Lipid and protein oxidation in female patients with chronic fatigue syndrome

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Submitted: 17 November 2010
Accepted: 4 April 2011

Arch Med Sci 2012; 8, 5: 886-891
DOI: 10.5114/aoms.2012.31620

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Abstract

Introduction: Chronic fatigue syndrome (CFS) is a widely recognized problem, characterized by prolonged, debilitating fatigue and a characteristic group of accompanying symptoms, that occurs four times more frequently in women than in men. The aim of the study was to determine the existence of oxidative stress and its possible consequences in female patients with CFS.

Material and methods: Twenty-four women aged 15-45 who fulfilled the diagnostic criteria for CFS with no comorbidities were recruited and were age matched to a control group of 19 healthy women. After conducting the routine laboratory tests, levels of the lipid oxidation product malondialdehyde (MDA) and protein oxidation protein carbonyl (CO) were determined.

Results: The CFS group had higher levels of triglycerides ($p = 0.03$), MDA ($p = 0.03$) and CO ($p = 0.002$) and lower levels of HDL cholesterol ($p = 0.001$) than the control group. There were no significant differences in the levels of total protein, total cholesterol or LDL cholesterol.

Conclusions: The CFS group had an unfavorable lipid profile and signs of oxidative stress induced damage to lipids and proteins. These results might be indicative of early proatherogenic processes in this group of patients who are otherwise at low risk for atherosclerosis. Antioxidant treatment and life style changes are indicated for women with CFS, as well as closer observation in order to assess the degree of atherosclerosis.

Key words: chronic fatigue syndrome, oxidative stress, malondialdehyde, protein carbonyl, atherosclerosis.

Introduction

Chronic fatigue syndrome is defined as fatigue lasting more than 6 months, associated with secondary symptoms, and inducing a 50% decreased ability to participate in ordinary activities [1]. It was first defined in 1988 by the US Center for Disease Control and Prevention (CDC), but the definition was later revised by Fukuda *et al.* by excluding any active or suspected medical or psychiatric affection and substance abuse as well as severe obesity as defined by a body mass index equal to or greater than 45 kg/m^2 [2]. The disorder is now a widely recognized problem and the diagnosis can be made after alternative medical and psychiatric causes of chronic fatiguing illness have been excluded [3]. Although there is still lack of a specific laboratory test or marker to identify it, chronic fatigue syndrome (CFS) should be considered when a patient presents with prolonged fatigue lasting 6 or more months (major criteria) and with at least

four of the following symptoms (minor criteria): self-reported impairment in short-term memory or concentration difficulties, sore throat, tender cervical or auxiliary lymph nodes, muscle pain, multi-joint pain without joint swelling or redness, headaches of a new type, pattern, or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 h [1].

In the primary care setting, the prevalence of CFS ranges from 3% to 20% depending on the diagnostic criteria used [4]. People of every age, gender, ethnicity and socioeconomic group can have CFS but it affects women at six times the rate of men [5]. Moreover, it seems that the symptoms of CFS are more severe in women: in the Chicago community-based sample, gender predicted fatigue severity, with women exhibiting higher fatigue scores than men. Also, within this sample women had significantly poorer physical functioning, more bodily pain, poorer emotional functioning, significantly more severe muscle pain, and significantly greater impairment of work activities [6, 7]. More than 80% of CFS sufferers go undiagnosed [8].

Despite the controversy surrounding CFS, there are numerous studies on the pathogenesis and treatment options for this condition [9]. Viral triggers and immune dysregulation, neuroendocrine or central nervous system dysfunction and muscular function disturbances have all been considered as pathophysiological factors [3, 5, 9-13]. There is mounting evidence that oxidative stress plays an important part in the pathogenesis of CFS by affecting different systems and tissues [14-21]. Moreover, the level of oxidative stress correlates with the severity of symptoms [19]. Investigations of the type and severity of oxidative stress in CFS have mostly dealt with lipid peroxidation (LPO) [17, 19, 28]. One of the most important LPO products is malondialdehyde (MDA), which serves as a potential biomarker of oxidative damage and disease severity [22-29].

Oxidative attack on proteins results in the formation of protein carbonyls (CO), often with the loss of functionality of the parent protein. To our knowledge, there has been only one study concerning protein oxidation and CFS, which showed a significant elevation of protein carbonyl levels in sera of CFS [30]. Protein carbonyls (CO) are reported to be the most frequently studied marker of oxidative damage to proteins [31] and the use of protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and stability of carbonylated proteins [32, 33].

