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Rheum emodi ameliorates glutamate toxicity in neuronal cells by up-regulating Nrf2/HO-1 expression

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ABSTRACT

Glutamate neurotoxicity has been implicated in various neurodegenerative diseases. Reduction of glutamate toxicity is one of the most essential therapeutic strategies for the treatment of these disorders. The present study was carried out to investigate the effectiveness of Rheum emodi against glutamate induced toxicity in neuronal cells. Treatment with Rheum emodi (100 and 200µg/ml) for 24 hrs *was able to revert glutamate-induced changes in nitric oxide (NO) and glutathione (GSH) levels back to normal. Moreover, Nrf2 and HO-1 mRNA levels were also significantly increased with the Rheum emodi treatment.*

Keywords: Glutamate toxicity, Rheum emodi, Nrf2, HO-1, GSH, NO

I. INTRODUCTION

Glutamate is a well known neurotransmitter responsible for communication between nerve cells under normal conditions. It is maintained at an intracellular concentration of 10^{-3} M in the brain, whereas extracellularly it is present at a concentration of 10^{-5} to 10^{-6} M [1] [2]**.** But under certain pathological conditions such as ischemia, hypoxia, and brain injury, there is huge elevation in its concentration in the brain [3], and at these excitotoxic concentrations, it can elicit damage and death of neurons [4] [5]. Glutamate toxicity has been implicated in stroke, alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), parkinson's disease (PD) and other neurodegenerative conditions. However, the molecular mechanisms responsible for glutamate-induced toxicity have not been fully elucidated [6]. Though, over-activation of glutamate receptors during glutamate toxicity has been hypothesized to impair calcium homeostasis by increasing the influx of calcium especially in mitochondria which results in energy failure, enhanced reactive oxygen species (ROS) and nitric oxide (NO) production and ultimately cell damage and death [7] [8] [9]. The increased levels of ROS and NO initiate certain neurotoxic cascades that are responsible for mediating neuronal cell death in many neurodegenerative disorders [10] [11] [12]. Therefore, the anti-oxidant substances that can scavenge ROS and NO could prove to be effective in either preventing the onset or delaying the progression of diverse neurodegenerative diseases. However, it has been observed that better cytoprotection is conferred by enhancement of endogenous anti-oxidant defense genes rather by supplemental anti-oxidants. Nuclear factor

(erythroid-derived 2)-like 2 (Nrf2) is a promising and upcoming endogenous anti-oxidant that is known to regulate a set of anti-oxidant and phase II detoxification genes, which defends cells against oxidative stress. Consequently, the targeted induction of Nrf2 by novel, natural and non-cytotoxic substances may represent a novel and effective approach to combat glutamate toxicity.

Herbal medicines are being extensively used for the treatment of various diseases and dysfunctions from ancient times, and have been hypothesized to combat various inflammatory conditions in combinatorial therapeutic approach [13] [14] [15]. *Rheum emodi* (*Himalayan rhubarb)* which is locally known as "*Pam chalan*" is a well known herb with immense medical potential in the Indian Ayurvedic system of medicine. It is an effective antispasmodic, antiseptic, anticholesterolemic, antitumor, aperients, astringent, cholagogue, diuretic and stomachic. Its chemical constituents are mainly hydroxyanthracene derivatives (emodin, rhein, aloe emodin and physcion, chrysophanol and their glycosides) [16]. Aqueous extract of *Rheum emodi* has been shown to protect the proximal tubule segments of kidneys against cadmium chloride, potassium dichromate, mercuric chloride and gentamicin-induced nephrotoxicity in rats [17]. So, *Rheum emodi* was chosen for present study as it possesses tremendous therapeutic potential and may pave way for amelioration of excitotoxic signaling pathways in diverse neurodegenerative conditions.

II.MATERIALS AND METHODS

2.1. Preparation of the methanolic extract of *Rheum emodi*

The dried rhizome of plant was powdered and extracted with methanol. The alcohol was distilled off at 45 °C and aqueous part was dried to obtain the extract. The extract of *Rheum emodi* was prepared in dimethyl sulfoxide (DMSO) for cell line studies.

2.2. Cell culture and treatments

IMR 32 cells were purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were grown in dulbecco's modified eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% pencillin-streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Cells were sub-cultured by trypsinization and cultured in plates according to the requirement of the experiment. After 24 hrs of seeding, IMR 32 cells were treated with methanolic extract of *Rheum emodi* (MER) (100 and 200µg/ml) for 24 hrs in presence/absence of glutamate (5mM).

