A neuroprotective role of TRPM2 channel blocker and ROCK2 inhibitors to curtail epileptogenesis in an experimental model of traumatic brain injury.

Shammy Chandel[1], Apinderpreet Singh [1], Rohit Rajput[2], Alka Bhatia[1], S.K. Gupta[2], Parampreet Kharbanda[1], Amit Kumar[1], Ajay Prakash[1], and Bikash Medhi[1]

[1]Postgraduate Institute of Medical Education & Research
[2]Post Graduate Institute of Medical Education and Research

April 16, 2024

Abstract

Background and Purpose: Post-traumatic epilepsy is the confirmed progression of unprovoked seizures after head trauma in the general population. This study was designed to control the devastating chronic consequences of post-traumatic epileptogenesis. Experimental Approach: Cerebral trauma was experimentally induced in Wistar rats to challenge the seizure susceptibility for futuristic PTE. The efficiency of Flufenamic acid, and fasudil hydrochloride, were assessed and compared with valproic acid for abnormal neurobehavioural symptoms, biomolecular levels, and apoptotic changes through histopathology. Key Results: Initial brain insult was successfully exacerbated the normal pathophysiology and neurobehavior changes in an experimental model. The lowered seizure threshold confirmed the epileptogenesis progression when a jab of Pentylenetetrazol(35mg/kg), gave the first successive seizure. Disrupted BBB permeability, neuronal infarction, and brain edema were found restored to normal after the treatment when compared to disease controls. The altered levels of neurotransmitters, mitochondrial complexes, cytoplasmic biomolecules/cytokines, different genes, and channel proteins were also restored to normal after 14-days treatments. No seizures have been observed with the test dose of Pentylenetetrazol. We analyzed the histopathological changes of hippocampus/cortex regions and found a significant decrease in the count of non-evident nucleoli, nuclear pyknosis, vascular congestion, and perineural vacuolization in treatment groups compared to injury controls. Conclusion and Implications: All the drugs greatly minimized the epileptogenesis cascade of futuristic PTE, confirms their neuroprotective capabilities. Flufenamic acid was found more effective with many potential abilities to minimize epileptogenesis compared to fasudil hydrochloride but its combination with valproic acid has much neuroprotection and efficacy.

INTRODUCTION

Traumatic brain injury is a global health concern. It is an unexpected diversified condition for most enervative consequences like post-traumatic epilepsy. TBI is the foremost reason for death worldwide and disability in the youths, sportspersons, and army veterans. It has been estimated that nearly 69 million population worldwide get brain insult annually(James et al., 2019; Maas et al., 2008) but in India, road traffic injuries cost 60% of total TBI cases(Gururaj, 2008; Murray & Lopez, 1996). Brain trauma in the general population, reports 20% of symptomatic epilepsy and 5% of all epilepsy(Immonen et al., 2019). Primary brain insult confirms brain hemorrhage, an increase in intracranial pressure, and cellular swelling which results in brain edema and blood-brain barrier damage. The cerebral bruises and tissue damage, complicate the secondary cascade by exacerbating neuronal inflammation and mitochondrial ETC dysfunction(Chen et al., 2021). Activated immune cells of inflamed sites initiate other catastrophic mechanisms by overproduction of proteases, ROS/RNS, and NF-$\varkappa\beta$ that interfere with the expressions of different inflammatory markers and pro-inflammatory cytokines(Shao et al., 2021). This pathophysiology counted for the progression of

the epileptogenesis cascade which lowers down the seizure threshold of the injured brain and gives the first post-traumatic successive seizure(Pitkänen et al., 2007).

Fluid Percussion Injury and Cortical Compact Injury studies were performed on mice models of brain trauma and they have detected increased seizure susceptibility for sub convulsive doses of pentylenetetrazole (i.e. non-convulsant at 35mg/kg)(Mukherjee et al., 2013). Disturbed Na^+ and Ca^{2+} influx through Transient Receptor Potential Melastatin-2 (TRPM2) channels prop up membrane depolarization, induce production of prostaglandins, disrupt the mitochondrial and endoplasmic reticulum functions which further led to enhance the intracellular Ca^{2+} through TRPM2 channels(Perraud et al., 2001). Rho signaling pathway was found highly activated in brain injuries due to inflammation and injury in the neuronal cytoskeleton(Brabeck et al., 2004). Inflammatory markers like TNF-α and glutamate also contribute to early cell death following TBI, by activating Rho kinases i.e. Rho-associated Protein Kinase (ROCK2)(Neumann et al., 2002). An experimental rat model of weight drop injury produces a diffuse type of injury by mimicking clinical complication and its complex pathophysiology provide post-traumatic complications(Chandel et al., 2016; Ye Xiong et al., 2013).

FDA-approved anti-seizure therapies include Valproic Acid, carbamazepine, lamotrigine, phenytoin but we still lack anti-epileptogenic therapy(Romoli et al., 2018). It was investigated that valproic acid influences rat hippocampus for the levels of glutamate and GABA transporter proteins during epileptogenesis(Ueda & Willmore, 2000). Flufenamic Acid belongs to the fenamate class of Non-Steroidal Anti-Inflammatory Drug, COX enzyme inhibition, and TRPM2 channel blocker which was found neuroprotective in *in-vitro*studies(Khansari & Coyne, 2012). Fasudil Hydrochloride is a selective ROCK2 inhibitor that induces neuroprotection *in-vitro* and also a specific inhibitor of NF-ϰB and protects against axonal degeneration and neuronal apoptosis(Fujimura et al., 2011; Xiao et al., 2014). The altered TBI pathophysiology is described in figure-1.

So, this study hypothesized that the recruitment of Ca^{2+} antagonists, TRPM2 channel blockers, and ROCK2 inhibitors might be effective for the initiation of neuroprotective responses after initial brain insult to stop or minimize the activated TBI associated epileptogenesis consequences. Hence this study was aimed to explore the *in-vivo* effects, efficacy, and potential of flufenamic acid and fasudil hydrochloride for the treatment of TBI induced epileptogenesis in experimental weight drop injury model of TBI.

MATERIAL AND METHODS

Adult Wistar rats were procured from Institutional Advanced Small Animal Facility PGIMER, Chandigarh, India. The necessary approvals were collected before experimental studies. All the experimental animals were accommodated in the groups of 2/3 under standard polypropylene cages under controlled hygienic environmental conditions and had proper access to food and water*ad libitum* at temperature 23 ± 4^{o}C under a 12-hour light-dark cycle throughout the experiment.

Experimental Design and Statistical Analysis:

TBI Model Standardization by Marmarou's weight-drop method: TBI instrument was designed with essential modifications to stabilize the actual energy impact for model standardization which included 6 subgroups of Wistar rats (150-450gm). The standardization was done by calculating the difference in the impact of injury concerning the adjustments of height and the weight falls. The impact of freefalling brass weights was correlated with by physical formula of Impact force and the reading of the impactometer. The rats were anesthetized with*ketamine* and *xylazine* (i.e. 100mg/kg and 10mg/kg) before inducing the diffuse type injury by Marmarou's weight drop method on Day0. The injury progression and neurobehavioural tests were assessed on Day0, Day1, Day3, Day7, and Day14. The treatment experiments were continued with the selected group having a lower seizure threshold and less mortality after TBI.

Epileptogenesis confirmation for Post-traumatic epilepsy: After following the TBI procedure of Marmarou's weight-drop method, rats were administrated with a sub-convulsive dose of pentylenetetrazole, 35mg/kg after 2 weeks of surgery i.e. on Day14. The rats were assessed for 2 hours for seizure detection in a plexiglass chamber(Racine et al., 1972). The effect of Post-traumatic complications was assessed for molecular changes in the rat hippocampus and final results were correlated with histological changes.

