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POST ISCHEMIC ADMINISTRATION OF DIHYDROKAINATE, A GLT-1 BLOCKER FAILS TO ATTENUATE TRANSIENT FOCAL CEREBRAL ISCHEMIA EVOKED NEURODEGENERATION IN RATS

P. KALAIVANI[1,2], GIRISH RAMESH[3,1], R. BARATHIDASAN[1] AND C. SARAVANA BABU[1]*

[1]Centre for Toxicology and Developmental Research, Sri Ramachandra University, Porur, Chennai-600 116, Tamilnadu, India
[2]Department of Biochemistry, PRIST University, Vallam, Thanjavur-613 403, Tamilnadu, India
[3]Department of Nephrology, Sri Ramachandra University, Porur, Chennai – 600 116, Tamilnadu, India

ABSTRACT

Recently modulation of the GLT-1 transporter has been extensively focussed in various models of ischemic injury for evaluating neuroprotection. Therefore an attempt was made to study the neuroprotective effect of dihydrokainate (DHK) – a GLT-1 transporter blocker in transient focal cerebral ischemia model of Sprague Dawley rats during post ischemic phase. 2 h of ischemia – 70 h of reperfusion significantly induced neurological deficit, elevated glutamate levels, depleted bioenergetics levels (NAD^+ and ATP), displayed heinous oxidative stress (increase of nitrite/nitrate and TBARS content, reduced glutathione and superoxide dismutase levels), increased expression of PARP1, Caspase-3 and decreased expression of SIRT1 along with neuronal damage with decreased nissl positive cells. Post ischemic administration of DHK significantly reversed neurological deficits; however failed to reverse the biochemical alterations, bioenergetics levels and expression of bioenergetics regulators. Based on our present findings, the authors concluded that post ischemic phase is not the suitable therapeutic regimen for DHK.

KEY WORDS: Dihydrokainate (DHK), Ischemia, GLT-1 transporter, neuronal damage, oxidative stress.

C. SARAVANA BABU
Centre for Toxicology and Developmental Research, Sri Ramachandra University(SRU), Porur, Chennai-600 116, Tamilnadu, India

INTRODUCTION

Glutamate is the major excitatory amino acid which plays a major role in homeostasis and is compartmentalized in neurons; however the rise of glutamate in the extracellular region produces neurotoxic[1]. Cerebral ischemia/stroke is the second most common disease after ischemic heart disease in most part of the world[2, 3]. Glutamate induced excitotoxicity and ionic imbalance are the majority of the events happening upon ischemic induction. Ischemic induction mainly restricts the blood flow which might lead to the restriction of oxygen and glucose supplement to important brain regions. The induced energy crisis activates the release of glutamate and other neurotransmitters like serotonin, dopamine and aspartate mediated by the Ca^{2+} dependent channels from the presynaptic terminal, which follows biochemical alterations and energy depletion leading to increased extracellular glutamate, following subsequent activation of calcium influx into the post synapse N-methyl-D-aspartate receptors (NMDARs) activation, ultimately leading to cell death[4,5,6,7]. Hence in order to protect the neurons from death, the excess glutamate has to be removed by the neurons / astrocytes itself. Glutamate uptake is mainly regulated by five known sodium dependent transporters namely Excitatory Amino Acid Transporter 1 or Glutamate Aspartate Transporter and Excitatory Amino Acid Transporter 2 or Glutamate transporter 1 (GLT-1). GLT-1 predominantly achieves 90% glutamate uptake in adult forebrain[8]. So, Modulation of the GLT-1 transporter has been in prime focus for the treatment of ischemia. Previous studies have focussed on evaluating the neuroprotective effects of ischemic insult at different time points, mostly during pre ischemic or during the ischemic condition using various anion channel blockers, NMDAR blocker etc and shown promising findings[9,10,11,12,13]. However, no reports have shown the post ischemic treatment with any class of compounds. Previous studies reported that GLT-1 mediates neuroprotection in animal models of stroke[7,11,14]. Hence this present study was carried out to evaluate the neuroprotective effects of dihydrokainate (DHK), a GLT-1 blocker during post-ischemic phase in transient focal cerebral ischemic model in SD rats.

MATERIALS AND METHODS

Chemicals & Reagents

Dihydrokainate was procured from Sigma, St. Louis. MO., USA. Primary antibodies for SirT1, Parp1, Caspase 3 and β-actin were purchased from Santa Cruz Biotechnology, Inc, Santa Cruz, CA. The BCIP/NBT substrate for the alkaline phosphatase kit was obtained from GeNei™, Mumbai, India. 4/0 nylon monofilament (Ethicon®) was procured from Pharmacy of Sri Ramachandra University, Chennai. All other chemicals, reagents and solvents were purchased from SISCO Research Laboratories, Mumbai, India and were of analytical grade.

