Role of antioxidant and oxidative stress levels in multiple sclerosis Iraqi patients

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Abstract. Multiple Sclerosis is a multifactorial disease characterized by demyelination and inflammation of the central nervous system. Accumulating data indicate that changes in the balance of antioxidants and oxidants contributed to the MS pathophysiology. The aim of the study to obtain a better understanding of the oxidative stress process to select a proper method of predicting the prognosis of the relapsing-remitting MS (RRMS). Serum of 97 patients (30 newly diagnosed RRMS patients and 67 RRMS patients underwent treatment) and 35 healthy individuals as control group. Serum levels of 25-hydroxycholecalciferol [25(OH)D3], vitamin E, vitamin C, malondialdehyde, protein carbonyl, and 8-hydroxydeoxyguanosine. There was a negative correlation between MDA and antioxidant (vitamin C and vitamin E), whereas a positive correlation between MDA and other oxidative stress (PC and 8-OHdG). Receiver operator characteristic analysis showed that PC was an ideal marker while vitamin C, MDA, 8-OHdG were excellent markers. Our data showed that the above markers as a mean could be used to predict MS disease and the extent of its progression.

Keywords: Multiple sclerosis, Antioxidants, Vitamin C, Vitamin E, 25(OH)D3, MDA, 8-OHdG.

1. Introduction

Multiple sclerosis (MS) is a mechanism of progressive demyelination and thought to be an autoimmune disorder of uncertain etiology. MS is characterized by infiltration of immune cells, mainly macrophages and T lymphocytes, loss the sheath of myelin that covers and protects nerve fibres and accelerates the transmission of the electrical signal, leading in numerous neurological disorders including deficits in sensory, motor, cognitive and psychology. It is believed that the underlying mechanism of causing MS is either the damage caused by the immune system or oligodendroglia cell apoptosis, also oxidative stress and dysfunction of mitochondria may be involved in the MS pathophysiology [1]. The active form of vitamin D3 1,25(OH)2D3 can be synthesized directly in the cell’s cytoplasm from 25(OH)D3 present in the blood, and it binds to a specific receptor found in many cells, including neurons and immune cells [2]. There are two types of 1,25(OH)2D3 receptors, a nuclear receptor through which the action of target genes is regulated by Inhibition or activation of transcription and expression of those genes and thus regulates protein synthesis. Therefore, the immune cells involved in causing MS and that have 1,25(OH)2D3 receptors may increase or decrease the manufacture of pro- or anti-inflammatory cytokines related to the inflammatory component of MS depending on the genetics and the level of 25(OH)D3 [3,4]. 1,25(OH)2D3 can also link to the membrane receptor that could be accountable for the actions of non-genomic mechanisms of 1,25(OH)2D3 [5]. Recently, one of the main factors accountable for demyelinating has also been oxidative stress (OS) and reactive oxygen species (ROS). The imbalance between OS factors and antioxidants lead to the stimulation of inflammatory processes by OS [6]. Therefore, consideration is given to the equilibrium between the level of compounds including content of protein carbonyl, peroxidation levels of lipid, oxidative harm of DNA and catalase, superoxide dismutase, vitamin C and vitamin E [7]. The formation of free radicals is the consequence of hyperactivity of the enzyme OS [8]. These free radicals attack the various groups of biomolecules (carbohydrates, proteins, lipids, DNA, and RNA), causing modifications of these biomolecules, damage to the mitochondria and ion channels, enabling activates the apoptosis pathways. In addition, the energy...
balance of neurons is disrupted and this leads to neurodegeneration. Therefore, understanding the connection between OS and MS is so important [7].

2. Experimental section

2.1. Study group
This study included 132 volunteers living in urban and rural areas of Iraq. All subjects have given written consent to share in this study which was conducted in the department of multiple sclerosis in Baghdad Teaching Hospital/Medical City from June 2018 to January 2019. The MS diagnosis was according to the criteria of McDonald, and all patients had RRMS. The patients of RRMS were divided into two groups. Group I consisted of 30 newly diagnosed RRMS patients within their ages ranging 19-47 years, while group II involved 67 RRMS patients with treatment (interferon β1b or fingolimod according to medical guidelines) their ages ranging 26-60 years. A total of 35 healthy subjects volunteers served as controls (group III) with age ranged 21-48 years.

2.2. Blood collection
Five milliliters of venous fasting blood samples were obtained from each RRMS patients and control by using 5 mL plastic disposable syringes, the sample let to clot for 30 minutes at 25˚C in Gel & Clot activator tube, after the centrifugation at 1500 xg for 10 minutes, the serum was collected and stored at -80˚C until assayed the time of analysis of 25(OH)D3, vitamin C, vitamin E, MDA, PC and 8-OHdG. Patients suffering from chronic or acute diseases, such as diabetes mellitus, hypertension, kidney or liver diseases were excluded from this study.

