

A STEREOLOGICAL STUDY OF HIPPOCAMPUS IN EXPERIMENTAL EPILEPTIC RAT MODEL TREATED WITH *ACORUS CALAMUS* AND BETA-ASARONE

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ABSTRACT

Objective: Epilepsy is the fourth most prevalent neurodegenerative disorder that affects about 1–2% of people round the world. Epilepsy cannot be cured even with modern medication, but the medications can control the seizures. Even this cannot be achieved in nearly 30% of epileptic population. At this point, we felt the need of some natural supplements to protect the nervous system against neurodegeneration and so created an equivalent model of epilepsy with kainic acid lesion and studied the novel role of *Acorus calamus* in protecting the neurons.

Methods: For this study, we divided the animals into four groups, based on the drugs used. We also produced a stereotaxic model of epilepsy by inducing kainic acid into the right hippocampus of all the animals except CO (control) group. Then, we conducted a stereological study both on the 2nd and 7th day after surgery, to rule out the neuroprotective and neuroregenerative ability of the drugs employed.

Results: The results were amazing. Stereological study on the 2nd day revealed a very large lesion on the hippocampus of lesion control (LC) animals, and the lesion was very much smaller in both drug group animals. On the 7th day also the LC animals showed a large lesion, but the lesion on the drug group animals diminished due to a number of new cells, probably of neurons grown in the place of lesion.

Conclusion: The results proved the neuroprotective and regenerative ability of both drugs, but as a fact, beta-asarone had an upper hand in this study.

Keywords: Epilepsy, Seizure, Neurodegeneration, Stereotaxic model, Kainic acid.

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INTRODUCTION

Epilepsy is a neurodegenerative disorder characterized by seizure, affecting around 1–2% of world's population. It is of two types the generalized and focal epilepsy. Mesial temporal lobe epilepsy (MTLE) is the major form of focal epilepsy, and hippocampal sclerosis is the most common cause of MTLE. Modern therapies can control epilepsy, but they cannot cure it completely. Over 30% people with epilepsy do not have seizure control even with best available treatment [1].

The hippocampus, which is present inside the mesial temporal lobe, plays important roles in short-term, long-term memory, spatial navigation, and initial learning [2]. Hippocampal sclerosis is the most common type of tissue damage in temporal lobe epilepsy. Hippocampal damage was a frequent result in experimental settings [3], where artificial repetitive seizures induced in animals.

According to the legends of Ayurveda such as Charaka and Susruta (600BC), *Acorus calamus* (AC) was a drug of choice for epilepsy, promotes intellect in children, memory, and used to boost up the activities of brain in the form of brain tonic. The good olden medicinal ghee or the nervine tonic had AC, *Bacopa monnieri*, and *Alpinia speciosa* and used for mostly all the neuronal disabilities [4]. The rhizome has around 1.5–3.5% of essential oil [5]. Aromatherapists use this oil for curing memory loss, epilepsy, and shock [6].

The extract of AC has beta-asarone (BA) as one of the major phytochemicals. BA is a powerful central nervous system stimulant and Waller's British Herbal says: It is of great service in all nervous complaints, vertigoes, and headaches. Oral administration of BA said to improve cognitive function by suppressing neuronal apoptosis in the hippocampus of beta-amyloid injected rats [7].

All the above said scenarios instilled the idea of creating a model of epilepsy and hauled us to study the preventive and therapeutic upshots of AC and BA.

METHODS

We created a lesion model by injecting kainic acid locally into the right hippocampus with the help of stereotaxic frame. We purchased the frame from INCO (Instruments and Chemicals Pvt., Ltd.) Ambala, Haryana.

Kainic acid

We purchased Kainic acid from Cayman Chemicals - USA and dissolved it in 0.9% NaCl (Sodium chloride) [8] just before lesion surgery to get 1 µg Kainic acid in 1 µl. Kainic acid is an analog of glutamic acid and a potent neuroexcitotoxin. Previous researchers observed free radical formation in brain, 1 h after kainic acid administration [9]. According to Manikandan *et al.*, reactive oxygen species are the major risk factors [10] that promote neurodegeneration [11], and AC is very well-known for its radical scavenging activity [12].

Ethanol extract of AC preparation

We prepared ethanol extract of AC by soxhletion method [5]. Elayaraja *et al.* proved that ethanol extract has more antioxidant activity than any other extracts of AC.

