Research Article

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STUDY OF NITRIC OXIDE SYNTHESIS AND ITS OXIDATIVE EFFECTS ON SERUM PROTEINS IN LEPROSY

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ABSTRACT:

Leprosy is a disease of great antiquity & it still continuous to be a significant health problem in few countries including India. Leprosy is a chronic infectious disease caused by mycobacterium leprae. As nitric oxide is a part of immunity, its level in infectious disease is increased. Higher amount of nitric oxide has bactericidal, viricidal & tumericidal action. However, it is toxic to self cells &leads to tissue death. Considering this the study was planned to observe the status of nitric oxide synthesis & its toxic effects on proteins in leprosy. In present study 50 leprosy patients & 50 age and sex matched healthy controls were included. Out of 50 leprosy patients 34 were multibacillary (MB) leprosy patients and16 were paucibacillary (PB) leprosy patients. The levels of nitric oxide end products, Nitrothiol, Protein Carbonyl (PC) were found to be significantly increased in leprosy patients than controls. Again the level of nitric oxide end products, Nitrothiol, was found to be significantly increased in multibacillary leprosy patients than paucibacillary leprosy patients. The positive correlation among nitric oxide, Protein carbonyl, Nitrothiol was observed among patients. Thus, increase of nitric oxide end products leads to protein modifications in leprosy which may be responsible for disease progression.

KEYWORDS:

Leprosy, Multibacillary (MB), Nitrothiol, Nitric oxide end products ($NO^{\bullet}$), paucibacillary (PB),Protein carbonyl (PC).

INTRODUCTION

Leprosy is a chronic infectious disease caused by mycobacterium leprae [1]. M.leprae is an obligatory intracellular parasite with affinity for the cells of Schwann and reticuloendothelial system mainly macrophages. The defence mechanism has primordial participation of macrophages, lymphocytes and its cytokines, regulating the production, release & modulation of diverse & important cellular immunity reactions [2].The microbicidal ability of phagocytes through reactive nitrogen intermediates is a basic mechanism of human host against microbial infection.The production of Nitric oxide($NO^{\bullet}$) from the amino acid L-arginine by the cytokine inducible nitric oxide synthase is one of the key defence mechanism of mammalian phagocytes [3].Macrophages synthesizes $NO^{\bullet}$and high concentration of $NO^{\bullet}$are responsible for the cytotoxicity of macrophages to tumour cells & bacteria[4] .Cells like macrophages which are capable of producing both $NO^{\bullet}$& $O^{\bullet}$ are likely host of very powerful deleterious Reactive oxygen species (ROS) .The peroxynitrite anion ($ONOO^{-}$) formed by reaction of $NO^{\bullet}$& $O^{\bullet}$ anion is a very long lived ROS [5].The $NO^{\bullet}$& peroxynitrite produced by macrophages in skin lesions are shown to be involved in nerve damage in borderline leprosy patients[6]).In contrast to cytokines, the interaction of nitric oxide is not restricted to a single defined receptor, rather it can react with other inorganic molecules, structures in DNA, prosthetic group of proteins leading to S-nitrosylation of thiol group, nitration of tyrosine residues[7]. Oxidative changes to proteins due to $NO^{\bullet}$ can lead to diverse functional consequences such as inhibition of enzymatic activities, proteolysis, & altered immunogenicity. Protein carbonyl serves as a marker of protein oxidation [8] .The target molecule of $NO^{\bullet}$ are intracellular thiols &metal containing proteins & low molecular weight thiols like glutathione & cystein etc. Nitrothiol, the nitrosylated products of thiols have been extensively investigated as an important intermediate for $NO^{\bullet}$ mediated biological actions [9]. Thus $NO^{\bullet}$ may cause damage to proteins in leprosy patients. Considering this, the study was planned to observe the status of $NO^{\bullet}$ synthesis and toxic effects on proteins in patients with leprosy.