Evaluation of neuroprotective effects of Flufenamic Acid, Fasudil Hydrochloride and Valproic Acid in TBI induced Epileptogenesis model: Four treatment groups have been completed with the administration of Fasudil Hydrochloride (FH), Flufenamic Acid (FFA), Valproic Acid (VPA), and Valproic Acid + Flufenamic Acid (FFA). FH was dissolved and diluted in normal saline before use and was administered by i.p. route of 10 mg/kg. FFA was dissolved in CMC and was administered by the oral route as 20mg/kg after standardization to injured rats. VPA was dissolved and diluted in normal saline before use and was administered by i.p. route as 350 mg/kg animals. Forth treatment group has been designed with the combination of a half dose of VPA and the half dose of more effective treatment between FH and FFA. All the drugs were administered to TBI-induced rats after 6 hours of the injury and once every 24 hours for 14 Days. An equal volume of vehicle was administered in control and sham animals i.e. normal saline. No drugs have been given to TBI and EPLT group. All the groups were examined for seizure scoring with sub convulsive dose of PTZ i.e. 35mg/kg after Day14 for 2 hours except control and sham groups. At the end of the animal experiment, the rats were sacrificed under ether anesthesia by decapitation procedure. The brains were washed by saline perfusion method before collection and stored in appropriate storage conditions till molecular experiments.

Experimental Parameters Analysis:

Assessment of Animal's Weight: Difference in weight was assessed for the abnormal metabolism after injury. And the comparison of the differences has been made to analyze the weight loss in injury controls and weight maintained in treatment groups.

Neurological Severity Scale Testing: A 28-point Neuroscore test was performed for each animal in this study, which ranging from simple observation to traversing a horizontal bar, involve in general condition, reflexes, behavior, motor tests, and grip strength [Stanford Behavioral and Functional Neuroscience Laboratory, Version 4.0]

Rotarod Test: Rotarod tests were employed for muscle coordination with a latency time of 120 sec.

S**eizure confirmation of Epileptogenesis:** Modified Racine scale was used for the assessment of neurobehavioral progression of seizures and monitored up to 2 hours following PTZ (35mg/kg). The maximum stage of seizure activity was analyzed in each 5-minute interval. Diazepam 4 mg/kg, i.p. was given an intraperitoneal route to terminate seizures if convulsions last for more than 2 hours.

2,3,5-Triphenyltetrazolium Chloride Staining: Whole-brain tissue viabilities and lesion size were estimated by 2,3,5- triphenyl tetrazolium chloride (TCC) staining. The excised brains were sliced into coronal sections of 2mm thickness and were incubated in a 2% TTC solution for 20-30min at 37°C.

Blood-Brain Barrier Permeability Estimation: A 2% (4 mL/kg i.p. of body weight) solution of Evans Blue in normal saline was injected and was measured by spectrophotometer (Shimadzu Japan) at 610nm. The brain tissue was quantified according to a standard curve and results were presented as ng/100mg(Manaenko et al., 2011).

Brain Edema Estimation: Whole-brain water content was measured with the wet-dry method for quantifying brain edema. The brains were weighed immediately after sacrifice the animals to yield wet weight. The tissues were placed for drying in a desiccating oven for 48 hours at 70°C and reweighed after 24 and 48 hours to calculate the weight loss. The %age of lost water content in the tissues was calculated by the average readings.

Biochemical Estimations : Biochemical imbalance alters the pathophysiology of TBI in the brain and such conditions open the doors for treatment check and development of antioxidant strategies to optimize brain insult i.e. tissue used was hippocampus and cortex.

Protein Assay: To estimate protein content in brain homogenates, the Biuret method was used in which bovine serum albumin was used as standard(GORNALL et al., 1949).

Acetylcholinesterase (AChE) activity: AChE activity has been determined by photometric method for cell suspensions, homogenates, and tissue extracts(Abass, 2014).

Catalase Activity: The activity of catalase was measured by the Luck, 1963 method.

Malondialdehyde (MDA): Thiobarbituric acid-reactive substances were measured to evaluated tissue LPO levels. MDA is a product of LPO that gives red light absorbance at 535nm after reacting with thiobarbituric acid.

Super Oxide Dismutase (SOD): The activity of SOD was estimated by the Kono *et al.* method which was developed in 1978.

Reduced Glutathione Assay (GSH): Jollow *et al.,* in 1974 used DTNB as a substrate to developed yellow color and estimated reduced glutathione levels immediately at 412 nm as µmol GSH/g tissue.**Enzyme-Linked Immunosorbent Assay Method (** ELISA) was proceeded in biological samples (i.e. brain tissue i.e. hippocampus, and cortex & blood serum) and analyzed over LISA Plus Microplate Reader in the form of concentration (pg/mL). The ELISA kits were procured from Diaclone and QAYEE-BIO.**Gene Expression by Real-Time PCR:** Brain tissues (especially hippocampus) were subjected to isolate total RNA from all experimental groups. The positive control of housekeeping gene β-actin was run. Different genes were standardized as per definite melting temperatures. The RT-PCR reaction conditions were set at 95°C for 15 min, 40 cycles of 95°C for 15 sec, and 61°C for 30sec. The termination step was set at 72°C for 30 sec. A sensitive and quantitative technique Real-time Polymerase chain reaction (i.e. TaqMan real-time QRT-PCR Applied Biosystem StepOnePlus, ThermoFisher) was used to simultaneously measure relative mRNA expression i.e. relative standard curve and comparative CT value of different biomarkers in the rat hippocampus.**Histopathological Estimation:** A 4% cold paraformaldehyde and PBS was perfused transcardially in experimental rats before their brains were removed and immersed in paraformaldehyde for 2–3 Days. Sagittal sections were selected after cut to study the hippocampus and cortex part in embedded paraffin blocks which were mounted and used for Hematoxylin and eosin staining H&E(10µm) and immunohistochemistry(5µm). Each group was observed under an optical microscope (OPUS) at magnifications of 20X & 40X and results have been shown @100µm scale. The scoring of apoptotic neurons in H&E staining was done based on Nuclear Pyknosis & Non-evident Nucleoli (NP&NN), Perineural Vacuolization (PV), Vascular Congestion (VC), and Brain Edema (BE)(Mena et al., 2004; Schmued et al., 1997; Schmued & Hopkins, 2000). All the primary antibodies were procured from Sigma-Merck and were diluted to the manufacturer's protocol to optimized again for the perfect dilutions. The following primary Abs were used having sensitivity for Rat: a rabbit polyclonal anti-glial fibrillary acidic protein GFAP(Sigma-Aldrich Cat# G4546, RRID:AB_1840895) for astrocytes, a mouse monoclonal anti–ionized calcium-binding adaptor molecule-1, Iba1 (Sigma-Aldrich Cat# SAB2702364, RRID:AB_2820253) for microglia, a rabbit polyclonal anti-ROCK2(Sigma-Aldrich Cat# HPA007459, RRID:AB_1079828) for axonal marker protein, and a rabbit polyclonal anti-TRPM2(Sigma-Aldrich Cat# SAB2103193, RRID:AB_10665621) for neuronal receptor protein. For enzymatic detection (HRP or AP secondary conjugates) anti-goat anti-rat IgG H&L (Alexa Fluor® 488) was used. The scoring of neurons in IHC was done based on apoptotic and necrotic neurons. Examination Scale includes Negative (-), Weak (+), Moderate (++), and Strong (+++)(Moriyama et al., 1997; Seeger et al., 2003).

Statistical Analysis: Results of this study were expressed as mean +- SD. Intergroup difference was estimated by one-way analysis of variance, ANOVA test which was followed by Post hoc Bonferroni and Post hoc Dunnett test. The data for biochemical, histology and molecular studies were analyzed with appropriate or suitable parametric tests. The $p<0.05$ was considered significant. Graph-Pad Prism 9 software was used to analyze the records.

RESULTS

The rat model development has been confirmed when it mimicked the clinical pathophysiology of futuristic PTE in Wistar rats (200-250 grams). The brass weight of 500 grams was dropped over the exposed skull from the height of 1.4 meters. This impact generated 0.70 joules of force which gives a moderate type of primary injury in the head. The initial brain insult exacerbated the epileptogenesis cascade in the form of

secondary injury by lowering the seizure threshold in the Wistar rats and they became susceptible to the first successive seizure with a sub-convulsive dose of PTZ(35mg/kg).

