J Biochem Tech (2014) 5(4):782-787
ISSN: 0974-2328

Enhanced safety and protection of glutamate induced hippocampal neuronal cells damage by Neurotol

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Received: 23 December 2013 / Received in revised form: 15 July 2014, Accepted: 23 July 2014, Published online: 03 November 2014

Abstract

We investigated the neuroprotective effect of Neurotol (mannitol 10% + glycerin 10% with excipient RD011) against neuronal damage induced by glutamate compared to Neurotol M (mannitol 20%), Kratol (mannitol 10% +glycerin 10%) and Zemisol (mannitol 10% + glycerin 10%). Glutamate exposure of neuronal cells caused concentration dependent neuronal damage as is evident by significant increase in LDH activity ($P<0.05$ to $P<0.001$). The neuronal cell toxicity by exposure to glutamate involves an apoptotic process. Among the tested drugs, 100 μM of Neurotol significantly prevented neuronal cells from glutamate induced damage ($P<0.001$) compared to Neurotol M, Kratol and Zemisol ($P<0.01$) indicating Neurotol is highly effective in neuronal cell protection. When various drugs were compared for their involvement in regulation of MAP2 expression, Neurotol was found to significantly up regulated the expression of MAP2, after 6 h of treatment ($P<0.05$) and was continued upto 24 hrs which was comparable with normal cells at 24 hrs. However, cells treated with Neurotol M, Kratol and Zemisol failed to provide early up regulation of MAP2 expression and significant up regulation was observed only after ≥18 h ($P<0.05$). Based on the above results, it is concluded that Neurotol may be a potential therapy in brain disorders or damage.

Keywords: Glutamate induced toxicity, Neurotol, Neurotol-M, Oxidative stress.

Introduction

A number of neuropathological processes are linked with glutamate excitotoxicity and oxidative stress that lead to neuronal damage and death. Glutamate is believed to be a major excitatory neurotransmitter involved in various functions including learning, memory and motor function of brain. Additionally, it is involved in neuronal tissue damage during cerebral ischemic hypoxia caused by accumulation of excess glutamate in the central nervous system (Sahota and Savitz 2011; Bach et al., 2012; Gliyazova et al., 2013). Till date, two pathways for glutamate toxicity have been described: first is receptor mediated which involves activation of glutamatergic receptors and second is oxidative pathway, which includes disturbances of redox homeostasis of the cell (Elia et al., 2012).

The excess glutamate leads to excessive activation of glutamate receptors and is believed to play a role in the pathophysiology of many diseases (Gliyazova et al., 2013). Excess levels of glutamate allow the Ca2+ influx into the cytosol. Excess calcium in the cytosol of the cell triggers the activation of glutamate receptors through ionotropic N-methyl-D- aspartate (NMDA) receptors as well as kinases including Ca2+/calmodulin-dependent kinases (CaMK), mitogen-activated protein kinases (MAP), which causes changes in neuronal structure and function (Wang 2013; Higley and Sabatini 2010). Glutamate receptor activation also stimulates an increase in mitochondrial respiration (electron transport) to generate the ATP necessary to drive the activity of ion-motive ATPases that restore ion gradients across cellular membranes (Vermehren and Fern 2013). Mitochondrial Ca2+ uptake and increased mitochondrial respiration can result in production of damaging free radical superoxide anion (Llorente-Folch et al., 2013). These free radicals interact with the neuron's membrane structures, including nuclear, mitochondrial and cellular membranes that trigger neuronal cell death, a process called excitotoxicity (Janc and Muller 2014). An increasing number of reports have shown that reactive oxygen species (ROS) provoked by glutamate-linked oxidative stress are involved in brain injury (Gliyazova et al., 2013). ROS leads to oxidative stress which is involved in numerous neuropathological disorders such as ischemic stroke, traumatic brain injury (TBI), depression, Alzheimer's disease, Parkinson's disease (Shadrina et al., 2010; Fernandez-Checa et al., 2010; Rawdin et al., 2013). Neuronal injury is also associated with a decline in both total and free brain magnesium concentrations that could persist over several days. The neuroprotective efficacy of magnesium has been attributed to a variety of the effects of this molecule on patho-physiological mechanisms during and after cerebral ischemia, vasodilatation, inhibition of the NMDA-

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receptor and anti-convulsant properties (Westermaier et al., 2013).

