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Resveratrol modulates NMDAR-nNOS axis in the cerebellum of a neuroexcitotoxic model of Hepatic encephalopathy

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Abstract: Resveratrol (RSV), a natural plant derived phytoalexin, is known to exhibits its biological activity by activating Sirt1 which confers neuroprotection under a variety of neurological complications like ischemia, Alzheimer's disease etc. Hepatic Encephalopathy (HE) is a disorder of mental activity, neuromuscular function and consciousness that occurs as a result of liver dysfunction, involving N-methyl-D-aspartate receptor (NMDAR) over activation in brain cells, causing excitotoxicity. Therapeutic modulation of this glutamate receptor, therefore,needs to be investigated to define cerebral mechanism based management of HE. This article evaluates the effect of RSV as herbal supplement in modulation of NMDAR-nNOS (neuronal nitric oxide synthase) axis in the cerebellum of rats with moderate grade HE (MoHE) induced by administration of 100 mg/kg b.w. of thioacetamide i.p. for 10 days. In another set, the MoHE rats were co-treated with RSV 10mg/Kg bw i.p from 8th day of TAA treatment up to 14 days.As compared to the NR2A dominated NMDAR composition observed in the cerebellum of the control rats, the MoHE rats showedenhanced NR2B dominated NMDAR expression. The level of nNOS and NO are considered as a biochemical assay for the NMDAR activity. Both, nNOS expression and NO levels, as compared to the control rats, were found to be enhanced in the cerebellum of the MoHE rats. However, all these parameters were observed to regain their normal levels in the cerebellum of the MoHE rats treated with RSV. The findings suggest that resveratrol could act as a neuroprotectant against MoHE pathogenesis by modulating NMDAR-nNOS axis in cerebellum.

Index terms: Resveratrol, NMDAR, excitotoxicity, hepatic encephalopathy, cerebellum, nNOS

I. INTRODUCTION

Resveratrol is the most important stilbenephytoalexin, synthesized in certain plants as a part of their defence mechanism (Hasan&Bae, 2017). It is a polyphenolic compound found in fruits like; grapes, mulberries, blueberries, raspberries etc. and it has been described to exhibit multiple pharmaceutical properties (Hasan&Bae, 2017).Its therapeutic effects as anti-aging, anti-diabetic, cardioprotective and neuroprotecti agent are generally attributed to its anti-inflammatory and antioxidant activities (Baur& Sinclair, 2006; Caminset *al*, 2010). The high antioxidant potential

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of resveratrol has been found to show protection against oxidative damage in HepG2 cells by increasingall the antioxidant enzymes and lactate dehydrogenase (LDH) level, (Li *et al*, 2021). It has also been described to exhibit cardioprotective effects by decreasing myocardial hypertrophy, fibrosis and severity of heart failure in postinfarction heart failure model (Riba*et al*, 2017).Amongst a good number of herbal medicines available, many reports suggest that Resveratrol is a potent neuroprotectant and it does so by improving cognitive and motor functions in different models of neurodegenerative diseases (Chung *et al*, 2016; Tian*et al*, 2016).Most of the neurodegenerative brain disorders are now evident to implicate Glutamate-NMDAR activation as the major neurochemical aberration at cellular level. However, the efficacy of resveratrol against glutamate excitotoxicity by modulating NMDARsignalling remains largely unexplored. Therefore, in this study, we have selected NMDAR activation led excitotoxic model of hepatic encephalopathytoexplore the efficacy of resveratrol.