We decided the dosages of ethanol extract of AC and BA by sticking with the lethal dose 50 of the substances.

BA

We purchased BA from Sigma-Aldrich Ltd., St. Louis, USA.

We started giving the drugs 10 days before lesion surgery and continued till the end of the study after lesion to analyze the protective nature as

well as the therapeutic role of them in hippocampus and given around 10'o clock every day in IP.

Animals

For this study, we housed adult male Sprague Dawley rats (200–250 g) under standard laboratory condition and maintained in compliance with strict institutional guidance and ethical permission. We maintained the room environment at $20^{\circ}\text{C}\pm 2^{\circ}\text{C}$; alternating 12 h light-dark cycle with food and water *ad libitum* took greatest effort to lower the unwanted stress to the animals and cut the number of animals used for this study. We used four groups of animals for this study with six animals in each group. They are CO (control group), lesion control (LC), AC 35 (AC ethanolic extract 35 mg/kg body weight), and BA 20 (BA 20 mg/kg body weight). We used tween 80 3% solution as a vehicle [13] to dissolve and to make different concentrations of drugs and adjusted the volume to 1 ml for each animal.

Stereological study to measure the damaged area

We conducted a stereological study to find out the degenerated area in hippocampus and used a 1 cm^2 grid of square lattice containing intersections, known as reticule for this quantitative study. We fixed the reticule in the eyepiece of a light microscope and used it to calculate the degenerated area.

We conducted the study both on the 2nd and 7th day after lesion surgery to rule out the protective effect (2nd day) and the regenerative ability (7th day) of the drugs employed, as a few studies showed the regeneration of nerve cells in the hippocampal region [14] even in adult human. The brain tissues collected on the 2nd day processed and stained with Trypan blue live cell exclusion technology and on the 7th day stained with Vectra staining technology with ABC-elite kit from Japan.

Methodology

The reticule was placed into the eyepiece of the light microscope

The grids of the lattice fixed in focus with the degenerated area of brain.

The corners of the grids that hit the degenerated area counted (P = number of points hitting the profile).

The total corner points over the test area also counted (P_t = total number of reference points).

Degenerated area or area fraction degenerated = P/P_t

Example

Calculation

Number of points hit the degenerated area (profile) = $P = 56$

Number of points hit over the test area (total number of reference points) = $P_t = 373$

Conversion factor = $373/100 = 3.73$

Degenerated area or area fraction degenerated = $56/3.73 = 15.01\%$

The result showed 15.01% degenerated area.

RESULTS

Limbic status epilepticus and convulsion, the results of LC group in the 1st h after lesion surgery. Over the following days, the animals were ferocious and that made handling them tough. The drug group animals did not show any gross epileptic changes as both drugs were very effective in protecting from the deleterious effects of kainic acid.

Stereology for analyzing the area of damage

This parameter deals the area fraction damaged due to kainic acid lesion and so the protective effect of the drugs in hippocampus with the help of a reticule on $\times 10$ magnification.

Fraction of degenerated area - on the 2nd day of lesion

The CO animals showed normal histological pattern of hippocampus. The LC group animals had a very large lesion ($15.36\pm 1.42\%$) in the hippocampal region. The AC 35 group animals showed only a small lesion with $7.09\pm 1.01\%$ damaged area that was much smaller when compared with LC group. With the animals treated with BA 20, only a very small lesion with $5.89\pm 0.35\%$ degenerated area observed. This was much less when compared with LC group.

These results clarify (Fig. 1) that the drug BA 20 was more effective against nerve cell degeneration in comparison with AC 35.

Fraction of degenerated area - on the 7th day of lesion

On the 7th day also the hippocampus belongs to LC group had a very large lesion with $15.05\pm 0.76\%$ damaged area and was equal with the damaged fraction of LC group on the 2nd day. However, the degenerated area reduced in the drug groups AC 35 ($4.82\pm 0.38\%$) and BA 20 ($4.28\pm 0.37\%$), on the 7th day as the lesion area covered with newly formed cells.

For BA 20 group animals, the lesion was almost covered with newly formed cells (Fig. 2), and they also formed connections with the nearby neurons.

We have drawn a bar diagram to compare the degenerated area among the 2nd and 7th day to analyze the preventive and therapeutic roles of drugs employed.

