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AN EXPERIMENTAL STUDY TO EVALUATE THE ANTI-EPILEPTIC ACTIVITY OF KSHEERAKALYANAKA GHRITA BY PTZ INDUCED KINDLING AND STAGING METHOD

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ABSTRACT

Kalyanaka Ghrita[1] is widely used to treat conditions like Unmada, Apasmara etc. Along with the reference of Kalyanaka Ghrita, Acharya Chakrapanidatta has quoted the reference of Ksheerakalyanaka Ghrita.[2] The ingredients of Kalyanaka Ghrita and Ksheerakalyanaka Ghrita is the same except for the addition of four parts of milk and two parts of water to it. Gokshira[3] is said to possess karmas like Rasayana, Medhya, Balya and Alakshmighna according to classical references; which is considered very essential in chiravyadhi like Apasmara.[4] **Aims and Objectives:** To evaluate the Antiepileptic activity of Ksheerakalyanaka Ghrita by PTZ Induced Kindling method. **Methodology:** For kindling induction, Pentylenetetrazol (PTZ) was freshly dissolved in normal saline and a sub-convulsive dose (35 or 40 mg/kg, *ip*) was administered every other day for a total of around 12 injections (which would in normal control mice induce convulsion of maximum intensity of grade 5). After each

injection of sub-convulsive dose of PTZ, mice in different groups were observed for 30 min and PTZ-induced seizures were evaluated and classified according to the scoring system of Fischer and Kittner -1998. Data was expressed as mean ± SEM and the data was analysed by one way ANOVA followed by Dunnet's Multiple't' test as a post HOC test for parametric data. After Kindling, the brains were excised and subjected to Histopathological studies. **Results:** Ksheerakalyanaka Ghrita showed statistically non-significant improvement in PTZ induced Kindling study. Histopathological reports depicted the protective action on brain tissues. **Conclusion:** Ksheerakalyanaka Ghrita can be advised to cope up with the side effects of commonly prescribed A.E.Ds as it imparts a protective action on brain tissues.

KEYWORDS: Ksheerakalyanaka Ghrita, Kindling, PTZ, Antiepileptic activity.

INTRODUCTION

Ksheera is one among the drava-dravyas which has been explained in the context of Sneha Kalpana. It can be inferred that different drava-dravyas are mentioned in the classics based upon capability of a particular solvent to imbibe certain therapeutically active principles into it; for e.g water soluble extracts, alcohol soluble extracts etc. Kalyanaka Ghrita[1] is widely used to treat conditions like Unmada, Apasmara etc. Along with the reference of Kalyanaka Ghrita, Acharya Chakrapanidatta has quoted the reference of Ksheerakalyanaka Ghrita.[2] The ingredients of Kalyanaka Ghrita and Ksheerakalyanaka Ghrita is the same except for the addition of four parts of milk and two parts of water to it. Gokshira[3] is said to possess karmas like Rasayana, Medhya, Balya and Alakshmighna according to classical references; which is considered very essential in chiravyadhi like Apasmara.[4]

AIMS AND OBJECTIVES

To evaluate the anti-epileptic activity of Ksheerakalyanaka Ghrita by PTZ Induced Kindling and Staging Method.

METHODOLOGY[5]

TEST DRUG

The sample of *Ksheerakalyanaka Ghrita* was prepared as per standard references in Department of Rasasashtra and Bhaishajya Kalpana, Sri Dharmasthala Manjunatheswara College of Ayurveda and Hospital, Udupi.

EXPERIMENTAL ANIMAL

18 Swiss albino mice were randomly selected and divided into 3 groups.

INCLUSION CRITERIA

a) Adult Swiss albino mice having weight from 30- 40 g.
b) Animals selected were of both sexes.
c) Active and healthy mice.

EXCLUSION CRITERIA

a) Weight range below 30g and above 40g.
b) Mice, which were used for other studies previously.
c) Pregnant and diseased mice.