The aim of this study was to determine the existence of oxidative stress and its possible consequences in female patients affected with CFS.

Material and methods

Study population

Consecutive patients who had been referred to the University Clinic for Infectious Diseases with a complaint of fatigue lasting for more than 6 months were considered for the study. As CFS is more frequent in female patients and the symptoms are more pronounced in this group, only females of generative age (15-45) were considered for the study. Also, the aim was to form a homogeneous group of patients regarding level of activity, body composition and general health status in order to limit influences on oxidative stress levels.

Patients were included in the study if they met the 1994 CDC definition: two major criteria (disabling fatigue lasting more than 6 months and exclusion of all other potential causes of fatigue) and at least four minor criteria (fever, sore throat, lymphadenopathy, muscle weakness, myalgia, headache, sleep disturbances and neuropsychological complaints).

Patients with the following conditions were also excluded from the study: acute severe illness during the previous 6 months, hypo or hypertension, pregnancy, chronic liver disease, thyroid dysfunction, suprarenal dysfunction, hypertension, diabetes mellitus, obesity (body mass index (BMI) greater than 30 kg/m^2), autoimmune disorders, a history of malignancy, alcohol or drug abuse and the presence of any psychiatric disorders. Also, patients who fulfilled the CFS criteria but had received any kind of medication for the condition were excluded from the study.

The control group comprised female subjects who had no medical complaints and had volunteered to participate in the study. They underwent the same medical evaluation process as the CFS group and were confirmed to be healthy. The control group was matched to the CFS group in terms of age and smoking habits.

The study was approved by the relevant ethical committees and all patients signed informed consent before entering the study.

The subjects entering the study underwent a complex medical evaluation. This included detailed history of the present illness, past medical history and a physical examination. Height and weight measurements were recorded for all subjects and BMI was calculated.

The levels of CFS symptoms were estimated using the Fibro Fatigues Scale (FFS), which is a self assessment 16-item questionnaire. Fibro Fatigues Scale has been used previously in numerous studies and has proved to be a useful and valid scale for assessing the degree of the symptoms in CFS in clinical studies [34-36].

After the examination, routine laboratory tests were performed. These included a complete blood count, erythrocyte sedimentation rate, C reactive protein, tests for autoantibodies, thyroid, kidney, suprarenal and liver function tests, as well as levels of total cholesterol (TC), triglycerides (TG), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C). Also, ELISA tests were performed to exclude the most important acute viral and bacterial infections that can influence oxidant status (Coxsackie B, adenovirus, EBV, CMV, HSV, HIV, HBV, HCV and *Borrelia burgdorferi*).

Oxidative stress markers

After inclusion in the study, 5 ml of venous blood was obtained from each patient and sera were immediately centrifuged and then refrigerated at –20°C.

Levels of MDA were measured using the OxiSelect MDA Adduct ELISA kit, one of the first commercial ELISA tests for research use only. The manufacturer revised protocol was followed. BSA standards or protein samples (10 µg/ml) were adsorbed onto a 96-well plate for 2 h at 37°C. The MDA-protein adducts present in the sample or standard were probed with an anti-MDA antibody, followed by an HRP conjugated secondary antibody. The MDA protein adducts content in an unknown sample was determined by comparing with a standard curve that was prepared from predetermined MDA-BSA standards. Sensitivity of the method varies from 1,875 pmol/mg to 120 pmol/mg MDA adduct.

Levels of CO were measured using the OxiSelect Protein Carbonyl ELISA kit, by Cell Biolabs. This test was developed for rapid detection and quantization of protein carbonyl. The quantity of protein carbonyl in protein samples was determined by comparing the absorbance with that of a known reduced/oxidized BSA standard curve. The sensitivity of the test ranges from 0.375 nmol/mg to 7.5 nmol/mg.

Statistical analysis

Data were analyzed using SPSS 8.0 software. Differences between the 2 groups were assessed by χ^2 tests, the Mann-Whitney test and ANOVA for several groups. The relationship between the various parameters was assessed by correlation. Data were expressed as mean (95% confidence interval for mean) and two-tailed *p*-values less than 0.05 were considered statistically significant.

