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Research Article

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Targeting the Glutamate Amino Acid by Alpha-Ketoisocaproic Acid and L-2-Oxothiazolidine-4-Carboxylic Acid as a Neuroprotective Approach in Rat Model of Cerebral Ischemia

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ABSTRACT

Background: Neuronal death following ischemic stroke is primarily attributed to glutamate excitotoxicity. Objective: The aim of the present study is to attenuate the glutamate excitotoxic effect in rat model of transient focal cerebral ischemia via two approaches. First by using alpha-ketoisocaproate (αKIC), a ketoacid of leucine that reacts with glutamate in glutamate/branched chain amino acid cycle. Second by using l-2-oxothiazolidine-4-carboxylate (OTC), a prodrug of cysteine and an oxoproline analog which is involved in gamma-glutamyl cycle of glutathione (GSH) biosynthesis. Method: Immediately after induction of transient left MCAO for 90 min. in rats, αKIC (300mg/kg) or OTC (8 mmol/kg) was injected intraperitoneally. Quantification of plasma glutamate, glycine, cysteine and leucine amino acids and reduced GSH was done 150 min after reperfusion. The post-infarct behavioral functioning and the infarction size were detected 24 hours after reperfusion. Results:The infarction caused by tMCAO was associated with significant increase in the plasma glutamate, glycine and cysteine and significant decrease in the plasma leucine and reduced GSH levels. Either αKIC or OTC caused significant decrease in the infarct size and improvement of the neurological outcome with significant decrease in the plasma glutamate level. OTC also had a significant anti-oxidant effect via increasing cysteine and GSH plasma levels. Conclusion: the use of these neuroprotective agents to prolong time window prior to reperfusion or to prevent reperfusion injury may represent a future therapeutic clinical application in ischemic stroke.

Keywords: OTC; αKIC; Transient focal cerebral ischemia; Glutamate; GSH

INTRODUCTION

Stroke is a major cause of morbidity and mortality worldwide [1]. Until now, thrombolytic reperfusion is the effective and only approved treatment for the ischemic stroke. The short time window (3- 4.5 hours) for this therapy limits its use for patients who reach medical attention during the first hours after the onset of stroke and have no contraindications to it [2]. Therefore, it would be important to develop treatment that targets downstream events in the ischemic cascade [3]. The use of the neuro-protective agents to prolong time windows prior to reperfusion or to prevent reperfusion injury may present a future therapeutic target for the treatment of ischemic stroke [4].

Immediately after ischemia, the excessive glutamate release and activation of its receptors initiate the excitotoxic neuronal damage [5]. The increased brain extracellular glutamate concentration was associated with poor neurological outcome and larger infarct volume either experimentally [6,7] or clinically [8]. High glutamate concentration during the first 24 hours in ischemic stroke patients suggested a wider window for neuroprotective approach targeting the glutamate excitotoxicity [9].

The aim of the present study is to attenuate the glutamate excitotoxic effects in rat model of transient focal cerebral ischemia via two approaches. The first approach is by using alpha-ketoisocaproate (αKIC). αKIC is the ketoacid of leucine that reacts with glutamate in a cycle called glutamate/branched chain amino acid cycle in the brain [10]. The glutamate is oxidized via glutamate dehydrogenase and aspartate aminotransferase enzymes forming alpha-ketoglutrate (α-KG) which enter tricarboxylic (TCA) cycle as an energy substrate in neurons and astrocytes. To maintain a steady level of glutamate in the brain, the branched chain amino acids (BCAAs) especially the leucine cross into the brain. Leucine is catalyzed by the branched chain aminotransferase (BCAT) to replenish the inevitable glutamate lost in these oxidative processes [11]. In the astrocyte, leucine is transaminated with alpha-ketoglutarate (α-KG) by mitochondrial BCAT producing glutamate and αKIC. The αKIC and glutamate are released to the extracellular space and taken by the neurons where transamination occurs between glutamate and α–KIC yielding α–KG and leucine by cytosolic BCAT [12].