MATERIAL AND METHODS

Present study was carried out in department of biochemistry, Dr.V.M.Govt.Medical College, Solapur in collaboration with Shree Chhatrpati Shivaji Maharaj general hospital Solapur (Maharasthra).The protocol was approved by ethical committee of institute. The consent form was obtained from patients. A total of 100 individuals were included in this study. Out of that 50 were clinically diagnosed leprosy patients and 50 healthy controls. The persons having disorders associated with heart, lung, kidney, other pivotal organs were excluded from the study. All the patients were comprised of clinically diagnosed leprosy patients of age group 21-60 years. Diagnosis and classification of leprosy was done by skin specialist as per WHO formula. The venous blood was collected from all the subjects under aseptic condition by venipuncture using 5 ml sterile syringe. Serum was obtained from this, and this serum was used for the estimation of NO•, Protein carbonyl (PC), Nitrothiol .The serum NO• end products was determined by a kinetic cadmium granule reduction method [10], PC by Levine method [11], and Nitrothiol by cook method.[12]

All the values of biochemical parameters in leprosy patients as well as in healthy controls were expressed as mean ± SD. All parameters in study groups were statistically compared with those estimated in controls and within the groups by using Z test and Tukeys test. Correlations between the variables were estimated by pearsons correlation coefficient. The difference was considered significant, when the $p<0.05$.

RESULTS

The present study was aimed to study the biochemical parameters, Nitric oxide end products (NOx), protein carbonyl, nitrothiol as a marker of protein oxidation in leprosy. Present study shows that there is statistically significant increase (P<**0.01**) in the level of NOx (**Figure.1**), Protein carbonyl (**Figure.2**), nitrothiol (**Figure.3**), in paucibacillary as well as multibacillary leprosy patients when compared to controls. Furthermore, NOx, nitrothiol levels in MB leprosy patients are significantly higher (P<**0.01**), than in PB leprosy patients. A positive correlation was observed between NOx, Protein carbonyl and nitrothiol in MB and PB leprosy patients. The results are depicted in **Table No.1& 2**.

Figure 1: Indicates serum Nitric oxide (NOx) levels in healthy controls and patients with PB and MB Leprosy

