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BIOCHEMICAL, HISTOLOGICAL AND GENETIC EVALUATION OF THE EFFECTS OF CAPTOPRIL ON DEMENTIA IN WISTAR ALBINO MICE

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ABSTRACT

In the elaborate search of the recent times for an effective drug against dementia, Angiotensin Converting Enzyme Inhibitors (ACEI) have become a debated class of drugs due to the existence of both supporting and opposing arguments regarding their benefits in Alzheimer's type dementia. In this study aimed to bring more clarity in this aspect, the centrally acting ACEI, captopril was evaluated for its biochemical, histological and genetic effects on $AlCl_3$ induced mice model of memory impairment. Administration of captopril in two different doses of 5mg/kg and 10 mg/kg for 29 days produced a dose dependent and significant reversal of the $AlCl_3$ induced adverse effects in the level of different biomarkers like acetyl cholinesterase ($P<0.0001$), glutathione ($P<0.0001$), serotonin ($P<0.001$), and malondialdehyde ($P<0.001$) in the brain homogenate of Wistar albino mice. Histological examination of the mice hippocampus also exhibited reversal of the $AlCl_3$ induced neuronal damage by captopril. Moreover the decreased and increased expression of BACE-1 and BDNF gene produced by captopril also support its possible beneficial effects in conditions of dementia.

KEYWORDS: Angiotensin Converting Enzyme Inhibitors, Dementia, Alzheimer's disease, Captopril.

INTRODUCTION

Neurodegenerative diseases (NDD) are a group of conditions involving progressive degeneration and death of neurons. NDD such as Parkinson's disease, Alzheimer's disease (AD) and Huntington's disease affect many activities such as movement, cognition, learning

and memory etc. Dementia is a condition which primarily affects memory, communication and thinking. According to WHO, dementia can be defined as a chronic or persistent disorder of the mental processes caused by brain disease or injury and marked by memory disorders, personality changes and impaired reasoning.[1]

Worldwide estimate of dementia burden indicates that, by 2030, 75.6 million people will be suffering from dementia and it is expected to rise to an estimate of 135.5 million in 2050. [2] In the state of Kerala, 33.6 people per 1000 are affected by dementia, among which, 54% suffer from Alzheimer-type dementia, 39% from vascular dementia and others due to various factors like infection, tumor, trauma etc.[3]

AD is a type of dementia that distorts memory, thinking and behavior. In this case, the symptoms usually develop slowly and get worse over time, becoming severe enough to interfere with daily life activities.[4] The difficulty in remembering recent events is considered to be one of the early symptoms of this kind of dementia. Usually, the disease has been found to be predominant in old age (beginning at 60 years or above).

AD is associated with two pathologic hallmarks:

- Extracellular beta amyloid deposits.
- Intracellular neurofibrillary tangles.

These two changes can lead to loss of synapses and neurons which ultimately results in gross atrophy of the affected areas of the brain. The mechanisms behind the formation of beta amyloid peptides and neurofibrillary tangles are not completely understood. But, many theories are presently under investigation.[5] Two most relevant theories regarding AD are the amyloid hypothesis and tau hypothesis.

According to amyloid hypothesis, otherwise called amyloid cascade, which is the major theory regarding AD, deposition of amyloid-β peptide in the brain is considered to be a crucial step in the pathogenesis of the disease.[6] But, there is no compelling evidence that, once initiated, the disease is continuously driven by Aβ deposition.

The second hypothesis under investigation is the tau hypothesis. Tau is a microtubule-associated protein which regulates the stability of tubulin assemblies. The tau gene is located in chromosome 17. The normal tau is soluble and catalyzed easily. But the abnormal tau is

insoluble and is deposited around neurons. This deposition causes the loss of microtubules which in turn reduces the acetylcholine synthesis. Eventually, this leads to loss of memory.[7,8]

As dementia is a multifactorial disease it has been closely studied with many other diseases. Hypertension is one among these diseases which is believed to be a contributing factor for dementia. In this context, many antihypertensive medications are under observation for their beneficial effect in dementia.[9] Antihypertensive drugs like Angiotensin Converting Enzyme Inhibitors (ACEI) and Angiotensin Receptor Blockers (ARB's) have shown some promising effects in Alzheimer-type dementia, but some also argue that they can lead to Aβ accumulation which in turn worsens AD. ACEIs are one of the most debated Class of antihypertensive in connection with dementia. Even though many studies are being conducted, it is still a controversy whether ACEIs are beneficial in dementia. The reason for this conflict is the disparity that exists in the understanding of whether or not the ACEI enhance Aβ deposition. Different hypotheses exist regarding the same.