2.3. Anthropometric measurements
Body Mass Index (BMI) was determined by dividing weight (kg) by height^2 (m^2). Patients were considered obese if their index of body mass was 29.9 (kg/m^2) or more [9].

2.4. Laboratory analysis
Serum levels of 25(OH)D3, vitamin C, vitamin E, MDA, PC and 8-OHdG were assayed by a double-antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kits obtained from (Melsin Medical Co., Limited, China) according to the provided assay procedure. Each assay was calibrated using its standard curve following the manufacturer's instructions and used the instruments (Reader and Washer, HUMAN, Germany).

2.5. Statistical Analysis:
All study data are shown as mean±standard division (SD) and analysed statistically with social sciences (SPSS) statistics version 23 program. One-way variance analysis (ANOVA) was used to measure the difference among groups. In addition, MedCalc version 19.4.1 used ROC to evaluate the area under the curve (AUC). The best cut off point of the studied markers, sensitivity, specificity, PPV and NPV were also calculated. A P value of <0.05 was considered as statistically significant.

3. Results
Table 1 showed the demographic characteristics of RRMS patients and control groups which participated in the current study. No significant differences (P>0.05) were observed in age, weight, height and BMI.

Table 1. Demographic characteristics of the study groups.

| Parameters  | Group I, N=30 Mean ± SD | Group II, N=67 Mean ± SD | Group III, N=35 Mean ± SD | P-value |
|-------------|------------------------|--------------------------|---------------------------|---------|
| Age (Years) | 31.633 ± 8.206         | 34.597 ± 7.877           | 34.171 ± 8.375            | NS      |
Weight (Kg) 77.100 ± 8.991 74.761 ± 14.085 78.100 ± 16.755 NS
Height (m) 1.661 ± 0.086 1.671 ± 0.075 1.652 ± 0.110 NS
BMI (Kg/m²) 28.018 ± 3.521 26.711 ± 4.377 28.810 ± 6.698 NS

Group I: newly diagnosed RRMS patients without treatment, Group II: RRMS patients with treatment, Group III: Control group, N: number of subjects, SD: standard deviation, NS: non-significant at P<0.05.

Table 2A revealed that there was highly significant difference (P<0.05) in all parameters which used in this study except, in the serum level of 25(OH)D3 which found a non-significant difference (P>0.05) among the study groups. On the other hand, the levels of 25(OH)D3, vitamin C, vitamin E, PC and 8-OHdG did not show any significant differences (P>0.05) between group I and group II, except for MDA level, which showed a significant difference at P<0.05 (Table 2B).

**Table 2A. The comparison of antioxidant vitamins and oxidative stress parameters in serum in the study groups**

| Parameters | Group I | Group II | Group III | P-value |
|------------|---------|----------|-----------|---------|
| 25(OH)D3 (ng/mL) | 10.287 ± 2.333 | 10.117 ± 2.548 | 10.964 ± 4.918 | NS |
| Vitamin E (µmol/L) | 16.751 ± 1.837 | 13.856 ± 2.872 | 25.807 ± 14.356 | 0.0001** |
| Vitamin C (µmol/L) | 19.128 ± 2.908 | 17.514 ± 3.778 | 42.413 ± 8.894 | 0.0001** |
| MDA (nmol/mL) | 1.646 ± 0.322 | 1.540 ± 0.521 | 0.504 ± 0.251 | 0.0001** |
| PC (nmol/L) | 1.775 ± 0.513 | 1.828 ± 0.803 | 0.577 ± 0.305 | 0.0001** |
| 8-OHdG (ng/mL) | 2.873 ± 0.572 | 2.752 ± 0.932 | 1.417 ± 0.356 | 0.0001** |

SD: standard deviation, **highly significant at P<0.05, NS: non-significant at P>0.05.