The second approach is by using l-2-oxothiazolidine-4-carboxylate (OTC). OTC, an oxoproline analog, acts as a competitive inhibitor of 5-oxoprolinase and converted by this enzyme to cysteine [13]. Cysteine is the rate limiting amino acid for GSH biosynthesis [14]. The first step in glutathione (GSH) synthesis is the formation of γ-glutamylcysteine from glutamate and cysteine catalyzed by γ-glutamylcysteine synthetase. The second step is the formation of GSH from glutamyl-cysteine and glycine catalyzed by GSH synthetase [15]. GSH degradation occurs in the extracellular space via glutamyl-transpeptidase producing cysteinyl-glycine and a γ-glutamyl residue which is transferred on another acceptor amino acid (cysteine mainly). The γ-glutamyl cysteine is taken by the cell and converted to oxoproline and the cysteine by γ-glutamyl cyclotransferase. 5-oxoprolinase hydrolyses oxoproline to yield glutamate which is used again to form GSH [16].

MATERIALS AND METHODS

Drugs and chemicals

L-2-Oxothiazolidine-4-carboxylic (OTC; Procysteine) and Sodium alpha-ketoisocaproate (αKIC; Ketoleucine sodium salt; Sodium 4-methyl-2-oxovalerate; 4-Methyl-2-oxopentanoic acid sodium salt) were obtained from Santa Cruz (Germany) with purity ≥ 98%. OTC solution was freshly prepared by dissolving OTC powder in phosphate-buffered saline (PBS) and adjusting the pH to 7.2 with 3 N NaOH. αKIC solution was freshly prepared by dissolving αKIC powder in isotonic saline.

Animals

Male Sprague–Dawley rats weighing 300-350 grams were obtained from Mansoura Experimental Research Centre. They were maintained under standard conditions of temperature 22 ± 2^{o}C with regular 12 h light/12 h dark cycle and allowed free access to standard laboratory food and water. All experimental procedures were performed in accordance with guidelines of the Institutional Animal Care and Use Committee of Mansoura University. The mortality rate was 50% due to cerebral hemorrhage and cardiorespiratory arrest 3-6 hours postoperative.

Twenty four rats were divided into four groups (6 rats each).

Group (I) SHAM group:

Rats were exposed to all steps of middle cerebral artery occlusion (MCAO) except the step of occlusion.

Group (II) Non-treated ischemic group:

Rats were exposed to left MCAO for 90 minutes followed by reperfusion and injected by 1ml PBS solution.

Group (III) αKIC-treated ischemic group:

Ischemic rats received αKIC sodium salt dissolved in 1ml saline at a dose 300mg/kg [17].

Group (IV) OTC-treated ischemic group:

Ischemic rats received OTC dissolved in 1ml PBS solution at a dose 8 mmol/kg [18]. The drugs or solvent were injected intraperitoneally (IP) immediately after reperfusion.

Surgical Procedure of transient left middle cerebral artery occlusion (tMCAO)

Transient focal cerebral ischemia was induced in rats by MCAO using the endovascular monofilament suture as described by Longa *et al*. [19] with some modifications [20]. Rats were anesthetized with xylazine (10 g/kg body weight) and ketamine hydrochloride (100 mg/kg) IP. Under an operating microscope, the common carotid (CCA), external carotid (ECA) and internal carotid (ICA) arteries were exposed through a midline incision of the neck. 3cm

4-0 monofilament nylon suture (Doccol Corp., Redlands, CA, USA) was introduced into the ECA lumen down toward the CCA where the microsurgical clip was located. The nylon suture was advanced from the ECA to ICA lumen to the middle cerebral artery (MCA) until resistance was felt. Typically, 22 mm insertion of the intraluminal suture beyond the CCA bifurcation produced blocking of MCA**.** The occlusion start time was recorded and the micro-clip on the CCA was removed. The incision was closed with 4-0 silk suture and the rat was carefully placed in a recovery cage. Ninety minutes later, reperfusion was done after re-anesthesia and reopening of the incision site by removal of the occluding suture from the ICA. the incision was then closed with silk suture.
Two and half hours after the reperfusion, rats were anesthetized with halothane inhalation then blood sample from the medial canthus of eye was withdrawn on heparinized tube. The heparinized blood was spun at 1500 g for 20 min. The separated plasma was stored at -80°C until biochemical measurement. Twenty four hours after tMCAO, five-point scoring scale for recording the post-infarct behavioral functioning was done. Scarification was then done by overdose of anesthesia and the brains were quickly removed for determination of the infarction size.