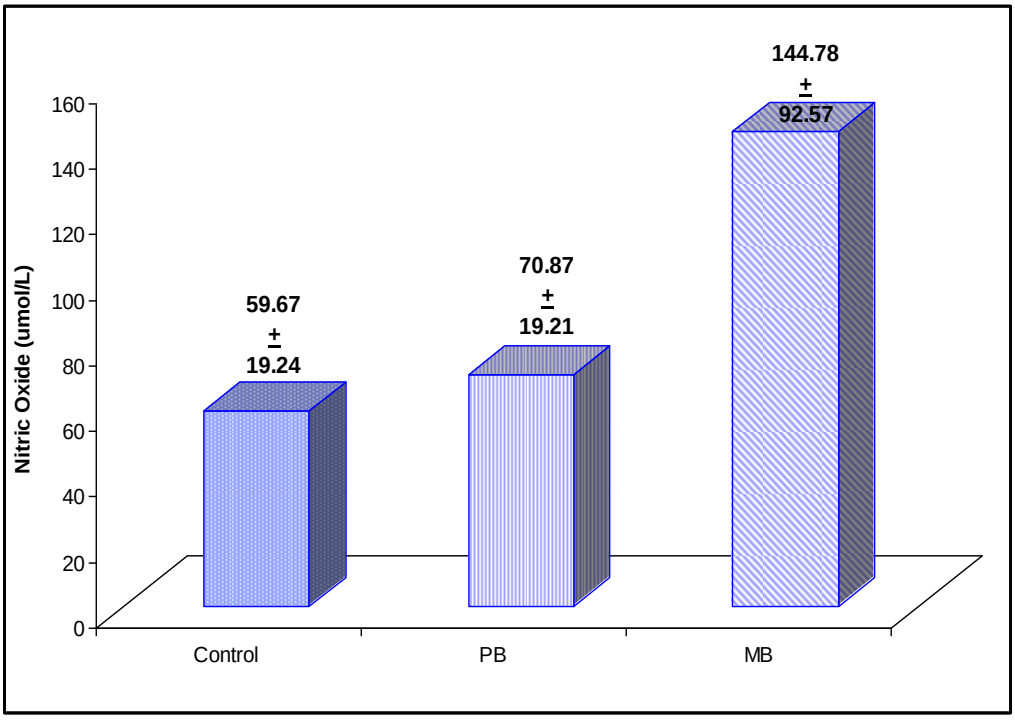

Figure 2: Shows serum Protein Carbonyl (PC) level in healthy controls and patients with PB and MB Leprosy

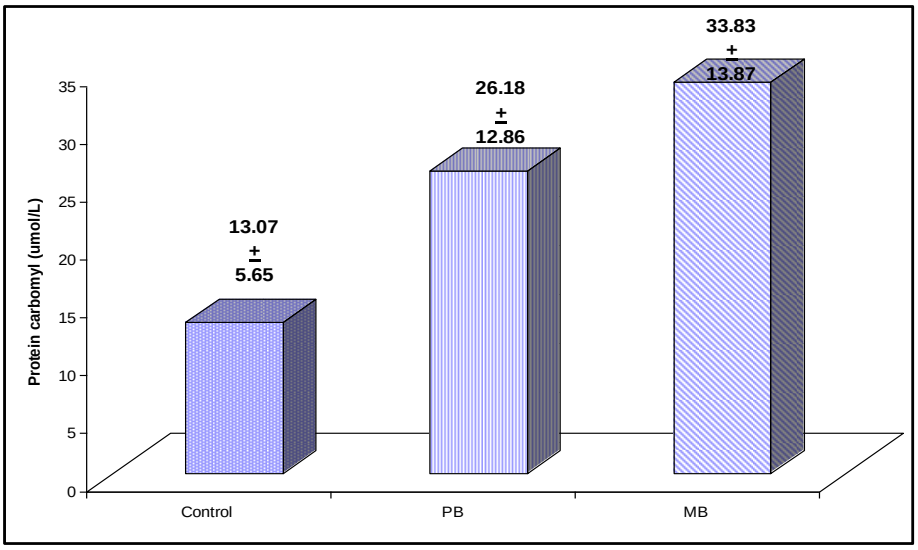

Figure 3: Indicates serum Nitrothiol level in healthy controls and patients with PB and MB Leprosy.