**Table 2B. Post hoc analysis of multiple comparisons in the study groups.**

| Parameters | Group I&Group III | Group II&Group III | Group I&Group II |
|------------|------------------|--------------------|------------------|
| 25(OH)D3 (ng/mL) | NS | NS | NS |
| Vitamin E (µmol/L) | 0.0001** | 0.0001** | NS |
| Vitamin C (µmol/L) | 0.0001** | 0.0001** | NS |
| MDA (nmol/mL) | 0.0001** | 0.0001** | 0.035* |
| PC (nmol/L) | 0.0001** | 0.0001** | NS |
| 8-OHdG (ng/mL) | 0.0001** | 0.0001** | NS |

**highly significant at P<0.05, *significant at P<0.05, NS: non-significant at P>0.05.**

Our results reported in Table 3 a negative significant correlation between MDA level and antioxidant vitamins (vitamin E and vitamin C) in Group I and Group II. The results also showed a positive significant correlation between MDA level and oxidative stress levels (PC and 8-OHdG). No correlation was found between MDA level and 25(OH)D3 level in both groups of RRMS patients.

**Table 3. The correlation between MDA level and 25(OH)D3, vitamin E, vitamin C, PC and 8-OHdG in Group I and Group II.**

| Parameters | Group I | Group II |
|------------|---------|----------|
| MDA vs 25(OH)D3 | r | P-value |
| Group I | -0.033 | NS |
| Group II | 0.153 | NS |
The results in Table 4 showed that there was negative significant correlation between PC level and antioxidant vitamins levels (vitamin E and vitamin C) in Group I and Group II, the results also revealed that there was positive significant correlation between PC level and oxidative stress levels (MDA and 8-OHdG). While the results did not show a significant correlation between PC level and 25(OH)D3 level in both of RRMS patients groups.

**Table 4.** The correlation between PC level and 25(OH)D3, vitamin E, vitamin C, MDA and 8-OHdG in Group I and Group II.

| Parameters | Group I          | Group II         |
|------------|------------------|------------------|
|            | r                | P-value          | r                | P-value          |
| 25(OH)D3   | 0.089            | NS               | 0.034            | NS               |
| Vitamin E  | -0.397           | 0.001**          | -0.452           | 0.0001**         |
| Vitamin C  | -0.722           | 0.0001**         | -0.648           | 0.0001**         |
| MDA        | 0.799            | 0.0001**         | 0.646            | 0.0001**         |
| 8-OHdG     | 0.779            | 0.0001**         | 0.675            | 0.0001**         |

The receiver operating characteristic (ROC) curve is a plot of the sensitivity (true positive rate) at y-axis against the 100-specificity (false positive rate) at x-axis for the different possible cut-points of diagnostic test. When blue curve is closer to follows the left-hand border and then the top border of the ROC space then the test is more accurate [10]. All data for ROC curve of RRMS patients groups were showed in Table 5 and Table 6 respectively.

**Table 5.** Receiver operator characteristic analysis results for 25(OH)D3, vitamin E, vitamin C, MDA, PC, 8-OHdG in serum of Group I.

| Parameters | Cut-off point | AUC | P-value | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|------------|---------------|-----|---------|-----------------|-----------------|---------|---------|
| 25(OH)D3   | >9.21 ng/mL   | 0.561| NS      | 86.67           | 48.57           | 59.1    | 81      |
| Vitamin E  | ≤19.549 µmol/L | 0.757| 0.0002  | 100             | 68.57           | 73.2    | 100     |
| Vitamin C  | ≤25.994 µmol/L | 0.971| <0.0001 | 100             | 97.14           | 96.8    | 100     |
| MDA        | >0.923 nmol/mL | 0.986| <0.0001 | 100             | 97.14           | 96.8    | 100     |
| PC         | >0.991 nmol/L  | 1   | <0.0001 | 100             | 100             | 100     | 100     |
| 8-OHdG     | >1.786 ng/mL   | 0.984| <0.0001 | 100             | 97.14           | 96.8    | 100     |

AUC: area under the curve, NS: non-significant at P<0.05, PPV: positive predictive value, NPV: negative predictive value.

**Table 6.** Receiver operator characteristic analysis results for 25(OH)D3, vitamin E, vitamin C, MDA, PC, 8-OHdG in serum of Group II.
Parameters & Cut-off point & AUC & P-value & Sensitivity (%) & Specificity (%) & PPV (%) & NPV (%)  
25(OH)D3 & >8.255 ng/mL & 0.536 & NS & 88.06 & 37.14 & 72.8 & 61.9  
Vitamin E & ≤19.17 µmol/L & 0.892 & <0.0001 & 97.01 & 71.43 & 86.7 & 92.6  
Vitamin C & ≤25.101 µmol/L & 0.973 & <0.0001 & 100 & 97.14 & 98.5 & 100  
MDA & >0.923 nmol/mL & 0.969 & <0.0001 & 97.01 & 97.14 & 98.5 & 94.4  
PC & >0.991 nmol/L & 1 & <0.0001 & 100 & 100 & 100 & 100  
8-OHdG & >1.786 ng/mL & 0.961 & <0.0001 & 95.52 & 97.14 & 98.5 & 91.9  

AUC: area under the curve; NS: non-significant at P<0.05; PPV: positive predictive value; NPV: negative predictive value.