Scoring scale for recording the post-infarct behavioral functioning
The neurologic findings were scored on a five-point scale [20]: Score (0) No neurologic deficit: able to fully extend both forepaws; Score (1) mild focal neurologic deficit: Failure to extend contralateral side forepaw fully; Score (2) moderate focal neurologic deficit: Circling to the contralateral side; Score (3) severe focal deficit: Falling to the contralateral side and Score (4) very severe: Did not walk spontaneously and had a depressed level of consciousness. Before the surgery all rats were healthy with no neurologic deficit (0). Very severe deficit (4) was observed 90 minutes post MCAO in the present study.

Separation and quantification of amino acids (Glutamate, glycine, cysteine and leucine) by high-performance liquid chromatography (HPLC) [21]
A 50µL volume of the plasma (or the aqueous standard) was pipetted into a tube, and the mixture was vacuum-dried using the Speed Vat concentrator. A 10 µL of a solution of methanol- water-triethylamine (2: 1: 1, v/v). and 20 µL of a solution of methanol, water, triethylamine, and phenyl isothiocyanate (PITC) (7:l:l:1, v/ v) were added to the tubes. The tubes were vacuum-dried using the Speed Vat Concentrator. The residue was reconstituted in 500 µL of mobile phase A adjusted to pH 7.5, and 20 µL were injected into the HPLC system. Hewlett Packard HPLC model 1984 B equipped with variable UV detector (Hewlett Packard, 1050 series, USA) adjusted at wavelength 254 nm was used. The separation was done on reversed phase (RP 18 C Lichrosorb, 15 cm - 4.6 mm i.d., Hibar, Merck, Darmastadt, Germany) column. Mobile phase A was prepared from ammonium acetate (0.05 M, pH 6.8)-acetonitrile (98:2, v/v), and mobile phase B was water-acetonitrile (40:60, v/v). Linear gradient program was used for the successful separation of the amino acids derivatives starting with 100% from mobile phase A and ending at 29 minutes with 52% from mobile phase A and 48% from mobile phase B. The standard mixture was diluted to 100 µM concentration as a working solution. HPLC analyses were conducted at a flow rate of 0.7 ml/min.). Quantification was based on integration of peak areas and compared to the standard calibration curves. (Figure 1)

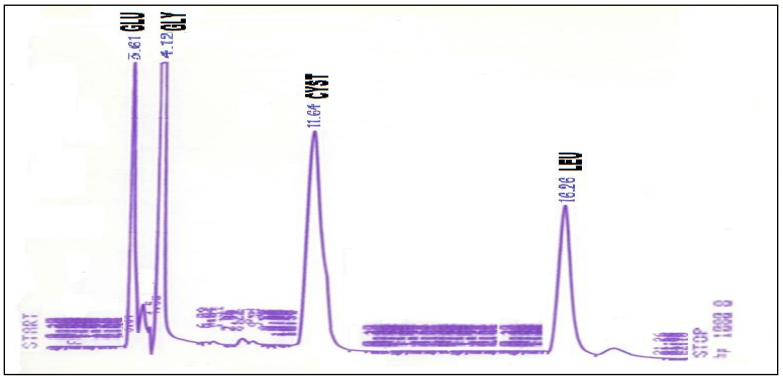

Figure 1: HPLC separation of PITC derivatives of a standard mixture of the four amino acids. Retention time for Glu.: 3.61, Gly.: 4.12, Cyst.: 11.64, Leu.: 16, 26 minutes

Measurement of reduced glutathione
The plasma level of reduced of glutathione (GSH) was determined colorimetrically using the Glutathione Assay Colorimetric Kit (Abnova, USA). GSH substrate 5,5 dithiobis 2-nitrobenzoic acid (DTNB) and GSH react to

generate 2-nitro-5-thiobenzoic acid which has yellow color. The GSH concentration can be determined by measuring absorbance at 412 nm and is expressed as µmol/L.

