responsible for male infertility and hampers the spermatogenesis and sperm motility.

Mammalian spermatozoa membranes are rich in high unsaturated fatty acids and are sensitive to oxygen induced damage mediated by lipid peroxidation. The excessive generation of ROS (Reactive Oxygen Species) by abnormal spermatozoa and by contaminating leukocytes has been identified as one of the few etiologies for male infertility (2).

A controversy has evolved as to whether the source of ROS in semen of subfertile men originates in the spermatozoa themselves, in the germ cells or in infiltrating leukocytes (3). However leukocytospermia is associated with poor semen quality and possible subsequent infertility. Further it is also known that fertile men may have highly variable numbers of WBC (4) suggesting that a factor besides leukocytospermia may be responsible to affect seminal quality.

The nitrogen derived free radicals nitric oxide (NO[•]) and peroxynitrite anion (ONOO⁻) also play significant role in the reproduction and fertilization. Nitric oxide may play a part in tissue damage for it may be cytostatic or cytotoxic not only for invading microorganism but also for the cells that produce it and for neighbouring cells (5).

In a normal situation the seminal plasma contains antioxidant mechanisms which are likely to quench these ROS and protect against the damage to spermatozoa. However, during genitourinary infection/inflammation these antioxidant mechanisms may downplay and create a situation called oxidative stress (6).

The present study is planned to evaluate the role of reactive nitrogen species (RNS) in spermicidal effect

Author for Correspondence :

Dr. P.S. Shukla
Head
Department of Biochemistry,
Krishna Institute of Medical Sciences,
Karad. (MS.) India.

with respect to leukocytospermia and also to study the status of total antioxidant power in seminal plasma.

MATERIAL AND METHODS

Semen Samples

Eighty-three semen specimens were obtained from healthy controls and patients of age group 21-45 years visiting the Infertility Clinic, at Krishna Hospital and Medical Research Centre, Karad. They were advised to observe an abstinence of 3 days and to deliver sample on 4th day.

Semen Analysis

After liquefaction, ejaculates were analyzed for volume, pH, Sperm Count, motility, morphology, leukocyte count and sperm membrane integrity test according to the guideline prescribed by World Health Organization (7).

Leukocyte Count

Round cells (immature germ cells and WBC) were counted in a hemocytometer and referred to $\times 10^6$ round cells /ml. of semen and number of leukocytes with respect to 100 round cells was determined in stained slides at 1000 X magnification. For this, a drop of semen sample was applied to glass microscope slide, and after drying, the smear was fixed in methanol and stained by MayGrunwald Giemsa. The number of leukocytes/ml semen was determined multiplying number of round cells/ml by percentage of leukocytes (8).

As per the WHO criteria semen specimens were divided into two group of normospermic $>20 \times 10^6$ sperms/ ml. with normal morphology and motility with leukocytospermic (Leukocyte count $>1 \times 10^6$ WBC/ ml. of semen) and nonleukocytospermic (Leukocyte count $<1 \times 10^6$ WBC/ ml of semen)

After centrifugation seminal plasma were used for assays of nitric oxide by Griess reaction (9). Nitric oxide is a labile and diffusible molecule which forms stable metabolites (nitrite/nitrate; NO_2^- and NO_3^-) detected by Griess reaction. In kinetic method nitrate is reduced to nitrite by copper coated cadmium granules. This nitrite produced was determined by diazotization of sulfanilamide and coupling to naphthylenediamine. Total antioxidant power in seminal plasma was measured as ferric reducing ability of plasma (FRAP) assay of Benzie I.F.F. (10). Antioxidant power converts ferric to ferrous ion by reduction at low pH and causes a coloured ferrous tripyridyltriazine complex.

Leukocytospermic patients were followed for next month after antibiotic and antioxidant treatment.

Hypoosmotic Swelling Test

Healthy viable sperm having good fertilizing potential have the property of curling of tails when exposed to the stress of hypoosmotic pressure. The unhealthy poor quality sperm do not have curling property under the hypoosmotic pressure (11).

1.0 ml of Hypoosmotic solution (HOS) (composition: 1.351 gm. fructose and 30.735 gm sodium citrate in 100 ml. distilled water) was taken in tube and warmed at 37°C for 10 minutes .0.1 ml. of semen sample was then added to HOS , mixed well and incubated at 37°C for 30 minutes. 10 μ l (Microlitre) of incubated mixture was taken on a labelled clean glass slide covered with a cover slip and examined under microscope at 400 X magnification.