Over 30 years, mannitol has been used clinically as an osmotherapeutic drug on brain injury which protects the brain by reducing blood viscosity (Shawkat et al., 2012; Diringer et al., 2012). Despite its neuroprotective action, it is associated with the "rebound phenomenon" (Palma et al., 2006). Glycerol is another agent that has been used in the treatment of brain oedema caused by ischaemia or trauma (Pynnönen et al., 2013). Apart from its hypertonic nature it also act as a free radical scavenger, antioxidant and activator of plasma prostaglandin resulting in vasodilation (Diringer et al., 2012; Shawkat et al., 2012; Soni et al., 2009).

Hippocampus, a major component of human brain plays an important role in learning and memory (Epp et al., 2013). It has been reported that hippocampus is the most sensitive to various neurological insults such as hypoxia–ischemia, seizure and prolonged stress (Wang 2013). Despite the clinical importance of neuronal injury, currently there are no effective medicines to combat diseases caused by glutamate excitotoxic cascade. Therefore, the present study was designed to investigate the neuroprotective effect of Neurotol (mannitol 10% + glycerin 10% with RD011) against neuronal damage induced by glutamate in rat hippocampal neuronal cell line with respect to Neurotol M (mannitol 20%), Kratol (mannitol 10% + glycerin 10%) and Zemisol (mannitol 10% + glycerin 10%).

Materials and Methods

Chemicals and kits

L-glutamate and Hank's balanced salt solution (HBBS) without Ca2+ and Mg2+ were obtained from Hi-Media (Mumbai, India). Fetal bovine serum was purchased from Invitrogen (USA). The lactate dehydrogenase (LDH) kit was purchased from Sigma-Aldrich (USA). MAP-2 kit was purchased from Wuhan Huamei Biotech Co., Ltd., (China). Neurobasal media and B27 were purchased from Invitrogen (USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was obtained from Calbiochem (EMD Chemicals, Inc., Aandiego, California, USA).

Neuronal cell cultures

Rat hippocampal neuronal cell line was purchased from Invitrogen BioServices Pvt. Ltd, Bangalore, India and grown in poly-L-lysine coated 25 cm2 culture flasks using Neurobasal medium supplemented with 0.5 mM glutamine and 2% B27 at 37°C in an atmosphere of 5% CO2 in air, as recommended by the supplier. B27 provides optimal growth and long- term survival of rat hippocampal neurons. Cells were sub-cultured to a fresh culture flask when growth reached 70–90% confluence.

Effect of glutamate on neuronal cells

To study the effect of various concentrations of glutamate on neuronal cells, the neuronal cells were seeded in poly-L-lysine coated 96-well plates (1x104cells/well). After 24 h, the cells were washed once with HBSS and added to new neurobasal media and plate was incubated in the absence and presence of different concentrations of glutamate (10, 25, 50, 100 and 150 µM) for 2-12 h to asses neuron damage. After incubation media was changed and neurons were incubated in fresh neuronal culture media for another 2-12 hours. After incubation, culture medium was collected into centrifuge tube and centrifuged to remove cell debris. The cells were collected into centrifuge tubes by adding 500 µl phosphate buffer saline (pH 7.4, 0.05 M) and cell homology was prepared using ultra-sonication for 30 second thrice and centrifuged at 12000 rpm for 5 min to remove cellular debris. LDH activity was estimated in the medium and cells lysate. Furthermore, in order to establish the mechanism of neuronal cell toxicity by exposure to glutamate, the cells were treated with only 100 µM of glutamate for 4 h and subsequently incubated in glutamate free medium for 20 h and subjected to TUNEL assay.

Determination of membrane integrity using lactate dehydrogenase (LDH) release assay

LDH release is largely accepted marker of cellular injury (Zitta et al., 2010). It is a stable cytosolic enzyme that is released upon cell membrane damage or cell lysis. LDH was measured using a cytotoxic detection kit according to manufacturer's instructions in the medium and cell homology. Results are presented as percentage of LDH released into the medium compared to total LDH (medium + cells).

TUNEL assay

Briefly, culture medium was removed and cells were washed twice with HBBS and added 100µl of terminal deoxynucleotidyl transferase (TdT) equilibration buffer into each well, incubated at 37°C for 30 mins and then removed the TdT equilibration buffer from wells. After addition of 60µl of (TdT) labelling reaction mixture into each well plate was incubated at 37°C for 1hr. Neuronal cells were washed with 100 µl of 1x tris buffer saline (TBS) and added 100 µl of Hoechst 33258 dye (1µg/ml). The stained cells were observed under fluorescent microscope using blue filter and total numbers of TdT-positive cells were counted. The results are expressed as ratio of TdT-positive to TdT-negative cell numbers.