Evaluation of neuroprotective effects of different treatments in TBI induced Epileptogenesis in rats:

Model standardizing studies confirmed the altered neurobehavioral and brain complications results in futuristic PTE. Fasudil Hydrochloride (FH, 10mg/kg i.p.), Flufenamic Acid (FFA, 20mg/kg oral), valproic acid (VPA, 350mg/kg i.p.), and Valproic Acid + Flufenamic Acid (VFA, 175mg/kg i.p. + 10mg/kg oral) treatment groups were compared against disease and normal healthy controls to check the efficacy in TBI progression. TBI was induced into 6 treatment groups in which 2 were TBI control, EPLT group, and 4 were selected for treatment studies. One control and one surgery group i.e. Sham were also considered for comparisons to conclude the surgery effect. All the groups were analyzed for bodyweight difference, neurological severity score, and motor coordination on Day0, Day1, Day3, Day7, and Day14 before proceeding towards molecular estimation.

Animal Body Weight Difference: The weight severity profile was observed followed by the head injury. The line graph of weight difference on Day3, Day7, and Day14 showed a significant difference in weight difference (grams). All treatment groups i.e. FH, FFA, VPA, and VFA were found to be significantly effective to maintain the rat weight compared to disease controls i.e. TBI/EPLT groups on Day7. There was no significant difference observed between intra-treatment groups on Day7 (Figure-2).

Neurological Severity Scale Estimation: The NSS observed in the VFA group was more significant to restoring the neurological score on Day3, Day7, and Day14 when compared to the TBI and EPLT group. The other treatment groups showed a significant increase in NSS scores on Day3, Day7, and Day14 when compared to disease controls (Figure-2).

Rotarod Test for Motor Co-ordination: The latency to fall (in seconds) was found to be significant for all treatment groups when compared to disease controls on Day7 and Day14 (Figure-2).

Seizure Score assessment after Brain Trauma: A sub-convulsive dose of PTZ(35mg/kg) was injected on day14 and injured rats were observed for seizure scoring for 2hours. Increased susceptibility for seizures was confirmed in the EPLT group with a low seizure threshold. EPLT group showed behavioral arrests, complex partial, dileptic, and tonic-colonic seizures. But no seizures were observed in any treatment groups (Table in Figure-2).

Brain Infarction by TTC Staining: The infarction was visible in the striatum and cortex leaving the hippocampus. Control and Sham group observed deep red in staining with normal histology with no infarction in the brain. TBI and EPLT groups were showed hypoperfusion significantly in the area of infarct (white pale color) when compared to normal controls. FH, FFA, VFA treatment groups showed a significant difference to the disease control in red staining i.e. less hypoperfusion and rich red stain when compared to TBI and EPLT groups. But VPA significantly shows nearly red staining of injured brain tissue with very less area of infarct (Figure-3).

Evan's Blue concentration: Evan's blue (2%, 4mL/kg i.p.) was administered in rats and an abrupt increase of Evan's blue was seen in TBI and EPLT groups. But the Evans concentration in FH, FFA, VPA, and VFA groups was found significantly less compared to injury controls. VPA alone and in combination with FFA was found very effective as compared to FH and FFA alone (Figure-3).

Brain Edema difference: Surgically removed rat brains were weighed just after the sacrifice and after 24 and 48 hours when placed in an incubator at 70°C. The brain edema in all the treatment groups showed no significant differences when compared to disease controls after 24 hours but there is a significant difference after 48 hours i.e. the value of water content in the treatment groups was found less as compared to the TBI/EPLT groups (Figure-3).

Oxidative Stress Parameters:

A biochemical assay was used to detect, quantify the activity of biological molecules in the brain tissue samples homogenate (Figure-4).

1. **Acetylcholinesterase:** Acetylcholinesterase activity was found to be significantly decreased in TBI ($0.38x10^{-4}$+-$0.046x10^{-4}$) and EPLT ($0.32x10^{-4}$+-$0.026x10^{-4}$) groups when compared to control ($0.98x10^{-4}$+-$0.166x10^{-4}$) and sham ($1.05x10^{-4}$+-$0.112x10^{-4}$) group. The treatment group include VPA alone ($1.02x10^{-4}$+-$0.217x10^{-4}$) and in combination with FFA were ($1.02x10^{-4}$+-$0.316x10^{-4}$) significantly increased the acetylcholinesterase activity as compared to injury groups. The FH & FFA groups were found significant i.e. ($0.72x10^{-4}$+-$0.217x10^{-4}$) & ($0.82x10^{-4}$+-$0.217x10^{-4}$) compared to disease groups.
2. **Catalase:** Catalase enzyme activity was assayed by measuring the degradation rate of H_2O_2. The levels were measured in U/g and the catalase enzyme activity was found to be significantly increased in the injured brain i.e. TBI (0.261 +- 0.04) and EPLT (0.264 +- 0.05) groups as compared to Control (0.127 +- 0.02) and Sham (0.133 +- 0.03) group. The treatment group include FH (0.154 +- 0.05), FFA (0.125 +- 0.04), VPA (0.137 +- 0.05) and VFA (0.124 +- 0.02) significantly restored the level of Lipid peroxidation as compared to disease controls.
3. **Lipid peroxidase:**Malondialdehyde (MDA, a by-product of LPO) helps in the measurement of free radical levels (nmols/g) which were significantly increased in the injured brain i.e. TBI (0.62 +- 0.2) and EPLT (0.66 +- 0.24) groups as compared to Control (0.76 +- 0.25) and Sham (0.71 +- 0.15) group. The treatment group includes FH (0.68 +- 0.18), FFA (0.72 +-0.27), VPA (0.78 +- 0.38), and VFA (0.73 +- 0.27) significantly restored the level of Lipid peroxidase as compared to disease controls.
4. **Superoxide dismutase:**The levels were significantly increased in the injured brain i.e. TBI (1.24 +- 0.15) and EPLT (1.27 +- 0.13) groups when compared to Control (0.89 +- 0.18) and Sham (0.91 +- 0.17) group. The treatment group includes FH (1.00 +- 0.18), FFA (0.95 +- 0.12), VPA (0.97 +- 0.17) and VFA (00.98 +- 0.21) significantly restored the level of reduced glutathione as compared to disease controls.
5. **Reduced Glutathione Assay:** The levels of reduced glutathione were found to be significantly decreased in the injured brain i.e. TBI (3.67 +- 1.5) and EPLT (3.32 +- 1.75) groups when compared to Control (12.5 +- 2) and Sham (12.3 +- 1.4) group. The treatment group includes FH (8.33 +- 1.4), FFA (8.83 +- 1.3), VPA (10.5 +- 2.26), and VFA (10.5 +- 1.6) significantly restored the level of reduced glutathione as compared to disease controls.

