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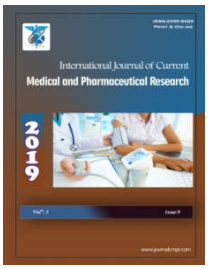

Research Article

EVALUATION OF SERUM ASCORBIC ACID, ALPHA-TOCOPHEROL AND PYRIDOXAL PHOSPHATE IN SENILE DEMENTIA

Balasaheb.H. Jadhav[1], Madhav. G. Kalekar[2] and Vaishali Saigaonkar[3]

[1]Dept of Biochemistry, Bidar institute of Medical Sciences Bidar
[2]Dept of Biochemistry, Grant Medical College Mumbai
[3]Medical Officer Primary Health Centre. Andur. Bidar

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ABSTRACT

The neurodegeneration disease is a senile dementia characterized by loss of memory, attention and cognitive functions. The oxidants- antioxidant plays important role by several mechanism and role which reduces β-amyloid toxic to neurons, thus have been interest in dementia. Serum levels of Malondialdehyde (indicator of Lipid peroxidation), Ascorbic acid, alpha-Tocopherol and Pyridoxal phosphate were measured using standard methods in 50 patients of dementia and 50 non-demented age and sex matched subjects. There was significant difference observed between healthy controls and Alzheimer dementia, decreased serum levels of Ascorbic acid, alpha-Tocopherol and Pyridoxal phosphate and increased levels of serum levels of malondialdehyde when compared with healthy controls ($p < 0.001$). Average of MDA, Ascorbic acid, Alpha-Tocopherol and Pyridoxal phosphate for healthy Control group were 2.51with SD ±0.21, 1.77 with SD ±0.20, 8.16 with SD±0.47 and 27.95 with SD±0.21. Average of MDA, Ascorbic acid, Alpha-Tocopherol and Pyridoxal phosphate for Alzheimer dementia were 4.14 with SD ±0.41, ($p < 0.001$), 0.79 with SD ±0.02, ($p < 0.001$), 6.03 with SD±0.47, ($p < 0.001$) and 18.36 with SD ±1.92 ($p < 0.001$). Thus, altered oxidative stress markers may play important role in neurodegenerative diseases like Senile dementia.

INTRODUCTION

"Senile Dementia is a neurodegenerative progressive loss of neurons leads loss of cognitive function, or disease of normal brain aging. In the brain there may be loss of memory attention, language, problem solving and disoriented in time, day, week, month, year, place and person in later stages of diseased person''. [(1)]

The diagnosis of dementia clinically made by Diagnostic and statistical manual of mental disorders, 4th edition (DSM IV).[(2)] Dementia can be classified by various types like Alzheimer's disease, vascular dementia, dementia due to head trauma, HIV, Parkinsons disease etc.

The 3.7 million people are affected by dementia at present in India according to ARDSI and it will double by 2030. The estimated cost of dementia patient for taking is huge about 43,000 annually. So we can't ignore challenges posed by dementia on the grounds of health and social issues, despite immensity there is gross of unawareness abandon and lack of services for dementia patients and their families.[(3)]

The main culprit of Alzheimer's disease is Amyloid-β(Aβ), Aβ aggregation amyloidogenesis and deposition of Aβ leads to plaque formation. Aβ induces tau phosphorylation leads in to ROS formation through peptidyl radicals' damages mitochondrial DNA, RNA, lipids and protein that results in to synapse damage and death of neuronal cell. This is taking place at memory centre, the hippocampus of the brain.

