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Research Article

ISCHEMIA MODIFIED ALBUMIN AND NITRIC OXIDE: NOVEL BIOMARKERS OF OXIDATIVE STRESSAND INFLAMMATION IN BONE TUMORS

Monica Verma[1], Kiran Dahiya[2], Vasudha Dhupper[3], Umesh Yadav[4], Sushil Kumar[5], Sanghpriya Pal[6] and Abhishek Soni[7]

[1,2,3,5,6]Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana- India
[4]Department of Orthopedics, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana- India
[7]Department of Radiotherapy, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana- India

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A B S T R A C T

Background: Primary bone tumors are extremely rare neoplasm, accounting for less than 0.2% of all tumors but mortality related to them is disproportionately high, especially among teenagers and young adults. Oxidative stress plays a major role in the pathogenesis of bone tumors. Nitric oxide (NO) is a well proven marker of oxidative stress and in recent years, Ischemia Modified Albumin (IMA) has also emerged as a new biomarker in some cancers. Hence, this study was planned to determine the role of oxidative stress in primary bone tumor patients by measuring NO and IMA levels together, before and after treatment.

Methods: A total of 98 subjects were included in the study. Out of these, 50 were healthy controls and 48 were histopathologically proven patients of bone tumors. Out of 48, 14 were benign and 34 were malignant tumors. The NO level [measured as nitrite-plus-nitrate {NO(x)} concentration] and IMA were estimated before (group I) and 2 weeks after standard treatment. Data was compared among different groups using appropriate statistical analysis.

Results: The levels of NO and IMA were found to be statistically significantly higher in cases than controls ($p<0.05$). The levels were significantly more in patients with malignant tumor as compared to benign tumor ($p<0.01$). A strong positive correlation ($r=0.84$; $p<0.01$) was found between IMA and NO in cases.

Conclusions: Increased levels of IMA and NO strongly favor the increased oxidative stress in primary bone tumor patients and may act as potential biomarkers for diagnosis and prognosis.

INTRODUCTION

Primary bone tumours are heterogenous group of malignancies which are quiet uncommon. Of primary tumors, osteosarcoma is the commonest tumor accounting for 35% of total [1, 2] chondrosarcoma (30%) and Ewing's sarcoma (16%) are the next 2 most common forms of bone cancer [2]. Bone tumors are further divided into benign and malignant category. Benign tumors of the bone are not life threatening and would not metastasize to other regions of the body [3]. The global incidence of primary malignant bone tumors shows a specific age distribution pattern with two incidence peaks at 10-20 year and a steady increase from 40 year to the age of 80 year. There is a significant difference between males and females, with males being affected almost 1.5 times more frequently than females[4]. To attain maximum response rate, early diagnosis and treatment of bone tumors is essential. It involves a multidisciplinary diagnostic and therapeutic approachinvolving surgeons, radiologists, pathologists, medical and radiation oncologists. So, as soon as the tumour is suspected, early referral to a specialist centre is critical [1]. For a low-grade tumor, the primary treatment is surgery. The goal of surgery is to remove the tumor and a margin of healthy bone or tissue around the tumor to make sure all of the cancer cells are gone. For high grade tumors, a combination of treatments is given [5].

Oxidative stress plays a major role in the pathogenesis of bone tumors. Various studies have demonstrated the correlation between two. The immediate microenvironment of bone tumor is an important determinant of growth of bone tumor. Nitric oxide is a short lived gas produced endogenously. It is highly reactive substance with a free electron and possesses the

Corresponding author:* **Monica Verma
Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana- India

capability to generate reactive oxygen species (ROS) which may produce oxidative damage to molecules, lipids, protein, and deoxyribonucleic acid (DNA). Thus, it acts as a marker of oxidative stress [6] ROS produced over a long time under sustained environmental stress may produce significant damage to structure and function of cell which may induce somatic mutations and neoplastic transformations [7].

Ischemia modified albumin (IMA) has emerged come out as a new biomarker for oxidative stress and inflammation in recent years. Its role as a sensitive biomarker is well proven in myocardial ischemia. The generation of ROS and free radicals brings about a change in the cobalt binding capacity of the N-terminal of albumin which is, then, known as IMA [6]. Chronic inflammation is pinpointed as a causative factor for development of cancer. In 1863, Virchow hypothesized that the origin of cancer at sites of chronic inflammation [8] So, IMA could have some role in causation of cancer. Recent studies have suggested the role of IMA in various cancers too like gastric, prostate, neuroblastoma and soft tissue cancer [9-11].But no published study was found in literature suggesting the role of IMA in bone tumors. Hence, this study was planned to determine the role of oxidative stress in primary bone tumor patients by measuring NO and IMA levels before and after treatment.

