

PROTECTIVE EFFECT OF PIFITHRIN-ALPHA ON BRAIN ISCHEMIC REPERFUSION INJURY INDUCED BY BILATERAL COMMON CAROTID ARTERIES OCCLUSION IN GERBILS

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Abstract : Involvement of p53 has been implicated in apoptosis induced cell death in ischemic reperfusion injury. In the present study, we have investigated neuroprotective potential of pifithrin-alpha, a p53 inhibitor in bilateral common carotid arteries occlusion (5 min) model of global cerebral ischemia in Mongolian gerbils. Gerbils were treated with pifithrin-alpha 3 mg/kg, ip. 30 min prior to occlusion. There was a significant increase in neurological symptoms and locomotor activity in ischemic animals as compared with the sham-operated animals. Increase in neurological symptoms and locomotor activity was attenuated by pifithrin-alpha 3 mg/kg, ip. Significant increase in the number of the surviving neurons in the hippocampal CA1 pyramidal region was observed in ischemic animals treated with pifithrin-alpha 3 mg/kg, ip. This study demonstrates the neuroprotective effect of pifithrin-alpha in global cerebral ischemia in gerbils.

Key words : pifithrin-alpha global cerebral ischemia gerbil
hippocampus neuroprotection p53

INTRODUCTION

Global cerebral ischemia is a clinical outcome occurring as a consequence of conditions like cardiac arrest, coronary artery bypass surgery causing deprivation of blood supply and energy in the brain due to

blockade of carotid arteries. The most vulnerable region for loss of blood supply is the CA1 pyramidal neurons of the hippocampus region (1). The neurodegeneration process in hippocampus is multifactorial which involves decrease in intracellular pH and ATP levels, increased levels of

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extracellular glutamate, elevated intracellular levels of calcium and increased generation of free radicals (2). Oxidative stress can activate apoptotic pathways in cerebral ischemia (3-6) and can lead to DNA damage initiating the activation of transcription factor p53 and activation of caspases (7).

The tumour suppressor and transcription factor p53 plays a central role in the regulation of cell cycle arrest and apoptosis (8). The factors that cause p53-mediated apoptosis involve mitochondrial impairment and increased oxidative stress (9). p53 induces apoptosis by the control of translation of various genes. This includes upregulation of pro-apoptotic genes bax, p53 inducible genes, p21^{WAF}, DNA damage-inducible gene 45 (GADD45) and downregulation of anti-apoptotic gene bcl 2 (10, 11). Recent findings have implicated p53 in renal ischemic injury and in the death of neurons observed in Parkinson's disease and cerebral ischemic injury (8, 9, 11, 12).

In the present study, we have investigated neuroprotective potential of pifithrin-alpha; a novel inhibitor of p53 in bilateral carotid artery occlusion (BCAO) induced global cerebral ischemia in Mongolian gerbils.

METHODS

Animals

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60-80 g were obtained

from Central Animal Facility (CAP), NIPER for this study. They were provided with standard diet and water *ad libitum* and were maintained at $22 \pm 2^\circ\text{C}$ and a 12:12 h light:dark cycle. All the procedures used in this study were approved by Institutional Animal Ethics Committee, NIPER.

Treatment schedule

Gerbils were divided into sham, ischemic and pifithrin-alpha treated group. Pifithrin-alpha was administered intraperitoneally 30 min before occlusion at a dose level of 3 mg/kg.

Induction of transient global cerebral ischemia

Transient global cerebral ischemia was induced according to the method of Gupta and Sharma, 2006 (13). Surgery was always carried out between 8:00 a.m. and 1:00 p.m. Overnight fasted gerbils were anesthetized with 2.0% halothane in a gas mixture of 70% N₂O and 30% O₂, followed by maintenance at 1.5% halothane (Gas anesthesia system, Harvard Apparatus). During occlusion anesthesia was maintained between (0.5–1%) halothane. Rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with homeothermic blanket control unit (Harvard Apparatus U.K.). Ventral neck incision of 2 cm was made; the left and right common carotid arteries were separated carefully from vagus nerve and were occluded simultaneously for 5 min with bulldog clamps. Five min later, clamps were removed and reflow was verified visually. Then the neck incision area

was sutured and iodine tincture was applied. The gerbils were kept under heating lamp for recovery to prevent post-ischemic hypothermia. Sham-operated non-ischemic animals underwent same surgical procedures, except common carotid arteries were not occluded.

Neurological symptoms

3 h after ischemia and reperfusion the gerbils were assessed for neurological symptoms, according to the stroke index as described by McGraw 1977 with some modifications, (14) which is as follows: no symptom = 0, hunched posture or hair roughed up = 1, ptosis = 2, circling behaviour = 3, splayed-out hindlimb = 4 and seizures = 5. Higher the score higher was the ischemic insult.

