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ORIGINAL PAPER

Lipid peroxidation, erythrocyte antioxidants and plasma antioxidants in osteoarthritis before and after homeopathic treatment

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Objective: **This study attempts to evaluate the status of oxidative stress in osteoarthritis (OA), by measuring some parameters of oxidant stress and antioxidant defenses in blood, before and after homeopathy treatment, and to asses the role, if any, of homeopathic treatment in modulating free radical toxicity in OA.**
Methods: **Erythrocyte lipid peroxidation (LP), erythrocyte antioxidants viz., glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CT) and plasma antioxidants viz., ceruloplasmin, glutathione-S-transferase (GST), vitamin C, total antioxidant activity (AOA) were determined in eighty one patients with OA and fifty three normals. Forty seven patients, who were treated with homeopathic remedies were considered for the follow-up studies.**
Location: **Father Muller Homeopathic Hospital, Mangalore, South Karnataka, India.**
Results: **Erythrocyte LP (0 hour, $p < 0.001$; 2 hours, $p < 0.01$; and susceptibility to LP, $p < 0.05$) and SOD ($p < 0.05$) were significantly higher, whereas plasma vitamin C ($p < 0.01$) and AOA ($p < 0.001$) were significantly lower in OA patients when compared to controls. In follow-up patients the erythrocyte LP (0 hour, $p < 0.01$; 2 hours, $p < 0.01$; and susceptibility to LP, $p < 0.01$) and SOD ($p < 0.01$) were significantly lower when compared to their pretreatment values. Plasma vitamin C attained a normal range. The AOA activity after treatment was not significantly different from that observed before treatment.**
Conclusion: **Oxidative stress increased in OA as indicated by increased LP, SOD, decreased vitamin C and AOA. On homeopathic treatment the LP has decreased in the erythrocytes which shows and reduced oxidative stress. This is further evidenced by returning of plasma vitamin C and erythrocyte SOD to the normal levels, but oxidant stress has not been completely overcome as plasma AOA remained low after treatment.**
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Keywords: Free radicals; Homeopathy; Osteoarthritis

Introduction

Homeopathy, is a system of alternative medicine that strives to treat "like with like".[1,2] Homeopathy rests on the premise of treating sick persons with extremely diluted agents that in undiluted doses are deemed to produce similar symptoms in a healthy individual. Belief in the effectiveness of homeopathy in general is wide-spread and growing among the physicians and public.[3]

Osteoarthritis (OA) is an inflammatory disorder of the joint. The principal pathologic features of this disease include progressive focal degradation of the articular cartilage which is associated with chronic pain and loss of knee function.[4] The underlying mechanism of cartilage matrix degradation in osteoarthritis is poorly understood but the reactive oxygen species (ROS) are implicated as causative factors.[4–8] The

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main ROS produced by chondrocytes are nitric oxide and superoxide anion that generate derivative radicals including peroxynitrite and hydrogen peroxide.

As oxidative stress has been implicated in the etiology of OA an attempt has been made in the present study to assess the oxidant-antioxidant status in OA, before and after homeopathic treatment.

Materials and methods

Study design

The study plan was approved by the Ethics Committee of the Medical Faculty, and all subjects volunteered for the trial. Blood samples were obtained from 81 OA patients (males 31, females 50), aged 30 to 75 years (mean age 51.89 ± 1.25). Of the 81 patients, 68 patients suffered from OA of both the knees, six suffered from OA of right knee and remaining seven from OA of left Knee. The mean duration of OA was 4.14 ± 0.656 years. Classical homeopathy was followed, a comprehensive homeopathic history was taken, followed by prescription of a single individualized remedy in response to changing symptoms. These patients suffered from one or more of the following symptoms: stiffness, swelling and pain in the knee joints. For follow-up studies, only 47 patients were available. From these patients, another blood sample was collected after 3 months of treatment. Different oxidant and antioxidant parameters were estimated in blood samples obtained before and after treatment. The results were compared with those obtained in age and sex matched healthy, non hospitalized individuals who were considered as normal controls. The control group consisted of 53 individuals (36 males, 17 females); 24 to 64 years (mean age 45.42 ± 1.36).

