Original Article

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Free Radicals and Antioxidant Status in Chronic Osteomyelitis Patients: A Case Control Study

Biochemistry Section

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ABSTRACT

Introduction: Osteomyelitis (OM) is a local or generalized infection of the bone and bone marrow which may be multifactorial in its causation. Chronic infection is characterised by sequestrum and involucrum formation.

Aim: The present study has been carried out for assessing the oxidative stress in chronic OM by measurement of serum oxidants {such as malondialdehyde (MDA), protein carbonyl (PC), nitrite} and the serum antioxidants {such as ascorbic acid, superoxide dismutase (SOD), ceruloplasmin (Cp), blood glutathione} by spectrophotometric method.

Materials and Methods: This was a case control study. About 5 ml of venous blood was collected for the estimation of biochemical parameters. This study comprised of 50 OM patients diagnosed at SSLH Hospital, Varanasi and 50 healthy ages (15-35 y) and sex matched individuals.

Results: Significantly increased ($p<0.0001$) levels of serum oxidants and significantly decreased ($p<0.0001$) levels of all serum antioxidants except ceruloplasmin indicated significantly increased ($p<0.0001$) levels in response to infections in chronic OM patients as compared to the healthy controls.

Conclusion: These results suggest that there occurs an imbalance between oxidants and antioxidants, especially an increase in oxidative stress, as measured by the levels of the parameters: serum MDA, serum protein carbonyl and serum nitrite.

Keywords: Ceruloplasmin, Malondialdehyde, Nitric oxide synthase, Nitric oxide, Protein carbonyl, Superoxide dismutase

INTRODUCTION

Osteomyelitis (OM) is an infection of the bone or bone marrow [1]. It is sub classified on the basis of the causative organism (pyogenic, mycobacterial, fungal, etc), route (hematogenously either from the lungs or bones), duration (chronic OM is OM that has been present for more than one month) and anatomic location of the infection (tibia, femur etc). OM by blood stream is most frequently seen in children and nearly 90% of the cases are caused by *Staphylococcus aureus* [2].

OM is seen more in males than females. In spite of treatment, up to 30% of bone infections become chronic [3]. Pus spreads into the bone's blood vessels, hindering the blood flow, and areas of devitalised infected bone, known as sequestra, form the basis of a chronic infection. A new bone is formed around the areas of necrosis called an involucrum [1]. Chronic infection results in various sequelae like chronic sinuses with exposed bone, loss of structural integrity and growth disturbances [4]. OM may be due to intracellular bacteria (inside bone cells). Bacteria may escape and invade other bone cells. Intracellular bacteria become resistant to some antibiotics leading to difficulty in eradication of this disease and the infection becomes chronic. The tibia, femur, humerus, vertebra, the maxilla and mandibular bodies are especially susceptible to OM because of the particularity of their blood supply [5]. Weiland et al., has defined chronicity, as a wound with exposed bone, positive bone cultures, and drainage for six months [6].

Neutrophils are the first line of host immune defense against many bacterial infections and they play a crucial role in the pathogenesis of OM. Circulating neutrophils are increased in OM and they accumulate at the focus of bone infection, along with bacteria. Serum and bone fragments from these patients have increased levels of inflammatory cytokines [7]. Pro-inflammatory cytokines such as interleukin (IL-1) and tumour necrosis factor (TNF-α) cause activation of the inducible isoform of nitric oxide synthase (iNOS or iNOS-2), and nitric oxide (NO) derived from this pathway stimulates the bone loss [8,9]. Also in the presence of bacteria, increased production of NO occurs due to more endothelial nitric oxide synthase (eNOS or eNOS-3) activity [10]. NO leads to increased reactive oxygen species (ROS) production which damages lipids, proteins, etc leading to oxidative stress. Inducible and endothelial NO causes bone loss by increasing bone resorption, and may account for the osteolysis that is characteristic of OM.

Ceruloplasmin (Cp) is one group of serum protein which rises after any form of tissue injury [11]. Its plasma concentration is increased in a variety of conditions like tissue damage, infection [12]. Cp acts as a ferroxidase by catalyzing the oxidation of (Fe^{2+} to Fe^{3+}) [13]. Cp by ferroxidase effect keeps iron in the oxidized ferric state thus preventing its conversion from ferric (Fe^{+3}) to ferrous (Fe^{+2}) necessary for bacteria to initiate their toxic effects. Through this action, it inhibits bacterial cell growth.