One group of researchers suggest that different forms of Aβ have different neurotic effects. Aβ 42 is suggested to have neurotoxic potential whereas Aβ 40 is thought to be neuroprotective. According to this group, in addition to converting angiotensin I to angiotensin II, ACE also has the potential to convert Aβ 42 to Aβ 40. Consequently, the inhibition by ACE inhibitors would aggravate the disease.[10]

On the other hand, another group suggests another pathway. While ACE causes hypertension, heart failure and atherosclerosis peripherally, it acts centrally causing neurodegeneration and stroke. Consequently, the inhibition of ACE might prove to be beneficial in Alzheimer type dementia.[11,12]

As both the above assumptions seem convincing, further research is definitely required so as to confirm the effect of ACEI in dementia. To fill this gap the present study entitled as 'Biochemical, histological and genetic evaluation of the effects of captopril on dementia in Wistar albino mice", aiming to bring more clarity in this aspect was selected. It was to determine whether the ACEI are beneficial / harmful in mice model of dementia and to find out the possible mechanism behind the beneficial / harmful effects, if any. In this study Alzheimer type dementia was produced in Wistar albino mice by the intraperitoneal injection

of the neurotoxic agent Aluminium chloride ($AlCl_3$) for 29 days.[13] The first and widely used centrally acting agent captopril was selected as the ACE inhibitor.

MATERIALS AND METHODS

The study protocol was approved as per the IAEC NO.03/09/2019/IAEC/ Govt. Medical College, Thiruvananthapuram dated 26/10/2019.

Captopril was purchased from Yarrowchem Products and $AlCl_3$ from Kanton Laboratories.
28 healthy adult male Wistar albino mice weighing 20-30 g were used for the study. They were obtained from University of Kerala, Kariavattom and animal house of Govt. Medical College, Thiruvananthapuram. The animals were acclimatized to laboratory conditions for seven days before the experiment.

Grouping of Animals
Group I- (A1) – Distilled water PO (Normal Control) od for 29 days.
Group II-(A2) – $AlCl_3$ 40 mg/kg IP. (Disease control) od for 29 days.
Group III–(A3)–$AlCl_3$ 40 mg/kg IP and Captopril 5 mg/kg in water PO od for 29 days.
Group IV- (A4)-$AlCl_3$ 40 mg/kg IP and Captopril 10 mg/kg in water PO od for 29 days.
After the completion of the drug administrations, the animals were sacrificed by cervical dislocation, skull was cut opened and the brain was exposed from its dorsal side. Immediately the brain was excised and transferred to a petri dish containing normal saline. The isolated brain was used for further studies.

I. Biochemical Investigations

Preparation of brain homogenate:
The hippocampal region of brain was separated and homogenized with 0.1 M phosphate buffer of pH 7.4 (100 mg / mL of phosphate buffer), using Polytron homogenizer. The homogenate was used for the estimation of acetylcholine esterase (Ach E), malondialdehyde (MDA) and glutathione (GSH).

a. Estimation of Acetylcholine esterase level[14]

Being the primary cholinesterase in the body, Ach E catalyzes the breakdown of the neurotransmitter acetylcholine. It is important to check Ach E levels in dementia as people with dementia often have some kind of cholinergic deficits in their neurons.

Principle

The Ach E activity in the brain homogenate was determined according to the Ellman's method for Ach E estimation. In this method, acetylthiocholine iodide, a synthetic substrate was used for the estimation of Ach E activity. Acetylthiocholine iodide gets converted into thiocholine and acetate by the enzyme Ach E. The formed thiocholine reacts with dithio-bisnitrobenzene acid (DTNB) to produce yellow color, which is a measure of Ach E activity.

Procedure

The brain homogenate was centrifuged at 2000 rpm and the supernatant was taken. 100 µL of supernatant was added to a cuvette containing 2.8 mL of 0.1 M phosphate buffer and 100 µL of 0.01 M DTNB solution. Immediately added 20 µL of 0.075 M acetyl thiocholine iodide and absorbance was measured at 412 nm every 60 sec for 4 min against blank.

b. Estimation of Malondialdehyde level[15]

MDA is the major product of lipid peroxidation which acts as a marker of oxidative stress. The variations in the level of MDA in biological samples indicate the extent of oxidative stress or lipid peroxidation.

Principle

MDA reacts with thiobarbituric acid (TBA) at low pH and elevated temperature to form a complex with absorption maxima at 532 nm.

Procedure

Brain homogenate was centrifuged at 4600 rpm for 10 min. To 0.1 mL of the supernatant, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of TBA and 0.2 mL of 8.1 % SDS was added and made up to 4 ml using distilled water. The tubes were mixed and heated to 100 ^{o}C for 60 min on a water bath and cooled under tap water. 5 ml n-butanol- pyridine mixture was added followed by 0.1 ml of distilled water. The tubes were shaken vigorously for 1 min and the mixture was centrifuged at 4000 rpm for 10 min. The upper organic layer was removed and the absorbance was measured at 532 nm against the blank (Pure sample of butanol-pyridine mixture).

c. Estimation of Glutathione level.[16]

Principle

GSH is an antioxidant which is capable of protecting the cells from damage caused by free radicals. Studies indicate that people with dementia have lower levels of glutathione thereby making the brain more vulnerable to neuronal dysfunction and neurodegeneration.

The assay involves optimized enzymatic recycling method using Glutathione reductase and Ellman's reagent (DTNB). Glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). DTNB reacts with GSH to form yellow coloured chromophore, 5- thionitrobenzoic acid (TNB) with absorbance maxima at 412 nm.

