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The Study of Homocysteine and Its Relationship with Oxidative Stress Biomarkers in Alzheimer's Disease

Ms. Santoshi R. Ghodake[1], Dr. Adinath N. Suryakar [2,] Dr. Prabhakar M. Kulhalli [1], Dr. Dhananjay A.Sangle[1], Dr Abdul Kayyum Shaikh [3]

[1]Maharashtra University of Health Sciences, Nashik, Maharashtra, India.
[2]D.Y.Patil Medical College and Hospital, Pune, Maharashtra, India.
[3]Ashwini Rural Medical College and Hospital, Solapur, Maharashtra, India

Corresponding Author: - Ms. Santoshi Ram Ghodake, UDIRT, Maharashtra University of Heath Sciences, Nashik, Maharashtra, India.

Abstract :

Background: Increasing evidence suggests that oxidative stress is associated with normal aging and several neurodegenerative diseases, including Alzheimer's disease. Alzheimer's disease is a progressive neurodegenerative disorder whose clinical manifestations appear in old age. Lipid peroxidation plays an important role in the development of dementia and AD. Elevated plasma homocysteine has been associated with pathogenesis of AD. The aim of the present study was to determine relationship between Hcy level and AD and its relationship oxidative stress biomarkers. Thirty AD patients and thirty healthy controls participated in the study. Plasma total Hcy and serum Malondialdehyde, activity of RBC-SOD, RBC-GSH (reduced Glutathione), serum Total thiol proteins and plasma Total Antioxidant Capacity were measured both in patients and controls using spectrophotometric methods. Competitive chemiluminescent immunoassay was used to analyse the total level of homocysteine in plasma. The lipid peroxidation in serum was measured by the level of TBARS. RBC-Superoxide dismutase activity and GSH were assayed by the method of Das and Burtis-Ashwood respectively. Serum thiol proteins (-SH) was determined by Habeeb method. TAC in plasma was analyzed by FRAP assay. Results: The plasma levels of Hcy and serum MDA in AD group are significantly higher than that of normal controls. The activity of RBC-SOD in AD group is significantly lower than that of normal control group. The significant decrease in the levels of RBC-GSH, total thiol proteins and TAC was observed in AD patients compared to controls. Furthermore, there were significant positive correlations between the plasma Hcy and MDA levels (r = +0.42, p= 0.01) and significant negative correlations between the levels plasma Hcy and TAC (r = -0.40, p= 0.02) in AD group. Conclusion: Hcy participate in pathogenesis of AD and elevated Hcy can promote significant oxidant damage and enhance oxidative stress in AD. This may represent additional risk factor pathways which conspire to produce AD. Increased oxidative stress and decreased antioxidants indicated its association with AD progression.

Key words: Alzheimer's disease, Homocysteine, Oxidative stress, Malondialdehyde, Total antioxidant capacity.

Introduction :

Alzheimer's disease (AD) is a fatal and progressive neurodegenerative illness and the most common, complex one of the main causes of dementia. Genetic and lifestyle-related risk factors

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for AD are associated with an increase in oxidative stress, suggesting that oxidative stress is involved at an early stage of the pathologic cascade [1]. Moreover, oxidative stress is mechanistically and chronologically associated with other key features of AD, namely, metabolic, mitochondrial, metal, and cell-cycle abnormalities [2]. One of the probable mechanisms of AD is the vascular injury. Hyperhomocysteinemia is considered as a risk factor of the vascular diseases because it is detected in 25% of the patient with the vascular disorders [3]. A defining feature of AD pathology is the presence of amyloid beta known as A-beta (Ab) within neuritic plaques of the hippocampus and neocortex of the brain [4].

Free radicals play a very important role in the structural and functional changes of neuronal membrane. This could be responsible for the development or aggravation of the basic diseased condition. Increasing evidence suggests that oxidative stress is associated with normal aging and several neurodegenerative diseases, including AD [5].