Free radicals dismissed, non-existent for many years in biological system but now their existence and importance in living system is accepted. Free radical are important biochemical intermediates of all metabolism linked with at cellular homeostasis. [(4)] In Neurological disorder the oxidative stress has been important role in recent research. The pathophysiology of neurodegenerative disorders involved by oxidative stress. [(5)]

The β-amyloid peptide produced free radicals are produced outside the neurons and these free radicals become neurotoxic to synaptosomal membrane and the hippocampal cells. [(6)] Such type of reactions is catalysed by the transition metals (iron), iron also produces free radicals of all organs in the body, but CNS is more oxidative damage by free radical as it is very sensitive as compared with other organs, due to more consumption fraction of oxygen (20%) and high metabolic rate in brain which leads in to increasing the amount of free radicals. [(7,8)]

Corresponding author:* **Balasaheb.H. Jadhav
Dept of Biochemistry, Bidar institute of Medical Sciences Bidar

The brain membranes contain proteins and a greater number of phospholipids. These phospholipid (PUFAS), like docosahexaenoic acids and archidonic acid, these PUFAS contain hydrogen ions held together by weak double bonds are very much vulnerable to attack by free radicals that serve as a target for reactive oxygen radicals and leads to oxidative damages. [9] It has been recommended that due to oxidative damage leads to alterations of phospholipids in the brain may play role in AD. [10] This study evaluate the oxidative stress markers in senile dementia patients.

MATERIAL AND METHODS

Aims and Objective

1. To Evaluate Malondialdehyde (indicator of Lipid peroxidation), Ascorbic acid, alpha-Tocopherol and Pyridoxal phosphate in Senile dementia.
2. To understand the role of oxidative stress markers in Senile dementia.

Study design

This study was designed as randomised controlled study.

Subjects and Method

The total number of subjects included in this study was 100 out of which 50 patients of Alzheimer's type of dementia diagnosed by DSM-IV and 50 age and sex matched controls. The informed written consent was taken from the subjects and study was approved by institute ethical committee.

Inclusion Criteria

- Newly diagnosed cases, not on treatments
- Male subjects, above 50 to 70 years.
- MMSE Score of less than 26.

Exclusion Criteria

- Patients addicted to alcohol or drug abuse.
- Patients suffering from major psychiatric disorder, chronic illness.
- Any other concurrent drug intake

Estimation of Oxidative stress markers

The 5ml blood samples from patients and control were collected from anticubital vein afterb12 hours fasting, with all aseptic precautions in plain polythene tubes containing EDTA for the estimation of oxidative stress markers. Plasma was separated by centrifuging the samples at 3000rpm for 10 minutes. These plasma samples were preserved in freezer till the laboratory estimation proceeds.

Malondialdehyde (MDA) was determined by using Thiobarbituric acid method.[11] 2.5ml of 20% trichloroacetic acid was added to 0.5ml of plasma in a test tube and allowed to stand for 10 minutes at room temperature. After centrifugation at 3500rpm for 10 minutes, the supernatant was decanted and the precipitate was washed once with 2ml of 0.5mM sulphuric acid . 2ml of 0.5M sulphuric acid and 3ml of TBA in 2M sodium sulphate were added to this precipitate and the coupling of lipid peroxide with TBA was carried out by heating in a boiling water bath for 30 minutes. After cooling in cold water, the resulting chromogen was extracted with 4ml of n-butanol by vigorous shaking. Separation of the organic phase was facilitated by centrifugation at 3000rpm for 10 minutes and its absorbance was measured at 530nm. The values were expressed in terms of malondialdehyde (nmol/ml).

The estimation of Ascorbic acid was done by the colorimetric method of Ayekyaw (1978).[12] 2ml serum sample was taken in a test tube (test) and 2ml distilled water was taken in another tube (blank). 2ml colour reagent was added in both tubes. Mixed thoroughly and allowed to stand for 30 minutes at room temperature, (Reaction is completed and is stable).The tubes were centrifuged at 300 r.p.m for 10 minutes. The clear blue supernatant was taken in cuvette (without disturbing the precipitate) and absorbance was read at 700 nm. against the blank. A standard graph of optical density versus concentration was plotted using 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 ml of working standard solution (making final volume to 10 ml with distilled water).The unknown test concentrations were extrapolated from the standard graph using their respective optical densities.

The estimation of Alpha-Tocopherol was done by colorimetric method of Baker and Frank (1949). [13] In to three stoppered centrifuge tubes 1.5 ml serum, 1.5 standard, and 1.5 ml water (blank) was taken respectively. Then in test and blank 1.5 ml Xylene was added, stoppered and mixed well and centrifuged. 1 ml of the Xylene layers was transferred into stoppered tubes. 1 ml α,α-Dipyridyl reagent was added to each tube. Add extinction of test and standard was read against the blank at 460 nm. Then in turn beginning with the blank 0.33ml ferric chloride solution was added mixed and after exactly 1.5 min extinction test and standard was read against the blank at 520 nm.