Inflammatory Markers Estimation:

1. **Dopamine:** Brain injury significantly increased the Dopamine levels in brain tissue of disease control [i.e. TBI (25.91 +- 1.08), EPLT (26.46 +- 0.87) groups as compared to normal controls [i.e. Control (19.95 +- 1.99), Sham (20.29 +- 1.45). The treatment groups showed a significant decrease in the levels of NSE when compared to disease controls. The decrease in the concentration of NSE treatment groups was calculated as [i.e. FH (20.95 +- 1.01), FFA (20.4 +- 0.93), VPA (20.29 +- 1), VFA (20.2 +- 1.3)]. The levels of Dopamine were significantly restored by the treatments applied.
2. **Mitochondrial complex-I:** Brain injury damaged the ETC machinery of mitochondria which results in apoptosis of the neurons. Brain levels for Mitochondrial complex-I were markedly found to be significantly increased in injury treated [i.e. TBI (6.77 +- 0.47), EPLT (6.73 +- 0.45) groups as compared to normal controls [i.e. Control (4.33 +- 0.69), Sham (4.46 +- 0.73). The decrease in the concentration of treatment groups was calculated as [i.e. FH (5.40 +- 0.55), FFA (4.99 +- 0.72), VPA (4.78 +- 0.49), VFA (4.8 +- 0.48)] which significantly restored the levels compared to injury groups.
3. **Neuron-Specific Enolase:** Brain and serum levels for NSE were markedly found to be significantly increased in injury treated [i.e. brain tissue: TBI (22.33 +- 2.64), EPLT (22.44 +- 3.2) and blood serum: TBI (10.4 +- 0.8) EPLT (10.8 +- 1.2)] groups as compared to normal controls [i.e. brain tissue: Control (14.58 +- 1.04), Sham (15.25 +- 1.37) and blood serum: Control (7.5 +- 1.2), sham (7.7 +- 0.6)]. But the treatment groups were shown a significant decrease in the levels of NSE [i.e. brain tissue: FH (16.8 +- 2.3), FFA (16 +- 2.04), VPA (15.8 +- 2.3), VFA (16.13 +- 1.98), and blood serum: FH (8.7 +- 1.01), FFA (8.3 +- 1.01), VPA (7.8 +- 0.9), VFA (8.2+- 1.1)] when compared to disease controls.

4. **Nerve Growth Factor-Beta:** Brain levels for Nerve Growth Factor-Beta (NGF-b) were markedly found to be significantly increased in injury treated [i.e. TBI (94.9 +- 6.1), EPLT (96.9 +- 6.8) groups as compared to normal controls [i.e. Control (52.1 +- 5.1), Sham (53.47 +- 6.2). But the decrease in the NGF-b concentration in treatment groups was calculated as significant [i.e. FH (77.58 +- 8.2), FFA (68.56 +- 7.7), VPA (66.1 +- 7.6), VFA (60.43 +- 8.6)] when compared to disease controls.
5. **Ubiquitin Carboxyl-terminal Hydrolase Isozyme L1:** Brain and serum levels for UCHL-1 were markedly found to be significantly decreased in injury treated [i.e. brain tissue: TBI (7.58 +- 0.92), EPLT (7.28 +- 1.13) and blood serum: TBI (4 +- 0.78) EPLT (3.96 +- 0.46)] groups as compared to normal controls [i.e. brain tissue: Control (11.92 +- 1.29), Sham (12.18 +- 1.4) and blood serum: Control (6.9 +- 0.71), sham (6.45 +- 0.7)]. The increase in the concentration of UCHL-1 has been found significant in treatment groups [i.e. brain tissue: FH (9.11 +- 0.75), FFA (9.46 +- 1.10) and blood serum: FH (4.69 +- 0.36), FFA (5.19 +- 0.47)]. The concentration values to standard drug therapy VPA, and its combination with FFA [i.e. brain tissue VPA (10.91 +- 0.52), VFA (11.31 +- 1.18) and blood serum VPA (5.61 +- 0.5), VFA (5.79 +- 0.6)] was found more significantly increased when compared to disease controls.
6. **Interleukin-1beta:** Brain and serum levels for IL-1β were markedly found to be significantly increased in injury treated [i.e. brain tissue: TBI (29.117 ± 2.34), EPLT (29.517 ± 3.69) and blood serum: TBI (11.23 ± 1.4) EPLT (11.83 ± 1.5)] groups as compared to normal controls [i.e. brain tissue: Control (9.97 ± 1.9), Sham (10.47 ± 1.46) and blood serum: Control (5.72 ± 1.11), sham (5.87 ± 1.36)]. The decrease in the concentration of IL-1β treatment groups [i.e. brain tissue: FH (14.95 ± 3.13), FFA (12.122 ± 3.08) and blood serum: FH (7.4 ± 1.11), FFA (6.6 ± 0.97)] was found less when compared the concentration values to standard drug therapy VPA, and its combination with FFA [i.e. brain tissue VPA (12.017 ± 1.8), VFA (11.27± 1.61) and blood serum VPA (6.33 ± 0.9), VFA (5.98 ± 0.68)]. The overall treatment groups were shown a significant decrease in the levels of IL-1β when compared to disease controls.
7. **Interleukin-10:** Blood serum levels for IL-10 were markedly found to be significantly increased in injury treated [i.e. TBI (279.1 ± 4.3), EPLT (289.5 ± 9.6) groups as compared to normal controls [i.e. Control (200.41 ± 9.1), Sham (210.83 ± 11.2)]. The treatment groups were shown a significant decrease in the levels of IL-10 [i.e. FH (232.08 ± 20.4), FFA (226.25 ± 16.1), VPA (221.6 ± 21.7), VFA (221.43 ± 15.9)] when compared to disease controls.
8. **Tumor necrosis factor-alpha:** Brain and serum levels for TNF-α were markedly found to be significantly increased in injury treated groups [i.e. brain tissue: TBI (63.6 ± 5.8), EPLT (63.9 ± 4.8) and blood serum: TBI (17.8 ± 1.87) EPLT (18.13 ± 2.6)] as compared to normal controls [i.e. brain tissue: Control (26.2 ± 6), Sham (35.2 ± 6) and blood serum: Control (13.5 ± 3.71), sham (14 ± 3.22)]. The decrease in the concentration of TNF-α treatment groups [i.e. brain tissue: FH (38.13 ± 3.12), FFA (34.91 ± 6) and blood serum: FH (15.81 ± 3.45), FFA (14.94 ± 1.6)] was found less when compared the concentration values to standard drug therapy VPA, and its combination with FFA [i.e. brain tissue VPA (36.22 ± 3.8), VFA (36.1± 4.4) and blood serum VPA (15.43 ± 2.87), VFA (14.66 ± 2.6)]. But the overall treatment groups were shown a significant decrease in the levels of TNF-α when compared to disease controls.

Relative mRNA Expression: The relative mRNA expression of pro-inflammatory/inflammatory genes has been assessed for overall change (Figure-6).

1. **Cyclooxygenase-2:** The overall fold change in COX-2 expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham. A significant decrease in the expression of the COX-2 gene has been found in injury treatment groups i.e. FH, FFA, VPA, and VFA. The overall change in expression was similar in all treatment groups.
2. **Heme Oxygenase-2:** The overall fold change in HO-2 expression was found to be significantly less in TBI and EPLT groups when compared to Control and Sham. But a significant increase in the expression of the HO-2 gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA.
3. **Nuclear factor (erythroid-derived 2)-like 2:** The overall fold change in Nrf-2 expression was

found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the Nrf-2 gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA.

4. **Nuclear Factor-:** The overall fold change in NF-β expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. And a significant decrease in the expression of the NF-β gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA.
5. **Ιντερλευκιν-1β:** The overall fold change in IL-1β expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham. A significant decrease in the expression of the IL-1β gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. FH drug was found less effective than FFA, VPA, and VFA in treatment groups while compared to disease controls.
6. **Interleukin-6:** The overall fold change in IL-6 expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the IL-6 gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The overall change in expression was similar in all treatment groups but FFA efficacy was found less compared to FH, VPA, and VFA.
7. **Interleukin-10:** The overall fold change in IL-10 expression was found to be significantly increased in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the IL-10 gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The VPA was found more effective to reduce the change in expression in all treatment groups.
8. **Tumor necrosis factor-alpha:** The overall fold change in TNF-α expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the TNF-α gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The overall change in expression was similar in all treatment groups.
9. **Protein Kinase-B:** The overall fold change in Akt expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the Akt gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The overall change in expression was similar in all treatment groups but combination therapy VFA was less effective than other treatments.
10. **Phosphatase and tensin homolog:** The overall fold change in PTEN expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the PTEN gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The change in expression was high in the combination VFA group in all treatment groups.
11. **Phosphatidylinositol 3-kinase:** The overall fold change in PI-3k expression was found to be significantly high in TBI and EPLT groups when compared to Control and Sham groups. A significant decrease in the expression of the PI-3k gene has been found in disease treatment groups i.e. FH, FFA, VPA, and VFA. The overall change in expression was similar in all treatment groups FH was found less effective.