It is now recognized that subjects with cardiovascular risk factors and a history of stroke have an increased risk of both vascular dementia and AD [6]. Research has shown that an elevated blood level of the sulphydryl amino acid homocysteine- termed hyperhomocysteinemia is an independent risk factor for vascular disease [6, 7].

Elevated total Hcy levels have been associated with an increased risk of atherosclerotic sequelae, including death from cardiovascular causes, coronary heart disease, carotid atherosclerosis, and clinical stroke [8, 9]. These observations led to the hypothesis that elevated plasma Hcy may be a risk factor for dementia and AD [10].

Oxidative stress and Hcy play an important role in the development of dementia and AD, but the pathogenesis was still not clear. Hcy, a non-protein amino acid reversibly formed and secreted during metabolism, is a potent neurotoxin [11]. Hcy is the demethylated metabolic product of methionine, an essential amino acid found in animal protein (meat, fish, eggs and milk) and to a lesser extent in vegetable proteins. In human plasma it is present in both reduced and oxidized forms [7].

It has been proposed that elevated plasma total Hcy promotes atherogenesis and thrombogenesis. A possible mechanism for atherogenesis caused by Hcy may be oxidative damage to the vessel wall, with migration and proliferation of vascular smooth muscle cells to the intima. In AD patients dysregulation of reactive oxygen species (ROS) metabolism, as detected by abnormal activities of critical antioxidant enzymes and other indicators—lipid peroxidation in plasma, red blood cells, blood platelets, and cerebrospinal fluid is observed [5, 12].

Oxidative stress induced by free radicals disrupts the equilibrium of biological systems by damaging the major constituent molecules, including proteins, lipids and DNA, leading eventually to cell death. Antioxidative enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and substances like reduced glutathione (GSH), they dispose the free radicals and when they are generated thereby protecting the cells and tissues from oxidative attack [13].

Normal aging triggers induce pathogenically high amyloid precursor protein (APP) gene expression in exposed, hypomethylated APP, individuals, which in turn produce more APP, which is further cleaved to build up amyloid β (Aβ) levels. Large–scale age–related change in gene expression levels has been previously documented in human brains. The buildup in Aβ has multiple consequences. It can produce disruption of synaptic function as a diffusible ligand; it can aggregate to form plaques, or it can promote reactive oxygen species (ROS) production [14, 15].

Several lines of evidence indicate that oxidative damage plays an important role in the pathogenesis in AD in the last decade, and

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interesting results have been found. Protein oxidation may affect neuron function by damaging enzymes that are critical to neuron metabolism (Markesbery and Carney 1999). Lipid oxidation can cause structural membrane damage and produce secondary bio-reactive aldehydes, such as 4-hydroxy- 2-nonenal and acrolein [(5)].

The most important mechanism underlying the tissue damage caused by free oxygen radicals is peroxidation of lipids within the cellular membrane (11). Final product of lipid peroxidation is (MDA). Assessment of serum MDA levels can be used as an indicator of tissue damage mediated by free oxygen radicals. Lipid oxidation also leads to formation of isoprostanes and neuroprostanes, and studies show that the concentration of isoprostanes in the CSF of AD subjects is higher than in controls. Moreover, in schizophrenic patients reduced status of plasma total antioxidant capacity was observed [(10, 14, 17, 18)].

TAC is a useful estimate of the activity of antioxidants in a medium. Measurement of TAC can provide information on an individual's overall antioxidant status, which may include those antioxidants not yet recognized or not easily measured [(19)].

To our knowledge there have been several studies that investigated total antioxidant capacity and some other studies evaluated the oxidative and antioxidative parameters (MDA, F2-isoprostanes, 4-hydroxynonenal (HNE), NO,SOD, GSH, vitamin E and C, TAC) [(8-10, 18, 20)].

