The Neuroprotective Approach of Amide Analogue of Kynurenic Acid at Mid-Early Stages of Traumatic Brain Injury: A pre-clinical study in mice

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ABSTRACT

Background: Traumatic brain injury (TBI) is a major cause for fatality and disability across the world. Primary injury represents immediate mechanical damage which is non-reversible and non-treatable whereas secondary injury is the delayed consequences of primary injury and life-long disability that can be therapeutically influenced. After many efforts, little success has been achieved toward the evolution of efficacious pharmacological invention to lessen the devastating effects of TBI. The neuroprotective approaches of Kynurenic acid (an endogenous metabolite of tryptophan metabolic pathway) have been found in an experimental model of migraine, Alzheimer's, and Huntington's disease. Therefore, In the present study, we evaluated the potential of Kynurenic acid amide analogue (KAA) at 72 h in delayed secondary consequences of TBI in mice. **Materials and methods:** The injury was used swiss albino mice (25-30 g) and analyzed biochemical parameters (Oxidative stress, BBB permeability, and edema formation, mitochondrial dysfunction), and Histopathological studies. **Results:** Our data showed significant rise in oxidative stress, % water content and BBB permeability at 72 h of injury. Meanwhile, cortical neuronal cell death was also evident at 72 h of injury. KAA administration (100, 200, 400, mg/kg, ip) to animals (swiss albino mice; 25-30 g) administered with KAA after 30 minutes of TBI showed a significant and dose dependent neuroprotective effect on oxidative stress, edema and BBB permeability. KAA also showed improvement in neuronal survival and neurological functions significantly. **Conclusion:** Overall, our data shows that KAA depicted neuroprotection against TBI-induced secondary cascades and improves neurological damage in the mice model of TBI.

Keywords: Oxidative stress, BBB permeability, mitochondrial dysfunction, neurological dysfunctions, edema, Kynurenic acid analogue

Traumatic brain injury (TBI) is one of the major health concerns in today's world. TBI happens by an outside mechanical impact which results in the disruption of brain tissue following various delayed secondary complications leads to wide neuronal damage and degeneration (Gaetz, 2004). It is a heterogenous medical issue, can be varying according to the type of injury, location of damage and mechanism of neuronal damage (Albert-Weissenberger and siren, 2010). As per the present scenario, TBI can be understood in two ways i.e. primary and secondary damage. Primary injury is irreversible brain cell damage, can be focal or diffuse. The moderate to severe primary injury leads to various secondary cascades i.e. oxidative stress, cerebral edema, changes in BBB permeability, inflammation, mitochondrial dysfunction, DNA fragmentation etc. It is reversible in nature which can be treated with suitable surgical methods and intensive therapeutic interventions (Khatri et al., 2021). TBI is the leading cause of fatality and disability across individual worldwide. It is recorded that near 69 million people injured every year, consists 55.9 million mild cases whereas 54.8 million moderate to severe cases (Dewan et al., 2018).

Various studies have been conducted to prevent secondary neurodegenerative cascades and fasten recovery after TBI. In spite of these rigorous efforts, an efficacious neuroprotective intervention to stop secondary consequences cannot be evolved. In this study, we investigated the therapeutic efficacy of amide analogue of Kynurenic acid. As per the previous studies, Kynurenic acid (KYNA) was found to be effective in various neurodegenerative disorders like Alzheimer's, Parkinson's and Huntington's disease (Ostapiuk and Urbanska, 2022). Due to the physicochemical and pharmacokinetic nature, there will be in vivo use of KYNA is troublesome. KYNA is not sufficiently crosses the blood brain barrier (BBB) and its solubility is a massive issue in high doses. It is rapidly removed from the body by organic anion transporters (Fukui et al., 1991; Bahn et al., 2005). To beaten these hurdles, various derivatives of KYNA have been prepared chemically. In these, some of the derivatives acts like prodrugs and others imitate the action of KYNA in brain (Fulop et al., 2009). In the present study, derivative is amide analogue i.e. N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, was proven effective by suppressing in vitro field excitatory post-synaptic potential (fEPSPs) in the CA1 region of hippocampus. It was also found inhibiting the rearing in acute open field test (Nagy et al., 2011). It was also found neuroprotective molecule in various neurodegenerative disorders such as trigeminal activation (Knyihar-Csilik et al., 2008), Migrain (Vamos et al., 2010), epilepsy (Marosi et al., 2010) and Huntington's disease (Zadori et al., 2011). The mechanism of action of this amide derivative is still under discussion. The two major probabilities are there; 1. Either the complete structure is imitate the actions of KYNA; 2. Or it acts as prodrug analogue which first convert into KYNA and then execute the actions of KYNA.

For the lighting of this concept, a pharmacokinetic study was executes till date (Zadori et al., 2011a). In that study, the serum level of KYNA and amide analogue i.e. N-(2-N, N-

dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride were estimated using HPLC. The study showed that a rapid increase in serum concentration and then abrupt fall in first hour. Meantime, the concentration of KYNA increases from the basal level but not to the considerable level. Therefore it can be conducted that analogue has good serum stability and only small amount is being converted to its parent compound i.e. KYNA.

Methodology

Animals

Swiss albino mice (25-30 g), of either sex were taken for the present study. They were acquired from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India after the approval of the Institutional Animal Ethics Committee (IAEC) of Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India. Animals were kept in groups of five in polypropylene cages (29×22×14 cm) lined with proper bedding. They were conserved under standard conditions (natural light and dark cycle; temperature 25±2˚C, Humidity 60-65%) and had free access to standard rodent feed and water *ad libitum.*

Experimental TBI model: One of the non-penetrating or impact acceleration models of traumatic brain injury was used in the present study in which the animal's head has been fixed. Weight drop model uses the gravitational forces of free-falling weight on the exposed skull of the animal. The grade of injury i.e. mild, moderate, severe and ultra severe can be developed by changing the height and weight of impact. Here, the moderate injury was produced in mice with the help of the following parts of model: **Metallic pipe**: (Length: 1 m; Diameter: 2.7 cm); **Metallic disc**: (Thickness: 3 mm; diameter: 1 cm); **Metallic spherical weight** (Weight: 70 g; diameter: 2.5 cm) (Foda and Marmarou, 1994; Marmarou et al., 1994; Chown et al., 2010).