MICE MAINTENANCE

Swiss Albino mice were obtained from animal house attached to S.D.M Centre for Research in Ayurveda and Allied Sciences, Udupi, Karnataka. They were maintained on feed of "Sai Durga feed and food, Bangalore" and tap water was given *ad-libitum*. The temperature and humidity were kept at optimum and animals were exposed to natural day-night cycles. The experiment were carried out in conformity with guidelines of the Institutional Animal Ethical Committee (SDMCRA/IAEC/UD/RS-01) after obtaining its permission.

EXAMINATION OF THE ANIMAL PRIOR TO THE EXPERIMENT

All Swiss Albino mice were subjected to general check for weight. Weight of each animal was checked by using weighing machine and the dose was calculated according to Paget and Barne's formula(Paget and Barne's 1964) involving body surface area ratio and human dose. The cages were labelled with name of the group and drug.

Reference Standard Drug: The reference standard drug used for antiepileptic activity evaluation was Diazepam. It was purchased from the market with the trade name Paciquil 5mg/ml, Mfd- 12.06.16, Exp- 12.06.20 Manufactured by Stadmed pvt. Ltd, Kolkata, India.

Chemical for inducing seizure: Pentylenetetrazole was used as a chemical for inducing the seizures in mice for evaluating the anti-epileptic activity experimentally. Chemicals were procured from HiMedia laboratories Pvt. Ltd, Mumbai, India with the name Pentamethylene tetrazole (5 gm), PKD:04/2011 and is of analytical grade regularly used in the laboratory.

GROUPING OF ANIMALS

A day prior to dosing, the selected animals were divided into different groups by randomization method. Each group consisted of 6 albino mice each.(Fig.1)

- Group 1-Positive control Group
- Group 2-Reference Standard Group (Diazepam)
- Group 3-Test Drug Group-KKG group

DOSE FIXATION[6]

Dose calculation of trial drug for mice = 0.0026*human dose*50/Kg body weight.

Here the dose of *Ksheerakalyanaka Ghrita* is 48 ml (1 pala).

Therefore, Dose= Human dose * 0.0026*50/ Kg body weight

= 48* 0.0026*50/ Kg body weight

= 6.24 ml/Kg body weight.

Dose Calculation for Reference Standard (Diazepam)

Mice dose = 8 mg/Kg body weight

Dose calculation for Generalized PTZ induced seizure **(**Pentylenetetrazole)

Mice dose = 40mg/Kg body weight

PTZ SOLUTION

A stock solution containing 40mg of PTZ and 10 ml of distilled water was prepared.

ROUTE OF DRUG ADMINISTRATION

Positive control, standard drug and test drug was administered according to body weight of the animals by oral route and PTZ was injected intraperitoneally for inducing seizures.(Fig.2).

PENTYLENETETRAZOLE INDUCED KINDLING METHOD

For kindling induction, Pentylenetetrazole (PTZ) was freshly dissolved in normal saline and a sub-convulsive dose of 40 mg/kg, *ip* was administered every other day for a total of around 12 injections. After each injection of the sub-convulsive dose of PTZ, mice in different groups was observed for 35 min and PTZ-induced seizures was evaluated and classified according to the scoring system of Fischer and Kittner[7] -1998.(Fig.3).

- 1 – Ear and facial twitching, head nodding;
- 2 – Myoclonic jerks;
- 3 – Generalized clonic convulsions rearing, jumping, and falling down,

- 4 – clonic-tonic convulsions, tonic hind limb extensions.

The mean seizure stages were calculated for all groups after each PTZ injection. The scores from the test formulation and reference standard groups were compared to Positive Control group.

STATISTICAL ANALYSIS

All the values were expressed an MEAN ± SEM (Standard error of mean). The date were analysed by one-way ANOVA followed by Dunnet's multiple't' test as post hoc test. Graph pad Inst 3 was used for this purpose. A level of $p \leq 0.05$ was considered statistically significant. Level of significance was noted and interpreted accordingly.

