Effect of Memantine and Hespiridine on Monosodium Glutamate Induced Excitotoxicity in Rats

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ABSTRACT

Background: Glutamate mediated excitotoxicity is proved to be involved in neurodegenerative diseases like ischemia, trauma, diabetic retinopathy, glaucoma, seizures. Development of specific glutamate antagonists favors a better treatment opportunity of these neurodegenerative diseases. Hesperidin, a proven antioxidant and memantine a known NMDA antagonist were selected and evaluated for their neuroprotective property against monosodium glutamate induced excitotoxicity model both *in vitro and in vivo*. **Materials and methods:** Monosodium glutamate commonly known as Ajinomoto which is used as flavoring agent was exposed to chick retina (*In vitro* model) and subcutaneously administered to rats (*In vivo* model). Various biochemical parameters including oxidative stress related parameters and histopathology studies were conducted. **Results:** A significant cell death through a process mediated by excitotoxicity was reported. Memantine and hespiridin have produced protective effects in our studies. As hespiridin is a natural antioxidant and it elicited its protective effect by decreasing lipid peroxidation and elevation of superoxide dismutase, catalase activity. **Conclusion:** Both memantine and hespiridin have shown protective effect against monosodium induced excitotoxicity in both *in vitro* and *in vivo* experiments. Food intake and body weight increased due to glutamate was antagonized by memantine and hespiridin. Biochemical parameters and histopathological studies also supported the above claim. However, further investigations are necessary to find mechanisms involved.

Key words: Glutamate, Excitotoxicity, Ajinomoto, Hesperidin, Memantine.

INTRODUCTION

Glutamate is an excitatory neurotransmitter which is involved in principle brain functions such as cognition, learning and memory process. It is highly toxic and causes excitotoxicity when its concentration exceeds beyond the limit. Excitotoxicity is defined as a mode of neural cell death triggered by prolonged overstimulation of the receptors by excitatory neurotransmitter glutamate, aspartate and amino acids. Initial studies were conducted on glutamate excitotoxicity in retinal ganglion cells by the scientist Lucas and New house in the year 1957.[1]

Excitotoxicity can be seen in brain trauma, neurodegenerative diseases such as Alzheimer's, Dementia, Parkinson's disease, Amyotrophic lateral sclerosis, Glaucoma, Diabetic retinopathy.[2] Primary event in the excitotoxic process may be vascular or ischemic, genetical as in Huntington's, metabolic (hyperglycinemia), or immunologic as in Human immuno deficiency virus encephalopathy.

Reasons for excitotoxicity is calcium overload, free radical generation, activation of caspases and transcription factors leading to neuronal cell death. Blockade of such receptors is a competent way to overcome the damage. Overstimulation of the NMDA receptors, leads to influx of sodium and calcium ions that are involved in neuronal depolarization caused by elevated extracellular glutamate concentrations hence activates the voltage gated calcium channels for the

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influxed calcium ions thereby excessive calcium overload is the contributing factor involved in excitotoxic neuronal damage.[3] Apart from these events, dysfunction of glutamate transporters may trigger a mephitic reaction cascade via glutamate receptors.

Development of newer antagonists to treat glutamate induced excitotoxicity is the need of the hour and in this direction, we identified hespiridin, an antioxidant with its proven neuroprotective effect. In this study, a known glutamate receptor antagonist memantine was also used.

A suitable experimental model using monosodium glutamate was standardized for the purpose of the above study, which mimics the human condition of glutamate induced excitotoxicity. We recommend this animal model for study of mechanisms involved in possible toxicity of monosodium glutamate and screening of effective antidotes.