MATERIALS AND METHODS

The study was conducted in the Department of Biochemistry, Pt B D Sharma, University of Health Sciences Rohtak in collaboration with Department of Orthopedics and Department of Radiotherapy, Rohtak. Forty eight histopathologically proven patients of bone tumors who had presented at Orthopaedics OPD were included in the study. Informed consent was taken from all the participants and the study was approved by Institutional Ethical Committee. Out of fifty, 34 patients were with benign and 14 with malignant bone neoplasm. Fifty apparently healthy controls, irrespective of age and gender, were also enrolled as controls. Cases with a known past history of any chronic disease like diabetes, cardiac, renal, hepatic or endocrine disease were excluded from the study. None of the participants in the present study were on dietary supplements. Besides plain X-ray and biopsy, all the patients underwent computerized tomography (CT) scan and magnetic resonance imaging (MRI), whenever necessary, for establishing the diagnosis and staging of the tumors. Standard treatment protocol followed was extended curettage with or without bone grafting for benign tumors and surgery (limb salvage or amputation), chemotherapy and/or radiotherapy depending on the type of malignant tumor. Study population was divided into 3 groups-

Group I : Healthy Controls (n=50)
Group II : Benign bone tumors (n=34)
IIa : Before treatment
IIb : After treatment
Group III: Malignant bone tumors (n=14)
IIIa : Before treatment
IIIb : After treatment

Five mL of venous blood sample was collected from all the participants under proper aseptic conditions. Two samples were collected from cases-one at the time of diagnosis and another 2 weeks after completion of respective treatment. Serum was separated and stored at -20°C for analysis of IMA and NO subsequently. The NO level [measured as nitrite-plus-nitrate {NO(x)} concentration] was estimated by Griess reaction. In this method nitrite reacts under acidic conditions with sulfanilic acid ($HO_3SC_6H_4NH_2$) to form a diazonium cation ($HO_3SC_6H_4– N N^+$) which subsequently couples to the aromatic amine 1-naphthylamine ($C_{10}H_7NH_2$) to produce a red– violet coloured (λmax≈ 540 nm), water-soluble azo dye ($HO_3SC_6H_4–N N–C_{10}H_6NH_2$) [12]. Reduced cobalt to albumin-binding capacity (IMA level) was measured using the rapid and colorimetric method developed by Bar-Or *et al* [13].

All statistical analyses were performed using the Microsoft office excel worksheet. Values shown in the text, tables and figures are mean ±SD. Paired and unpaired student's t test was applied for comparison of means of study groups. p value <0.05 was considered significant and <0.01 as highly significant. Correlations between groups were analyzed using Pearson correlation coefficient (r) formula.

RESULTS

In the present study, mean age was comparable between two groups. The mean age of cases was found as 28.08 ±17.01 years ranging from 6-75 years and of controls as 27.87± 15years. Out of 48 cases, 33 (68.75%) were males and 15 (31.25 %) females. The mean age was found to be 24.91 years and 35.78years in patients with benign and malignant tumors; respectively. The mean age of the patients with osteosarcoma, which was the most common primary malignant bone tumor was 18.83 (14–39) years. In total, there were 34 benign tumors and 14 malignant, details of which is given in table 1.

Table 1 Clinical data of patients with Bone tumor

Behaviour	Tumor type	No. of cases
Benign (n=34)	Osteochondroma	7
	Osteoid osteoma	2
	Giant cell tumor	13
	Synovial chondromatosis	2
	Hamartoma	3
	Enchondroma	2
	Aneurysmal Bone Cyst	1
	Eosinophylic granuloma	1
	Exophytic lesion	1
	Hemangioma	1
	Chondroblastoma	1
	Lytic lesion	1
Malignant (n=14)	Osteosarcoma	7
	Chondrosarcoma	3
	Metastasis	2
	Plasmacytoma	1

The levels of NO and IMA were compared between groups I, IIa, IIb, IIIa and IIIb and the results are given in table 2, 3 and figure 1, 2.

Table 2 The results of paired *t* test in benign and malignant tumors before and after treatment.

Parameter	Group IIa	Group IIb	Group IIIa	Group IIIb	p value (Group IIa& IIb)	p value (Group IIIa &IIIb)
IMA (ABU)	0.54 ± 0.07	0.37 ± 0.07	0.86± 0.07	0.50 ± 0.05	<0.01**	<0.01**
NO (µmol/L)	13.18 ± 1.27	3.95 ± 1.21	26.04 ± 3.50	4.2 ± 0.95	<0.01**	<0.01**

*Significant; **Highly significant; all values are in mean ± SD.

Table 3 The results of paired *t* test in cases and controls.

Parameter	Group I	Group II	Group III	p value (Group I & II)	p value (Group II & III)
IMA (ABU)	0.45 ± 0.03	0.63 ± 0.16	0.41 ± 0.09	<0.05*	<0.01**
NO (µmol/L)	11.33 ± 0.21	16.93 ± 6.27	4.025 ± 1.13	<0.05*	<0.01**

*Significant; **Highly significant; all values are in mean ± SD.