Effect of drugs on neuronal cells

The drugs used for the study procured from Indian market were as follows: Neurotol (Mannitol 10% + Glycerin 10% with excipient RD011 1.5%) and Neurotol M (mannitol 20%) of Venus Remedies Limited, Panchkula, India, Kratol (mannitol 10% +glycerin 10%) of Parenteral Drugs India, Indore, and Zemisol (mannitol 10% +glycerin 10%) of Claris Life Sciences Limited, Ahmedabad. The neuronal cells were incubated in absence (control) or presence of 100 µM of glutamate alone or in combination with 10-250 µM of each of the drugs, Neurotol, Neurotol M, Kratol and Zemisol for 4 hours. After incubation, cells were washed twice with HBBS and then re-incubated in culture media lacking any drugs for an additional 20 hrs. At last, cultured neurons were harvested and LDH activity in the medium and cells was estimated. The neuronal cells exposed to glutamate along with drugs were also subjected to TUNEL assay.

Enzyme-linked immunosorbent assay (ELISA) for MAP-2

To study the effect of glutamate and drugs on neuritic degeneration, we performed ELISA for MAP2 protein expression. MAP2 protein expression study was performed in cell homology using the kit following the manufacturer instructions. For MAP2 expression study, neuronal cells were seeded in poly-L-lysine coated 96-well plates (1 × 104 cells/well) and treated with glutamate and drugs as for LDH assay. After treatment, culture medium was removed and added 0.3 ml phosphate buffer saline (pH7.4, 0.05 M) and cell

homology was prepared as for LDH assay. Values are shown as mean ± SD of 3 experiments. Results are presented as percentage of MAP2 in control compared to treatment.

Statistical analysis

Results are expressed as mean ± SD values. Statistical evaluations were carried out using one way analysis of variance (ANOVA) followed by tuke'kramer multiple comparison between control and treatments groups. A value of * P<0.05; ** P<0.01; *** P<0.001, compared to controls was considered significant.

Results

Effect of glutamate

To determine whether glutamate induces neuronal cell toxicity, we measured LDH release into the media following 2-12 h glutamate exposure. At low glutamate concentrations (10 µM), glutamate-induced cell toxicity was not significant (P>0.05), however, cells treated with high concentrations of glutamate (25 to 150 µM) caused a concentration dependent neurotoxicity as evident by significant (P<0.05 to P<0.001) increase in LDH activity up to 100 µM of glutamate exposure and further increasing in concentration had no further significant increase in LDH release. When we compared the time needed for glutamate to induce toxicity, we found that up to 2 hrs of glutamate exposure, no toxicity was observed whereas neuronal cells treated for 4 hrs caused a significant increase in glutamate induced toxicity (Figure 1), further increasing the incubation time did not increase the toxicity as evidenced by LDH release. TUNEL assay revealed that at low concentration of glutamate (10 µM), TUNEL positive apoptotic nuclei were not observed, however, neuronal cells treated with higher concentrations of glutamate (25 to 150 µM), showed a significant increase in number of TUNEL positive apoptotic nuclei. Interestingly, 6-7 fold increase in the number of apoptotic nuclei (in comparison to control) at 100 µM concentration of glutamate was noted (Figure 2). These results confirm that neuronal cell toxicity by exposure to glutamate involves an apoptotic process.

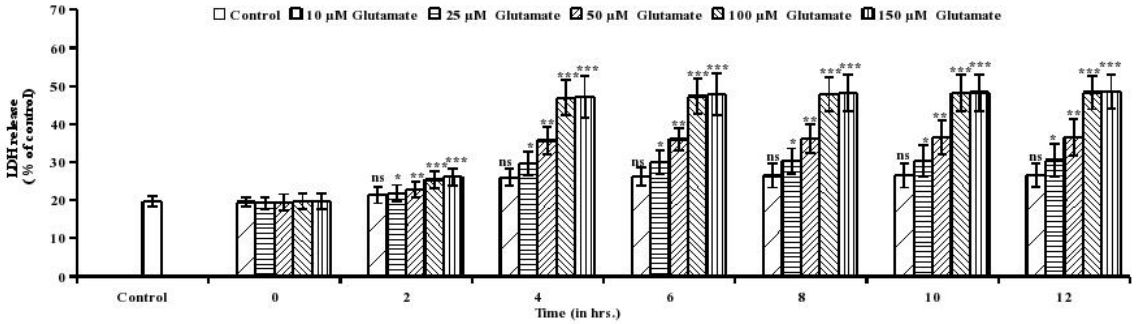

Figure 1: Release of LDH activity in neuronal cell after exposure to various concentrations of glutamate. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

