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Preclinical Study on Expression of Bax and Bcl-2 Apoptotic Regulatory Proteins on Amyloid Beta (Aβ 25-35) Induced Oxidative Stress in Alzheimer's Mice

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ABSTRACT

Alzheimer's disease (AD) becomes global threat as it prevails very rapidly in the developing countries like India. Progression of AD predicted majorly by deposition of protein called amyloid beta which is a hallmark protein majorly responsible for triggering most of the inflammatory episodes in the etiology of the AD. Chronic inflammation and oxidative stress ultimately contributes to the neural cell death which in turn leads to galvanizing of caspases. Being a protease enzyme caspases constantly involved in fragmenting the core cellular components such as DNA and other functional proteins in the neurons essential for learning and memory. Proportion of bcl-2 protein (Anti-apoptotic) with respect to Bax protein (Pro-apoptotic) regulates the survival or death of the neuronal cell after apoptotic stimuli in amyloid induced neurodegenrated mice model. Ipomoea aquatic Forsk is a novel medicinal herb traditionally used for treating nerve debilities throughout India but still now there is no proper documentary evidence available on its molecular action against neurodegeneration. This prompted us to peruse the present investigation on this versatile herb. The main aim of the present study is to determine the effect of hydroalcoholic extract of Ipomoea aquatica (HAEIA) on protein expression of anti-apoptotic BCL-2 gene and a pro-apoptotic BAX gene in amyloid beta (Aβ 25-35) induced Alzheimer's mice. Swiss albino mice were treated with HAEIA for periods of 4 weeks dose-dependently (200 and 400 mg/kg) then received a single intra cerebro ventricular (i.c.v) injection of Aβ25-35 (10 µg/mouse). At the end of the study animals were sacrificed and their brains were isolated to study the expression of Bax and Bcl-2 gene by PCR technique. Results of the present investigation clearly projects that brain sample of mice treated with HAEIA has shown significantly increased level of expressing anti-apoptotic protein Bcl-2 further it was observed that there is remarkable decease in the expression of pro-apoptotic protein gene Bax and this become an evidence based data that the herb Ipomoea can able to limit the progression of apoptosis caused by amyloid injection. From the data's of the present investigation it was concluded that the herb Ipomoea aquatic may serve as an valuable lead for the effective treatment and clinical management of neurodegenerative disorders like AD in near future.

Keywords: Alzheimer's disease; Beta-amyloid; *Ipomoea aquatica*; Neuron; Apoptosis; BAX; BCL-2

INTRODUCTION

Alzheimer's disease (AD) is considered to be one of the prime reasons for neurodegeneration apart from stroke, diabetes and other polygenic disease. Cholinergic neurons of brain are actively involved in regulating sensory parameters such as learning, memory, reasoning, taught, perception and intelligence. Excessive deposition of macro molecular amyloid protein significantly disturbs the physiology of memory and learning in AD patients and further it had been proved through several research findings that accumulation of amyloid in the most significant areas of brain results in dementia. An amyloid plague potentiates the neurons to undergo oxidative stress and triggers the event of lipid peroxidation, inflammation, apoptosis, atrophy etc. Membrane destabilization of neural cells disturbs the process of glucose and glutamate transportation which ultimately ends in ATP energy depletion [1].

Human brain is an extremely versatile organ which has potential immunological and disciplined support in preventing neurons undergoes cell death. As it may be known that neural cells once degenerated will not become functional underneath any circumstances. Apoptosis is a serious event behind the pathophysiology of several diseases, whereas the scenario of necrosis differs from apoptosis since the later stimulates the inflammatory response in several aspect and leads to tissue injury [2]. Focus of research towards prediction and development of therapy towards apoptosis has gained paramount importance in recent years. Caspase mediated cell death shares a typical house with regard to etiology of cancer and neurodegeneration, because the failure of cell death triggers abnormal cell proliferation in cancer and at a same time excessive caspase mediated cell death promotes neural degeneration in AD, together with stroke and Parkinsons [3].