Monosodium glutamate has the capability of exhilarating and lesioning circumventricular ventricular organs, as it does not cross blood brain barrier. Food intake and body weight was increased by effecting circumventricular organs when injected orally.[3,4] Apart from its central nervous system effects it also produces a damaging effect to kidney, liver reproductive dysfunction Glutamate mediated excitotoxicity is classified into two types namely acute and delayed which is differentiated by time course and ionic dependent.[5]

MATERIALS AND METHODS

Reagents and Chemicals

Magnesium sulphate and Potassium dihydrogen phosphate were procured from SD Fine-chem limited, India. Sodium chloride, Calcium chloride and Glucose were obtained from Finar chemicals limited, India. Sodium hydrogen carbonate was from Hi media laboratories limited, India. Potassium chloride was from Merck private laboratories limited, India. MTT and Xylene was from SRL chemicals private limited, India. Glutamate, Hespiridin and Memantine was from Sigma Chemical Co, St. Louis, MO. Haematoxylin and Eosin were procured from Bio lab diagnostics, India.

In vitro Method : White Leghorn chick *(Gallus domesticus)* embryos were used for this study[6] The eggs collected from local commercial hatchery were collected and incubated at 37ºC for 11 days. On 12 day the embroyo's were collected from eggs in aseptic condition and covering layer around the eyeball was removed using scissors. The eye ball was separated using fine smooth tipped forceps and placed in a sterilized petriplate containing Krebs like balanced salt solution (KBSS) supplemented with aeration. Petriplates are placed in a darkened area until separation of retina from the eye ball. Eyeball was gently squeezed out for protrusion of the lens; the hollow ball was then freed of cornea and vitreous humor by making small cut at the edges of the ball, then the thin hollow glass like whitish layer *i.e* was collected.[7]

Experimental set up

Twenty four retinas were divided into four groups, each group containing six retinas are taken and placed in a 24 well titre plate in which each well contains 1 ml of KBSS. Then the retinas are exposed to following drugs and vehicles:

1) Control–1%DMSO (Dimethyl sulfoxide) .2) Glutamate induced group–Glutamate (10 mM concentration from which 0.1 ml of glutamate was added to the wells containing retina with KBSS).3) Memantine treated group–Memantine (3 μM concentration from which 0.1 ml was added to the wells containing retina with KBSS. 4) Hespiridin treated group–Hespiridin (3 μM concentration from which 0.1 ml of hespiridin was added to the wells containing retina with KBSS). After addition of drugs and vehicle the 24 well titre plates were placed in an incubator for 24 h. Temperature was maintained at 30⁰C. After incubation retinas were subjected to MTT assay by treating them with 0.25 mg/ml of MTT and incubated for 1 h at 37⁰C. Only viable cells are able to reduce MTT into a blue formazan product that is soluble in DMSO and measured at 570 nm. Percentage cell viability was counted by using the formula below.

$$Cell\ viability = \frac{OD\ of\ treated}{OD\ of\ control} \times 100$$

Values are then entered in the graph pad prism for mean ± SEM; ANOVA followed by Bonferroni's comparison test was used for statistical evaluation.[8,9]

In vivo: Sprague dawley rats were collected from Sanzyme enterprises private limited, Hyderabad and maintained with free access to food and water at 28 ± 2ºC and 12 h light/day cycles. The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986 were followed throughout the study. The research protocol was approved by the Institutional Animal Ethical Committee (IAEC, 05/SPIPS/IAEC/2013).[10]

20 Rats were divided into four groups, each group comprising of six rats segregated as following groups:

1. Control group–Receiving normal saline solution.
2. Glutamate treated group-(6 mg/kg.)
3. Glutamate+Hespiridin treated group–36 mg/kg.
4. Glutamate+Memantine treated group–10 mg/kg.
5. After 2 months period of treatment orally, food intake and body weight were observed.

Histopathology

Retina was placed in 90% formalin for 24 h. Then the tissue was stained with haematoxylin and Eosin dye and mounted with distyrene plasticizer xylene and observed under microscope at a magnification of 60X.

The results were presented as the mean ± SEM. Statistical analysis was done by ANOVA followed by Bonferroni's tests using Graph pad PRISM software version 6. $P < 0.05$ was considered as statistically significant.

