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Effect of Vitamins C, E and *Nigella sativa* Seeds on Antioxidant Activity in Fibromyalgia Patients

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Abstract.- Fibromyalgia syndrome (FMS) is characterized by musculoskeletal pain of unknown etiology and is often accompanied by many psychological symptoms. This study is aimed at evaluating the importance of antioxidants (vitamin E, vitamin C and *Nigella sativa* seeds) therapy in management of this disease. Fifty female patients having mean age of 37.87±1.68 years suffering from FM were enrolled in the study. Role of oxidative stress was determined by measuring antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), antioxidant capacity by ABTS (2,2'- azinobis-(3-ethylbenzo-thiazoline-6-sulphonic diammonium salt) and ferric reducing antioxidant capacity (FRAP) assay in plasma and catalase in erythrocytes. The FM patients were also supplemented with antioxidants (Vitamin C, Vitamin E and *Nigella sativa* seeds) for two months to assess the impact on activity of FM with the help of VAS (visual analogue scale) (0 being no pain and 100 being severe pain).It was found that FM patients had low activity of SOD, GPx and low antioxidant capacity (AOC) than healthy controls. It was further observed that after two months supplementation with antioxidants (vitamin C, vitamin E and Nigella sativa seeds) the level of SOD, GPx and AOC increased and their mean VAS 90.30±1.52 at baseline decreased to mean VAS of 77.80±1.65 after supplementation with antioxidants.The antioxidant supplementation for two months resulted in significant improvement in FM patients and it may help in minimizing the effects of the oxidative stress.

Key words: Fibromyalgia, Oxygen free radicals, antioxidants.

INTRODUCTION

Fibromyalgia (FM) is characterized by chronic widespread pain in all the four quadrants of body and the axial skeleton along with presence of 11/18 tender points on physical examination. FM can occur alone called primary FM or, in patients having diseases like Rheumatoid Arthritis (RA) or Systemic Lupus Erythmatosus (SLE), where it is called secondary FM. Up to 25% of patients with established autoimmune disorders like RA, SLE and ankylosing spondylitis also meet American College of Rheumatology (ACR) criteria for FM. The other clinical features of FM includes tenderness on palpation, sleep disturbance, fatigue, depression, anxiety, memory and concentration problems, headaches, urinary complaints, vaginal pain and dryness and others. It may also be associated with diarrhea and constipation (Wallace and Wallace, 2002). FM is to be common worldwide (White and Harth, 2001). It has been reported that FM is most common in women than men between the ages of 30 to 60 years (Ataoglu *et al.*, 2003). Prevalence of FM has been studied in different countries which ranges from 7.3% to 12.9% (Buskila *et al.*, 2000; Wolfe *et al.*, 1995). To our knowledge, no one study has analyzed antioxidant status in FM patients in Pakistan.

Several factors have been proposed to cause FM but the exact cause is still unknown.

The free radicals which may belong to reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in a number of metabolic and other degenerative diseases (Halliwell, 2001; Omar *et al*., 1999). It was pointed out, imbalance in the antioxidant system in FM as well as in chronic fatigue syndrome (CFS) (Manuel *et al*., 2000). The human body has developed a wide

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range of antioxidant systems to limit production of free radicals, inactivate them and repair cellular damage (Rauch *et al.*, 2006*)*. Vitamin E (α-tocopherol) is fat-soluble vitamin. It is the most powerful biological antioxidant (Traber, 2007). The vitamin C (ascorbic acid) is an electron donor and, therefore, a reducing agent. All known physiological and biochemical actions of vitamin C are due to its action as an electron donor (Aysun, 2009). It also acts as an antioxidant, protecting cells from the harmful effects of free radicals (Carr and Frei, 1999). Nazıroğlu *et al.* (2010) found vitamin C and vitamin E may protect against FM. Moreover, vitamin C is the water soluble vitamin, it is scavenger for free radicals and also help to convert vitamin E to its active form (Sies and Stahl, 1995).A large number of clinical studies in healthy individuals, populations at risk for certain diseases, and patients undergoing disease therapy indicate that supplementation with vitamin C or E may result in changes in either oxidative status, disease risk, or disease outcome (Sudesh *et al.*, 2006). *Nigella sativa*, commonly known as black cumin and kalunji has been extensively investigated in recent years. Various studies have implicated the role of black cumin seeds and their oil as antioxidants (Burits and Bucar, 2000; Suboh *et al.*, 2004).

On the basis of above we hypothesized that FM patients may have decreased levels of antioxidant enzymes including SOD, GPx and catalase leading to decreased total antioxidant capacity (TAC). Secondly we hypothesized that supplementation with antioxidants like vitamin E, vitamin C and *N. sativa* seeds would lead to improvement in FM symptoms by increasing the oxidative capacity in terms of increase in antioxidant enzymes.

