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Tryptophan-enriched antioxidant cereals improve sleep in children with autistic spectrum and attention deficit hyperactivity disorders
Carmen Galán, Soledad Sánchez, Lourdes Franco, Rafael Bravo, Montserrat Rivero, Ana Beatriz Rodriguez, Carmen Barriga
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B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry, and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD⁺ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson’s and Alzheimer’s diseases)

D- Gene and Oxidative Stress
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Levels of leukocyte oxidative DNA damage (8-OHdG), serum coenzyme Q10 and lipid peroxidation in the formation attacks of patients with multiple sclerosis

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Abstract
Multiple sclerosis (MS) is a demyelinating disease of the nervous system. Evidence about oxidative stress plays an important role in the pathogenesis of MS is increasing day by day. In our study, we aimed to investigate the effect of oxidative DNA damage and oxidative stress in the pathogenesis of MS disease. Blood samples were obtained from during an attack (Group 1), between attacks (Group 2) of MS patients (20 male and 10 female) and 30 healthy volunteers (Group 3). Malondialdehyde (MDA) levels as indicator of oxidized lipids were detected using fluorescence detector with high pressure liquid chromatograph (HPLC). DNA was extracted from leukocytes of control and patients with MS and then we measured 8-hydroxy-2′-deoxyguanosine (8-OHdG) and deoxyguanosin (dG) by using HPLC method with electrochemical and UV detector, respectively. Measurement of oxidized coenzyme Q10 (CoQ10) and reduced CoQ (CoQ10H) was performed by using UV detector with HPLC method. Serum MDA level of group 1 was significantly higher than those in group 2 and group 3 (p< 0.001). 8-OHdG/10^6 dG ratio of group 1 was significantly higher than those in group 2 and group 3 (p< 0.001). CoQ10/CoQ10H rates of group 1 were significantly increased compared with group 2 and group 3 (p<0.001). In conclusion, we observed that oxidative DNA damage, lipid and mitochondria oxidative damage were high in blood of patients with MS. It seems that oxidative stress acts a play role the pathogenesis of MS patients as well as induces attacks.

Keywords: Coenzyme Q10; Lipid peroxidation; Oxidative DNA damage; Multiple sclerosis
Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) and characterized by re-activation of antigen-specific cells, microglia activation, recruitment of systemic immune-competent cells and production of cytotoxic mediators leading to neural tissue damage (Gonsette, 2008). Four different forms were found in terms of clinic progress in MS. These forms are called relapsing-remitting, secondary progressive, primary progressive and progressive relapsing form. The most frequently seen form is the relapsing-remitting form (Miller, 1999). Today, MS’s reasons could not be fully understood. In MS pathogenesis, there are genetic factors and environmental factors (Lassmann et al., 2012). The studies conducted on MS stated that reactive oxygen species (ROS) could particularly play an important role in demyelination in MS pathogenesis (Koch et al. 2006). ROS are formed in normal physiological process in the body. ROS are minimized by the antioxidant system and converted into harmless structures and this process constantly continues in balance. Along with the effect of the environmental factors, oxidant stress occurs when ROS-antioxidant balance shifts towards ROS. ROS affect biological molecules such as proteins, lipids and DNA (Frohlich et al., 2008). ROS attack on lipids and cell membrane lipids and lipid peroxidation products are formed due to ROS-mediated oxidation of cell membrane lipids. The most common and abundant lipid peroxide product is malondialdehyde (MDA) (Huyut et al., 2016a and 2016b). Therefore, MDA levels are widely used as an indicator of lipid peroxidation (Irshad and Chaudhuri, 2002).