Evaluation of the brain infarct area

After scarification, the brain of each rat was quickly removed and placed in ice-cold saline for 5 minutes. The cerebellum and brain stem were dissected and the remaining forebrain was cut into 3-mm coronal slices. Sections were incubated in TTC- containing saline solution (sigma chemical Co., St. Louis, Mo, USA) for 20 minutes. Then, the slices were refrigerated in 10 % formalin over-night. The viable area was colored red, while the infarcted area appeared white indicating severely compromised mitochondrial function [22]. The slices were photographed on white background using Olympus® digital camera with a ring flash at high resolution and converted to TIFF. The % infarction in each image was analyzed on Intel® Core I3® based computer using VideoTest Morphology® software Russia with a specific built-in routine for area and % area measurement.

Statistical analysis

Data are presented as mean ± SEM. Differences among groups within an experiment were analyzed by the one-way ANOVA analysis of data followed by post hoc test of Tukey HSD. A *P* value of <0.05 value was considered significant.

RESULTS

Brain sections stained with TTC stain 24 hrs after tMCAO showed a mean infarct area of 37.5 ± 2.8% including dorsolateral cortex and caudatoputamen**.** Treated tMCAO groups with either αKIC or OTC showed significant decrease in the mean infarct area percentage in comparison to the non-treated ischemic group (21.2±2.1% & 20.1±1.9%, respectively). No significant change between treated groups was noticed (figures 2 and 3).

The mean score of neurological deficit was 3.33±0.3 in non-treated tMCAO. Treated tMCAO group with either KIC or OTC showed significant decrease in the mean score of neurological deficit in comparison to the non-treated ischemic group (1.5±0.1 & 1.3±0.1, respectively) (figure 4).

tMCAO caused significant increase in the mean plasma level of glutamate, glycine and cysteine and significant decrease in the mean plasma leucine level as compared to the SHAM group. αKIC-treated tMCAO group showed significant decrease in the mean plasma level of glutamate and glycine; significant increase in the mean plasma leucine level and non-significant change in the mean plasma cysteine level in comparison to non-treated ischemic group. Meanwhile, OTC-treated tMCAO group showed significant decrease in the mean plasma level of glutamate and glycine; significant increase in the mean plasma cysteine level and non-significant change in the mean plasma leucine level in comparison to non-treated ischemic group (figures 5-8).

tMCAO produced significant decrease in the reduced GSH level in comparison to SHAM group. The plasma reduced GSH level was non-significantly affected by αKIC treatment in tMCAO group. While treatment of tMCAO group with OTC showed significant increase in the reduced GSH level in comparison to the non-treated ischemic group (figure 9).

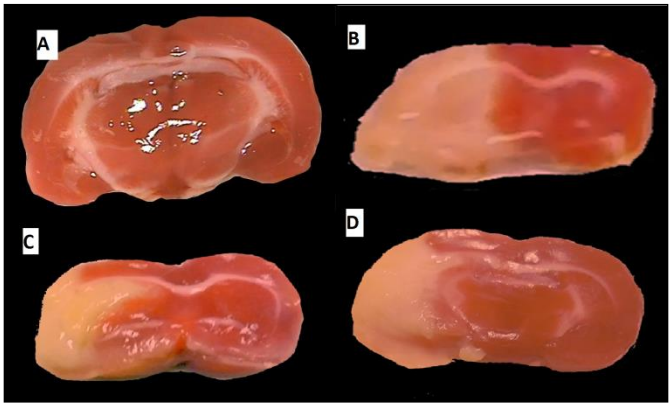

Figure 2: TTC stained coronal sections of the brain 24 hours after tMCAO of SHAM group (A), Non-treated ischemic group (B), αKIC-treated ischemic group (C) and OTC-treated ischemic group (D)

