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Role of neurosteroids in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked neuronal injury and behavioral deficits in mice

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ABSTRACT

To investigate the role of neurosteroids in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked neuronal injury and behavioral deficits in mice. Mice were randomly divided into four groups. Mice were subjected to 13min global cerebral ischemia followed by three episodes of 10s of ischemia and reperfusion for 24h. Metyrapone (100mg/kg), an inhibitor of 11β-hydroxylase, was administered 30 min prior to global cerebral ischemia and ischemic postconditioning. Finasteride (50mg/kg), an inhibitor of 5α-reductase, was administered 30 min prior to Metyrapone 100mg/kg and 1h before induction to global cerebral ischemia and ischemic postconditioning. The mice were exposed to elevated plus maze test for assessing transfer latency time to evaluate the short term memory. The rota rod, inclined beam walking test were used to evaluate motor coordination. Ischemic postconditioning significantly attenuated the ischemia-reperfusion-induced increase in cerebral infarct size measured by volume and weight method. Administration of metyrapone (100 mg/kg; i.p.), an inhibitor of 11β-hydroxylase, 30min before the induction of cerebral ischemia and ischemic postconditioning, significantly attenuated ischemia-reperfusion-induced increase in cerebral infarct size. It may be concluded that neurosteroids may be involved in neuroprotective mechanism of ischemic postconditioning as neurosteroids may be responsible for decrease in infarct size, improvement in motor performance and short term memory.

Key words: Ischemic Postconditioning, Global cerebral ischemia, $GABA_A$, Neurosteroids, Metyrapone, Finasteride.

INTRODUCTION

Stroke is the third leading cause of death and the main cause of disability worldwide. Thrombolytic therapy with tissue-type plasminogen activator (tPA) is the only FDA approved treatment of stroke. tPA clinical use is limited to a narrow time window of safe administration and associated with dangers of intracranial hemorrhage [1]. Thus, there is great need for further therapies for stroke.

Brain injury following stroke develops from a complex cascade of cellular events[2]. After ischemia, decreased blood flow decreases phosphocreatine and ATP which disrupt membrane ionic gradient leading to neuronal depolarization [3] and produces the massive release of glutamate from presynaptic nerve terminals [4]. The released glutamate activates postsynaptic N-methyl-D-aspartate (NMDA) receptors leading to abnormally high Ca^{2+} influx [5]. This increase in cytosolic Ca^{2+} activates μ-calpain [6], phospholipase A_2, nitric oxide synthase, endonuclease [7] and other proteins such as cyclophilin A and B [8]. Increase in cytosolic Ca^{2+} promotes the formation of free radicals

in cytoplasm and in the mitochondria [9]. Ca^{2+} overload-induced inhibition of mitochondrial electron transport chain and activation of phospholipase A_2 are mainly responsible to generate reactive oxygen species (ROS) during cerebral ischemia and reperfusion [10]. ROS have been thought to be an important contributor to reperfusion injury. Upon the onset of reperfusion there is a ''respiratory burst'' lasting several minutes that originates from a number of cellular sources including endothelial cells, [11] and activated neutrophils [12]. This oxidative ''burst'' is followed by a moderately but persistently elevated production of superoxide anions. The ROS such as peroxynitrite [13], hydroxyl radical [14], superoxide anion and to lesser extent nitric oxide [15] are reported to produce DNA nicking. Additionally, mitochondrial damage due to ROS releases proapoptotic factors such as cytochrome C, caspase 9 and apoptosis inducing factor, which may contribute to delayed cell death after cerebral ischemia and reperfusion [16, 17].

Different neuroprotectants were introduced for the treatment of ischemic brain injury but none of the neuroprotective agent has proven effective in clinical trial for last two decades. Preconditioning and postconditioning are two innovative strategies for protecting the brain from ischemic reperfusion injury. Preconditioning can be defined as a sub-threshold ischemic insult applied to an organ and it activates cellular pathways that can help to reduce damage caused by subsequent severe ischemic episodes [18]. Postconditioning is a series of brief mechanical interruptions of reperfusion following a specific prescribed algorithm applied at the very onset of reperfusion leading to neuroprotection. Ischemic postconditioning reduces ischemic injury by blocking the overproduction of reactive oxygen species and lipid peroxidation, and by inhibiting apoptosis [19]. Akt/PKB survival pathway [20], K_{ATP} channel activity [21], MAPK pathway [22], PKC [23], Cytochrome C/caspase-mediated apoptotic pathways [24] and NMDA [25] contribute to its protective effects. Postconditioning is clinically more applicable than preconditioning in that therapy would not have to be administered prior to an ischemic episode, but could be administered at the time of reperfusion [26].

Neurosteroids are the neuroactive steroids, synthesized *de novo* from cholesterol or in situ from sterol precursors imported from peripheral sources [27]. Development, growth, maturation and differentiation of brain are strongly influenced by steroid hormones. Neurosteroids are potent modulators of ligand-gated ion channel-receptors, such as the $GABA_A$, glycine, NMDA and $5HT_3$ (serotonin) receptors, as well as voltage gated Ca^{2+} channels and distinct G-protein coupled receptors, acting *via* nongenomic mechanisms [28, 29]. In genomic mechanism, neurosteroids activate or repress the multiple genes. Their genomic actions are mediated through binding to their ubiquitously expressed cytoplasmic glucocorticoid receptors (GRs) [30]. During stress, synthesis of neurosteroids increases that undergoes sequential metabolic reduction by 5α-reductase and 3α-hydroxysteroid oxidoreductase to form 5α-dihydrodeoxycorticosterone (DHDOC) and allotetrahydrodeoxycorticosterone (THDOC) which are neuroprotective [31]. Several protective mechanisms like Akt/PKB survival pathway, K_{ATP} channel activity, MAPK, PKC, Cytochrome c/caspase-mediated apoptotic pathways and NMDA have been shown to be involved in neuroprotection by postconditioning. As neurosteroids are involved in neuroprotection, the present study has been designed to study the role of neurosteroids in the protective effects of postconditioning.

