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Oxidative Stress in Relapsing-remitting Multiple Sclerosis Patients

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ABSTRACT

Introduction: In multiple sclerosis (MS), oxidative stress (OS) plays a vital role in the neurodegeneration process. Cholesterol and lipids in the myelin sheath supplied by low-density lipoprotein (LDL) are also vital for nerve cells. In OS, lipid peroxidation occurs in LDL.

To investigate the OS biomarker such as prooxidant-antioxidant balance (PAB), malondialdehyde (MDA) and their correlation with LDL and oxidized LDL (Oxi-LDL) in patients with relapsing-remitting MS.

Methods: Blood samples from 18 patients with relapsing-remitting MS and 18 healthy subjects were collected to measure the OS biomarkers.

Results: In the patients' group in comparison to the control group: PAB, white blood cells (WBC), and neutrophils significantly increased ($P<0.05$), but there was no difference between the relapsing and remitting phase; MDA significantly increased in the relapsing phase ($P=0.013$) but was marginally significant in the remitting phase ($P=0.068$). There was no significant difference in LDL and Oxi-LDL between the two groups. Only the lymphocytes were different between the relapse and remission phases.

Conclusion: The importance of OS in the process of MS disease was confirmed and a PAB assay can be used to determine OS levels.

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Introduction

Multiple sclerosis (MS), a chronic autoimmune disorder of the central nervous system (CNS), is one of the most prevalent disabilities in young adults, identified by oligodendrocyte loss, demyelination, inflammation, and axonal damage (1).

MS can present in different forms, like primary progressive (PP-MS), relapsing progressive (RP-MS), secondary progressive phenotypes (SP-MS), and relapsing-remitting (RR-MS), which is the most prevalent form (80% of cases) (2). In MS patients, myelin in the CNS is damaged, and a large majority of myelin-producing oligodendrocytes are lost (3). Progressive loss of myelin and deterioration of its component proteins may further inflame the autoimmune response (4). As myelin consists of 70% lipids, human serum lipoproteins are implicated in the transportation of lipids, modulation of membrane lipid distribution, and regulation of signal transduction in CNS (5). Under normal conditions, elevated low-density lipoprotein (LDL) levels are present in CNS to transport across the brain-blood barrier (BBB) (6). Dyslipidemia may contribute to inflammatory processes, leading to the generation

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of adhesion molecules and the recruitment of leukocytes. The recruitment of immune cells in the activated endothelium of the BBB is critical in MS pathogenesis (7). Shreds of evidence on oxidative stress and clinical involvements have shown that the formation of free radicals lead to oxidative stress, which performs a role in neurodegeneration pathogenesis (8). In a healthy condition, the prooxidants and antioxidants remain in a balance status (9). Oxidative stress (OS) is an imbalance status between antioxidants and prooxidants in favor of the prooxidants that damage cellular ingredients, including proteins, lipids, and DNA. For ROS generation, the major exogenous factors are environmental pollution, radiation, cigarette smoking, certain foods, and drugs. The major endogenous factors are mitochondrial respiratory chain and various intracellular enzymes (10).

Reactive oxygen species (ROS) induce peroxidation of biological molecules, particularly lipoproteins that are involved in the pathogenesis of MS (11). The CNS is sensitive to oxidative stress because of the brain's high oxygen consumption, rich lipid content, and lack of antioxidant agents (12). Since many studies have demonstrated that oxidative stress has a main role in multiple sclerosis (8,12), in this study, prooxidant-antioxidant balance (PAB), malondialdehyde (MDA), Oxi-LDL, as markers of OS, and LDL, were measured in relapsing-remitting MS (RR-MS) patients; and their correlations were evaluated, which may provide evidence for diagnostic and therapeutic implications.