Table 4 The results of unpaired *t* test in benign and malignant tumors.

Parameter	Group II	Group III	p value (Group II & III)
IMA (ABU)	0.54 ± 0.07	0.86± 0.07	<0.01**
NO (µmol/L)	13.18 ± 1.27	26.04 ± 3.50	<0.01**

*Significant; **Highly significant; all values are in mean ± SD.

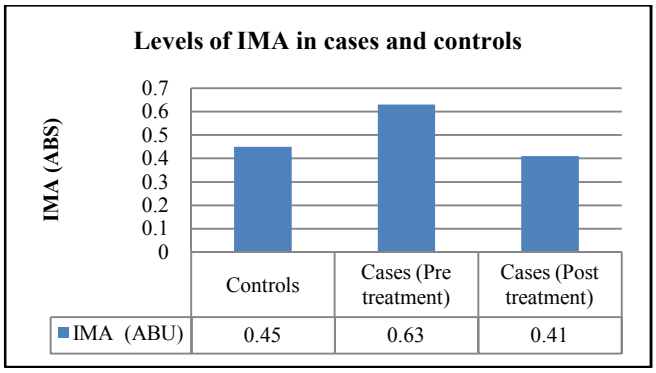

Figure 1 Bar diagram showing levels of IMA in cases (before and after treatment) and controls.

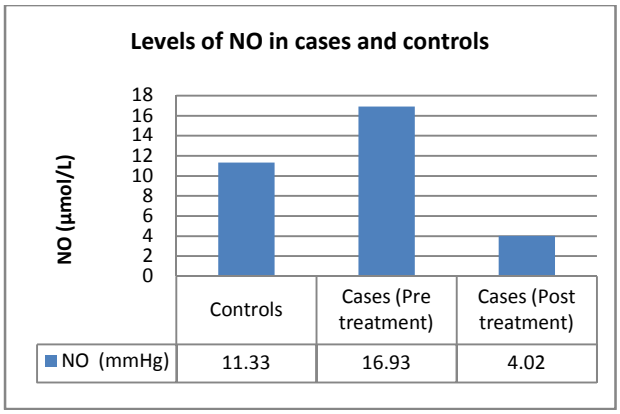

Figure 2 Bar diagram showing levels of NO in cases (before and after treatment) and controls.

The mean levels of IMA in group I was 0.45 ± 0.03 ABS and in group II was 0.63 ± 0.16 ABS. Statistical analysis showed that the difference in the mean values in the group I and group II was found to be significant ($p<0.05$). Similarly, levels of NO in group I and II were11.33 ± 0.21µmol/L and 16.93 ± 6.27 µmol/L respectively with statistically significant differences in the mean values ($p<0.05$). As shown in table 3, the levels of IMA ($p<0.01$) and NO ($p<0.01$) were found more in malignant cases as compared to benign with statistically significant differences. Pearson's correlation coefficient between IMA and NO was 0.842 ($p<0.01$) indicating a strong positive correlation. Figure 3 shows linear regression plot between IMA and NO with linear equation, y=31.73x-3.75; showing positive relationship between two variables.

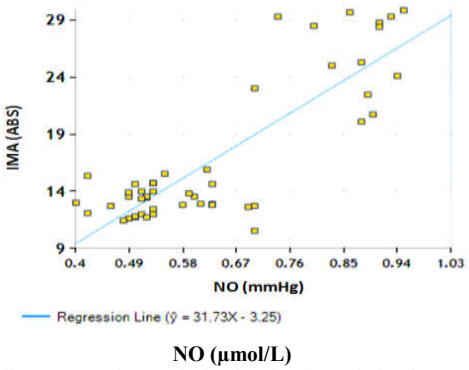

Figure 3 A linear regression analysis plot showing relation between IMA and NO in cases.

DISCUSSION

Results of the present study showed increased levels of oxidative stress markers (IMA and NO) in bone tumors as compared to healthy controls ($p<0.05$) and the levels decreased after treatment. A significant correlation was found between IMA and NO levels ($r=0.842$; $p<0.01$).Also, the levels were significantly more in malignant cases as compared to benign tumors ($p<0.01$).To the best of our knowledge, till date there is no published report about serum concentrations of IMA and NO together in patients with primary bone tumors.