According to the results of the ROC analysis of Group I patients, PC was shown to be the best potential diagnostic biomarker for RRMS (Figure 1 and Figure 2) with an AUC= 1 and a specificity (100%) (Table 4). In addition, MDA, 8-OHdG and vitamin C with an AUC of 0.986, 0.984, 0.971 respectively are also promising biomarkers of RRMS. For Group II of patients, it has been also shown that PC was shown to be the best potential diagnostic biomarker for RRMS with an AUC of 1 and a specificity (100%) (Table 5). In addition, vitamin C, MDA, 8-OHdG and vitamin E with an AUC of 0.973, 0.969, 0.961 and 0.829 respectively are also promising biomarkers of RRMS (Figure 3).

Figure 1. The Receiver Operation Characteristic (ROC) curve of serum levels of PC and MDA of Group I patients.

Figure 2. The Receiver Operation Characteristic (ROC) curve of serum levels of 8-OHdG and vitamin C of Group I patients.
Figure 3. The Receiver Operation Characteristic (ROC) curve of serum levels of vitamin C, MDA, 8-OHdG and vitamin E of Group II patients.

4. Discussion
25(OH)D3 can have immune regulating role, which is critical for inhibiting inflammation that is predominant in pathophysiology of MS [11]. The active form of vitamin D3 1,25(OH)2D3 has dual impact on immune system by enhancing the innate system response and inhibiting the activity of adaptive immune [11]. An association was found between decreased of 25(OH)D3 level and risk of MS [11]. Collectively, 1,25(OH)2D3 has several immunomodulatory actions that are potentially relevant to the MS pathogenesis, including down regulation of antigen presenting cells [12], inhibits proliferation and differentiation of B cell, therefore regulating production of antibody via plasma cells [13]. Vitamin D3 also reduces interleukins production (IL-1 and IL-21) and other pro-inflammatory cytokines [14].

Our results are compatible with several authors concluded that no significant difference in serum 25(OH)D3 level was observed in MS patients or in healthy control groups [15,4]. The antioxidants, which are synthesized exogenously or endogenously, act as ROS-neutralizing agents to prevent it from the destruction of various biomolecules. In the sera of patients with RRMS, the levels of nonenzymatic antioxidant defenses like vitamin E, and vitamin C decreased substantially in comparison with the control group. The decrease of antioxidants substantial contribution to the development neurodegenerative diseases because oxidative damage to components of the cell [16]. Vitamin E regeneration is performed by vitamin C, therefore, the low vitamin C levels in RRMS patients refer that protection versus peroxidation of lipid won't occur due to the levels of these vitamins are lower than the normal level, which are important to prevent the peroxidation of lipid. In addition, vitamin C plays an antioxidant role versus the oxidation of thiol groups that may not occur as a result of reduced in its levels [16]. In addition, our results were consistent with the results that found lower levels of sera vitamin E, vitamin C and the levels of vitamin C were negative correlation with lipid peroxidation in MS patients vs control group [4]. The reduction in nonenzymatic antioxidantin patients of MS is due to the lack of such antioxidants or depleted it by increased levels of oxidative stress markers as attempt to reduce inflammation and immune mediated tissue damage [16]. Our results were consistent with other studies from [17, 81, 19, 20]. The ROS is continuously produced by cells as part of their metabolism, under normal conditions, this production of ROS balanced by inherent nonenzymatic and enzymatic antioxidants [17]. Macrophages constitute a major factor responsible for the production of ROS due to
high oxygen consumption [21, 22]. However, under pathological circumstances like MS, high ROS generation overcomes the natural defenses of antioxidants and causes oxidative stress and consequent harm [17]. The inflammatory response increases production of both reactive nitrogen species and ROS, ROS production, due to monocyte interactions endothelium of brain, causes cytoskeletal rearrangements, loss of integrity of blood brain barrier, alterations of tight junction and the extravasation of leukocytes into the CNS [23] and this eventually leads to chronic neuroinflammation, oxidative stress and inflammation are thought to enhance damage in MS [7].

5. Conclusion
In contrast to healthy controls, substantially lower levels of antioxidant vitamins (vitamin C and vitamin E) and elevated levels of oxidative stress are associated with an increased risk of disease in MS patients. we confirmed a strong negative correlation between the PC and antioxidant in MS patients with highly significant P value <0.0001. On the other hand, a strong positive correlation between the PC and oxidative stress (MDA and 8-OHdG) in MS patients with highly significant P value <0.0001. Our data showed that vitamin C, vitamin E, MDA, and 8-OHdG are markers as a mean could used to predict MS disease and the extent of its progression.