MATERIALS AND METHODS

Experimental

Swiss albino mice (either sex), weighing 25-30g were employed in the present study (procured from the Central Research Institute (CRI), Kasauli). They were maintained on a standard laboratory diet (Ashirwaad feeds Ltd., Kharar, Chandigarh, India) and tap water *ad libitum*. They were housed in the animal house of Rayat and Bahra Institute of Pharmacy (RBIP), Sahauran and were exposed to natural photoperiod. All the animals used in the study were naive to the elevated plus-maze test. The experiments were conducted in a semi sound proof laboratory between 10:00 am to 5:00 pm. The experimental protocol of the study was duly approved by the Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No-1380/a/10/CPCSEA).

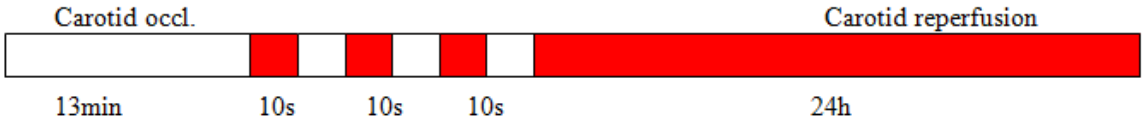
Experimental Design

Animals were randomly separated into four groups and each group comprised of 5 mice. Each animal was subjected to the elevated plus-maze, beam walking and rota-rod tests as described above. The sequence of tests performed on each animal was elevated plus-maze test, rota-rod test and inclined beam walking test. The gap of 10min was kept between each test. These tests were performed in the above described sequence 3h before global cerebral ischemia

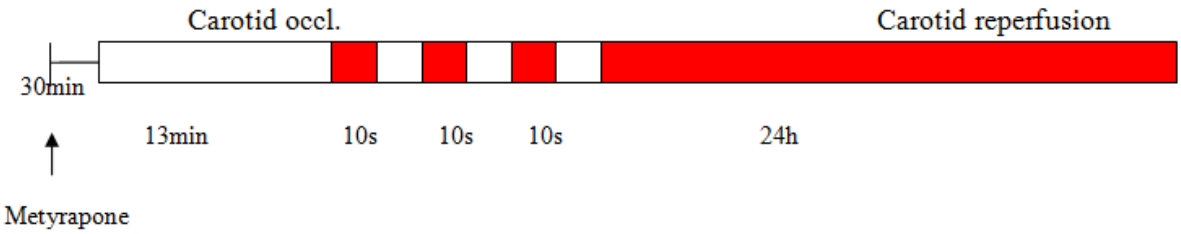
and ischemic postconditioning and 24h after global cerebral ischemia and ischemic postconditioning episodes followed by 24h reperfusion.

Control Group (group I; n=5)
Mice were subjected to 13min global cerebral ischemia followed by reperfusion for 24h.

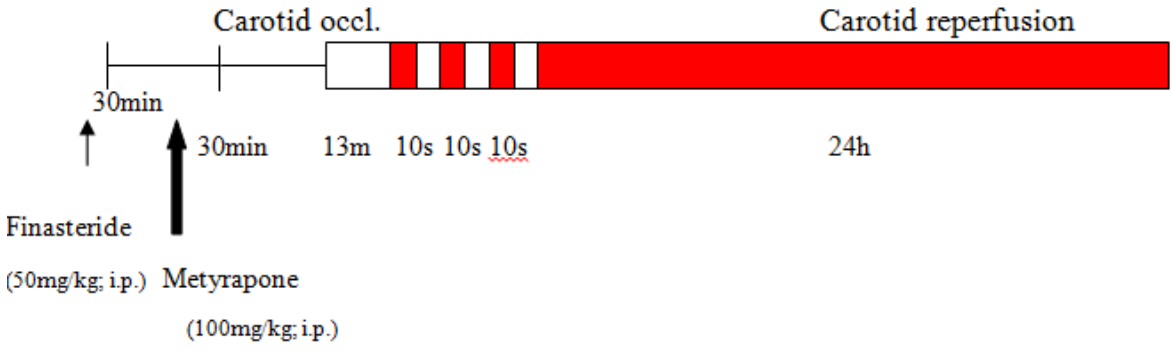
Ischemic Postconditioning Group (group II; n=5)
Mice were subjected to 13min global cerebral ischemia followed by three episodes of 10s of ischemia and reperfusion for 24h.



Metyrapone and ischemic postconditioning treated Group (group III; n=5)
Mice were administered Metyrapone 100mg/kg 30min prior to global cerebral ischemia. The rest of the procedure was same as described for group II.



Mice were administered Finasteride 50 mg/kg 30min prior to Metyrapone 100 mg/kg and 1h before induction of to global cerebral ischemia. The rest of the procedure was same as described for group II.



Induction of Global Cerebral Ischemia-Reperfusion injury and ischemic postconditioning episodes
Mice were anesthetized using chloral thiopental sodium (40mg/kg, i.p.). A midline ventral incision was made in the throat. Right and left common carotid arteries were located and freed from surrounding tissue and vagus nerve. A

cotton thread was passed under each of the carotid artery. Global cerebral ischemia was induced by pulling the ends of thread with constant weight. After 13 min of global cerebral ischemia, weight on the thread was removed to allow the reflow of blood through carotid arteries. The incision was sutured back in layers [31]. The sutured area was cleaned with 70% ethanol and was sprayed with antibiotic (Neosporin) dusting powder. Body temperature of mice was maintained at 37ºC by heated surgical platform.

