NEUROPROTECTIVE EFFECT OF ACETOGENIN ON EXPERIMENTALLY INDUCED CEREBRAL ISCHEMIC RATS

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ABSTRACT

Aim of the present study was to investigate the possible neuroprotective effect of acetogenin in experimentally induced cerebral ischemic rats. Cerebral ischemia was produced by bilateral common carotid artery occlusion (BCCAO). Results indicated that cerebral ischemia group developed a significant infarction and oxidative stress which indicates that cellular leakage and loss of functional integrity of cell membrane in brain. The different doses of acetogenin have significantly improved the altered levels of lipid peroxidise (LPO), acetyl cholinesterase (AChE), reduced glutathione (GSH) and total protein levels in treatment groups which prior undergone occlusion procedure for 10 min in rat. The results obtained here study suggested that the neuroprotective effect of acetogenin was mediated through the antioxidant, free radical scavenging activity and also return of biochemical marker level near to normal values. Histopathological observation supports the prevention in architecture of the brain due to the treatment with acetogenin against occlusion induced cerebral ischemia in rat. The finding of the present study provides the strong evidence of acetogenin may be beneficial in the patient suffering from stroke (cerebral ischemia) due to various reasons.

KEYWORDS: Acetogenin, neuroprotective, carotid artery occlusion, cerebral ischemia, acetyl cholinesterase.

INTRODUCTION

Acetogenins are fatty acid derivatives obtained naturally from plants belonging to the Annonaceae family.[1] Structurally acetogenins are long chain aliphatic fatty acids with C35-

C37 with terminal methyl substituted α, β- unsaturated γ lactone ring with 1-3 tetrahydrofuran rings in hydrocarbon chains. These natural products exhibit a broad range of biological properties, such as cytotoxic, immunosuppressive, pesticidal, antiparasitic and antimicrobial activities, and their potential to inhibit cells that are multiple drug-resistant has attracted increasing interest.[2] In a study acetogenins showed a strong DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical-scavenging activity, similar to that of ascorbic acid.[3] The DPPH radical-scavenging activity of the ethanol extract, fractions and pure acetogenins revealed that they present antioxidant capacity, with IC50 at the same level as ascorbic acid.

Most of the brain disorders are due to oxidative stress in the brain as the brain tissue involved in high metabolic rate and low antioxidant defences[4] Neuroprotection within the nervous system protects neurons from apoptosis or degeneration, for example following a brain injury or as a result of chronic neurodegenerative diseases. Cerebrovascular diseases include some of the most common devastating disorders such as ischemic stroke, hemorrhagic stroke, cerebrovascular anomalies, etc. Stroke is the leading cause of adult disability with approximately one third of patients who survive 5-7 months are dependent on others. Neurodegeneration is the term for the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases including Parkinson's, Alzheimer's and Huntington's occur as a result of neurodegenerative processes.[5] Cerebral ischemia is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain and it leads to various pathophysiological modalities such as reactive oxygen species (ROS), calcium overload, mitochondrial damage, neuronal cell death and also associated with oxidative stress and DNA fragmentation. The most common form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space.[6] Over production of ROS is a central feature of all neurodegenerative disorders. Reperfusion after cerebral ischemia further add to the complications of stoke by releasing various mediators such as pro-inflammatory cytokines and free radical generation. This increases the oxidative stress to the brain and ultimately leading to neuronal cell death.[7] Analysis of community surveys has shown that India shows a crude stroke prevalence rate of about 203 per 100,000 populations above 20 years of age, amounting to a total of about 1 million cases. The male to-female ratio was estimated to be 1:7 Most studies carried out in India have shown that about 10% to 15% of strokes occur in the population below 40 years, which is a higher proportion compared with other countries.[8]

However, it was estimated that stroke represented 1.2 % of the total deaths in the country, when all ages were included. The proportion of stroke death increased with age, and in the oldest group (>70 years of age) stroke contributed to 2.4% of all deaths.[9] According to the World Health Organization, 15 million people suffer stroke worldwide each year Of these, 5 million die and another 5 million are permanently disabled. The concept of neuroprotection is derived from the studies of ischemic brain injury. It has been well documented that abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades, leading to spread of neuronal death of the numerous pathways identified, excessive activation of glutamate receptors, accumulation of intracellular calcium cations, abnormal recruitment of inflammatory cells, excessive production of free radicals and initiation of pathological apoptosis are believed to play critical roles in ischemic damage, especially in the penumbral zone. Thus, it is logical to suggest that if one is able to interrupt the propagation of these cascades, at least part of the brain tissue can be protected.[10]