MATERIALS AND METHODS

Fifty patients with chronic OM, in the age range of 15–35 years, who presented to outpatient department of SSLH in Varanasi, attached to Government Medical College, I.M.S, BHU Varanasi Uttar Pradesh, India, were included in the study. It was conducted for two years (2012-2013). Patients with other associated co-morbidities like diabetes mellitus, hyperlipidemia, and hypertension, etc. were excluded from the study. Following exclusion criteria were kept in consideration: postoperative infections, malunion, tubercular abscess disease or metabolic disorder, non healing ulcer, tumour with secondary infection, with a previous medical history of any chronic illness, patients taking any antioxidant drugs.

Before the start of the study, the approval of the institutional ethical committee was obtained. A group of 50 normal healthy individuals, age and sex matched, from the same population served as the controls. Malondialdehyde (MDA) is one of the products of lipid peroxidation. It can be estimated by the thiobarbituric acid (TBA) test [14,15].

Protein carbonyl (PC) content is calculated from the peak absorbance at 370 nm of a stable dinitrophenylhydrazone product, a method described by Reznick and Packer [16].

The nitrite concentration of serum sample was estimated from the standard curve. Its short half life makes its direct measurement impractical, though in aqueous solutions NO decays to yield equal amounts of nitrite and nitrate [17], which are used as indices of NO synthesis in vitro. This assay relies on a diazotization reaction that was originally described by Griess [18]. The absorbance of the magenta coloured azo compound formed was measured at 540 nm [19]. Standard curve was used for estimation of nitrite concentration of unknown serum sample. The ascorbic acid concentration of serum sample was estimated from standard curve. Ascorbic acid can be estimated by dinitrophenyl hydrazine (DNPH) method at 520 nm Roe [20]. Standard curve was used for estimation of ascorbic acid concentration of unknown serum sample. Super Oxide Dismutase (SOD) was assayed by the method of Marklund and Marklund [21] modified by Nandi Chatterjee [22]. This method is based on the ability of SOD to inhibit autoxidation of pyrogallol under specific conditions. Enzyme activity of SOD is expressed as the amount of enzyme required to cause 50% inhibition of pyrogallol auto oxidation per 3 ml of assay mixture.

The glutathione determination was performed by the method described by Beutler et al., [23] using 5, 5'-dithio bis- (2 nitro) benzoic acid. The reduced glutathione concentration was estimated from standard curve. Cp has a ferroxidase activity. In this reaction dye p-phenylene diamine used for its estimation. It catalyzes the oxidation of a dye p-phyenelene diamine to a violet coloured oxidation product, the absorbance of which is measured at 546nm by the method of Ravin [24]. Clinical examination and biochemical analysis was carried out in all the subject. During selection of case and control, exclusion and inclusion criteria were taken. Diagnosis of osteomyelitis was done by orthopaedic surgeon after doing clinical evaluation and x-ray of particular bone. Informed consent was taken from patient and was ethically approved by Institutional ethics committee (IEC).

STATISTICAL ANALYSIS

All the results were expressed as mean± SD. The statistical analysis was done by using the independent t-test. The p-values which were <0.0001 was considered as highly significant.

RESULTS

[Table/Fig-1] shows that mean serum MDA in chronic OM patients and the controls were 1.40 ± 0.054 (µ mol/l) and 1.12 ± 0.19 (µ mol/l); PC in patients 5.27 ± 0.38 (nano mol/mg protein) and controls 4.18 ± 0.73 (nano mol/mg protein); Nitrite in patients 35.12 ± 19.3 (µ mol/l) and controls 13.6 ± 2.62 (µ mol/l). [Table/Fig-2] shows that mean serum ascorbic acid in patients 0. 746 ± 0.33(mg/dl) and controls 1.62 ± 0.212 (mg/dl); SOD in patients 0.537 ± 0.28 (unit/0.1 ml) and in the controls 1.08 ± 0.087 (unit/0.1 ml); reduced glutathione (GSH) in patients 31.68 ± 12.5(mg/ml) and controls 56.8 ± 23.3 (mg/ml); Cp in patients 49.99 ± 13.15 (mg/dl) and controls 26.06 ± 6.03 (mg/dl). The level of the antioxidant Ascorbic acid, GSH and enzyme SOD was significantly decreased while Cp increased in the chronic OM patients as compared to that in the controls. The serum lipid peroxide concentration in the form of MDA, PC, Nitrite was significantly higher in the OM patients (p< 0.0001) as compared to that in the controls [Table/Fig-1]; while the serum concentration of antioxidants ascorbic acid, SOD and GSH was significantly decreased in patients (p< 0.0001) as compared to that in the controls [Table/Fig-2] and antioxidant Cp was significantly higher in patients (p< 0.0001) as compared to that in controls [Table/Fig-2].