Animals, husbandry and ethics approval

Male Sprague Dawley rats (270-320g) were obtained from the Central animal facility, Sri Ramachandra University, Chennai, Tamilnadu, India. Animals were housed individually in polypropylene cages in a well-ventilated room (air cycle: 15 changes/h; recycle ratio 55:45) under an ambient temperature of 22±3°C and 30–70% relative humidity, with an artificial photoperiod of 12h light/dark cycle. Animals were fed with a standard rodent pellet (Provimi Animal Nutrition Pvt. Ltd., India) and purified water *ad libitum* (RiOs Water Purification Systems, Millipore, USA). Animals were acclimatized for a period of 7 days to the laboratory conditions prior to the initiation of the study. The animals were housed, cared and experimented in accordance with the National Institute of Health guidelines to the care and use of laboratory animals[15]. The study was approved by Institutional Animal Ethical Committee (IAEC/XXXXII/SRU/400/2015).

Experiment design

The animals were divided into three groups (n=12).

Group I: Sham Operated rats (0.9% saline as a vehicle; 10ml/kg)

Group II: 2h Ischemia + 70h reperfusion (0.9% saline as a vehicle; 10ml/kg)

Group III: 2h Ischemia + 70h Reperfusion [DHK treated with DHK 3h after the onset of reperfusion (Post-ischemic 3h after)]
DHK dose was fixed based on the earlier findings [16]. DHK (10 mg/kg) was dissolved in 0.9% saline and administered intraperitoneally.

Middle cerebral artery occlusion (MCAO) Surgical Procedure

Transient focal cerebral ischemia was induced in rats by middle cerebral artery occlusion (MCAO) method as described earlier[17] with minor modifications. Rats were anaesthetized with chloral hydrate (350mg/kg, i.p) and right common carotid artery was exposed to the level of external and internal carotid artery bifurcation. 4-0 nylon monofilament was used and its tip was made round-headed by exposing it to flame. The filament was coated with 0.01% poly-l-lysine and inserted into the external carotid artery and advanced to the internal carotid artery for a length of about 20–21mm until a slight resistance was felt. After occlusion, the filament was held in place with a ligature and skin was temporarily sutured. After 2h of ischemia, the rats were again anaesthetized, suture was released, and the filament was withdrawn to establish ischemic reperfusion (I/R) that was visually ensured. Throughout the surgical procedure, body temperature was monitored by inserting a rectal thermometric probe (Harvard Apparatus, USA) and maintained at 37±1°C by a thermostatically controlled heating blanket (Harvard Apparatus, USA). The animals were then housed in a cage with heating lamp to maintain the temperature at 29±1°C for another 1h to counteract any possible hypothermia effect. In the sham operated (SO) group, external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted. Postoperative care was provided to all the experimental animals following procedures described[15].

Assessment of neurological deficit

An evaluator blinded to experimental design was assigned to perform the procedure. Neurological deficit scoring was performed as previously described[18]. Animals from group I, II, and III were scored for neurological deficits as follows.

Score 0 — no apparent neurological deficits
Score 1 — contralateral forelimb flexion
Score 2 — decreased resistance to lateral push
Score 3 — spontaneous movement in all directions and contralateral circling when lifted by tail
Score 4 — spontaneous circling

Estimation of Glutamate

Cortical, striatal and hippocampal tissues were homogenized in 0.1N hydrochloric acid in 80% ethanol (for every 10mg tissue / 200µl) in manual homogenizer. Homogenates were transferred to polypropylene tubes and centrifuged at 4500 rpm for 20 min. Glutamate content in the supernatant was estimated using high performance thin layer liquid chromatography (HPTLC, CAMAG – version 1.3.4, USA)[19].

Estimation of Glutamate synthetase activity

Cortical, striatal and hippocampal tissue were homogenized with 10% KCl in manual homogenizer and used for the estimation of glutamine synthetase activity as described previously [9]. The intensity of blue colour was read at 640 nm using a spectrophotometer (Perkin Elmer, λ 25, USA) against a blank that contained all the reagents without the supernatant. Results were expressed in nano moles of inorganic phosphorus liberated/min/ mg of protein.

Estimation of NAD^+ and ATP content

Cortical, striatal and hippocampal tissue were homogenized with 10% KCl in manual homogenizer and used for the estimation of ATP and NAD^+ level as described previously[20] with slight modifications. Aliquots of homogenates were sonicated immediately in ice-cooled per chloric acid (0.1N) to inactivate ATPases. After centrifugation (14,000 g, 4°C, 5 min), supernatants were neutralized with 1N sodium hydroxide and stored at – 80°C until taken for analysis. NAD^+ level in supernatants were quantified using a reversed-phase high performance liquid chromatography (RP-HPLC) (Perkin Elmer, India). RP-HPLC determination was performed on a reversed-phase Hypersil C18 (4.6mm×250 mm, 5µ) column (Elite, Dalian, China) attached to two LC-10ATvp pumps (Shimadzu, Kyoto, Japan),

equipped with UV–Vis detector. The mobile phase was 100mM KH2PO4–K2HPO4 buffer solution (pH 6.0), the flow rate was 1.2ml/min, the column temperature was 25°C and the detection wavelength was 254 nm. Reference solution of NAD^+ and ATP was used and prepared for calibration (Sigma, St. Louis, MO, USA).