Ischemic postconditioning was induced at the beginning of reperfusion by occluding the carotid arteries. For the ischemic postconditioning episode, the carotid arteries were re-occluded for a period of 10s followed by 10s of reperfusion time. Three such cycles of ischemia and reperfusion were allowed immediately after the bilateral carotid artery occlusion performed for 13 min. All the surgical instruments used in the surgical procedure were sterilized by incineration prior to use [32]. After completion of the surgical procedure, the animals were shifted individually to their home cage and were allowed to recover.

Assessment of Cerebral Infarct Size

At the end of 24-h reperfusion after the global cerebral ischemia and ischemic postconditioning episodes, animals were sacrificed by cervical dislocation and the brain was removed. The brain was kept overnight at -4ºC. Frozen brain was sliced into uniform coronal sections of about 1mm thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37ºC in 0.2 M Tris buffer (pH 7.4) for 20 minutes. TTC is converted to red formazone pigment by NAD and dehydrogenase and thereof stained the viable cells deep red. The infarcted cells have lost the enzyme and cofactor and thus remained unstained dull yellow. The brain slices were placed over a glass plate. A transparent plastic grid with 100 squares in 1 cm2 was placed over it. Average area of each brain slice was calculated by counting the number of squares on either side. Similarly, numbers of squares falling over non-stained dull yellow area were also counted. Infarcted area was expressed as a percentage of total brain volume [33].

Estimation of Thiobarbituric Acid Reactive Substance (TBARS)

At the end of 24 h of reperfusion after the global cerebral ischemia, animals were sacrificed by cervical dislocation and the brain was removed. The brain was homogenized in 5 ml of 30 mM Tris-HCl and 2.5 mM CaCl2 buffer (pH 7.6 at 50C). Homogenate was centrifuged at 750g to separate cellular debris. The supernatant was accurately divided into two parts. Both portions were centrifuged at 8200g to obtain the mitochondrial fraction. One fraction was utilized for determination of TBARS [34] and the other portion was employed for protein estimation [35].

Rota Rod Test

Rota rod is used to evaluate motor coordination by testing the ability of mice to remain on a revolving rod. The apparatus consists of a horizontal rough metal rod of 3cm diameter attached to a motor with a variable speed. The rod is 70cm in length and is divided into four sections by wooden partitions, thereby allowing the simultaneous testing of four mice. The rod is kept at a height of about 50cm above the tabletop in order to discourage the animals from jumping off the roller. The rate of rotation of the rod was adjusted such that the normal mouse was able to stay on the rotating rod for a period of five minutes. The difference in the fall off time from the rotating rod between the control and treated animals is taken as an index of motor incoordination. Each mouse was given four or five trials before the actual reading was taken. The mice that were able to stay on the rotating rod for a period of five minutes before global cerebral ischemia were selected and the test was again performed after global cerebral ischemia and ischemic postconditioning episodes followed by 24h reperfusion [36].

Inclined beam-walking test

Inclined beam-walking test was employed to evaluate fore and hind limb motor co-ordination. Each animal was individually placed on a metallic bar 55 cm long and 1.5 cm wide, inclined at an angle of 60° from the ground. The motor performance of mice was graded on a scale ranging from 0 to 4. A grade of 0 was assigned to an animal that could readily traverse the beam, grade 1 was given to animal demonstrating mild impairment, grade 2 was assigned to animal demonstrating moderate impairment, grade 3 was given to animal demonstrating severe impairment and grade 4 was assigned to animal completely unable to walk on the beam. Inclined beam-walking test was performed before global cerebral ischemia and ischemic postconditioning and the animals which readily transversed the beam (grade 0) were selected. The test was again performed after global cerebral ischemia and ischemic postconditioning episodes followed by reperfusion for 24h [37].

Short Term Memory Evaluation Using Elevated Plus Maze

Elevated plus maze consisted of two open (16 × 5 cm) and two enclosed (16 × 5 × 12 cm) arms, connected by a central platform (5 × 5 cm). The apparatus was elevated to a height of 25 cm above the floor. A fine line was drawn in the middle of the floor of each closed arm. All the animals were given a single trial on plus maze. Each mouse was individually placed at the end of open arm facing away from a central platform of the maze. The time taken by the mouse to enter from the open arm with all the four legs into the enclosed arm was taken at transfer latency time (TLT). In case the animal did not enter the enclosed arm within 90s, it was gently pushed into the closed arm and TLT of 90s was assigned to it. The animal was allowed to explore the maze for an additional 10s after the measurement of TLT. The exposure of the elevated plus-maze was repeated on day 2 and 3 to obtain a low level of TLT. After 3rd training trial animals were subjected to global cerebral ischemia and ischemic postconditioning for 13 minutes followed by reperfusion for 24 h. The animals were again exposed to elevated plus maze to measure the TLT on day 4 after 24h of reperfusion. TLT measured on 3rd training trial (Day 3) serves as index of learning or acquisition, whereas TLT recorded 24 h after global cerebral ischemia and reperfusion (Day 4) served as an index of retrieval or memory. Utmost care was taken not to change the relative location of plus-maze with respect to any object serving as a visual clue in the laboratory [38].

Statistical Analysis

All the results were expressed as mean± standard error of mean (S.E.M.) followed by one way ANOVA along with Tukey's multiple comparison test. The $p<0.05$ was considered to be significant.