Procedure

The brain homogenate was mixed with an equal amount of 10% TCA and centrifuged at 2000 rpm for 10 min. The supernatant was used for GSH determination. To 0.1 mL of the supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 ml DTNB and 0.4 mL of double distilled water were added. The mixture was shaken vigorously and vortexed. The absorbance was read at 412 nm within 15 min against the blank (phosphate buffer).

d. Estimation of Serotonin level.[17]

Serotonin, which is the neurotransmitter having a role in well-being and happiness has been widely correlated with normal functioning of brain in areas including learning, memory and mood behavior.

Principle

The serotonin content was estimated based on the reaction between serotonin and o-phthaldialdehyde resulting in the formation of a fluorophore, which can be determined spectrofluorimetrically.

Procedure

Preparation of brain homogenate

Homogenized the brain in HCl- butanol (100mg/mL) for about 1 minute, centrifuged for 10 minutes at 2000 rpm. Supernatant (1 mL) was removed and added to centrifuge tube containing 2.5 mL heptane and 0.31 mL 0.1 M HCl. After 10 minutes of vigorous shaking, the tube was centrifuged for 10 minutes at 2000 rpm in order to separate the two phases and upper organic phase was discarded. The aqueous phase was then taken for estimation.

To 0.2 mL aqueous phase, 0.25 mL of O-Phthaldialdehyde reagent was added. The fluorophore was developed by heating to 100 ^{o}C for 10 minutes and the absorbance was measured at 470 nm.

II. Histological examination of brain.[18]

After sacrifice, the brain was immediately isolated and fixed in 5 mL of 10% neutral buffered formalin solution overnight. The hippocampal region was sliced and placed in the cassette and processed by soaking it in different concentrations of isopropyl alcohol. Later it was cleaned by xylene, embedded in paraffin wax and sectioned using microtome. Sections of 5-6 µm thickness were stained with Hematoxylin and Eosin stain and examined for histopathological changes under high power microscope.

III. Gene expression studies.[19, 20]

Gene expression studies were done to elucidate the effect of captopril on the mRNA levels of BACE 1 and BDNF in the hippocampus of albino mice.

a. BACE

BACE-1 (Beta-site Amyloid precursor protein Cleaving Enzyme-1) is an aspartic acid protease expressed in the brain and is encoded by the BACE-1 gene. Its major function is myelin sheath formation in the peripheral nerve cells. BACE is also a major factor for the cleavage of APP to amyloid β peptides and the level of BACE-1 is elevated in Alzheimer's dementia and therefore, it is considered as a biomarker of the disease.

b. BDNF

BDNF (Brain-Derived Neurotropic Factor) gene is responsible for the production of a protein, Brain-Derived Neurotropic Factor, which is mainly found in the brain and spinal cord. It belongs to a family of neurotrophins that have a crucial role in survival and differentiation of neuronal populations during development.

Neurotropic factors also have a protective role against amyloid beta toxicity. Reduced expression of BDNF has a crucial role in the pathogenesis of Alzheimer's disease.

Procedure

At the end of the 29 days, mice were killed by cervical dislocation and the brain tissue was isolated from the different groups, washed in PBS, transferred to RNA later solution and chilled. Just before homogenization tissue samples were thawed, homogenized in RNA-

Xpress reagent at the rate of 1mL/ 100 mg and the total RNA were extracted as per manufacturer's instruction (Himedia Pvt. Ltd, India). Purified RNA was used for reverse transcription and amplification using MMLV one step RT PCR kit (Merck Genei). PCR was performed using the following primers.

BDNF- F: 5'-AGCTGAGCGTGTGTGACAGT-3'
R: 5'- ACCCAATGGGATTAACACTG-3'
BACE - F: 5'-TGGGTGAAGTCACCAATCAG-3'
R:-5'-CACTGGCCGTAGGTATTGCT-3'
ß Actin- F: 5'-CTGACCGAGCTGGCTAC-3'
R: 5'-CCTGCTTGCTGATCCACA-3'

(ß Actin was used as the house keeping gene in the expression analysis of both BACE and BDNF).

cDNA synthesis was performed at 70 ^{o}C for 5 minutes followed by inactivation at 95 ^{o}C for 3 minutes. 35 cycles of amplification were performed using a thermal cycler (Eppendorf Master Cycler, Germany) as per the following pattern. Denaturation at 94 ^{o}C for 30 seconds, annealing at 63 ^{o}C for 45 seconds and extension at 72 ^{o}C for 45 seconds. A final extension was performed for 15 minutes at 72 ^{o}C. The PCR products were run on a 1.5% agarose gel containing ethidium bromide. Band intensity was normalized to values for ß-Actin that was used as the internal control using Image J analysis software and expressed as arbitrary units.