Neuronal excitotoxicity is caused by an abnormal increase in the synaptic glutamate level resulting into calcium overload led mitochondrial dysfunction generating ROS and causing energy deficit in the brain cells. Excitotoxicity is mediated by glutamate receptors like NMDAR, AMPA or KA receptors. But activation of NMDAR triggers more lethal injury than AMPA and KA receptors, as it has greater ability to induce calcium influx (Prentice *et al*, 2015). NMDA receptor performs two contrasting functions in the brain. On one hand, NMDAR is is involved in controling critical events in the formation and development of synaptic organization and synaptic plasticity. On the other hand, the over activation of NMDARs can promote neuronal death in neuropathological conditions due to enhanced Ca^{2+} permeability (Carvajal *et al*, 2016). Deregulation in the Ca_2^+ influx through NMDA receptor is associated with several neurological diseases such as Alzheimers disease (AD), Parkinson's disease (PD), Schizophrenia, Amyotrophic Lateral Sclerosis (ALS) and also Hepatic Encephalopathy (HE).

Structurally, NMDAR is a tetrameric complex consisting of two obligatory NR1 and two regulatory NR2 subunits (Franchini *et al*, 2020), which, in particular, are encoded by four genes NR2A-D accountable for differential NMDAR channel properties and pharmacology (Girling& Wang, 2016). Thus, variations in the NR2 subunit lead to the differential receptor kinetics, properties and downstream signaling pathways (Zhang *et al*, 2022). Also, it has been reported that NR2A containing NMDAR have primary role in cell survival signaling, whereas, NR2B containing NMDAR triggers excitotoxic neuronal death by inducing apoptotic cascades demonstrated both *in vitro* and *in vivo*(Liu *et al*, 2007).It has been reported that GluN2A subunits and GluN2A/GluN2B subunit ratio were increased in PD patients as well as in levodopa-treated dyskinetic rats and monkeys (Zhang *et al*, 2019). Further, it is suggested that GluN2B promotes cell death in cerebral ischemia by activating pro-death signalling cascade mediated by neuronal nitric oxide synthase (nNOS) and death-associated protein kinase 1 (DAPK1) (Mao*et al*., 2022). Besides this, it has also beenreported that there occurs a significant decline in the expression of NR2A vs NR2B and PSD-95 in the PFC of the depressed human subjects with no change in the NR1 subunit (Feyissa *et al*, 2009). Hence,it is now speculated that alterations in the sub unit composition of NMDAR deserve a scientific merit for evaluating efficacy of a neuroprotectant against a neurological disorder developed due to NMDAR over activation.

Hepatic Encephalopathy (HE) is a serious type of neuromuscular dysfunction and impaired consciousness that occurs in the patients suffering from the persistent liver dysfunction (Felipo, 2009). The neuropsychiatric complications of HE has been described to be associated with the hyperammonemia (HA)induced derangement of NMDAR function ((Butterworth, 2000;Felipo, 2009). Thus, the fate of ammonia in brain ultimately constitutes initial event of ammonia neurotoxicity as the primary etiology of HE. Since brain lacks urea cycle enzymes, brain cells operate ammonia- Glutamine-glutamate cycle which gets disrupted to finally accumulate excess of glutamate in the synaptic cleft to induce HE pathogenesis (Liere *et al*, 2017). This situation could over activate NMDA receptor, causing influx of Ca^{2+} ion into the post synaptic neurons accountable for

inducing a series of neurochemical derangements in those neurons (Felipo, 2013).

Additionally, motor function of the brain is considered to be affected the most during HE pathogenesis and therefore, cerebellum neurochemistry is argued to be affected first (Felipo, 2009). Indeed, it has been described that cerebellum undergoes a cascade of changes related to nNOS biochemistry in the model of MoHE (Singh &Trigun, 2010). Also, alterations in the Glutamine-glutamate cycles have been demonstrated in case of the acute HE rats (Singh *et al*, 2014). Importantly, Bacopa extract (CDRI-08) has been described to modulate NMDAR-nNOS axis in the cerebellum of the rats with minimum hepatic encephalopathy (MHE) (Mondal & Trigun, 2015).

The present article therefore, intends to explore whether resveratrol, evolving as a novel natural neuroprotectant, is able to modulate the NMDAR-nNOS axis in the cerebellum during MoHEpathogenesis. The hypothesis is well supported by the neuromodulatory effects of RSV demonstrated in the MoHE rat model (Khanna &Trigun, 2016; Khanna *et al*, 2020).