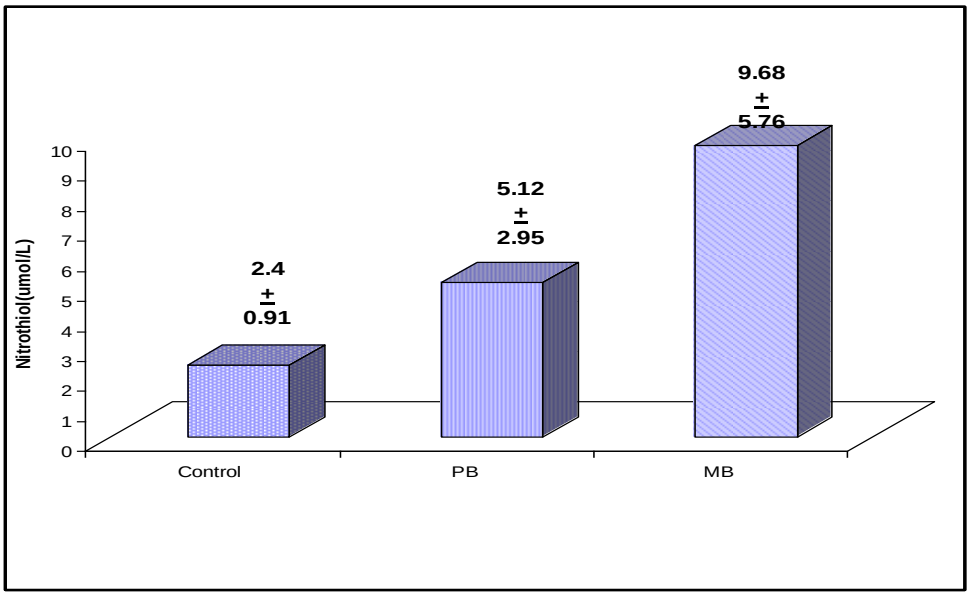

Table 1: Correlation among NOx and Protein modifications in MB Leprosy patients

	NOx	Proteincarbonyl	Nitrothiol
NOx	--	r = +0.935	r = +0.845
Protein carbonyl	r = +0.935	--	r = +0.897
Nitrothiol	r = +0.845	r = +0.897	--

Table 2 : Correlation among NOx and Protein and Lipid Modifications in PB Leprosy patients

	NOx	Proteincarbonyl	Nitrothiol
NOx	--	**r = +0.817**	**r = +0.914**
Protein carbonyl	**r = +0.817**	--	**r = +0.674**
Nitrothiol	**r = +0.914**	**r = +0.674**	--

DISCUSSION

NO• is a molecule of multiple effects on different tissues. NO• plays important role in vasodialation, bacterial challenges and cytokine stimulation, regulation of mineralized tissue function, neurotransmission and platelet aggregation. However, under pathological conditions NO• has damaging effects. i NOS is closely related to pathophysiological characteristics of inflammatory diseases.[13] NO• formation may have originated as a first line defense against invading microbial organisms including parasites, bacteria, viruses. The cytotoxic effects of NO• provide non specific immunity not only for invading organisms, but also for the killing the cells[14]. The induction of iNOS in response to excessive cytokines production is a nonspecific event which will occur in a wide variety of cell types. [14] Activated macrophages play an important role in host resistance to the development of clinical leprosy and limitation of growth of mycobacterium leprae. Recently it has been reported that NOx can also be produced during the infection of macrophages with gram positive bacteria. Macrophages activated with bacterial stimuli can secrete a variety of cytokines including TNFα, IFN-β [13]. An appropriate stimulus, such as IFN-γ, interleukin IL-l, TNF-α, endotoxin or exotoxin, lipopolysaccharides, inflammatory cytokines, iNOS is induced in the relevant cell type by gene transcription, probably via the transcription factor nuclear factor – kappa B (NF-KB). [13,15,16] Macrophages-infected with viable intracellular pathogen alone or in combination with IFN-γ or whole killed bacterial particles in presence of interferon β (IFN- β), has been shown to produce nitrite.Our results are in accordance with The Khanolkar et al[17], Kesar K. Mohanthy et. al.[18], Linda B. Adams et.al.[19], Schon T. et al. [20], Khanolkar et al. results are immune cytochemical localization demonstrating its presence in tuberculoid leprosy lesions, are consistent with a role for iNOS in killing of M. leprae in the form of the disease. Expression of iNOS in reactional lesions is correlated with an activation of bacterial killing mechanisms during reversal reactions. It will be interestingly determine if iNOS expression is activated in cells initially present within the lesions or whether is due to influx of iNOS-positive cells during the reactional episode and may destroy the host tissues and invading microorganisms [21]. Thus NOx concentration in serum can be used as an inflammatory marker for disease status and progression [22]. Kesar K. Mohanthy et. al suggested that, NO•/NOM i.e. NO• metabolites excretion is increased in leprosy patients during ENL episodes, with antireactional therapy and clinical improvements the levels are reduced [18].Linda B. Adams et.al. demonstrated that, macrophages from iNOS knockout mice are deficient in the iNOS gene, but not deficient in the production of ROI, but in the absence of reactive nitrogen intermediates (RNI), ROI alone is not sufficient to kill M. leprae[19].Schon T. et al. concluded that, iNOS and nitrotyrosine are expressed in granulomas from borderline leprosy patients with and without reversal reaction (RR) and propose that RNIs might be involved in nerve damage following RR in leprosy[20]. Present study agrees with above authors.

