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Araştırma Makalesi / Research Article

Deformability of Erythrocytes and Oxidative Damage in Alzheimer Disease

Alzheimer Hastalığında Eritrosit Deformabilitesi ve Oksidatif Hasar

Mükerrem Betül Yerer[1], Sami Aydogan[2], Emel Köseoğlu[3], Recep Baştuğ[4]

[1]University of Erciyes, Department of Pharmacology, [2]Department of Physiology, [3] Department of Neurology, Medical Faculty, KAYSERİ

[4]İskenderun State Hospital, Department of Neurology, HATAY

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ABSTRACT

Purpose: A lowered cerebral perfusion as a consequence of hemodynamic microcirculatory insufficiency is one of the factors underlying in Alzheimer's disease, which is a neurodegenerative disorder leading to progressive cognitive impairment. Erythrocyte deformability is one of the major factors affecting the microcirculatory hemodynamics which is closely related to the oxidative damage. The aim of this study is to investigate the relationship between the erythrocyte deformability, nitric oxide levels and oxidative stress in Alzheimer's disease.

Methods: The blood samples of 30 elderly people in three groups consisting of healthy control and different severities of the disease (low and severe) were used. Then the erythrocytes were isolated and the deformability of erythrocytes was determined by Rheodyne SSD evaluating the elongation indexes of the erythrocytes under different shear stress. The catalase, glutathione peroxidase and plasma nitric oxide levels were measured spectrophotometric ally.

Results: The plasma nitric oxide levels, catalase activities were found significantly higher and glutathione peroxidase activity was significantly lower in severe Alzheimer's disease patients compared to the control group. However, the deformability of erythrocytes was not significantly affected from these alterations.

Conclusion: the oxidant-antioxidant status is dramatically changed in Alzheimer's disease patients with the severity of the disease and similar alterations were seen in the nitric oxide levels without any significant change in erythrocyte deformability.

Keywords: Alzheimer 's disease, erythrocyte deformability, nitric oxide, antioxidant enzymes.

ÖZET

Amaç: Progresif kognitif bozukluğa yol açan bir nörodejeneratif hastalık olan Alzheimer hastalığı'nın altında yatan faktörlerden biri hemodinamik mikro dolaşım yetmezliğe bağlı gelişen azalmış serebral perfüzyondur. Oksidatif hasarla yakından bağlantılı olan eritrosit deformabilitesi, mikro dolaşım hemodinamiğini etkileyen başlıca faktörlerden biridir. Bu çalışmanın amacı Alzheimer hastalığı'nda eritrosit deformabilitesi, nitrik oksit seviyesi ve oksidatif stres arasındaki bağlantıyı araştırmaktır.

Yöntem: Sağlıklı kontrol grubu ve farklı şiddette hastalığı (hafif, ağır) olan gruplar şeklinde üç grup yaşlı kişinin kan örnekleri kullanıldı. Sonrasında eritrositler izole edildi ve eritrosit deformabilitesi, Rheodyne SSD ile farklı shear streste eritrosit uzama indeksleri çalışılarak belirlendi. Katalaz, glutathion peroksidaz ve plazma nitrik oksit seviyeleri spektrofotometrik olarak belirlendi.

Bulgular: Kontrol grubu ile karşılaştırıldığında şiddetli Alzheimer hastalığı olan hastalarda plazma nitrik oksit ve katalaz aktivitelerinin önemli derecede yüksek ve glutathion peroksidaz aktivitesinin ise önemli oranda düşük olduğu bulundu. Bununla birlikte eritrosit deformabilitesinin bu değişikliklerden önemli şekilde etkilenmediği gözlendi.

Sonuç: Alzheimer hastalığı'nda hastalığın şiddetlenmesi ile oksidan-antioksidan durumu dramatik olarak değişmekte ve benzer değişimler önemli oranda eritrosit deformabilitesi eşlik etmeden nitrik oksit seviyelerinde de görülmektedir.

Anahtar kelimeler: Alzheimer hastalığı, eritrosit deformabililitesi, nitrik oksit, antioksidan enzimler

INTRODUCTION

Alzheimer's disease (AD) neuropathology is characterized by presence of extracellular senile plaques with amyloid beta (Ab) peptide deposition and intraneurofibrillary tangles with abnormally phosphorylated tau protein[1,2]. A large body of data suggests that Ab causes neuronal degeneration and death by mechanisms that involve reactive oxygen species[3,4,5,6] and evidence has been accumulated that oxidative stress participates in the neuronal loss in AD[7,8,9,10].