RESULTS

Effect of various interventions on cerebral infarct size

Global cerebral ischemia of 13min followed by reperfusion for 24h produced a significant increase in cerebral infarct size measured by volume and weight method (Table no. 1) (Fig. 1 and 2). Ischemic postconditioning significantly attenuated the ischemia-reperfusion-induced increase in cerebral infarct size measured by volume and weight method (Table no.1) (Fig.1 and 2). Administration of metyrapone (100 mg/kg; i.p.), an inhibitor of 11β-hydroxylase, 30min before the induction of cerebral ischemia and ischemic postconditioning, significantly attenuated ischemia-reperfusion-induced increase in cerebral infarct size measured by volume and weight method (Table no.1) (Fig. 1 and 2). Also, administration of metyrapone (100 mg/kg; i.p.), significantly enhance the effect of ischemic postconditioning on ischemia-reperfusion-induced cerebral infarction as measured by volume and weight method (Table no. 1) (Fig. 1 and 2).

Table no. 1 Effect of various interventions on cerebral infarct size

S.no	Experimental groups	% CEREBRAL INFARCT SIZE	
		By volume method	By weight method
1.	Control	80.25±0.4787	80.35±0.7563
2.	IPostC group	55.70±0.2299	55.05±0.3849
3.	Metyrapone+ IPostC group	49.36±0.1836	39.133±0.1492
4.	Fin+Met+IPostC group	63.1925±0.4182	63.19±0.4299

For all groups (n=5). All the results were expressed as mean±standard error of mean (S.E.M.) followed by one way ANOVA along with Tukey's multiple comparison test.

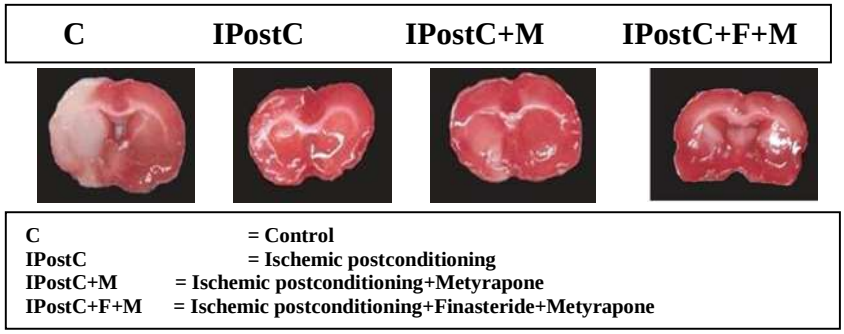


Fig. 5.1: Cerebral infarction after 24 hours of ischemia-reperfusion-injury

Finasteride (50mg/kg; i.p.), an inhibitor of 5α-reductase inhibitor, administered 30min prior to metyrapone (100mg/kg; i.p.) and 1h before induction of cerebral ischemia and ischemic postconditioning significantly reversed the effect of metyrapone and ischemic postconditioning on ischemia-reperfusion-induced cerebral infarction as measured by volume and weight method (Table no. 1) (Fig. 1 and 2).

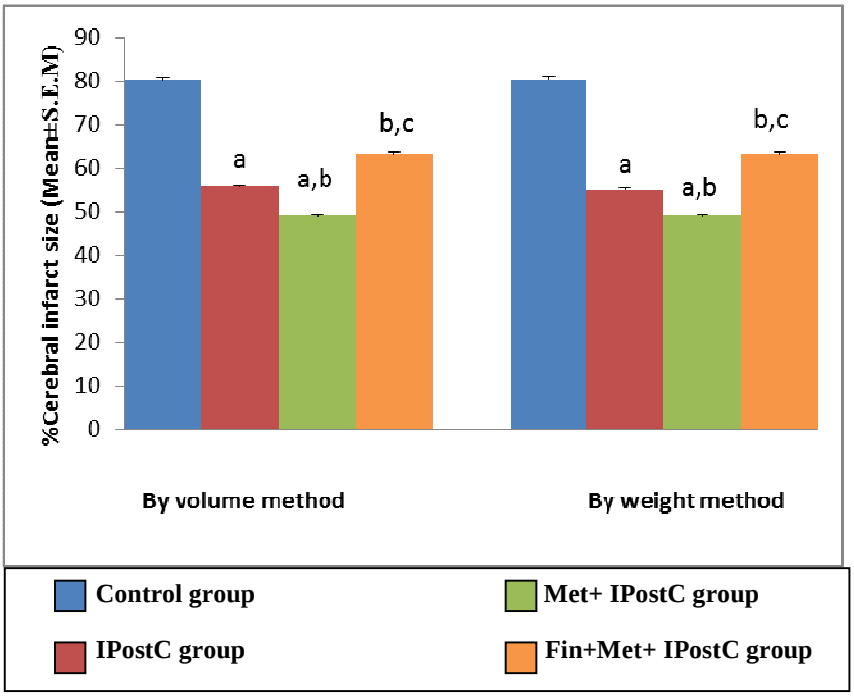


Fig. 2 Effect of ischemic postconditioning on ischemia-reperfusion induced cerebral infarct size in mice

Control represents that mice were subjected to cerebral ischemia for 13min followed by reperfusion for 24h. Values were expressed as mean *(n=5)* ± SEM. a=$p<0.05$vs control. b=$p<0.05$vs ischemic postconditioning. c=$p<0.05$vs metyrapone and ischemic postconditioning. IPostC stands for Ischemic postconditioning group; Fin stands for Finasteride group; Met stands for Metyrapone group.