Oxidative stress markers

Oxidative stress markers, namely Nitrate/Nitrite; Reduced glutathione; Superoxide dismutase content were estimated in the cortical, striatum and hippocampus tissues as described previously [21, 22, 23]. The extent of lipid peroxidation (LPO) was estimated by measuring the levels of thiobarbituric acid-reactive substances (TBARS) as described previously [24]. The total protein content was estimated by Lowry's method [25].

Estimation of PARP1, SirT1 and Caspase 3

Tissue was homogenized in lysis buffer containing 0.1M sodium chloride, 0.01M Tris buffer, and 0.1mM ethylene diamine tetra acetic acid, and the protease inhibitors such as chymostatin 2mg/ml, leupeptin 2mg/ml, pepstatin 2mg/ml, and phenyl methyl sulfonyl fluoride 100mg/ml. Lysates were centrifuged at 14,000 rpm for 30 min at 4°C and boiled in loading buffer for 3 min. 50µg of protein samples were loaded on a 12% sodium lauryl sulphate-polyacrylamide gel (SDS-PAGE), separated electrophoretically, and transferred to a Polyvinylidene difluoride (PVDF) membrane (Millipore Corp) using the semi dry transfer unit (Hoefer, USA). The transferred membrane was incubated overnight in 5% skimmed milk powder and then washed with 1X PBS (phosphate buffered saline with tween 20) for 3-5 times. Primary antibody against β-actin, SirT1, Parp1 and caspase 3 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 1:500 dilution was added and incubated at room temperature for 1 h. Following incubation, it was washed three times in PBS containing 0.1% Tween 20 (PBST), the secondary antibody was then added at 1:2000 dilutions for 1 h. Then it was washed in PBST for 3 times over 25 min and then incubated in substrate buffer containing 4M sodium chloride, 1M magnesium chloride and 1M Tris buffer. Following 10 min incubation, the membrane was incubated in dark with chromogen substrate. The bands were photographed and semi quantified by densitometry using Bio ID software in gel documentation unit (Vilber Loumart, France).

Histopathological Examination

Coronal sections of the rat brains (5µm thickness) were cut and stained with Cresyl violet for semi-quantitative evaluation of neuronal degeneration[26,27] and cell loss in the ipsilateral hemispheres of cerebral cortex, CA1 region of hippocampus and corpus striatum at 400X magnification using Motic Images assisted light microscopy (Motic DMB1 – 2MP, China). Grading of lesions in brain regions were scored as follows.

0-10%: No morphological changes and few dark stained cells - Score 1

11-30%: Mild oedema or dark neurons or pyknotic cells - Score 2

31-50%: Moderate number of dark neurons - Score 3

51 -70%: Moderate edema, necrosis and severe morphological changes - Score 4

71-100%: Severe oedema, necrosis and infarction - Score 5

The semi-quantitative grading of cerebral I/R injury was expressed as percentage of nissl positive cells.

Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Mean difference between the groups for biochemical analysis were analysed by one way ANOVA followed by Tukey's multiple comparison test and neurological scoring by Mann-Whitney U test using Graphpad Prism 4.0 (San Diego, USA) software. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of DHK on neurological deficit

The postural reflex test scores measured after 3 days of MCAO ischemic rats treated with DHK at post ischemia/ reperfusion (I/R) is shown in Figure 1. A significant increase (*p<0.01*) in the neurological deficits was observed in I/R rats at 72h when compared to Sham Operated (SO) rats. Post-ischemic treatment with DHK significantly (*p<0.01*) decreased the neurological score compared to vehicle treated I/R.

Fig. 1. Neurological Deficit

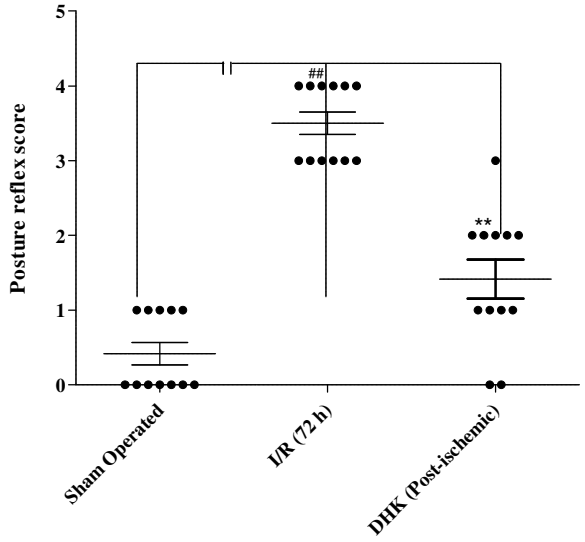

Figure 1

Effect of Dihydrokainate (DHK), on neurological score in I/R induced rats. Values were expressed as mean ± SEM (n =12); ## - p< 0.01 Vs sham operated; ** - p<0.01 Vs I/R

Effect of DHK on glutamate content

A significant elevation in the glutamate level was observed in the ipsilateral cortex (*p<0.01*), striatum (*p<0.01*) and hippocampus (*p<0.01*) regions of I/R rats when compared to the SO group. Post-ischemic administration of DHK altered these changes in all the regions when compared to vehicle treated I/R rats but not to the significant level (Table 1).