Protein oxidation is the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary byproducts of oxidative stress. Amino acids, the building blocks of peptide and proteins are targets of free radical attack. Oxidation of amino acids in proteins leads invariably to physical changes in proteins themselves [21].The accumulation of oxidized proteins might have impair cell function. It has been suggested that, oxidative damage to proteins might be particular importance in vivo by affecting the function of receptors enzyme, transport proteins and perhaps generating new antigens that provoke immune response [22]. Oxidative damage to several amino acids including histidine, proline, arginine and lysine or to the peptide backbone of proteins can generate Protein carbonyl products [22]. Oxidation of amino acids in proteins leads invariable physical changes in proteins themselves. These physical changes are categorized into three groups, fragmentation, aggregation, susceptibility to proteolytic digestion. Fragmentation of albumin, collagen and α- globulins occurring during the oxidative damage has been well documented. Furthermore, free radical damage of collagen and albumin is selective for proline residues because of the greater susceptibility of proline to OH• radical attack. Selective attack by OH• on histidine or arginine residues also occurs. Since, these amino acids are often in close association with transition metals, which serve as catalysts in the generation of OH• [23]. The aggregation of proteins could be related to the ability of OH• to form cross linkages. These aggregates consist of native cross-linked proteins rather than a nonspecific aggregation of fragmented proteins. Because oxidation causes gross protein conformational alterations, such

damaged proteins themselves become more susceptible to proteolytic degradation. Thus protein denaturation increases protein digestion by various proteolytic enzymes. [23]

In many pathological conditions, the elevation in protein carbonyl groups correlates well with the progression and severity of the disease [24]. Oxidative damage to several amino acids or to the peptide backbone of proteins can generate PC products. Fragmentation of polypeptide chains, formation of protein-protein cross-linkages as well as modification of amino acid side chains to hydroxyl or carbonyl derivatives are possible outcomes of oxidation reactions [22]. However, reaction of some unsaturated hydroxyaldehyde with proteins has been suggested to form products that are detectable in the carbonyl assay [25]. Finally in addition to oxidatively susceptible amino acids, protein oxidation may also be influenced by the conformational and tertiary structure of the protein molecule [26]. The potential mechanisms by which $NO^{\bullet}$ and other RNIs affect , modification of bacterial proteins and lipids at microbial surfaces, deamination of bacterial DNA and direct interaction with accessory protein targets, resulting in enzymatic inactivation or other protein malfunctions to initiate intracellular mycobacterial killing[27]. Oxidative changes to proteins due to $NO^{\bullet}$ can lead to diverse functional consequences such as inhibition of enzymatic activities, proteolysis and altered immunogenicity[28].Reactive Nitrogen Intermediates (RNIs) has damaging effects on proteins converting them into protein carbonyls. Oxidatively modified proteins are not repaired and must be removed by proteolytic degradation. A decrease in the efficiency of proteolysis will cause an increase in the cellular content of oxidatively modified proteins, hence shown to increase in the disease process.

On statistical evaluation it has been observed that positive correlation between NO_x and protein carbonyl in both MB and PB leprosy patients. This clearly indicates that elevated levels of $NO^{\bullet}$ increases protein oxidation, hence there is tight relationship between $NO^{\bullet}$ and protein carbonyl.

According to Vijayraghvan R .et.al.PC increases due to ROS in leprosy, the carbonyl content of cell proteins is a useful indicator of oxidative protein damage during drug and chemically induced toxicities. Since range of lipophilic carbonylating agents produced during the membrane peroxidation that accompanies chemically induced oxidative stress, integral membrane proteins seem especially vulnerable to adduction by these species [29].Ponczek M.B. et.al reported that reaction of a variety of free radicals ROS and RNS with proteins can lead to oxidative modifications of proteins[30]. Thus present study agrees with Vijayraghvan R.et.al.