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6. References
[1] Kocot J, Luchowska-Kocot D, Kiełczykowska M, Musik I and Kurzepa J 2017 *Nutrients*. 9, 659.
[2] Matías-Guiu J, Oreja-Guevara C, Matias-Guiu J A and Gomez-Pinedo, U 2018 *Neurologia (English Edition).* 33, 177–186.
[3] Pierrot-Deseilligny C and Souberbielle J C 2017 *Multiple Sclerosis and Related Disorders.* 14, 35–45.
[4] Evans E, Piccio L and Cross A H 2018 *JAMA Neurology.* 75, 1013–1021.
[5] Pytel V, Matías-Guiu J, A, Torre-Fuentes L, Montero-Escribano P, Maietta P, Botet J, Álvarez S, Gómez-Pinedo U and Matías-Guiu J 2019 *Brain and Behavior.* 9, 1–15.
[6] Adamczyk, Bożena, Kozierska D, Kasperczyk S and Adamczyk-Sowa M 2018 *Free Radical Research.* 52, 1083–1093.
[7] Adamczyk, Bozena, Wawrzyniak S, Kasperczyk S and Adamczyk-Sowa M 2017 *Oxidative Medicine and Cellular Longevity.* 2017
[8] Islam M. T. 2017 *Neurological Research.* 39, 73–82.
[9] Denise R.F. 2017 Lippincotts illustrated reviews. In: Biochemistry 7th ed Chapter 26 pp 349–350.
[10] Ciobotariu D, Ghiaciu C M and Lupușoru C E 2015 *Substance Abuse Treatment, Prevention, and Policy.* 10, 1–14.
[11] Khosravi-Largani M, Pourvali-Talatappeh P, Rousta A M, Karimi-Kivi M, Noroozi E, Mahjoob A, Asaadi Y, Shahmohammadi A, Sadeghi S, Shakeri S, Ghiyasvand K T and avakoli-Yaraki M 2018 *European Journal of Medical and Cellular Longevity.* 2018
[12] Koch M W, Metz L M, Agrawal S M and Yong V W 2013 *Journal of the Neurological Sciences.* 324, 10–16.
[13] Koduaah P, Paul F and Dörr J -M 2017 *Epilepsy Research.* 8, 313–325.
[14] Rodney C, Rodney S and Millis R M 2020 *Autoimmune Diseases.* 25, 1083-1093
[15] Kusumadewi W, Imran D, Witjaksono F, Pakasi T A, Rusmana A I, Pangeran D, Marwadhani S S, Maharani K and Estiasari R 2018 *Multiple Sclerosis and Related Disorders.* 25, 329–333.
[16] Polachini C R N, Spanevello R M, Zanini D, Baldissarelli J, Pereira L B, Schetinger M R C, da Cruz I B M, Assmann C E, Bagatini M D and Morsch V M 2016 *Neurotoxicity Research.* 29, 230–242.
[17] Juybari K B, Ebrahimii G, Momeni Moghaddam M A, Asadikaram G, Torkzadeh-Mahani M, Akbari M, Mirzamohammadi S, Karimi A and Nematollahi M H 2018 *Multiple Sclerosis and
Related Disorders. 19, 79–84.
[18] Zhang S -Y, Gui L-N, Liu Y-Y, Shi S and Cheng Y 2020 Frontiers in Neuroscience. 14, 823.
[19] Padureanu R, Albu C V, Mititelu R R, Bacanoiu M V, Docea A O, Calina D, Padureanu V, Olaru G, Sandu R E, Malin R D and Buga A-M 2019 Journal of Clinical Medicine. 8, 1815.
[20] Cokluk E, MILANLIOĞLU A, Huyut Z, Çilingir V, Alp H H, AYDIN M N, Şekeroğlu M R and Balahoroğlu R 2017 J Cell Neurosci Oxid Stress. 9, 601–607.
[21] Adamczyk, Bozena and Adamczyk-Sowa M 2016 Oxidative Medicine and Cellular Longevity. 2016.
[22] Castaneda O A, Lee S-C, Ho C-T and Huang T-C 2017 Journal of Food and Drug Analysis. 25, 111–118.
[23] Tasset I, Agüera E, Sánchez-López F, Feijóo M, Giraldo A I, Cruz A H, Gascón F and Túnez I 2012 Clinical Biochemistry. 45, 440–444.