PROCEDURE FOLLOWED TO PREPARE HISTOPATHOLOGICAL SLIDES

After completion of Kindling, the brains were excised out immediately after sacrificing the animals(Fig.4), cleaned of extraneous tissue, cut into pieces of appropriate thickness and were transferred to 10% formalin solution(Fig.5). The tissues were allowed to remain in it till they are taken up for tissue processing. After tissue processing and section cutting, the slides were viewed under binocular research Carl-Ziess's microscope (Germany) at various magnifications to note down the changes in the microscopic features of the tissues studied.

RESULTS

The effect of Ksheerakalyanaka Ghrita on PTZ Induced Kindling and Staging on parameters such as Face twitching/head nodding, myoclonic jerks, generalised clonic convulsions, clonic-tonic convulsions/tonic hind limb extension is tabulated in Table 1,2,3,4 respectively.

The histopathological study report of Ksheerakalyanaka Ghrita group is tabulated as Table 5.

In PTZ Induced Kindling and staging study the results were statistically non-significant. The histopathological reports clearly depicted the protective action of Ksheerakalyanaka Ghrita on brain tissues. Mild to moderate decrease in the degenerative features and an increase in number of purkinje cells were observed in the slides of Ksheera Kalyanaka Ghrita group. (Fig.6, Fig.7, Fig.8).

TABLES

Table 1: Effect of Ksheerakalyanaka Ghrita On Ptz Induced Kindling Convulsion- No of Face Twitching/Head Nodding.

Groups	No of Face Twitching/Head Nodding	Percentage Change
POSITIVE CONTROL GROUP	33.2±3.231	-
STANDARD GROUP	73.6±9.943*	121.68↑
KSHEERAKALYANAKA GHRITA	61.2±6.938	84.33↑

Data: Mean±S.E.M **p<0.01,*p<0.05.

Table 2: Effect of Ksheerakalyanaka Ghrita On Ptz Induced Kindling Convulsion- No of Myoclonic Jerks.

Groups	No of Myoclonic Jerks	Percentage Change
POSITIVE CONTROL GROUP	102.6±24.808	-
STANDARD GROUP	7.6±1.030*	92.5↓
KSHEERAKALYANAKA GHRITA	198±35.766*	92.98↑

Data: Mean±S.E.M **p<0.01,*p<0.05.

Table 3: Effect of Ksheerakalyanaka Ghrita On Ptz Induced Kindling Convulsion- No of Generalised Clonic Convulsions.

Groups	No of Generalised Clonic Convulsions	Percentage Change
POSITIVE CONTROL GROUP	5.8±1.463	-
STANDARD GROUP	0**	100↓
KSHEERAKALYANAKA GHRITA	2.2±0.8602*	62.06↓

Data: Mean±S.E.M **p<0.01,*p<0.05.

Table 4: Effect of Ksheerakalyanaka Ghrita On Ptz Induced Kindling Convulsion- No of Clonic-Tonic Convulsions, Tonic Hindlimb Extension.

Groups	No of Clonic-Tonic Convulsions, Tonic Hindlimb Extension	Percentage Change
POSITIVE CONTROL GROUP	1.6±0.6782	-
STANDARD GROUP	0.2±0.2600	87.5↓
KSHEERAKALYANAKA GHRITA	0.4±0.2449	75↓

Data: Mean±S.E.M **p<0.01,*p<0.05.

Table 5: Histopathological Study Report-Ksheerakalyanaka Ghrita.