AD majorly affects the neural population at cerebral cortex and the limbic lobe which regulates both motor and sensory function, Degeneration of neurons in these biologically active regions of the brain ends in cognitive impairment and also causes motor incoordination. Brain of healthy individual capable of balancing between expression of an anti-apoptotic Bcl-2 gene and a pro-apoptotic Bax gene. Previous research suggested that there are several factors which directly and indirectly influence misbalancing of bax/Bcl2 ratio of the brain of AD subjects. In this oxidative stress, inflammation and apoptosis plays a remarkable role on worsening the condition clinically. Oxidative stress often associated with increased expression of Bax and decreased expression of Bcl-2. It has been hypothesized that Bax induces the release of cytochrome C by inhibiting Bcl-2 function through binding of the BH1, BH2, and BH3 domains. There is, however, considerable evidence to support the alternative hypothesis that Bax and Bcl-2 function independently in regulating apoptosis [4].

As there is always a constant research opportunity in bridging the gap between the neurodegeneration and medicinal herbs. In the present research wok we made an effort of exploring an alternate complimentary therapy of Indian medicinal origin towards ailment of neural inflammation by characterizing its effects at molecular level. India being a place of potential biodiversity of 45,000 known plant species has offered versatile herbs with indigenous medicinal property for treating neurodegenerative disease like Alzheimer's and Parkinson's [5]. One such potential medicinal herb is Ipomoea aquatic Forsk also known by its name water spinach.

Ipomoea aquatic Forsk is cultivated for commercial and medical usage in Hong kong, Taiwan, China and also in rural part of India [6]. There are very limited numbers of scientific studies have been conducted on its medicinal aspects. Ipomoea possess numerous pharmacological activity which includes hypoglycemic [7], anti-oxidant [8], hepatoprotective [9], constipation [10] and also for treating intestinal disorders [11]. Ipomoea further claimed for the treatment of high blood pressure [12], emetic [13], anti-helminthic activity [14] and also for treatment of eye diseases [15]. Still now there is no proper documentary evidence available on anti-apoptotic potential of this herb in preclinical and clinical level. The main objective of the present study is to investigate the expression of Bcl-2 and Bax in the brain of AD mice pretreated with Ipomoea leaf extract, with special attention to the possible relationship between Bcl-2 and Bax in neurofibrillary degeneration and senile plaques.

MATERIALS AND METHODS

Preparation of the Plant Extract

The fresh leaf of Ipomoea was collected and washed with running water. It was shade dried at room temperature and 1 kg of the dried leaf was made in to coarse powder. The powder was passed through a 60 No mesh sieve. Air dried Powdered drug was extracted with mixture of Ethanol: water (6:4) (hydro-alcoholic extract) by using soxhlet extraction. Hydroalcoholic extract of *Ipomoea aquatica* (HAEIA) was filtered, concentrated by rotary vacuum pump to get the solid mass.

Experimental Animal

Swiss albino male mice weighing between 20–25 g, acquired from C. L. Baid Metha College of Pharmacy was used for the present investigation. The animals were kept under standard laboratory conditions maintained at 25 ± 2°C, 12 h light/dark cycle and given standard pellet diet (Hindustan lever, Bangalore) and water provided ad libitum. The animals were acclimatized to the laboratory conditions for about one week prior to the start of experimentation. All the animals were housed in polypropylene cages with adequate ventilation and maintained at 12:12 dark light cycle by using paddy husk bedding. Principles of animal handling were strictly obeyed during animal handling and was under the supervision of animal ethics committee of the institute. The experimental protocol was approved by Institutional animal ethics committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). C. L. Baid Metha College of Pharmacy, Chennai, Tamil Nadu, India.

Intra Cerebro Ventricular injection of Aβ Peptide

Administration of amyloid beta protein fragment (Aβ 25-35) was performed by specifically identifying the bregma point on the skull, each mouse was injected with a 10 µl volume of fragment suspension using Hamilton micro syringe. The needle was inserted unilaterally 1 mm to the right of the middle point equidistant from each eye slightly angled towards 450 1 mm to the right of the middle point equidistant from the eyes perpendicular to the plane of the skull. Mice exhibited normal behavior within 1 min after injection [16].