Treatment	Glutamate content (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	2.43±0.09	3.43±0.08	2.24±0.07
I/R	6.98±0.65$^{##}$	9.94±1.03$^{##}$	8.66±0.13$^{##}$
Post ischemic treatment with DHK	5.46±0.17	8.35±0.12	8.29±0.09

Table 1

Effect of DHK on Glutamate level in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p< 0.01 Vs sham operated

Effect of DHK on glutamate synthetase activity

Ischemic/ reperfusion injury increased the GS activity in ipsilateral cortex (*p<0.01*), hippocampus (*p<0.01*) and striatum regions (*p<0.01*) in comparison with SO rats ($p<0.01$). Post- ischemic treatment with DHK did not attenuate the I/R effects in all the regions in comparison to I/R treated group (Table 2.).

Treatment	Glutamine synthetase activity (nmol of inorganic Phosphorus liberated /min /mg protein)		
	Cortex	Striatum	Hippocampus
Sham operated	2.31±0.12	2.05±0.11	2.51±0.09
I/R	4.53±0.09$^{##}$	4.92±0.13$^{##}$	5.13±0.13$^{##}$
Post ischemic treatment with DHK	4.30±0.10	4.80±0.17	4.80±0.15

Table 2

Effect of DHK on Glutamine Synthetase Activity in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p< 0.01 Vs sham operated

Effect of DHK on NAD$^+$ content

I/R rats exhibited a significant decrease in NAD^+ content in the ipsilateral cortex (*p<0.01*), striatum (*p<0.01*) and hippocampal (*p<0.01*) regions in I/R group in comparison to the SO rats. However, no change in NAD^+ was observed in post ischemic treatment with DHK when compared to vehicle treated I/R rats (Table 3).

Treatment	NAD^+ content (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	17.04±0.10	18.19±0.56	19.38±0.84
I/R	8.49±0.79$^{\#\#}$	9.96±0.21$^{\#\#}$	7.33±0.38$^{\#\#}$
Post ischemic treatment with DHK	8.79±0.51	7.96±0.42	6.31±0.51

Table 3

Effect of Dihydrokinate on NAD$^+$ level in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p<0.01 Vs sham operated

Effect of DHK on ATP content

The effect of DHK on brain ATP content in the experimental groups is represented in Table 4. When compared to the SO group, ATP content was found to be significantly decreased in the ipsilateral cortex (*p<0.01*), striatum (*p<0.01*) and hippocampal (*p<0.01*) regions of the I/R group. Administration of DHK in post-ischemic phase failed to increase the ATP content when compared with vehicle treated I/R group.

Treatment	ATP content (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	15.05±0.64	13.41±0.28	16.93±0.38
I/R	8.66±1.20$^{\#\#}$	6.45±0.48$^{\#\#}$	8.83±0.56$^{\#\#}$
Post ischemic treatment with DHK	8.14±0.26	7.32±0.95	8.14±0.46

Table 4

Effect of DHK on ATP level in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p< 0.01 Vs sham operated

Effect of DHK on SOD activity

A significant decrease in SOD activity was observed in the ipsilateral cortex (*p<0.05*), striatum (*p<0.01*) and hippocampus (*p<0.01*) of the I/R rat brain when compared to the Sham operated group. Post ischemic treatment with DHK did not reverse the I/R induced changes significantly as shown in Table 5.

Treatment	SOD (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	4.24±0.21	4.11±0.45	4.83±0.41
I/R	0.57±0.09$^{\#\#}$	0.47±0.11$^{\#\#}$	0.44±0.14$^{\#}$
Post ischemic treatment with DHK	1.43±0.20	1.81±0.15	1.52±1.26

Table 5

Effect of Dihydrokinate on SOD level in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p<0.01 Vs sham operated

Effect of DHK on GSH content

The effect of DHK on brain GSH content is shown in Table 6. A significant decrease of GSH content in ipsilateral cortex (*p<0.01*), striatal (*p<0.01*) and hippocampal (*p<0.01*) region was observed in the I/R rat brain when compared to the SO rat brain. Post-ischemic administration of DHK produced no significant change in cortex, striatum and hippocampus in comparison to vehicle treated I/R rats.

Treatment	GSH (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	10.13±0.41	10.42±0.83	10.36±0.43
I/R	5.14 ±0.12##	5.78±0.11##	5.32±0.25##
Post ischemic treatment with DHK	4.43±0.09	4.55±0.09	5.66±0.17

Table 6

Effect of DHK on GSH content in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p < 0.01 Vs sham operated

Effect of DHK on LPO activity

Induction of ischemia and reperfusion significantly altered the LPO levels in the different brain regions of the experimental rat brain. A significant elevation in TBARS content was observed in the ipsilateral cortex (*p<0.01*); striatum (*p<0.01*); hippocampus (*p< 0.01*) of the I/R rat brain when compared to the SO rat brain. Post ischemic treatment with DHK reduced the TBARS content in all the brain regions but not to significant level in comparison to vehicle treated I/R group (Table 7).