Mice no and section	Cerebrum	Cerebellum	Hippocampus	Remark
1	Many degenerated neurons.	Purkinje cells increased compared to Positive control group. One area shows reduced purkinje cells	Few degenerated neurons.	Moderate degenerative features
2	Few Degenerated neurons	Reduction of purkinje cells in some areas	No degenerated neurons	Mild degenerative features
3	Very few degenerated neurons	Reduction of purkinje cells in one area	Few degenerated neurons	Mild degenerative features
4	Few degenerated neurons	Increase of purkinje cells. And mild reduction in some areas	No degenerated neurons	Mild degenerative features

FIGURES

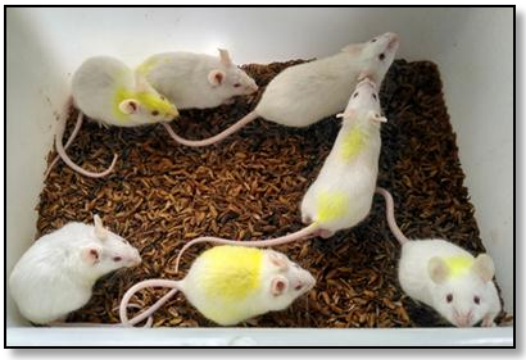

Fig 1: Grouping of Swiss Albino Mice.

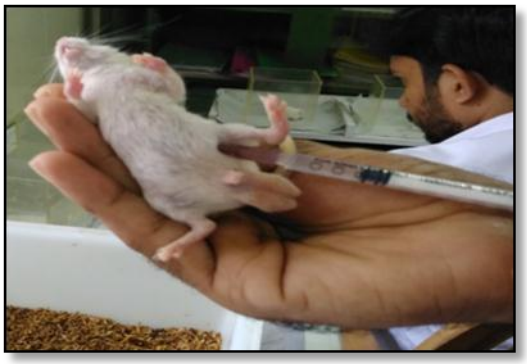

Fig 2: Administration of PTZ.

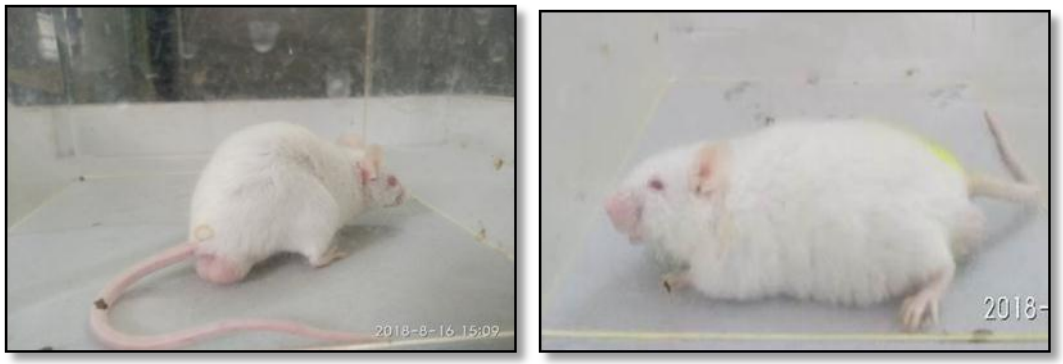

Fig 3: Observing the assessment criteria.

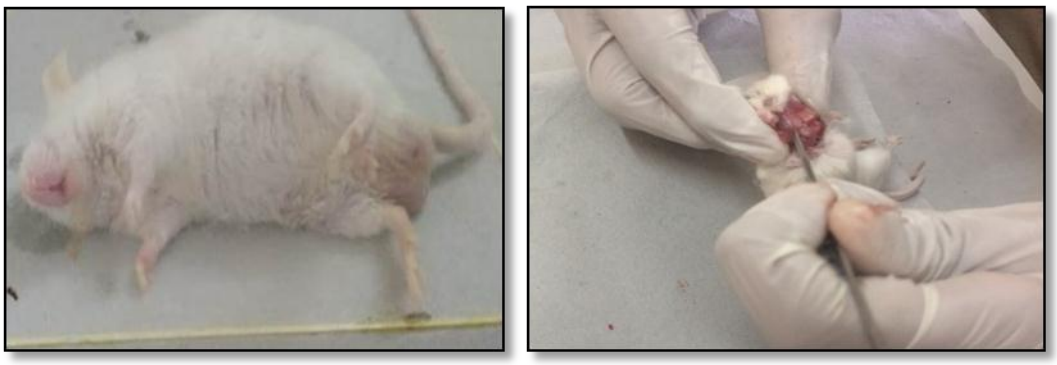

Fig 4: Excision of brain.