RESULTS AND DISCUSSION

I. Biochemical Investigations

a. Estimation of Acetylcholine esterase level

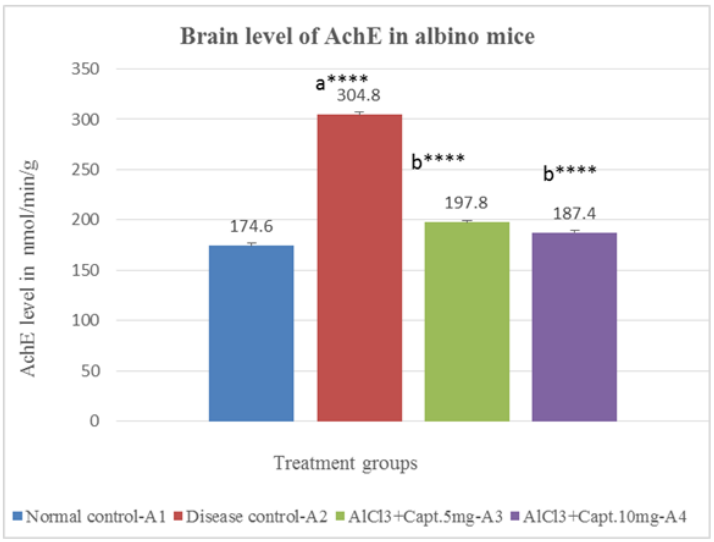

Fig. 1: Effect of Captopril treatment on brain Ach E levels of albino mice.

*All values are Mean + SEM, n=5. Statistical analysis is by One-way ANOVA followed by Post hoc Tukey's. a**** denotes p< 0.0001 compared to control and b**** denotes p<0.0001 compared to disease control ($AlCl_3$ 40 mg/kg) on the same day.*

Fig. 1 represents the Ach E levels in the brain homogenate of albino mice after different treatments for 29 days. The group A2 ($AlCl_3$ 40 mg/kg) showed an increase in level of Ach E (304.8 ± 3.5) nmol/min/g tissue when compared to control (174.6 ± 2.01) nmol/min/g tissue. This increase in Ach E caused by $AlCl_3$ was reversed by Captopril treatment to a statistically significant (p<0.0001) level of (197.8 ± 2.5) nmol/min/g tissue for group A3 ($AlCl_3$ 40 mg/kg + Captopril 5 mg/kg) and to (187.4 ± 2.5) nmol/min/g tissue for group A4 ($AlCl_3$ 40 mg/kg + Captopril 10 mg/kg).

b. Estimation of Malondialdehyde level

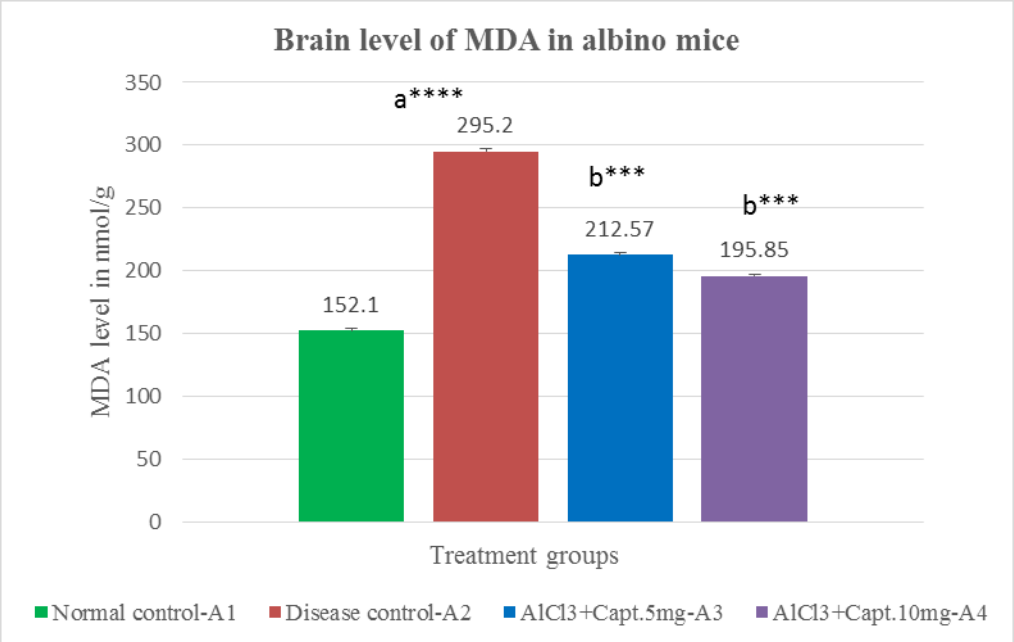

Fig. 2: Effect of Captopril treatment on brain Malondialdehyde levels of albino mice.

*All values are Mean + SEM, n=5. Statistical analysis is by One-way ANOVA followed by Post hoc Tukey's. a**** denotes p< 0.0001 compared to control and b*** denotes p< 0.001 compared to disease control ($AlCl_3$ 40 mg/kg) on the same day.*

Fig. 2 represents the MDA level in the brain homogenate of albino mice after different types of treatment for 29 days. The MDA level in A2 (295.2 ± 0.4) nmol/g tissue was significantly (p<0.0001) higher than that of the control (152.1 ± 2.4) due to oxidative stress. This increase in MDA level is an indication of brain damage mainly due to oxidative stress.