2.3. Nitric oxide (NO) assay

NO estimation was performed as per standard Griess assay. After treatment of cells, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100µl of cell culture medium was first mixed with 100µl of Griess reagent 1 (Sulfanilamide) and incubated at room temperature for 10 min. Subsequently, 100µl of Griess reagent 2 (NED)

was added to the mixture and the absorbance was measured at 540 nm in a microplate reader. Fresh culture medium was used as a blank in every experiment. The concentration of nitrite was determined from a sodium nitrite standard curve.

2.4. GSH assay

IMR 32 cells were cultured in a 12-well plate for overnight. After treatment of cells, media was removed and cells were rinsed four times with 1x phosphate buffer saline (PBS). Cells were collected by centrifugation (3000rpm for 4 minutes), supernatant was removed and 200µl of ice-cold [(10% trichloroacetic acid (TCA) and 0.01 N HCl)] was added to the cell pellets. Tubes were vortexed, kept on ice for 10 minutes and centrifuged (12,000 rpm for 15 min at 4°C). Supernatant was extracted five times with diethyl ether to remove TCA, followed by measurements of GSH [18].

2.5. RNA isolation, cDNA Synthesis and qRT-PCR

For qRT-PCR analysis, IMR 32 cells were treated with 5mM glutamate in presence/absence of MER for 24 hrs before RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen, USA). The integrity of RNA was checked on 1% agarose gel and cDNA was generated using cDNA synthesis kit (Thermo, USA) according to the manufacturer's protocol. It was followed by qRT-PCR performed with SYBR Green Supermix in Real time PCR system (Applied Biosystems, USA). The primers for RT-PCR (IDT, USA) are listed below.

Nrf2	FP: 5'-CCTCAACTATAGCGATGCTGAATCT-'3
	RP: 5'-AGGAGTTGGGCATGAGTGAGTAG-'3
HO-1	FP: 5'-TCCGATGGGTCCTTACACTC-'3
	RP: 5'-TAAGGAAGCCAGCCAAGAA-'3
β –actin	FP: 5'-AGGCATCCTCACCCTGAAGTA-'3
	RP: 5'-TAAGGAAGCCAGCCAAGAA-'3

2.6. Statistical analysis

In the present study, results for each experiment are given as mean of triplicates ± SE. Statistically significant differences between sample groups were determined using Student's t-test. A p value of <0.05 was considered significant.

III.RESULTS

3.1. *Rheum emodi* impairs NO production in glutamate-activated IMR 32 cells.

NO has been observed to contribute to neurotoxicity in various neurodegenerative diseases like Alzheimer's disease and has been observed to rise to a considerable level during stress conditions. Thus its regulation can have therapeutic role in various neurodegenerative diseases. We accordingly investigated the effect of *Rheum*

emodi on elevated NO levels during glutamate-induced toxicity in IMR 32 cells. As NO is secreted out by cells, we estimated its level after various treatments in culture supernatant (media) by standard Griess assay (described in materials and methods).

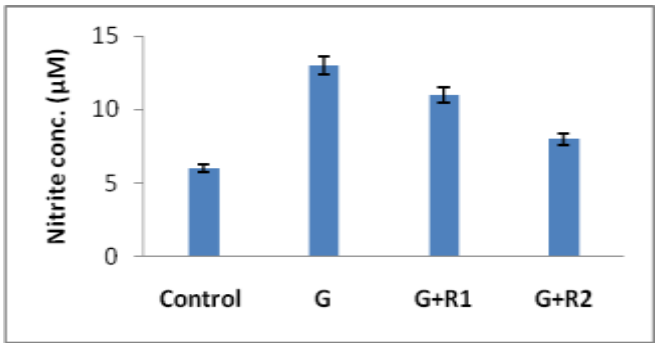

Figure 1: Estimation of NO levels in IMR 32 cells by various treatments determined by Griess assay. Exposure of neuronal cells to the glutamate stress resulted in the appreciable rise in NO levels after 24 hrs (lane G), compared to control untreated cells (lane Control). Moreover, treatment with *Rheum emodi* resulted in significant decrease ($p<0.01$) in NO levels of IMR 32 cells after 24 hrs of glutamate exposure (lane G+R1 and G+R2). The results also clearly depicts more effective NO impairment upon treatment with higher concentration of MER (200µg/ml) (lane G+R2) than lower concentration (100µg/ml) (lane G+R1).