Treatment	TBARS (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	2.70±0.95	2.92±1.57	2.78±1.29
I/R	10.37±1.05##	10.58±1.03##	10.86±1.03##
Post ischemic treatment with DHK	8.11±1.01	7.59±0.42	8.46±1.01

Table 7

Effect of DHK on TBARS content (LPO activity) in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p<0.01 Vs sham operated

Effect of DHK on nitrate / nitrite content

The effect of DHK on brain Nitrite content is shown in Table 8. A significant increase in ipsilateral cortex (*p<0.01*), striatal (*p<0.01*) and hippocampal (*p<0.01*) nitrite content was observed in the I/R rat brain when compared with the Sham Operated group. An insignificant decrease in nitrate/nitrite content in cortex, striatum and hippocampus region was observed in post-ischemic DHK treated group in comparison to vehicle treated I/R group.

Treatment	Nitrite/nitrate (nmol/mg tissue)		
	Cortex	Striatum	Hippocampus
Sham operated	179.04±9.42	191.43±5.81	166.02±8.19
I/R	303.98±6.09##	290.54±8.53##	294.07±9.62##
Post ischemic treatment with DHK	271.71±10.31	279.38±5.99	281.95±9.57

Table 8

Effect of DHK on Nitrate/Nitrite content in various brain regions following I/R. Values were expressed as mean ± SEM (n =3); ## - p<0.01 Vs sham operated

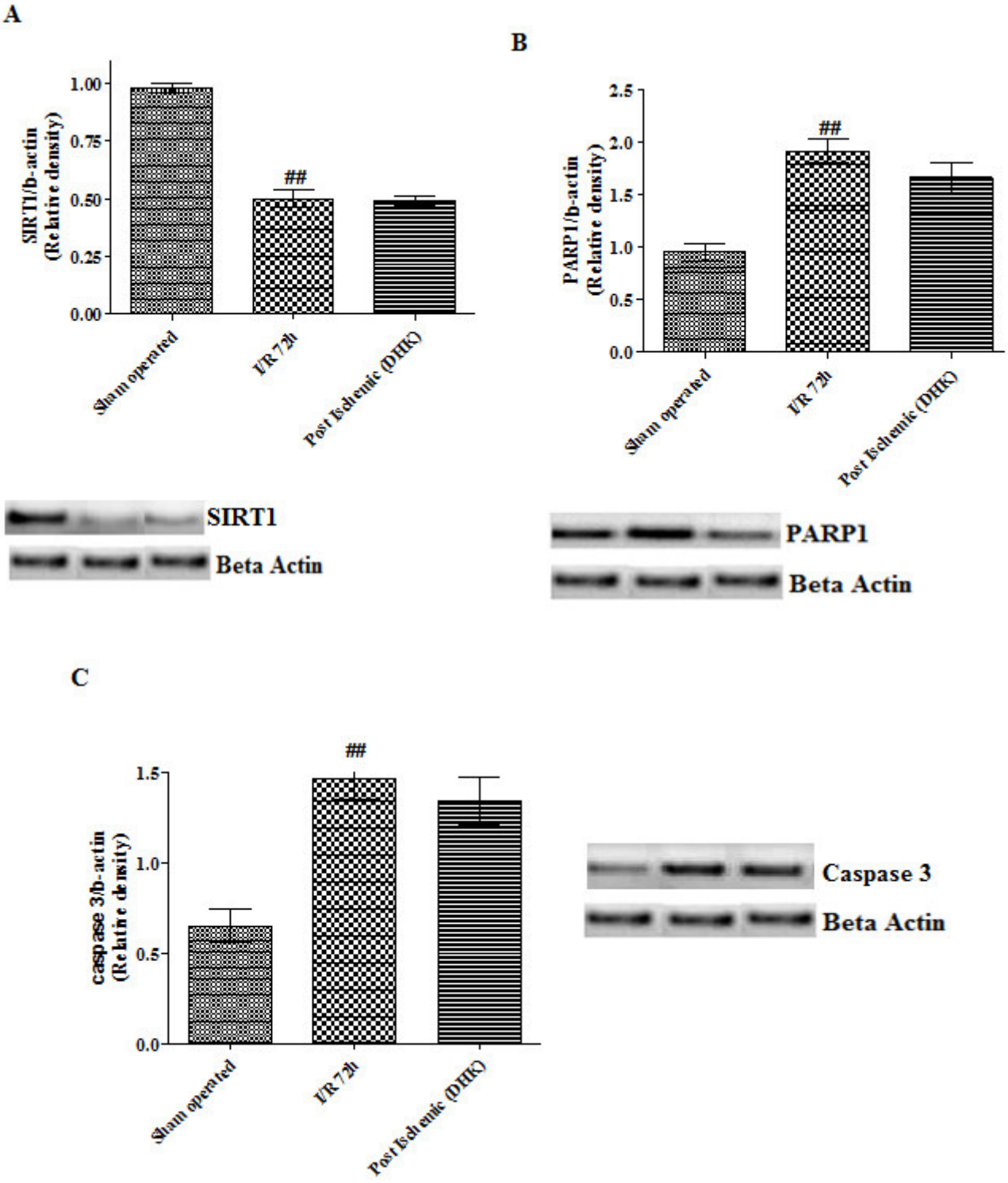

Figure 2

Western blot analysis. A. SIRT1, B. PARP1 and C. Caspase 3. Values were expressed as mean ± SEM (n =3); ## indicates p<0.01 Vs sham operated

Effect of DHK on expression of SIRT1, PARP1 and Caspase 3

A significant increase in the expression of PARP1 and Caspase 3 was observed in ischemia/ reperfusion induced group (*p<0.01*), while a significant decrease in the expression of SIRT1 (*p<0.01*) in comparison to sham operated group. Post ischemic administration of DHK neither lowered the expression levels of PARP1 and caspase 3 nor elevated the expression of SIRT1. Western blot analysis of protein is illustrated in Figure 2.