The estimation of Pyridoxal phosphate was done by enzymatic Bhulmann method.[14] Samples and controls have to be diluted 1:40 with dilution buffer. Diluted samples and controls are not stable. Thus, prepare dilution immediately before usage.E.g. Pipette 25 µl of patient sample or control into a disposable polypropylene microtube, add 975 µl of Dilution Buffer and mix well (vortex). The test should be carried out in duplicates. Pipet 50 µl of Substrate into wells of Microtiter plate. Pipet 50 µl of Calibrator 0 nmol/L (Blank), Calibrator 20 nmol/L, Calibrator 20 nmol/L, Control Low (diluted), Control Normal (diluted), Diluted patient samples. Add 50 µl of Apo-Enzyme to each well. Mix shortly (10-15 seconds) with a microtiter plate shaker. Incubate the Microtiter plate for 30 minutes at 37^0C (+5 minutes) in a plate incubator. Pipet 100 µl of enzyme with a multipipette (with disposable tips) in to wells of microtiter plate. Shake the plate gently (5-10 seconds) with microtiter plate shaker. Incubate the microtiter plate for 15 minutes at 37^0C (+3 minutes) in a plate incubator. Read the OD at 546 nm (or at 520-595 nm) in a microtiter plate reader within 3-5 minutes. Use endpoint mode with two calibrators (20 and 200 nmol). Calibrator 0 is used as Blank. Read absorbances (OD) for Calibrator 0 (Blank), calibrators, controls and samples. Have the duplicates averaged for each calibrator, control, and sample and subtract the average Blank. Have a standard curve created by using linear curve fitting.

RESULT

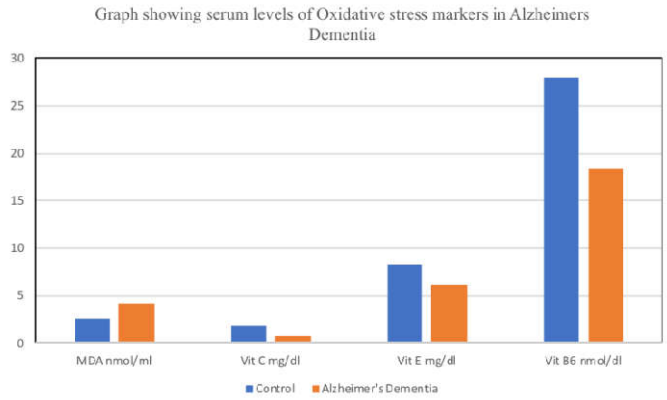
The study was done on male subjects with mean age of cases being 73.42 ± 3.72 and that of controls 74.56 ± 4.30. The Ascorbic acid levels were lower in Alzheimer's dementia patients (0.79 with SD. ±0.02, P value < 0.001) compared to healthy controls (1.77 with SD ±0.20). Similarly Alpha-Tocopherol and Pyridoxal phosphate levels were lower in

Alzheimer's dementia (6.03 with SD±0.47 & 18.36 with SD ±1.92, P value < 0.001) as compared to healthy controls (8.16 with SD±0.47 & 27.95 with SD±0.21). The levels of MDA were higher in Alzheimer's dementia patients (4.14 with SD ±0.41nmol/ml, (p < 0.001) as compared to controls (2.51with SD ±0.21nmol/ml).

Table 1 Serum Levels of Oxidative Stress markers in Control and Alzheimer's dementia.