Induction of traumatic brain injury:

Mice were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.), put down on a sponge pad and a small longitudinal midline incision was specified over the scalp of mice, a metallic disc was centrally fixed on the exposed skull and mouse was placed appropriately under the metallic pipe. Then the metallic spherical weight was freely fall through the metallic pipe over the skull of mice. Then, the metallic disc was detached and closed the incision of scalp immediately. Finally, antibacterial (Neosporin) powder (GlaxoSmithKline Pharmaceuticals Ltd., Bangalore, India) was spread over the site of surgery and then mice were returned to their home cage for recovery. Sham group gone through all surgical procedures, except the impact (Marmarou et al., 1994)

Synthesis of Kynurenic acid amide analogue {N-(2-N, N dimethylaminoethyl)-4-oxo-1H quinoline-2-carboxamide hydrochloride}:

Kynurenic acid amide analogue (N-(2-N, N dimethylaminoethyl)-4-oxo-1H quinoline-2-carboxamide hydrochloride) was prepared in our laboratory using Kynurenic acid as a precursor compound. The amidation reaction was being carried out between kynurenic acid and an amine,

N, N-dimethylethylene diamine using dimethylformamide as a solvent. 1-hydroxybenzotriazole (coupling additive) and diisopropylcarbodiimide (coupling agent) are used for the completion of reaction. The reaction was performed at 25˚C for 16 hours by stirring with a mechanical stirrer. The reaction mixture was passed through the resin (benzenesulphonic acid) column to separate the by-products and was followed by solvent extraction in diethyl ether to isolate the analogue. The product was then analyzed by IR and NMR spectroscopy (US Patent Scott, US6362351B2, 26th Mar 2002).

Experimental Plan:

The present study consisted of 07 groups each consisting of 6 animals.

Group 1: Control; **Group 2:** N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (400 mg/kg, i.p.); **Group 3:** TBI+Vehicle treated (Distilled Water); **Group 4:** Sham (Surgery without TBI); **Group 5:** TBI+N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (100 mg/kg, i.p.); **Group 6:** TBI+N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (200 mg/kg, i.p.); **Group 7:** TBI+N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (400 mg/kg, i.p.)

The N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride were administered by intraperitoneal route after half an hour of injury and repeated after every 24 hours. In present study, Biochemical and histopathological parameters were done at 72 h.

Oxidative stress parameters:

Malondialdehyde level: Lipid peroxidation is a free radical arbitrated event and acts as a marker of oxidative stress in cells and tissues. The primary products of such event are a complex mixture of peroxides which then disassociate to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl product. This MDA content was estimated in the form of thiobarbituric acid-reactive substances following the method given by Ohkawa et al (1979) and modified by Gupta et al (2005). In this method, 1.5 ml of acetic acid (20%) pH 3.5, 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml of sodium dodecyl sulphate (8.1%) were added to 0.1 ml of the processed tissue sample. Then, the mixture was heated at 100˚C for 60 min. The mixture was then cooled with tap water, and 5 ml of n-butanol:pyridine (15:1 v/v) and 1 ml of distilled water were added. The mixture was shaken vigorously and centrifuged at 4000 rpm for 10 min. The pink colour organic layer was produced. This pink colour layer was separated and its absorbance was read at 532 nm using a double beam UV-Visible spectrophotometer. MDA concentration was measured using a standard plot prepared with tetramethoxy propane and expressed as nmol/mg protein.

Reduced Glutathione: Reduced glutathione content in tissue homogenate was measured using Ellman (1959) method. This assay is based on the reduction of 5, 5-dithiobis-(2- nitro benzoic acid) (DTNB) by SH groups of glutathione to form 2-nitro-S-mercaptobenzoic acid per mole of glutathione. The sample was mixed with an equal amount of 10% trichloroacetic acid (TCA) and then centrifuged at 2000 rpm at 4˚ C for 10 min to remove out the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5'-dithiobis (2- nitrobenzoic acid)

(DTNB) and 0.4 ml of distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. Another set with standard glutathione was also analyzed under same conditions and used to plot the standard curve. The concentration of glutathione (GSH) was measured by the standard graph and expressed as µg/mg protein.

Catalase: Catalase was tested by Sinha method (1972). The 0.1 ml of brain homogenate was taken and 1ml phosphate buffer (pH 7.4) and 0.4 ml of distilled water were added to it. To this mixture, H_2O_2 solution (0.5 ml) was added and incubated for 1 min at room temperature. Then, 2ml potassium dichromate –acetic acid reagent was added to it. This solution was then kept in boiling water bath (Narang Scientific Works, Pvt. Ltd., New Delhi, India) for 15 min and then cooled. Absorbance was then measured at 570 nm against control. Control set was analyzed under the identical conditions except the addition of hydrogen peroxide. Catalase activity calculated from the standard plot and the outcome was expressed as µmol H_2O_2 consumed/mg protein.

Superoxide dismutase:

SOD activity was analyzed using the method of Kostyuk and Potapovich (1989). This method allows the superoxide drivenauto-oxidation of quercetin at pH 10 in the presence of TMEDA (*N*,*N*,*N*′*N*′-*N*′-tetramethylethylenediamine) and EDTA. The standard reaction mixture having 0.8 mM TMEDA and 0.08 mM EDTA in 0.02 M potassium phosphate buffer pH 10 with sample and blank. The reaction was initiated by the addition of 0.1 ml of quercetin 0.44 mM in dimethylformamide., and the oxidation of quercetin was observed at 406 nm. The SOD activity present in the tissue sample was found out and standard graph using known amounts of purified SOD under similar conditions was created.

