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ORIGINAL ARTICLE

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Antioxidant status, lipid peroxidation and nitric oxide in fibromyalgia: etiologic and therapeutic concerns

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Abstract We proposed to assess the oxidant/antioxidant status, lipid peroxidation and nitric oxide (NO) in untreated fibromyalgia (FM) patients and controls. The effect of amitriptyline (A, 20 mg daily) and sertraline (S, 100 mg daily) treatment on patients' superoxide dismutase (SOD), xanthine oxidase (XO), adenosine deaminase (ADA) enzyme activities, thiobarbituric acid reactive substances (TBARS) and NO levels was investigated. Thirty female patients with primary FM and age-matched 16 healthy female controls were included. Patients received an 8-week course of treatment with either A or S. FM patients had higher serum levels of TBARS (particularly malondialdehyde) and lower levels of nitrite compared to controls whereas enzyme activities were similar. A and S significantly improved Fibromyalgia Impact Questionnaire (FIQ) pain scores, Hamilton anxiety and depression rating scales. But neither A nor S had significant effects on measured oxidative stress parameters, except SOD activity that was significantly reduced after S treatment. Total myalgic scores negatively correlated with XO activity, and depression scales negatively correlated with levels of TBARS. Our results indicate that patients with FM are under oxidative stress. These findings represent a rationale for further research assessing the effect of free radical scavengers or antioxidant agents like vitamins and omega-3 fatty acids on peripheral and central mechanisms in FM.

Keywords Fibromyalgia · Superoxide dismutase · Lipid peroxidation · Nitric oxide · Oxidative stress

Abbreviations FM: Fibromyalgia · NO: Nitric oxide · S: Sertraline · A: Amitriptyline · SOD: Superoxide dismutase · XO: Xanthine oxidase · ADA: Adenosine deaminase · TBARS: Thiobarbituric acid reactive substances · FIQ: Fibromyalgia Impact Questionnaire · ROS: Reactive oxygen species · CFS: Chronic fatigue syndrome · O_2: Dioxygen · NOS: Nitric oxide synthase · PPT: Pain pressure threshold · HDRS: Hamilton Depression Rating Scale · HARS: Hamilton Anxiety Rating Scale · PUFAs: Polyunsaturated fatty acids · NBT: Nitroblue tetrazolium · PDE: Phosphodiesters · MDA: Malondialdehyde · NO_2^-: Nitrite · $NO^{\cdot}$: Nitric oxide radical

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Introduction

Fibromyalgia (FM) is a common, chronic widespread pain syndrome usually associated with other somatic and psychologic symptoms including fatigue, sleep disturbances, cognitive difficulties (memory problems, diminished mental clarity and concentration difficulties), psychologic distress of the accompanying anxiety and depressive disorders. The etiology and pathophysiology of FM has not been clearly understood and that makes the disease a frustrating condition for the patients and the physicians [1, 2].

Reactive oxygen species (ROS) are highly reactive chemical species with an unpaired electron and formed

by catalyzing transition metals like iron, copper or manganese. These ROS have been suggested to play important roles in rheumatologic conditions like rheumatoid arthritis (RA), ankylosing spondylitis (AS) and chronic fatigue syndrome (CFS) as well [3–6].

There is a balance between ROS and antioxidants within the cell, in the membranes and in the extracellular space. Endogenous free radical scavengers, namely antioxidants, are overwhelmed by excessive production of ROS. The ROS attack the polyunsaturated fatty acids (PUFAs) in the membrane lipids, thereby leading to lipid peroxidation resulting in loss of fluidity of the membranes, changes in membrane potentials and eventually rupture leading to the release of cell and organelle contents. Assessment of thiobarbituric acid reactive substances (TBARS) or 4-hydroxynonenal is probably the most commonly applied method for the measurement of lipid peroxidation [7, 8].