Potential molecular targets of $NO^{\bullet}$ include transition metals, thiols, lipids and DNA. Under physiological condtions, $NO^{\bullet}$ may react with thiol containing molecules such as glutathione (GSH) to form S-nitrosothiols. S-nitrosothiols have been detected in human bronchoalveolar lavage fluid, plasma, platelates and polymorphonuclear neutrophils with higher concentrations measured in inflammatory states. S-nitrosothiols have been recognized to possess antimicrobial activity. These compounds have also been proposed to mediate $NO^{\bullet}$ transfer to outer membrane thiols in bacillus, which inhibits spoure outgrowth. Differences in stability and target response relative to $NO^{\bullet}$ lead credence to the suggestion that S-nitrosothiols are important physiologic redox forms of $NO^{\bullet}$. N-nitrosamine and S-nitrosothiols are formed by the addition of nitrosonium equivalent (NO^{+}) to amine and thiol respectively. Activation of rodent macrophages with both IFN-γ and LPS, results in iNOS expression and nitrosation of target molecules present in the culture medium [31]. As far as our knowledge no study would be done on nitrothiol in leprosy. The $NO^{\bullet}$ attacks on thiol groups to form nitrothiols, Nitrothiol acts as $NO^{\bullet}$ carrier. Thus in leprosy increase in $NO^{\bullet}$ may increase nitrothiol formation. Thus, increased NOx leads to lipid and protein modifications in leprosy which may be responsible for disease progression.

A positive correlation was observed between NO_x ,PC and nitrothiol in MB and PB leprosy patients.This is clearly indicated that elevated levels of nitric oxide increase nitrothiols, protein carbonyl formation.The increase in $NO^{\bullet}$ level affects on protein molecules, leading to their oxidation. Thus structure and activity of protein get disturbed in leprosy patients. Thus, increased NOx leads to protein modifications in leprosy which may be responsible for disease progression.

REFERENCES

[1]. Wikipedia (Free encyclopedia) Leprosy

[2]. Lima E. S. , Roland I. A., Maroja M. F., Marcon J. L.2007 Vitamin A and lipidperoxidation in patients with different forms of leprosy. *Rev Inst Med trops Paulo (49 (4)* : 211-214.

[3]. Qadoumi M, Becker I, Donhuser N, Rollinghoff M, Bogdan C.,2002. Expression of iNOS in skin lesions of patients with American cutaneous Leishmaniasis. *Infection and immunity. 70(8)*: 4638 – 4642

[4]. Davies M., Fulton F, Hagen P., 1995. Clinical biology of nitric oxide. *British J surgery, 82*: 1598-1610

[5]. Sen H.K., 1995.Oxygen toxicity and antioxidants: state of the art, *Indian I physical pharmacol : 39 (3):* 177-196.

[6]. Schon T , Hernandez R.,Negesse Y,Leekassa R, Sund Vist T, Brittion S.,2001 Expression of inducible nitric oxide synthase and nitrotyrosine in borderline leprosy lesions. *British J Dermatol . 145(5)* : 809-817

[7]. Bogdan C. 2000.Nitric Oxide and immune response. *Nature*.,907-915

[8]. Lancy A. Z.,. Lisowska M. S,. Szygula Z, Witkowski K.2008 Association of proantioxidant status with immunological response in healthy men after oral N – acetyl. L-Cysteine administration.*Med sport* 12(4) : 129-135

[9]. Pacher P., Beckman J, Liaudet L., 2007. Nitric Oxide and peroxynitrite in health and disease. *Physiol Rev. 87* : 315-424.

[10]. Cortas N, Wakid N., 1990.Determination of inorganic nitrite in serum and urine by a kinetic cadmium reduction method. *Clin Chem 36(8):*1440–1443.