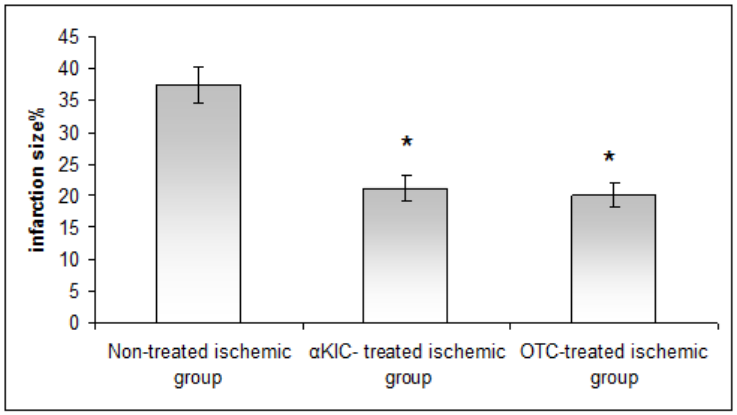

Figure 3: Effect of α-KIC (300mg / kg IP) and OTC (8 mmol / kg IP) on the mean infarct area percentage 24 hours after tMCAO in rats (n=6)

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. *****Indicates significant change from non-treated ischemic group values at $p < 0.05$

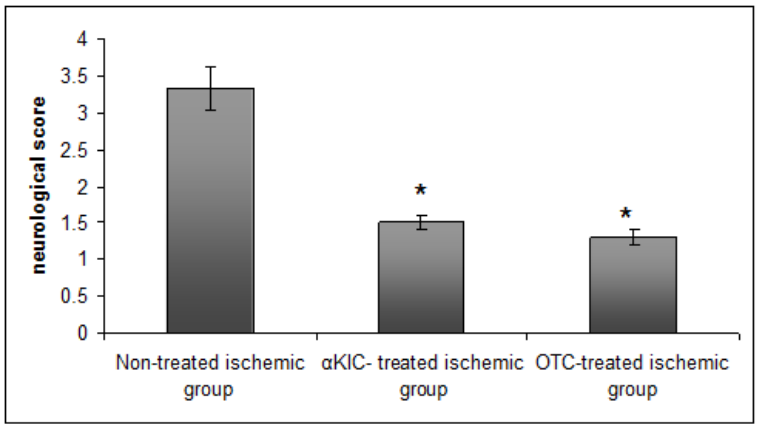

Figure 4: Effect of α-KIC (300mg / kg IP) and OTC (8 mmol / kg IP) on the mean score of neurological deficit 24 hours after tMCAO in rats

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. *****Indicates significant change from non-treated ischemic group values at $p < 0.05$

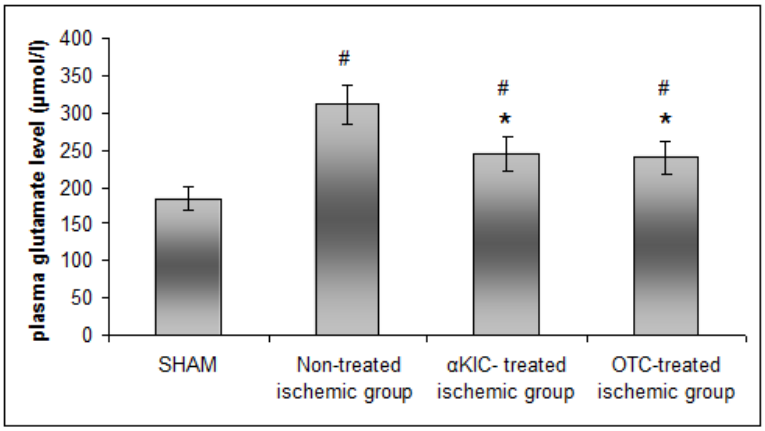

Figure 5: Effect of α-KIC (300 mg / kg IP) and OTC (8 mmol / kg IP) on the mean plasma glutamate level (µmol/L) after tMCAO in rats

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. # Indicates significant change from SHAM values at $p < 0.05$ *****Indicates significant change from non-treated ischemic group values at $p < 0.05$.