Sr. No.			Groups		
			Healthy Control Mean Age (73.42 ± 3.72)	Alzheimer's Dementia Mean Age (73.42 ± 3.72)	P value
1	Ascorbic Acid mg/dl	Mean/ SD	1.77±0.20	0.79±0.02	< 0.0001
2	Alpha-Tocopherol mg/dl	Mean/ SD	8.16±0.47	6.03±0.47	< 0.0001
3	Pyridoxal phosphate nmol/L	Mean/ SD	27.95±0.21	18.36±1.92	< 0.0001
4	MDA nmol/L	Mean/ SD	2.51±0.21	4.14±0.41	< 0.0001

Statistically significant difference was observed between healthy controls and Alzheimer's dementia i.e decreased levels of Ascorbic acid, Alpha-Tocopherol, Pyridoxal phosphate when compared with healthy controls. (p < 0.001) and increased levels of MDA when compared with healthy controls. (p < 0.001).



DISCUSSION

The present study evaluated oxidative stress markers in Alzheimer's dementia patients in comparison with age and sex matched controls. The results showed that a decrease in blood levels of antioxidants such as Ascorbic acid (Vit C), Alpha-Tocopherol (Vit E) and Pyridoxal phosphate (Vit B6) in patients with Alzheimer's dementia, with higher levels of MDA. Most of the previous studies on evaluation of these oxidative stress in brain region. This is in contrast of our study were the levels of these markers assayed in blood and not in brain region.

In Neurological disorder the oxidative stress has been important role in recent research. The pathophysiology of neurodegenerative disorders like Alzheimer's disease involved by oxidative stress.[15] In the brain, production of free radicals in AD patients are unique which comes from sources of AD affected brain. It has been shown that the free radical production by β-amyloid peptide and advanced glycation end products. [16,17]

The components of cells and mitochondrial DNA, RNA, Proteins and lipids damages by increased ROS which leads in to synapse damage and finally neuronal cell death. This whole process taking place in to the memory centre, the hippocampus, and related key brain areas of AD patients. The critical step in the pathogenesis of several diseases is lipid peroxidation. The underlying mechanism of oxidative damage due to free radicals for many pathological conditions. The cellular component is damaged by free radicals. Lipid peroxidation in often the first parameters to which researchers turn when they wish to prove the involvements of free radicals in cell damage. Free radical involvement in neurodegenerative disorder is through distinct stages; initiation, elongation & chain propagation. [18]

Table no 7 & Graph no- 11 clearly shows significantly elevated levels of lipid peroxide and decreased levels of Ascorbic acid (Vit C), Alpha-Tocopherol (Vit E) and Pyridoxal phosphate (Vit B6) in Alzheimer's dementia patient compared to control groups.

Baldeiras *et al*. Greilberger *et al* 2008 studies found that higher levels of lipid peroxidation in the central nervous system and peripheral tissues both in patients and Alzheimer's dementia and mild cognitive impairement.[19] Hence one can postulate the accumulation of free radicals which leads in to stimulate antioxidant defences, leads to depletion of antioxidant reserves. Hence our study showed there was increased lipid peroxidation (MDA). Thus we conclude that the free radical produced by this β-amyloid protein is neurotoxic and vascular endothelial cells produce abundance superoxide radicals by interacting with β-amyloid causing lipid peroxidation. The surrounding of senile plaque contains deactive microglia cells which become active macrophage of microglia can generate ROM and increases susceptibility of lipid peroxidation membrane causing loss of cholinergic neurodegeneration and dopaminergic neurons in the Alzheimer's brain.

Riviere S *et al*, Montilla lopez P *et al*. studies showed that like other vitamins the plasma levels of vitamin C were significantly lowered in patients of Alzheimer's disease even after adequate amount of this vitamin in the diet. Further invitro and invivo research showed that vitamin C can decrease oxidative stress and inhibits structural progression of Alzheimer's disease by arresting the oligomerization of Aβ peptides.[20] Our results also showed similar of this research. Therefor we suggest that due to increased oxidative stress by free radical increases consumption of Ascorbic acid (Vit C) for countering the free radicals, decreases the antioxidants like Ascorbic acid (Vit C) and regeneration of Alpha-tocopherol (Vit E).