Locomotor activity

Spontaneous locomotor activity was recorded using Opto-Varimex (Columbus, Ohio 43204, USA). The instrument consisted of a perspex cage crisscrossed with infrared light sensitive photocells and record locomotor activity as a total count. Gerbils were placed individually in each cage and acclimatized for 5 min before the study. For the time-course study, locomotor activity of the sham-operated, ischemic and drug treated animals was assessed for 15 min at 1st and 4th day of reperfusion (15). Whole experiment was performed under dark sound attenuated and ventilated conditions. The readings were always recorded between 10:00 a.m. and 1:00 p.m.

Histological studies

The gerbils were euthanised 4 days after reperfusion by decapitation. The brains were removed, fixed in 10% formalin for 24 h and then paraffin embedded. Representative coronal sections (5 μ m) were obtained with the help of rotary microtome (Leica RM2145, Germany), which included dorsal hippocampus at the level of 1.5 mm to 1.7 mm posterior to bregma (16) and stained with 1% acid fuchsin and 1% celestine blue. CA1 neurons with blue staining, intact round shaped nuclei were viable neurons, while red stained, pyknotic cell shaped and nuclear condensed were damaged neurons. Bilateral counts were done of either side of hippocampal region and a total of 8–10 images were captured for each animal for taking average. CA1 cell counting of viable neurons was done at a magnification of 400X using a CCD camera attached microscope (Leica, Germany) in the medial portion of CA1 of dorsal hippocampus by an observer blind to the treatment condition. The cell counting was expressed as % of normal cells per mm as compared with sham group (17, 18).

Statistical analysis

Statistical analysis was done using Sigma Stat 2.0 software. Neurological score parameter was analysed using unpaired t-test followed by non-parametric Mann-Whitney U test, represented as Median \pm 95% confidential limits. Locomotor activity parameter was analysed by Two-way analysis of variance (ANOVA) followed by

Bonferroni's multiple comparison t-test. Histological changes was analysed by one-way ANOVA followed by Bonferroni's multiple comparison t-test. Parametric data are reported as mean \pm S.E.M. Statistical significance was considered when $P < 0.05$.

RESULTS

Bilateral common carotid artery occlusion (BCAO) produced a significant increase in the neurological score (3 ± 0.78) and locomotor activity and significant ($P < 0.05$) decrease in the number of CA1 viable hippocampal neurons (84%) in ischemic animals when subjected to BCAO as compared with sham-operated animals. Pifithrin-alpha treatment significantly decreased neurological score (2 ± 0.97 , $P < 0.01$) (Fig. 1).

We observed significant increase in locomotor activity on 1st day and 4th day after global cerebral ischemia. Increased locomotor activity in ischemic animals was significantly ($P < 0.01$) attenuated on pifithrin-alpha treatment.

The dead cells as evident from photographs of histology (Fig. 3 and 4) were shrunken with red staining, while viable neurons were round shaped with blue staining. Pifithrin-alpha (3 mg/kg) treatment significantly increased the number of viable hippocampal CA1 neurons 35.48% ($P < 0.05$) respectively as compared to ischemic animals.

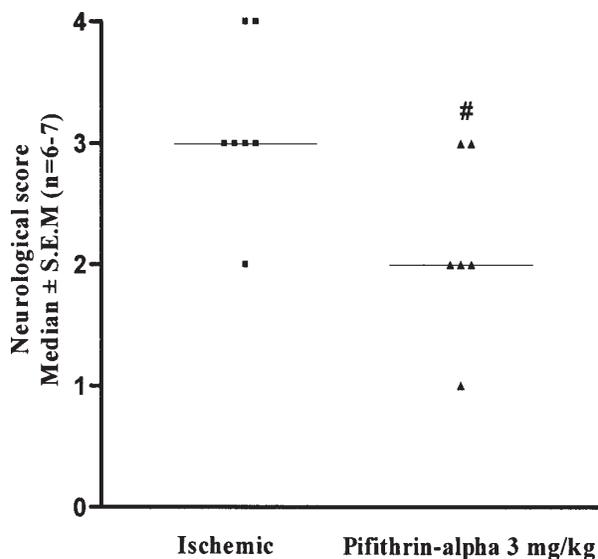


Fig. 1: Effect of pifithrin-alpha on neurological score in global ischemic gerbils. Neurological scores were assessed 3 h after ischemia and reperfusion. # $P < 0.01$ Vs ischemic group. All values are expressed as median \pm 95% confidential limits tested using unpaired t-test followed by non-parametric Mann-Whitney U test).