Because of its high rate of oxygen consumption and its high content of polyunsaturated fatty acids, the brain exhibits increased vulnerability to oxidative stress. Elevated lipid peroxidation, as found in the brains of AD patients, not only reveals oxidative stress, but also exerts secondary effects on protein modification, oxidation and conformation[11,12].

An increase in DNA, lipid and protein oxidation products has been found in blood and in post-mortem brain samples obtained from AD patients in comparison with controls. The measurement of peripheral antioxidants is considered an appropriate way of looking at oxidative stress in various disease states in humans as in AD[13].

The recent research into Alzheimer's disease is mainly focused in post-mortem characterization of pathological and biochemical alterations in the brain. Finding peripheral markers that could be associated with the changes observed in the Alzheimer's brain would be of interest in the field of primary cognitive disorders[11,13,14,15,16].

AD's vascular component may also involve nitric oxide (NO)[17]. Nitric oxide is a compound with important physiological functions, such as neurotransmission required for memory ability, and notably the relaxation of smooth muscles in arterial walls; consequently it is important in controlling blood flow and pressure[18]. Therefore the relationship between NO and oxidative damage can be a crucial factor in the pathogenesis of the disease. It may be NO's antioxidant abilities that are at work, eliminating free radicals and superoxide anions that maintain or encourage the production of Ab as an inflammatory signal[19]. Other studies have found a significant connection between AD and oxidative stress[20]; the antioxidant properties of basal NO may be vital to combating the progression of AD[21]. On the other hand, in excessive amounts the NO may cause an oxidative damage in the cells and tissues[22].

Mammalian erythrocytes consist of a flexible and filamentous protein network named 'skeleton' and that it is structurally distinct from the lipid bilayer. This specialized submembrane is responsible for the rheological properties of normal erythrocytes such as deformability and mechanical resistance to shear stress[23,24]. However, the protein-protein interactions as well as the lipid peroxidation in the erythrocyte membrane can be triggered by oxidative stress which therefore leads to the diminished tissue perfusion and cell death including the neurons that can be another substantial factor in neurodegeneration in AD. Furthermore, NO plays a regulatory role in the deformability of erythrocytes in basal levels controversy to its oxidative damage effects in excessive amounts. Therefore, the NO levels may also play a crucial role in AD patient erythrocytes and may affect the deformability of erythrocytes.

Therefore, this study was performed for three aims. First of all, the erythrocyte deformability properties of AD patients in different stages of the disease in elderly people were investigated. Secondly, the peripheral antioxidant enzyme activities and the nitric oxide level alterations were measured. Finally, the relationships between these parameters were evaluated to find out if there was any oxidative damage to the erythrocytes which can alter the deformability of erythrocytes probably leading to the impairment in tissue perfusion in neuron cells in AD.

MATERIAL and METHODS

Patients

Ten age matched controls (7 men, 3 women), aged 60 to 72 years, and 20 AD patients (12 men, 6 women), aged to 65-82 who scored above the severity-related cut-off in Mini-Mental State Examination (MMSE) test were selected to participate in the study. The patients were recruited from the neurology clinic at the Erciyes University Hospital. The severity of cognitive impairments was assessed using the Mini Mental State Examination and the Global Deterioration Scale [25]. MMSE cut-off scores were: >24, for control subjects, >18 for subjects with low severity, 13-18 for individuals with high severity. A full medical history was taken and, physical examination performed, and patients with evidence of other significant medical problems were excluded. This study was approved by Institutional Ethics Committee. The nature and the purpose of the study was explained to all participants, and to the relatives/caregivers of AD patients and prior to consent being given all subjects or responsible caregivers, whichever appropriate, signed informed consent forms. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Sample Collection

Heparinized antecubital venous blood samples were washed with saline and centrifuged three times at 3000 ´g for 10 min at room temperature; plasma was separated and white cells were discarded by aspiration. The deformability of erythrocytes, erythrocyte glutathione peroxidase, catalase activities and plasma total nitrit/nitrate levels were measured.