The LC group animals have shown near equal degenerated area both on the 2nd and 7th days of lesion. In AC 35 animal groups, the degenerated area got reduced on the 7th day than the 2nd day. The degenerated area was the smallest with BA 20 group on the 2nd day and was further reduced (Fig. 3) on the 7th day too due to the regeneration of large number of nerve cells.

DISCUSSION

Research in China has shown the essential oil of AC rhizome had neuroprotective activity [15]. Legradi, 2010 [16], studied the adult

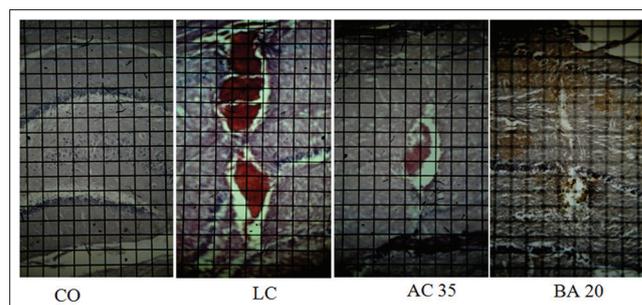


Fig. 1: The comparison of fraction of degenerated area in hippocampus belongs to CO, lesion control, *Acorus calamus* 35, and BA 20 on the 2nd day of lesion ($\times 10$ magnification)

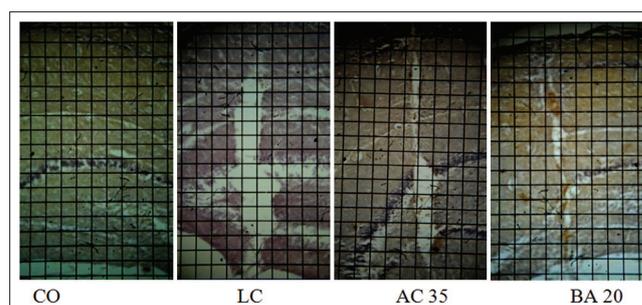


Fig. 2: The comparison of fraction of degenerated area in hippocampus belongs to CO, lesion control, *Acorus calamus* 35, and BA 20 on the 7th day of lesion ($\times 10$ magnification)

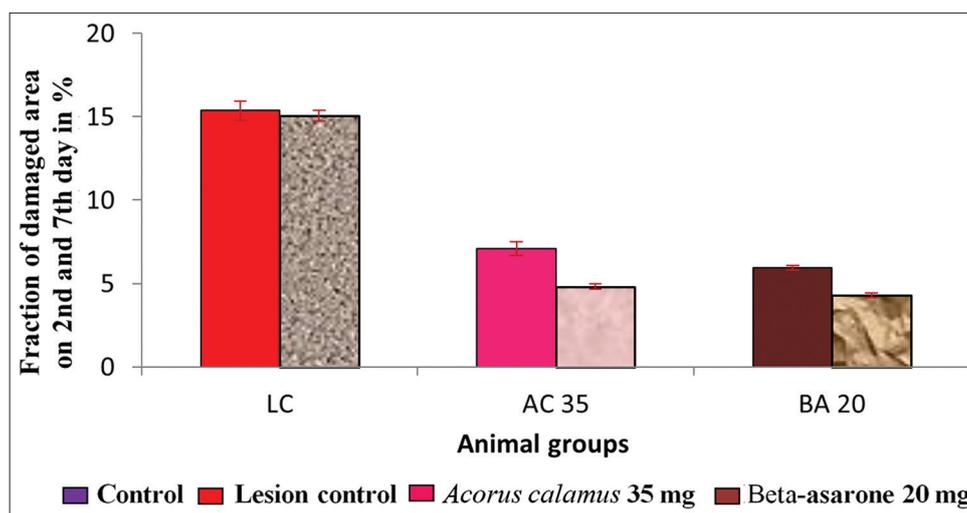


Fig. 3: The comparison of degenerated area in the hippocampus of rats on the 2nd and 7th day of lesion

hippocampal slice cultures as a model of apoptotic aspects of neurodegeneration and used Trypan blue staining to analyze the slice viability. Here, also, we used the same technique live cell exclusion technology to analyze the dead cells and so the degenerated area. In LC group animals, the hippocampus showed a very lesion stained by Trypan blue both on the 2nd and 7th day as there were no drugs either to prevent the neuronal damage or to stimulate new cells to form. The AC 35 group animals exhibited a comparatively smaller Trypan blue-stained area, but it was smallest in BA 20 group that stated the efficacy of the drugs and confirmed the rolls of both drugs in preventing nerve cell degeneration.