Animals were divided into six groups.

GROUP I : Animals received 10 µl phosphate buffered saline through I.C.V

GROUP II : Animals received 10 µl of Aβ peptide fragment (25-35) by I.C.V

GROUP III : Animals received 10 µl of Aβ peptide fragment (25-35) by I.C.V and treated with HAEIA 200 mg/kg (p.o.)

GROUP IV : Animals received 10 µl of Aβ peptide fragment (25-35) by I.C.V and treated with HAEIA 400 mg/kg (p.o.)

GROUP V : Animals injected with Aβ peptide (10 µl) by I.C.V and treated Donepezil 5 mg/kg (p.o.)

Induction of amnesia in mice proceeded with Intra Cerebro Ventricular injection (I.C.V.) injection of Aβ peptide (25-35) preparation to II, III, IV and V groups on the 21st day after pretreatment with HAEIA and continues for the period of about 7 days post amyloid exposure. Control group mice were injected with only phosphate buffered saline.

Reverse Transcription (RT)-PCR

The mRNA expression for Bax and Bcl2 genes were analyzed in mice brain using a reverse transcription (RT)-PCR approach as described previously [17]. Single-stranded cDNA was synthesized from 5 µg of total cellular RNA using reverse transcriptase [18]. Amplification of β-actin served as a control for sample loading and integrity. PCR products were detected by electrophoresis on agarose gel containing ethidium bromide. Size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized and images were captured using a gel documentation system. Expression of all the genes was assessed by generating densitometry data for band intensities in different sets of experiments and was generated by analyzing the gel images on the Image J program (Version 1.33, Wayne Rasband, NIH, Bethesda, MD, USA) semi-quantitatively. The band intensities were compared with constitutively expressed β--actin.

Statistical Analysis

All values are expressed as mean SEM. Data were analyzed by one way ANOVA followed by Dunnett's test, and other data were evaluated using Graph Pad PRISM software. A p-value<0.05 was considered significantly different. Comparisons were made between: a: Group I (control) vs. Group II (negative control), b: Group II (negative control) vs. Group III (HAEIA 200 mg/kg), IV (HAEIA 400 mg/kg) and V Donepezil (5 mg/kg). Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001.

RESULTS

Effect of HAEIA on mRNA Expression of Bax Protein

It was observed form the data's obtained from the present investigation that there was a significant upsurge in mRNA expression of Bax protein in group II (Beta amyloid alone injected) which serves as disease control group with the ratio of 2.24 ± 0.14 when compare to that of normal control group I with the ratio 0.47 ± 0.12. Pretreatment of mice with HAEIA at the dose of 200 mg and 400 mg/kg has shown notable decrease in the expression of Bax protein with the ration of 1.85 ± 0.31 and 1.35 ± 0.24 respectively. Similarly treatment with standard marketed drug Donepezil 5 mg/kg has shown significant decrease in Bax protein expression with the ratio of 0.92 ± 0.18. As shown in Figure 1. The results were tabulated in Table 1 and illustrated in Figure 2.

Table 1: Estimation of bax protein expression

Group	Treatment	Bax/β actin ratio
I	Animals injected with phosphate buffered saline (10 µl)	0.47 ± 0.12
II	Animals injected with Aβ (25-35) peptide (10 µl) by I.C.V	2.24 ± 0.14 [a]*
III	Animals injected by Aβ (25-35) peptide (10 µl) by I.C.V and treated with HAEIA 200 mg/kg (p.o.)	1.85 ± 0.31 [b]*
IV	Animals injected with Aβ (25-35) peptide (10 µl) by I.C.V and treated with HAEIA 400 mg/kg (p.o.)	1.35 ± 0.24 [b]*
V	Animals injected with Aβ (25-35) peptide (10 µl) by I.C.V and treated Donepezil 5 mg/kg (p.o.)	0.92 ± 0.18 [b]*

Values are expressed as mean ± S.D, comparisons were made between: a: Group I (control) vs. Group II (negative control), b: Group II (negative control) vs. Group III (HAEIA 200 mg/kg), IV (HAEIA 400 mg/kg) and V Donepezil (5 mg/kg). Symbols represent statistical significance: *p<0.05