Therefore this study tested serum levels of MDA, total homocysteine, RBC-SOD, GSH, Total thiol proteins and TAC in AD. The relation between oxidative markers and serum levels of homocysteine has been analyzed in this study to clarify the pathogenic role of homocysteine in AD. Parameters were correlated and results were compared with normal subjects in the control group. These findings may lead to attempt novel therapeutic approaches towards appropriate use of antioxidants to alleviate or slows down the progression of disease.

Materials and Methods

Type of study :

The case control study was conducted at the department of Biochemistry and Psychiatry, PDVVPF's Medical College and Hospital, Ahmednagar.

Inclusion criteria: The total of 30 patients with Alzheimer's disease and 30 age and sex (age of Alzheimer's disease patients here chosen is from 55 to 80 years old) matched controls were included in the present study.

Alzheimer's disease was diagnosed when subjects met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association for definite, probable, or possible Alzheimer's disease [(21)]. All the patients and controls were free of any medication at least one month.

Exclusion criteria: Patients with a history of drug abuse or dependence, serious medical illness, severe head injury or seizure disorders were excluded from the study. None of the control subjects had a history of psychiatric disorders, severe head injury, seizure, diabetes mellitus, chronic renal failure, hypertension etc both groups consisted of non smokers.

Ethical approval: The overall study was carried out in accordance with Helsinki declaration made in 1975 revised in 2000 (22) and was approved by our institutional ethical committee. Written informed consent was obtained from all participants and study partners who had knowledge of participants' functional activities.

5 ml of blood samples was taken from each subject collected in plain and heparinized bulbs and centrifuged (by centrifugation at 3000 g for 15 minutes) immediately; separated plasma and serum were frozen and stored at -20^{0}C. Serum was

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used for the estimation of MDA and thiol proteins. Plasma was used for Hcy and TAC determination. The buffy coat was removed and the packed cells were washed three times with physiological saline.

Oxidative stress markers including oxidants MDA and plasma total Hcy and antioxidants including RBC-SOD, RBC-GSH, thiol proteins and TAC were assessed.

The erythrocytes suspension was prepared by the method of Dodge et. al. [23], modified by Quist [24]. SOD activity was measured in hemolysate, according to the method of Kajari Das [25] and RBC-GSH was estimated by the method Burtis-Ashwood using dithio-bis- nitro benzoic acid [26]. Serum MDA concentrations was determined as the measure of TBARS [27] and plasma Hcy was measured by kit method, using direct chemiluminescent immunoassay [28]. Serum total thiol proteins and TAC were measured by Habeeb's method [29] and FRAP assay [30] respectively. All the reagents used were of analytical reagent grade.

Statistics

Statistical analysis between controls and patients was performed by students 't' test (paired and unpaired) using Graph Pad Prism, Version 3.02 software. Quantitative data were expressed as mean ± SEM. Simple correlation analysis (Pearson correlation coefficient) was used to compare continuous variables. $P<0.05$ was considered as significant.

Results

Levels of plasma Hcy, oxidative biomarkers in healthy controls and AD patients are given in the Table 1.

Table 1. Comparison of total level of homocysteine (Hcy) and the level of selected biomarkers of oxidative stress in plasma of healthy subjects and AD patients (Mean ± SEM)

Parameters	Control subjects (n=30)	Patients (n=30)	P values
Hcy (µmol/L)	7.800± 0.81	27.73±2.51	<0.0001
MDA (nmol/dl)	278.16 ± 6.06	309.02±8.68	0.0052
RBC-SOD (U/mg of Hb)	28.72±0.74	23.35±0.63	<0.0001
RBC-GSH (mg/dl of Hb)	31.62 ± 0.65	25.45 ± 0.82	<0.0001
Total thiol proteins (µmol/L)	0.675±0.02	0.367±0.02	<0.0001
TAC (µmol/L)	852.60 ± 17.29	716.33 ± 16.59	<0.0001

n=Indicates the no. of subjects
p<0.0001= Highly significant, p< 0.01= Significant (compared to Control group)

The plasma Hcy and serum MDA in patient group are significantly higher than that of control subjects (P <0.0001 and P= 0.0051, respectively); The activity of erythrocytic- SOD, GSH and thiol proteins levels in patients group are significantly lower than that of in normal group (P < 0.0001, for all). Plasma TAC also decreased significantly in patients compared to controls (P < 0.0001).