Effect of various interventions on thiobarbituric acid reactive substance (TBARS)

Global cerebral ischemia of 13min followed by reperfusion of 24h significantly increased the thiobarbituric acid reactive substances (TBARS) (Table no. 2) (Fig.3). Ischemic postconditioning significantly attenuated ischemic-reperfusion-induced increase in TBARS (Table no. 2) (Fig. 3). Administration of metyrapone (100mg/kg; i.p.), an inhibitor of 11β-hydroxylase, 30min before induction of cerebral ischemia and ischemic postconditioning significantly attenuated ischemia-reperfusion-induced increase in TBARS (Table no. 2) (Fig. 3). Also, administration of metyrapone (100mg/kg; i.p.), significantly enhanced the effect of ischemic postconditioning on ischemia-reperfusion-induced TBARS (Table no. 2) (Fig. 3). Finasteride (50mg/kg; i.p.) administered 30min prior to metyrapone (100mg/kg; i.p.) and 1h before induction of cerebral ischemia and ischemic postconditioning significantly reversed the effect of metyrapone and ischemic postconditioning on ischemia-reperfusion-induced increase in TBARS (Table no. 2) (Fig. 3).

Table no. 2 Effect of various interventions on thiobarbituric acid reactive substances (TBARS)

S. No	Groups	TBARS (nm/mg of proteins)
1.	Control	3.2575±0.05413
2.	IPostC group	1.3547±0.06116
3.	Metyrapone+IPostC group	1.084±0.04942
4.	Fin+Met+IPostC group	2.0096±0.1177

For all groups (n=5). All the results were expressed as mean±standard error of mean (SEM) followed by one way ANOVA along with Tukey's multiple comparison test.

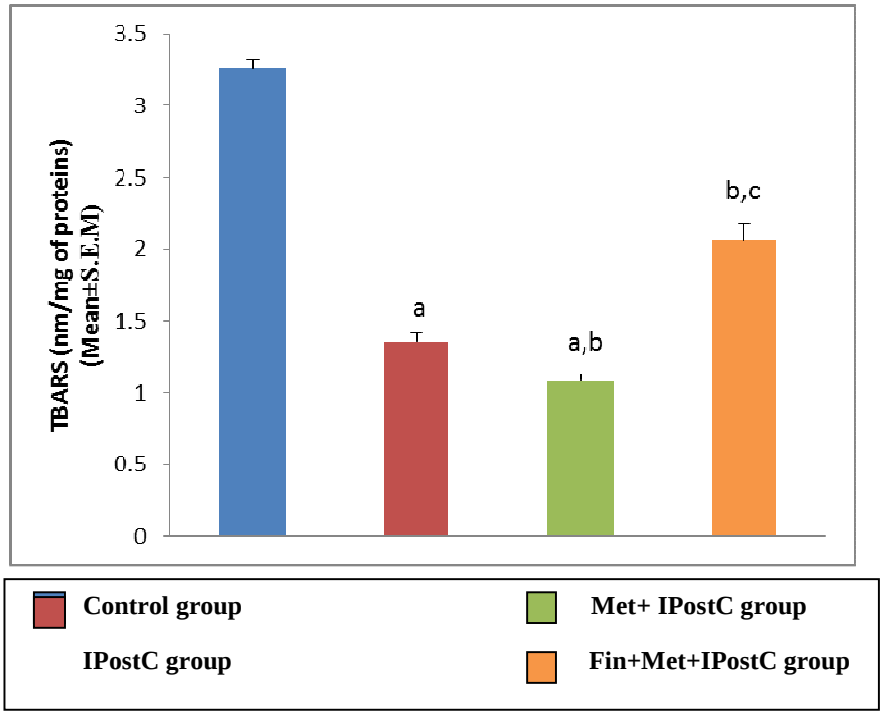


Fig.3 Effect of various interventions on thiobarbituric acid reactive substances (TBARS)

Control represents that mice were subjected to cerebral ischemia for 13min followed by reperfusion for 24h. Values were expressed as mean *(n=5)* ± SEM. a=p<0.05vs control. b=p<0.05vs ischemic postconditioning. c=p<0.05vs metyrapone and ischemic postconditioning. IPostC stands for Ischemic postconditioning group; Fin stands for Finasteride group; Met stands for Metyrapone group.

Table no. 3 Effect of various interventions on motor coordination

S. No.	Groups	Rota Rod (Time In Sec.)	Inclined Beam Walking (Score Of Motor Performance)
1.	Control	2.25±0.9574	2.8±0.5831
2.	IPostC group	5.54±0.4778	0.8±0.3774
3.	Metyrapone+ IPostC group	6.0±0.4472	0.5±0.3774
4.	Fin+Met+IPostC group	5.0±0.4472	1.2±0.4874

For all groups (n=5). All the results were expressed as mean±standard error of mean (SEM) followed by one way ANOVA along with Tukey's multiple comparison test.

Effect of various interventions on motor co-ordination:

Global cerebral ischemia of 13min followed by reperfusion for 24h produced a marked impairment of motor performance using inclined beam walking and rota-rod test (Table no. 3) (Fig. 4 and 5). Ischemic postconditioning significantly attenuated the ischemia-reperfusion-induced impairment of motor performance (Table no. 3) (Fig. 4 and 5). Administration of metyrapone (100mg/kg; i.p.), an inhibitor of 11β-hydroxylase, 30min before induction of cerebral ischemia and ischemic postconditioning significantly attenuated the ischemia-reperfusion-induced impairment of motor performance (Table no. 3) (Fig. 4 and 5). Also, administration of metyrapone (100mg/kg; i.p.), significantly enhanced the effect of ischemic postconditioning on ischemia-reperfusion-induced impairment of motor performance (Table no. 3) (Fig. 4 and 5). Finasteride (50mg/kg; i.p.), an inhibitor of 5α-reductase, administered 30min prior to metyrapone (100mg/kg; i.p.) and 1hour before induction of cerebral ischemia and ischemic postconditioning significantly reversed the effect of metyrapone and ischemic postconditioning on ischemia-reperfusion-induced cerebral impairment of motor performance (Table no. 3) (Fig. 4 and 5). The motor

performance assessed by inclined beam walking and rota-rod tests demonstrated identical results with all treatments (Table no.3) (Fig. 4 and 5).