Figure 1: Quantitative representation of mRNA expression of Bcl-2 and Bax

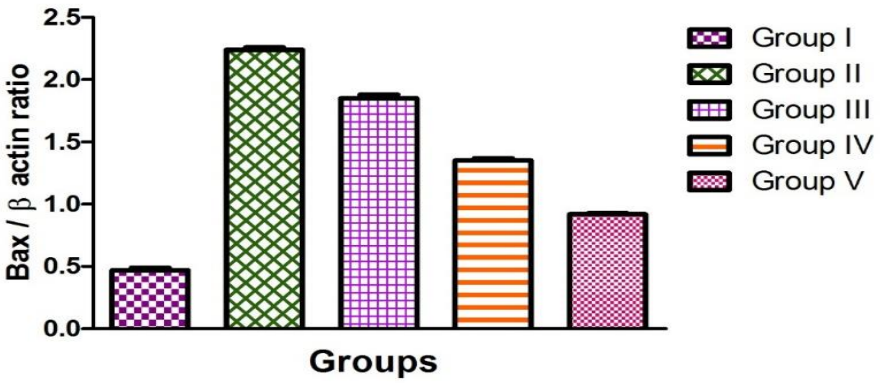

Figure 2: Ratio of Bax/Beta actin

Effect of HAEIA on mRNA Expression of Bcl-2 Protein

From the results analysis of the present investigation it was justified that there was a significant decrease in mRNA expression of Bcl-2 protein observed in group II beta amyloid alone injected group with the ratio of 0.93 ± 0.12 when compare with control group I with the ratio 3.01 ± 0.4. Treatment with HAEIA at the dose of 200 mg and 400 mg/kg has shown significant increase in the expression of Bcl-2 protein with the ration of 1.69 ± 0.24 and 1.94 ± 0.18 respectively. Similarly treatment with Donepezil 5 mg/kg has shown tremendous increase in Bcl-2 protein expression with the ratio of 2.26 ± 0.42. As shown in Figure 1. The results are tabulated in Table 2 and illustrated in Figure 3.

Table 2: Estimation of Bcl-2 protein expression

Group	Treatment	Bcl-2/β actin ratio
I	Animals injected with phosphate buffered saline (10 µl)	3.01 ± 0.4
II	Animals injected with Aβ peptide (10 µl) by I.C.V	0.93 ± 0.12 a*
III	Animals injected by Aβ peptide (10 µl) by I.C.V and treated with HAEIA 200 mg/kg (p.o.)	1.69 ± 0.24 b*
IV	Animals injected with Aβ peptide (10 µl) by I.C.V and treated with HAEIA 400 mg/kg (p.o.)	1.94 ± 0.18 b*
V	Animals injected with Aβ peptide (10 µl) by I.C.V and treated Donepezil 5 mg/kg (p.o.)	2.26 ± 0.42 b*

Values are expressed as mean ± S.D, comparisons were made between: a: Group I (control) vs. Group II (negative control), b: Group II (negative control) vs. Group III (HAEIA 200 mg/kg), IV (HAEIA 400 mg/kg) and V Donepezil (5 mg/kg). Symbols represent statistical significance: *p<0.05