Erythrocyte Deformability

The deformability of erythrocytes was determined by Rheodyne SSD (Myrenne GmbH, Germany) at 37˚C evaluating the elongation indexes of the erythrocytes under different shear stress (0.30, 0.60, 1.20, 3.00, 6.00, 12.00, 30.00 and 60 Pa) in 2ml of Dextran 60 (viscosity: 24 mPas, osmolality 290 mOsm, pH=7.4) suspension. The dextran 60 solution is the solution which has been standardized by the company for the measurement of elongation index (EI) by Rheodyn SSD (the device which we measure the deformability) so we used this solution as the commercial firm has confirmed and produced for these measurements. The company of the device Rheodyn SSD uses this Dextran 60 solution which has 24 mPa viscosity (osmolality 290 mOsm, pH=7.4) for the elongation index (EI) measurements in this device. The deformability of erythrocytes was calculated via the software by using the formulation below[24].

Elongation Index (EI)= A-B/A+B

A: Vertical diameter of the erythrocyte under shear stress

B: Horizontal diameter of the erythrocyte under shear stress

Catalase Activity

Catalase activity was determined by measuring the decrease in absorption at 240nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.0) and 37.5 mM hydrogen peroxide as described before. The results are expressed as catalase activity (nmol/min.ml) in per mg of hemoglobin[26]. Hemoglobin content was measured by the conversion of hemoglobin to cyanomethemoglobin at 540nm using the Drabkin's reagent. The results are expressed as g% hemoglobin[27].

Glutathione Peroxidase Activity

GSH-Px activity was measured spectrophotometrically as previously described [28]. Briefly, 50 mM Tris HCl (pH= 7,6), 5 mM EDTA, 1 mM GSH, 0.22 mM β-NADPH, 0.4 U/ml glutathione reductase were prepared as a reaction buffer. The GSH-Px activity was followed for 3 min at 340 nm in the presence of 0,22 mM Tert-Butyl Hydroperoxyde. The activity was then calculated by using the formulation below:

nmol NADPH oxidized / min = 1mU GSH-Px NADPH ε= 6.22 cm^2 / µmol Activity (nmol/min .ml) = mA/min . 1000/6.22

Total nitrite/nitrate levels

For the total plasma nitrite/nitrate level, first of all the samples were deproteinized by 0.5 N NaOH ve %10 $ZnSO_4$. 0.05U/ml nitrat reductase 200µmol/L reduced β-NADPH ve 10 µmol/L FAD were added and the samples were incubated for 15min at 37°C. Then Griess reagents (%2 p-aminobenzensulfonamide and %0.2 N-(1-naphtyl) etilen diamin dihidroklorid) were added and the total nitrite/nitrate level was estimated at 540 nm spectrophotometrically. The results were calculated via standard curve of the nitrate standard dilutions and calculated as the µmol/L[29].

Statistical Analysis

SPSS 13.0 was used for the statistical analysis of the data. The data were expressed as the mean ± SD and the number of subjects is indicated by n. The data obtained were analyzed by the help of Student-t and Mann-Whitney U tests for the possible significant differences between the groups. The significance levels were considered significant at $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Deformability of erythrocytes in AD has slightly changed under physiological shear stress with the severity of the disease but these changes were not statistically significant (Figure 1).

The alterations in erythrocyte deformability under different shear stress are presented in Figure 2. When the shear stresses of 0.30, 0.60, 1.20, 3.00, 6.00, 12.00, 30.00 and 60.00 Pa were applied to the erythrocytes, respectively, the elongation indexes has changed especially in the severe AD group however these changes were not significant (Figure 2).

The descriptive statistics and the alterations in plasma total nitrite/nitrate levels are presented in Figure 3 as a marker of nitric oxide levels in these patients. Plasma total nitrit/nitrate levels were significantly increased in either the low or the severe AD groups (Figure 3, $p<0.05$).

Erythrocyte catalase activities in different stages of AD are presented in Figure 4. The activity of catalase was significantly higher in severe AD ($p<0.001$), compared to the control group within 3 groups (Figure 4).

GSH-Px activity was decreased by the severity of the disease. These reduction in GSH-Px activity in low AD patients ($p<0.05$) was significantly different from control where as it was more dramatically in severe AD patients' erythrocytes ($p<0.001$). Furthermore the GSH-Px activity of the severe AD group was also significantly lower from low AD group (Figure 5, $p<0.05$).

When we evaluated the correlations between antioxidant enzymes, NO levels and elongation indexes of erythrocytes throughout all groups we found out GSH-Px and CAT activities were negatively correlated ($p<0.01$) and elongation index and CAT activities were positively correlated (Figure 6, $p<0.05$).