The objective of the present study was to determine the role of oxidative stress in pathogenesis of FM and to access the role of antioxidants (vitamin E, vitamin C, *N. sativa* seeds) in patients with FM.

MATERIALS AND METHODS

The study was conducted in the outpatient's clinics of Division of Rheumatology, Fatima Memorial College of Medicine and Dentistry, Lahore, Pakistan.

Study design

This study was conducted as per good clinical practice (GCP) guidelines (declaration of Helsinki). Formal approval was taken from Ethical Committee of the Hospital and informed consent was also taken from all the study subjects. The study was Prospective, observational and descriptive.

Fifty female patients with age ranging from 30 to 55 years (mean age 37.87±1.68 years) who met the American College of Rheumatology criteria (ACR's criteria) (Wolfe *et al.*, 1990) for the classification of FM and 16 age matched healthy controls were enrolled for this study. Patient's detailed history study including physical examination was undertaken by consultant rheumatologists. Tender points were assessed by digital/thumb palpation (4 Kg) on specific points of the muscles and the number of tender points was recorded accordingly. Each patient was asked to quantify their pain on a Visual Analogue Scale (VAS) of 0-100 mm with 0 as no pain and 100 as the worst pain. The patients with any chronic illness like diabetes, hepatic or renal insufficiency, malignancies or any other illness which can cause diffuse musculoskeletal pain were excluded from the study. None of the FM patient was taking any type of medicine (analgesic or antidepressant) and only those patients were included which have stopped taking them at least 8 weeks before the study.

In the present study different parameters of total antioxidant activity such as TAC (Total Antioxidant Capacity) by ABTS (2, 2'- azinobis-(3-ethylbenzo-thiazoline-6-sulphonic diammonium salt) assay and total reducing power in terms of Ferric Reducing Antioxidant Power (FRAP) values were measured for plasma in FM patients and the results were compared with those of healthy individuals. We also measured the levels of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) in plasma and catalase in erythrocytes of FM patients and healthy persons treated as controls. Furthermore, we also investigated the effects of supplementation of vitamin C, vitamin E and *N. sativa* seeds on the oxidative stress level in the FM patients.

Blood samples of total 50 patients were collected as baseline at the start of the study. After eight weeks of supplementation with vitamin C (Cecon; 500 mg tablets, Abbot Laboratories) recommended one tablet daily, vitamin E (Evion; 200 mg; capsule Merck pharmaceuticals) recommended one capsule every day and *N. sativa* (13 mg; 4-5 seeds daily) to each patient. After 8 weeks of supplementation blood samples were again drawn for the analysis. During study patients were not taking any medicine except vitamin C, vitamin E and *N. sativa* seeds. The blood samples of 16 age and sex matched controls were also collected. All the blood samples (each 4 ml in size) were collected in heparanized tubes, in fasting state. Soon after collection the blood samples were spun at 4000 rpm for 15 min. The plasma was separated and the erythrocytes were washed three times in ice-cold saline (0.15 M NaCl). The cell lyses was performed in 4x packed cell volumes of cold and deionized water. The samples were then stored at –70°C for further analysis. All the patients completed the study period and no patient was dropped.

SOD activity was determined in plasma as described by Liu *et al.* (1997) with minor modifications. Superoxide radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), which contained 78 µM *β*-nicotinamide adenine dinucleotide (reduced form, NADH), 50 µM nitroblue tetrazolium (NBT), 10 µM phenazine methosulfate (PMS), and 50 µL of plasma sample. The color reaction of superoxide radicals and NBT was detected at 560 nm using UV-1700 PharmaSpect UV-Visible spectrophotometer (Shimadzu, Japan). Ascorbic acid was used as the standard antioxidant in this assay.

The GPx activity level was determined by the method of Paglia and Valentine. (1967) using a commercially available kit (The NWLSS™ Glutathione Peroxidase Assay). The catalase activity in erythrocytes was assayed using a kit (Randox, UK) which is based upon the methods of Aebi (1984) and Beers and Sizer (1957).

TAC was determined by ABTS assay as developed by Re *et al.* (1999). The ABTS radical cation was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate (final concentration). The ABTS radical cation solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. The reduction in absorbance of a specified volume of ABTS radical cation solution at 734 nm was noted after adding 10 µl of plasma samples after each minute for 8 minutes.

The reducing capacity of plasma samples was measured using method demonstrated by Benzie and Strain (1996). The FRAP reagent was prepared by using 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ solution in 40 mM HCl solution and 2.5 ml of 20 mM ferric chloride solution. The FRAP reagent (3 ml) was mixed with 100 µl of sample and 300 µl of distilled water. The absorbance was taken at 593 nm after every minute for 4 minutes. The results were compared with the standard curve obtained by using ferrous sulphate as standard reducing agent. For accuracy every experiment was repeated 3 times.