II. MATERIALS AND METHOD

A. Animals and Chemicals

Adult male Charles foster rats weighing 130 to 160g were used in this study. The rats were kept in separate cages, fed with the recommended diet, and maintained at standard conditions of 12h light and dark period at room temperature (25±2∘C) in an animal house. Rats were maintained in following the guidelines of laboratory animal care approved by the institutional animal ethical committee for the care and use of laboratory animals (Dean/11-12/CAEC/331).

All chemicals used were of analytical grade obtained from E-Merck and Sisco research Laboratory, Mumbai (India) except N,N'-methylenebisacrylamide, Acrylamide, N,N,N'N'-tetramethylethylenediamine(TEMED), phenylmethylsulphonyl fluoride (PMSF), Ponceau and bromophenol blue, purchased from Sigma-Aldrich, USA. Primary antibodies used were supplied from the following companies: Rabbit polyclonal nNOS from Santa Cruz Biotechnology, Rabbit monoclonal anti-NR2B from Invitrogen, Mouse monoclonal NR2A, and Lamin B from Abcam. Goat anti-Rabbit horseradish peroxidase (HRP) conjugated secondary antibodies were supplied from Genei, Bangalore. ECL western blotting detection kit was procured from Thermo Scientific. The trans-Resveratrol was supplied from the Cayman chemicals company.

B. Induction of Moderate grade Hepatic Encephalopathy

The MoHE model of neuroexcitotoxicity in adult male albino rats was developed by the administration of thioacetamide (TAA) as standardized in our lab (Singh & Trigun, 2010). The rats were divided into three groups with 6 rats in each. Group A: control (C), administered with 0.9% NaCl i.p, once daily for 10 days; Group B: Moderate Hepatic Encephalopathy (MoHE), administered with 100 mg/Kgbw TAA (prepared in 0.9 % NaCl) i.p once daily for 10 days; Group C: MoHE + Resveratrol (MoHE+ RSV) administered with 10mg/Kgbw RSV i.p once daily, dissolved in 1% DMSO, starting from 8th day onwards till 14th day. The resveratrol was administered 4h after the TAA treatment. After 24 h of the last dose given, all the rats were sacrificed on 15th day. The cerebellum was dissected out and stored at −80˚C for further studies.

C. Preparation of cerebellum extracts

Nuclear and cytosolic extracts were prepared according to the protocol of Dignam et al (1983). Two types of buffer (a) Homogenizing buffer containing- 10 mM HEPES pH =7.9, 1.5 mM MgCl2, 10 mM KCl, 1% NP40, 0.5 mM DTT, 0.5 mM spermidine, 0.5 mM PMSF, 4µg/ml Aprotinin, 0.1 mM EDTA and 12 % glycerol was added. Cerebellum extracts were homogenized in 4 volumes of ice cold homogenizing buffer and centrifuged at 1000 X g for 10 min at 4^{o}C. Pellet contained mainly nuclei and hence, it was preceded for nuclear fractionation. Supernatant was transferred to a new tube and cleared by centrifugation at 14000 rpm for 45 min at 4^{o}C. Aliquots of supernatant were stored at -80^{o}C. This was treated as the cytosolic fraction. (b) Nuclear

extraction buffer containing 20 mM HEPES pH=7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and Protease inhibitor cocktail. Pellet was washed twice with nuclear extraction buffer and re-suspended in ~ 1.2µl / mg of pellet in cold buffer. Then it was sonicated for 30 sec at 4°C, Centrifuged at 14000 rpm for 15 min at 4°C and aliquots of the nuclear fractions were stored at -80°C.

D. Assay of NO (nitrate + nitrite)

Nitric oxide is extremely unstable due to its short half life period of 3-5 sec. It undergoes rapid oxidative degradation to nitrite and nitrate, which can be determined spectrophotometrically.