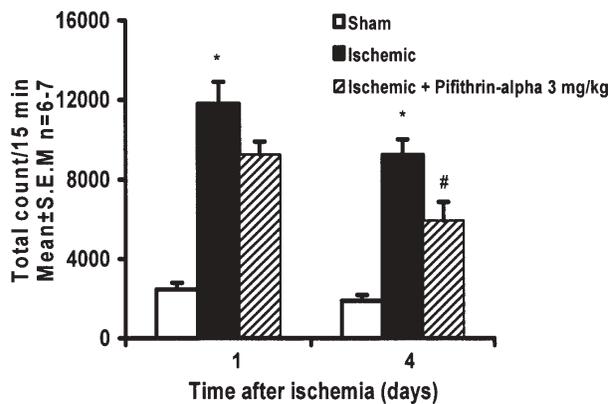


Fig. 2: Effect of pifithrin-alpha on locomotor activity in global ischemic gerbils. Locomotor activity was assessed on 1st and 4th days after reperfusion. * $P < 0.05$ of the respective group Vs sham-operated group, # $P < 0.05$ of the respective group Vs ischemic group. All values are expressed as mean \pm S.E.M (tested using Two-way ANOVA followed by Bonferroni's multiple comparison t-test).

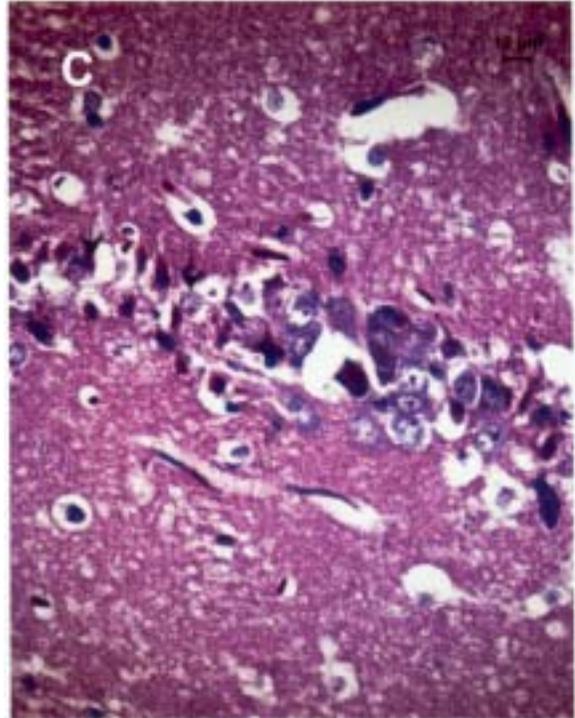
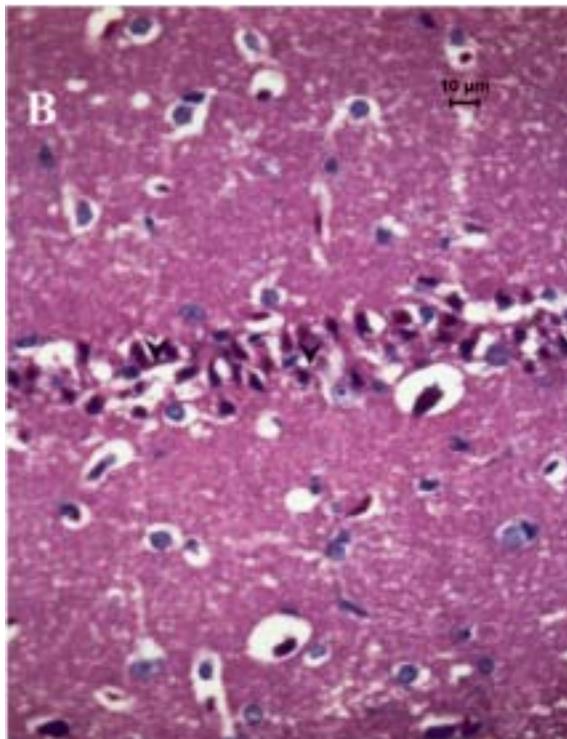
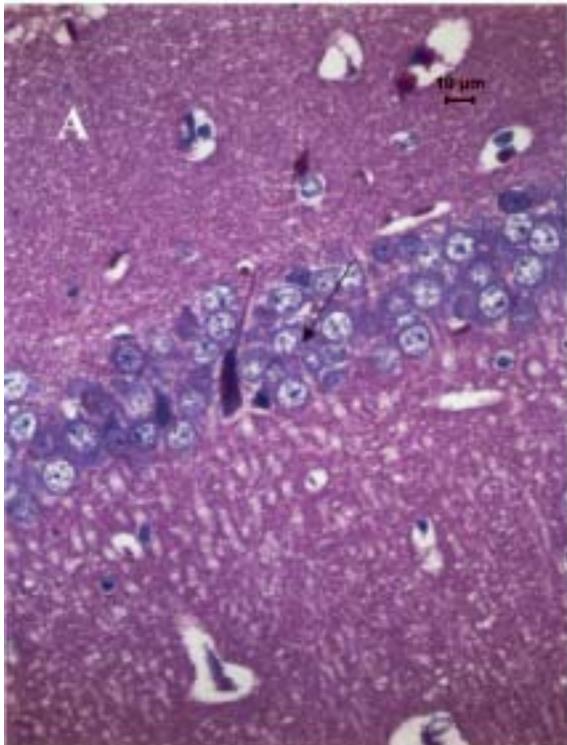