Results

Twenty-four women who fulfilled the CFS criteria and 19 healthy women, aged 15-45 years, were included in the study. According to the design of the study the two groups, i.e. the CFS group and the control group, were similar in age and smoking habits. Subjects in both groups were nonobese and the difference in BMI between the two groups was not significant (Table I). ANOVA statistical analysis showed a significant difference between groups in all 12 questionnaire items ($p < 0.0001$). The median of the total FFS score was higher in the CFS group than in the control group ($X = 21.90$,

Table I. Lipid profile and oxidative stress markers in patients with CFS and in healthy controls

Variable	CFS group ($n = 24$) Mean (min.-max.; SD)	Control group ($n = 19$) Mean (min.-max.; SD)	Value of *p*
Age	33.33 (24-47; 5.65)	33.37 (25-44; 5.28)	0.9
Smokers:	9	7	0.8
Heavy	4	3	1.0
Light	5	4	1.0
BMI [kg/m^2]	22 (19-25; 0.3)	24 (18-28; 0.4)	0.9
C-reactive protein [mg/l]	1.05 (1-8.2; 1.4)	1.2 (0-21; 3.3)	1.0
Fibrinogen [g/l]	3.1 (1.8-5.3; 0.67)	3.4 (2.2-6.2; 0.9)	0.9
Glucose in blood [mmol/l]	4.5 (3.1-5.8; 0.8)	4.8 (3.2-5.6; 0.6)	0.9
Triglycerides [mmol/l]	1.038 (0.5-2.0; 0.39)	0.798 (0.5-1.4; 0.27)	0.03
Total cholesterol [mmol/l]	4.636 (3.0-6.7; 1.1)	4.895 (3.9-6.7; 0.81)	0.2
LDL cholesterol [mmol/l]	3.253 (2.2-4.7; 0.83)	3.024 (0.3-4.7; 0.91)	0.6
HDL cholesterol [mmol/l]	1.181 (0.9-1.6; 0.23)	1.474 (0.9-1.9; 0.28)	0.001
MDA [pmol/mg]	15.05 (0.0-30.1; 9.12)	10.13 (0.0-22.4; 8.92)	0.03
Protein carbonyl [nmol/mg]	3.18 (0.3-5.9; 1.41)	1.78 (0-4.2; 1.09)	0.002

**To convert mmol/l of blood glucose to mg/dl, multiply by 18; to convert mmol/l of total cholesterol, HDL or LDL cholesterol to mg/dl, multiply by 39; to convert mmol/l of triglycerides to mg/dl, multiply by 89*

SD = 5.23 vs. X = 2.93, SD = 1.87, $p < 0.0001$), as expected.

There were no significant differences in the routine laboratory tests (complete blood count; erythrocyte sedimentation rate; tests for autoantibodies; and tests of thyroid, kidney, suprarenal and liver function). The results of all tests were within the normal range for both groups, which was expected as patients with co-morbidities were not included in the study.

C-reactive protein, fibrinogen and blood glucose levels were also in the normal range and did not differ between the groups (Table I).

However, in the case of the levels of TG and HDL-C, there was a difference between groups (Table I). The CFS group had higher levels of TG and lower levels of HDL-C. No such difference was found in the levels of TC and LDL-C. The MDA levels were significantly higher in the CFS group. Levels of CO were also higher in the CFS group than in the control group. The FFS score was correlated with both MDA and CO levels and no significant correspondences were found.

Discussion

In this study we found increased levels of both MDA and CO levels in the CFS group. We can therefore conclude that oxidative stress is present in our group of female CFS patients.

Although there are still many controversies concerning CFS, it is now a well-established condition with several million sufferers. It is likely that physicians avoid making this diagnosis due to the fact that CFS is still a *diagnosis per exclusionem* and that it has well-known overlapping syndromes. There is also the fact that many CFS patients do not seek medical attention due to their social and/or economic status. Nevertheless, many scientists are investigating the answers to the numerous questions CFS still poses: epidemiology, etiology, pathogenesis and treatment have not been completely defined.

Among the possible mechanisms of pathogenesis, oxidative stress is especially interesting as it can lead to potential therapeutic options and secondary prevention. It appears to play an important role in the development of, and also contributes to, some of the symptoms in CFS [14-21].

The MDA is a product of lipid peroxidation of polyunsaturated fatty acids and it can be found in physiological conditions in lower concentrations. High levels of MDA are indicative of oxidative stress. The MDA is potentially very noxious [22] and its toxicity can be directed towards cardiovascular stability. Primarily, MDA reacts with apoB fractions of oxidized lipoproteins (LDL), which causes impaired interaction of the modified lipoproteins and macrophages [22, 37]. This phenomenon is the basis of atherogenicity. Another probable toxic action of MDA involves collagen. Even if the nature of the cross-link has not yet been determined in detail, the inter-molecular cross-linking of collagen through MDA may significantly contribute to the stiffening of cardiovascular tissue [22]. It is quite possible that the presence of MDA at higher levels may predict the insurgence of vascular pathologies. Polidori and collaborators found higher levels of antioxidants and lower levels of MDA in healthy subjects when compared in a case-control study with congestive heart failure patients [23]. Plasma from atherosclerotic patients was richer in MDA studied by Tamer *et al.* [38]. In two studies reported by Boaz *et al.* [39, 40], serum MDA values were markedly higher in hemodialysis patients with cardiovascular complications than in those without such complications.