To further explore the mechanism oxidative damage, the role of scavenging antioxidants and its relationship with Hcy levels in AD patients, correlation analysis between Hcy and oxidative biomarkers had been done here. At the same time, linear correlation analysis also was done between Hcy and oxidative biomarkers in controls subjects.

When we analyzed the correlation between plasma Hcy and serum MDA levels, RBC-SOD activity, RBC-GSH total thiol proteins and TAC levels (Table 2.) ,

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Table 2. Correlation between Hcy levels and other oxidative stress markers

	Hcy levels of plasma	
	Patients	Control subjects
MDA	+0.42*	+0.34
RBC-SOD	-0.11	-0.001
RBC-GSH	-0.25	-0.15
Total thiol proteins	-0.35	+0.14
TAC	-0.40*	-0.17

+ positive correlation, - negative correlation,
* P<0.05 was considered as statistically significant.

a significant positive correlation was found between Hcy and MDA (r = +0.421, P < 0.05) (Figure 1).

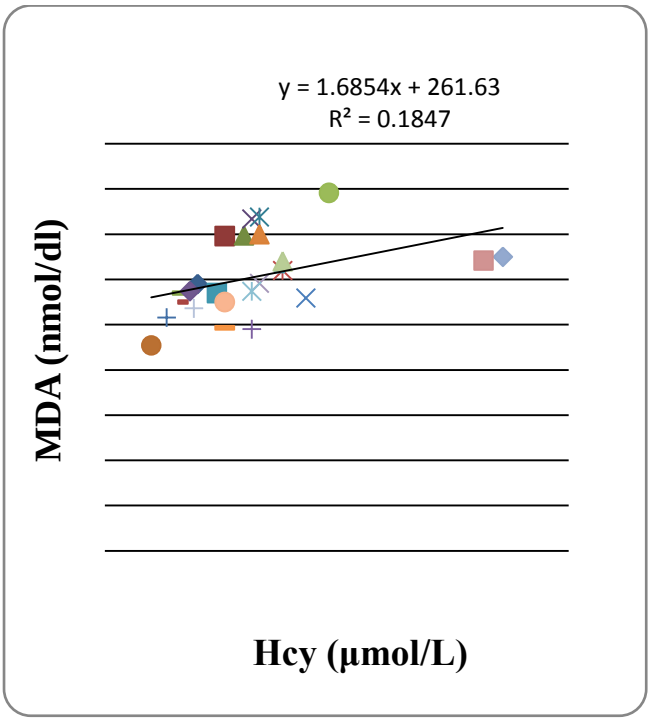

Figure 1. Correlation analysis between Hcy and MDA

There was significant negative correlation found between Hcy and TAC levels (r = -0.411, P < 0.05) (Figure 5).