ROS cause DNA damage such as strand breaks and base modifications, including the oxidation of guanine residues into 8-hydroxy-2′-deoxyguanosine (8-OHdG). Thus, 8-OHdG can serve as a sensitive biomarker of oxidative DNA damage (Arı et al., 2011). One of the sources of ROS in the body is the electrons which leak from the electron transport chain (ETC) in the mitochondria internal membrane. One of the proteins that transport electron in ETC is coenzyme Q10 (ubiquinone). The main task of coenzyme Q10 (CoQ10) is to transport electrons between nicotinamide dinucleotide and succinate dehydrogenase in ETC (Ostman et al., 2012). CoQ10 is also used as supplementary in the treatment of some diseases such as diabetes and Parkinson (Chinnery et al., 2006; Litarru and Langsjoen, 2007). CoQ10 is found in two forms which are oxidized and reduced (Cobanoglu et al., 2011). Reduced form of CoQ10 is also known as ubiquinol-10 and it is the first defense against the oxidative damage of low-density lipoprotein (Mracoff and Thompson, 2007). Due to this antioxidant effect of CoQ10, ubiquinol-10/ubiquinon-10 ratio can be considered as a marker to determine oxidative damage (Huyut et al., 2016b). Plasma or serum CoQ10 concentration is usually used as the indicator for CoQ10 status in the human being.

We suggest that the oxidative DNA damage, lipid peroxidation and mitochondrial oxidative damage levels will be helpful in prognosis of these patients. In the current study, we aimed to investigate levels of leukocyte 8-OHdG, serum CoQ10 and MDA levels and ubiquinol10/ubiquinon-10 ratio in the during and between attack in MS patients.

Materials and Methods

Patients and Samples

The study was performed on the blood samples of 30 MS patients aged between 22-60 (34.85 ± 10.55) (20 male, 10 female) and 30 healthy control patients aged between 26-57 (33.46±9.54) (17 male, 13 female). The patients were elected among the patients who were diagnosed with MS and treated in the department of Neurology in Yuzuncu Yil University Medical Faculty. MS was diagnosed based on McDonalds’ criteria (McDonald et al., 2001) and only relapsing-remitting type MS patients were taken to the study. Whole blood samples were taken during the attack (group 1) and in minimum 1 month after the same patients’ attack (group 2). Healthy control group (group 3) was made up of healthy individuals who did not have any chronic and acute disease. The study was approved by Human Ethics Committee of Yuzuncu Yil University (REC number: 11/09.05.2013).

Biochemical Assays

Measurement of MDA

Analysis of MDA in serum was done by high-performance liquid chromatography (HPLC, Agilent 1200 Series system, Agilent Technologies, Waldbronn, Germany) as described by Khoschsorur et al., (2000).
Fluorometric detection was performed with excitation at 527 nm and emission at 551 nm. The peak of the MDA-TBA adduct was calibrated as a 1,1,3,3 tetraethoxypropane standard solution, carried out in exactly the same process as with the plasma sample. MDA levels were expressed as µM.

**Reduced and oxidized CoQ10 analysis**

Analyses of oxidized and reduced CoQ10 were performed according to Litarru et al. (Litarru et al., 2004; Litarru et al., 2007). The HPLC (Agilent 1200 Series system) was used to analyses of total CoQ10 and oxide CoQ10 levels. For HPLC measurement, octadecyl sulfonate (ODS) reversed phase supercoil LC 18 (15 x 0.46 cm i.d. 3 µm) colon was used. Oxidized and reduced CoQ10 were measured by electrochemical detector at 0.35V.

**DNA isolation and hydrolization from leucocytes**

Total DNA of leukocytes was extracted by used method of Ates et al., (2010). 2 ml of blood was mixed with 3 ml of erythrocyte lysis buffer, and incubation for 10 min in ice was followed by centrifugation (10 min at 1500 xg). The supernatant was decanted, and the pellet was resuspended thoroughly in sodium dodecyl sulfate (10%, v/v), proteinase K (20 mg/ml) and 1.9 ml leukocyte lysis buffer (4 M NaCl, 0.5 M EDTA). The mixture was incubated at 65 °C for 1 hour and then mixed with 0.8 ml of 9.5 M ammonium acetate. After centrifugation at 1500 xg for 25 min, the clear supernatant (2 ml) was transferred to a new sterile tube and DNA was precipitated by the addition of 4 ml ice-cold absolute ethanol. DNA samples were dissolved in Tris EDTA buffer (10 mm, pH 7.4).