Hematoxylin-Eosin Staining for Neuronal Injury Scoring: It has been found that the morphology of neurons was intact and good in the case of the Control and Sham groups. They exhibited normal size, shape and were arranged compactly with a prominent nucleus i.e. no apoptosis was observed. The disease groups i.e. TBI and EPLT showed diffuse neuronal injury with features such as rarefaction of neuropil, eosinophilic cytoplasm, shrunken and pyknotic nuclei in hippocampus and cortex region i.e. moderate and severe apoptosis was observed. In the case of treatment groups, we have seen fewer apoptotic neurons as compared to disease controls. The scoring system revealed mild apoptosis in the case of FH, FFA, and VFA but no proper distinguished apoptotic neurons were found in the VPA group (Figure-7).

Along with it, no significant histopathological changes have been found in vital organs i.e. liver and kidneys of injury controls when compared to treatment groups.

Protein Expressions by Immunohistochemistry method:

1. **GFAP, Astroglial Marker protein:** In this protocol, the injured brain sections were incubated with anti-GFAP Antibody (1:100). The injured brain significantly expressed up-regulated GFAP in the injured hippocampus and cortex. It has been observed that the treatment therapy applied i.e. FH, FFA, VPA, and VFA significantly reduced the GFAP protein expression in astroglial cells in the Cerebral Cortex and hippocampus. The standard drug treatment along with the combination of a standard with FFA was more effective in the hippocampus when compared to FH and FFA. Moreover, all the treatment effect was noticeable in reducing the expression of GFAP in cortex region (Figure 8).
2. **Iba-1, Microglial Marker protein:** The injured brain sections were incubated with anti-Iba-1 Antibody (1:100). Iba-1 is predominantly expressed in the injured brain and is significantly found to be up-regulated in brain-injured tissues especially the hippocampus and cortex. It has been observed that the treatment therapy applied i.e. FH, FFA, VPA, and VFA significantly reduced the Iba-1 protein expression in microglial cells of the Cerebral Cortex and hippocampus. VFA combination was found less effective when compared to FH, FFA, and VPA in both the regions likewise FH in the cortex (Figure 8).
3. **ROCK2, Axonal Marker protein:** In this IHC procedure, the injured brain sections were incubated with anti-ROCK2 Antibody (1:200). ROCK2 is predominantly expressed in the injured brain i.e. hippocampus and cortex and this also was found to be significantly up-regulated. It has been observed that the treatment therapy applied i.e. FH, FFA, VPA, and VFA significantly reduced the ROCK2 protein expression in axon cells of the hippocampus and cortex. FH, FFA, and VFA combinations were found less effective when compared to VPA in Cerebral Cortex likewise FH and FFA combinations in the cortex (Figure 8).
4. **TRPM2, Neuronal Receptor protein:** The **s** ections were incubated with anti-TRPM2 Antibody (1:100). TRPM2 was predominantly expressed in the injured brain and was found to be significantly up-regulated in brain-injured tissues. It has been observed that the treatment therapy applied i.e. FH, FFA, VPA, and VFA significantly reduced the TRPM2 protein expression in the neurons of the hippocampus and cortex. FH, FFA, and VFA combinations were found less effective when compared to VPA in Cerebral Cortex likewise FH combination in the cortex (Figure-8).

DISCUSSION AND CONCLUSION

Progression periods of head injury studies showed a difference in brain physiology and a decrease in body weight(Aadal et al., 2015). The neurological abnormalities confirmed the disturbed brain pathophysiology in injury controls, as reported in previous studies(Umschwief et al., 2010; Villasana et al., 2014). This study also confirmed the weight loss, abnormal grip strength, poor motor coordination, disturbed beam-walk, and abnormal routine functions in injury groups due to abnormal brain conditions. PTZ challenge confirms the progression of epileptogenesis and animals were reported for behavioral arrests, complex partial dileptic, and tonic-clonic seizures. Brain edema induces ICP due to excessive accumulation of fluid in intra/extracellular spaces i.e. due to ruptured blood vessels and damaged neurons(Shiozaki et al., 2005). In this study, the treatments applied significantly restored the body weight, neurological abnormalities, and no seizures were confirmed after 2 weeks for PTZ challenge in treatment groups. FH and FFA significantly reduced the brain edema volume but VPA and VFA combination were more effective to restore the neurophysiological and behavioral abnormalities.

Post-injury BBB disruption abruptly increases Evan's blue penetration to parenchyma(Rákos et al., 2007) and VPA is reported to restore BBB in transient focal brain ischemia rat model(Xuan et al., 2012). We detected a very less amount of dye in the brain homogenates after treatment. Especially in VPA and VFA has significantly more impact to restore the BBB. TTC staining confirms tissue viability i.e. less stained area means, more number of infarct cells(Bederson et al., 1986). Infarction was significantly visible with a high number of infarct cells in the striatum hippocampus and cerebral cortex area in injury controls. But treatments reduced the apoptotic neuronal count i.e. less hypoperfusion and a rich red stain. Secondary insult of CNS exacerbates the free radical's formation i.e. ROS/RNS in the brain, resulting in oxidative stress(Lipton, 1999). These activities alter neurotransmitters and enzyme levels in the brain. AChE activity was reported acutely elevated in the brain of ischemic/blast injury but lowered AChE activity was seen in the

neo-cortex of TBI patients with chronic cognitive symptoms(Östberg et al., 2011). There was a significant decrease in the levels of AChE after a head injury but the treatments significantly elevated the AChE levels in the hippocampus and cerebral cortex. Increased catalase activity has also been restored with treatment drugs. The injured brain degrades LPO into aldehydic malondialdehyde(Ostergard et al., 2016). But the increased level significantly came to normal with our treatments. When SOD enzyme concentration was calculated, significant elevation due to injury was estimated which has been reduced by the treatments applied. Antioxidants such as GSH which acts as an intracellular buffer for ROS and get increased in injury conditions were also significantly restored after treatments.

CNS inflammation due to dopamine metabolism was altered at 28-Days post-injury along with microglial activation(Van Bregt et al., 2012). But altered levels of dopamine were significantly recorded stable after the treatment applied confirmed lesser loss of neurons in the injured brains. Mitochondrial dysfunction enhances ROS production, brain apoptosis, and elevated levels of Mitochondrial complex-I in injury(Sullivan et al., 2005; Y. Xiong et al., 1997). The present study has found that the treatment groups significantly decrease the levels of Mitochondrial complex-I when compared to injury controls. NSE plays a crucial role in erythrocytes, neuronal cell glycolysis(Chabok et al., 2012). We have found the increased NSE levels have been significantly restored by treatment to maintain the normal functional amount of enzyme in the blood and brain. It has been reported, hypoxia and brain insult induce local up-regulation of NGF-β(Kossmann et al., 1996). But a significant decrease in the levels of NGF-β in the brain was recorded in this study. Previous literature showed robust and significant elevation of UCHL-1 in the acute phase which is the main marker for Parkinson's Disease and over the Day7 of the study period when compared the serum and CSF levels in TBI patients(Mondello et al., 2012). But a significant decrease in the levels of UCHL-1 in the brain and serum was assessed after 2 weeks in injury controls which significantly elevated after the treatment. This treatment may help in PD pathophysiology. Our findings have found the overlapping insults with brain damage by pro-/inflammatory cytokines upregulation. CSF and serum concentrations showed IL-10 elevation in severe trauma patients up to Day22 to 6 months(Csuka et al., 1999). The present study confirms blood serum levels for IL-10 significantly increased after 2 weeks in injury controls which got a significant decrease after treatment applied. TNF-α was reported elevated in Marmarou's model of hypoxic injury(Yan et al., 2011). But this study significantly confirmed elevated TNF-α levels in injury controls and was significant reduced after treatment. The functional deficits result in disturbed COX-2 levels following diffuse TBI(Cernak et al., 2002). HO-2 has been found elevated in the adult rodent brain(Ewing and Maines, 1997). Nrf2 was found able to regulate HO-1 via the phosphorylated PI3K/Akt/GSK3β pathway(Singh et al., 2017). But the overall fold change in HO-2 gene expression was significantly less in injury groups which get increased in treatment groups. But COX-2 and Nrf-2 expression was found to be significantly high in injury groups and treatment restored it to normal. NF-kβ activation may potentially involve long-term inflammation following TBI(Nonaka et al., 1999). Increased IL-1β expression after the primary insult exacerbates epileptogenicity(Semple et al., 2017). The significantly elevated overall fold change in NF-β and IL-1β expression in injury groups were decreased after treatment. IL-6 was also reported elevated in mTBI(Goodman et al., 2011) and our study and it was significantly decreased by the treatment applied. Initial brain insult increased IL-10 overproduction by resident microglia(D'Mello et al., 2009). But the overall fold change in IL-10 expression was found significantly low in injury controls which got significantly elevated in disease treatment groups. TNF-α was found elevated in our experiment and also reported elevated in the FPI injury model(Knoblach et al., 1999) but the overall fold change in TNF-α expression was found to be significantly decreased in treatment groups. Glial cells and hippocampal neurons also involved in post-traumatic dementia and neuroinflammation by releasing TNF-α and IL-1β via PI3K/AKT/NF-κβ signaling pathway(Zhao et al., 2014). The phosphorylation of Protein Kinase-B was found to increase in rat hippocampus at Day1 after the initial blast and last for at least 6 weeks(Wang et al., 2017). Phosphatase and tensin homolog expression was found upregulated after TBI(Ding et al., 2013). In the present study, we have found the overall fold change in PK-B-Akt/ PTEN and PI-3k expression was found to be significantly high in TBI and EPLT groups which were recorded significantly decreasing in treatment groups. It confirmed the neuroprotection abilities of these drugs in dementia comorbidities also.

