CHANGE OF ANTIOXIDANT ENZYME ACTIVITIES, SOME METALS AND LIPID PEROXIDATION IN ALZHEIMER'S DISEASE

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ABSTRACT

***Introduction**: Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive regression and memory loss. It has been claimed that oxidative stress and factors such as metal accumulation in the brain play important roles in the etiopathogenesis of the disease.*

***Material and methods**: The subjects of this study consisted of 24 individuals with Alzheimer's disease and 15 healthy age-matched controls. Blood samples were withdrawn from the patients and healthy controls, and the activities of antioxidant enzymes SOD (Superoxide Dismutase), GSH (Glutathion), GSHPx (Glutathion peroxidase), GST (Glutathion S-Transferase) and MDA (Malondialdehyde) levels were determined by Spectrometer. Some metals and heavy metals were measured by atomic absorption spectrometry (AAS).*

***Results**: Biochemical analyses showed a significant decrease of the main enzymatic antioxidant defences (SOD, GSH, GST and GSHPx) and increased production of lipid peroxidation marker (MDA) in the serum of AD patients, compared to age-matched control group ($p<0.001$). Also the levels of Zn, Mg, and Mn was lower and Fe, Pb, and Cd was higher in the patient group, compared to the control group. Serum Cu and Co levels did not differ significantly between the patient and control groups ($p>0.001$).*

***Conclusion**: These results supports the theory that in AD there is a defect in the antioxidant defense system, which may lead to oxidative damage. Also alterations in some trace metals and their related enzymes may play a role of etiopathogenesis in AD.*

***Keywords**: Alzheimer's Disease, Antioxidants, Serum Metals, Heavy Metals, Lipid peroxidation.*

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Introduction

Alzheimer's disease is a neurodegenerative disorder characterized by progressive cognitive regression and memory loss. The etiology of AD is complex and consist of the combination of genetic and environmental factors. In this neurodegenerative disease, aging is the major risk factor. Also oxidative stress and accumulation of metals and protein deposits including extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT) in the brain play an important role in the etiopathogenesis of the disease[1-4]. It has been reported that amyloid beta (Aβ) is a major source of reactive oxygen species (ROS) and others toxic radicals in the AD brain[5].

Oxidative stress is a consequence of an imbalance between the antioxidant system, and the ROS

like superoxide, hydrogen peroxide, and hydroxyl radicals[6]. The free radicals produced in the body are harmfull, and if not removed, they react with cellular component and damage cellular functions[7]. The brain is especially sensitive to oxidative stress, since it contains high levels of iron and polyunsaturated fatty acids (this exists as a convenient state for the reactions of free radicals), also due to increased oxygen needs and relatively low levels of antioxidants[8]. Growing evidence from experimental studies suggest that oxidative stress plays an important role in the pathogenesis of dementia, including Alzheimer's disease[9].

Lipid peroxidation is one of the major outcomes of free radical-mediated injury and generates a number of secondary products including aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal[10]. MDA is the most abundant aldehyde resulting from lipid peroxidation and can be assessed as a marker of lipid peroxidation[11]. It has been reported high levels of MDA are important indicator of oxidative stress[12]. Numerous studies have shown increased lipid peroxidation in patient with AD[13,14].

Metals and metalloid elements contribute to many cellular processes in the biological systems and play important roles. Metals have various and important biological functions, such as enzymatic activity, mitochondrial function, myelination, and neurotransmission[15]. The brain is an organ that concentrates the metal ions and current findings demonstrate that an impairment in metal homeostasis may be an important factor in the development of various neurodegenerative diseases related to aging, including the Alzheimer's disease[16]. Additionally, it has been claimed that abnormal accumulation and distribution of different metals induce oxidative stress; this causes damage to the macromolecules, leading to the development of AD[17].

In this study, we measured (SOD, GSH, GSHPx, GST) activity and MDA levels in AD patients and in healthy nondemented controls with similar ages to the patients in order to evaluate the degree of oxidative stress in patients with AD. Furthermore, in order to evaluate the potential relationship between some metals and AD, the serum metals (Mn, Mg, Zn, Fe, Cu, Co, Cd, Pb) were also assayed.