Nitric oxide (NO) is a highly diffusible and labile, gaseous messenger molecule, involved in various biological functions such as vasodilatation or vascular regulation, modulation of nociception, immune function, neurotransmission and excitation–contraction coupling [9, 10]. Nitric oxide also acts as a metabolic regulator during exercise [9]. The production of nitric oxide ($NO^{\cdot}$) from L-arginine is catalyzed by the dioxygenase, nitric oxide synthase (NOS), which has three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Recently, NO has been shown to modulate levels of ROS in a variety of cells [11]. Larson et al. [12] suggested the possible role of NO in pain modulation in FM.

We proposed to assess the oxidant/antioxidant enzyme activities, lipid peroxidation and NO in untreated FM patients in comparison to healthy controls. Additionally the effect of amitriptyline (A) and sertraline (S) treatment on patients' superoxide dismutase (SOD), xanthine oxidase (XO), adenosine deaminase (ADA) enzyme activities, TBARS and NO levels was also investigated.

Methods

Thirty female patients with a mean (SD) age of 37.7 (8.9) years who met the 1990 American College of Rheumatology (ACR) [13] criteria for the classification of FM and 16 healthy female controls matched for age [33.3 (8.8) years] were enrolled. The patients and controls volunteered to participate in the study and gave their informed consent. None of the patients were under the treatment of antidepressants, analgesics or antioxidant drugs and were only included if they had stopped using them at least 3 weeks before the study. All the participants were sedentary living women. The inclusion criteria for the study group comprised a negative history for heavy cigarette smoking, neuropsychiatric disorders (dementia, cerebrovascular disease, alcohol abuse, severe depression), psychoactive drug treatment and other neurological or endocrinological (i.e., diabetes mellitus, hypo or hyperthyroidism) disorders.

The patients were randomized into two study arms to receive either amitriptyline ($n = 12$, group A, 20 mg daily) or sertraline ($n = 18$, group S, started with 50 mg daily for the first 3 days and continued with 100 mg daily) for an 8-week period. No supplementary therapies, special diets or aerobic exercise programs were allowed during the study period. Compliance was assessed by tablet counting and subjects with less than 70% compliance were excluded from the analyses.

Assessing myalgic scores

The pain pressure threshold (PPT) measurements of patients were performed in the same room in the early afternoon with a mechanical algometer. The same doctor carried out all the measurements and tests throughout the study. Before the evaluations, subjects were informed of the procedure. Pain threshold was explained as the amount of pressure adequate to induce a sensation of discomfort, and the subjects were warned that the aim was to determine the pain threshold but not pain tolerance.

Eighteen tender points (TPs) and three control points (CPs) were evaluated using the methods, which were previously defined and used elsewhere [14–16]. A positive TP was defined as a point at which the subject had mild or great pain with < 4 kg/cm^2 pressure. The sum of the PPTs of 21 points (18 TPs, 3 CPs) was calculated as the total myalgic score (TMS in kilogram per square centimeter) [14–16].

The Hamilton Depression Rating Scale (HDRS) [17] and the Hamilton Anxiety Rating Scale (HARS) [18] were used to evaluate the affective condition of patients with FM. The cut-off score for mild depression on the HDRS was 27. Patients were administered the Turkish version of the Fibromyalgia Impact Questionnaire (FIQ) [19]. All these measurements were performed at the beginning and after an 8-week course of treatment.

Biochemical assays

Fasting blood samples were drawn into heparin-free tubes during routine blood sampling for biochemical and hematological analyses. After immediate centrifugation (1,000*g* for 10 min at $+4$°C), serum samples were stored frozen at -30°C.

The TBARS level was determined by a method based on the reaction with thiobarbituric acid (TBA) at 90–100°C [20]. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances (i.e., the byproduct of lipid peroxidation process of the PUFAs) and TBA react together for production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) tri-

chloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm (Ultra spec Plus, Pharmacia LKB Biochrom Ltd, England). The results were expressed as micromole per liter serum sample (μmol/l).