3.2. GSH levels are recovered by MER in glutamate-activated cells.

Since the depletion of GSH (body's master anti-oxidant) is followed by increase in ROS and in the light of this effect, it has been hypothesized to be responsible for the detrimental effects of glutamate. So, we determined GSH levels after treatment with MER for 24 hrs in glutamate-activated IMR 32 cells.

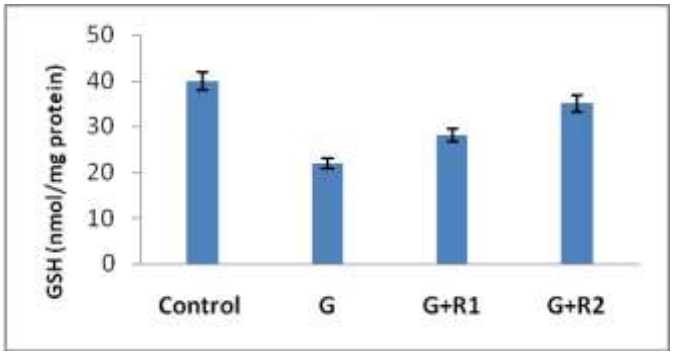

Figure 2: Determination of GSH levels after various treatments in IMR32 cells. GSH levels were determined by Elman's method after treatment of IMR32 cells with two effective concentrations of MER for 24 hrs in presence or absence of glutamate. The endogenous levels of GSH were seen to decrease significantly in glutamate-challenged cells. However, MER (200 µg/ml, lane G+R2) was able to recover this anti-oxidant thiol (GSH) to a significant level, comparable to control than MER (100 µg/ml, lane G+R1).

3.3. MER induces Nrf2 mRNA expression in glutamate-activated cells.

Since Nrf2 regulates the expression of a set of anti-oxidant and cyto-protective proteins that protect against oxidative stress, drugs having potential to stimulate Nrf2 pathway are being focused for treatment of diverse diseases caused by oxidative stress. Since *Rheum emodi* has been reported to possess certain anti-oxidant properties, we revealed the influence of *Rheum emodi* on the expression of Nrf2 mRNA. Briefly, we treated IMR 32 cells with MER in presence/absence of glutamate and subjected to qRT-PCR to quantify the Nrf2 mRNA expression levels in these cells.

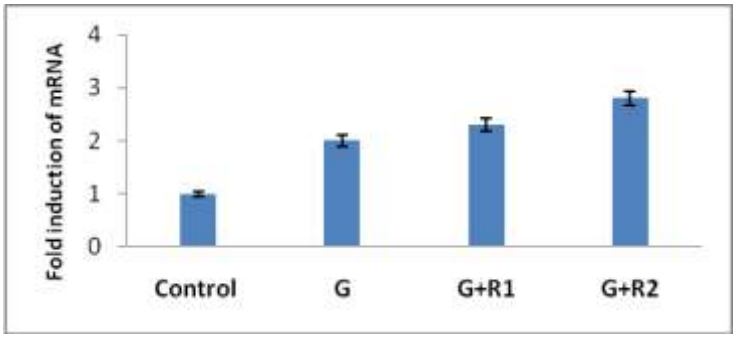

Figure 3: *Rheum emodi* up-regulates Nrf2 mRNA expression in glutamate activated IMR 32 cells: RT-PCR results reveal a significant increase in Nrf2 mRNA expression after exposure to glutamate as compared to control (lane G). On treatment with MER, Nrf2 mRNA was further seen to increase in glutamate-stimulated IMR-32 cells in a concentration-dependent manner, with much pronounced up-regulation with higher concentration of MER (200µg/ml, lane G+R2) than its lower concentration (100µg/ml, lane G+R1)

3.4. MER induces HO-1 mRNA significantly in glutamate-stimulated IMR 32 cells.

To confirm the up-regulation of HO-1 mRNA, an anti-oxidant downstream target of Nrf2, we first investigated the mRNA expression of HO-1 upon treatment with same selected concentrations of MER in presence/absence of glutamate stimulus. To achieve this, total RNA was isolated from these cells and subjected to qRT-PCR in order to quantify HO-1 mRNA expression in control as well as MER treated cells that were challenged with glutamate.