As described previously from our lab (Singh & trigon, 2010), the protocol of (Miranda *et al*, 2001) was used to assay NO in the tissue samples; where the level of NO was measured by the reduction of Nitrate to Nitrite by Vanadium (III) chloride in combination with Greiss reagent (composed of equal volume of 100 µl each, 1% sulfanilamide in 2.5% H3PO4 and 0.1 % NEDD prepared in distilled water). The sulfanilamide in acidic media first reacts with nitrite to form transient diazodium salt. This intermediate then reacts with coupling reagent NEDD to form a stable azo compound. The intense purple colour of the product is measured by recording OD at 540nm.

Briefly, the tissue extracts were deproteinised using absolute alcohol (sample: ethanol; 1:2 ratio) and centrifuged at 10000 x g at 4°C. The supernatant was collected and equal volume of vanadium (III) chloride was added. This was followed by addition of Griess reagent and incubation at RT for 40 min. The OD was measured at 540 nm. Nitrite concentration was determined by using a standard plot constructed against sodium nitrite. The nitrite content was expressed as µmole/mg protein.

E. Western blotting

Briefly, 60 µg of cytosolic protein was loaded in each lane and subjected to 10% denaturing polyacrylamide gels. Electrophoresis was carried out at constant voltage of 100 volts for 2 hours followed by transferring the protein bands on nitrocellulose membrane over night at 50 mA at 4°C. Efficiency of Protein transfer was checked via Ponceau S staining. The membrane was then placed in a blocking solution (5% non fat dried milk in 1X PBS) for 2 hours. The membrane was processed for immunodetection of NR2A, NR2B and nNOS antibody (1:1000). HRP-conjugated secondary antibody was used for final detection of the respective proteins using ECL western blotting detection kit. For loading control, monoclonal anti-β-actin peroxidase antibody (1:10,000) was used. The normalized densitometric values of each protein vs β-actin, was recorded using gel densitometry software Alpha Imager 2200.

F. Statistical analysis

The statistical analysis of experimental data was done by applying Student's't' test, expressed as mean ± SEM and P<0.05 was taken as level of significance between control and experimental sets.

III. RESULT

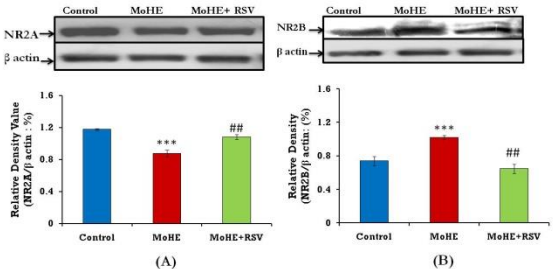

Fig1. Profile of NMDA receptor subunit NR2A in cerebellum of control, MoHE and MoHE rats treated with resveratrol. (A) & (B) show representative western blot photographs of NR2A and NR2B with 60µg protein in each lane in cerebellum respectively. Lower panels show densitometric values of NR2A and NR2B/β-actin. Values are represented as mean ± SEM; from 5 western blot repeats; ***p<0.001 (Control vs. MoHE), ##p<0.01 (MoHE vs MoHE+RSV).

A. Effect of Resveratrol on the expression level of NR2A and NR2B

NMDAR complex is a tetrameric protein complex, comprising of two glycine binding NR1 subunit and two glutamate binding NR2 subunit which can be NR2A, 2B, 2C or 2D. Specific combination of

NMDAR subunits have differential role in mediating excitotoxicity or neuroprotective functions in the brain. The NR2A combination of NMDAR is neuroprotective while NR2B combination is neurodegenerative driving neurons towards apoptosis during excitotoxicity. This differential role of NMDAR subunit emphasizes about the importance of functional composition of this glutamate receptor in neuropathology. As depicted, in figure 1(A), in comparison to control, the NR2A protein is significantly declined (p<0.001) in the cortex of MoHE rats. However, this pattern was seen to be recovered back with a significant increase in NR2A level (p<0.01) in the cerebellum of MoHE on resveratrol treatment.