Since NO is a very labile molecule, its direct measurement in the biological samples is very difficult. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite (NO_2^-) and nitrate (NO_3^-) ions. Therefore, the stable oxidation end-products of NO, NO_2^- and NO_3^- can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of NO production. Therefore, plasma total nitrite concentration was accepted as an index of NO production. For total nitrite detection, serum was treated with copporized cadmium (Cd) granules to reduce NO_3^- to NO_2^-. Nitrite concentrations were quantified by a colorimetric assay based on the Griess reaction [21]. Briefly, a chromophore with a strong absorbance at 545 nm is formed by the reaction of nitrite with a mixture of *N*-naphthylethylenediamine and sulphanilamide. A standard curve was established with a set of serial dilutions (10-8 to 10-3 mol/l) of sodium nitrite. Results were expressed as micromole per liter serum.

The principle of the total SOD (EC 1.15.1.1) activity method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by $O_2^{-\cdot}$ generated by xanthine/xanthine oxidase system [22]. Activity was assessed in the ethanol phase of the plasma after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the plasma and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per milliliter (U/ml).

Serum XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm [23]. A calibration curve was constructed using 10–50 mU/ml concentrations of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 μmol uric acid formed per minute at 37°C, pH 7.5. Results were expressed in units per liter (U/l).

Plasma ADA activities were estimated spectrophotometrically by the method of Giusti [24], which is based on the direct measurements of the formation of ammonia, produced when ADA acts in excess of adenosine. Results were expressed as units per liter serum (U/l).

Statistical analyses

Results are expressed as mean ± SD, differences between the two groups at baseline were assessed using Mann–Whitney *U* test. The changes observed before and after amitriptyline or sertraline were assessed using Wilcoxon signed rank test. Spearman rank correlation and Pearson correlation coefficients were used to assess relationship between parameters. Two-tailed *P* value of <0.05 was considered statistically significant. The Statistics Package for Social Sciences (SPSS Inc., Chicago, IL, USA) was used for the analyses.

Results

All the patients and controls had normal erythrocyte sedimentation rate and C-reactive protein levels. Routine biochemical parameters including glucose, urea, creatine, uric acid and liver function tests were within normal limits.

Oxidants/antioxidants in patients versus controls

FM patients had higher serum levels of TBARS (Fig. 1) and lower levels of nitrite (Fig. 2) compared to control patients. Other parameters had no significant difference between patients and controls (Table 1).

Patients' SOD enzyme activity significantly correlated with nitrite ($r=0.58$, $P=0.001$) and ADA activities ($r=0.44$, $P=0.014$). There was no significant correlation between biochemical parameters and clinical measures (TMS, HARS, HDRS, FIQ-pain and FIQ-fatigue items) except TBARS correlated negatively with HDRS ($r=-0.40$, $P=0.03$) (Fig. 3) and XO activity negatively correlated with TMS ($r=-0.46$, $P=0.009$) (Fig. 4).

Effects of antidepressants on oxidative stress and antioxidant markers

Amitriptyline and sertraline significantly improved patients' pain VAS scores, HARS and HDRS after an 8-week course of treatment except fatigue VAS, which did not significantly improve with the treatment in amitriptyline group, and TMS, which did not significantly change in both treatment groups (Table 2). But neither amitriptyline nor sertraline had significant effects on measured oxidative stress parameters except SOD that was significantly reduced after sertraline treatment (Table 2).

Discussion

The present results indicate that FM patients are under oxidative stress demonstrated with increased levels of TBARS, an end-product of lipid peroxidation, and lower levels of nitrite. Patients' TMS negatively correlated with the activity of potent oxidative enzyme XO and HDRS scores of patients negatively correlated with TBARS. Recent study by Eisinger et al. [25] reported significantly increased protein carbonyls and decreased plasma thiols and glutathione in FM patients compared