Materials and methods

24 Alzheimer's patients were included in this study according to the criteria of NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) in the Van Regional Training and Research Hospital Neurology Department[18]. The following criteria were considered in selection of the cases for study: the presence of dementia determined by clinical examination and tests for mental state, disorder in at least two cognitive states, the presence of progressive cognitive degradation including the memory, the presence of normal conscious state, and the absence of other medical and neurological diseases that might cause this disease. Furthermore, the patients with systemic or brain diseases that would affect the cognitive functions, and those with diabetes, advanced stage of cardiac failure, chronic obstructive pulmonary disease and malignant diseases were excluded from the study.

The standardized mini mental test (MMSE) was administered for the entire study group; individuals with MMSE scores between 7 and 23 were determined as the patient group, and they were included in the study. The MMSE scores of the subjects in the control group was over of 30. The patient group included four female and 20 male cases, and the mean age of the group was 75.36±4.124 years (min: 65 years, max: 86 years). The control group included 15 cases who were determined not to have any neurological disease according to the clinical and radiological investigations; they were in the same age group as the AD cases. Five female and ten male cases were included in the control group, and the mean age was 70.46±5.237 years (min: 65 years, max: 87 years). The mean durations of education in the patient and control groups were 5.25±4.1 years and 6.65±4.4 years, respectively. All patients were informed about the details of the study and written consent was obtained. All procedures were performed in accordance with the ethical standards of the Declaration of Helsinki. Permission was obtained from the Van Regional Training and Research Hospital Ethics Committee for Non-interventional Clinical Researches.

Biochemical analyses (enzymatic activities, MDA measures, and levels of some metals) were performed by the Biochemistry Laboratory of the Chemistry Department, Faculty of Science at Yuzuncu Yil University. The venous blood samples of the patients and controls were drawn from the antecubital fossa vein, in accordance with the guidelines mentioned in the Declaration of Helsinki. Fasting blood samples were obtained from all sub-

jects and collected into tubes with anticoagulant. Serum was separated by centrifugation (at 2500 rpm for 15 minutes), and the samples were processed immediately. The serum samples were placed in deionized polyethylene tubes and held at -80˚C in a deepfreeze until the day of study.

The serum levels of metals and heavy metals (Mn, Mg, Zn, Fe, Cu, Co, Pb, and Cd) were determined in all study subjects with an Atomic Absorption Spectrophotometer (AAS: UNICAM-929 Spectrophotometer, Unicam Ltd., York Street, Cambridge, UK).

The serum MDA levels were determined as the markers of oxidative stress. Activities of SOD, GSHPx, GST, and GSH in erythrocytes were also measured as markers of the antioxidant system. The red blood cells, separated from serum, were washed three times with cold physiological saline and with demineralized water, prior to analyses of hemoglobin and enzyme activities.

Measurements of antioxidant enzyme activities and MDA levels

SOD activity was measured in red blood cell. 0.1 ml of blood was hemolyzed in 0.9 ml of ice cold water (0˚C - 4˚C). The hemoglobin was removed by adding 0.25 ml of chloroform and 0.5 ml of ethanol, followed by vigorous shaking, and then the mixture was centrifuged at 18,000 rpm for 60 min. The rate of inhibition of the superoxide reaction by SOD was calculated according to the definition of McCord and Fridovich[19].

GSHPx activity was measured in erythrocyte using a Hitachi 902 Autoanalyzer (Hitachi, Brisbane, CA) with Randox's reagent (San Diego, CA). Results were indicated per milliliter of whole blood. When the oxide glutathione is reduced, NADPH is oxidized and turned into NADP. This change was observed at 340 nm wave and activation of GSHPx was measured. The intraassay and interassay CV for GSHPx were 5.2% and 7.2%, respectively. Units of GSHPx activity were calculated following NADPH oxidation at 340 nm using cumene hydroperoxide as the substrate[20].