DNA samples that were obtained for 8-OHdG analysis were hydrolyzed by using with formic acid at 150 °C for 30 minutes according to Kaur and Halliwell (1996). The hydrolysed DNA samples were dissolved in pure acetonitrile (final volume: 1 ml). 8-OHdG and dG levels were measured by using ECD and UV detector in the HPLC device, respectively. The reverse phase C-18 (RP-C18) analytical column was used as the column (250 mm x 4.6 mm x 4.0 µm, Phenomenex, CA). The mobile phase was prepared with mixed 0.05 M potassium phosphate buffer (pH: 5.5) and acetonitrile (97:3, v/v) and flow rate was set to 1 ml/min. The amount of 8-OHdG and dG was determined by using the ECD adjusted to 600 mV, and absorbance measurement at 245 nm with the UV detector, on the HPLC apparatus, respectively. For measurement of 8-OHdG and dG, 8-OHdG and dG standards were used (Sigma Aldrich). The oxidative DNA damage values were expressed as the number of 8-OHdG per 10^6 dG (8-OHdG/10^6dG) (Tarng et al., 2000).

**Statistical analysis**

Statistical analyses were done by using SPSS-15. The statistical significance was calculated using the ONE-Way ANOVA test. In the post-hoc analysis which was made in the statistical examination, the groups were compared between themselves. P-value of less than 0.05 was considered statistically significant. All the results were expressed as mean scores with their standard deviation (mean ± SD).

**Results**

Of all the patients, 66.7 % were males, while 56.7 % of the control group were males. The mean age of the patient group was 34.85±10.55 years, and the mean age of the control group was 33.46±9.54 years. The age and gender distributions of the patient and control groups did not significantly differ (p >0.05) (Table 1).

Median level of EDSS scores of the patients was 2.66 and ranges were 1 to 6. The changes in all the parameters are shown in Table 2.
Table 1: Demographic characteristics of individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male (%)</th>
<th>Female (%)</th>
<th>Age</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>56.7</td>
<td>43.3</td>
<td>33.46±0.54</td>
<td>30</td>
</tr>
<tr>
<td>Patients with MS</td>
<td>66.7</td>
<td>33.3</td>
<td>34.85±10.55</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2: Comparison of levels of MDA, CoQ10 and DNA damage in during attack and between attack of MS patients according to the healthy control.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDA (μmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During attack</td>
<td>3.54 ± 2.67*</td>
<td>3.05</td>
<td>2.61</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td>Between attack</td>
<td>2.95 ± 0.68</td>
<td>2.88</td>
<td>1.62</td>
<td>2.99</td>
<td>0.045</td>
</tr>
<tr>
<td>Healthy control</td>
<td>2.31 ± 1.82</td>
<td>1.64</td>
<td>2.69</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td><strong>S-OHdG/10^6 dG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During attack</td>
<td>2.16 ± 1.05*</td>
<td>1.84</td>
<td>1.80</td>
<td>2.53</td>
<td></td>
</tr>
<tr>
<td>Between attack</td>
<td>1.08 ± 0.34</td>
<td>1.02</td>
<td>0.95</td>
<td>1.21</td>
<td>0.001</td>
</tr>
<tr>
<td>Healthy control</td>
<td>0.75 ± 0.038</td>
<td>0.75</td>
<td>0.61</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td><strong>CoQ10/CoQ10H</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During attack</td>
<td>0.090 ± 0.017*</td>
<td>0.09</td>
<td>0.084</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>Between attack</td>
<td>0.041 ± 0.023#</td>
<td>0.03</td>
<td>0.032</td>
<td>0.05</td>
<td>0.001</td>
</tr>
<tr>
<td>Healthy control</td>
<td>0.012 ± 0.006</td>
<td>0.011</td>
<td>0.01</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td><strong>CoQ10 (μmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During attack</td>
<td>0.065 ± 0.009#</td>
<td>0.067</td>
<td>0.065</td>
<td>0.071</td>
<td></td>
</tr>
<tr>
<td>Between attack</td>
<td>0.033 ± 0.014#</td>
<td>0.031</td>
<td>0.027</td>
<td>0.038</td>
<td>0.001</td>
</tr>
<tr>
<td>Healthy control</td>
<td>0.009 ± 0.004</td>
<td>0.0094</td>
<td>0.0081</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

*: It is statistically significant compared to other groups. #: It is statistically significant compared to the healthy control groups.