[11]. Levine L,.Garland D, OliverAmici N .Climent I,LenzG ,Ahn B, Stad man B.,1990.Determination of carbonyl content in oxidatively modified proteins. *Methods enzmol:186;*464-466

[12]. Cook J.A., . Kim S.Y, Teague D, Krishna M.C, Pacelli P, Mitchell J.B.,1996.Convenient colorimetric and fluorometric assays for s-nitrosothiols. *Anal Biochem . 238(2)*: 150-158

[13]. Hackam D. J., Rotstein O.D., Zhang W. J., Gruenheid S.,.Gross P,Grinstein S., 1998.Host resistance to intracellular infection: Mutation of natural resistance associated macrophage protein I impairs phagosomal acidification. *J Exp Med 188(2)* : 351-364.

[14]. Cankal D. U. , Ozmeric N.,2006. A multifaceted molecule, Nitric oxide in oral and periodontal diseases. *Clin Chim Acta. 366 :* 90-100

[15]. Clancy R, Amin R, Abramson S.,1998 The role of nitric oxide in inflammation and immunity. *Arthritis and Rheumatism. 41(7)* : 1441-1451.

[16]. Jiang J, Malaria M, Suresh V, George S.,2009.Nitric oxide gas phase release in human small airway epithelial cells. *Respire res 10(3)* : 1-8

[17]. Davies M, Fulton F, P Hagen P. Clinical biology of nitric oxide.1995. *British J surgery .82*: 1598-1610

[18]. Mohanthy K, Gupta M., Girdhar B. Girdhar A.Chakma J, Sengupta U. 2007.Increased level of urinary nitric oxide metabolites in leprosy patients during type -2 reactions and decreased after antireactional therapy. *Lepr. Rev ; 78*: 386 –3 90.

[19]. Adams L.B, Job C. K., Krahenbuhl J. K., 2000. Role of inducible nitric oxide synthase in resistance. *Infection and immunity .68(9):* 5462-5465

[20]. Schon T, Hernandez R, Negesse Y,Leekassa R,Sund Vist T, Brittion S.,2001. Expression of inducible nitric oxide synthase and nitrotyrosine in borderline leprosy lesions. *British J Dermatol. 145(5)* : 809-815

[21]. Cals Grierson M.M., Ormerod A.,2004. Nitric oxides function in skin. *Nitric oxide 10 (4) :* 179-193

[22]. Uzun H, Konukaglu D, Gelisgen G, Zengin K, Taskin M.,2007, Plasma protein carbonyl and thiol stress before and after laproscopic banding in morbidly obese patients. *Obesity surgery. 17*: 1367-1373.

[23]. Yu B.P,Cellular Defense against damage from reactive oxygen species.,

[24]. Rudolph V, Freman B.,2009 Cardiovascular consequence when Nitric Oxide and lipid signaling converge. *Circ Res*: 105-11

[25]. A. Z. Renzick A.Z., Cross C.E., Hu M.L., Suzuki Y.J., et.al 1992, Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J. 286:* 607-611.

[26]. Stadtman E. R..1992. Protein oxidation and aging. *Science 257 (5074)*: 1220-1224.

[27]. Kulkarni A, Madrasi N.A...2008. Relationship of nitric oxide and protein carbonyl in tuberculosis.*Indian J. Tuberc. 55:* 138-144.

[28]. Niki E., 2009. Lipid peroxidation: physiological levels and duel biological effects. *Free Radic Biol*

[29]. R. Vijayaraghavan R, Suribabu C, Sekar B, Oommen P.,et.al 2005. Protective role of vitamin E. on oxidative stress in Hansen's disease patients. *European J. Clin. Nutrition. 59(10)* : 1121-1128

[30]. Ponczek M, Wachowicz B., 2005. Interaction of reactive oxygen and nitrogen species with proteins post *Biochem 31(2):* 140-145

[31]. Espey M. Miranda K, Pluta,Wink D.,2000. Nitrosative capacity of macrophages is dependent on nitric oxide synthase induction signals. *J Biol chem.275 (15)* : 11341-11347

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