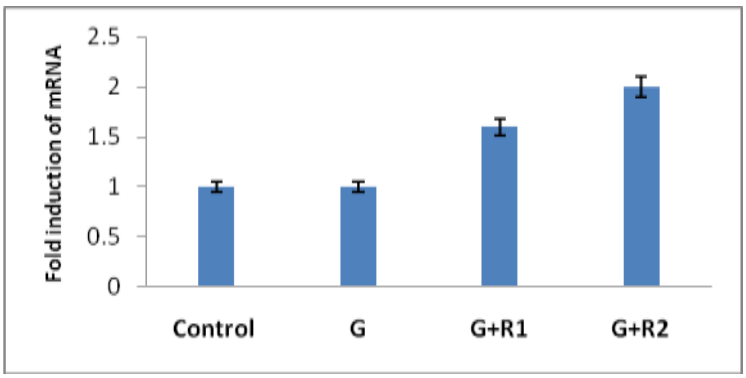

Figure 4: Changes in HO-1 mRNA expression upon MER treatment in glutamate activated IMR 32 cells: RT-PCR results clearly reveal that there was no significant change in mRNA expression of HO-

1 upon stimulation with glutamate (lane G). However, a significant increase in HO-1 mRNA levels was observed upon treatment with MER in presence of glutamate stimulus in a dose-dependent manner, with higher concentration of MER (200 µg/ml, lane G+R2) being more efficient than its lower concentration (100 µg/ml, lane G+R1).

IV. DISCUSSION

Elevated levels of glutamate lead to neuronal death and have been implicated in a wide range of neurological diseases [19] [20] [21]. Two pathways for glutamate toxicity have been described [22]: (i) excitotoxicity, which occurs through the activation of glutamatergic receptors [23] [24], and (ii) oxidative glutamate toxicity which is mediated via a series of disturbances to the redox homeostasis of the cell [25]. These pathways are however incompletely characterized, yet both result in the production of free radicals [19] [25] which can cause damage to all the essential biomolecules, eventually leading to neuronal death. Moreover, various antioxidant substances have been observed to protect cells against glutamate-induced toxicity [26], so the free radical production has been hypothesized to be mainly involved in detrimental effects of glutamate. Consequently, the substances capable of scavenging free radicals can have therapeutic potential for managing diverse neurodegenerative conditions.

Calcium overload has been hypothesized to be the main culprit responsible for the detrimental effects of glutamate toxicity. Increased intracellular Ca^{2+} levels after glutamate exposure have been attributed to calcium ion leakage from mitochondria [27] [28], and as such mitochondrial dysfunction has been partly suggested to be responsible for the increased intracellular concentration of Ca^{2+} ions, and consequent immense ROS production and neuronal damage and death. Furthermore, depletion of master anti-oxidant, GSH has been suggested to add to the miseries during glutamate toxicity. Reduced GSH levels may interfere with the ROS scavenging ability of cells, hence augmenting oxidative stress and ultimately leading to death of neurons [29]. We therefore evaluated effect of MER primarily on NO and GSH levels. Our study also confirmed increase in potent ROS species, NO during glutamate stress in IMR 32 cells, with simultaneous decrease in GSH levels. However, we demonstrate the ability of MER to significantly increase GSH levels in glutamate-activated cells with concomitant reduction in NO levels. Thus MER seems to reduce toxic effects of glutamate by recovering the GSH levels, which in turn scavenges ROS as seen by decreased levels of NO.

Additionally, targeted induction of cytoprotective genes has been postulated to be of immense therapeutic potential by conferring protection to the cells during stress. MER was seen to increase the expression of Nrf2 and HO-1 mRNA significantly, two important components of endogenous defence mechanism. Up-regulation of these enzymes in a cell decreases the stress. So, our study provides an insight towards the therapeutic potential of MER and can pave way for the treatment of diverse neurodegenerative diseases.

V. CONCLUSION

In conclusion, treatment with MER increases master anti-oxidant defence regulator gene (Nrf2) and its main downstream anti-oxidant target gene (HO-1) and GSH in glutamate-stimulated cells with simultaneous decrease

in stress parameter like NO, implicating its potential protective role against glutamate toxicity. Further research into the molecular mechanism of this neuroprotection mediated by MER may prove valuable therapeutic agent to combat various neurological disorders.

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