Mangialasche F, Xu W, Kivipelto M *et al* studies reported that decreased levels of plasma Vitamin E with increased risk of associated neurodegenerative disorders like Alzheimer's disease and mild cognitive impairement.[21] Further Aoki K, *et al* postulate that vitamin E deficiency leads in to destruction of neurons causes cerebral atrophy.[22] Alpha-Tocopherol (Vit E) act as chain breaking antioxidant which reduces the progression of Alzheimer's disease. Increased oxidative stress by Aβ plaques is well known risk factor for neuronal damage. Alpha-Tocopherol act as scavenger for these free radical and provides neuroprotection. Therefore Alpha-Tocopherol (Vit E) plays a role in protective plasma lipids from oxidative stress. In our study due to increased oxidative stress, consumption of Alpha-Tocopherol (Vit E) is increased. It breaks the chain reaction by trapping free radicals that damages cells. This leads decreased antioxidant Alpha-Tocopherol (Vit E) in Alzheimer dementia may cause oxyradical mediated injury.

Thus, the important role of Alpha-Tocopherol (Vit E) is in inhibition of lipid peroxidation in Alzheimer dementia.

Pyridoxal-5-Phosphate (Vit B_6) involved in cell metabolism, amino acids synthesis, and their degradation, transformation, supply of one carbon unit, transsulfurartion, synthesis of polyamines, have role in regulation of transcription factors. [23] PLP-dependent takes part in the of neurotransmitters metabolism such as dopamine, epinephrine, norepinephrine serotonin, glycine, gamma-aminobutyric acid, D-serine, L-glutamate, and histamine, levels of this byproducts affected by PLP deficiency leading to loss of body functions such as poor sleep, behaviour, cardiovascular function, loss of hypothalamus pituitary control of hormone secretion. It has been recently shown that Vitamin B_6 is highly antioxidant effects. It was manifested that Vitamin B_6 act as a quencher of hydroxyl radical (*OH) and further up to scavenging of eight (*OH) molecules. [24] Pyridoxine deficiency leads to fatty acids biosynthesis, and impaired defence mechanism and increases lipid peroxidation. [25]

M. Keles, B. *et al* and B. K. Ohta *et al* showed that lower levels of Vitamin B_6 in patients suffering from mild cognitive impairment (MCI) or Alzheimer disease (AD) leads into progression of disease, they further found improvement in cognitive function by supplementation of pyridoxal phosphate (Vit B_6) in their patients groups. [26,27]Therefor due to antioxidant property of pyridoxal phosphate (Vit B_6) we suggest the hypothesis that oxidative stress may reduce vitamin B_6 due to high rate of consumption, which results in to deficiency of pyridoxal phosphate (Vit B_6) leads to increased Homocystein in circulation which results in to toxicity of neurons by generation of ROS and increase the oxidative stress.

In conclusion our results suggest that there is change in production and metabolism of reactive oxygen species. In normal biological process generation of oxidants, which are powerful molecules is balanced by antioxidants. In neurodegenerative disorders like Alzheimer dementia there is imbalance of oxidant-antioxidant, which results in elevated oxidative stress. Elevated oxidative stress may be responsible for loss of cholinergic, noradregenic, dopaminergic neurons in Alzheimer dementia.

Initial increase in lipid peroxidation is due to β-amyloid protein is neurotoxic and vascular endothelial cells produce abundance superoxide radicals, with reduction in Vitamin C and Vitamin E and Vitamin B6 by exposing the cells to the effect of superoxide radical, hydroxyl radical and hydrogen peroxide. The Oxidant mediated neurotoxicity and loss of neurons occur in neurodegenerative disorders, thus the initiation of progression of Alzheimer dementia is due to free radical injury is important, then the therapy of agument endogenous antioxidant to reduce oxidative injury which might prevent delay the disease process.

Thus, we suggest that antioxidant agents may be useful in treatment of neurodegenerative disorders like Alzheimer dementia. In Alzheimer dementia β-amyloid is toxic to neurons, which can be diminish by antioxidants such as Vitamin C and vitamin E and vitamin B6.

Ascorbic acid (Vitamin C) with multidrug therapy may be useful for neurodegenerative disorders like Alzheimer dementia. Thus, we suggest antioxidant therapy is most promising field in oxygen free radical research.

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