Methodology

Blood samples were collected in heparinized bottles from normal subjects and OA patients. Plasma and RBCs were separated. 50% erythrocyte suspensions was prepared according to the method of Kartha and Krishnamurthy.[9] These suspension were used for some of the assays performed. The assays performed in the erythrocytes were lipid peroxidation (LP), Superoxide dismutase (SOD) glutathione (GSH), glutathione reductase (GR), catalase (CT), and in plasma were glutathione-S-transferase (GST), vitamin C, ceruloplasmin, antioxidant activity (AOA).

The hemoglobin content of the erythrocytes was determined by the cyanmethemoglobin method. Erythrocyte LP was determined by incubating RBC suspension in saline phosphate buffer containing 0.44 M H_2O_2 at 0 h and 2 h. Aliquots were drawn from the above mixture at 0 h and 2 h. LP in RBC was determined by estimating malondialdehyde (MDA) produced using thiobarbituric acid.[10] Erythrocyte GR activity was determined by recording the decrease in absorbance due to depletion of NADPH for a period of 5 min at 340 nm.[11] Superoxide dismutase (SOD) was determined according to the method of Beauchamp and Fridovich[12] based on inhibition of nitrozolium reduction. CT activity in the hemolysate was determined by adopting the method of Brannan *et al.*[13] The assay is based on the disappearance of H_2O_2 in the presence of the enzyme source at 26° C. The GSH content of erythrocytes was determined as described by Beutler *et al.*[14]

Plasma ceruloplasmin was determined by p-phenylene diamine oxidase activity.[15] Plasma vitamin C was determined chemically using dinitrophenyl hydrazine as a colour compound.[16] Plasma GST was determined by incubating CDNB (1 chloro 2, 4 dinitro benzene) with reduced GSH in the presence of serum containing GST. 2,4-dinitrophenylglutathione (adduct) formed was read at 340 nm.[17] AOA activity was measured per Koracevic *et al.*[18]

The package used for statistical analysis was SPSS/PC+ (version 11.0).

Results

Erythrocyte LP and susceptibility towards LP in OA patients was significantly high compared to normal controls (Table 1). After treatment a significant decrease was observed in LP. Susceptibility was also decreased significantly (Table 2). This shows that the treatment has effectively reduced the LP and susceptibility to LP.

SOD activity was increased significantly in OA patients when compared to normal controls at trial entry (Table 3), and decreased significantly in post-treated patients when compared to corresponding pretreated subjects (Table 4). A comparison of erythrocyte GSH, CT and GR in OA patients with those in normal controls showed no significant differences (Tables 3, 4).

Plasma vitamin C level and AOA activity were significantly decreased in OA patients compared to normal controls (Table 5). A comparison of vitamin C levels in OA before and after treatment showed an increase but it was not statistically significant (Table 6). After treatment, AOA remained significantly low when compared to normal subjects. There was no significant difference in the ceruloplasmin and GST activity in OA patients when compared to

Table 1 *In vitro* RBC lipid peroxidation in osteoarthritis (Mean ± SEM)

Group	*TBARS as nmol MAD/g Hb*		
	0 Hour	*2 Hours*	*Susceptibility to LP*
Normal Controls (NC) n = 53	77.8 + 4.46 (20.8–181.6)	384.5 + 18.54 (102.8–898.7)	306.0 + 16.65 (72.0–735.6)
Osteoarthritis n = 81	110.6 + 6.11*** (13.3–260.0)	480.0 + 24.00** (115.2–1127.8)	369.2 + 21.55* (36.3–963.5)
% change	42.15% > NC	24.83% > NC	20.65 > NC

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Range in parentheses, n = sample size, (Kruskal Wallis Test – Mann Whitney Test).