GROUPS	MDA (µ mol/l)	PC (nano mol/ mg protein)	nitrite(µmol/l)
CONTROLS	(1.12 ± 0.19)	4.18 ± 0.73	13.6 ± 2.62
PATIENTS	(1.40 ± 0.054)	5.27 ± 0.38	35.12 ± 19.3
Significance (p)	< 0.0001	< 0.0001	< 0.0001

[Table/Fig-1]: Serum MDA, PC, nitrite in controls and patients of chronic OM (mean ± S.D, n =50 controls, n= 50 patients).
MDA, malondialdehyde; PC, protein carbonyls; OM, chronic osteomyelitis

GROUPS	Ascorbic acid (mg/dl)	SOD (unit/0.1 ml)	GSH (mg/ml)	Cp (mg/dl)
CONTROLS	1.62 ± 0.212	1.08 ± 0.087	56.8 ± 23.3	(26.06 ± 6.03)
PATIENTS	0.746 ± 0.33	0.537 ± 0.28	31.68 ± 12.5	(49.99± 13.15)
Significance (p)	<0.0001	<0.0001	<0.0001	<0.0001

[Table/Fig-2]: Serum Cp, ascorbic acid, SOD, GSH levels in controls and patients of chronic OM (mean ± S.D, n=50 controls, n=50 patients).
Cp,ceruloplasmin; ascorbic acid; SOD,superoxide dismutase; GSH, reduced glutathione; OM, chronic osteomyelitis

DISCUSSION

Ascorbic acid is a radical scavenging antioxidant present in all cells and can also act as a reducing agent [25]. Thus protects the cell against the toxic oxygen free radicals. The lowered values of Vit C indicate the severity of infection. Ascorbic acid level decreases by scavenging the free radicals and preventing lipid peroxidation. The findings in the present study were in accordance with the observation of various authors like D' Silva et al., [26] who had detected serum ascorbic acid levels in osteomyelitis patients.

Decline in antioxidant SOD might be due to the severity of infection or may occur with the progression of lipid peroxidation. The findings in the present study were in accordance with the observation of various authors like Shetty et al., [27] who had detected SOD levels in osteomyelitis patients. Matskevich et al., [28] had detected SOD levels in 12 patients of chest osteomyelitis and found lowering of SOD activity by 37-40%. Chakraborty et al., [29] carried out a study regarding antioxidant enzyme status in lymphocytes and detected that SOD was significantly ($p < 0.05$) diminished in lymphocytes due to VSSA and VRSA infection as compared to control mice group and was significantly ($p < 0.05$) increased due to nanoconjugated vancomycin treatment.

We observed a significant decrease in the levels of reduced glutathione (GSH) in erythrocyte of patients with chronic OM compared to controls. The decrease in the levels of these non enzymatic antioxidant parameters may be due to the increased turnover, for preventing oxidative damage in these patients suggesting an increased defense against oxidant damage. Previously work has been done on glutathione reductase and glutathione peroxidise on 12 chest OM patients by Matskevich et al., [28] but no study has been done on reduced glutathione in human OM. Chakraborty et al., [29] concluded that glutathione is an important antioxidant in cellular system. To determine the level of glutathione, they have to measure both forms of glutathione, glutathione-reduced and oxidised in Swiss male mice. In blood lymphocytes Glutathione (GSH) level and redox ratio (GSH/GSSH)were significantly (p<0.05) reduced due to VSSA and VRSA infection as compared to control mice group which was treated with non conjugated vancomycin.