Estimation of oedema formation:

The % water content was determined by the wet weight-dry weight method. After 24 h of TBI, mice were sacrificed, brain from each mouse was isolated and put on the pre-weighed glass plate and weighed to noted wet weight of the brain. Then, dried these brains by set down them in a hot air oven at 100˚C for 24 h and reweighed to yield dry weight. Then, the % water content was calculated using the formula (Bareyre et al., 1997):

$$\%\ \text{water content} = \frac{\text{Wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

Estimation of Blood Brain Barrier (BBB) permeability:

The blood brain barrier permits the transit of few gases and lipid soluble molecules through passive diffusion and the active transport of molecules such as amino acids and glucose crucial for neurons. Traumatic brain injury causes the disruption of the tight junctions and diminishes the integrity of the basement membrane following increased paracellular permeability. This permeability was determined by estimating albumin content in cerebrospinal fluid. A borosilicate glass capillary (length 10 cm, outer diameter 1 mm, and inner diameter 0.75 mm) was divided into two parts using

flame. The tip of the capillary was cut with a scissor to form its internal diameter of about 0.5 mm. The neck area of the anaesthetized mouse was shaved and wiped with 70% ethanol using a cotton plug. A longitudinal incision was given over the neck and subcutaneous muscles were extracted to expose the dura mater of cisterna magna. The mouse was put down with the head at a 135˚ angle to the body for CSF collection. The prepared capillary was inserted to the cisterna magna via the dura mater. After collection of CSF in a capillary, connect the capillary to a 3 ml syringe and collect the CSF into a 2 ml vial. After CSF sampling, suturing was done and 1 ml of normal saline was injected subcutaneously to prevent dehydration. Then 50 µl of CSF was mixed with 5 ml of 2.5 mg/dl strength of bromocresol green working reagent which produced green colour. This coloured solution was analyzed at 630 nm against blank using a UV-visible spectrophotometer. The concentration of albumin content in CSF was calculated using the standard curve method. The data was expressed as mg/dl (Kietduriyakul and Riddhimat, 1990).

Mitochondrial dysfunction:

Complex-I activity:

0.1 ml of brain mitochondrial sample was taken in different test tubes. Glycyl glycine buffer (0.2 M, pH 8.5), NADH (6 mM) in (2 mM) glycyl glycine buffer and cytochrome c (10.5 mM) was added in equal quantity in test tubes. 0.1 ml of distilled water was taken in blank test tube in place of mitochondrial sample and reading was taken at 550 nm using UV-Visible spectrophotometer. The activity of enzymes was calculated on the basis of absorbance index of cytochrome c and results were expressed as nmol NADH oxidized /min/mg protein (Kumar et al., 2011).

Complex II activity:

0.1 ml of brain mitochondrial sample was taken in different test tubes and 0.1 ml of distilled water was taken in blank test tube in place of mitochondrial sample. Phosphate buffer (0.2 M, pH 7.8), succinic acid (HPLC Pvt. Ltd., Mumbai, India) (0.6 M), BSA (1%) and potassium ferricyanide (HPLC Pvt. Ltd., Mumbai, India) (0.03 M) was mixed in equal quantity in all test tubes containing mitochondrial sample. Then, reading was taken at 420 nm using UV-Visible spectrophotometer and results were expressed as nmol succinate oxidized/min/mg protein (Kumar et al., 2011).

Complex IV activity:

0.1 ml of brain mitochondrial sample was taken in different test tubes and 0.1 ml of distilled water was taken in blank test tube in place of mitochondrial sample. 100µl of reduced cytochrome c (0.3 mM) in phosphate buffer (75 mM) was added in all test tubes containing mitochondrial sample. Then, reading was taken at 550 nm using UV-Visible spectrophotometer and results were expressed as nmol cytochrome c oxidized/min/mg protein (Novarro et al., 2008).

Catalase activity:

100µl of brain mitochondrial sample was taken in test tubes and 2.90 ml hydrogen peroxide (30%) was added in each test tube. 3 ml phosphate buffer (pH 7.4) was taken in blank test tube and reading

was taken at 240 nm for 1 min using UV-Visible spectrophotometer. Results were expressed as mmol H_2O_2 oxidized/min/mg protein (Novarro et al., 2008).

Histopathological studies:

Brains were isolated and kept in Bouin's fixative (mixture of saturated solution of picric acid, formaldehyde and glacial acetic acid in ratio of 70:25:5 ml respectively) for 72h. After 24h of fixation, the tissues were divided into two cerebral hemispheres then again fixed in fresh Bouin's fixative for the rest of the 36h. Tissues were then put under washing using tap water to remove the excess of Bouin's fixative for 2h. After that, tissues were dehydrated with different grades of alcohol. Then, tissues were washed two times with a mixture of alcohol and xylene (50:50) for 4 min. The tissues were put in pure xylene for 6 min. Then, the tissues were leaved in a mixture of xylene and wax for 12h in a hot air oven at 60°C. After 12h, the brain tissues were fixed in pure wax and placed in a pre-set oven for 24h at 60°C. After 24h, brain samples were embedded into wax blocks and then cut sections of 7 µm using microtome. The slides were wiped and smeared with the adhesive solution (Mixture of glycerol and egg albumin in equal quantity and small amount of thymol crystals). Sections were then mounted on these slides, stretched, and then dried. Then stained the slides with haematoxylin and eosin dye and visualized at 40 X magnification under a light microscope (Bancroft and Gamble, 2008; Kamat et al., 2011).

Results:

Oxidative stress:

Malondialdehdye:

Malondialdehyde (MDA) level has modulated after brain injury and its modification by administration of KAA was measured at 72 h of injury. A significant ($p<0.05$) increase in MDA level was shown in vehicle treated group as compared to the control group as well as the sham group. Drug treated groups were found to reduce the MDA level dose dependently as compared to vehicle treated group.