Materials and Method

In this case-controlled study, following informed consent, 18 RR-MS patients, and 18 control subjects were enrolled from Qaem hospital, Mashhad. RR-MS patients had been diagnosed to be in the secondary progressive phase and referred to Qaem hospital for corticosteroid therapy. Since this study is the first study to determine PAB in MS patients, therefore, sample size was determined with at least 18 participants in each group for this pilot study. Exclusion criteria were the use of corticosteroids within the past 30 days, the presence of infections or fever in the past 30 days, pregnancy, use of vitamin supplements, obesity, diabetes, thyroid dysfunction, and renal disorders. A 5 cc blood sample was obtained in the relapsing phase before corticosteroid therapy in Qaem hospital. Plasma was isolated from red blood cells by centrifugation at 2500 rpm, 4°C, and 15 minutes. Aliquots of supernatant (0.5–1 mL) were immediately frozen at -20°C and not thawed until analysis. Three months later, another blood sample was taken from the same patients who were in the remitting phase in the same situation. The control group consisted of 18 subjects from the identical geographic region, who did not show either laboratory or clinical attributes of autoimmune, liver, heart, or renal disorder. Also, the control subjects expressed that they were not consuming any antioxidant supplements or anti-inflammatory drugs. The patients' nutritional status did not vary from the control group, and none of the subjects were under a particular diet. Parameters like age, ethnicity, sex, body mass index (BMI), and smoking were matched in the patient and control groups.

Biochemical parameters

Prooxidant-antioxidant balance (PAB) method

The PAB assay was done according to the method explained by Alamdari et al. (13). A low PAB value indicates that AO exists at greater concentrations than prooxidants (OX); a high level of PAB indicates more prooxidants exist than AO.

Serum Oxi-LDL Evaluation

Oxi-LDL was measured by the ELISA kit (EASTBIOPHARM, CK-E10869) using anti-Oxi-LDL monoclonal antibody FOH1a/DLH3, the capture antibody, and an anti-human apolipoprotein B (apoprotein B) monoclonal antibody labeled with horseradish peroxidase.

MDA Measurement

Samples were added to the reaction mixture including phosphate buffer and $FeCl_3$ (pH=7.4). The reaction was arrested by adding 10% trichloroacetic acid (TCA), followed by 0.67% TBA, and the tubes were placed in boiling water for 20 minutes. The tubes were then transferred to an ice bath, and the contents were centrifuged for 10 minutes. The amount of MDA formed in each of the samples was evaluated by measuring the supernatant's optical density using tetra ethoxy propane (TEP) as a standard. MDA content was indicated as $nmol.mg^{-1}$ protein.

Serum LDL Measurement

Serum LDL level was measured by a biochemical autoanalyzer (BT3000, Parsazmoon, Iran) through an enzymatic reaction using cholesterol esterase and peroxidase. The procedure was carried out based on the manufacturer's instructions, and the results were shown in mg/dL.

Hematology analysis

CBC count was performed by the Sysmex XS800i, a hematology analyzer with fluorescence technology (Diamond Diagnostics-USA).

Statistical analysis

The data were assessed by the statistical analysis software SPSS version 16. Descriptive statistics were used to analyze data. The distribution of sex, smoking, and ethnicity was analyzed using a chi-square test. Comparisons between the control group and MS patients were done with the Mann–Whitney and independent t-test (for non-parametric and parametric variables, respectively). Comparisons between MS patients in the relapsing and remitting phase of the disease were performed using the Wilcoxon test and paired t-test (for non-parametric and parametric variables, respectively). All the results were considered meaningful when $p<0.05$.

Results

After neurological examinations, RR-MS was diagnosed in 18 patients. The mean age of participants was 29.21 (22-42) years. The demographic characteristics of RR-MS patients and the control group are presented in Table 1.

The mean PAB value in MS patients was 157.550±12.31 in the relapse phase, 156.766±13.81 in remission, and 118.539±9.58 in the control group. A significant increase in PAB value in both phases of MS was seen in comparison to the control group (Table 2).

MDA increased significantly in the relapsing phase (0.314±0.089) in comparison to the control group (0.119±0.043, P=0.013), but was marginally significant in the remission phase (0.218±0.054, P=0.068). There was no meaningful difference in MDA between the two phases (Table 3).

The LDL in MS patients was 81.89±3.087 in the relapse phase, and 82.17±3.037 in remission, which is significantly less than in the control group (112.96±7.321). There was no significant difference in LDL in the relapse and remission phases (Table 4).