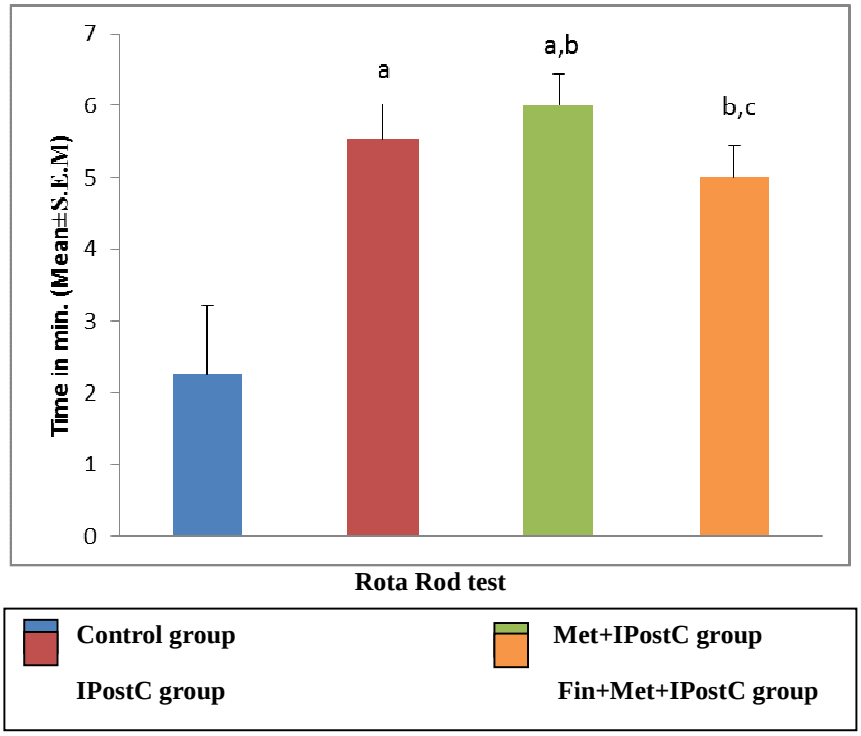


Fig. 4: Effect of various interventions on ischemia-reperfusion-induced changes in motor performance

Control represents that mice were subjected to cerebral ischemia for 13min followed by reperfusion for 24h. Values were expressed as mean *(n=5)* ± SEM. a=p<0.05vs control. b=p<0.05vs ischemic postconditioning. c=p<0.05vs metyrapone and ischemic postconditioning. IPostC stands for Ischemic postconditioning group; Fin stands for Finasteride group; Met stands for Metyrapone group.

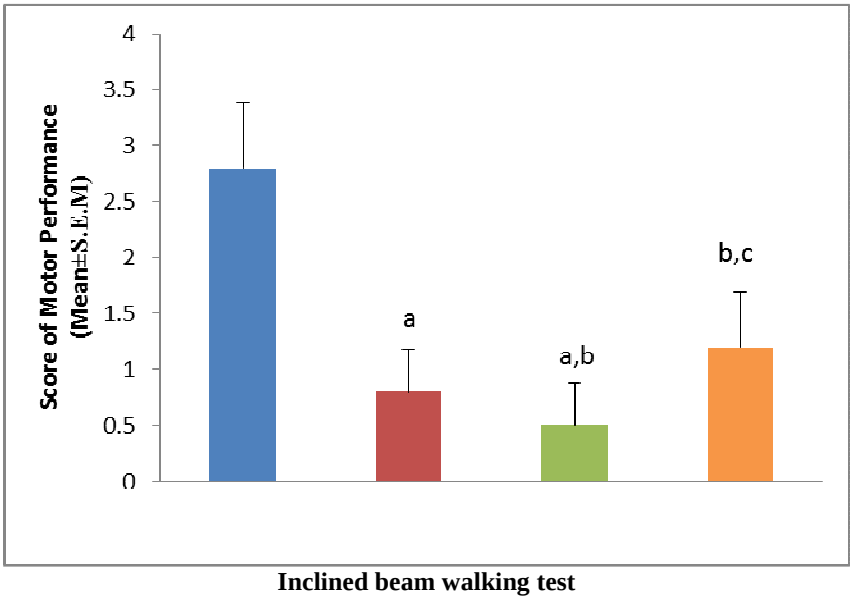


Fig. 5: Effect of various interventions on ischemia-reperfusion-induced changes in motor performance

Control represents that mice were subjected to cerebral ischemia for 13min followed by reperfusion for 24h. Values were expressed as mean *(n=5)* ± SEM. a=p<0.05vs control. b=p<0.05vs ischemic postconditioning. c=p<0.05vs metyrapone and ischemic postconditioning. IPostC stands for Ischemic postconditioning group; Fin stands for Finasteride group; Met stands for Metyrapone group.

Effects of various interventions of short term memory

The training trials performed on day 2 and day 3 significantly decreased the transfer latency time (TLT) as compared to TLT recorded on day 1 trial using elevated plus maze (Table no. 4) (Fig 6). Global cerebral ischemia of 13 min followed by reperfusion of 24h conducted after day 3 trial significantly increased the transfer latency time (TLT) demonstrating impairment of short term memory (Table no. 5) (Fig. 7). Ischemic postconditioning attenuated the ischemia-reperfusion-induced increase in TLT (Table no.5) (Fig.7).

Table no. 4: Effect of trials on acquisition response

TRIAL DAY	TLT in sec.
Day 1	20.64±3.602
Day 2	14.107±1.755
Day3	10.85±2.669

TLT denotes transfer latency time in seconds. Values were expressed as mean *(n* = 25*)* ± standard error of mean (S.E.M.) followed by one way ANOVA along with Tukey's multiple comparison test.