Percentage of sperm having coiled tails was calculated in two fields and the mean was calculated.

Statistical Analysis

Mean values were compared using the unpaired student's 't' test.

RESULTS

Samples were grouped according to number of WBC as nonleukocytospermic ($<1 \times 10^6$ WBC/ ml.) or leukocytospermic ($>1 \times 10^6$ WBC/ ml). Leukocytospermic samples were followed for next month after treatment of antibiotic (Ciprox TZ 500 mg.1bd. -7 days) combined with antioxidant treatment (A-Z antioxidants) (1 tab/day for 1 month).

It was observed that nitric oxide level was significantly ($P<0.001$) increased in leukocytospermic samples than in the nonleukocytospermic specimens. Also in HOS test percentage of coiled tail was reduced to 58.60 % along with significantly decreased sperm motility was observed and total antioxidant capacity was found to be significantly reduced (Table 1).

After antibiotic and antioxidant treatment leukocyte were found to be decreased and appeared to be normal while HOS test significantly improved and total antioxidant power increased significantly ($P<0.001$). Nitric oxide levels were found to be significantly reduced ($P<0.001$) after treatment as compared with pretreatment level.

DISCUSSION

Unexplained infertility is a challenging problem for the treating doctor but is a frustrating experience for infertile couple. The routine semen analysis repeatedly shows apparently normal report, but one does not know whether the functional property of sperms is normal or not. So the purpose of this study was to find out the effect of nitric oxide on the sperm function in normospermic leukocytospermia subfertile patients in

Table 1. Sperm function ability, nitric oxide level and total antioxidant power in semen with Normospermic Nonleukocytospermic and Normopospermic Leukocytospermic specimen before and after treatment

Groups	Treatment Status	N	Sperm count 10 ⁶ sperm/ml	Sperm Motility %	HOS test '% of tail curling	Nitric Oxide Mol/Lit	TAP Mol/Lit
Nonleukocytospermic (Control)		31	72.48±49.17	73.59±8.28	77.37±5.70	25.46±5.30	1240.96±62.56
Leukocytospermic	Before	26	53.68±27.65*	42.92±4.64*	58.60±5.29*	48.33±5.09*	886.69±73.82*
	After	26	56.20±24.52*	54.70±4.30*	69.91±5.24*	26.88±4.76*	1145.17±97.82*
Values are expressed as Mean±SD; 'n' : Indicates the number of cases; * P<0.001 TAP: Total Antioxidant Power; Leukocytospermic means WBC >10 ⁶ cells/ml.							

comparison to fertile control with non-leukocytospermic subjects.

Hypoosmotic swelling test evaluates the physiologic integrity of plasma membrane of sperm. Semen samples with abnormal hypoosmotic swelling test had significantly high debris suggesting of infection, which is known to affect sperm function and membrane integrity (11).

Our results show that normozoospermic leukocytospermic samples have significantly high levels of nitric oxide, suggesting that leukocyte concentration in the semen may be responsible for increase in the nitric oxide levels in the seminal plasma. These increased levels of nitric oxide may produce cytotoxic effect, which damage the sperm membrane integrity causing decrease in sperm motility. Hypoosmotic swelling test is also found to be decreased as compared with normozoospermic specimens suggesting that nitric oxide may hamper the sperm function.

Several inflammatory mediators are produced within the normal testis, where they are believed to be involved in regulating Leydig cell function and spermatogenic development (12, 13). Production of these mediators is increased by inflammatory stimuli. A number of recent in vitro studies have indicated that the inflammatory vasodilator nitric oxide (NO) is capable of inhibiting steroidogenesis by the Leydig cells, the granulosa luteal cells, and the adrenal cortex (14-15). Production of NO occurs through the action of one of three nitric oxide synthase (NOS) enzymes. Immunohistochemical studies have shown that the endothelial (type III) isoform is present in both human Leydig and Sertoli cells (16).

A balance between the benefits and risks from reactive oxygen species and antioxidants appears to be necessary for survival and normal functioning of spermatozoa (17,18). Increased production of lipid

peroxide is associated with decrease in Zinc as antioxidants .An imbalance in this harmony may be responsible for the inactivation of hyaluronidase activity thereby leading to severity of oligozoospermia (19). In the present study it was observed that decrease in total antioxidant power correlates with abnormal sperm membrane integrity by HOS and increased nitric oxide concentration. This suggests that imbalance between antioxidants and reactive nitrogen species results in sperm membrane integrity.