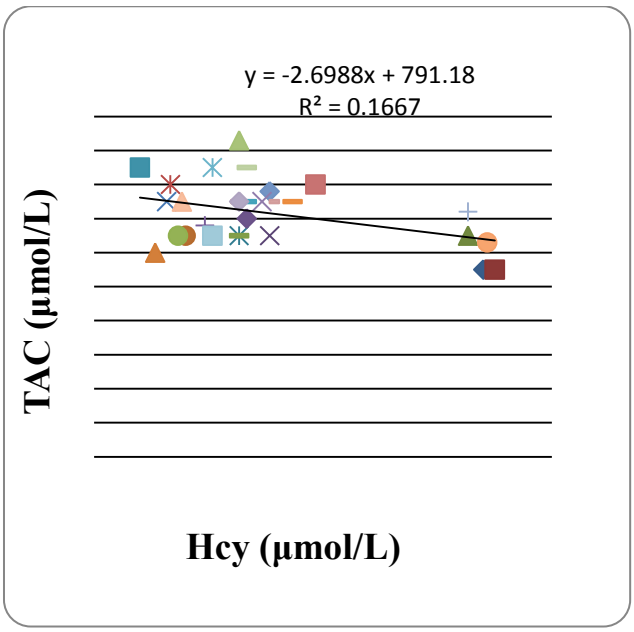

Figure 5. Correlation analysis between MDA and TAC

Correlation analysis showed that Hcy levels of patients has no correlation with activity of RBC-SOD, RBC-GSH and thiol protein levels (r = - 0.11, P > 0.05; r = - 0.25, P > 0.05 and r = - 0.35, P > 0.05; respectively) (Figure 2, 3 and 4).

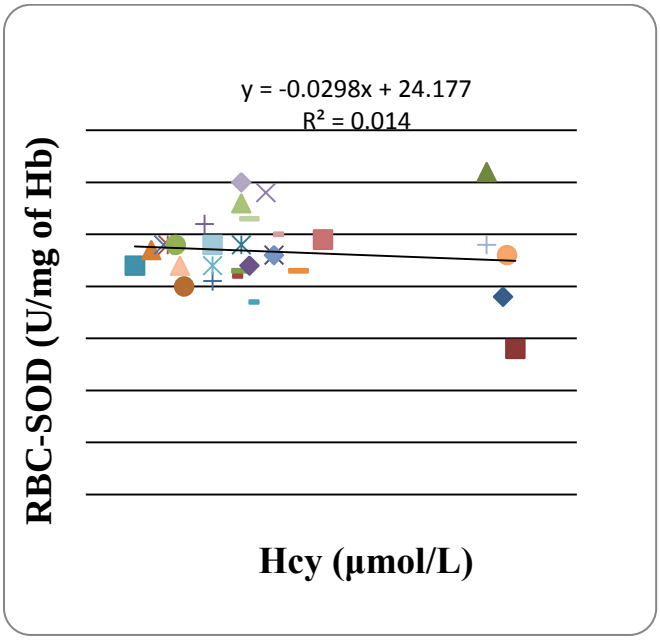

Figure 2. Correlation analysis between Hcy and RBC-SOD

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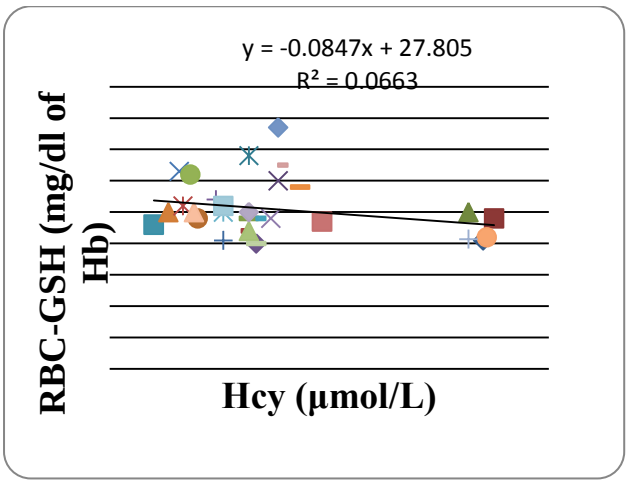