RESULTS

As glutamate mediated excitotoxicity is involved in causing several neurodegenerative disorders, an approach to establish a simple, accurate pre-clinical *in vitro* and *in vivo* method to screen glutamate antagonists was established. Retinal excitotoxicity studies using MTT assay was done *in vitro* method. Biochemical parameters, food intake, body weight was observed in *in vivo* method. Protective effect of memantine and hespiridin were evaluated by using the established models and following are the results obtained.

In vitro method: Percentage cell viability by MTT assay

Only 35% cells were viable in glutamate induced group, but the memantine (84.167 ± 0.792) and hesparidine (65.333 ± 0.882) protected the loss of viable cells in MTT assay. The NMDA antagonist, memantine has shown better results when compared to hesperidine. (Table 1).

In vivo method

Glutamate induced stimulation of food intake was observed after administration of 6 mg/kg of glutamate for a period of 5 h. Food intake was approximately 3.5 gms at 5th h test period; Both hespiridin and memantine were able to antagonize the effect of glutamate at a dose of 36 mg/kg and 10 mg/kg respectively. (Table 2) Glutamate increased body weight in a time dependent manner at a dose of 6 mg/kg. This effect was antagonized by hespiridin and memantine at a dose 36 mg/kg and 10 mg/kg respectively (Table 3).

Biochemical parameters

Catalase is an antioxidant enzyme and its activity has been expressed in % inhibition rate. The antioxidant activity of the enzyme was inhibited in glutamate treated group when compared with normal control group. Hespiridin and Memantine protected the brain from oxidation by preventing inhibition of enzyme activity. Hespiridin has produced a better effect than memantine (Table 4).

The treatment of glutamate significantly increased MDA levels compared to control group indicating lipid peroxidation. However hespiridin 36 mg/kg and memantine 10 mg/kg prevented the rise in MDA levels indicating antioxidant property in providing protection against oxidative damage induced by the glutamate (Table 5).

The anti-oxidant activity of the enzyme was inhibited in glutamate treated group when compared with normal control group. Hespiridin and protected the brain from oxidation by preventing inhibition of enzyme activity when compared to glutamate treated group (Table 6).

Histopathology studies of chick retina
Control

Stained section shows stroma composed of round to oval cells with centrally placed nucleus and large amount of clear to eosinophilic cytoplasm. All the layers are intact (Figure 1).

Glutamate

Stained section shows superficial layer undergone mild necrosis with loss of polarity of cells, cell size altered and plenty of acute inflammatory cells (neutrophils and occasional lymphocytes) indicated by black arrow (Figure 1).

Memantine

Stained section shows superficial layer undergone minimal necrosis, with fewer acute inflammatory cells (neutrophils and occasional lymphocytes) indicated by the black arrow and normal round to oval cells with centrally placed nucleus and large amount of clear to eosinophilic cytoplasm indicated by white arrow (Figure 1).

Hespiridin

Stained section shows stroma composed of round to oval cells with centrally placed nucleus and large amount of clear to eosinophilic cytoplasm with no necrosis, similar to that of control. It shows no loss of polarity of the cells. The stained section shows no inflammatory

Table 1: Percentage cell viability by MTT assay

Groups	Mean ± SEM
Control	100.000 ± 0.000
Glutamate	35.833 ± 1.014[*]
Glutamate+ Memantine	84.167 ± 0.792[^]
Glutamate+ Hespiridin	65.333 ± 0.882[#]

Values are expressed as mean ± SEM, [*] P<0.05 when compared with control, [^]P<0.05 when compared with glutamate, [#]P<0.05 when compared with glutamate.

Table 2: Effect of glutamate on food intake

	1 h	2 h	3 h	4 h	5 h
Control	0.860 ± 0.024	1.040 ± 0.040	1.340 ± 0.060	1.640 ± 0.040	1.800 ± 0.000
Glutamate	1.020 ± 0.049[*]	1.920 ± 0.282[*]	2.540 ± 0.437[*]	2.840 ± 0.536[*]	3.460 ± 0.589[*]
Hespiridin	0.920 ± 0.0826[#]	1.100 ± 0.126[#]	1.480 ± 0.073[#]	1.700 ± 0.126[#]	2.000 ± 0.032[#]
Memantine	0.980 ± 0.037[$]	1.240 ± 0.060[$]	1.620 ± 0.020[$]	1.740 ± 0.060[$]	2.020 ± 0.020[$]

Values are expressed as mean ± SEM, [*] P<0.05 when compared with control, [#]P<0.05 when compared with glutamate, P[‡]<0.05 when compared with glutamate.