Nitric oxide is produced in large quantities during host defence and immunologic reactions. Because it has cytotoxic property and is generated by activated macrophages, it is likely to have a role in nonspecific immunity. The L-arginine nitric oxide pathway in murine macrophages constitutes a primary mechanism of defence against extracellular and intracellular microorganisms (20).

High levels of ROS in semen have been correlated with reduced sperm motility and damage to sperm nuclear DNA (18). Determining the levels and the sources of excessive ROS production in human semen and precise evaluation of the scavenger system may be useful tools to develop therapeutic strategies for male infertility (21).

A controversy about seminal nitric oxide and sperm parameters was raised. According to Ravella *et al.* (22) in vivo nitric oxide synthesis does not affect the sperm function. But present study finds that increased level of nitric oxide correlates with leukocytospermia and affects the sperm membrane integrity.

Male genital tract inflammation typically results in leukocytospermia. Approximately 82% leukocytes are granulocytes. Small number of lymphocytes (T and B cells) and monocytes/macrophages may also be present (23). In semen granulocytes are major producer of reactive oxygen species (24). Also

lymphocytes releases nitric oxide has been reported (25). Nitric oxide may play a part in tissue damage for it may be cytostatic or cytotoxic not only for invading microorganisms but also for the cells that produce it for neighbouring cells (5). Furthermore, although nitric oxide is cytostatic and cytotoxic in its own right, in some situation it may interact with oxygen derived radicals to generate molecules that could enhance its cytotoxicity (26).

In vitro data have indicated that nitric oxide (NO) inhibits Leydig cell testosterone production, suggesting that NO may play a role in the suppression of steroidogenesis and spermatogenic function during inflammation. Potential role for inducible nitric oxide synthase (iNOS) in regulating the normal functions of a particular organ, i.e., the testis had been reported. A role for iNOS in mediating inflammatory damage on the testis during inflammation is also suggested (27).

Thus, the concentration of nitrite in seminal plasma is significantly increased with leukocyte concentration in semen and decreased sperm membrane integrity is also observed suggesting that nitric oxide released by leukocytes may be responsible for defect in sperm membrane integrity, which may be improved after antioxidants treatment. Our study suggests that supplementary treatment of antioxidant along with antibiotic have beneficial effect in preventing damage to sperm membrane integrity and inhibiting the reactive oxygen species.

Further studies are clearly warranted to find mechanism of nitric oxide synthase in spermicidal effect and role of inhibitors of nitric oxide synthase in reducing sperm membrane damage by pro-inflammatory processes.