On the contrary, as depicted in Figure 1(B), a significant increase was observed in protein level of NR2B in the cerebellum of MoHE rats (p<0.001) in comparison to control. Whereas, the enhanced NR2B level was restored back to the normal level on resveratrol treatment (p<0.01) in the cerebellum of Moderate HE rats.

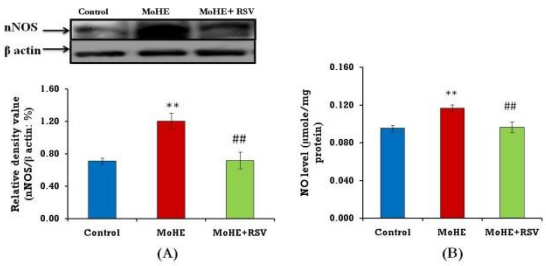

Fig 2. Expression of nNOS in the cerebellum of control, MoHE and MoHE rats treated with resveratrol. (A) show representative western blot photographs of nNOS with 60µg protein in each lane in the cerebellum region. Lower panels show densitometric values of nNOS/β-actin. Values are represented as mean ± SEM; from 5 western blot repeats; **p<0.01 (Control vs. MoHE), ##p<0.01 (MoHE vs. MoHE+RSV). (B) Represents level of NO in cerebellum of control, MoHE and MoHE rats treated with resveratrol. Values are represented as mean ± SEM, where n=5. **p<0.01(Control vs MoHE), ##p<0.01 (MoHE vs MoHE+RSV)

B. Effect of Resveratrol on nNOS expression and NO production

Molecular connection between nNOS, PSD95 and NR2B subunit of NMDAR is known to forms a ternary complex in neuronal cells which is important for regulating NMDAR over activation led neurophysiology. Since nNOS and NMDAR are directly linked to a molecule PSD-95, the level of nNOS and NO serve as measure for NMDAR activation. Therefore, as seen in Figure 2(A), the level of nNOS was significantly increased (p<0.01) in the cerebellum of MoHE rats, however, with its recovery on resveratrol treatment (p<0.01).

Further, the above data was consistent with a significant increase (p<0.01) in the NO level in MoHE rats, which was observed to be decreased significantly on resveratrol treatment in the cerebellar region of those rats (Fig. 2 B).

IV. DISCUSSION

It is now becoming clearer that modulation of the NMDAR activity by exogenous factors constitutes one of the therapeutic mechanisms to recover neurological complications associated with the glutamate excitotoxicity. The efficacy of resveratrol as a potent neuroprotectant is an evolving concept. In a report from our group, it has been demonstrated that resveratrol, by activating SIRT1, could recover the neurostructural aberrations associated with the MoHE pathogenesis (Khanna *et al*, 2020). To explore further the neurochemical basis of the RSV effect at cellular level, we attempted to investigate whether RSV could mediate the neuro-recovery via modulating NMDAR activity. We focused this study at cerebellum neurochemistry as resveratrol has already been reported to improve the motor function deficits in the MoHE rats (Khanna *et al*, 2020).

The change in the NMDAR composition vs neurochemical signaling is considered the primary event of neurological outcomes. It has been reported that excessive NR2B expression results in cognitive deficits and its down regulation resulted in alleviation of this effect, in a hypoxia model (Huo*et al*, 2014). In the present study, the treatment with resveratrol could restore the MoHE associated disturbed NR2A and NR2B levels, causing a remarkable shift from a declined ratio of NR2A/NR2B towards increased NR2A/NR2B ratio in the cerebellum of MoHE rats (Fig.1 A & B). This data suggested that resveratrol is able to modulate the NMDAR composition resulting into transition from a NR2B dominated neurodegenerative composition towards a NR2A dominating neuroprotective composition.