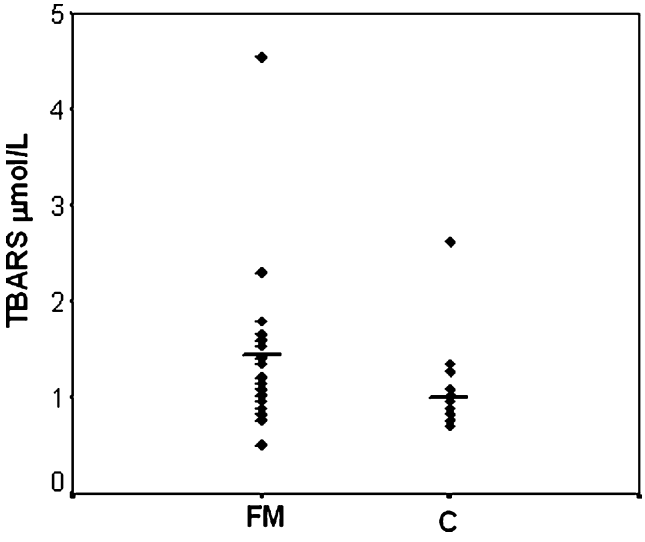

Fig. 1 Serum TBARS (thiobarbituric acid reactive substances) levels in patients and controls (baseline values)

to controls. Decreased nitrosothiols, which reflect NO, have been reported in accordance with our results of decreased nitrite levels. But contrary to our increased TBARS levels, this study reported similar MDA levels in FM compared to controls [25].

Efforts to explain the pathogenesis of FM and other chronic pain conditions by oxidative stress mechanisms are not new. Lund et al. [26] showed abnormal capillary microcirculation at the tender point sites in FM; Bennett et al. [27] showed muscle blood flow abnormalities and reduced aerobic fitness in patients with fibrositis. Another recently published study by Hein and Franke [28] pointed out increased levels of pentosidine, which was an advanced glycation end-product-modified protein and accelerated by oxidative stress in fibromyalgia. Oxidative enzymes were studied in muscle biopsy specimens by Lindh et al. [29] and 3-hydroxy CoA dehydrogenase and citrate synthase were found to be lower in FM patients compared to controls. Abnormal microcirculation has been reported in the skin above the tender points of FM patients using Laser Doppler flowmetry technique [30]. This report supports local hypoxia and possibly concomitant decrease in high-energy phosphate concentrations resulting from oxidative stress and membrane lipid peroxidation. Oxidative damage resulting from oxidative stress in vastus lateralis muscle has been reported in patients with CFS, a disease which has overlapping symptoms with FM [31].

Table 1 Biochemical parameters of the fibromyalgia (FM) patients and controls (C)

	FM ($n=30$)	C ($n=16$)
SOD (U/ml)	6.2 ± 2.1	6.4 ± 2.4
XO (U/l)	2.4 ± 0.9	2.6 ± 1.0
TBARS (µmol/l)*	1.4 ± 0.7	1.0 ± 0.5
Nitrite (µmol/l)*	51.9 ± 24.5	69.5 ± 10.9
ADA (U/l)	219 ± 73	181 ± 67

Values are means ± SD
SOD superoxide dismutase, *XO* xanthine oxidase, *ADA* adenosine deaminase, *TBARS* thiobarbituric acid reactive substances
*$P < 0.05$

To the best of our knowledge, estimation of serum levels of NO and TBARS and activities of XO, SOD and ADA and the effect of antidepressant treatment on these parameters have not yet been studied in this particular combination in FM.

In a newly published report, Sackner et al. [32] found that whole body periodic acceleration, which increased shear stress to the endothelium causing release of NO into the circulation, diminished symptoms of fibromyalgia and CFS. Our results of decreased NO levels support the hypothesis by Sackner et al.; however, we did not show any relationship between severity of FM symptoms and NO levels.