Nitric oxide levels were found to be increased in cases as compared to controls and the levels decreased after completion of treatment. When compared in benign and malignanttumors the role of NO· in cancer is complex and spans the range from cause to cure. Human body is constantly bombarded by stressful factors both from outside and inside. Factors affecting from outside are known as exogenous such as UV rays, tobacco, smoking etc. and those attacking from inside are called endogenous and they mainly attack at mitochondrial level resulting in synthesis of ROS. When there occurs imbalance between oxidative stress and oxidant-reduction system of body, mutations may result manifesting in the form of carcinogenesis. Tumor is a stress like condition. Under hypoxic stressful conditions, mitochondria produce NO, which is a marker of oxidative stress. NO further, can generate other reactive nitrogen species (RNS) such as nitrite and nitrate, S-nitroso-thiols or peroxynitrite. RNS attacks on cellular biomolecules like DNA, phospholipids, proteins, carbohydrates etcand generates other reactive species like reactive aldehydes-malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), etc which acts as biomarkers [14]. Peroxynitrites(ONOO-), nitrates and dinitrogen trioxide (N_2O_3) produceoxidation, nitrosation and nitrationof DNA by attacking on sugar phosphate backbone and induces single stranded breaks. Nitrosation (addition of NO^+) of amines present in DNA bases produces diazonium ions and subsequent deamination and cross linking. DNA when oxidized induces genetic mutations [15].Peroxynitrite and its conjugate acid ONOOH are potent cytotoxic oxidants oxidizing thiols or thioethers, nitrating tyrosine residues, nitrating and oxidizing guanosine, degrading carbohydrates, initiating lipid peroxidation and cleaving DNA,which has important implications in cancer [16].

NO is produced from arginine by the action of enzyme nitric oxide synthase (NOS). It also requires the cofactors NADPH, FMN, BH_4, and FAD. There are three isoforms of NOS,

inducible (iNOS), endothelial (eNOS) and neuronal form (nNOS). All the forms have been detected in tumour cells from a wide range of isolates. iNOS is an inducible form which can betranscriptionally regulated by various factors such asbacterial endotoxin (LPS), oxidative stress and cytokines (e.g. interferon- α (IFN-α), interleukin-1(IL-1) and tumour necrosis factor-α (TNF-α) [15]. Unlike eNOS and nNOS, induction of iNOS results in continuous production of NO which may be for many hours or even days [17]. The role of iNOS in cancer pathogenesis has been reported by various researchers [18-20].

NO stimulates the synthesis of cyclooxygenase-2 (COX-2) which has been associated with angiogenesis in cancer [21]. NO mediates up-regulation of vascular endothelial growth factor (VEGF) which increases vascularisation in the xenograft tumours. So, NO generated by NOS promotes new blood vessel formation. vascularization This neovasculaturization not only enhances the ability of the tumour to grow, but also increases its invasiveness and metastatic ability [15]. In present study too, levels of NO are more in malignant bone tumors than benign one stating the role of NO in metastasis and angiogenesis. These two factors, i.e. induction of NOS activity and increased oxidative stress, seen in cases may be responsible for increased NO levels in our patients.

IMA has been approved as first serum marker by the United States Food and Drug Administration, for detecting early stages of myocardial ischemia [22]. There have also been studies, indicating the role of raised IMA, in states of ischemia, hypoxia or oxidative stress with non-cardiac origin such as systemic sclerosis, glaucoma, skeletal muscle ischemia, peripheral vascular disease, and diabetes mellitus [23]. But there are not many studies evaluating the relationship between IMA and cancer. In present study, the levels of IMA were found to be more in cases as compared to controls and these levels decreased after treatment. Also, a positive correlation was found between IMA and NO levels which was statistically significant. Oxidative stress and hypoxia are two factors which induce changes in structure of albumin which reduces its binding capacity for cobalt cations. A study conducted in 2009 showed increased IMA levels in prostate cancer [24]. Similarly, in 2011 and 2012, Stachowicz-Stencel *et al*. and Fidan *et al*. demonstrated increased IMA levels in pediatric neuroblastoma, soft tissue sarcomas and gastric carcinoma respectively [25, 26]. Yasar Ellidag *et al* in 2013 conducted two studies, one on 40 bladder carcinoma patients and another on 40 colorectal carcinoma patients. In both studies, they found that there was an impaired oxidative/antioxidant status in favor of oxidative stress and IMA could be a new biomarker of oxidative stress in colorectal and bladder carcinoma patients [23, 27]. To the best of our knowledge, no study till date has demonstrated the role of IMA in bone tumors.Inflammation and oxidative stress plays a key role in pathogenesis of bone cancer [14]. The relationship between tumor and inflammation is a well known fact. Inflammatory cells located in tumor microenvironment play a major role in neoplastic processes. [28]. Increased levels of IMA and NO in present study are pointing towards inflammatory and oxidative stress conditions. Further studies are needed to establish the relationship of IMA and NO with inflammatory and oxidative stress parameters in Bone tumors.

COCLUSION

Increased levels of IMA and NO strongly favor the increased inflammation and oxidative stress in primary bone tumor patients and may act as potential biomarkers for diagnosis and prognosis of disease.

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