Table 3: Effect of glutamate on body weight

Control	160.000 ± 5.477	164.000 ± 5.099	172.000 ± 4.899	180.000 ± 0.000	185.000 ± 2.499
Glutamate	180.000 ± 6.325[*]	192.000 ± 8.602[*]	204.000 ± 7.483[*]	220.000 ± 7.071[*]	230.000 ± 3.742[*]
Glutamate+Hespiridin	168.000 ± 7.348[#]	174.000 ± 6.000[#]	180.000 ± 5.099[#]	180.000 ± 3.742[#]	190.000 ± 4.000[#]
Glutamate+Memantine	154.000 ± 2.449[$]	168.000 ± 3.742[$]	182.000 ± 2.000[$]	192.000 ± 3.742[$]	192.000 ± 2.449[$]

Values are expressed as mean ± SEM, [*] P<0.05 when compared with control, [#]P<0.05 when compared with glutamate, P[‡]<0.05 when compared with glutamate.

Table 4: Effect of hesperidin and memantine on catalase activity

Groups	%H_2O_2 scavenging activity
Control	65.956 ± 0.678
Glutamate	37.756 ± 0.706[*]
Hespiridin	44.798 ± 0.512[#]
Memantine	43.522 ± 0.369[$]

Values are expressed as mean ± SEM, [*]P<0.05 when compared with control, [#]P<0.05 when compared with glutamate, P[‡]<0.05 when compared with glutamate.

Table 5: Effect of hesperidin and memantine on lipid peroxidation

Groups	nmoles/gm brain tissue
Control	22.656 ± 0.986
Glutamate	46.134 ± 1.745[*]
Hespiridin	22.502 ± 0.912[#]
Memantine	21.016 ± 0.447[$]

Values are expressed as Mean ± SEM, [*]P<0.05 when compared with control, [#]P<0.05 when compared with glutamate, P[‡]<0.05 when compared with glutamate.

Table 6: Effect of hesperidin and memantine on SOD activity

Groups	%SOD scavenging activity
Control	64.690 ± 0.413
Glutamate	43.610 ± 0.454[*]
Glutamate+Hespiridin	55.314 ± 0.673[#]
Glutamate+Memantine	52.974 ± 0.35[8][$]