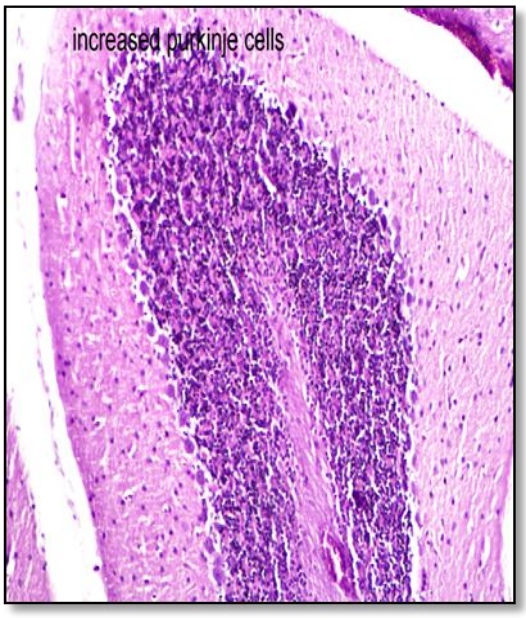

Fig 5: Excised brain. Fig 6: 10x: cerebellum (Histopathological slide).

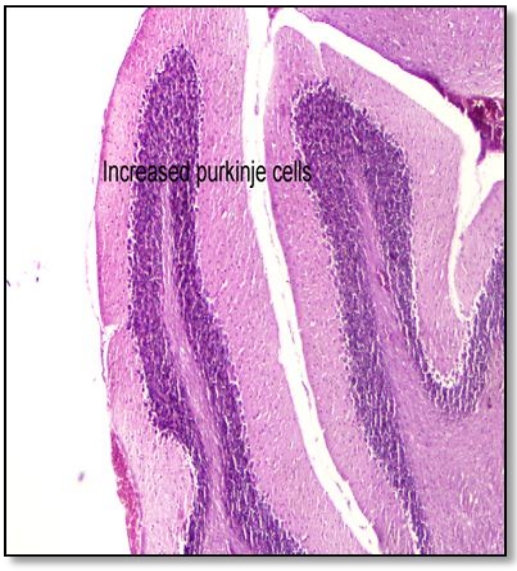

Fig 7: 4x: cerebellum (Histopathological slide).

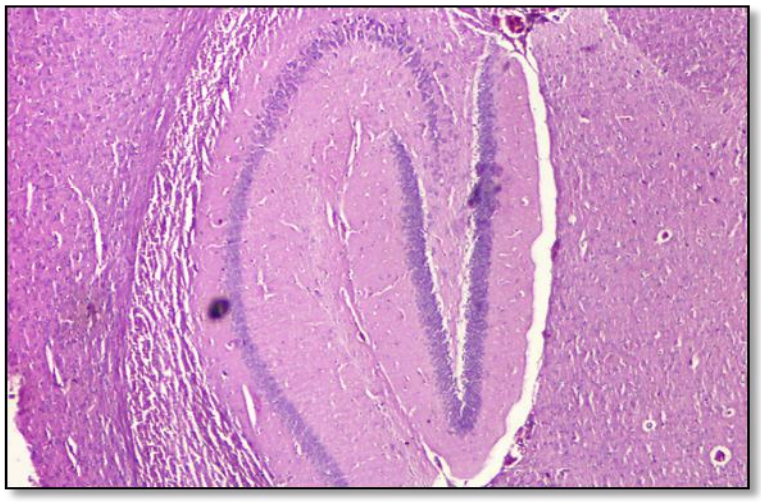

Fig 8: 2x: Hippocampus (Histopathological slide).