Singh, 2008 [17], described that AC can delay brain aging and stimulate regeneration of neurons. Nakatomi *et al.*, 2002 [15], proved stimulation of endogenous progenitors led to the massive regeneration of hippocampal pyramidal neurons after ischemic brain injury. Bendel *et al.*, 2005 [18], suggested the endogenous capacity of the brain to form new nerve cells after injury and proved regeneration of neurons in the CA1 hippocampal region after ischemic insult. This present study demonstrated regeneration of nerve cells in the hippocampus of animals belonging to both AC 35 and BA 20 that was the reason for the reduction in the damage area fraction on the 7th day in both the drug groups.

CONCLUSION

Although there were few new cells on the periphery of the lesion in LC animals on the 7th day, the degenerated area was equal with the 2nd day as the newly formed cells were negligible. The degenerated area belongs to AC 35 was very much reduced on the 7th day as more new cells grown in lesion. In higher magnification, they appeared more like big multipolar nerve cells (Fig. 4) with visible processes.

Lesion on BA 20 animals was almost diminished (Fig. 5) on the 7th day due to large number of newly formed cells. The cells were more like neurons that made connections with the nearby neurons.

From all the above said, we came to a conclusion that both the drugs AC 35 and BA 20 had a very good action on hippocampus in terms of protection with a step ahead action with BA 20. This study will be further continued to confirm whether the newly formed cells are glia or nerve cells.

As prevention is always better than cure, we end that both AC and BA can effectively use as food supplements in a minimal amount to prevent neurodegeneration as degeneration of neurons is the major cause for most of the nervous system related incurable disorders.

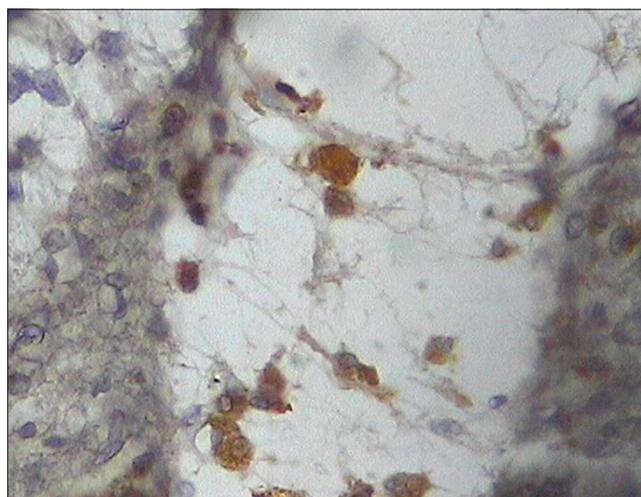


Fig. 4: The large newly formed cells with processes in lesion area belongs to *Acorus calamus* 35 mg group on the 7th day ($\times 40$ magnification)

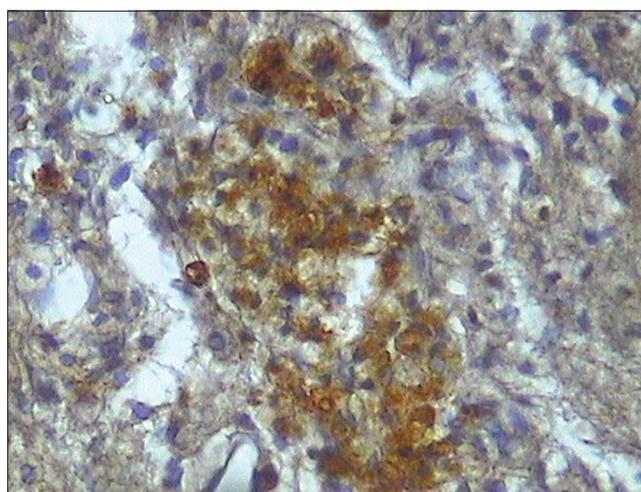


Fig. 5: Many large newly formed cells with processes covered the area of lesion belongs to beta-asarone 20 mg group on the 7th day ($\times 40$ magnification)

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AUTHOR'S CONTRIBUTIONS

The whole work done was by Dr. Venkataramaniah. C, the author of this manuscript. Assistance of staining the histology sections and bar diagrams was done by the corresponding author Dr. Mary Antony Praba. A.

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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