This finding is supported by a similar study conducted on neuronal culture cells showing a neuroprotective effect of resveratrol against NMDA-induced excitotoxicityby activating SIRT1 activation (Yang *et al*, 2017). Besides this, recently, the efficacy of resveratrol has been shown to improve the quality of life of patients suffering from MHE (Malaguarnera*et al*, 2018). Thus, it is argued that resveratrol mediated SIRT1 activation might be considered as an important modulator of NMDAR activity in the brain cells challenged with stress and neuropathology.

Also, It is suggested that NMDAR over activation led increased intracellular calcium generates oxidative stress and energy deficits at cellular level (Hasam-Henderson *et al*, 2018). This could in turn decline SIRT1 activity in cerebellum of MoHE rats. Resveratrol, being a strong antioxidant and protectant of the metabolic homeostasis, could be able to recover the cerebellar cells of MoHE rats from a metabolically deranged status by activating SIRT1. This might be executed via deacetylation of several protein substrates. Some literature does suggest improvement of metabolism by SIRT1 dependent deacetylation of certain proteins (Li, 2013).

Another mechanism could be the modulation of nuclear transcription activity due to deacetylation of certain transcription factors. Indeed, being nuclear in localization, SIRT1 is reported to modulate functions of some critical transcription factors under a variety of neuropathology (Jęśko*et al*, 2017). Though, this argument needs to be substantiated from other study, but it is speculated that resveratrol is able to alter glutamatergic NMDAR activity by SIRT1 activation via altering subunit composition of this receptor complex in cerebellum of MoHE rats. This argument gets support from a report describing reversal of NR2A/2B ratio in the cortex and cerebellum of the MoHE rats due to the treatment with *Bacopamonnieri* (Mondal&Trigun, 2015).

Irrespective of the mechanism by which NMDAR composition gets altered, downstream signalling of NMDAR over activation constitutes an important neuropathological outcome. The nNOS and NO level thus, serve as a biochemical parameter to assay the activity of NMDAR. This is because of the coupling of NR2B subunit of NMDAR to nNOS by PSD-95 via their respective PDZ domains (Mungrue&Bredt, 2004; Christopherson*et al*, 1999). Hence, it acts as an effector molecule to relay the information from NMDAR to downstream pathway, after influx of Ca_2^+ in the post synaptic neurons (Mondal&Trigun, 2015).

NO is a pleiotropic molecule which, if produced in excess in brain, adapts numerous mechanisms to induce neuronal derangement (Monfort*et al*, 2002). Thus, the activity of NMDAR was assessed by studying the expression of nNOS and level of NO in the brain. According to Fig.2A& B, the increase in the nNOS level in the cerebellum of MoHE rats was observed, which coincided with the increment in the NO level. However, administration of resveratrol to MoHE group normalized the expression of nNOS in the cerebellum region with a similar pattern of change in NO level. Thus, it suggests that resveratrol treatment to MoHE rats resulted into the declined NMDAR activity. This is supported by a report which suggests that *Bacopamonnieri* extract could not only reverse the NR2A/NR2B ratio but also normalized the nNOS level in the cerebellum of MHE rats (Mondal&Trigun, 2015).

CONCLUSION

In the present study, resveratrol is able to alter the constitution of the functional NMDAR composition by reciprocal expression of the two glutamate binding subunits, NR2A and NR2B, in cerebellum of the MoHE rats. This is consistent with a similar decline in NMDAR-nNOS activity in the cerebellum of those MoHE rats. Thus these findings provide a neurochemical basis of the

resveratrol dependent recovery in the MoHE associated limb and motor coordination deficits (Khanna &Trigun, 2020).

FUTURE DIRECTIONS

As pathogenesis of many neurodegenerative brain disorders involve Glu-NMDAR over activation, modulation of this neurotransmitter receptor is considered a relevant therapeutic target. Thus, transition from a neurodegenerative NMDAR composition in the cerebellum of the MoHE rats towards a neuroprotective one due to the resveratrol treatment, provide a neurochemical basis to examine this mechanism in other brain disorder involving NMDAR over activation led excitotoxicity.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest about this paper.

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