Figure 3. Correlation analysis between MDA and RBC-GSH

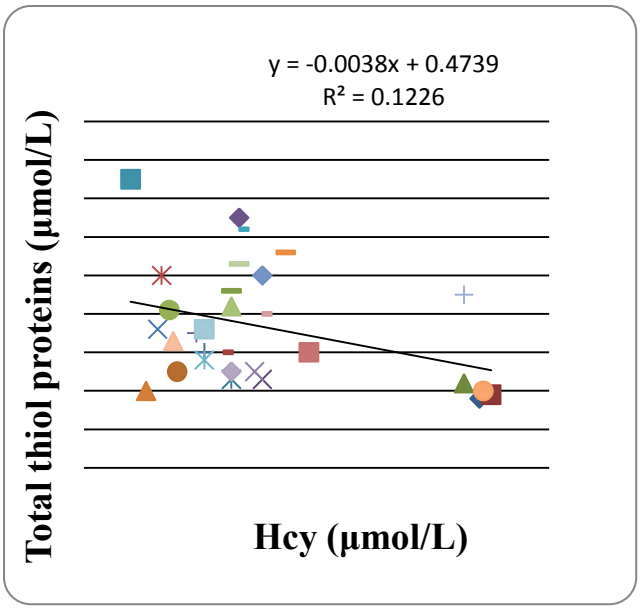

Figure 4. Correlation analysis between MDA and Total thiol protein

Therefore, elevated plasma homocysteine enhanced oxidative stress by lipid peroxidation in AD. At the same time, linear correlation analysis showed that the effect of oxidative stress in the pathogenesis of AD has no specificity. No correlation was found in between Hcy and oxidative biomarkers in controls subjects.

Discussion

The present study demonstrated that the plasma total Hcy and serum MDA were significantly higher in patients with AD compared to healthy control group. Erythrocyte-SOD activity and the levels of erythrocyte-GSH, total thiol proteins and TAC levels were depleted significantly in AD patients compared to healthy controls. A significant positive correlation was found between Hcy and MDA (Figure 1) and negative correlation was found between Hcy and TAC levels ($r = -0.411$, $P < 0.05$) (Figure 5). Correlation analysis showed that Hcy levels of patients has no correlation with activity of RBC-SOD, RBC-GSH and thiol protein levels The findings of the present study suggest a strong association between elevated plasma homocysteine, lipid peroxidation and depleted antioxidants defense system in AD patients.

There is substantial evidence implicating oxidative stress mechanism in AD. Evidence for oxidative stress derives from both human (post-mortem and living patients) studies, and transgenic mouse models of the disease. There is a long list of surrogate markers of reactive oxygen species-mediated injury that have been found increased in the brain and cerebrospinal fluid of AD patients. It includes, just to mention a few, MDA, 4-hydroxynonenal, F2-isoprostanes (lipid peroxidation); protein carbonyls, nitrotyrosine (protein oxdidation); 8-hydroxy-2'-deoxyguanosine (DNA oxidation) [8, 17, 18, 31].

Amyloid theory gives more importance to the presence of oligomers than to amyloid plaques and although it has been not described what is the molecular pathway by which amyloid-beta (Aβ) can achieve its supposed toxic function [1].
Ozcankaya et al , Balazs et al and Palmer et al reported that MDA levels are elevated in Alzheimer's patients compared to controls. The study also reported that increased lipid peroxidation may promote the formation of additional ROS and enhance protein and DNA oxidative damage [32-34].

Zhao et al showed that the serum levels of MDA in hyperlipidemia group with AD (AH) group are significantly higher than that of in normal group (N). The serum levels of MDA in AD group

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without hyperlipidemia (A) and hyperlipidemia group without AD (H) groups compared with N group have no significant change; MDA levels have no significant change among A and H groups [10].

The results of our prospective, observational study indicate that there is a strong, graded association between plasma total homocysteine levels and the risk of dementia and AD. Elevated concentrations of homocysteine in human tissues, definied as hyperhomocysteinemia have been correlated with some diseases, such as cardiovascular, neurodegenerative, and kidney disorders. The elevated level of homocysteine has been repeatedly observed in patients with AD. The substantial evidence that total Hcy is an independent vascular risk factor supports the role of hyperhomocysteinemia in AD [6].