The GSH level was measured spectrophotometrically at 412 nm by a glutathione disulfide reductase recycling method at room temperature. The reference rate was established using a freshly prepared GSH standard (30 mmol)[21].

GST activity was determined according to Habig et al.[22].

MDA was estimated by measuring TBARS (thiobarbituric acid-reactive substances) in serum samples, according to a modified method (23). 0.2 ml of serum was added to the reaction mixture containing 1 ml of 1% ortho-phosphoric acid, 0.25 ml alkaline solution of thiobarbituric acid-TBA (final volume 2.0 ml) followed by 45 min heating at 95˚C. The results were expressed as nmol MDA per milliliter of plasma.

Statistical analysis

Descriptive statistical data for the continuous variables were expressed as the mean ± standard deviation. The categorical variables were expressed as the numbers and percent values. The mean values of continuous variables were compared between groups, using the student's t test. The chi-square test was used to determine the relationships between groups and categorical variables. Pearson's correlation coefficient was used to evaluate the relation between the antioxidant enzyme levels and lipid peroxidation. The results were considered to be statistically significant when $p<0.05$. The data were analyzed using the SPSS® 13 (SPSS Inc., Chicago, IL, USA).

Results

Antioxidant enzyme activities and MDA levels in the patient and control groups are shown in Table 1. As shown in the table, antioxidant enzyme activities (SOD, GSH, GSHPx and GST) in the patients with AD were significantly lower than the control group ($p<0.001$). In contrast, the MDA levels in the cases with AD were significantly higher compared to control group ($p<0.001$).

	AD (n=24)	Control (n=15)	p
SOD (EU/mL)	13.92 ± 2.34*	28.31± 3.91	0.001
GSH (mmol/g Hb)	33.38 ±2.17*	60.19 ±1.73	0.001
GSHPx (EU/mL)	31.05±2.77*	58.76±3.47	0.001
GST EU/L	2.19±0.53 *	6.01 ± 0.78	0.001
MDA (nmol/ml)	33.40±3.27*	13.50±1.40	0.001

Table 1: Antioxidant enzymes activity and MDA level in AD group and control group.
*SOD: superoxide dismutase; GSHPx: glutathione peroxidase; GST: glutathione-S-transferase; MDA: malondialdehyde; GSH: glutathione. Values are expressed as mean and (±) standard deviation. * It implies the statistical significance between the patients and the control group (P < 0.001)*

The correlations between MDA and antioxidant enzyme activities in the AD group are shown in Table 2. A negative correlation (-0.414) was determined between the SOD activity and MDA level. In other words, these results reveal that MDA level increases as the SOD activity decreases (p<0.05).

	SOD	GSHPx	GST	MDA	GSH
SOD	1				
GSHPx	0.212	1			
GST	0.189	-0.192	1		
MDA	-0.414(*)	0.057	-0.142	1	
GSH	-0.127	0.11	-0.003	-0.115	1

Table 2: Pearson's correlation coefficients between MDA and antioxidant enzyme activities in AD group.
** P< 0.05*

The correlations between MDA and antioxidant enzyme activities in the control group are shown in Table 3. A positive correlation (0.626) was detected between GST and GSHPx in the patient group (p< 0.001).

	SOD	GSHPx	GST	MDA	GSH
SOD	1				
GSHPx	0.032	1			
GST	-0.127	0.626*	1		
MDA	0.042	0.266	0.351	1	
GSH	0.248	-0.292	-0.156	0.12	1

Table 3: Pearson's correlation coefficients between MDA and antioxidant enzyme activities in control group.
**p< 0.001*

	AD group (n=24)	Control group (n=15)	p
Mn (µg/dl)	0.012 ±0.053*	0.089±0.061	0.001
Mg (µg/dl)	25.420±6.22*	47.076±3.70	0.001
Zn (µg/dl)	0.937±0.20*	2.204±0.44	0.001
Fe (µg/dl)	1.717 ± 0.031*	0.651 ± 0.24	0.001
Cu (µg/dl)	1.097 ± 0.35	0.901 ±0.30	0.087
Co (µg/dl)	0.0014±0.0007	0.0011±0.0001	0.102
Cd (µg/dl)	0.0032±0.0005*	0.0011±0.0002	001
Pb (µg/dl)	0.037 ± 0.012*	0.0053 ± 0.0011	0.001