Table 2 Follow up studies of *In vitro* lipid peroxidation in osteoarthritis (Mean ± SEM)

Group	*TBARS as nmol MAD/g Hb*		
	0 Hour	*2 Hours*	*Susceptibility to LP*
Before treatment n = 47	110.1 ± 8.43 (13.4–259.9)	480.6 ± 30.70 (134.6–887.6)	370.5 ± 27.98 (56.6–774.9)
After treatment n = 47	81.3 ± 5.80** (16.10–182.7)	362.0 ± 26.52** (91.90–782.0)	280.7 ± 22.81** (32.70–599.4)
% change	26.15% < before treatment	24.67% < before treatment	24.23% < before treatment

**$p < 0.01$, Range in parentheses, n = sample size, (Paired *t*-Test).

Table 3 Erythrocyte antioxidant levels in osteoarthritis (Mean ± SEM)

	Normal Controls (NC)	*Osteoarthiritis*	*% change*
GSH (μmol/gHb)	4.8 ± 0.21 (2.4–10.3) n = 53	4.9 ± 0.23 (1.3–10.0) n = 81 NS	2.08 > NC
SOD (units/gHb)	9214 ± 492.5 (4046–21990) n = 53	11331 ± 589.2* (1168–26716) n = 81	22.97 > NC
Catalase (units/gHb)	245996 ± 10410.2 (27920–413385) n = 53	276936 ± 16859.0 (91962–848860) n = 81 NS	12.56 > NC
GR (units/gHb)	1.8 + 0.15 (0.1–4.09) n = 51	2.0 + 0.21(0.1–10.36) n = 73 NS	11.29 > NC

* $p < 0.05$, NS = Not Significant, Range in parentheses, n = sample size, (Kruskal wallis Test - Mann Whitney Test).

Table 4 Follow up studies of various erythrocyte antioxidants in osteoarthritis patients (Mean ± SEM)

	Normal Controls (NC)	*Osteoarthiritis*	*% change*
GSH (μmol/gHb)	5.1 + 0.30 (2.0–10.0)	5.0 + 0.36 (1.9–13.4) n = 47 NS	2.76 < before treatment
SOD (units/gHb)	12208 ± 752.9 (3920–26716)	9378 ± 657.7** (1347–25851) n = 47 NS	23.18 < before treatment
Catalase (units/gHb)	284827 + 227673.0 (102042–848860)	240474 + 16103.4 (107356–583728) n = 47 NS	15.57 < before treatment
GR (units/gHb)	1.7 + 0.22 (0.1–5.8)	2.3 + 0.40 (0.0–10.5) n = 44 NS	35.00 < before treatment

NS = Not Significant, ** $p < 0.01$, Range in parentheses, n = sample size, (Wilcoxon Signed Rank test).

normal controls both before and after homeopathic treatment (Tables 5, 6).

Homeopathic treatment

A total of 208 prescription lines were prescribed for 81 patients ie 2.4 medications per patient, on average. One prescription line corresponds to one medication prescribed to one patient at the inclusion visit. Medications were given simultaneously or sequentially depending on the condition of the patient. Homeopathic treatment was prescribed for all the patients.

Table 7 shows the 10 homeopathic medications most prescribed in the study group (ie 87 patients) during this study; the most frequent were: *Calcarea fluorica*, *Rhus toxicodendron*, *Pulsatilla*, *Natrum muriaticum*, *Bryonia alba* and *Thuja occidentalis*. *Rhus toxicodendron*, *Natrum muriaticum* and *Bryonia alba* were most often prescribed at a dilution of 30C, whereas *Calcarea fluorica* at 6X and *Thuja occidentalis* was prescribed at 200C. 65.4% of the 81 patients received *Calcarea fluorica* and 43% received *Rhus toxicodendron*, *Pulsatilla*, *Natrum muriaticum*, *Bryonia alba* and *Thuja occidentalis*

Table 5 Plasma antioxidant levels in osteoarthritis patients (Mean ± SEM)