The highly elevated levels of MDA caused by $AlCl_3$ treatment was decreased in a statistically significant ($p<0.001$), and dose dependent manner, compared to A2, in the groups A3 (212.57 + 1.64) nmol/g and A4 (195.85 + 1.31) nmol/g with the co administration of Captopril.

c. Estimation of Glutathione level

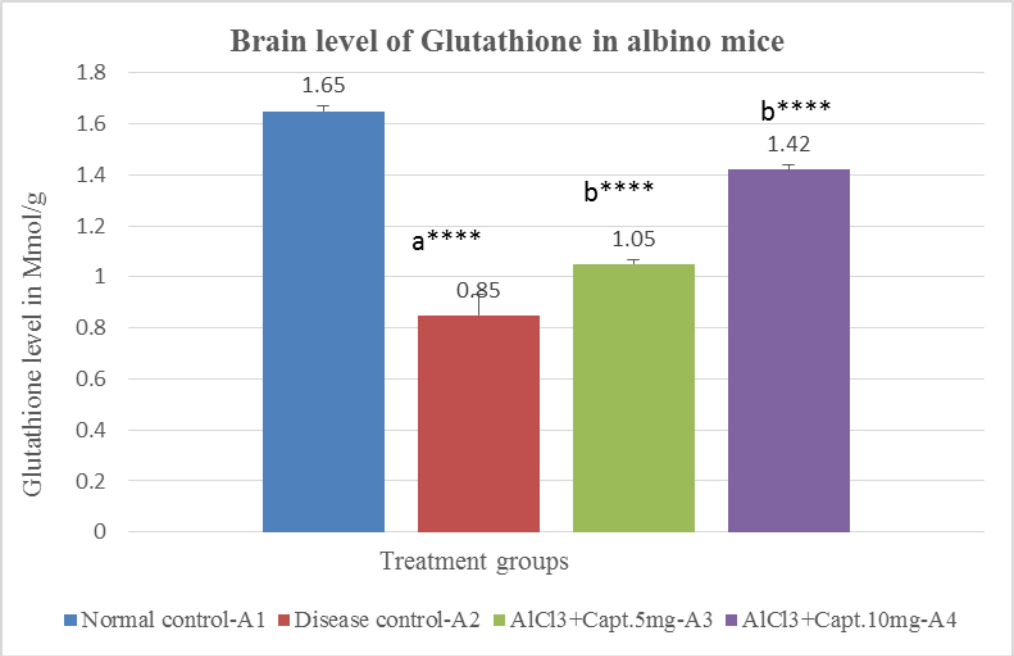

Fig. 3: Effect of Captopril treatment on brain glutathione levels of albino mice.

*All values are Mean + SEM, n=5. Statistical analysis is by One-way ANOVA followed by Post hoc Tukey's. a**** denotes p< 0.0001 compared to control and b**** denotes p< 0.0001 compared to disease control ($AlCl_3$ 40 mg/kg) on the same day.*

Fig. 3 represents the glutathione level in the brain homogenate of albino mice after different types of treatment for 29 days. The group A2 (AlCl3 40 mg/kg) showed a significant ($p<0.0001$) decrease in Glutathione level (0.85 ± 0.08) Mmol/g, compared to control (1.65 ± 0.02) Mmol/g. The dose dependent potential of Captopril to reverse the $AlCl_3$ induced glutathione deficit is evident from the increased values of glutathione in group A3 (1.05 ± 0.02) Mmol/g, and in group A4 (1.42 ± 0.02) Mmol/g, both significant compared to $AlCl_3$ treated group ($p<0.0001$).

d. Estimation of Serotonin level

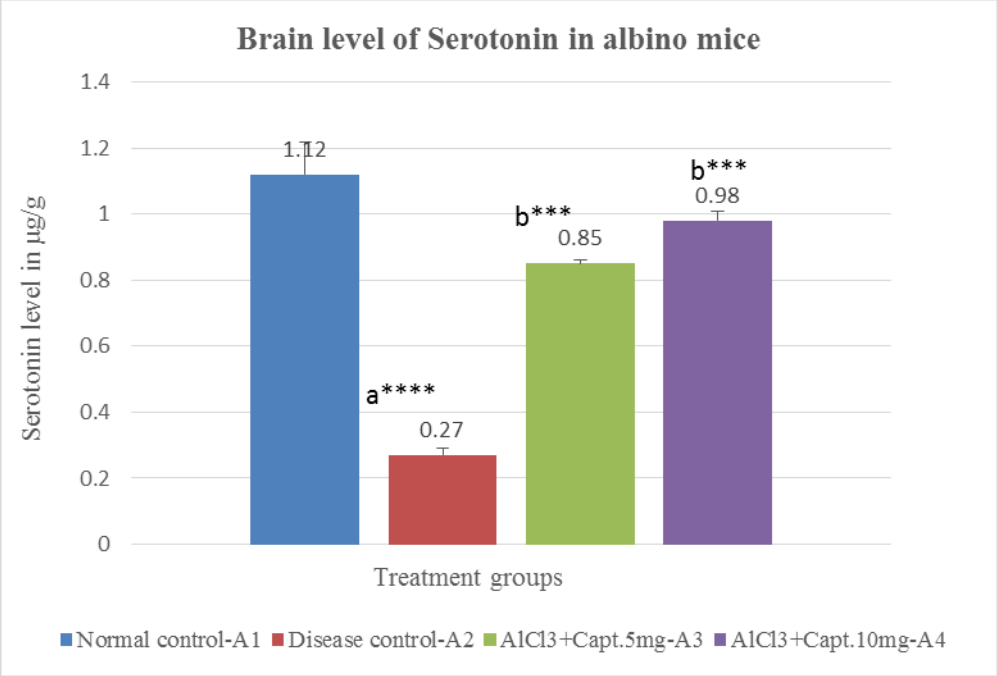

Fig. 4: Effect of Captopril treatment on brain serotonin levels of albino mice.