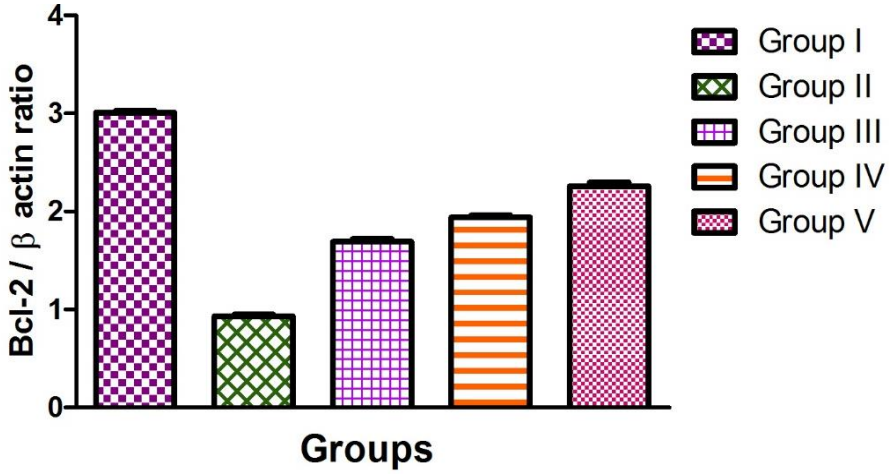

Figure 3: Ratio of Bcl-2/Beta actin

DISCUSSION

Apoptosis involves a series of biochemical events leading to sequential changes in neural cell morphology and death. These changes include neural protrusion, asymmetrical membrane, cellular atrophy, fragmented nucleus, chromosomal condensation [19]. Bax being nuclear-encoded proteins prevails mostly in higher eukaryotes that are capable to piercing the mitochondrial outer membrane to mediate cell death by apoptosis. Thus, organelles recruited by nucleated cells to supply energy can be recruited by Bax for cellular destruction [20].

The results of the study clearly shown that there was a significant increase in mRNA expression of Bax protein observed in group II beta amyloid alone injected group with the ratio of 2.24 ± 0.14 when compare with control group I with the ratio 0.47 ± 0.12. Treatment with HAEIA at the dose of 200 mg and 400 mg/kg has shown significant decrease in the expression of Bax protein with the ration of 1.85 ± 0.31 and 1.35 ± 0.24 respectively. Similarly treatment with Donepezil 5 mg/kg has shown highly significant decrease in Bax protein expression with the ratio of 0.92 ± 0.18.

Bcl-2 (B-cell lymphoma-2) group of heterogeneous genes majorly involved in arresting the progression of cell apoptosis [21]. Bcl-2 proteins are located on nuclear membrane, endocytoplasmic reticulum and mitochondrial outer membrane. Overexpression of Bcl-2 can halt cell apoptosis without any impact on cell proliferation. From the results of the present investigation it was evident that there was a significant decrease in mRNA expression of Bcl-2 protein observed in group II beta amyloid alone injected group with the ratio of 0.93 ± 0.12 when compare with control group I with the ratio 3.01 ± 0.4. Treatment with HAEIA at the dose of 200 mg and 400 mg/kg has shown significant increase in the expression of Bcl-2 protein with the ration of 1.69 ± 0.24 and 1.94 ± 0.18 respectively. Similarly treatment with Donepezil 5 mg/kg has shown tremendous increase in Bcl-2 protein expression with the ratio of 2.26 ± 0.42.

The result obtained from the present study indicates that there is a significant increase in mRNA expression of Bax and decrease in Bcl-2 protein were observed in group II beta amyloid alone injected group. Bax is considered as an apoptotic promoter increase in the level of this protein in group II mice indicates the accelerated level of apoptotic induction caused due to beta amyloid. Similarly decrease in the levels of mRNA expression of anti-apoptotic protein Bcl-2 observed in group II. Treatment with HAEIA and standard drug donepezil shows significantly increased level of anti-apoptotic protein Bcl-2 and decrease in pro-apoptotic protein Bax in mice belongs to group III, IV and V this justifies the anti-apoptotic activity of active phytoconstituents present in HAEIA. Traditional medicines of herbal origin are considered as a major healthcare provider around the globe in particular with rural areas since years together. Modern research findings also recognized the importance of herbal medicine [22].

CONCLUSION

Indian medicinal herbs served as a primary source of new drug discovery and lead identification for treatment against dreadful disorders since several centuries. There are growing evidence that people of developed and

developing countries relies more on herbs and other traditional medicine for their health care needs. Clinical trials on plant based medicine have proven evidence that herbal preparations act as efficient curative substance either alone or in combination with others allopathic drugs. Further it was evident from the present investigation that the brain sample of mice treated with extract of Ipomoea has significantly increased expression of the anti-apoptotic protein Bc1-2 and decrease level of Bax gene which justifies the clinical usage of this plant towards the management of neural debilities.

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