Figure 1. The comparison of all measured parameter values in between of healthy controls, during attack and between attack in patients with multiple sclerosis.

*: It is statistically significant compared to other groups. #: It is statistically significant compared to the healthy control groups.
Serum MDA level of group 1 was significantly higher than those in group 2 and group 3 (Figure 1). The lowest MDA level was detected in group 3, but there was no significant difference between group 2 and 3. The level of oxidative DNA damage (8-OHdG/10^6 dG) of group 1 (2.16±1.05) was significantly higher than those in group 2 and group 3 (p<0.001) (Figure 1). The lowest 8-OHdG/10^6 dG ratio was detected in group 3 but there was no significant difference between group 2 and 3. The CoQ10/CoQ10H ratio of group 1 was significantly increased when compared to those in group 2 and group 3 (group 1: 0.09±0.017, group 2: 0.04±0.023 and group 3: 0.012±0.006 respectively; p<0.001) (Figure 1). In addition, the serum CoQ10 levels were also significantly increased when compared to those in group 2 and group 3, respectively; p<0.001) (Figure 1). The lowest CoQ10 level was detected in group 3 and this difference was statistically significant.

As a result, it was observed that all measured parameter values in group 1 were very high compared to those in other groups and were parallel to each other in during attack. In addition, these increases in CoQ10, CoQ10/CoQ10H and oxidative DNA damage, were about 65-70% ratios in during attack according to the healthy control group.

Discussion
In demyelinating diseases including MS, the inflammation in the demyelinating area causes increase in the level of oxygen and nitrogen free radicals and the reason for this increase is substantially known as active macrophages (Mahad et al., 2015). There are studies that show that oxygen and nitrogen free radicals which are produced by macrophages intermediate axonal damage and demyelination in MS (Gonsette, 2008).

Lipid peroxidation is the name given to the oxidative modification process which is created by ROS on lipids. Reactive aldehyde structures such as MDA, acrolein 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal develop as a result of lipid peroxidation. MDA is the second most frequently developing structure as a result of lipid peroxidation. It is used as the determinant of lipid peroxidation because of its easy measurement and availability (Grotto et al., 2009). In primary studies, it was found that there were lipid peroxidation products in the cerebrospinal liquids and plasma of MS patients (Gilgun-Sherki et al., 2004; Besler et al., 2002). We investigated the serum MDA levels and evaluated the results to compare the lipid peroxidation levels of relapsing-remitting MS patients during the attacks and after the attacks with the healthy control group in our study. We found that the MDA levels which were found in the samples taken from MS patients during the attack were significant when compared to the MDA levels found in the samples taken from the healthy control group (p<0.045). And no significant difference was found between the MDA levels of the patients and between the attacks and the MDA levels of the healthy control group in the results obtained. Our results were in harmony with the literature when compared to the studies in literature. Acar et al., (2012) have shown that the MDA levels were higher in the relapsing-remitting MS patient group when compared to the healthy control group. However, the MDA levels during the attacks were not studied in by Acar et al., (2012). Korpela et al., (1989) could not find significant difference on the MDA levels between the MS patient and healthy control groups (Korpela et al., 1989). We suppose that this different result can be probably associated with the changes in attack or remission phases of the patients at the time when the study was done (for example, exacerbation phase, beginning or end of relapse phase or remission phase).