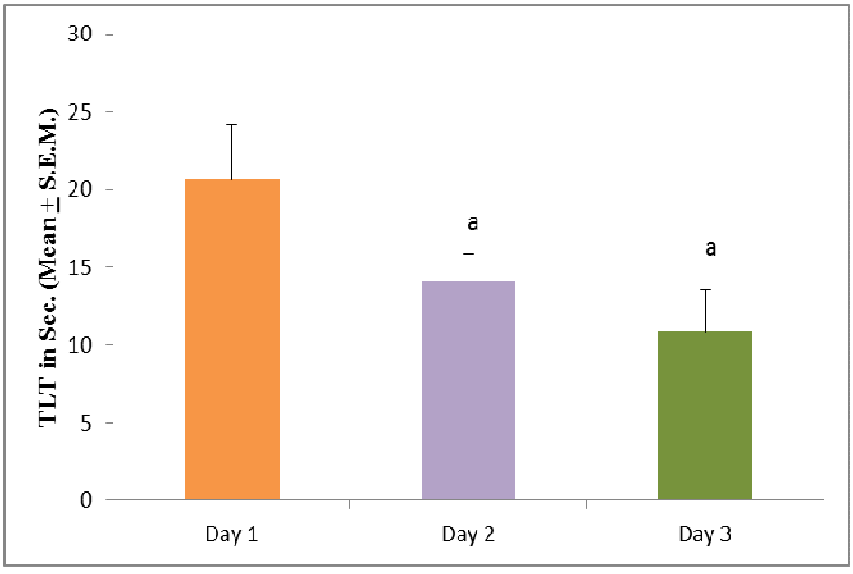


Fig. 6: Effect of trials on acquisition response
TLT denotes transfer latency time in seconds.
Values were expressed as mean (n = 25) ±S.E.M. a = P < 0.05vs. Day1.

Administration of metyrapone (100mg/kg; i.p.), an inhibitor of 11β-hydroxylase, 30min before induction of cerebral ischemia and ischemic postconditioning significantly attenuated the ischemia-reperfusion-induced increase in TLT (Table no. 5) (Fig.7). Also, administration of metyrapone (100mg/kg; i.p.), significantly enhanced the effect of ischemic postconditioning on ischemia-reperfusion-induced increase in TLT (Table no. 5) (Fig. 7). Finasteride (50mg/kg; i.p.) administered 30min prior to metyrapone (100mg/kg; i.p.) and 1h before induction of cerebral

ischemia and ischemic postconditioning significantly reversed the effect of metyrapone and ischemic postconditioning on ischemia-reperfusion induced increase in TLT (Table no. 5) (Fig. 7).

Table no. 5: Effect of various interventions on short term memory

S.No.	GROUPS	DAY 4 (Time in sec.)
1.	Control group	27 ± 5.801
2.	IPostC group	12.25 ± 2.217
3.	Metyrapone + IPostC	9.25 ± 2.5
4.	Fin+Met+IPostC treated	13 ± 2.16

For all groups (n=5). All the results were expressed as mean± standard error of mean (S.E.M.) followed by one way ANOVA along with Tukey's multiple comparison test.

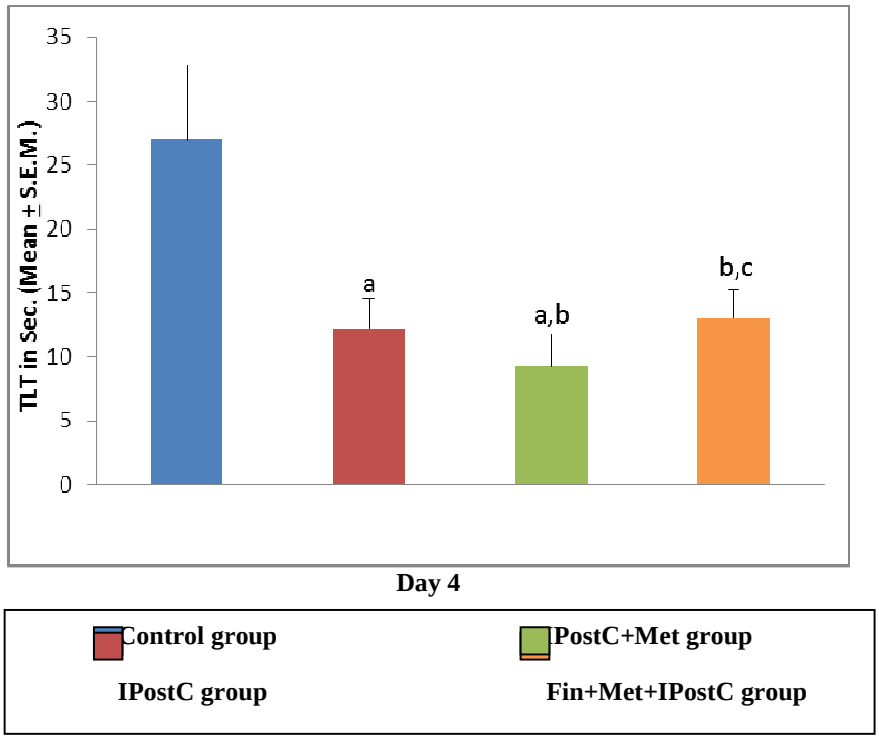


Fig. 7: Effect of various interventions on short term memory recorded on day 4 in mice

Control represents that mice were subjected to cerebral ischemia for 13 min. followed by reperfusion for 24 hrs. Values were expressed as mean *(n* = 5*)* ± S.E.M. a = *P* < 0.05vs. Control. b = *P* < 0.05vs. ischemic postconditioning. c=*p*<0.05vs metyrapone and ischemic postconditioning. TLT denotes transfer latency time in seconds. IPostC stands for Ischemic postconditioning group; Fin stands for Finasteride group; Met stands for Metyrapone group.