Fig. 3: Figures depict microphotographs of the hippocampal CA1 region in the gerbil 4 days after 5 min of global cerebral ischemia. The neurons are stained with Acid Fuchsin/Celestine blue. Arrows indicates blue stained round-shaped neurons as viable, while arrowheads indicate red stained shrunken neurons as dead. CA1 regions of (A) sham-operated; (B) ischemic; (C) pifithrin-alpha 3 mg/kg single administration Magnification (A-C) is at 40X.

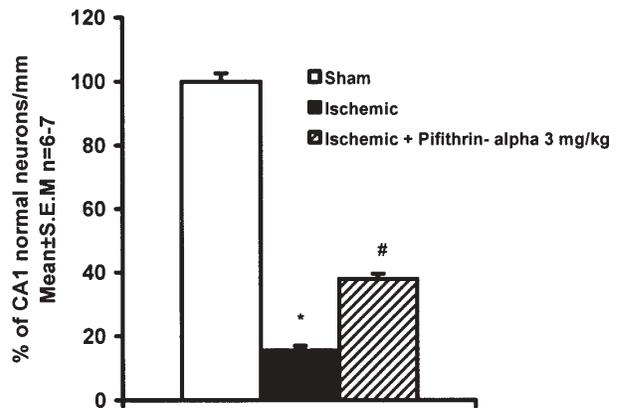


Fig. 4: Histological analysis of sham-operated, ischemic and pifithrin-alpha treated gerbils when subjected to 5 min of BCAA, assessed 4 days after reperfusion. *P<0.001 of the respective group Vs sham-operated group. #P<0.01 of the respective group Vs ischemic group. All values are expressed as mean ± S.E.M. (tested using ANOVA followed by Bonferroni's multiple comparison t-test).

DISCUSSION

In the present study, pifithrin-alpha, a p53 inhibitor produced significant neuroprotection in Mongolian gerbils. Gerbils were used for inducing global ischemia. Gerbil shows features of global cerebral ischemia just after brief occlusion of common carotid arteries, as they lack posterior communicating arteries between the carotid arteries and vertebral arteries, which constitute an incomplete Circle of Willis (19). BCAA for 5 min in gerbils resulted in selective loss of pyramidal neurons in the CA1 area of hippocampus within 96 h to become apparent morphologically. There was substantial hippocampal neuronal death (80–85%) in ischemic animals as compared with the sham-operated animals. Ischemic animals showed hyperlocomotion on 1st and 4th day of reperfusion. This was found to be consistent with the findings stating that on the first day after reperfusion, ischemia induced increase in locomotor activity is prominent, following two days it starts decreasing (15, 20). Thus based on this analysis the day 1st and 4th was selected for assessment of effect of ischemic insult on gerbil's locomotor activity.

Pifithrin-alpha is small, stable, and lipophilic molecules that have octanol to water partition coefficient (log P value of 1.75). It reaches brain within 30 min after a single ip. dose of 2 mg/kg (9). Pifithrin-alpha as such does not downregulate p53 synthesis, but inhibits the translocation of p53 into nucleus and prevents its binding to its

specific DNA sites (7).

Pifithrin-alpha treatment showed a significant improvement in the neurological behavior and locomotor activity. The number of normal CA1 neurons was significantly increased and damaged neurons were decreased as compared to ischemic animals indicating neuroprotective effect of Pifithrin-alpha. Its neuroprotective effects have been reported in transient focal cerebral ischemia (10). Over expression of p53 neurons has been shown to cause neuronal death with features of apoptosis. This expression is inhibited by Pifithrin-alpha (8). Pifithrin-alpha decrease cytochrome c release from mitochondria and apoptotic cell death (21). Pifithrin-alpha inhibited production of Bax, a proapoptotic protein induced by p53, in nigrostriatal cells and preserved motor function in a mouse model of Parkinson's disease (9). Pifithrin-alpha has also shown to reduce Amyloid β induced apoptosis in PC 12 cell line (22). Pifithrin-alpha protected mice from lethal genotoxic stress associated with anticancer treatment without promoting the formation of tumors (23).

In conclusion, pifithrin-alpha produced neuroprotective effects in global cerebral ischemia as evident from reduction in neurological score, hyperlocomotion and neuronal damage.

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