One of the main sources of the ROS is the ETC in the mitochondria internal membrane. There is constant electron transportation in ETC and constant formation of ROS. The molecules in lipid structure in ETC act like an antioxidant. CoQ10 is the leading one among these structures. The main task of CoQ10 is to transport electrons between nicotinamide dinucleotide and succinate dehydrogenase (Ostman et al., 2012). In the experimental animal studies which were done on mitochondrial CoQ10, it was claimed that CoQ10 support had treating effect on Alzheimer disease and ischemia (Smith and Murphy, 2010; Manczak et al., 2010). In our study, we wanted to determine the reduced and oxidized CoQ10 levels in all groups and determine the oxidative damage in the mitochondria during the attacks and between the attacks of MS patients. In our study, we detected that the CoQ10/CoQ10H ratio which was found in the samples taken during the attack was higher than the other two groups. However, there was no significant difference between the CoQ10/CoQ10H ratio which was found in the samples taken during the
attacks and in the samples of the healthy control group. Based on these results, we can say that oxidative damage in mitochondria increases during attacks in MS patients. There are very few studies which are associated with the research on CoQ10 levels in MS patients in literature. In a experimental animal study, Mao et al., (2013) found that mitochondrial CoQ10 regulated some inflammatory associated genes and based on these results they stated that mitochondrial CoQ10 promises hope for the neuroprotective treatment in MS patients (Mao et al., 2013). Our study supports the results of Mao et al., (2013) in that CoQ10 level increases during the attack. Giving MS patients CoQ10 as a supplementary can be useful to reduce mitochondrial oxidative damage in these patients and it can also be useful due to its neuroprotective effect. However, the crucial point here is which CoQ10 (reduced or oxidized form) should be given. We believe that further studies are needed on this issue.

ROS, which plays a role in the pathogenesis of MS, leads to oxidative modifications in the nucleic acids on DNA. 8-OHdG is used as a biomarker in the determination of the oxidative DNA damage (Huyut et al., 2016b). Supposing that DNA synthesizes RNA and proteins, it is likely that the nucleic acids which are oxidized on DNA will lead to coding error and the proteins which are synthesized due to that process will lose their function or completely disrupt. Therefore, the damage which will be created by ROS in DNA can be severer and more permanent. 8-OHdG, which occurs under normal physiological conditions, gets into circulation and is excreted in the urine. Therefore, 8-OHdG is analyzed both in serum and urine. 8-OHdG concentration which is only found in serum and urine can only give us information about oxidative damage. And, the amount of 8-OHdG on DNA strand gives us information about mutation. There are very few studies which are similar to our 8-OHdG on MS patients. Therefore, results obtained on oxidative DNA damage in the pathogenesis of MS will provide important new information for the literature. We found the 8-OHdG/10^6dG ratios, which was determined in the samples that were taken during the attack statistically significantly higher when compared to the other two groups. Post-attack 8-OHdG/10^6dG ratios was also significantly higher than the level in the healthy control group. It is interesting that DNA damage is significantly higher. These data showed that antioxidant system reduced the MDA levels and CoQ10/CoQ10H ratio. Even if the patients returned to their normal lives after the attack, the oxidative DNA damage continued. The study by Tasset et al., (2012) supports our study. Tasset et al., (2012) divided the MS patient group into two groups which are namely expanded disability status scale (EDSS)<5 and EDSS>5 and compared them with the healthy control group and found that 8-OHdG levels were higher than the healthy control group (Tasset et al., 2012). Tasset et.al. analyzed 8-OHdG in the plasma samples and as a result they stated that oxidative DNA damage increased in the MS patients. The data which we obtained from our study have showed that guanine bases which are oxidized on DNA in MS patients can lead to severe complications if no antioxidant supplement is given.

In conclusion, we observed that oxidative DNA damage, lipid and mitochondria oxidative damage during attack in patients with MS were higher than those of healthy control group and after attack in MS patients. In the light of these data, we concluded that increased MDA, CoQ10 levels and oxidative DNA damage may play an important role in the pathogenesis of MS patients and they may be associated with attacks.

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The Roles of Authors
RB and RS researched literature and conceived the study. HHA, ZH and EC were involved in protocol development, gaining ethical approval, patient recruitment and data analysis. HHA, ZH, AM, VC and MNA wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Conflict of Interests
The authors declare, which they have no conflict of interest.
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