There was no significant difference in Oxi-LDL among patients in the relapse phase, remission phase, and control subjects. The Oxi-LDL was 3573.978±584.397 in relapse and 3932.897±647.158 in remission and in the control group 3677.669±626.268 (Table 5).

There was no correlation of PAB with MDA, LDL, and Oxi-LDL.

White blood cells (WBC) were significantly increased in the relapse phase 7.956±0.472 and the remission phase 8.500±0.557 in comparison to the control group 6.522±0.371 (P<0.05), but there were no significant differences between the relapse and remission phase (P>0.05).

The neutrophils (4.900±0.438) in the relapse and remission (5.467±0.477) were significantly more than the control group (4.200±0.325), but there were no meaningful differences between the relapse and the remission phase (P>0.05). There was no significant difference in lymphocyte (2.483±0.217) in the relapse phase, but significance existed in the remission phase (2.928±0.223), in comparison to the control group (2.300±0.140). There was no meaningful correlation of PAB with WBC, neutrophils, and lymphocytes (P> 0.05) (Table 6).

Table 1. Characteristics of the RR-MS patients and healthy controls

		Controls	RR-MS patients	P-Value
Age	y 20> y 20-30 y 30-40 y 40<	% 6.0 % 55.0 % 33.0 % 6.0	% 5.9 % 47.0 % 35.3 % 11.8	P>0.05
Sex	Male Female	% 22.5 % 77.5	% 22.2 % 77.8	P>0.05

RR-MS: Relapsing Remitting- multiple sclerosis.

Table 2. Mean level of PAB in MS and control group

Comparison	PAB, Mean ± SEM	P-value
MS (relapse) **Control**	157.550±12.31 118.539±9.58	0.017*
MS (remission) **Control**	156.766±13.81 118.539±9.58	0.029*
MS (relapse) **MS (remission)**	157.550±12.31 156.766±13.81	0.955

*Statistically significant, MS: Multiple Sclerosis, PAB: Prooxidant- Antioxidant Balance

Table 3. Mean MDA in MS and control group.

Comparison	Mean ± SEM	P-value
MS (relapse) **Control**	0.314 ± 0.089 0.119 ± 0.043	0.013*
MS (remission) **Control**	0.218 ± 0.054 0.119 ± 0.043	0.068
MS (relapse) **MS (remission)**	0.314 ± 0.089 0.218 ± 0.054	0.112

*Statistically significant, MDA: Malondialdehyde

Table 4. Mean LDL in MS and control group.

Comparison	LDL, Mean ± SEM	P-value
MS (relapse) **Control**	81.89 ± 3.087 112.96 ± 7.321	0.0001*
MS (remission) **Control**	82.17 ± 3.037 112.96 ± 7.321	0.0001*
MS (relapse) **MS (remission)**	81.89 ± 3.087 82.17 ± 3.037	0.927

*Statistically significant, MS: Multiple Sclerosis, LDL: Low Density Lipoproteins

Table 5. Mean Oxi-LDL in MS and control group

Comparison	Ox-LDL, Mean ± SEM	P
MS (relapse) Control	3573.978± 584.397 3677.669± 626.268	0.945
MS (remission) Control	3932.897 ± 647.158 3677.669± 626.268	0.963
MS (relapse) MS (remission)	3573.978± 584.397 3932.897 ± 647.158	0.349

Multiple Sclerosis, Ox-LDL: Oxidized Low Density Lipoproteins

Discussion

In this study, the increased markers of oxidative stress (PAB and MDA), WBC, and neutrophils were seen in both phases of the disease (relapsing and remitting) in comparison to the control group but it did not differ significantly between both phases. There was no significant difference in lymphocytes in the relapse phase, but significance existed in the remission phase in comparison to the control group. There was no significant difference in LDL, or Oxi-LDL among patients in the relapse phase, remission phase, and control group. No correlation of PAB was seen with MDA, Oxi-LDL, LDL, WBC, neutrophils, and lymphocytes.