Statistical analysis

Statistical Package for Social Sciences (SPSS) software, version 13.0 for windows was used for all statistical analysis. Differences between the two groups *i.e.,* FM patients and controls were evaluated by the Student's *t* test. Data was analyzed using one way ANOVA followed by Dunken's multiple range tests. The level of significance was set at $P < 0.05$.

RESULTS

The demographic and clinical data of the FM patients before and after supplementation with vitamins are summarized in Table I. There were no differences in age and body mass index (BMI) between FM patients.

Superoxide is produced during cellular metabolic reactions. SOD, an antioxidant enzyme, changes superoxide anion into hydrogen peroxide and oxygen. A comparison of the percent inhibition (% inhibition) of plasma samples of control and FM patients with pre-supplementation and post-supplementation (vitamin E, vitamin C, *N. sativa* seeds) is shown in Table II. Percent inhibition of SOD in healthy females was 23.44±0.69 which was found to be markedly higher than 13.23±0.39 in pre-supplementation FM patients. The percent inhibition

of SOD was increased to 18.07±0.54 after supplementation with vitamin E, vitamin C, *N. sativa* seeds in FM patients (P<0.05).

Table I.- Demographic and clinical data of FM patients pre and post supplementation.

Parameters	FM patients pre-supplementation (n=50)	FM patients post-supplementation (n=50)
Age (Years)	42.93±1.59	42.93±1.59
BMI(kg/m^2)	24.53±0.36	24.53±0.36
Married (%)	70	70
Pain score (0-100)	73.1±13.2	55.5±10.3
Rheumatoid arthritis (%)	80	80
Systemic lupus erythematosus (%)	60	60
Chronic fatigue syndrome (%)	70	70
Depression (%)	50	50

Values are means±standard deviation (SD), BMI= Body mass index, %= percentage

The GPx levels (618.33±4.24 mU/ml) were found to be significantly low (P < 0.05) in FM patients without supplementation as compared to healthy young females (646.63±3.77 mμ/ml). There was a statistically non significant (P>0.05) improvement in GPx (622.40±3.72 mU/ml) levels after supplementation with vitamin E, vitamin C, *N. sativa* seeds (Table II).

Catalase levels (Table II) were found to be lower in FM patients before supplementation (53800.00±1271.93 U/ml) as compared to controls (57856.25±1505.27 U/ml) but the difference was statistically not significant. After supplementation with antioxidants, a decrease was noticed in the mean catalase level (53290.00±1116.23U/ml) (P > 0.05). Employing ABTS and FRAP assays, TAC values in FM patients without supplementation were also found to be significantly lower (P < 0.05) than those of healthy subjects, but the values increased appreciably after supplementation (Table II). The TEAC values were found to be 1.48 ± 0.04 TEAC mM in normal healthy females. The levels of TAC (ABTS) were markedly lowered to 0.94 ± 0.03 mM TEAC in FM patients prior to supplementation which increased to 1.46 ± 0.05 mM TEAC after supplementation with vitamin E, vitamin C and *N. sativa* seeds. It was observed that the TEAC levels returned to almost normal values after supplementation. This clearly depicts the powerful effect of supplementation on the plasma TAC level of FM patients. This effect may be considered as a combined effect of the anti-oxidants used and the individual contribution of each component needs further studies.

TAC (FRAP) levels were 352.81±5.75 μM in healthy individuals. The AOC (FRAP) concentrations were markedly depressed to 187.77±7.11 μM in FM patients prior to supplementation and increased to 362.07±4.74 μM after supplementation in FM patients. There was also significant (P<0.05) difference between the TAC (FRAP) of healthy subjects and baseline levels of FM patients. The mean VAS in FM patients before supplementation for pain was 90.30±1.52 which dropped to 77.80±1.65 after supplementation (P > 0.05).

DISCUSSION

This study was first of its kind in which we have tried to introduce the therapeutic role of vitamins and *N. sativa* seeds among FM patients in Pakistani population. The intensity of oxidative stress is measured not only by the production of free radicals but also by antioxidant enzymes. Antioxidant enzymes help in decreasing the oxidative stress by inactivating the oxygen free radicals. In the present study, 8 weeks of supplementation with vitamin C, vitamin E and *N. sativa* was found to elevate the antioxidant enzymes in blood in comparison with pre supplementation.

The results of present study indicating that FM patients are under oxidative stress, demonstrated by decreased levels of the antioxidant enzymes. In our study we found SOD and GPx levels of FM patients prior to supplementation were significantly lower than healthy subjects. These findings are in line with previous findings where a decrease in SOD level was reported in FM patients in Turkeys and European population (Bagis *et al.,* 2005; Ozgocmen *et al.,* 2005; Sendur *et al.,* 2009). It has also been reported in many studies that arthritis patients have low GPx levels as compared to healthy subjects (Popovici *et al.,* 2001; Karatas *et al.,* 2003;

Table II.- Comparison of enzymatic antioxidants between FM patients (pre and post supplementation) and control.