Histopathological examination

The neuroprotective effect of DHK treatment during the post ischemic phase of ischemia was evaluated using cresyl violet staining method. Cresyl violet is a basic dye that stains the negatively charged nucleic acids. The nissl bodies appear purple-blue in colour and the nucleus appears light blue. Viable neuronal cells are said to contain intact nissl bodies. The representative photographs of cresyl violet stained sections of the striatum, cortex and hippocampus obtained from DHK treatment during post ischemic phase is shown in Figure 3. In the SO group, the structure of Nissl bodies were observed with intact nuclei and evenly distributed in the cytoplasm. In the I/R group, a decline in neuronal density, diffused disappearance of nissl bodies and swollen cell ruptures were observed. A significant decrease in percentage of nissl positive cells was observed in the ipsilateral cortex ($p< 0.01$); striatum ($p < 0.01$); hippocampus ($p < 0.01$) of the I/R rat brain when compared to the SO rat brain. Post- ischemic treatment with DHK could not preserve neuronal population at a higher percentage compared to I/R induced group. No significant change in percentage of nissl positive cells was observed in post ischemic treatment of DHK was observed when compared to vehicle treated I/R rats (Figure 4).

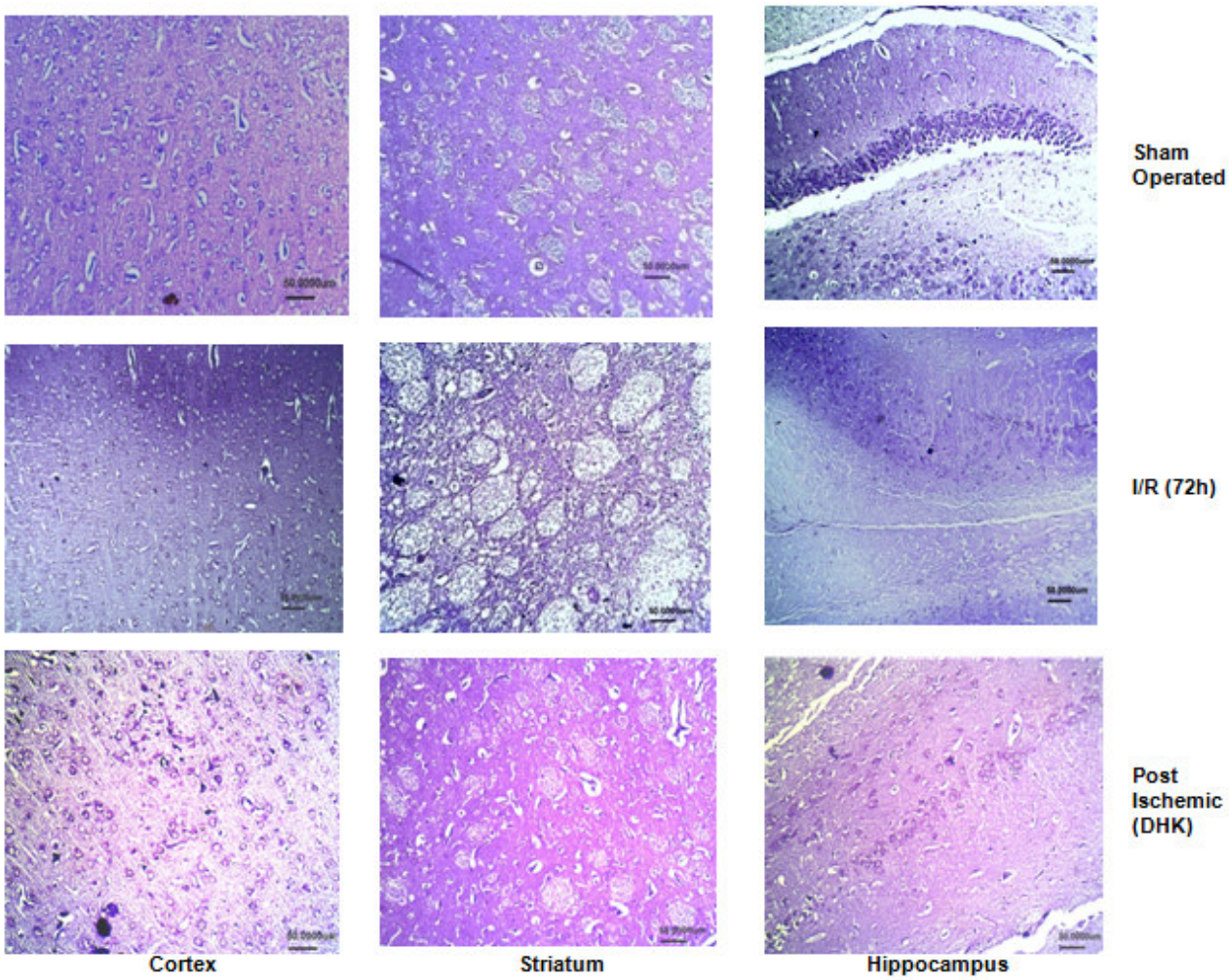

Figure 3

Histopathology. Cresyl violet staining in cortex, striatum and hippocampus region of Sham Operated, I/R and DHK treatment in rat brain at 400 X magnification.