As an antioxidant, Cp clears free oxygen radicals in a manner similar SOD. Cp keeps iron in the oxidized ferric state thus preventing it from undergoing the redox cycle {ferric (Fe^{+3}) to ferrous (Fe^{+2})} necessary for bacteria to initiate their toxic effects. This is referred to as the ferroxidase effect of Cp. Bacteria need iron in the ferrous state to be pathogenic. Through this action, Cp acts to inhibit bacterial cell growth. The findings in the present study were in accordance with the observation of various authors like Natesha et al., [30] who had detected serum Cp levels in OM patients. The determination of MDA is one of the most commonly used methods for monitoring lipid peroxidation. Serum MDA levels were significantly elevated (p<0.0001) in patients of chronic OM as compared to controls. The increase in the levels of MDA in our study indicates that lipid peroxidation is taking place in patients of OM. The end product of lipid peroxidation, MDA increases due to its high severity of infection in OM. The findings in the present study were in accordance with

the observation of various authors like D Silva et al., [26] who have found that there is a significant increase in serum lipid peroxidation in OM. Koruk et al., [31] carried out study on the activity of paraoxonase and arylesterase in OM patients and found increased concentrations of lipid peroxide observed in OM patients appear to be related to the increased oxidative stress and inflammatory conditions present in these patients, and may cause a much more severe status of the disease. Chakraborty et al., [29] found that lipid peroxidation is an important determinant to access the cellular damage. Lipid peroxidation in terms with malondialdehyde (MDA) level was significantly ($p < 0.05$) increased in lymphocytes due to VSSA and VRSA infection as compared to control mice group, which was significantly ($p < 0.05$) decreased due to treatment of nanoconjugated Vancomycin. PC is most commonly measured products of protein oxidation in biological samples. Among the various oxidative modifications of amino acids in proteins, carbonyl formation may be an early marker of protein oxidation. PC increases due to its high severity of infection in osteomyelitis. Previously work has been done by Chakraborty et al., [29] found that like lipid peroxidation, protein oxidation is also an important determinant to assess the cellular damage. Protein oxidation in terms of PC level was significantly ($p < 0.05$) increased in lymphocytes due to VSSA and VRSA infections in Swiss male mice as compared to control group mice, which was significantly ($p < 0.05$) decreased due to treatment of nanoconjugated vancomycin. But no study has been done regarding PC in human OM.

Serum nitrite levels were also significantly elevated ($p<0.0001$) in patients of chronic osteomyelitis as compared to controls. Nitric oxide (NO) produced by the inducible isoform of NO synthase (NOS) is an important mediator of inflammation. Peroxynitrite, a cytotoxic oxidant formed from the reaction of nitric oxide (NO) and superoxide is a mediator of cellular injury in ischaemia/reperfusion injury, shock and inflammation. Peroxynitrite initiates lipid peroxidation and this mechanism also contributes to superoxide radical and NO mediated cytotoxicity as reported by Radi et al., [32]. Chakraborty et al., [29] in Swiss male mice found that NO level was significantly ($p < 0.05$) increased in lymphocytes due to VSSA and VRSA infections as compared to control group mice, which was significantly ($p < 0.05$) decreased due to treatment of nanoconjugated vancomycin. Asensi et al., [10] carried the study on OM patients and found that OM patients homozygous for the NOS3 (27-bp repeat, intron 4 polymorphism) 4 allele are more susceptible to OM and showed significantly higher serum NO levels as compared to controls.

CONCLUSION

The study showed that there is a definite evidence of oxidative stress in patients of chronic osteomyelitis as evidenced by an increase in levels of all parameters of oxidative stress measured: serum MDA, serum protein carbonyl and serum nitrite. This oxidative stress has further led to a compensatory increase in ceruloplasmin level in patients, underscoring the role of ceruloplasmin as an antioxidant in chronic osteomyelitis. The results suggest the necessity for therapeutic co-administration of antioxidants with conventional drugs to such patients. Therefore, treatment with antioxidants in the initial stages of the disease may be useful as secondary therapy to prevent the oxidative damage and deterioration of the musculoskeletal tissues in osteomyelitis.

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