*All values are Mean + SEM, n=5. Statistical analysis is by One-way ANOVA followed by Post hoc Tukey's. a**** denotes p< 0.0001 compared to control and b*** denotes p< 0.001 compared to disease control ($AlCl_3$ 40 mg/kg) on the same day.*

Fig. 4 represents the serotonin levels in brain homogenate of albino mice after different types of treatment for 29 days. After 29 days of treatment, Serotonin level in $AlCl_3$ treated group (0.27 ± 0.02) µg/g, was significantly ($p<0.0001$) reduced, compared to that in the control group (1.12 + 0.10) µg/g.

At the same time, Captopril treatment has produced an increase of (0.85 + 0.01) µg/g, ($p<0.0001$) and (0.98 + 0.03) µg/g n, ($p<0.0001$) respectively for the lower and higher doses, when compared to group A2 ($AlCl_3$ treated).

Here, the learning and memory impairment of disease control group (A2) is evident from its decreased brain serotonin level. Also, Captopril has shown its dose dependent potential to reverse the $AlCl_3$ induced damage by improving the serotonin level in brain.

II. Histological examination of brain

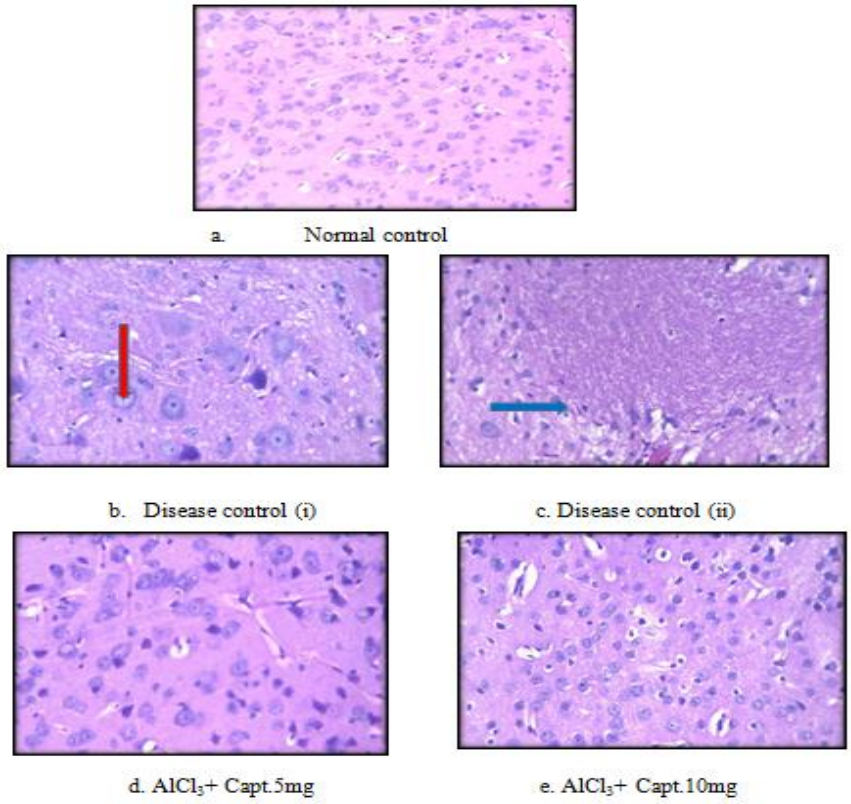

Fig. 5: (a to e): Brain sections of albino mice from different treatment groups (H and E x 400). (*The red arrow represents the inflammation and blue arrow represents amyloid plaques*).

a. Group I- Distilled water PO for 29 days.

b. & c. Group II - $AlCl_3$ 40 mg/kg IP for 29 days.

d. Group III- $AlCl_3$ 40 mg/kg IP + Captopril 5 mg/kg PO for 29 days.

e. Group IV- $AlCl_3$ 40 mg/kg IP + Captopril 10 mg/kg PO for 29 days.

Figure 5. (a to e) show the high power view of brain sections of albino mice treated with different drugs for a period of 29 days. Compared to the control group, large number of red neurons, indicating neurodegeneration, are seen in the $AlCl_3$ treated group. At the same time, the number of red neurons have progressively decreased in groups III and IV indicating the capacity of Captopril to provide neuroprotection in albino mice.