The second significant finding in the CFS group was raised levels of CO, the product of protein oxidation. As generation of carbonyl derivatives occurs by many different mechanisms, the level of CO groups in proteins is widely used as a marker of oxidative protein damage, often correlating well with the progression of the disease. To date, accumulation of CO has been observed in several human diseases including Alzheimer's, Parkinson's and Huntington's diseases; diabetes; inflammatory bowel disease; adult (or acute) respiratory distress syndrome; chronic renal and lung diseases; sepsis; arthritis; preeclampsia and amyotrophic lateral sclerosis [31, 41, 42]. The level of oxidized proteins also increases with aging and in age-related diseases [31-33], which is why it was important that the two groups studied were matched in terms of age. Protein oxidation products in CFS patients have so far been investigated only by Smirnova and Pall [30]. This group had similar results to ours but they found even more significantly elevated CO levels ($p < 0.0005$) in CFS patients compared to controls.

The third important finding of our study was the unfavorable lipoprotein profiles in the CFS group. We found significantly lower levels of HDL-C and higher levels of TG in the study group. HDL-C is the beneficial form of blood cholesterol which protects the artery wall from atherosclerosis [43]. Even in individuals whose LDL levels are low, HDL remains a strong independent predictor of coronary artery disease risk [44]. Besides preventing cholesterol accumulation in cells of the artery wall, HDL-C acts as an anti-inflammatory agent by degrading lipid oxidation products [43-47]. In this way, HDL-C acts against products of lipid peroxidation which are potentially atherogenic. We also found higher TG levels in the CFS group of patients. High levels of triglycerides are a well-known factor for atherosclerosis and are included in the Framingham charts

that have been widely used by clinicians to quantify an individual's absolute risk for coronary heart disease.

Oxidative stress plays an important role in atherosclerosis. The LDL carries cholesterol from the liver to the circulatory system. It is susceptible to oxidation by reactive oxygen species (ROS), and damage is seen both to the lipid and to the protein moiety [48, 49]. In an environment where oxidative stress is present and where there are lower levels of antioxidant HDL-C and higher levels of TG, oxidation of LDL might be more pronounced [50].

To date, only a few studies have been conducted that have implicated an increased risk of cardiovascular events in CFS patients with no comorbidities. Kennedy *et al.* used 8-Iso-prostaglandin $F_{2\alpha}$ as a lipid peroxidation marker with a potential potent biological activity [19]. They found significantly elevated levels of F2-isoprostanes and oxidized LDL and decreased HDL in nonobese, normotensive patients. Isoprostane levels were even greater in CFS patients who had additional cardiovascular risk factors. Similarly to our study, they concluded that CFS patients have a lipid profile and oxidant biology that is consistent with cardiovascular risk.

Three years later, the same group observed a relationship between inflammation and oxidative stress and augmentation index, a measure of arterial stiffness [51]. They have shown that patients with CFS have higher serum CRP levels, elevated levels of isoprostanes and oxLDL, and significantly increased AIx@75, which indicates a significantly increased risk of a future cardiovascular event in these patients.

We also suggest a follow-up of our group of patients which should include an imaging technique in order to assess possible early atherosclerosis.

One limitation of the present study is that we did not measure the levels of oxidized LDL, which is associated with the development of atherosclerosis. A second limitation is the small number of patients. This is mainly due to difficulties in recruiting patients due to the relatively low incidence and prevalence of CFS. Furthermore, we were faced with skepticism about CFS both from the patients and the medical community, which made it difficult to mobilize patients.

In conclusion, in the present study we used two reliable biomarkers of oxidative stress rarely used before with CFS patients. We found signs of lipid and protein oxidative damage, as well as an unfavorable lipid profile in nonobese premenopausal female CFS patients with no comorbidities. We suggest that in this otherwise low risk group there are signs of possible early atherogenesis. This would mean that women who have CFS are at an increased risk for cardiovascular events and are in need of early intervention including antioxidant supplementation and LDL lowering strategies.

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