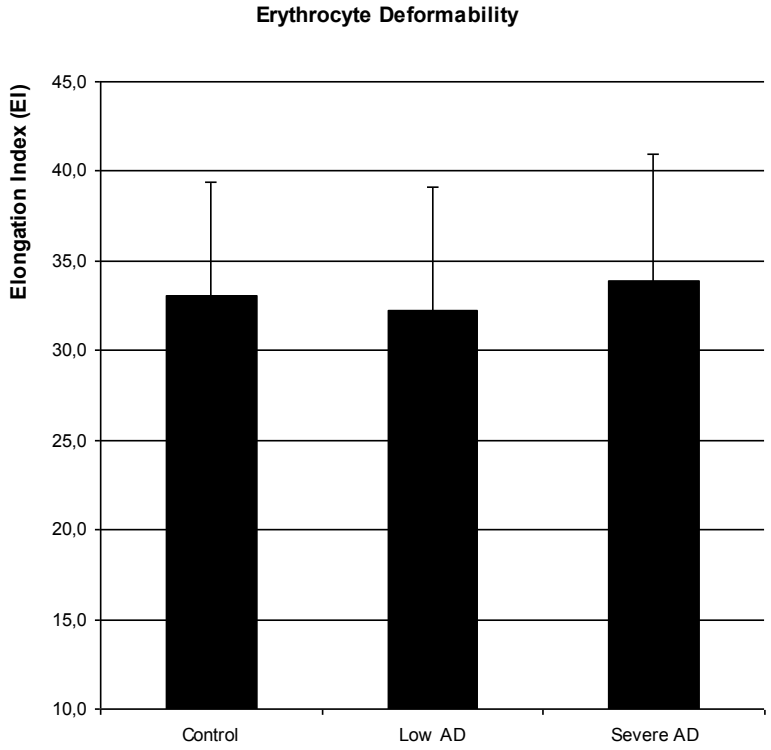

Figure 1. Deformability of erythrocytes under physiological shear stress at 30 Pa.

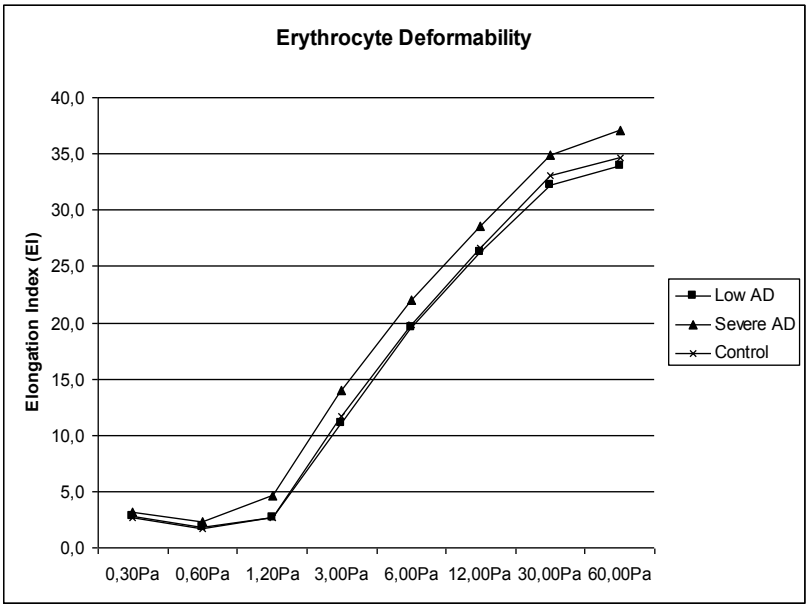

Figure 2. The alterations in erythrocyte deformability under different shear stress.