H&E is the further insight into necrosis and apoptosis and our study confirms the morphology of neurons in normal controls was normal size and intact shape with prominent nucleus like the old literature reported(Isaksson et al., 2001). But in the case of injury controls, DAI was seen with neutrophil rarefaction, eosinophilic cytoplasm, shrunken and pyknotic nuclei in the hippocampus and cerebral cortex. Our treatment altered the apoptotic scoring by reducing the nuclear pyknosis, karyolysis, and nuclear lacking cellular structures. We haven't found proper distinguished apoptotic neurons in the VPA group. The present study investigated the widespread heterogeneous distribution of different neuronal proteins. GFAP was found up-regulated when CNS insult was followed by reactive gliosis(Schiff et al., 2012). Iba-1 confirmed the activated microglial expression after TBI up to 2-3 weeks(Neri et al., 2018). The ROCK2 is an axonal protein expressed in axonal retraction balls and intermittent swellings from 6hours to 1-week post-injury(Zhang et al., 2016). Many studies reported elevated TRPM2 expression following experimental trauma in rats at Day3-5 post-trauma, especially in DG neurons(Cook et al., 2010). In our injury controls, GFAP and Iba-1 predominately over-expressed and significantly up-regulated in the hippocampus and cortex region. ROCK2 and TRPM2 are also predominantly over-expressed and significantly up-regulated. But the treatment therapy significantly reduced the protein over-expression in astroglial, microglial, and axon cells of the hippocampus and cerebral cortex. VPA and VFA were found much effective treatments to minimize the apoptotic neurons.

We also checked the toxicity profile in vital organs of all animals and observed the location, cell size and boundary area of organ tissues were intact and at the exact position in all the groups i.e. no major significant changes were observed.

The present study demonstrated that the fasudil hydrochloride(10mg/kg, i.p.) acts as a potent Rho-kinases inhibitor that stopped the post-injury neuronal apoptosis and flufenamic acid(20mg/kg, oral) with anti-inflammatory properties can modulate the inflammatory environment of post-traumatic epileptogenesis. We confirmed the neuroprotective nature of these drugs for holding and minimizing the epileptogenesis progression (Figure-9). VPA alone was observed much efficient compared to a combination of FFA in molecular and histopathological findings and the FFA was observed potentially more neuroprotective compared to FH. Our study was limited to cover few pathways of epileptogenesis but the complexity of this condition needs more studies on regulatory mechanisms of intracellular signaling molecules during epileptogenesis progression for PTE.

SUMMARY:

What is already known:

Post-traumatic epilepsy is the most provoking consequence after head trauma with no specific treatment measure.

Epileptogenesis is key term sequelae of altered brain pathophysiology after brain injury for futuristic PTE.

What this study adds

Potential novel targets have been selected for protecting external cytoskeleton and internal cytoplasmic infrastructure.

These proposed targets were tried to perform normal functions with specific potential drug regimens after TBI.

Clinical significance

Fasudil hydrochloride and Flufenamic acid have a significant role to minimize cerebral edema and inflammation.

The combination groups with Valproic acid have been found more effective to minimize epileptogenesis in futuristic PTE.

BIBLIOGRAPHY

Aadal, L., Mortensen, J., & Nielsen, J. F. (2015). Weight reduction after severe brain injury: A challenge during the rehabilitation course.*Journal of Neuroscience Nursing* . https://doi.org/10.1097/JNN.0000000000000121

Abass, K. S. (2014). A method for fast assessment of OP/CB exposure in the Japanese quail (Coturnix coturnix japonica) Using combined esterases enzyme activity as biomarkers. *Enzyme Research* . https://doi.org/10.1155/2014/812302

Bederson, J. B., Pitts, L. H., Germano, S. M., Nishimura, M. C., Davis, R. L., & Bartkowski, H. M. (1986). Evaluation of 2, 3, 5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats.*Stroke* . https://doi.org/10.1161/01.STR.17.6.1304

Brabeck, C., Beschorner, R., Conrad, S., Mittelbronn, M., Bekure, K., Meyermann, R., Schluesener, H. J., & Schwab, J. M. (2004). Lesional expression of RhoA and RhoB following traumatic brain injury in humans.*Journal of Neurotrauma* . https://doi.org/10.1089/0897715041269597

Cernak, I., O'Connor, C., & Vink, R. (2002). Inhibition of cyclooxygenase 2 by nimesulide improves cognitive outcome more than motor outcome following diffuse traumatic brain injury in rats.*Experimental Brain Research* . https://doi.org/10.1007/s00221-002-1245-z

Chabok, S. Y., Moghadam, A. D., Saneei, Z., Amlashi, F. G., Leili, E. K., & Amiri, Z. M. (2012). Neuron-specific enolase and S100BB as outcome predictors in severe diffuse axonal injury. *Journal of Trauma and Acute Care Surgery* . https://doi.org/10.1097/TA.0b013e318246887e

Chandel, S., Gupta, S. K., & Medhi, B. (2016). Epileptogenesis following experimentally induced traumatic brain injury - A systematic review. In *Reviews in the Neurosciences* . https://doi.org/10.1515/revneuro-2015-0050

Chen, W., Guo, C., Feng, H., & Chen, Y. (2021). Mitochondria: Novel Mechanisms and Therapeutic Targets for Secondary Brain Injury After Intracerebral Hemorrhage. In *Frontiers in Aging Neuroscience* . https://doi.org/10.3389/fnagi.2020.615451

Cook, N. L., Vink, R., Helps, S. C., Manavis, J., & Van Den Heuvel, C. (2010). Transient receptor potential melastatin 2 expression is increased following experimental traumatic brain injury in rats.*Journal of Molecular Neuroscience* . https://doi.org/10.1007/s12031-010-9347-8

Csuka, E., Morganti-Kossmann, M. C., Lenzlinger, P. M., Joller, H., Trentz, O., & Kossmann, T. (1999). IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury: Relationship to IL-6, TNF-α, TGF-β1 and blood-brain barrier function. *Journal of Neuroimmunology* . https://doi.org/10.1016/S0165-5728(99)00148-4

D'Mello, C., Le, T., & Swain, M. G. (2009). Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factora signaling during peripheral organ inflammation. *Journal of Neuroscience* . https://doi.org/10.1523/JNEUROSCI.3567-08.2009