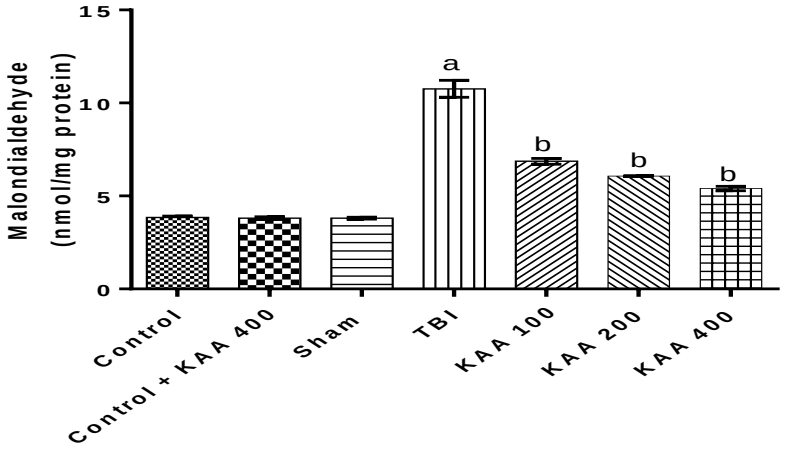

Fig. 1: Effect of Kynurenic acid amide analogue on brain MDA level at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Reduced Glutathione:

The GSH level gets decline and leads to oxidative stress in traumatic brain injury. The change in level of GSH in brain due to administration of amide analogue of Kynurenic acid was measured at 72 h after injury. GSH level was found to be significantly less ($p<0.05$) in vehicle treated group as compare to the control and sham group. A significant dose dependent increase in GSH level was shown in drug treated groups at 72 h of injury.

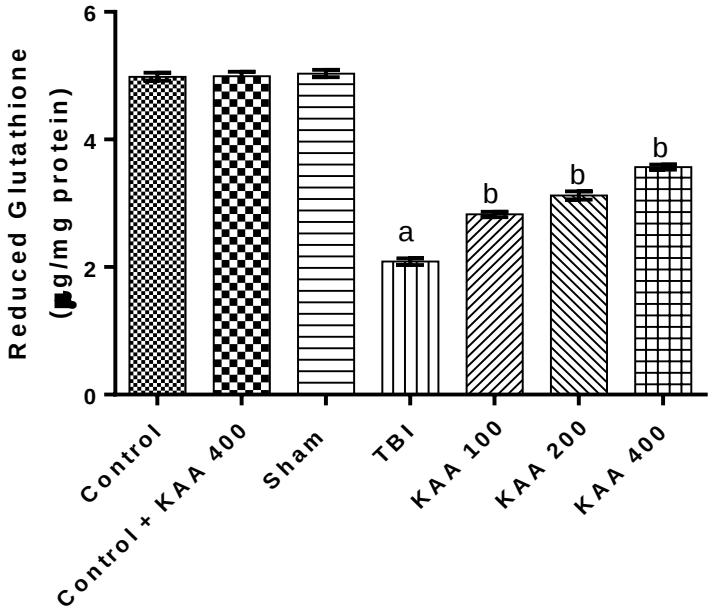

Fig. 2: Effect of Kynurenic acid amide analogue on brain Reduced GSH level at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Superoxide dismutase activity:

A decrement in the SOD activity was observed after a brain injury, resulting in increment in level of free radicals. The SOD level was estimated after 72 h of injury. A significant deduction

of SOD activity ($p<0.05$) in vehicle treated group was shown after injury in comparison to control and sham group. The drug treated groups (KAA 100, 200 & 400) at 72 h were shown a dose dependent rise in SOD activity.

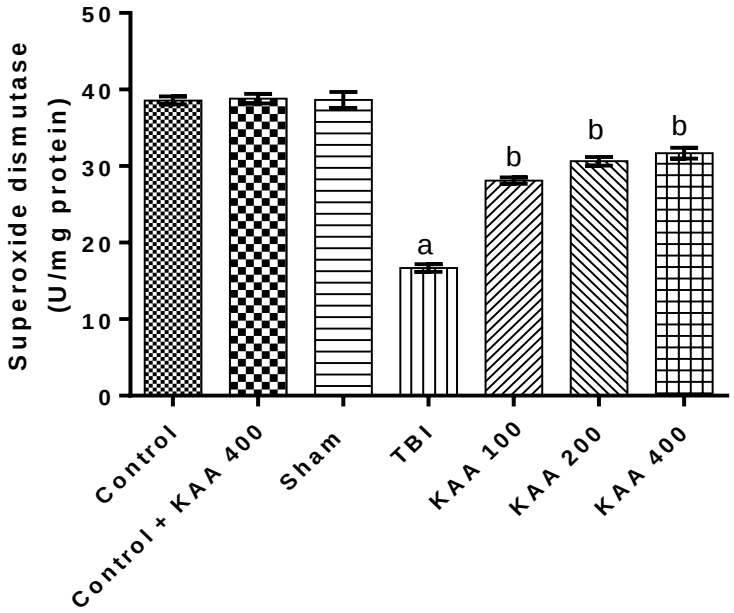

Fig. 3: Effect of Kynurenic acid amide analogue on brain SOD activity at 72 h of injury.
The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Catalase activity:

The catalase, an antioxidant enzyme disturbs after injury following rise in free radicals level. The catalase activity was found after 72 h of injury. A significant loss ($p<0.05$) of catalase activity was observed in TBI group in comparison to the control and sham group. Control and sham group were not significantly differ to each other. A dose dependent enhancement was found in drug treated group i.e. KAA 100, 200 & 400 when compared to vehicle treated group.