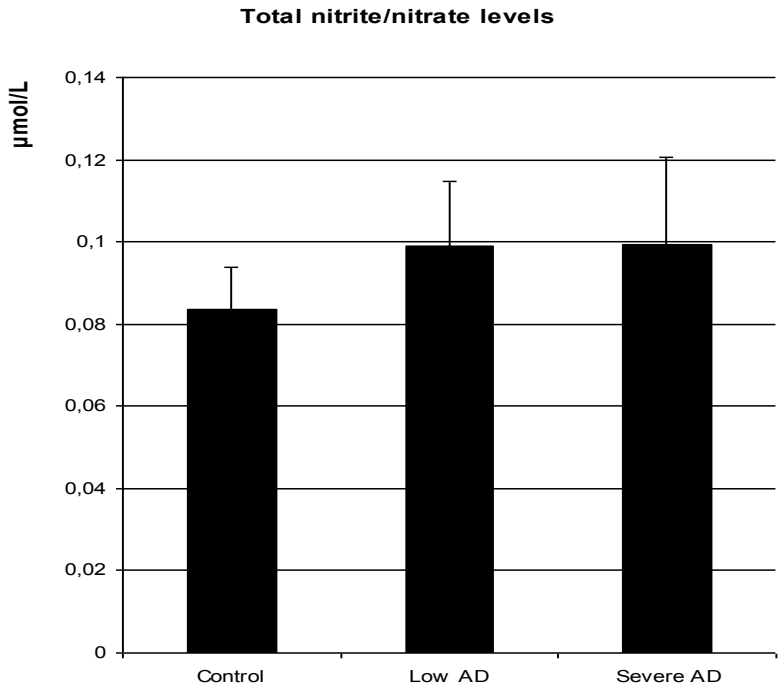

Figure 3. Total nitrite/nitrate levels as a marker of nitric oxide. *: Significantly different compared to control, p<0.05.

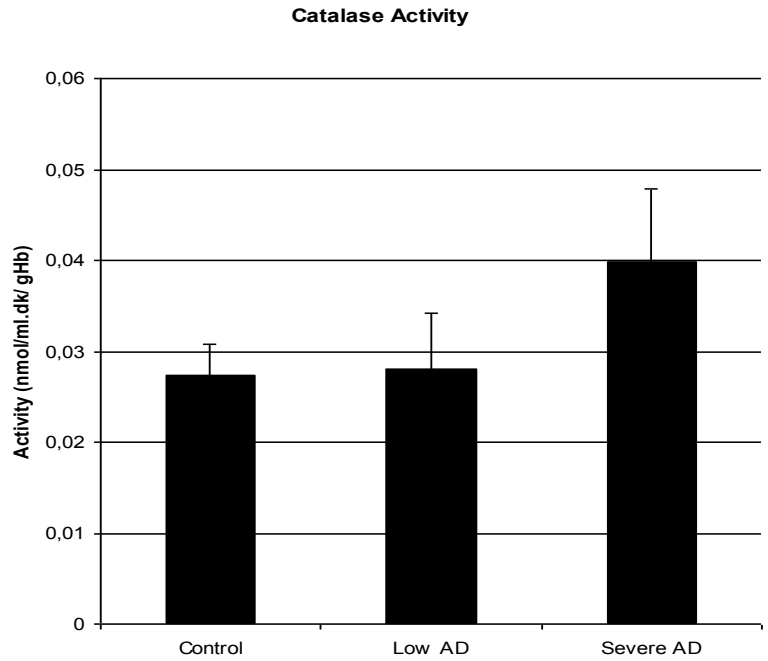

Figure 4. Erythrocyte catalase activity. *: Significantly different compared to controls, p<0.001. #: Significantly different compared to low AD, p<0.05.

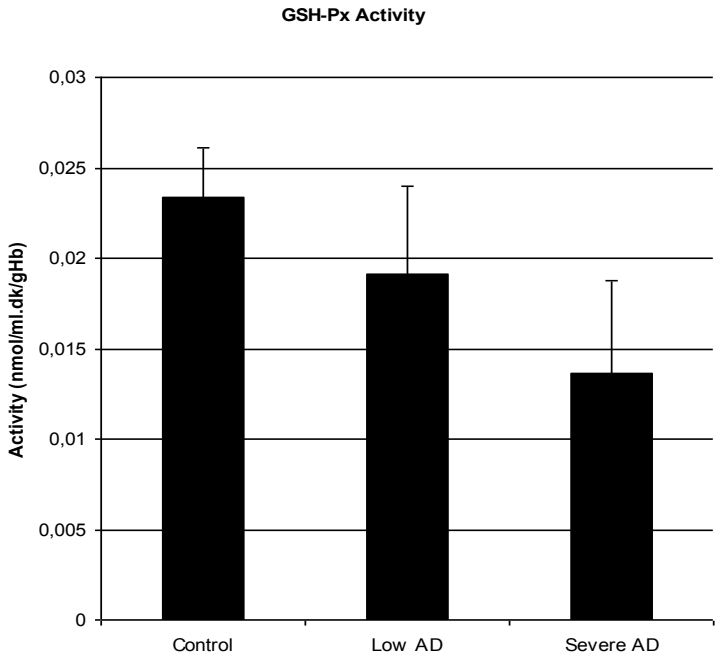

Figure 5. Erythrocyte GSH-Px activity. *: Significantly different compared to the control, p<0.05. #: Signifcantly different compared to the low AD group, p<0.05. **: Significantly different compared to the control, p<0.001.