Ding, J., Guo, J., Yuan, Q., Yuan, F., Chen, H., & Tian, H. (2013). Inhibition of phosphatase and tensin homolog deleted on chromosome 10 decreases rat cortical neuron injury and blood-brain barrier permeability, and improves neurological functional recovery in traumatic brain injury model. *PLoS ONE* . https://doi.org/10.1371/journal.pone.0080429

Ewing, J. F., & Maines, M. D. (1997). Histochemical localization of heme oxygenase-2 protein and mRNA expression in rat brain. *Brain Research Protocols* . https://doi.org/10.1016/S1385-299X(96)00027-X

Fujimura, M., Usuki, F., Kawamura, M., & Izumo, S. (2011). Inhibition of the Rho/ROCK pathway prevents neuronal degeneration in vitro and in vivo following methylmercury exposure. *Toxicology and Applied Pharmacology* . https://doi.org/10.1016/j.taap.2010.09.011

Goodman, M. D., Makley, A. T., Huber, N. L., Clarke, C. N., Friend, L. A. W., Schuster, R. M., Bailey, S. R., Barnes, S. L., Dorlac, W. C., Johannigman, J. A., Lentsch, A. B., & Pritts, T. A. (2011). Hypobaric hypoxia exacerbates the neuroinflammatory response to traumatic brain injury. *Journal of Surgical Research* . https://doi.org/10.1016/j.jss.2010.05.055

GORNALL, A. G., BARDAWILL, C. J., & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *The Journal of Biological Chemistry* .

Gururaj, G. (2008). Road traffic deaths, injuries and disabilities in India: Current scenario. In *National Medical Journal of India* .

Immonen, R., Harris, N. G., Wright, D., Johnston, L., Manninen, E., Smith, G., Paydar, A., Branch, C., & Grohn, O. (2019). Imaging biomarkers of epileptogenecity after traumatic brain injury – Preclinical frontiers. In *Neurobiology of Disease* . https://doi.org/10.1016/j.nbd.2018.10.008

Isaksson, J., Hillered, L., & Olsson, Y. (2001). Cognitive and histopathological outcome after weight-drop brain injury in the rat: influence of systemic administration of monoclonal antibodies to ICAM-1.*Acta Neuropathologica* , *102* (3), 246–256.

James, S. L., Theadom, A., Ellenbogen, R. G., Bannick, M. S., Montjoy-Venning, W., Lucchesi, L. R., Abbasi, N., Abdulkader, R., Abraha, H. N., Adsuar, J. C., Afarideh, M., Agrawal, S., Ahmadi, A., Ahmed, M. B., Aichour, A. N., Aichour, I., Aichour, M. T. E., Akinyemi, R. O., Akseer, N., ... Murray, C. J. L. (2019). Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology* . https://doi.org/10.1016/S1474-4422(18)30415-0

Khansari, P. S., & Coyne, L. (2012). NSAIDs in the treatment and/or prevention of neurological disorders. In *Inflammopharmacology* . https://doi.org/10.1007/s10787-011-0116-2

Knoblach, S. M., Fan, L., & Faden, A. I. (1999). Early neuronal expression of tumor necrosis factor-α after experimental brain injury contributes to neurological impairment. *Journal of Neuroimmunology* . https://doi.org/10.1016/S0165-5728(98)00273-2

Kossmann, T., Hans, V., Imhof, H. G., Trentz, O., & Morganti-Kossmann, M. C. (1996). Interleukin-6 released in human cerebrospinal fluid following traumatic brain injury may trigger nerve growth factor production in astrocytes. *Brain Research* . https://doi.org/10.1016/0006-8993(95)01501-9

Lipton, P. (1999). Ischemic cell death in brain neurons. In*Physiological Reviews* . https://doi.org/10.1152/physrev.1999.79.4.1431

Maas, A. I. R., Stocchetti, N., & Bullock, R. (2008). Moderate and severe traumatic brain injury in adults. *The Lancet Neurology* ,*7* (8), 728–741.

Maines, M. D., Eke, B. C., & Zhao, X. (1996). Corticosterone promotes increased heme oxygenase-2 protein and transcript expression in the newborn rat brain. *Brain Research* . https://doi.org/10.1016/0006-8993(96)00184-9

Manaenko, A., Chen, H., Kammer, J., Zhang, J. H., & Tang, J. (2011). Comparison Evans Blue injection routes: Intravenous versus intraperitoneal, for measurement of blood-brain barrier in a mice hemorrhage model. *Journal of Neuroscience Methods* . https://doi.org/10.1016/j.jneumeth.2010.12.013

McCoubrey, W. K., & Maines, M. D. (1994). The structure, organization and differential expression of the gene encoding rat heme oxygenase-2.*Gene* . https://doi.org/10.1016/0378-1119(94)90749-8

Mena, H., Cadavid, D., & Rushing, E. J. (2004). Human cerebral infarct: A proposed histopathologic classification based on 137 cases. *Acta Neuropathologica* . https://doi.org/10.1007/s00401-004-0918-z

Mondello, S., Linnet, A., Buki, A., Robicsek, S., Gabrielli, A., Tepas, J., Papa, L., Brophy, G. M., Tortella, F., Hayes, R. L., & Wang, K. K. (2012). Clinical utility of serum levels of ubiquitin C-terminal hydrolase as a

biomarker for severe traumatic brain injury.*Neurosurgery* . https://doi.org/10.1227/NEU.0b013e318236a809

Moriyama, M., Kumagai, S., Kawashiri, S., Kojima, K., Kakihara, K., & Yamamoto, E. (1997). Immunohistochemical study of tumour angiogenesis in oral squamous cell carcinoma. *Oral Oncology* . https://doi.org/10.1016/S1368-8375(97)00025-0

Mukherjee, S., Zeitouni, S., Cavarsan, C. F., & Shapiro, L. A. (2013). Increased seizure susceptibility in mice 30 days after fluid percussion injury. *Frontiers in Neurology* . https://doi.org/10.3389/fneur.2013.00028

Murray, C. J. L., & Lopez, A. D. (1996). Evidence-based health policy - Lessons from the global burden of disease study. In *Science* . https://doi.org/10.1126/science.274.5288.740

Neri, M., Frati, A., Turillazzi, E., Cantatore, S., Cipolloni, L., Di Paolo, M., Frati, P., La Russa, R., Maiese, A., Scopetti, M., Santurro, A., Sessa, F., Zamparese, R., & Fineschi, V. (2018). Immunohistochemical Evaluation of Aquaporin-4 and its Correlation with CD68, IBA-1, HIF-1α, GFAP, and CD15 Expressions in Fatal Traumatic Brain Injury. *International Journal of Molecular Sciences* . https://doi.org/10.3390/ijms19113544

Neumann, H., Schweigreiter, R., Yamashita, T., Rosenkranz, K., Wekerle, H., & Barde, Y. A. (2002). Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a Rho-dependent mechanism. *Journal of Neuroscience* .

Nonaka, M., Chen, X. H., Pierce, J. E. S., Leoni, M. J., McIntosh, T. K., Wolf, J. A., & Smith, D. H. (1999). Prolonged activation of NF-ϰB following traumatic brain injury in rats. *Journal of Neurotrauma* . https://doi.org/10.1089/neu.1999.16.1023

Östberg, A., Virta, J., Rinne, J. O., Oikonen, V., Luoto, P., Någren, K., Arponen, E., & Tenovuo, O. (2011). Cholinergic dysfunction after traumatic brain injury: Preliminary findings from a PET study.*Neurology* . https://doi.org/10.1212/WNL.0b013e318211c1c4

Ostergard, T., Sweet, J., Kusyk, D., Herring, E., & Miller, J. (2016). Animal models of post-traumatic epilepsy. In *Journal of Neuroscience Methods* . https://doi.org/10.1016/j.jneumeth.2016.03.023

Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P., & Scharenberg, A. M. (2001). ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology.*Nature* . https://doi.org/10.1038/35079100

Pitkänen, A., Kharatishvili, I., Karhunen, H., Lukasiuk, K., Immonen, R., Nairismägi, J., Gröhn, O., & Nissinen, J. (2007). Epileptogenesis in experimental models. *Epilepsia* , *48* (s2), 13–20.