Subjects with vascular risk factors and cerebrovascular disease have an increased risk of AD, and hyperhomocysteinemia has been related to cerebral macro- and microangiopathy, endothelial dysfunction, impaired nitric oxide activity, and may also enhance oxidative stress (10). Moreover, as shown in cell cultures, homocysteine can directly cause brain damage through several mechanisms: increased glutamate excitoxicity via activation of *N*-methyl-D-aspartate (NMDA) receptors, enhancement of β-amyloid peptide generation, impairment of DNA repair, and sensitization of neurons to β-amyloid toxicity [35].

There are conflicting reports shown by some the studies as increase in erythrocytic SOD activity in patients (31, 35) while some showed a decrease in SOD activity (10, 36). But our observations were founded as those studies documenting illustrating reduced activity of SOD in patients with neuropsychiatric disorders.

Decreased SOD activity suggests that increased generation of ROS associated with the oxidative stress may induce lipid peroxidation and consequently decreasing antioxidant defense AD [36, 37].

This study is in concurrence with most of the studies [38-40] which reported declined levels of GSH in Alzheimer's disease. But, very few studies [9] showed that GSH level does not changed. GSH maintains the redox status of sulfhydryl proteins which are necessary for DNA expression and repair. A primary decrease of GSH may explain both the glutamate hypofunction and the dopamine dysfunction hypothesis [38]. The cause of GSH depletion is not clearly known but it has been reported that the content of GSH in brain cells strongly depends on the availability of GSH precursors. This is due to consumption of GSH during oxidative stress [39].

Low levels of total thiol demonstrated an oxidative damage to protein in stress conditions. Decreased levels of total thiol proteins may be due to decreased GSH content. The decreased thiol content may suggest its effects by changes in protein conformation and altering the thiol groups present at enzyme active sites [40, 41].

Lowered TAC results in poor production of erythrocyte membrane from oxidative stress [42]. Depleted antioxidant status due to increased utilization with increasing oxidative stress has been reported [43]. Our results are consistent with the hypothesis that oxidative stress is a feature of Alzheimer's disease and the determination of oxidative stress parameters including MDA, Hcy and antioxidants levels could be used as a clinical index for diagnosis [10]. Moreover, our experiments indicate that the correlation between the increased amount of homocysteine and the oxidative stress exists. Considering the data presented in this study, we suggest that the elevated Hcy in AD patients may stimulate the oxidative stress.

The ideal study would be to evaluate oxidative stress parameters in brain tissue or the cerebrospinal fluid but this is not practical. The various oxidative-antioxidative system parameters

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that were measured are substances that pass from the brain to the blood and vice versa and many studies have analyzed these parameters only in the serum, as in the present study.

For future, it is interested to find out that should elevated plasma Hcy be proven to play a role in the pathogenesis of AD and evaluate the role of different antioxidants in the oxidative stress in their modification with antioxidants may offer therapeutic benefit.

Conclusion

In conclusion we found higher serum MDA and lower antioxidants (RBC-SOD and GSH, thiol proteins) and TAC Alzheimer's disease patients than in healthy controls.

Considering the data presented in this study, we suggest that the elevated Hcy in AD patients may stimulate the oxidative stress. The present study further emphasizing about the growing concepts that free radicals induced damage may have an important role in development of neuropsychiatric disease and suggests that decreased plasma antioxidant defenses may be related to the disease progression.

The relation between elevated plasma homocysteine levels and dementia must be evaluated in other cohort studies. If such studies confirm our findings, proof of a causal association between plasma Hcy and oxidative stress in AD will require further elucidation of the pathophysiologic mechanisms and direct evidence from controlled clinical interventions in humans that reduce plasma homocysteine levels can reduce the risk of AD. Further studies on Hcy and its association with oxidative stress in AD are still necessary to improve our understanding of the disease pathogenesis.

List of abbreviations

AD- Alzheimer's disease
MDA- Malondialdehyde
Hcy - Homocysteine
ROS- Reactive oxygen species
GSH- Reduced glutathione
TAC- Total antioxidant capacity
APP- Amyloid precursor protein
Aβ- Amyloid β
DNA- Deoxyribose nucleic acid

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