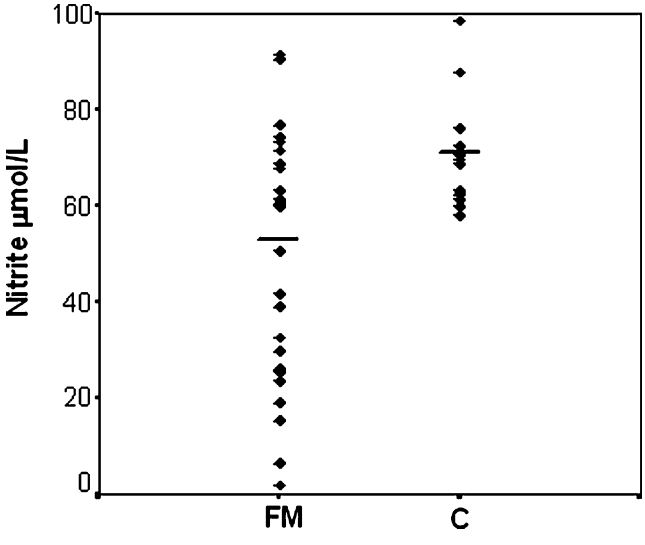

Fig. 2 Serum nitrite levels in patients and controls (baseline values)

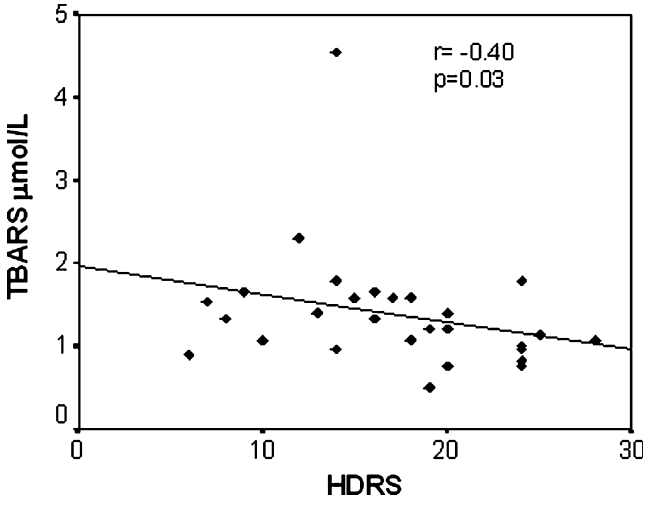

Fig. 3 Relationship between serum thiobarbituric acid reactive substances (baseline values) and Hamilton Depression Rating Scale in patients

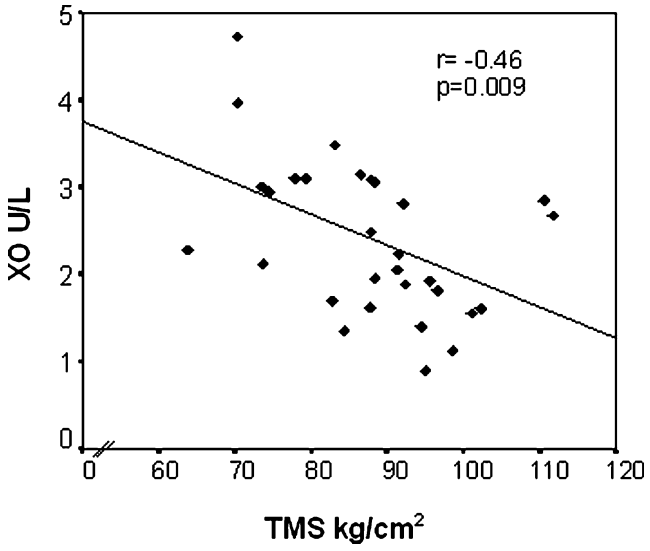

Fig. 4 Relationship between serum xanthine oxidase enzyme activity (baseline values) and total myalgic scores in patients

Bagis et al. [33] reported decreased SOD enzyme activity and increased MDA levels in patients with FM indicating that FM patients were under oxidative stress. Since the authors did not give any data of the treatment regimens of the patients, it is unclear whether decreased SOD enzyme activity was related to some of the antidepressants used by the patients or the disease process itself.