III. GENE EXPRESSION STUDIES

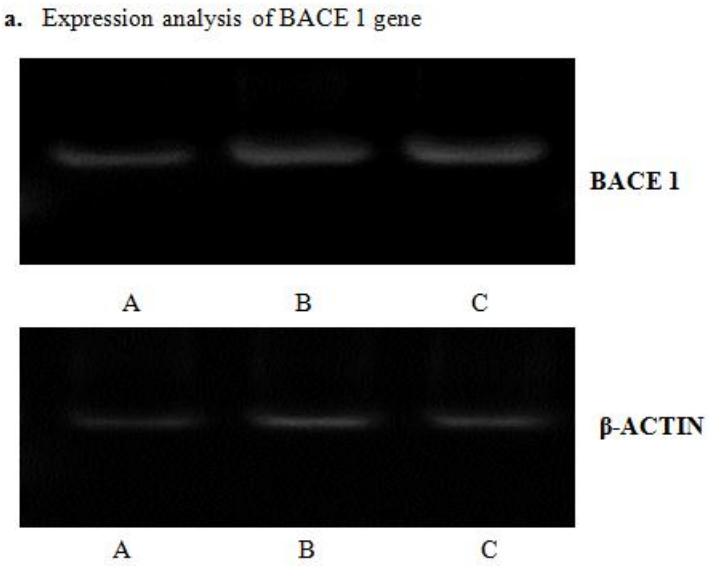

Fig. 6: Hippocampal expression of BACE-1 gene in different groups of albino mice.

(A) Control- Distilled water PO for 29 days.

(B) Treated with AlCl3 40 mg/kg IP for 29 days.

(C)Treated with AlCl3 40 mg/kg IP and Captopril 10 mg/kg PO for 29 days.

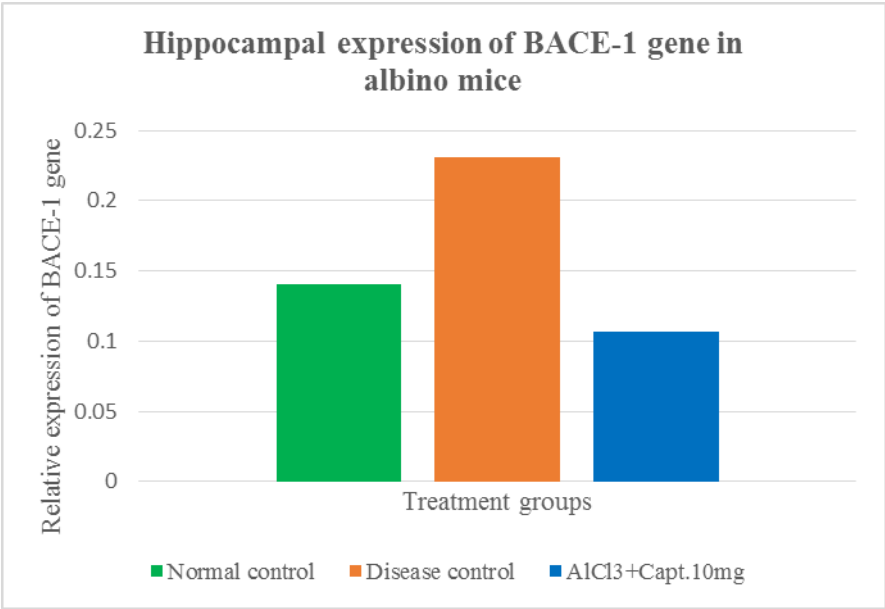

Fig. 7: Relative expression of BACE-1 gene in different groups of albino mice.

As seen in figures 6&7, the mRNA level of BACE-1 was increased in the hippocampus of the $AlCl_3$ treated mice compared to that of the control group. Furthermore, it is clear from the figures that when Captopril was given in a dose of 10 mg/kg PO for 29 days, the expression

of BACE-1 gene in the hippocampus was reduced even to a level less than that in the normal control. As BACE is a major factor for the cleavage of APP to amyloid β peptides, the reduced expression of BACE in the Captopril treated group may be considered as an indication of the potential of captopril to prevent the formation of amyloid plaques in brain from Amyloid Precursor Protein (APP).

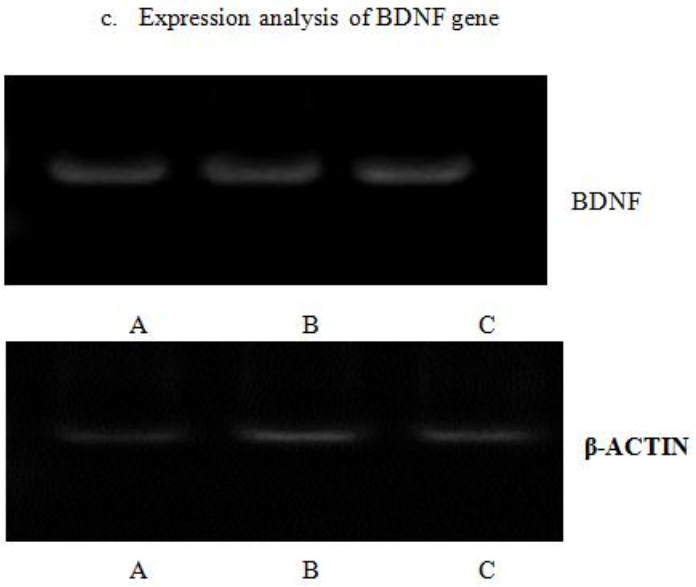

Fig. 8: Hippocampal expression of BDNF gene in different groups of albino mice.