Brain ischemia or cerebral ischemia is a condition which leads to alterations in brain metabolism, reduction in metabolic rates and energy crisis where the brain cannot perform aerobic metabolism due to lack of oxygen supply.[11] Though a large number of therapeutic agents like thrombolytics, NMDA receptor antagonists, calcium channel blockers, antioxidants, sodium-channel and potassium channel openers, glutamate antagonists, magnesium sulphate, glycine antagonists, GABAergic compounds (such as clomethiazole), growth factors (such as basic fibroblast growth factor), free radical scavengers (such as tirilazad), and anti-inflammatory compounds (such as enlimomab) have been used, there remains a large gap between the benefits by these agents and properties an ideal drug for stroke should offer.[12]

Acetogenin has powerful antioxidant effects and being used in the form of capsules as supplements for both a therapeutic and preventive agent. Hence, the present study was undertaken to evaluate possible neuroprotective effects of acetogenin in experimentally induced cerebral ischemic rats.

MATERIAL AND METHODS

Chemicals

Acetogenin was purchased from Puresources Inc, Ontario, Canada. Acetyl thicholine, 2-thiobarbituric acid, Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid), Sodium lauryl sulphate A.R (Sodium dodecyl sulphate), EDTA (Ethylene diamine tetra acetic acid) A.R,

Tris Hydrochloride A.R, 2,3,5 triphenyl tetrazolium chloride (TTC) (tetrazolium salt), Pyrogallol A.R. 98.5%, Sodium phosphate monobasic monohydrate A.R, Sodium phosphate dibasic dehydrate A.R and other chemicals were obtained from Himedia Laboratories, Mumbai, India.

Instruments

U.V. Spectrophotometer Shimadzu Corporation, Japan, Auto analyzer- Rapid Diagnostics, Delhi, pH meter- Micropro Labmate, India, Homogenizer and Centrifuge -Remi Motors Pvt. Ltd. India.

Animals

Swiss albino rats of either sex weighing 250-300g were used. The animals were purchased from Sri Venkateshwara Enterprises, Bangalore, India. They were maintained in the animal house of Soniya Education Trust's College of Pharmacy, Dharwad, India for experimental purpose. The animals were maintained under controlled conditions of temperature ($23\pm2°C$), humidity ($50\pm5\%$) and 12-h light-dark cycles. They were having free access to standard pellets as basal diet and water ad libitum. The animals were housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize if any of non-specific stress. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of Soniya Education Trust's College of Pharmacy, Dharwad, India (REG.No.112/1999/CPCSEA) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Induction of cerebral ischemia by bilateral common carotid artery occlusion (BCCAO)

Rats were anaesthetized with pentobarbitone (50mg/kg BW). A midline incision was made in the region between neck and sternum and trachea is exposed. Both the right and left common carotid arteries were located lateral to sternocleidomastoid, freed from surrounding tissues and vagus nerve separated. Cerebral ischemia was induced by clamping both the arteries with the help of aneurysm clips. After 10min of cerebral ischemia; the clips were removed from the arteries to allow the reflow of the blood through carotid arteries. The incision was sutured back in layers with surgical suture. While performing the surgical procedure; the body temperature was maintained at 37°C by heated IR lamp. All surgical

instruments used in procedure were sterilized prior to use.

Experimental Design

Animals were divided into five groups of six animals in each group (n=6) and Group I was normal control treated with only vehicle (Distilled water 1ml/p.o.). Group II was Sham, in this group surgery was performed but no cerebral ischemia was induced. Group III was cerebral ischemia group, in this group all the animals were operated to induce cerebral ischemia as per procedure mentioned in the previous section and treated with vehicle (Distilled water 1ml/p.o.). Group IV was acetogenin treated group, in this group acetogenin was administered (50mg/kg,b.w) 30 min before cerebral ischemia and 24 hours after 1st dose. Group V was acetogenin treated group, in this group acetogenin was administered (100mg/kg,b.w) 30 min before cerebral ischemia and 24 hours after 1st dose.