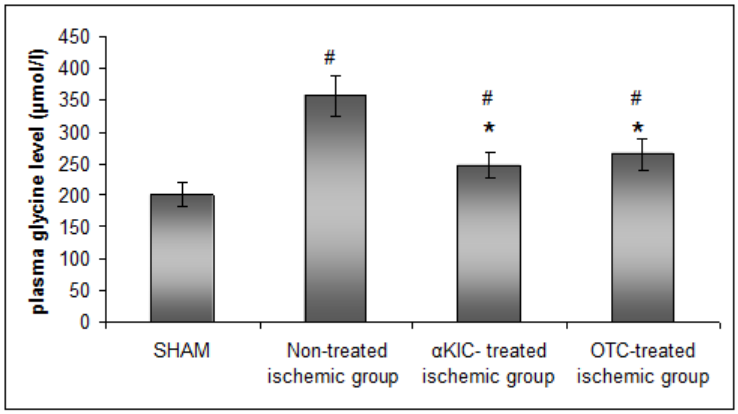

Figure 6: Effect of α-KIC (300mg / kg IP) and OTC (8 mmol / kg IP) on the mean plasma glycine level (µmol/L) after tMCAO in rats

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. # Indicates significant change from SHAM values at $p < 0.05$ *****Indicates significant change from non-treated ischemic group values at $p < 0.05$.

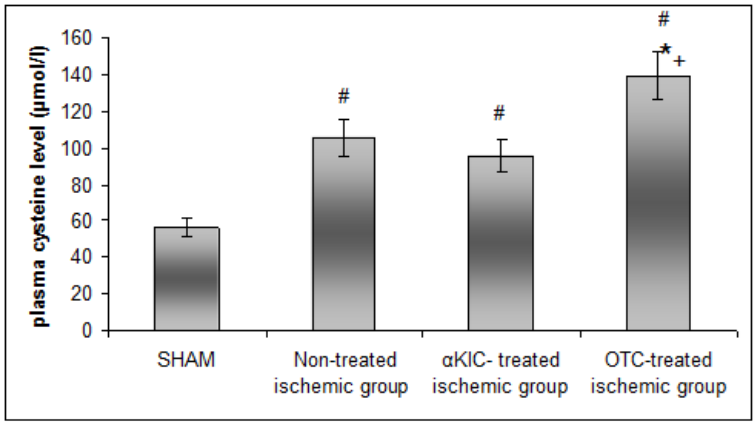

Figure 7: Effect of α-KIC (300mg / kg IP); OTC (8 mmol / kg) IP) & their combination on the mean plasma cysteine level (µmol/L) after tMCAO in rats

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. # Indicates significant change from SHAM values at $p < 0.05$ *****Indicates significant change from non-treated ischemic group values at $p < 0.05$ **+** Indicates significant change from αKIC - treated ischemic group values at $p < 0.05$.

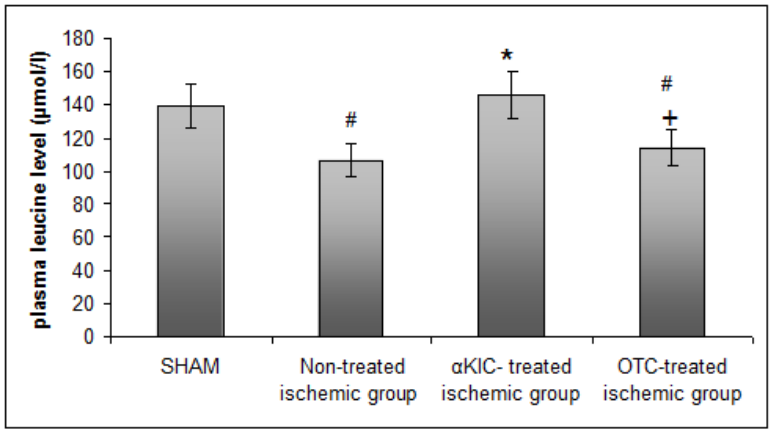

Figure 8: Effect of α-KIC (300mg / kg IP) and OTC (8 mmol / kg IP) & their combination on the mean plasma leucine level (µmol/L) after tMCAO in rats

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. # Indicates significant change from SHAM values at $p < 0.05$ ***** Indicates significant change from non-treated ischemic group values at $p < 0.05$ **+** Indicates significant change from αKIC-treated ischemic group values at $p < 0.05$.