	Normal Controls (NC)	*Osteoarthiritis*	*% change*
VitaminC (μmol/L)	22.5 ± 12.3 (3.5–49.5) n = 53	16.9 ± 1.28**$ (0.7–43.6) n = 81	24.8 < NC
Cerulop lalstin (g/L)	0.5 + 0.03 (0.2–1.4) n = 53	0.5 + 0.20 (0.1–1.1) n = 81 NS	1.03 < NC
GST (IU/L)	4.3 ± 0.45 (0.4–15.4) n = 53	4.0 ± 0.37 (0.0–15.8) n = 81 NS	8.35 > NC
AOA (mmol/L)	1.0 ± 0.06 (0.3–2.2) n = 53	0.6 ± 0.03***# (0.1–1.2) n = 70	39.8 > NC

**$p < 0.01$, *** $p < 0.001$, NS = Not Significant, Range in parentheses, n = sample size
($ = ANOVA-Dunnelt *t* Test, # = Krusal wallis Test - Mann Whitney Test).

Table 6 Follow up studies of plasma antioxidants in osteoarthritis patients (Mean ± SEM)

	Normal Controls (NC)	*Osteoarthiritis*	*% change*
VitaminC (μmol/L)	18.4 + 1.79 (0.7–43.6)	20.7 + 1.78 (2.2–48.6) n = 47 NS	12.50 > before treatment
Cerulop lalstin (g/L)	0.5 ± 0.23 (0.1–1.0)	0.5 ± 0.03 (0.1–0.9) n = 47 NS	0.43 > before treatment
GST (IU/L)	3.8 ± 0.44 (0.6–13.5)	3.6 ± 0.45 (0.3–13.3) n = 47 NS	6.00 < before treatment
AOA (mmol/L)	0.6 + 0.04 (0.1–1.2)	0.6 + 0.04 (0.2–1.2) n = 41 NS	1.66 < before treatment

NS = Not Significant, Range in parentheses, n = sample size, (Wilcoxon Signed Rank test).

Table 7 The 10 most prescribed homeopathic medications in 81 patients

Names of homeopathic medications	*Number of lines*	*Total homeopathic medications*	
		%	*Total (%)*
Calcarea fluorica	53	25.48	25.48
Rhus toxicodendron	35	16.83	42.31
Pulsatilla	20	9.62	51.93
Natrum muriaticum	16	7.69	59.62
Bryonia alba	14	6.73	66.35
Thuja occidentalis	12	5.77	72.12
Lycopodium clavatum	9	4.33	76.45
Kali Carbonium	9	4.33	80.78
Amica montana	7	3.37	84.15
Sepia officinalis	5	2.40	86.55
Other	28	13.45	100.00
Total medications	208	100.00	–

were prescribed for 25%, 19%, 17% and 15% of the patients respectively.

Table 8 shows that 10 homeopathic medications most prescribed for 47 patients whose follow-up blood sample was taken. *Calcarea fluorica*, *Rhus toxicodendron*, *Pulsatilla* and *Bryonia alba* were the main homeopathic treatments prescribed for the patients.

Discussion

The lipid profile of human articular cartilage, especially the concentration of polyunsaturated fatty acids, increases with normal aging of cartilage.[19] Previous studies have shown that normal articular chondrocytes express constitutive levels of LP activity, which is enhanced by treatment with agonists such as calcium ionophore A23187.[20] LP measured by MDA concentration, was found to be higher although not significant in synovial fluid (SF).[4] Results obtained in the present study indicate that the changes with regard to LP in the chondrocytes and SF are well reflected in the erythrocytes of OA patients. The LP in erythrocytes was increased at 0 h as well as after 2 h. The susceptibility towards LP was also significantly increased in osteoarthritis compared to normal controls. Similar observations have been made by Rubyk *et al*[21] and Maneesh *et al*.[22] who reported significantly increased serum MDA levels in osteoarthritis patients compared to controls. Thus, these findings are in keeping with possible evidence of free radical production and damage in OA.