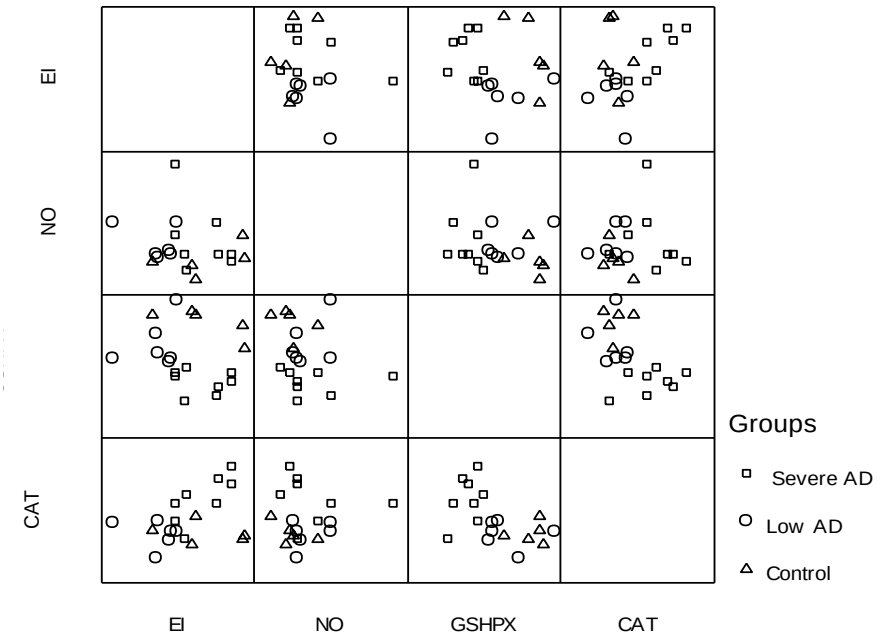

Figure 6. Correlations between the antioxidant enzymes, NO levels and elongation indexes in erythrocytes of AD patients. NO- CAT activity: p<0.05. CAT- GSH-Px: p<0.01.

DISCUSSION

The mechanism underlying age-related neurodegenerative diseases such as AD and Parkinson's disease is still an area of significant controversy. Increased evidence suggests that oxidative stress plays an important role in the neuronal cell death observed in these diseases. However, the mechanism underlying such an increase in oxidative damage of macromolecules is unclear.

GSH-Px and CAT activity alterations in AD patients' erythrocytes with the severity of the disease may be an attempt to control a primary increase of H_2O_2 production. On the other hand, this is especially important in the CNS because of its low levels of activity of antioxidant enzymes[30]. That means that above a certain point, GSH-Px loses its ability to protect from damage and increases peroxidation. Other investigators also described a GSH content decreases in AD patient's erythrocytes[31] and suggested that GSH-Px enzyme activity might be a peripheral marker of this condition. In this study, taken into account all these data when we measured the GSH-Px activity, there was a significant decrease in AD patients with the severity of the disease supporting the previous data on GSH content depletion in these patients and in controversy to the data revealed in another study[13]. However, subjects developing AD may have an inadequate antioxidant enzymatic activity unable to counteract the hyper production of free radicals during a recently established condition of oxidative stress. Supporting our results, lower activities of plasma and RBC SOD as well as of plasma GSH-Px were found previously in AD patients as compared to controls[16].

In addition to these findings, the probable Alzheimer patients also presented higher erythrocyte CAT activity than the control group as previously shown[15]. The changes induced in the redox status of tissues may initiate intracellular signal transduction processes that trigger the expression of the different proteins that constitute the enzymatic antioxidant defense system[32]. In this case, the consequence of the oxidative stress condition would be a compensatory up-regulation of catalase activity. In summary, the state of several peripheral markers used in this study showed evidence of the occurrence of oxidative stress in the blood of probable Alzheimer patients compared with the group of healthy individuals. The markers were associated with clinical parameters of the disease indicating that reactive oxygen species could play a role in the development of the pathology. Oxidative stress in the blood of probable Alzheimer patients could be a reflection of the brain condition, and suggests that oxygen free radicals could be partially responsible of the damage observed in this disease.