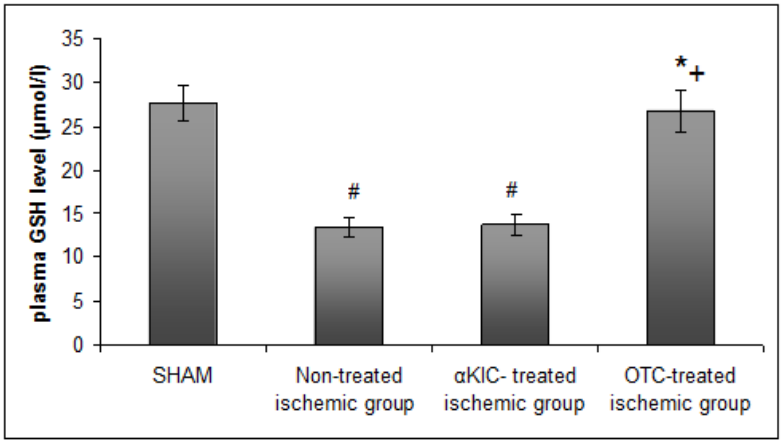

Figure 9: Effect of α-KIC (300mg / kg IP) and OTC (8 mmol / kg IP) on the mean plasma GSH level (µmol/L) after tMCAO

Statistical analysis was carried by ANOVA one way analysis variance followed by post hoc test of Tukey HSD. All values presented as mean of 6 rats ± SEM. # Indicates significant change from SHAM values at $p < 0.05$ ***** Indicates significant change from non-treated ischemic group values at $p < 0.05$ **+** Indicates significant change from αKIC-treated ischemic group values at $p < 0.05$

DISCUSSION

In the current study, focal cerebral ischemia was induced by using the endovascular monofilament suture occlusion of MCA 22 mm beyond the common carotid artery (CCA) bifurcation for 1.5 hours followed by reperfusion. This model is considered one of the best models to mimic human ischemic stroke [23]. Our model produced a neurological deficit and infarcted area including dorsolateral cortex and caudatoputamen. These results were previously reported by Zarow *et al*. [24] who reported that monofilament suture occlusion of MCA caused severe neurological deficit consistent with histological damage in the dorsolateral cortex and great damage in medial caudate.

The infarction caused by tMCAO in the present study was associated with significant increase in the mean plasma level of glutamate, glycine and cysteine and significant decrease in the mean plasma leucine and reduced GSH level. Wang *et al*. [25] showed that the glutamate and glycine increased from the basal level in the first 24 hours after reperfusion in the serum of rats exposed to tMCAO that correlated with larger ischemic insult. Clinically, in patients with acute hemispheric infarction, high plasma glutamate concentration in the first 24 hours was associated with early neurologic deterioration and greater infarct volume at 72 hours [8, 26]. Also high plasma cysteine level was significantly associated with early deterioration during the first 48 hours and poor outcome within 3 months in ischemic stroke patients [27]. Moreover, experimental and human studies detected that reduction in BCAAs, especially leucine, in plasma and CSF of ischemic rat and acute stroke patients was correlated with worse neurological outcome [28].

The excitotoxic neuronal injury related to glutamate excessive release plays a key role in the pathogenesis of cerebral ischemic damage [29]. Excessive Ca^{2+} influx through NMDA receptors during the ischemic condition is proved to be neurotoxic [30]. The excessive intra-neuronal Ca^{2+} ions results in activation of multiple signaling pathways and number of enzymes including proteases, kinases, lipases, endonucleases, calpains and caspases resulting in degradation of various cytoskeletal and membrane proteins, destabilizing the structural integrity and forcing the neurons to delayed death [31]. Also, increased cytosolic Ca^{2+} strongly activates phospholipase A_2 with subsequent release of eicosanoids and free radical production [32]. Lastly, the excessive Ca^{2+} influx through NMDA receptors targets mitochondria, leading to mitochondrial Ca^{2+} overload triggering mitochondrial membrane depolarization and mitochondrial dysfunction [33].