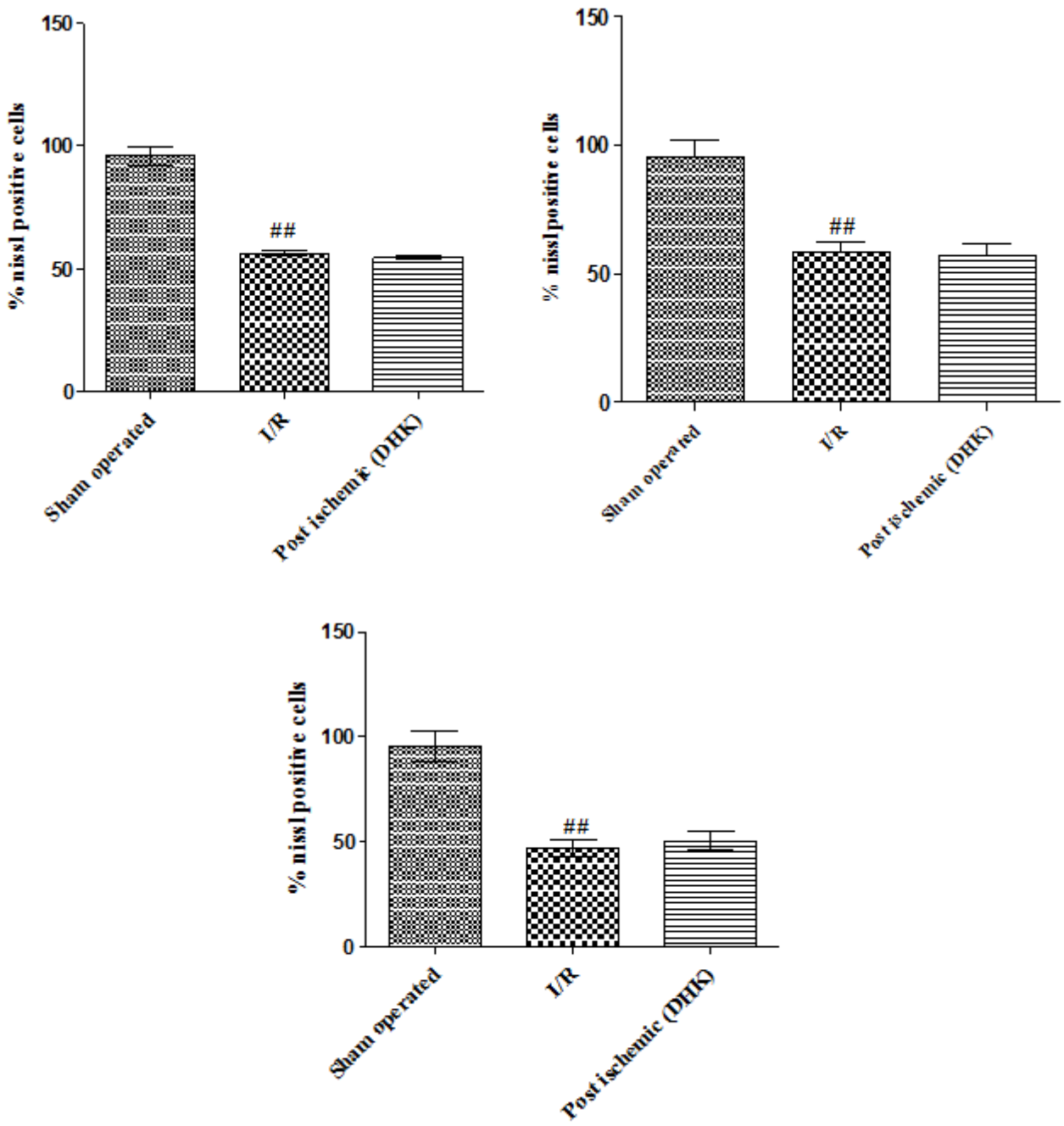

Figure 4

Effect of DHK on % Nissl positive cells in various brain regions following I/R. Values were expressed in mean ± SEM (n =3); ## indicates p<0.01 Vs sham operated