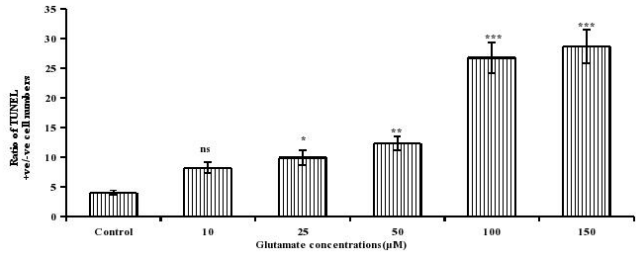

Figure 2: Formation of apoptotic nuclei in neuronal cells after 4 hours exposure to glutamate. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

Comparison of effect of drugs on glutamate induced neuronal damage

To determine the effective concentrations of drugs in neuroprotection, the neuronal cultures were exposed to 100 µM of glutamate alone (positive control) and in combination with various concentration of drugs to assess the effect of each drug on neuroptotection while being damaged at the same time. The LDH assay showed that exposure of neuronal cells with 100 µM glutamate resulted in 170.2 % increase in the LDH activity in comparison to control group (without treatment). Among drugs, 100 µM of Neurotol fully significantly prevented the neuronal cells from glutamate toxicity (P<0.001) indicating Neurotol is fully effective in neuronal cell protection. Contrary to this, Neurotol M, Kratol and Zemisol failed to significantly prevent the increase in LDH activity even though the concentration of respective comparator drugs were raised upto 250 µM. However, results indicate that 200 µM concentration of each of Neurotol M, Kratol and Zemisol is effective in neuroprotection to some extent (P<0.01) (Figure 3). These results were also confirmed by TUNEL assay where the ratio of TUNEL-positive and TUNEL-negative cell numbers were very less in Neurotol treated group (7.0 %) whereas it was very high in the groups treated with Neurotol M (76.5%) followed by Zemisol (73.7%) and Kratol (68.6%) (Figure 4).

In addition, neuroprotective effect of these drugs was also investigated for recovery after gluatamate induced damage by first exposing to 100 µM glutamate for 4 hrs for the damage, washed twice with HBBS and treated with drugs, Neurotol, Neurotol M, Kratol and Zemisol for 4 hrs and subsequently incubated for 24 hrs in drug free media subsequently LDH release was measured at 3, 6, 9,12, 15, 18, 21 and 24 hrs to check recovery.

As expected recovery of neuronal cell after glutamate toxicity was time dependent. Treatment of neuronal cells with Neurotol showed a significant neuroprotective activity as evident by decreasing LDH release when compared with glutamate induced neuronal cells. The significant neuroprotective activity of Neurotol started from 6 h (P<0.05) and reached to highly significant level at 18 h (P<0.001). Whereas other comparator drugs (Neurotol M, Kratol and Zemisol) showed significant (P<0.05) neuroprotective effect at 18 hrs in comparision to control (Figure 5).

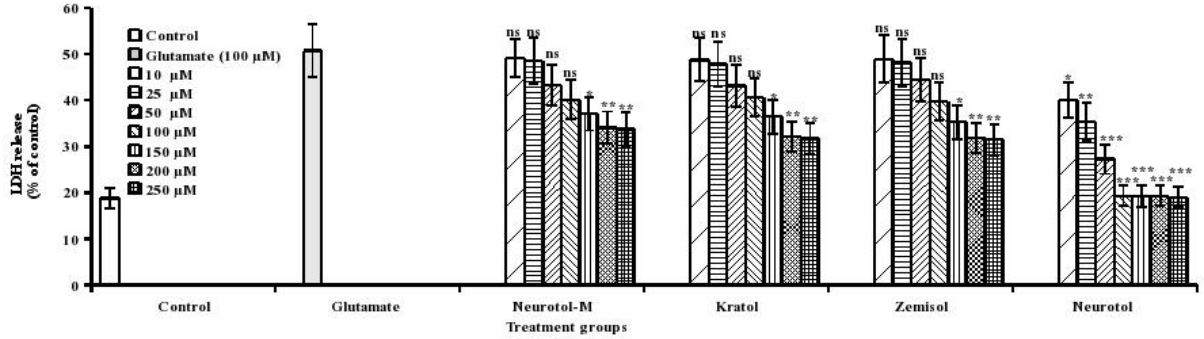

Figure 3: Optimization of the effective concentrations of drugs on neuroprotection. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