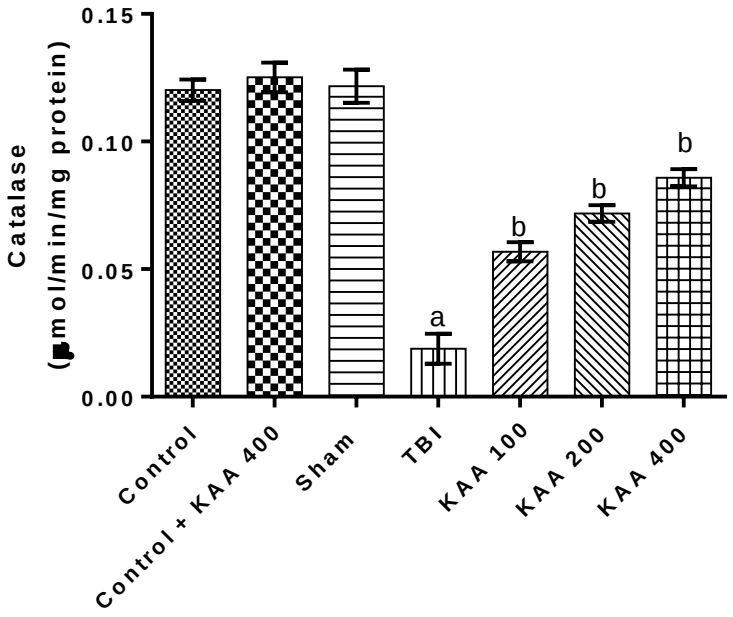

Fig. 4: Effect of Kynurenic acid amide analogue on brain catalase activity at 72 h of injury. The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Edema formation:

TBI results an elevation in brain tissue water content responsible for the formation of edema. This was measured by calculating % water content in brain of mice after 72 hours of TBI. The % water content was found to be significantly increased in vehicle treated group as compare to control group and sham group. After drug administration, the edema level was shown significantly less ($p<0.05$) in highest dose only.

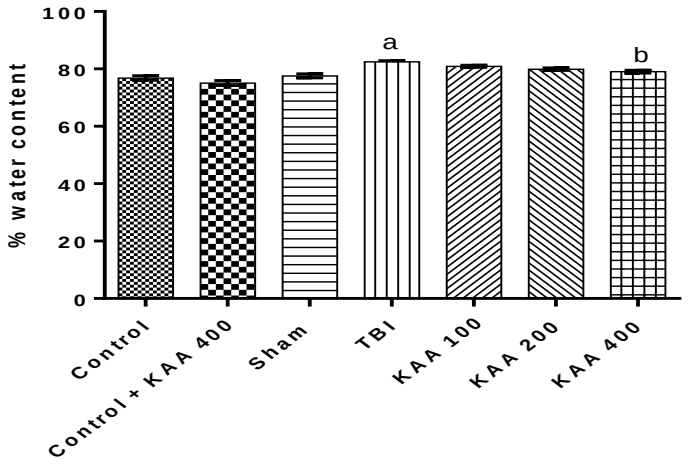

Fig. 5: Effect of Kynurenic acid amide analogue on edema formation at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.01$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

BBB permeability:

BBB allows the passage of small amount of albumin to diffuse in CSF. In response to the TBI, the entry of albumin in CSF rises due to damage of BBB. After TBI, albumin content was significantly higher ($p<0.05$) in TBI group when compared to control group and sham group. Control and sham groups didn't show any significant difference. Significant attenuation of albumin content in CSF was observed in drug treated groups as compared to TBI group.

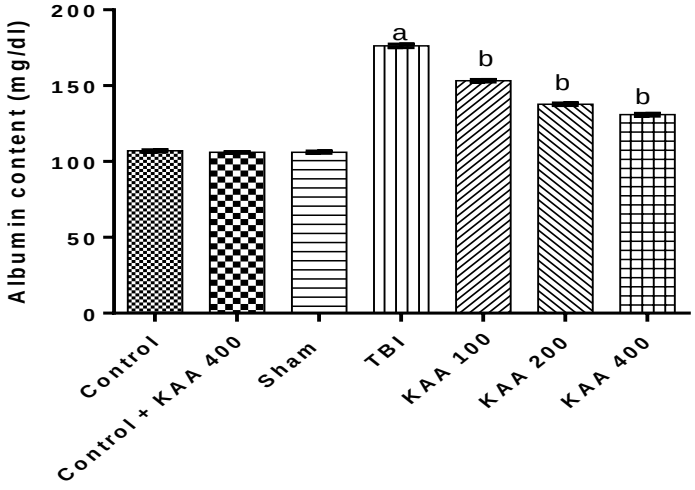

Fig. 6: Effect of Kynurenic acid amide analogue on BBB permeability at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** p<0.0001 vs control group; **b.** p<0.0001 vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Mitochondrial dysfunction:

Complex I activity:

Changes in mitochondrial complex I activity and its modification by administration of amide analogue of KYNA were recorded at 72 h of injury. Mitochondrial complex I activity was found significantly decrease (p<0.001) in vehicle treated group in comparison to control group and sham group. Drug treated groups showed dose dependent significant increase (p<0.001) in mitochondrial complex I.

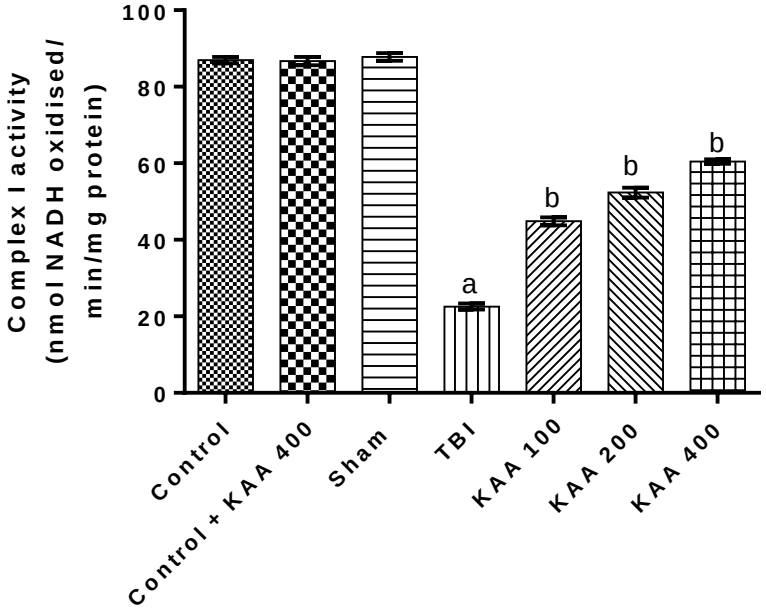

Fig. 7: Effect of Kynurenic acid amide analogue on mitochondrial complex I activity at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** p<0.0001 vs control group; **b.** p<0.0001 vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Complex II activity:

Mitochondrial electron transport chain (ETC) is highly sensitive target of oxidative stress. Mitochondrial complexes activities get decreased after TBI in mice brain mitochondria due to

the formation of free radicals and ROS. Mitochondrial complex II activity was found significantly decrease ($p<0.001$) in vehicle treated group in comparison to control group and sham group. Drug treated groups showed dose dependent significant rise ($p<0.001$) in mitochondrial complex II activity.