Preparation of post-mitochondrial supernatant

Following decapitation, the brain was removed and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized as 10% (w/v) in cold phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 1000×g for 10min at 4ºC and post-mitochondrial supernatant (PMS) was kept on ice until assayed.[13]

Biochemical Estimation

Estimation of Total Protein

The estimation of total protein content was done using kit of Erba as per Biuret Methods, End point method. 1 ml of this working reagent was pipetted out in each of the test tubes labeled as blank, standard, test. 20 μl of distilled water, 20 μl of standard and 20 μl of sample homogenate was pipetted out and added to test tubes labeled as blank, standard and test respectively. Test tubes were incubated for 10 mins at 37ºC. Reading was obtained by measuring the absorbance at 546 nm (520-560nm) in the autoanalyser of standard and each test against reagent blank (Tietz 1986).

Total Protein (g/dl) = (Absorbance of test/Absorbance of standard) X Concentration of standard (g/dl).

Estimation of Lipid peroxidase (LPO)

Lipid peroxidase activity was determined by taking sample in 15 ml centrifuge tubes. 100 μl was pipetted out into into the respective test tubes. To that 200 μl of 8.1 % SDS solution was

added, swirled and mixed. 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % aqueous solution of TBA was added to each tube. Final volume was made up to 4 ml with biological grade water. The tubes were heated at 95-100°C in boiling water bath for one hour. After one hour tubes were cooled immediately under running tap water for 10 min and centrifuged at 4000 rpm for 10 min. The supernatant was collected and optical density was read at 532 nm using UV-Visible Spectrophotometer.[14]

Estimation of Glutathione (GSH)

To the each samples of test and blank a 50 μl of Ellaman's Reagent was added in a 2.5ml reaction buffer and in order to measure GSH, 250 μl brain homogenate was added to test sample whereas 250 μl reaction buffer was added to blank samples. It was mixed well and incubated at room temperature for 15 minutes. Absorbance was measured at 412 nm on UV spectrophotometer to calculate GSH.[15]

Estimation of acetyl cholinesterase enzyme activity of whole brain

The brain was homogenized in a tissue homogenizer. 400mg of brain was homogenized in 0.1M Phosphate buffer pH 8 (10% w/v), the homogenized tissue was centrifuged to 10,000 rpm for 10 min. 0.4ml aliquot of the supernatant is added to a cuvette containing 2.6 ml phosphate buffer (0.1M, pH 8) and 100μl of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and absorbance is measured at 412 nm. When absorbance reaches a stable value, it was recorded as the basal reading. 20μl of substrate i.e., acetylthiocholine was dded and change in absorbance was recorded for a period of 10 mins at intervals of 2 min. Change in the absorbance per minute was thus determined. AchE activity was calculated using the formula.[16]

$$R = 5.74 \times 10^{-4} \times A/CO$$

Where,

R = Rate in moles of substrate hydrolyzed /minute /gm of brain tissue

A = Change in absorbance / min.

CO = Original concentration of the tissue (mg/ml)

Measurement of infarction area:

The infarction area was measured by 2, 3, 5-triphenyl terazolium chloride (TTC) staining method. After ischemia and reperfusion animals were sacrificed, and brains were removed within 2-3 min. Two coronal slices were made at 5 and 7 mm from the frontal pole, and brain

slices were immersed in 2% solution of TTC stain in normal saline at 37° C for 30 min. After which sections were fixed in 10% phosphate buffered formalin for photograph. Then the cerebral infarction area was observed and compared between various treatment group and negative control group.

HISTOPATHOLOGICAL EXAMINATION

The animals were deeply anaesthetized with pentobarbitone (50mg/kg BW). Following decapitation, the brains were taken out and fixed in 10% formalin. Multiple, paraffin-embedded, coronal sections (5-Am thick) were taken from each brain (spanning through striatum tocaudal hippocampus). Serial sections (spaced apart by 250 Am, 15 in total for each brain) were selected for histopathological analysis of neuronal damage. After staining with hematoxylin and eosin, the slides were examined using light microscopy by an observer blinded to experimental groups. The brain was subjected to histopathology and observed for infarct cells. The samples were submitted to Jeevan Lab Pvt Ltd. (Belgaum, India) for histopathological examination.[17]

STATISTICAL ANALYSIS

Results were expressed as mean ± S.E.M. Differences among data were determined using one-way ANOVA followed by Dunnet's Multiple Comparison Test.