Values are expressed as Mean ± SEM, [*]P<0.05 when compared with control, [#]P<0.05 when compared with glutamate, P[‡]<0.05 when compared with glutamate.
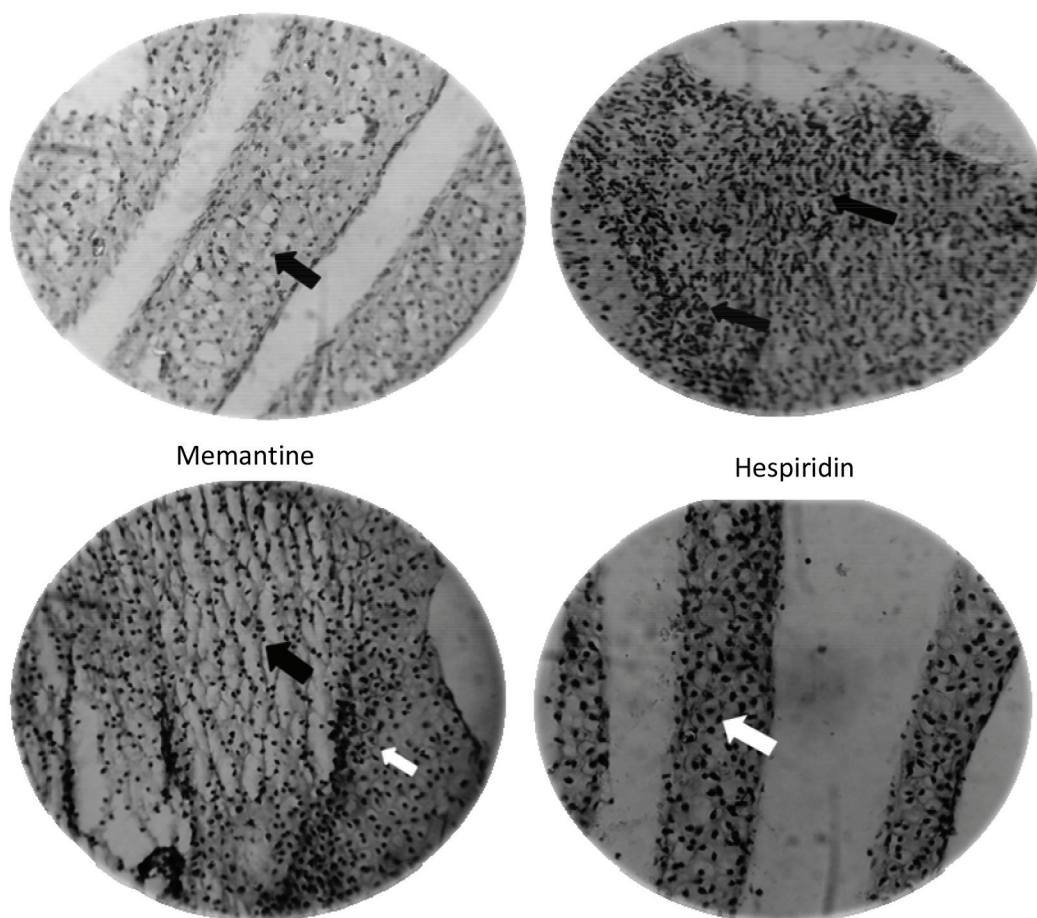
Memantine

Hespiridin

Figure 1: Histopathology studies of chick retina

cells compared to that of glutamate. All the layers are intact (Figure 1).

DISCUSSION

Monosodium glutamate is a widely used flavoring agent with many adverse effects emphasized on the hypothalamus-pituitary axis of the brain, leading to its neuro-excitatory, neuro-endocrine effects and induction of obesity.[11] It also has the potency of inducing oxidative stress in experimental animals at chronic usage and alter hepatic glucose metabolism.[11] Apart from these effects it causes damage to central nervous system by an action mediated by synaptic receptors[12] following excitotoxicity.

Glutamate mediated excitotoxicity is the major risk factor involved in neurodegenerative diseases such as Dementia, Parkinson's disease, Amyotrophic lateral sclerosis, Glaucoma, Diabetic retinopathy. The mechanism behind the excitotoxicity is imbalanced calcium homeostasis due to delay in neuronal calcium intensification; mitochondrial membrane depolarization in reverberation to glutamate is a progressive phenomenon in cell death[13] It is well known that Ca^{+2} mediate a delayed neuronal degeneration that is caused by the activation of NMDA and non-NMDA receptors.[14] The Ca^{+2} influx by glutamate receptor activation causes oxidative stress that leads to neuronal degeneration. Glutamate is the potent inducer of excitotoxicity in various tissues of the body[11] mostly brain, retina of the eye. The current research in glutamate receptor antagonist badly requires suitable screening models. So we have taken up this study to standardize *in vitro* and *in vivo* preclinical pharmacological screening models. *In vitro* model comprises of chick embryonic retina collection, incubation of the same using suitable buffer systems and test/standard drugs.

We also screened the effect of hesperidin, a known antioxidant for its efficacy against the glutamate induced excite toxicity in both the above models. We used memantine a known antagonist also to screen it efficacy.