Another important result of our study was the nearly unchanged oxidant–antioxidant parameters with treatment despite significant improvement in clinical parameters. If the oxidative stress associated mechanisms (i.e., impaired muscle metabolism and/or microcirculation, structural alteration of the mitochondria) were the sole satisfactory explanation of FM pathophysiology, an improvement in lipid peroxidation or NO-related products would be expected in parallel to the clinical improvement. On the other hand, lipid peroxidation may play an important role in CNS mechanism of depression, and the peroxidation-reducing effect of different selective serotonin reuptake inhibitors in major depression has been demonstrated by Bilici et al. [34]. We found a negative correlation between HDRS and TBARS but unlike their results we did not observe significant changes in TBARS with the treatment. Additionally cognitive difficulties in FM patients have been well established [16, 35]. Plasma MDA levels has been suggested to be an important marker of cognitive deterioration in patients with dementia of Alzheimer type [36] and further researches assessing the relationship between cognitive functions and lipid peroxidation in FM would be very valuable. Furthermore using plant-based diets or omega-3 fatty acids, which are also potent antioxidants, has been suggested to cause improvement in serum peroxidation products, pain scores, stiffness, depression and sleep quality in cases with fibromyalgia [37–39]. But these reports are usually pilot results or uncontrolled open label studies that needs further additional researches.

In addition it is very difficult to completely eradicate or match all the factors influencing the oxidative stress parameters like life style, nutritional and smoking habits, psychological and physical stress conditions, etc., in a study population. These are primary difficulties or major limitations of the studies assessing oxidative stress in vivo as well. Secondly, our data is not enough to conclude whether these findings are a feature of FM or caused by the unfit condition of these patients.

In conclusion, our results suggest a possible link between direct and indirect markers of oxidative stress and patients' susceptibility to pain and depression. These findings justify further researches to explore the oxidant/antioxidant-related mechanisms and the effect of free radical scavengers or antioxidant agents in FM.

Table 2 Comparison of pre- and post-treatment measurements of the patients in both groups

	Amitriptyline ($n=12$)			Sertraline ($n=18$)		
	I	II	*P*	I	II	*P*
Clinical parameters						
FIQ-pain	70.0 ± 16.2	38.1 ± 21.2	0.008	57.3 ± 29.7	34.9 ± 19.2	0.02
FIQ-fatigue	65.2 ± 24.1	45.6 ± 27.2	0.12	55.2 ± 30.1	26.9 ± 27.0	0.006
TMS	87.2 ± 14.0	95.2 ± 6.4	0.24	88.1 ± 10.0	91.3 ± 9.4	0.08
HDRS	17.3 ± 5.1	8.7 ± 4.7	0.005	17.3 ± 6.7	8.6 ± 5.4	0.001
HARS	18.0 ± 7.0	11.2 ± 8.2	0.005	21.5 ± 8.8	9.9 ± 6.0	0.001
Biochemical parameters						
SOD (U/ml)	6.3 ± 2.1	6.8 ± 2.4	0.56	6.2 ± 2.0	4.5 ± 1.9	0.04
XO (U/l)	2.6 ± 0.7	2.7 ± 1.1	0.79	2.3 ± 1.0	2.9 ± 1.2	0.13
TBARS (μmol/l)	1.6 ± 1.0	1.2 ± 0.3	0.28	1.2 ± 0.3	1.1 ± 0.3	0.44
NO (μmol/l)	60.6 ± 24.0	51.9 ± 23.2	0.28	46.2 ± 23.8	46.6 ± 25.1	0.90
ADA (U/l)	246 ± 99	271 ± 107	0.72	201 ± 43	195 ± 56	0.58

Values are means ± SD; *P* values represent Wilcoxon test results I (measurement at study entry) versus II (measurement after 8-weeks in each group)*FIQ-pain* Fibromyalgia Impact Questionnaire pain visual analog scale, *FIQ-fatigue* Fibromyalgia Impact Questionnaire fatigue visual analog scale, *TMS* total myalgic scores, *HDRS* Hamilton Depression Rating Scale, *HARS* Hamilton Anxiety Rating Scale

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