RESULTS

In the present study cerebral ischemia was successfully produced by bilateral common carotid artery occlusion. In order to find out the neuroprotective effect of acetogenin two doses were selected viz., 500 and 1000 mg/kg on the basis of toxicity studies.

Total protein activity (g/dL)

Samples containing proteins react with copper II ions in alkaline solution to form blue-violet complex as per biuret reaction and each copper ion complexion with 5 or 6 peptide bonds. However, the colour formed is stabilized by the addition of Tartarate to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546nm (520-560nm). The total protein activity showed significant decrease ($P<0.001$) in total protein level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant ($P<0.001$) increase in total protein level

compared to positive control group (Cerebral ischemia induced group). Results are summarized in Table1.

Lipid Peroxidase (LPO) activity (µmol/g)

Peroxidation of cellular lipids results into oxidative stress and which is determined by measurement of thiobarbituric acid reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results in increased of oxidative free radicals, which attacks the polyunsaturated fatty acids in cell membranes and cause lipid peroxidation. The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS. The Lipid peroxides (LPO) assay showed significant ($P<0.001$) increase in LPO activity of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham group. Acetogenin treated (500 and 1000 mg/kg p.o.) group showed significant ($P<0.01$) reduction in LPO activity compared to the positive control group. Results are summarized in Table 1.

Reduced Glutathione (GSH) activity (µmol/g)

GSH is a major non-protein thiol and endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reaction. The reduced glutathione assay showed significant decrease ($P<0.001$) in GSH level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant ($P<0.001$) increase in GSH level compared to positive control group (Cerebral ischemia induced group). Results are summarized in Table 1.

Acetyl cholinesterase (AChE) activity (µmols/min/mg)

The Acetyl cholinesterase assay showed significant increase in AChE level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant ($P<0.001$) decrease in AChE level compared to positive control group (Cerebral ischemia induced group). Results are summarized in Table 1.

Effect of Acetogenin on cerebral infarction

Bilateral carotid artery occlusion induced cerebral ischemia in rats resulted in cerebral tissue damage resulting in large infarction area, as indicated by lesser TTC staining when compared

to control. Ischemia followed by reperfusion led to further increase in infarction area, as indicated by further less TTC staining when compared to control.

Pretreatment with Acetogenin prevented tissue damage and therefore infarction induced by ischemia and reperfusion as shown by increase in TTC staining, when compared to cerebral ischemia group. Hence, pretreatment with Acetogenin showed marked reduction infarction area near to normal as indicated by further increase in TTC staining when compared to cerebral ischemia group.

Histopathology of brain

The brain sections of bilateral carotid artery occlusion induced cerebral ischemia in rats showed marked cerebral edema, moderate cerebral congestion and mild neutrophilic Infiltration. No change was observed in histoarchitecture of brain in control group. Ischemia followed by reperfusion resulted in moderate cerebral edema, marked cerebral congestion, moderate neuronal vacuolization and mild neutrophilic infiltration. Pretreatment with Acetogenin reduced these alterations as indicated by mild cerebral edema, cerebral congestion and mild neutrophilic infiltration. Pre-treatment with Acetogenin further prevented the pathological changes as shown by mild cerebral edema, cerebral congestion and focal neutrophilic infiltration (see figure 1).

Table 1. Results of total protein, lipid peroxidise (LPO), reduced glutathione (GSH) and acetyl cholinesterase (AChE) activities of various groups.

S.No	Groups	Total Protein(g/dL)	LPO (μmols/g)	GSH (μmols/g)	AChE (μmols/min/mg)
1	Normal (Vehicle)	1.31±0.032	0.17±0.07	4.21±0.214	7.87±0.10
2	Sham (Surgery)	1.87±0.033	0.22±0.02	3.70±0.121	9.01±0.07
3	Cerebral ischemia group	1.14±0.021###	0.37±0.03###	0.55±0.032###	10.08±0.021###
4	Acetogenin (500 mg/kg)	1.08±0.051***	0.30±0.002**	2.13±0.114***	8.14±0.074***
5	Acetogenin (1000 mg/kg)	1.61±0.032***	0.21±0.001**	3.19±0.101***	6.88±0.095***