Table 4: Concentrations of metals and heavy metals in the serum of study groups
*Values are expressed as mean and (±) standard deviation. * It implies the statistical significance between patients and control group (P < 0.001)*

The levels of some metals and heavy metals in the study groups are shown in Table 4. As shown in the table, serum levels of Fe, Pb, and Cd in the patient group were significantly higher compared to control group. In contrast, serum levels of Mn, Mg, and Zn were lower in the patient group compared to the control subjects. The serum Cu level was higher in the patient group; however, this result was not statistically significant. The serum Co level also did not differ between the two groups.

Discussion

Alzheimer's disease is the most common type of dementia and constitute about 50% to 80% of all demans cases. World-wide it leads to high health care costs with increase in the elderly population[24]. Currently there is no effective treatment of this disease, despite increased its prevalence and economic costs. Therefore much research has been conducted in recent decade in this field.

Oxidative stress develops as a result of the deficiency of enzymatic or non-enzymatic antioxidants, or the production of free radicals in excessive amounts. The excessive production of free radicals leads to damage in the protein, lipid, sugar, and nucleic acid molecules, which jeopardizes the cell viability and may induce cellular responses leading to cell death by apoptosis and necrosis[25]. The viable systems have developed various mechanisms to control the harmful effects of free radicals. The antioxidant defense system is the most important of these mechanisms[26]. Enzymatic antioxidants such as SOD (EC 1.15.1.1), GSHPx (EC 1.11.1.9) and CAT (Catalase: EC 1.11.1.6), and various non-enzymatic antioxidants like GSH, alpha tocopherol, and vitamin C are important agents in removing free radicals in the SOD is an important enzyme that protects cells from free oxygen radical toxicity and catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen[28]. SOD one of the most studied antioxidant enzymes in AD but the information obtained from many studies are contradictory. Rinaldi et al.[29] determined that erythrocyte SOD activity is significantly lower in patients with AD compared to controls ($P < 0.001$).

This result is in accordance with the current findings. In contrast, in the study by Serra et al.[30] erythrocyte SOD activity was higher in the AD group ($P < 0.001$). In another study was determined no difference in SOD between AD and controls[31]. These controversial results may imply that SOD is

induced or consumed by oxidative stress, depending on the stage of disease[32].

GSH is the most extensively present antioxidant in the brain, and it is found in many cells in millimolar concentrations. This molecule contains thiol, and it may react with reactive oxygen species, nucleophilic compounds and products of lipid peroxidation. Previous studies have reported that GSH plays an important role in the pathogenesis of age-related diseases, including AD[33,34]. It is also a matter of debate whether GSH might be a potential marker for Alzheimer's disease[35].

On the other hand, GSTs are a group of enzymes that catalyze the reactions between GSH and nucleophilic compounds (such as HNE- 4-hydroxy-2-transnonenal and acrolein)[36]. Products of lipid peroxidation like MDA and 4-hydroxynonenal, may decrease by GST (37). The current study determined that the erythrocyte GSH and GSH-dependent (GSHPx and GST) antioxidant enzyme levels decreased significantly in AD patients compared to control group ($p<0.001$). Cristalli et al.[38] demonstrated that the plasma GSH level is significantly higher in AD, compared to control group. On the contrary in postmortem studies related with AD showed, levels of GSH, GST and GSHPx in the specimens of the frontal cortex were significantly lower when compared with the control group[39]. It has been claimed that oxidative stress was alter GSH metabolism and to cause consumption of GSH and reduction of GST activity, resulting in a predisposition to the development of neurodegenerative diseases[13].