Furthermore, the negative correlation between GSH-Px and CAT activities is also meaningful since both of the enzymes try to eliminate the excessive amounts of H_2O_2 and the reaction rate of the CAT is more than GSH-Px. Therefore, the increases in CAT activity may probably result in the decrease in GSH-Px activity. However, we should also take the depletion of the antioxidant enzyme activities in elderly people into account as a possible mechanism underlying the decrease in GSH-Px activity.

In the central nervous system NO plays physiological and pathophysiological roles as a messenger molecule; it is involved in neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity, and regulation of gene expression[33,34]. Nevertheless, when generated in excess, NO is neurotoxic, leading to neuronal dysfunction and death [34]. Tarkowski et al[17] demonstrated an inverse correlation between the concentration of NO in the cerebrospinal fluid of AD patients and the degree of cognitive impairment. The reactive oxygen species react with nitric oxide (NO) to produce peroxynitrate, which can cause lipid peroxidation

that further accelerates degenerative changes including those leading to AD via b-amyloid/lipid interactions[35].

On the other hand, some reports indicate that NO has antioxidant abilities that are at work, eliminating free radicals and superoxide anions that maintain or encourage the production of Ab as an inflammatory signal[19]. Other studies have found a significant connection between AD and oxidative stress[20]; the antioxidant properties of basal NO may be vital to combating the progression of AD[21,22].

In the present study, the NO levels were increased in both the low and severe AD patients which can induce the oxidative stress in AD disease since there was a significant positive correlation between the NO levels and CAT activity ($p<0.05$). Therefore the role of NO levels in plasma in AD patients is more likely to cause oxidative damage than its protective effects in erythrocytes which differs from some brain reports[36].

In this study, we also evaluated the deformability of erythrocytes which is crucial for the tissue perfusion. The hypothesis of impaired deformability which may cause cell death may be another possible mechanism underlying the neurodegeneration in the AD. Taking into account all our previous studies, we can say that there is a strong relationship between the deformability of erythrocytes, NO levels and antioxidant enzymes[23,24,37,38]. NO plays a regulatory role in the deformability of erythrocytes. As previously shown, the increases in physiological ranges can protect the erythrocytes from impaired deformability. However, the excessive amount of NO causes oxidative damage in erythrocytes[39]. And one of the main factors affecting the deformability of erythrocytes is the oxidative damage caused by NO. Therefore in this study we tried to find out if the NO levels are elevated in AD and whether this increases in NO levels cause any oxidative damage in erythrocytes and therefore affect the deformability of erythrocyte which is crucial for a physiological perfusion of the neurons in AD or not. In this case, this is the first report to evaluate the erythrocyte deformability and elongation indexes of the erythrocytes under shear stress in AD patients and which indicates the relationships of NO and antioxidant enzyme levels and erythrocyte deformability in these patients. As a result, we found that the NO levels significantly increased which is positively correlated with the CAT activities in AD patients revealing that it may be one of the factors causing oxidative damage in these patients. However, the increases in the NO levels did not result in impaired deformability. This can be explained by the CAT activity elevation was probably sufficient for the protection of the erythrocytes from impaired deformability.

As a conclusion, the NO levels and oxidant-antioxidant status is dramatically changed in AD patients with the severity of the disease but the deformability of erythrocytes was slightly affected from these. In the severe AD group, the increase of the deformability was not significant. So this increase was only a little that has not been taken into consideration. Not only in the 30 Pa shear stress but also in all the shear rates the deformability changes were not any significant in both low and severe AD patients. The oxidation status has been changed dramatically with the severity of the disease, however these alterations did not effect the deformability results.

For a better understanding of the pathogenesis of the disease, the relationships of the possible factors likely the deformability of erythrocytes, oxidative damage and relationships between these parameters should be well identified by further investigations.

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Yazışma Adresi / Address for Correspondence:
Dr. Emel Köseoğlu
Erciyes University Faculy of Medicine
Deparment of Neurology
KAYSERİ
e-mail: emelk@erciyes.edu.tr
tel: 90 352 207 66 66/21755

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