Values are expressed as (mean± S.E.M) Significant differences between Sham group and Ischemia group defined with #, Significant differences between Surgery group and Treatment groups defined with *
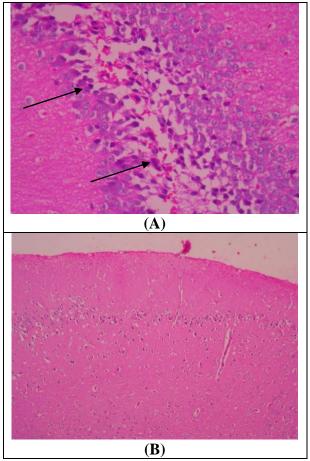
Figure 1: (A):Brain section of bilateral carotid artery occlusion induced cerebral ischemia in rats showing marked cerebral edema, moderate cerebral congestion and mild neutrophilic Infiltration; (B): A typical brain section of bilateral carotid artery occlusion induced cerebral ischemia in treated rat groups showing marked improvements.

DISCUSSION

Acetogenin is used as a both therapeutic and preventive agent due to its powerful antioxidant effects. In this study possible effect if acetogenin as a neuroprotective agent is evaluated in experimentally induced cerebral ischemic rats by measuring various supporting parameters such as biochemical and enzymes levels in the brain. It is proved in the literature that, when global cerebral ischemia is produced by carotid artery occlusion, a makeable change in histopathology of brain tissue (see figure 1), Marked destruction of different lobes and cells of brain could be seen (Olsson et al. 2003). The ischemic parts were confirmed by staining different regions of brain with hematoxylin and eosin stains. This study has shown following major findings: Cerebral ischemia is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain and it leads to various

pathophysiological modalities such as reactive oxygen species (ROS), calcium overload, mitochondrial damage, neuronal cell death and also associated with oxidative stress and DNA fragmentation. The process of neuroprotection within the nervous system protects neurons from apoptosis or degeneration, for example following a brain injury or as a result of chronic neurodegenerative diseases (Reynolds et al. 2007).The reduced glutathione assay showed significant decrease (P<0.001) in GSH level of positive control group (cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant (P<0.001) increase in GSH level compared to positive control group (Cerebral ischemia induced group). The Acetyl cholinesterase assay showed significant increase in AChE level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant (P<0.001) decrease in AChE level compared to positive control group (Cerebral ischemia induced group). The lipid peroxides (LPO) assay showed significant (P<0.001) increase in LPO activity of positive control group (cerebral ischemia induced group) compared to vehicle control group and sham group. Acetogenin treated (500 and 1000 mg/kg p.o.) group showed significant (P<0.001) reduction in LPO activity compared to the positive control group. The total protein activity showed significant decrease (P<0.001) in total protein level of positive control group (Cerebral ischemia induced group) compared to vehicle control group and sham control group. Acetogenin treated group showed significant (P<0.001) increase in total protein level compared to positive control group. During ischemia free radicals may be produced to such an extent that endogenous antioxidant systems are overwhelmed. Free radicals are demonstrated to promote lipid peroxidation. In the present study there was a significant increase in acetyl cholinesterase, lipid peroxidation levels and significant decrease in reduced glutathione, as well as total protein levels. Treatment with acetogenin in doses of 500 and 1000 mg/kg decreased acetyl cholinesterase, lipid peroxidation levels and increased in reduced glutathione as well as total protein levels. Histopathological observation supports the prevention in architecture of the brain due to the treatment with Acetogenin against occlusion induced cerebral ischemia in rat. Improvement in all antioxidant enzyme levels to significant values suggest antioxidant and free radical scavenger activity of Acetogenin against cerebral ischemia.

CONCLUSION

Bilateral common carotid artery occlusion significantly produces cerebral ischemia and caused neuronal damage. Treatment with Acetogenin in a dose of 500 mg/kg and

1000 mg/kg b.w orally showed significant neuroprotective effect. Various biochemical parameters were measured such as lipid peroxidase (LPO), reduced glutathione (GSH), Acetyl cholinesterase (AChE), and total brain protein were showed a significant improvement in the levels compared to that of bilateral common carotid artery occlusion induced cerebral ischemia group. Hence, present findings indicate the possible exploitation of acetogenin for neuroprotective activity.

ACKNOWLEDGEMENT

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