MDA is the end-product of lipid peroxidation; it is formed by the oxidation of membrane polyunsaturated fatty acids by free radicals and it is an important indicator of oxidative stress[40]. The current study revealed that serum MDA level in patient group (33.40±3.27) is significantly higher compared to the control group (13.50±1.40) ($p<0.001$). The current study also determined that a negative correlation between SOD and MDA level so the level of SOD activity decreases, MDA is increases ($p<0.05$). Padurariu et al.[41] determined that the serum MDA levels in AD patients (mean age=65 years) was higher than that of the control group ($p<0.05$). Also in the same study, a negative correlation was determined between MDA and SOD, and the level of MDA was shown to increase as the SOD activity decreased. This results are in accordance with the current study.

Also in this study determined that the levels of serum Zn, Mg, and Mn were lower whereas the levels of serum Fe, Pb, and Cd were higher in the AD group. Serum Cu and Co levels did not differ significantly between the patient and control groups ($p>0.001$).

Recently studies shows that beta-amyloid aggregation and toxicity in AD develop due to abnormal interactions with the metal ions, such as Cu, Fe, and Zn[42]. It has been considered that Fe leads to the production of free radicals by the Fenton reaction and thus causes oxidative stress, and copper has been reported to be a harmful metal contributing to the development of AD[43]. Zinc is also an important metal, playing a role in the processes related with antioxidants and detoxification. However, studies related to the role of zinc in AD revealed controversial results. It has been claimed that zinc in low concentrations protects from the beta amyloid toxicity, whereas in high concentrations it shows neurotoxic effect[44]. Our AD group had lower Zn level and this deficiency could account for a minor protection against the oxidative damage, being Zn the constituent of SOD enzyme. Dominguez et al.[45] determined that the serum Zn level lower in the AD group. They also found serum Fe and Cu levels do not differ between with AD and the control group. However, we found that serum Fe level higher in AD group and the serum copper levels did not differ between groups. In another study, serum Fe and Zn levels in the patients with AD were determined to be lower, and serum Cu level did not differ between the patient and control groups[46].

Another element considered to be strongly implicated in neurodegeneration is Mn. It has been claimed that Mn is neurotoxic, may impair mitochondrial metabolism, and leads to oxidative stress[47]. In the current study, serum Mn levels was lower in AD group, compared to the control group. Similar results were obtained in some studies, and serum Mn levels were lower in AD group[45]; however, plasma Mn levels were found higher in other studies[48].

Recent studies have demonstrated that the levels of Mg in the brain and serum decrease in patients with AD[49]. This information is consistent with the results of our study. However, the exact role of magnesium in the pathogenesis of AD is still unclear. On the contrary Bocca et al.[46] found that Mg levels higher in AD group. About Co, Gerhardsson et al.[48] found in plasma and cerebrospinal fluid did not differ between the patients and controls in the same direction as the present result. In another study found the serum Co levels lower in AD patients compared

to controls[46]. Studies in the literature have reported that Co is cytotoxic for many types of cells including neural cells, and may cause cell death by apoptosis and necrosis[50].

However, the relation between Co and Alzheimer's disease is yet unclear.

Experimental studies have reported that exposure to Pb leads to damage in white matter and cell death, which were considered to be related with cognitive impairment[51]. Likewise the studies related to Cd have determined that long-term exposure increase in lipid peroxidation and cause inhibition of SOD, which is an indicator of oxidative stress[52]. In agreement with this observations, our patients had an increased concentration of serum Cd and Pb and inhibiting SOD activity. In the studies by Bocca et al.[46] and Basun et al.[53] although the serum Cd level was significantly higher in the AD group than in the control group and Pb levels did not differ between patients and controls in the studies by Bocca et al.[46].

In conclusion, in this study showed that the main antioxidant activities are decreased and lipid peroxidation are increased in AD. Also it is observed that significantly deterioration of the metal balance in AD. A growing body of experimental studies show that a major role of oxidative stress and interaction of redox-active metals and amyloid beta in AD etiopathology. But the details of this mechanisms is not fully elucidated and further investigation is requred.

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