Racine, R., Okujava, V., & Chipashvili, S. (1972). Modification of seizure activity by electrical stimulation: III. Mechanisms.*Electroencephalography and Clinical Neurophysiology* . https://doi.org/10.1016/0013-4694(72)90178-2

Rákos, G., Kis, Z., Nagy, D., Lür, G., Farkas, T., Hortobágyi, T., Vécsei, L., & Toldi, J. (2007). Evans Blue fluorescence permits the rapid visualization of non-intact cells in the perilesional rim of cold-injured rat brain. *Acta Neurobiologiae Experimentalis* ,*67* (2), 149–154.

Romoli, M., Mazzocchetti, P., D'Alonzo, R., Siliquini, S., Rinaldi, V. E., Verrotti, A., Calabresi, P., & Costa, C. (2018). Valproic acid and epilepsy: from molecular mechanisms to clinical evidences. *Current Neuropharmacology* . https://doi.org/10.2174/1570159x17666181227165722

Schiff, L., Hadker, N., Weiser, S., & Rausch, C. (2012). A Literature Review of the Feasibility of Glial Fibrillary Acidic Protein as a Biomarker for Stroke and Traumatic Brain Injury. *Molecular Diagnosis & Therapy* . https://doi.org/10.1007/bf03256432

Schmued, L. C., Albertson, C., & Slikker, W. (1997). Fluoro-Jade: A novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Research* . https://doi.org/10.1016/S0006-8993(96)01387-X

Schmued, L. C., & Hopkins, K. J. (2000). Fluoro-Jade B: A high affinity fluorescent marker for the localization of neuronal degeneration.*Brain Research* . https://doi.org/10.1016/S0006-8993(00)02513-0

Seeger, T. F., Bartlett, B., Coskran, T. M., Culp, J. S., James, L. C., Krull, D. L., Lanfear, J., Ryan, A. M., Schmidt, C. J., Strick, C. A., Varghese, A. H., Williams, R. D., Wylie, P. G., & Menniti, F. S. (2003). Immunohistochemical localization of PDE10A in the rat brain.*Brain Research* . https://doi.org/10.1016/S0006-8993(03)02754-9

Semple, B. D., O'Brien, T. J., Gimlin, K., Wright, D. K., Kim, S. E., Casillas-Espinosa, P. M., Webster, K. M., Petrou, S., & Noble-Haeusslein, L. J. (2017). Interleukin-1 receptor in seizure susceptibility after traumatic injury to the pediatric brain.*Journal of Neuroscience* . https://doi.org/10.1523/JNEUROSCI.0982-17.2017

Shao, R., Sun, D., Hu, Y., & Cui, D. (2021). White matter injury in the neonatal hypoxic-ischemic brain and potential therapies targeting microglia. In *Journal of Neuroscience Research* . https://doi.org/10.1002/jnr.24761

Shiozaki, T., Hayakata, T., Tasaki, O., Hosotubo, H., Fuijita, K., Mouri, T., Tajima, G., Kajino, K., Nakae, H., Tanaka, H., Shimazu, T., & Sugimoto, H. (2005). Cerebrospinal fluid concentrations of anti-inflammatory mediators in early-phase severe traumatic brain injury. *Shock* . https://doi.org/10.1097/01.shk.0000161385.62758.24

Singh, A. K., Kashyap, M. P., Tripathi, V. K., Singh, S., Garg, G., & Rizvi, S. I. (2017). Neuroprotection Through Rapamycin-Induced Activation of Autophagy and PI3K/Akt1/mTOR/CREB Signaling Against Amyloid-β-Induced Oxidative Stress, Synaptic/Neurotransmission Dysfunction, and Neurodegeneration in Adult Rats. *Molecular Neurobiology* . https://doi.org/10.1007/s12035-016-0129-3

Sullivan, P. G., Rabchevsky, A. G., Waldmeier, P. C., & Springer, J. E. (2005). Mitochondrial permeability transition in CNS trauma: Cause or effect of neuronal cell death? *Journal of Neuroscience Research* . https://doi.org/10.1002/jnr.20292

Ueda, Y., & Willmore, L. J. (2000). Molecular regulation of glutamate and GABA transporter proteins by valproic acid in rat hippocampus during epileptogenesis. *Experimental Brain Research* . https://doi.org/10.1007/s002210000443

Umschwief, G., Na'ama, A. S., Alexandrovich, A. G., Trembovler, V., Horowitz, M., & Shohami, E. (2010). Heat acclimation provides sustained improvement in functional recovery and attenuates apoptosis after traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism* , *30* (3), 616–627.

Van Bregt, D. R., Thomas, T. C., Hinzman, J. M., Cao, T., Liu, M., Bing, G., Gerhardt, G. A., Pauly, J. R., & Lifshitz, J. (2012). Substantia nigra vulnerability after a single moderate diffuse brain injury in the rat. *Experimental Neurology* . https://doi.org/10.1016/j.expneurol.2011.12.003

Villasana, L. E., Westbrook, G. L., & Schnell, E. (2014). Neurologic impairment following closed head injury predicts post-traumatic neurogenesis. *Experimental Neurology* . https://doi.org/10.1016/j.expneurol.2014.05.016

Wang, Y., Sawyer, T. W., Tse, Y. C., Fan, C., Hennes, G., Barnes, J., Josey, T., Weiss, T., Nelson, P., & Wong, T. P. (2017). Primary blast-induced changes in Akt and GSK3β phosphorylation in rat hippocampus. *Frontiers in Neurology* . https://doi.org/10.3389/fneur.2017.00413

Xiao, W. D., Yu, A. X., & Liu, D. L. (2014). Fasudil hydrochloride could promote axonal growth through inhibiting the activity of ROCK.*International Journal of Clinical and Experimental Pathology* .

Xiong, Y., Gu, Q., Peterson, P. L., Muizelaar, J. P., & Lee, C. P. (1997). Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. *Journal of Neurotrauma* . https://doi.org/10.1089/neu.1997.14.23

Xiong, Ye, Mahmood, A., & Chopp, M. (2013). Animal models of traumatic brain injury. In *Nature Reviews Neuroscience* . https://doi.org/10.1038/nrn3407

Xuan, A., Long, D., Li, J., Ji, W., Hong, L., Zhang, M., & Zhang, W. (2012). Neuroprotective effects of valproic acid following transient global ischemia in rats. *Life Sciences* . https://doi.org/10.1016/j.lfs.2012.01.001

Yan, E. B., Hellewell, S. C., Bellander, B. M., Agyapomaa, D. A., & Morganti-Kossmann, M. C. (2011). Post-traumatic hypoxia exacerbates neurological deficit, neuroinflammation and cerebral metabolism in rats with diffuse traumatic brain injury. *Journal of Neuroinflammation* . https://doi.org/10.1186/1742-2094-8-147

Zhang, P., Zhu, S., Li, Y., Zhao, M., Liu, M., Gao, J., Ding, S., & Li, J. (2016). Quantitative proteomics analysis to identify diffuse axonal injury biomarkers in rats using iTRAQ coupled LC-MS/MS. In *Journal of Proteomics* . https://doi.org/10.1016/j.jprot.2015.12.014

Zhao, M., Zhou, A., Xu, L., & Zhang, X. (2014). The role of TLR4-mediated PTEN/PI3K/AKT/NF-κB signaling pathway in neuroinflammation in hippocampal neurons. *Neuroscience* . https://doi.org/10.1016/j.neuroscience.2014.03.039

Hosted file

`table, figure and ligends.docx` available at https://authorea.com/users/731846/articles/710607-a-neuroprotective-role-of-trpm2-channel-blocker-and-rock2-inhibitors-to-curtail-epileptogenesis-in-an-experimental-model-of-traumatic-brain-injury