Table 8 The 10 most prescribed homeopathic medications in for the 47 patients whose follow up blood sample were taken

Names of homeopathic medications	*Number of lines*	*Total homeopathic medications*	
		%	*Total (%)*
Calcarea fluorica	41	27.70	27.70
Rhus toxicodendron	30	20.27	47.97
Pulsatilla	11	7.43	55.40
Natrum muriaticum	9	6.08	61.48
Bryonia alba	8	5.41	66.89
Thuja occidentalis	8	5.41	72.30
Lycopodium clavatum	6	4.05	76.35
Kali Carbonium	6	4.05	80.40
Amica montana	6	4.05	84.45
Sepia officinalis	4	2.70	87.15
Other	19	12.85	100.00
Total medications	148	100.00	–

To prevent toxicity by ROS, chondrocytes possess a well-coordinated antioxidant enzyme system in humans as they constitutively express CT, GPX and Cu/Zn and Mn SOD.[23] Ostalowska *et al*[4] have reported an increase in total SOD and both isoenzymes Zn/Cu SOD and Mn SOD in SF of OA patients. In contrast, Ivanova and Ivanova[24] found that there was no SOD, no or low CT in SF from OA joints. Schumacher[25] found similar results of decreased SOD and CT activities in SF from OA patients, as compared to normal SF.

In the present study, SOD activity was significantly increased in the erythrocytes of OA patients compared to the normal healthy controls, but CT was not significantly altered. Maneesh *et al*[22] reported a significant increase in SOD activity and significant decrease in CT activity in the erythrocytes. But Sarbans *et al*[26] have shown no significant difference in erythrocyte SOD activity between OA patients and healthy subjects. It is possible that differences between our study and studies by other investigators, regarding antioxidant status, is due to differences in the stage of the disease. Chronic joint disease may deplete antioxidant defences whereas acute inflammation can upgrade them.[27]

Studies done on chondrocytes by Carlo and Loeser[28] *in vitro* have shown decreased activity of GR, thioredoxin reductase and glutathione peroxidase (GPx) but GSH levels remain unaltered. Studies done by Ostalowska *et al*[4] have shown increased levels of GST, GR and GPx in SF of OA patients. In contrast to this Schumacher[25] found decreased GPx activity in SF from OA patients, as compared to the normal SF. Very little GR and GST has also been reported[28] in SF of OA patients. In the presents study, neither GSH nor GR in erythrocytes nor plasma GST were significantly altered in OA patients. However, Maneesh *et al*[22] showed a decrease in erythrocyte GSH levels.

Mean vitamin C concentration in plasma of OA patients significantly decreased when compared to normal controls in the present study. Similar studies done by Maneesh *et al*[22] also showed a significant decrease in vitamin C level in plasma. There was also a significant decrease in the AOA, in the present study when compared to the control subjects. The decrease is probably due to the consumption of antioxidants in the neutralization of excess of ROS produced in the disease process.

After homeopathic treatment for three months, 85% of the patients who returned for follow-up studies, had improved with decrease in pain, stiffness and swelling of the joints, homeopathic treatment. With regard to biochemical changes, the erythrocyte TBARS levels decreased significantly both at 0 h and 2 h, thus suggesting an antioxidant role for which had the effect of reducing LP in OA. It is also possible that the drugs given may have activated antioxidant mechanisms in the patients without direct antioxidant effect.

The erythrocyte SOD significantly decreased after treatment compared to pre-treatment values almost reaching normal levels. Plasma vitamin C increased approaching normal range in the follow-up cases. But the plasma AOA remained low even after treatment. This suggests that other antioxidants, (not measured in the present study) contributing to the total AOA of the plasma may not have altered after treatment.

In conclusion, the oxidative changes in chondrocytes of OA patients are reflected in erythrocytes as evidenced by increased LP. Plasma antioxidants are decreased as shown by decrease in vitamin C and AOA. On homeopathic treatment the LP decreased in the erythrocytes which shows that homeopathic treatment has some effect in reducing oxidative stress. This is further evidenced by the return of erythrocyte SOD to the normal levels.

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