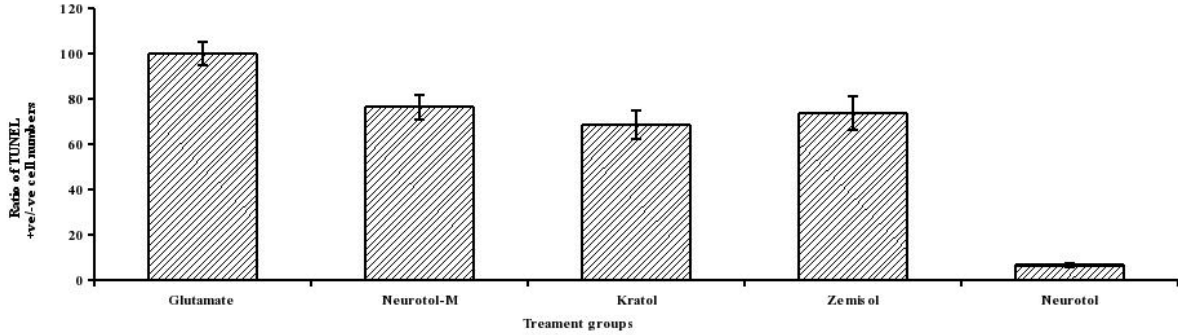

Figure 4: TUNEL assay in neuronal cells exposed to drugs. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

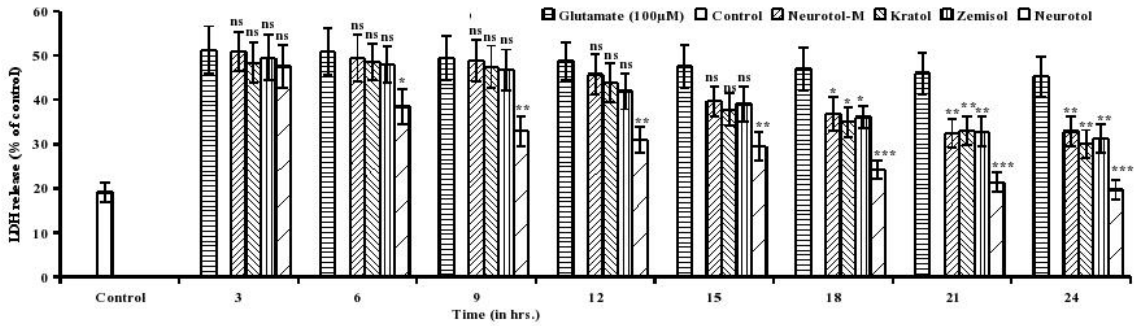

Figure 5: The comparative neuroprotective effects of drugs. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

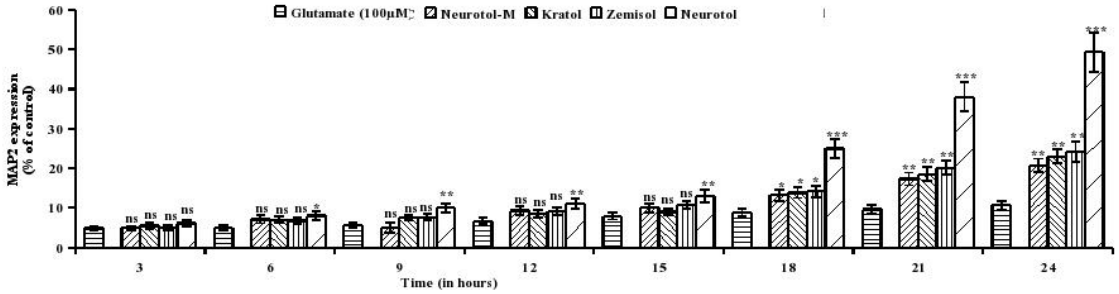

Figure 6: Comparative effect of drugs on MAP-2 expression. Level of Significance -* P<0.05; ** P<0.01; *** P<0.001, compared to controls.