DISCUSSION

Recent researches have thrown light on glutamate transporter, which is emerging as a potential therapeutic target in a wide range of neurological disorders. Glutamate transporter dysfunction has been shown to play a role in various neurological disorders like Alzheimer's disease, traumatic brain injury, epilepsy and ischemia/stroke[7,28]. Hence manipulation of glutamate transporter might have a therapeutic role in curing these disorders. Therefore, the present study was undertaken to evaluate the neuroprotective effects of DHK, a potential GLT-1 blocker, during post-ischemic phase in rat model of

cerebral ischemia / reperfusion in order to explore the best therapeutic time phase for treating stroke. Various parameters like neurological deficit, glutamate content, ATP, NAD^+ content, glutamine synthetase activity, oxidative markers such as superoxide dismutase, reduced glutathione content, lipid per oxidation activity, nitrite/nitrate content and histopathological examination have been considered for investigating the neuroprotective effects of DHK during post ischemic phase. In our earlier studies, marked neurological deficits were observed at 24 h following ischemic insult [29]. On this basis, we evaluated the neurological deficit at 24 h and terminated the experiment at 72 h. Neurological deficit was significantly attenuated in post ischemic administration of DHK when compared with vehicle treated I/R group, which illustrates that DHK could reverse the ischemic effects with respect to behaviour. The total glutamate content has acutely risen under ischemic condition when compared with sham operated group from the time point of middle cerebral artery occlusion. Post ischemic administration of DHK could not protect the glutamate excitotoxicity in comparison to I/R treated group. Previous studies with NMDA blocker, memantine also could not protect the glutamate rise during post ischemic phase[9,30], yet the reasons behind is still unclear. Impaired glutamate homeostasis of glutamate during stroke leads to neuronal death [31]. In our study, histopathological evidences revealed that ischemic insult reduced the number of nissl positive cells and produced more neuronal cell damage. This could be because of the excess of glutamate uptake by NMDA receptors. We expected that DHK during post ischemic phase could reverse the effects, however it could not. Previous studies reported that excitotoxicity, inflammation, apoptosis are the usual characteristics involved in ischemic neuronal cell death[32]. Altered bioenergy metabolism in neuronal cells is believed to play a vital role in the neuronal dysfunction and degeneration that occurs in cerebral stroke. In general, neurons require a large amount of energy to survive and maintain their functional homeostasis. The energy demand of neurons will get increased upon glutamate excitotoxicity. During stroke, there is an excessive release of glutamate from the presynaptic vesicles. Calcium dependent glutamate activation, upon depolarization, get released from their synaptic vesicles through glutamate transporters in the neurons leads to accumulation of glutamate in extracellular space and NMDA receptors gets activated and uptake excess glutamate resulting in disruption of cellular ion homeostasis. Since ATP is the principle source of energy, available ATP will be utilized due to glutamate overload in extracellular region of neuron. In our study also, glutamate activation caused a depletion in the ATP content in the striatum, cortex and hippocampus tissue, which was confirmed by analysis. NAD^+ levels are important for regulating various metabolic reactions like glycolysis, TCA cycle and mitochondrial phosphorylation, which are involved in the restoration of ATP levels. Prolonged ischemia followed by 70 h of reperfusion depleted NAD^+ levels in the present study. Decreased ATP levels lead to the collapse of ion gradients resulting in cellular oxidative stress. Excessive glutamate accumulation over-activates NMDA receptor causing an influx of Ca2+ ions, resulting in cellular oxidative stress that might lead to neuronal death[33]. In the present study, significant elevation of oxidative stress markers like superoxide dismutase, lipid per oxidation activity, nitrite/nitrate content and reduction in glutathione content was observed. Glutathione synthesis is mainly regulated by glutamate in astrocytes[34]. Post ischemic treatment with DHK showed no signs of restoration of oxidative stress, ATP levels and NAD^+ levels. Glutamine synthetase (GS) is also considered a critical enzyme for the regulation and scavenging of extracellular glutamate in the brain. The post-ischemic increase in astrocytic GS activity suggests that the capacity to take up glutamate and to convert glutamate to glutamine is enhanced after ischemia[7,35]. In our study, post ischemic treatment with DHK showed increase GS activity, which might illustrate that enhanced GS activity, is trying to compensate the glutamate increase in extracellular space. NAD^+ plays a predominant role in regulation of various enzymes involved in cell survival,

signalling and death. Poly (ADP - ribose) polymerase 1 (PARP1) activation and caspase pathway are well known to play a role in apoptosis[36]. SIRT1 and PARP1 regulation mainly depend upon the level of NAD^+ in the brain and it helps in protection against excitotoxicity. Previous studies have reported that PARP 1 is a crucial mediator of cell death in excitotoxicity, ischemia and oxidative stress. PARP1 has an important role in DNA repair; however, excessive PARP1 activation can lead to cell death[37]. In the present study, ischemia reperfusion resulted in elevated expression of PARP1 and caspase 3 and reduced SIRT1 expression which was evident from western blot and it showed that ischemia resulted in DNA damage thereby mediating cell death. Post ischemic treatment with DHK did not alter the expressions of PARP1, SIRT1 and caspase3.

CONCLUSION

Based on the present study findings, the authors suggest that treatment with DHK during post ischemic phase could not attenuate any changes happened due to I/R insult. It can be concluded that DHK treatment during post ischemic phase is not the best therapeutic regimen for the treatment of focal cerebral ischemia / stroke.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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