Parameters	Control (n=16)	FM patients	
		Pre-supplementation (n= 50)	Post- supplementation (n= 50)
% Inhibition of SOD	23.44±0.69	13.23±0.39*	18.07±0.54**
Plasma GPx (mU/ml)	646.63±3.77	618.33±4.24*	622.40±3.72*
Erythrocytes catalases (U/ml)	57856.25±1505.27	53800.0±1271.93	53290.0±1116.23
TEAC (mM)	1.48±0.04	0.94±0.03*	1.46±0.05**
FRAP value (μM)	352.81±5.75	187.77±7.11*	362.07±4.74**

Values are expressed as mean ± S.D; FM, fibromyalgia patients; * significantly different from controls, $P < 0.05$,
** significant difference from pre supplementation of patients= $P < 0.05$,
SOD, Superoxide dismutase; GPx, glutathione peroxidase; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant capacity.

Surapneni and Chandrasada, 2008). Current study also observed that SOD and GPx increased after supplementation with vitamin E, vitamin C and *N. sativa* seeds. It has been reported that higher vitamin C level with oral supplementation can improve tissues from oxidative damage (Thompson *et al.,* 2001).

Catalase concentration showed diversified behavior in FM patients. Lower level of catalase was observed which declined further after supplementation. Catalase is an enzyme, which catalyzes the decomposition of hydrogen peroxide to water and oxygen. The catalase levels declined significantly ($P < 0.05$) in FM patients, as has been reported in severe form of disease (Sendur *et al.,* 2009; Eisinger *et al.,* 2002) and also in arthritis patients (Popovici1 *et al.,* 2001; Surapneni and Chandrasada, 2008). In contrast to current findings increase in the catalase concentrations has been reported in arthritis patients (Vijayaraghavan *et al.,* 2005; Fulle *et al.,* 2000) demanding further investigations to confirm the role and levels of catalase in FM patients. The non effective role of vitamin C, E and *Nigella* seeds found on catalase activity in erythrocytes may be attributed to either weak solubility of vitamin C in lipids or two months supplement of these vitamins may be not sufficient to affect catalase activity. Similarly, the decrease in catalase activity in these patients may be attributed to either non sufficient doses or a non sufficient period of therapy.

In the present study, FRAP and ABTS assays were used to measure the total antioxidant capacity of plasma in control and patients (before and after supplementation). We demonstrated that AOC of plasma obtained from FM patients before supplementation was less than healthy subjects. The post- supplementation levels of AOC increased non-significantly in comparison to healthy subjects. Current findings are in line with previous findings in FM (Altindag and Celik, 2006) and arthritis patients. Cao *et al*. (1998) demonstrated effective role of vitamins in increasing AOC in FM.

However, VAS decreased markedly and significantly ($P<0.05$) in FM patients after supplementation. Nevertheless, it was still higher in comparison with healthy females. These observations are in accordance with the previous finding of European population in which moderate to high pain was recorded at VAS in FM patients, while no such observation was reported in normal individuals (Bagis *et al*., 2005). The decrease in pain at VAS scale has also been reported in FM patients treated with improved diet and vitamin supplementation in an open, non-randomized controlled study (Kaartinen *et al*., 2000). The difference between the baseline and post-supplementation VAS in FM patients was statistically not significant implying randomized double blind placebo controlled trials to evaluate the efficacy of vitamins in FM.

Furthermore, the study demonstrates that there is a strong correlation between FM and oxidative stress. Moreover, antioxidant supplementation plays a permissive role in the cure of oxidative stress, which appears to result in improvement of patient's health condition. More replicated studies are needed to evaluate the role of

antioxidants as therapeutic agent in FM. In parallel, the metabolic disturbances in FM might also have lowered the antioxidant levels which may be inadequate for scavenging the free radicals that arise. The enzymatic antioxidant levels in the present study were shown to decline with disease suggested that attacks by oxidative stress (may be due to decrease in endogenous antioxidant levels) may be altered with extracellular antioxidants (dietary antioxidants) and lifestyle changes, which may help in modulation of the disease (Booth *et al*., 2012).

CONCLUSION

To our knowledge current study is the first in Pakistani population which have tried to explore the therapeutic role of vitamin C and vitamin E and *Nigella sativa* seeds for lowering the oxidative stress among FM patients. However, further studies are needed in elaborating the role of antioxidant enzymes in FM. Moreover, randomized placebo controlled trials may explore the therapeutic role of vitamin E, vitamin C and *N. sativa* seeds.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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