About 85% of MS patients are in the RR-MS phase at the time of the diagnosis (14). The etiology of MS is unclear although numerous investigations have proposed that oxidative stress is a significant etiology of MS (15). Additionally, autoreactive lymphocytes are the primary inflammatory causes of CNS that begin the disorder process (16). Inflammatory signs were observed in biopsied plaques, including lymphocytes and macrophages, and the MS patient's serum, including myelin reactive T lymphocytes (17). Microglia cells are present in inflammatory situations by releasing cytokines, oxidative products, and free radicals, which are toxic to myelin (18). Shreds of evidence illustrate mitochondrial dysfunction and oxidative stress as critical factors of common progressive neurological disorders (19). Antioxidants are a promising treatment for decreasing and preventing the disease's progress (20). ROS damages lipids, proteins, and nucleic acids and renders them to cell death. Their generation elevates through various pathological situations (21, 22). ROS, by its possible role in tissue damage in MS, provokes inflammatory responses (23).

Oxidative stress causes neurodegeneration through bioenergetic failure, depletion of antioxidant defenses, damage of bio-molecules, microtubular disruption, ion channel activation, demyelination, neuroinflammation, mitophagy impairment, and apoptosis of the neuronal cell, in which these events contribute to MS pathogenesis (24). Although various mechanisms are engaged in the demyelination and neurodegenera-

Table 6. White blood cell (WBC) count and WBC differentiation

	MS patients (relapse)	MS patients (remission)	Control
WBC count	7.956 ± 0.472[a]	8.500 ± 0.557[b]	6.522 ± 0.371
Neutrophils	4.900 ± 0.438	5.467 ± 0.477[c]	4.200 ± 0.325
Lymphocyte	2.483 ± 0.217	2.928 ± 0.223[d]	2.300 ± 0.140

a, b, c and d: significant difference compared with control group (independent T-test), MS: Multiple Sclerosis

tion in MS, multiple research shows that oxidative stress has a vital role in MS pathogenesis related to myelin and oligodendroglia degeneration that ultimately causes neuronal death (25). Notably, high concentrations of prooxidant agents in the serum of MS patients are found (26). A study has shown a remarkably lower capacity ($p<0.001$) of total antioxidants in the serum of MS patients in comparison to healthy subjects (27).

Evidence implies that infiltrated macrophages are among the primary ROS sources in CNS inflammation in patients with MS (28). Infiltrated macrophages through ROS lead to neuronal damage via their interaction with lipids, proteins as well as nucleic acids, and disruption of membrane integrity of neurons (29). Therefore, high ROS generation is among the most critical factors in inflammation and neuronal damage, and there is a mutual correlation between oxidative stress and inflammation wherein they boost each other (30).

White blood cells are known as recourses of oxidative stress in inflammatory diseases (31) and the role of neutrophils in the pathogenesis of MS is complicated (32). They can play dual effects by omitting damaged myelin particles and secreting growth factors and on the other hand, they can adversely affect the disease by producing pro-inflammatory cytokines (33). It has been reported that lymphocyte levels are higher in MS patients and they are considered to be correlated with axonal injury (34). In our study, there were more lymphocytes in MS patients than in healthy controls, but just in the remitting phase it was significant in comparison to the control group.

Mariani et al. showed that lipid peroxidation products alter cell membrane permeability and induce cell dysfunction, and oxidation of lipids by prooxidants could perform a role in the pathogenesis of MS (35). Therefore, the product of lipid peroxidation such as MDA can be predictive of the disease stage. Our results showed MDA increased significantly in the relapsing phase but was marginally significant in the remitting phase.

The limitations of this study are the small sample size and conducting the research in one academic center. Hence, it is suggested that additional studies be run in multiple centers to substantiate the finding of this study. The strengths of study are the cost effectiveness, generalizability, and reliability of the PAB method, which could be done easily in every clinical laboratory.

Conclusion

Oxidative stress plays a main role in MS patients and PAB assay can be used to determine oxidative stress levels.

Statement of ethics

The procedures done in this study were approved by the Mashhad University of Medical Sciences (MUMS); project no.: 930202.

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Conflict of interest

The authors declare that there is no conflict of interest

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