In the current study, αKIC-treated tMCAO rats showed significant decrease in the infarct area size and improvement of the neurological outcome. This improvement was significantly associated with decrease in the mean plasma level of glutamate and glycine and increase in the leucine. These results came in agreement with a study of McKenna *et al*. [34] who showed a significant decrease in glutamate concentration in the medium from primary cultures of rat cerebral cortical astrocytes after addition of αKIC the culture with twofold increase in intracellular leucine compared with the control cells. This significant decrease in glutamate concentration attributed to the active uptake and/or metabolism of glutamate in these cells. Also, Dufour *et al.* [17] reported that injection of αKIC (300 mg/kg) in epileptic rats induced a decrease in the thalamic and cortical concentration of glutamate. The significant decrease of glutamate and increase of leucine plasma level by αKIC noticed in the present work could be explained by the transamination reaction of glutamate by αKIC producing leucine and α-KG in the neurons [12] and in the astrocytes [35]. Leucine readily crossed into the brain to act as nitrogen donor to replenish the inevitable glutamate lost [11]. αKIC increased the rate of glutamate oxidation by two fold with formation of α-KG. Consecutively the glutamate released in the extracellular space decreased with attenuation of the glutamate-induced excitotoxicity [36]. In turn the glutamate moved from the brain to the circulation decreased [37]. Attenuation of the glutamate excitotoxic stimulation of the neurons and the consecutive calcium-dependent vesicular release of neurotransmitters might provide an explanation to the decrease in the plasma glycine level in this group [38]. Notably, this transamination reaction with αKIC may also occur in other tissue throughout the body such as skeletal muscles, kidney and adipose tissue with the result of attenuating of plasma glutamate level [11].

In the present study, OTC-treated tMCAO rats showed significant decrease in the infarct area size and improvement of the neurological outcome. This improvement was significantly associated with decrease in the glutamate and glycine with increase in the cysteine and GSH plasma levels. The cause of the significant attenuation of plasma glutamate level by OTC in this study is that OTC is an oxoproline analog acts as a competitive inhibitor of 5-oxoprolinase, preventing the formation of glutamate from oxoproline in the gamma-glutamyl cycle for GSH synthesis [39].

OTC is also a precursor for cysteine which is the rate limiting amino acid for GSH biosynthesis [14]. Providing cysteine by OTC could replenish GSH level in the present study. Anderson and Meister [13] showed that OTC is an efficient vehicle for the transport of cysteine into the brain cells to restore the depleted GSH level in many regions of the rat brain. The same was noticed in many cells such as hepatic cells and lymphocytes [40]. GSH counteracts the oxidative stress through a cycle called the redox cycle in which GSH reacts with the free radicals and oxidized to GSSG. GSSG in turn is reduced back to GSH by GSSG reductase at the expense of NADPH [41]. Severe oxidative stress in ischemia overcomes the ability of the cell to reduce GSSG to GSH, consequently cellular GSH is depleted [15]. OTC is considered to be neuroprotective in the current study due to its anti-oxidant effect via replenishing the depleted GSH.

The subsequent addition of glycine catalyzed by glutathione synthetase to generate glutathione in the brain [18] might partially participate in reducing glycine level by OTC in the current work. Also, it may be due to attenuation of the glutamate excitotoxicity and consecutive decrease of Ca^{2+}-dependent vesicular release of neurotransmitters [38].

CONCLUSION

The current work revealed neuroprotective influence of αKIC or OTC in experimental rat model of transient focal cerebral ischemia. To the best of our knowledge, the use of either agent in this model is a novel study. αKIC was involved in the glutamate/branched chain amino acid cycle in the brain with the result of increasing consumption of glutamate attenuating its excitotoxic effect. The neuroprotective of OTC was partially due to interference with the gamma-glutamyl cycle leading to attenuation of glutamate synthesis and also due to its antioxidant effect via replenishing of the depleted GSH level during cerebral ischemia/reperfusion. The use of these neuroprotective agents to prolong time window prior to reperfusion or to prevent reperfusion injury may represent a future therapeutic clinical application in ischemic stroke.

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