Comparison of effect of drugs on MAP2 expression after glutamate exposure

Next we investigated, neuritic degeneration following exposure to glutamates (10-150 µM). Compared with untreated cells, MAP2 expression is down regulated following treatment of glutamate. At low concentration of glutamate (10 µM) there was no change in MAP2 expression. Contrary to this, when cells were treated with higher concentrations (25-150 µM) they exhibited a significant down regulation in MAP2 expression and similar to LDH release, high down regulation in MAP2 expression was observed with 100 µM glutamate (data not shown). When various drugs were compared for their involvement in regulation of MAP2 expression, Neurotol was found to significantly up regulate the expression of MAP2, after 6 h of treatment (P<0.05) and was continued upto 24 hrs which was

comparable with normal cells at 24 hrs. However, cells treated with Neurotol M, Kratol and Zemisol failed to provide early up regulation of MAP2 expression and significant up regulation was observed only after ≥18 h ($P<0.05$). These results suggest that with Neurotol treatment neuronal cells could recover from glutamate-induced toxicity and show proper functioning after ≥ 6h compared to other drugs which did not improve significant in MAP2 expression hence neuronal cells failed to show proper functioning even after 18 h (Figure 6).

Discussion

Glutamate induced neurotoxicity is believed to play an important role in neuronal cell death in many neurodegenerative diseases, including Alzheimer's disease (Lau and Tymianski 2010; Ye et al., 2013). Among these, oxidative pathway is a major source of glutamate-induced cell damage in vitro (Gliyazova et al., 2013). The present results of glutamate induced toxicity substantiated the previous reports regarding role of apoptosis in neuronal damage (Lee et al., 2012; Sundaram et al., 2012).