REFERENCES

1. Chander, P.P., Indira, H. and Kusum, Z. (2000) Need and feasibility of providing assisted technologies for infertility management in resource poor settings. *ICMR Bulletin* 30 (6-7), 55-62.
2. Wolff, H., Politech, J.A., Martinez, A., Haimovici, F., Hill, J.A. and Anderson, A.J. (1990) Leukocytospermia is associated with poor semen quality. *Fertile and Sterile* 53, 528-536.
3. Aitken, R.J. and West, K.M. (1984) Analysis of the relationship between reactive oxygen species production and leukocyte infiltration in fraction of human semen separated on percoll gradients. *Int.Jr.Androl.*13, 433-451.
4. Harrison, P.E., Barratt, Cir., Robinson, A.J., Kessopoalor, E. and Coolce, R.D. (1991) Detection of white blood cell population in the ejaculates of fertile men. *J. Reprod. Immunol.* 19, 95-98.
5. Moncada, S. (1992) The L-arginine: nitric oxide pathway. *Acta. Physiol. Scand.* 145, 201-227.
6. Sikka, S.C., Mahadevan, R.J., Wayne and J.G., Hellstrom (1995) Role of oxidative stress and antioxidant in male infertility. *Journal of Andrology* 16 (6), 464-468.
7. World Health Organization (1987) WHO Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction. 2nd Ed. Cambridge: The press syndicate of the university of Cambridge. 59, 1-59.
8. Gustavo, F.G., Graciana, K. and Alicia, B.M. (1992) Leukocytospermia and function of seminal vesicles on seminal quality. *Fertility and Sterility* 57 (5), 1058-1065.
9. Najwa, K.C. and Nabil, W.W. (1990) Determination of inorganic Nitrate in serum and urine by a kinetic cadmium reduction method. *Clin-Chem.* 36 (8), 1440-1443.
10. Benzie, I.F.F. and Strain, J.J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Analytical Biochemistry* 239, 70-76.
11. Suvarna, S.K. and Tayde, S.M. (2001) Role of sperm function test in unexplained infertility. *Journal of Obst. and Gyn. of India.* 51 (5), 146-149.
12. Jegou, B., Cudicini, C., Gomez, E. and Stephan, J.P. (1995) Interleukin-1, interleukin-6 and the germ cell-Sertoli cell cross talk. *Reprod. Fertil. Dev.* 7, 723-730.
13. Hales, D.B. (1996) Leydig cell-macrophage interactions: an overview. In: Payne AH, Hardy MP, Russell LD (eds.), *The Leydig Cell*. Vienna: Cache River Press. 451-465.
14. Adams, M.L., Meyer E.R. and Cicero, T.J. (1996) Effects of nitric oxide-related agents on opioid regulation of rat testicular steroidogenesis. *Biol. Reprod.* 54, 1128-1134.
15. Cameron, L.A. and Hinson, J.P. (1993) The role of nitric oxide derived from L-arginine in the control of steroidogenesis, and perfusion medium flow rate in the isolated perfused rat adrenal gland. *J. Endocrinol.*139, 415-423.
16. Zini, A., O'Bryan, M., Magid and M.S., Schlegel, P.N. (1996) Immunohistochemical localization of endothelial nitric oxide synthase in human testis,

- epididymis and vas deferens suggest a possible role for nitric oxide in spermatogenesis, sperm maturation, and programmed cell death. *Biol. Reprod.* 55, 935-941.
17. Alka, R., Chaudhari, A.R. and Ramji, S. (2003) Oxidative stress and antioxidant activity in normal and abnormal ejaculates. *J. MGIMS.* 8 (i), 25-27.
 18. Nicol, S. G. (2004) Pro-oxidative and antioxidant imbalance in human semen and its relation with male infertility. *Asian J. Androl.* 6, 54-65.
 19. Yadav, S.B., Sardeshmukh, A.S. and Suryakar, A.N. (2001) A study of seminal hyaluronidase, fructose, lipid peroxide and Zinc in primary male infertility. *Journal of Obst. and Gyn. of India* 51 (5), 142-145.
 20. Salvador, M. and Annie, H. (1993) The L- arginine -Nitric oxide pathway. *The New England Journal of Medicine* 329 (27), 2002-2012.
 21. Agarwal, A. and Saleh, R.A. (2002) Role of oxidants in male infertility: rational, significance and treatment. *Urol. Clin. North Am.* 29 (4), 817-827.
 22. Ravelli, A., Bergandi L., Massobrio M., Lindholm B., Bosia A. and Ghiga, D. (2001) The concentration of nitrite in seminal plasma does not correlate with sperm concentration, sperm motility, leukocytospermia or sperm culture. *Fertile sterile* 76 (3), 476-500.
 23. Aitken, R.J., Krausz, C. and Buckingham, D.W. (1994) Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation and the presence of leukocytes and precursor germ cells in human sperm suspension. *Mol. Reprod. Dev.* 39, 268-279.
 24. Sharma, R.K., Pasqualotto, F.F., Nelson, D.R., Thomas, A.J. and Agarwal, A. (2001) Relationship between seminal white blood counts and oxidative stress in men treated at an infertility clinic. *Journal of Andrology* 22, 575-583.
 25. Kirk, S.J., Regan, M.L. and Barbut, A. (1990) Cloned murine T lymphocytes synthesize a molecule with the biological characteristics of nitric oxide. *Biochem. Biophys. Res. Commun.* 173, 660-665.
 26. Beckman, J.S., Beckman, T.W., Chen J, Marshall, P.A. and Freeman, B.A. (1990) Apparent hydroxyl radical production by peroxynitrite implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA.* 87, 1620-1624.
 27. Moira, K.B., David, J.P., David, M.D. and Mark, P.H. (2000) Inducible Nitric Oxide Synthase in the Rat Testis. *Biology of Reproduction* 63, 1285-1293.