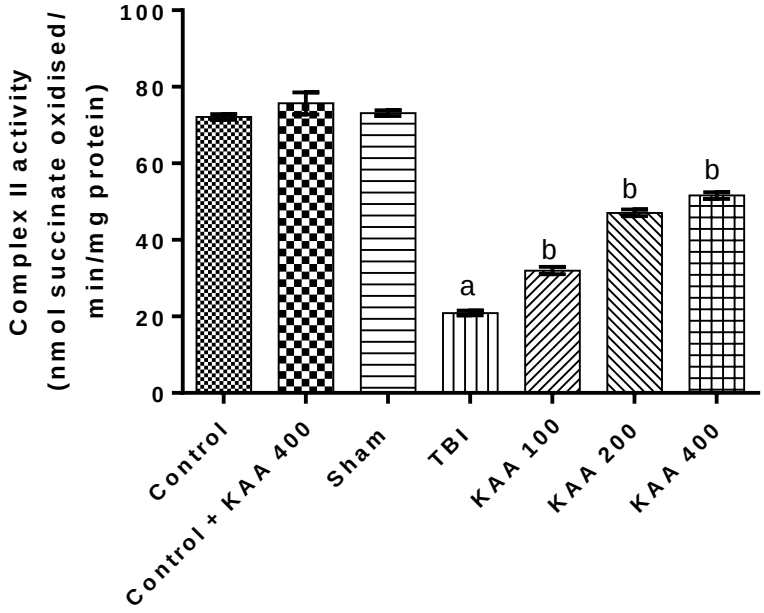

Fig. 8: Effect of Kynurenic acid amide analogue on mitochondrial complex II activity at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Complex IV activity:

Mitochondrial complexes activities get decreased due to inhibition of ETC after TBI which may lead to loss of membrane potential and increased ROS generation. Mitochondrial complex IV activity was found significantly decrease ($p<0.001$) in vehicle treated group in comparison to control group and sham group at 72 h. Drug treated groups showed dose dependent significant increase ($p<0.001$) in mitochondrial complex IV activity as compare to TBI group.

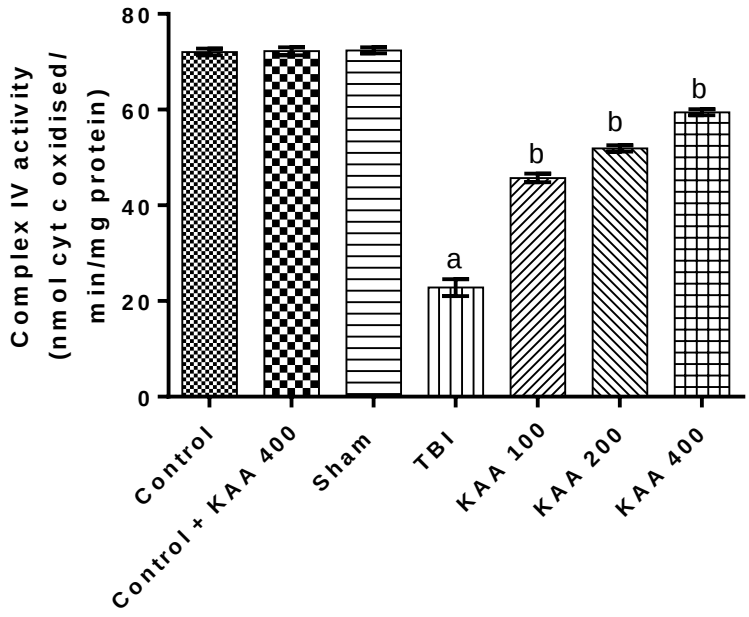

Fig. 9: Effect of Kynurenic acid amide analogue on mitochondrial complex IV activity at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Mitochondrial Catalase activity:

Functional impairment of mitochondria is also linked with the suppression of its free radical scavenging system. Catalase is an antioxidant removes H_2O_2 and protects mitochondria from cytotoxic ROS. Catalase activity gets decreased after injury and leads to rise in free radicals. A significant reduction ($p<0.001$) in vehicle treated group was observed after injury when compared to control group and sham group. There was no significant difference found in control and sham groups. Dose dependent increase in catalase activity was found in drug treated groups.

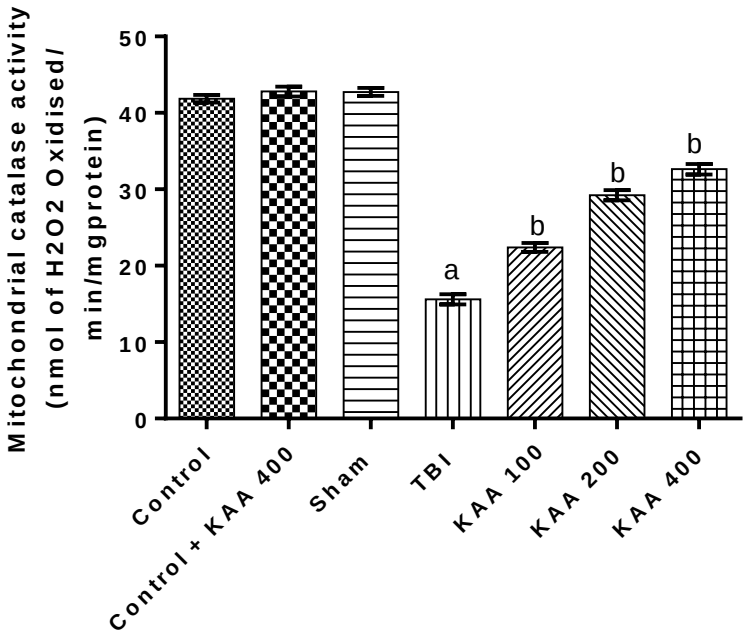

Fig. 10: Effect of Kynurenic acid amide analogue on mitochondrial catalase activity at 72 h of injury.