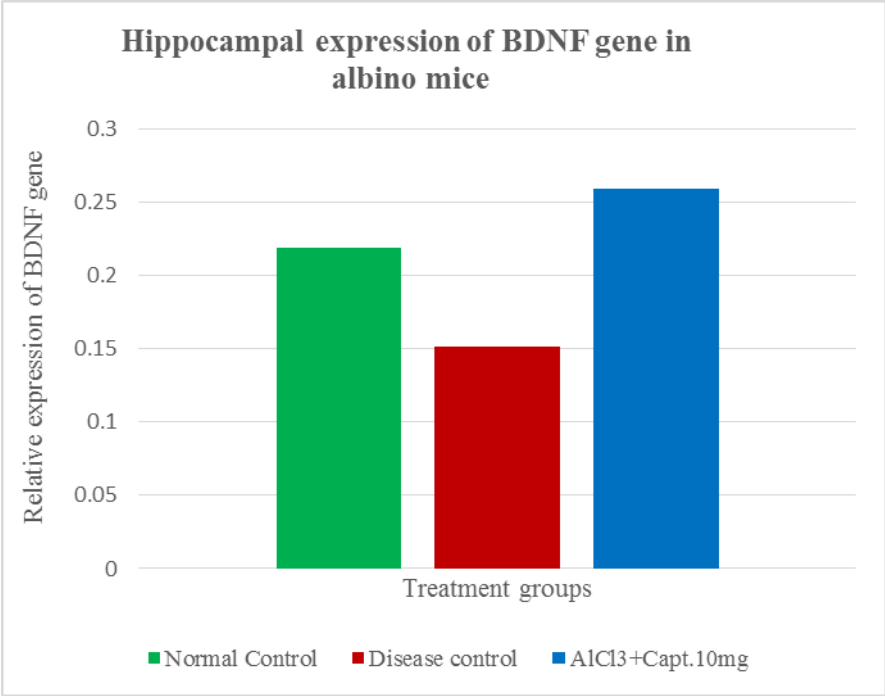

Fig. 9: Relative expression of BDNF gene in different groups of albino mice.

As seen in figures 8&9, expression of BDNF gene was down regulated in $AlCl_3$ treated mice hippocampus compared to the control group. On the other hand, up regulation of the BDNF gene occurred in hippocampus of mice co-treated with captopril in a dose of 10 mg/kg (i.e.; $AlCl_3$ 40mg/kg + Captopril 10 mg/kg), more than that in the control group indicate the possible capacity of Captopril to support survival of neurons.

CONCLUSION

Dementia is a progressive neurodegenerative disorder characterized predominantly by marked changes in learning and memory. Among the people with dementia, a large proportion is constituted by Alzheimer-type dementia. As of now, no proper treatments are available for the disease and many research works are under progress. Many drugs, including antihypertensive medications are looked upon for their effect in dementia. Among the antihypertensive, ACEI are the most debated class of drugs for dementia, especially for the Alzheimer-type. As per the available literature many arguments exist in the understanding of whether or not the ACEI enhance Aβ deposition. The present study was conducted to bring more clarity in this aspect. Here the first and widely used centrally acting ACEI, captopril was evaluated for its effect on $AlCl_3$ induced mice model of memory impairment. In this study 29 days of once daily $AlCl_3$ administration in a dose of 40mg/Kg body Wt. IP was shown to produce Alzheimer-type dementia in Wistar albino mice. The effect of co-administration of captopril was evaluated for two different doses of 10 mg/kg PO and 5 mg/kg PO.

Here the effects of captopril on learning and memory were assessed by biochemical estimations, histological and gene expression studies of the brain of treated mice. In all these tests, captopril was found to be significantly reversing the adverse effects of $AlCl_3$ in a dose dependent manner. It was found that captopril treatment normalized the Ach E level which was elevated by $AlCl_3$. Acetylcholine is essential for learning and memory, which in turn is cleaved by Ach E. This proves that captopril can reverse the damage caused by $AlCl_3$.

Besides, the reversal of $AlCl_3$ induced brain oxidative stress by captopril was evident from the decrease and increase in the level of brain MDA and brain GSH respectively, in the captopril treated groups. Finally, captopril significantly reversed the serotonin deficit induced by $AlCl_3$ in mice brain. Overall, the findings from biochemical estimations suggest that captopril was able to reverse the neuronal damage caused by $AlCl_3$.

To be clearer regarding the mechanism involved in the above mentioned action, gene expression studies were conducted. Two genes BDNF and BACE-1 were evaluated. As BACE-1 gene is considered as a major factor for the cleavage of amyloid precursor protein to amyloid β peptides, the down regulation of BACE-1 in captopril treated group suggests the possible potential of captopril to prevent the formation of β amyloid plaques in brain. Similarly, up regulation of BDNF by captopril indicates its positive role in learning and memory.

Moreover, in the histopathological evaluation of the brain from the different study groups, the captopril treatment was found to reverse the neuronal damage caused by $AlCl_3$. Thus, altogether the results of this study is in support of the second hypothesis regarding the effects of ACEI in Alzheimer's disease. According to this hypothesis centrally acting ACE can cause neurodegeneration and stroke. If so, the centrally acting ACEI like captopril can prevent the ACE induced neurodegeneration, making it a potential drug in the treatment of dementia. More detailed studies in this direction, especially in genetic models of dementia, are needed before approving the benefits of centrally acting ACEI in dementia.

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