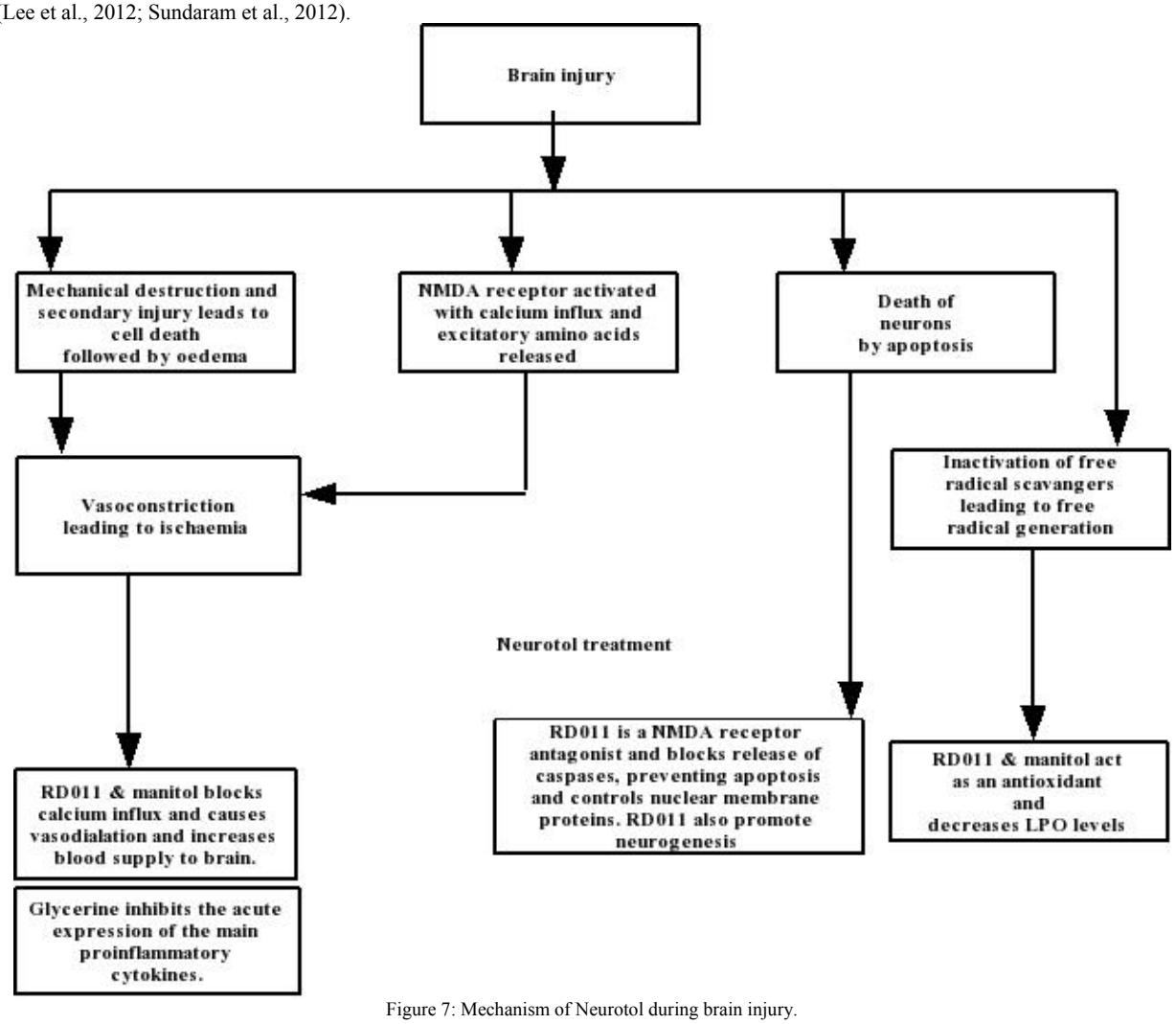

Figure 7: Mechanism of Neurotol during brain injury.

We observed that treatment of hippocampal neuronal cells after glutamate induced injury and during glutamate exposure with 100 µM of Neurotol prevented the apoptosis suggesting that Neurotol exerts its neuroprotective effect through its anti-apoptotic property. The Bcl-2 family of proteins includes both pro-apoptotic and anti-apoptotic members and relative balance of each other strongly influences fate of cells (Liu et al., 2012). It is possible that RD011 an adjuvant/ excipient in Neurotol catalyzes neuronal survival, either by increasing expression of Bcl-2 or by decreasing BAX, BAD and BNIP. Furthermore, our in house laboratory investigation concluded that RD011 probably acts by array of mechanisms viz. blocking of NMDA and voltage gated Ca2+ channels and inhibition of presynaptic exitatory neurotransmitter (data under publication).

It has been demonstrated that increasing toxicity of neuronal cell is associated with MAP2 expression (White et al., 2011). Our results revealed that Neurotol in glutamate-induced cell death significantly increased MAP-2 expression, suggesting that the regulation of MAP-2 is involved in the biological mechanism of the effect of Neurotol on neural recovery. Recent research findings suggested that glutamate toxicity in vivo may not be mediated entirely by glutamate receptor and certain other mechanisms may also be involved in it (Dutta and Trapp 2011). It has recently been confirmed that HT-22 hippocampal cell line is killed by glutamate via the oxidative pathway, as exposure of glutamate causes a decrease in glutathione levels and a concomitant increase in H_2O_2 levels which leads to neuronal death (Gliyazova et al., 2013).

The present report shows that Neurotol (mannitol 10% +

glycerin 10% with RD011) at 100 µM significantly abolish the increased LDH level and inhibit neuronal death caused by glutamate within 6 hrs. In vivo study has also proved the efficacy of Neurotol compared to individual glycerol and mannitol. Earlier, Soni et al. (2009) compared the effect of glycerol, mannitol and Neurotol on Albino rat ischemic brain injury and concluded that Neurotol is more effective than mannitol and/or glycerol alone. Our results hypothesize that neuroprotective effect of Neurotol is through its ability to act as an antioxidant, thus removing dangerous free radicals from the neurons. Our results suggest that administration of Neurotol during or after the brain injury may prevent sequels and can be a safer option compared to comparator drugs. The detailed mechanism of Neurotol during brain injury is depicted in Figure 7.

Conclusions

The present investigation of Neurotol (Mannitol 10% + Glycerin 10% with 1.5% RD011) on in vitro efficacy in glutamate induced neuronal toxicity confirmed that the Neurotol exerts neuroprotection by combined mechanisms of osmotic activity, free radical scavenging potential and NMDA and calcium channel blockage. Therefore, Neurotol may become a potential therapy candidate in brain disorders or damage.

Acknowledgement

This work was supported by Venus Medicine Research Centre,Werne, Germany.

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