Our findings indicate the possibility of isolation of chick embryonic retina, a very thin and sensitive tissue and using it for excitotoxicity studies. When glutamate

added to retina it produced neuronal damage within 24 h. Percentage cell viability was accessed using MTT (Tetrazolium salt) assay as proposed in the past studies.[15]

Glutamate has the potency of causing neuronal damage in the retinal ganglion cells and it was proved in our study. Glutamate has produced severe neurodegeneration as reported in MTT assay after incubation of retina with glutamate for 24 h. Only 34% of cells are viable after glutamate exposure for 24 h. But in contrast to our results, past literature reveals that the glutamate produce severe neurodegeneration in just 2 h time.[14,1] The reason is unknown and needs to be studied. In MTT assay, 84% protective effect was seen in memantine received groups whereas in case of hespiridin the protection was 65%.

In vivo studies were also conducted using Sprague dawley rats. Severe lesioning to the neurons was observed after administration of Glutamate in higher doses (6 g/kg) to rats. This effect can be attributed to stimulation of the circumventricular orga.[16,17] Food intake and body weight were increased in glutamate treated group in a time dependent manner compared to control.

Lipid peroxidation levels were also very high in glutamate administered group. Superoxide dismutase and catalase activity was decreased by glutamate compared to control. Protective effect from oxidative stress and prevention of loss of SOD and catalase was observed in rats after treatment with hesperidin. However mechanisms underlying hespiridin activity still need to be evaluated. Memantine showed a moderate protective effect in these studies. Thus the result illustrates that memantine and hespiridin may merit further investigation for their potency.[18,19]

CONCLUSION

Glutamate mediated excitotoxicity is the common pathway involved neuronal damage in many of the neurodegenerative diseases and ischemia, trauma, diabetic retinopathy, glaucoma, seizures. As glutamate is playing a central position in many of these disorders, developing its antagonistic drug favors a better achievement in treating neurodegenerative diseases. Hence Monosodium glutamate commonly known as Ajinomoto which is used as a flavoring agent was exposed to chick retina (*In vitro* model) and subcutaneously administered to rats (*In vivo* model). A significant cell death through a process mediated by excitotoxicity was reported. Memantine an NMDA receptor antagonist and hespiridin with its antioxidant and neuroprotective properties were screened for their efficacy. Hespiridin produced a better protective effect than that of memantine even at a lower dose

(3 µM) evidenced histopathologically after 2 h exposure period, but in the assay during the 24 h exposure memantine effect was greater than that of hespiridin. This area is one of the most immensely investigated field and the information obtained from this work will surely provide the basics, underlying mechanism of the diseases and the development of potent glutamate antagonists to treat the glutamate mediated excitotoxicity.

As hespiridin is a natural antioxidant and elicit its protective effect by decreasing lipid peroxidation and elevating superoxide dismutase, catalase activity. Increase in food intake and body weight due to glutamate was antagonized by memantine and hespiridin. Histopathological studies supported the biochemical data. Further investigations are necessary to find reasons and mechanisms involved.

CONFLICT OF INTEREST

The author have no conflict of interest.

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PICTORIAL ABSTRACT

White Leghorn chick (Gallus domesticus) embryo.

SUMMARY

- Glutamate mediated excitotoxicity is proved to be involved in neurodegenerative diseases like ischemia, trauma, diabetic retinopathy, glaucoma, seizures.
- Hesperidin, a proven antioxidant and memantine a known NMDA antagonist were selected and evaluated for their neuroprotective property against monosodium glutamate induced excitotoxicity model both *in vitro* and *in vivo.*
- Memantine and hespiridin have produced protective effects in our studies.
- Food intake and body weight increased due to glutamate was antagonized by memantine and hespiridin.
- Histopathological studies supported the biochemical data.

ABBREVIATIONS USED

DMSO: Dimethyl sulfoxide, **CPCSEA:** Control and Supervision of Experiments on Animals, **MDA:** Malondialdehyde, **NMDA:** N-methyl-D-aspartate receptor.

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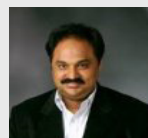
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