The present study having 7 groups and n = 6 in each group.Values are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by multi-comparison test. **a.** $p<0.0001$ vs control group; **b.** $p<0.0001$ vs TBI. Control + KAA 400 = *per se* group; KAA 100, 200, 400 stands for N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.

Histopathological studies:

In present study, we have observed the effect of amide analogue of Kynurenic acid on histopathological modifications in cerebral cortex of traumatic brain injury induced mice at 72 h. The sections stained by H & E showing intact neuronal cell with clear appearance of purple colour nuclei and pink cytoplasm (black arrows) in control group whereas in vehicle treated group, there was no clear difference observed between nuclei and cytoplasm. These neuronal cells were found to be shrinked and dark stained by eosin, represented as damaged cells (red arrows). At 72 h, most of the cells were found to be damaged. Drug treated groups (KAA 100, 200 and 400) were shown dose dependent improvement in neuronal cell damage in cerebral cortex as compared to vehicle treated group.

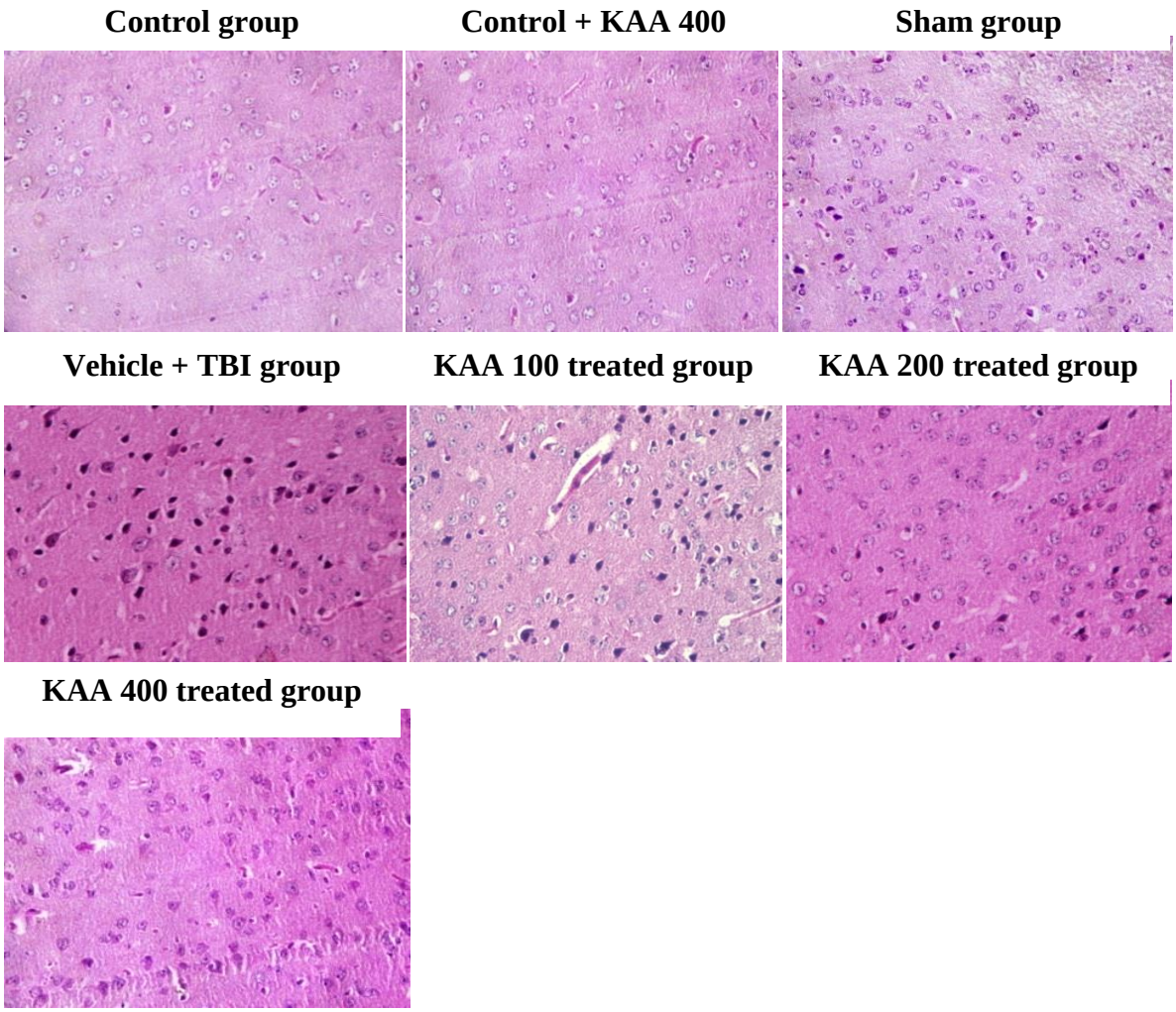

Fig. 13: Histopathological findings at 72 h of injury

Discussion:

TBI occurs due to the outside mechanical impact causing sudden disruption of brain tissue and delayed secondary consequences which collectively leads to widespread neurodegenration (Gaetz, 2004). It is one of the most common health issues occurring in one of the most complex organ of the body (McGinn and Povlishock, 2016). Depending on the duration of injury, it can be classified into primary and secondary injury. Primary injury is immediate brain deformation at the moment of head impact which can never be reversed by any therapeutic intervention. Brain deformation is including the damage of various neural elements like axons, neurons, glia and blood vessels in focal and diffuse manner. Secondary injury is depicted as secondary consequences of the primary injury including generation of free radicals, edema development, and changes in BBB permeability and integrity, mitochondrial dysfunction, inflammation etc and it can be reversed by some therapeutic treatment. In the current study, we focussed on the secondary complications and their treatments (Finnie, 2014). In this study, we evaluated the therapeutic potential of amide analogue of Kynurenic acid to improve the secondary cascades in

TBI. Kynurenic acid is produced endogenously through pathway of tryptophan metabolism and was proven to have neuroprotective potential. Besides this, it couldn't be used in neurodegenerative diseases because of complicated solubility profile, low BBB permeability and rapid excretion from the body. Therefore various derivatives have been prepared in which amide analogues are therapeutically potential agents. Our analogue is also the amide one i.e. N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride. This analogue having mechanism of action in two ways: either by imitating the action of Kynurenic acid or acts like a pro drug. As per the study of Zadori et al. 2011, this amide analogue is quite stable and doesn't dissociate into Kynurenic acid and not to act like pro-drug in much amount. In this study, TBI aggravates the production of oxygen free radicals including superoxide anion, hydrogen peroxide ion, nitric oxide and peroxynitrite ions at 72 h of injury. This bursting of free radicals causing peroxidation of vascular and cellular structure, oxidation of protein, DNA fragmentation, impairment of mitochondrial respiratory chain and hamper the energy production. These processes lead to neuronal cell dysfunction. These reactive radicals induced the excessive calcium influx by the over-activation of glutamate receptors by provoking the excitotoxic pathways and the exhausation of the anti-oxidant enzymes (GSH, SOD and catalase) (Bayir et al., 2005; Chong et al., 2005). In the present study, elevation in free radical ions as evidenced by remarkable rise in MDA level and decrease in GSH level, SOD and catalase activity at 72 h of TBI. MDA is the marker of lipid peroxidation which is mediated by the free radicals which consists of the production of hydroperoxides of highly unsaturated fatty acids as primary products, further converted to secondary products such as 4-hydroxy-2-nonenal and malondialdehyde (MDA) etc. Therefore, MDA is one of the parameters to measure the free radical formation in the brain. CNS has the high urge of oxygen and unsaturated lipid content so brain is the most promising site of lipid peroxidation (Ates et al., 2006). In our study, KAA treated group (100, 200 and 400 mg/kg) diminishes the MDA level significantly and dose dependently as compared to the vehicle treated group. GSH is an essential thiol containing tripeptidal antioxidant enzyme in the intracellular compartment which scavenges the free radicals such as singlet oxygen, hydroxyl and superoxide radical and prevents the cells from damage under normal physiological conditions. In the present study, there is significant depletion of GSH at 72 h of injury whose level was found to be enhanced in drug treated group significantly and dose dependently. Similarly SOD is an oxido-reductase enzyme which dismutase the superoxide radical ion whereas catalase enzyme dissociates the hydrogen peroxide radical to neutral ions and protects the neuronal cells from oxidative damage. In present study, the activities of SOD and catalase were depleted remarkably at 72 h of injury. After drug administration, the activities were found to be significantly and dose dependently restored. These result findings suggests that KAA may be responsible to decrease oxidative stress, probably by lessen lipid peroxidation and enhancing the antioxidative defensive capacity of brain to fight against oxidative stress in TBI. Previous studies involving the use of various antioxidants to reduce oxidative stress such as resveratrol (Ates et al., 2006), polyethylene conjugated superoxide dismutase (Muizelaar et al.,

1993) etc after TBI and helped in the management of TBI consequences. % water content and BBB permeability were also observed to enhance at 72 h of injury due to edema formation and loosen the strength of tight junctions in BBB respectively after TBI. After the drug administration, the % water content and albumin content in CSF were found to be reduced. These findings depicts that KAA may be helpful in the improvement edema formation and BBB permeability. Mitochondria are known as major site for ROS production and play important role for regulating energy metabolism in various cells. After TBI, glutamate excitotoxicity occur which starts excessive calcium influx through activated NMDA receptor and voltage gated calcium channel. Increased intracellular calcium activates various enzymes neuronal NOS and endothelial NOS enhance nitric acid production leading to lipid peroxidation, increases calcium content in mitochondria and eventually cellular necrosis. Due to mitochondrial calcium overload mitochondrial membrane permeability increased and complexes activity get supressed. Any decrease in the activities of mitochondrial complexes suggests inhibition of electron transport chain (ETC), which may leads to loss of membrane potential and increase ROS production (Fukui and Moraes, 2008).As a result ROS causing oxidative stress and the protein cytochrome-c released into the cytoplasm. This cytochrome-c binds to the apoptosis activating protein-1 (apaf-1) which in turns activating apoptosis inducing caspases pathway (Andriessen et al., 2010).

Functional impairment of mitochondria is also linked with the suppression of its free radical scavenging system. MnSOD dismutases superoxide radicals and catalase removes H_2O_2 hence both protect mitochondria from cytotoxic ROS (Prakash et al., 2017).

In the present study, the activities of mitochondrial complexes I, II, IV and catalase was suppressed after TBI at 72 h of injury. KAA (100, 200, 400 mg/kg) treated group showed increase in mitochondrial complex I, II and IV significantly and dose dependently. These findings shown that due to NMDA receptor blocking activity, the overloading of Ca^{2+} ion is being reduced which further suppresses the excitotoxic processes which may be responsible in reduction of oxidative stress, edema, BBB permeability and restoring the activities mitochondrial complexes and catalase enzyme. The damage induced by TBI was evidenced by histopathological studies. Haematoxylin and eosin stained sections showed shrink and darkly eosin stained cells which were considered as damaged cells, where this kind of damage was found to be less in KAA treated groups significantly and dose dependently. Therefore, the present amide analogue of Kynurenic acid was found depicted as good pharmacological candidate in the treatment of traumatic brain injury.

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