DISCUSSION

The positive control group mice developed all grades of convulsions and seizures were more severe. The seizure score after successive injections were increasing and it attained its peak by the 12th day. On the 12th day all mice in the positive control group were dead. Hence, the survival rate was nil. The observations clearly showed that repeated kindling decreased the resistance to sustain convulsions which ultimately led to their death.

The test drug Ksheera kalyanaka Ghrita exerted inhibition of kindling effect from the first day of injection. The stages of convulsions were very well controlled even though there was no complete cessation of seizure activity. Ear and facial twitching, myoclonic jerks and forelimb clonus/full rearing i.e., upto 3rd grade of convulsion were the stages of convulsions that were exhibited by most of the mice till the last injection.

There was an increase in number of face twitching/head nodding in Ksheerakalyanaka ghrita group when compared to positive control group. There was an increase in the number of face twitching/head nodding in standard group when compared to positive control group and the observed increase was found to be statistically significant.

There was an increase in number of myoclonic jerks in Ksheerakalyanaka ghrita group when compared to positive control group. The observed increase in value was found to be statistically significant for Ksheera kalyanaka ghrita group. There was a decrease in the number of myoclonic jerk in standard group when compared to positive control group.

The number of generalised clonic convulsions in Ksheerakalyanaka Ghrita group decreased when compared to positive control group. The observed decrease in Ksheerakalyanaka Ghrita group was found to be statistically significant. The decrease in the number of generalised clonic convulsions were extremely significant in case of standard group.

There was a decrease in number of clonic tonic convulsions/hind limb extension in Ksheerakalyanaka ghrita group and standard group when compared to positive control group but it was found to be statistically non-significant.

The mortality rate was 0/6 for Standard group and for the test group i.e., Ksheerakalyanaka ghrita group.

According to the present study, the test drug Ksheera kalyanaka Ghrita proved to be helpful in suppressing the development of Kindled seizures in mice. It also imparted protective action against a subconvulsant dose of PTZ in kindled mice which throws light on the action of test drugs Ksheerakalyanaka Ghrita on inhibiting epileptogenesis and developing a potential seizure threshold even when the disease was fully developed.

One of the major differences found in the the test group-K.K.G Group was that all the mice were active till the last day of Kindling indicating that long term administration of these drugs may not produce psychological or cognitive side effects unlike the commonly used A.E.Ds like C.N.S depression or sedation effect. It was observed that in standard drug group they were becoming weak day by day after kindling and fall of body hair in mice and signs of C.N.S depression and sedation effect was evident in standard group.

On analysing the histopathological reports; it was observed that in case of Positive control group, all the sections showed presence of many degenerated and necrosed neurons in cerebrum and hippocampus and also reduced purkinje cells in cerebellum were observed which indicated moderate to severe degeneration.

In case of Standard group, degenerated neurons have decreased in cerebrum, hippocampus in all sections compared with Positive Control group. There is an increase in purkinje cells of cerebellum compared to positive control group which directly indicated that it has mild degenerative features. Protective action is evident.

In the slides of K.K.G group, degenerated neurons have decreased in cerebrum, hippocampus in most of the sections compared with positive control group. There is a decrease in purkinje cells of cerebellum in most of the sections which indicated mild to moderate degenerative features. Protection is evident but it is less compared to Reference standard group.

CONCLUSION

Ksheerakalyanaka ghrita may not have highly significant role as an anti-convulsant i.e in controlling the number of seizures during an ictal event but the protective action of it is very much evident after analysing the histopathological study results after PTZ Induced Kindling. Thus Ksheerakalyanaka Ghrita may act upon the seizure threshold of the individual. It can also be administered as an adjuvant with other commonly used A.E.Ds to curb its cognitive and psychological side effects.

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