DISCUSSION

The global cerebral ischemia reperfusion model employed in the present study is reported to simulate the clinical situation of cerebral ischemia due to cardiac arrest, drowning and asphyxia. Therefore, global cerebral ischemia of short duration followed by reperfusion for a long time has been employed in the present study [39]. Triphenyltetrazolium chloride (TTC) staining has been employed in the present study to determine the area of infarction in brain tissue. The TTC is a water-soluble dye that is reduced to the formation by the enzyme succinate dehydrogenase and cofactor NAD, present in mitochondria and stains viable tissue deep red in color. Ischemic tissue with damaged mitochondria remains unstained [40, 41]. Therefore, the TTC staining technique has been used to measure the extent of infarct size in the present study.

Free radicals have been implicated in cerebral ischemia and reperfusion induced neuronal injury (42, 43, 44]. Free radicals promote lipid peroxidation, which results in the alteration in permeability and fluidity of membranes [45]. The neurons vulnerable to free radical damage during reperfusion are reported to demonstrate lipid peroxidation [46]. The concentration of MDA, which is an end product of lipid peroxidation, increases markedly after reperfusion of the brain as ROS also increase as a result of ischemia reperfusion injury. MDA reacts with thiobarbituric acid (TBA) and is thus estimated as TBARS [47]. Therefore, in the present study, estimation of MDA with TBARS has been used as a biochemical marker of lipid peroxidation.

Cerebral ischemia has been reported to impair memory because hippocampal neurons are susceptible to the deleterious effects of ischemia reperfusion and the hippocampus is involved in the regulation of memory. Therefore, in the present study, we employed the elevated plus maze test to evaluate the effect of global cerebral ischemia and reperfusion on short term memory [32, 37]. Cerebral ischemia is documented to impair sensory motor ability [48]. It is important for the development of drug therapies to establish the convenient method, and to evaluate impairment and the improvement of neurological and motor function in rodent models because muscle weakness is a common complaint after stroke in humans [49, 50]. Therefore, inclined beam walking test and rota rod test has been used in the present study to evaluate motor performance [51]. The Rota rod test is used to evaluate the activity of drugs interfering with motor coordination. It has been suggested that the skeletal muscle relaxation induced by a test compound could be evaluated by testing the ability of mice or rats to remain on a revolving rod.

Ischemic postconditioning was reported to prevent cerebral ischemic injury in rats [52, 53], and is involved in neuroprotection. Ischemic postconditioning invokes the activation of signal transduction cascades by autacoid triggers; these accumulate extracellularly in response to the postconditioning stimulus and act on cell surface receptors or other molecular targets [54]. Postconditioning attenuates ROS production and apoptosis, as early reperfusion is considered to cause significant ROS products leading to apoptosis. Postconditioning may also inhibit inflammation after stroke. During the inflammatory response, leukocytes extravasate into the brain tissue, releasing ROS, thus attacking lipid membranes, DNA, and proteins [55]. Ischemic postconditioning inhibits the Ca+2 overloads by suppressing the activity of NMDA receptor [56]. Decrease in intracellular Ca+2 initiates inhibiting effects of ROS leading to an improved activity of the Akt and KATP channels, which contributes to the protection of postconditioning [57]. Therefore, ischemic postconditioning induced attenuation of ischemia-reperfusion-induced increase in cerebral infarct size, TBARS and memory impairment noted in the present study is due to its various neuroprotective mechanisms.

Neurosteroids may exhibit neuroprotective effects in both central and peripheral nervous systems [58]. Neurosteroids have been shown to modulate expression of particular subunits of $GABA_A$ receptors. Neuroprotection offered by steroid hormones realized in both genomic (classical intracellular steroid receptor) and non genomic (ion channels and membrane receptors) mechanisms involve regulation of the pro- and anti apoptotic factor expression, intracellular signaling pathways, neurotransmission, oxidative, and inflammatory processes [29]. Neurosteroids have been proved to have antioxidant effects [59], hence they prevent the cell damage related to oxidative processes and activation of the mitochondrial apoptosis pathway [60]. Various studies have shown that neurosteroids may be involved in memory enhancement, behavioral actions, and neuroprotective effects [61, 62]. Neurosteroids modulate brain excitability primarily by interaction with neuronal membrane receptors and ion channels, principally $GABA_A$ receptors [63]. 11β-hydroxylase inhibitor, metyrapone (100mg/kg; i.p.) inhibits the synthesis of corticosterone from deoxycorticosterone (DOC) and increase the conversion of DOC to THDOC which has neuroprotective effects on the brain [64]. 5α-reductase inhibitor, finasteride (50mg/kg; i.p), inhibits the synthesis of neurosteroids.

Ischemic postconditioning affects the corticosteroid level and may increase the synthesis of neurosteroids which may be converted to the other prototypic $GABA_A$ receptor modulating neurosteroids [65]. So in the present study it was hypothesized that neurosteroids may also be involved in the protective effects of ischemic postconditioning.

Ischemia reperfusion injury significantly increased the levels of TBARS in mice brain. This may be due to increase in Ca^{+2} overload and increase in production of ROS, leading to oxidative damage and hence increase the levels of TBARS. Ischemic postconditioning attenuated the ischemia-reperfusion-induced increase in brain TBARS. This may be due to fact that ischemic postconditioning may increase the synthesis of neurosteroids and hence reduce the Ca^{+2} overload further leading to decrease in the production of ROS [9]. In the present study, metyrapone and ischemic postconditioning has attenuated ischemia-reperfusion-induced increase in brain TBARS. Administration of metyrapone enhanced the effect of ischemic postconditioning. As metyrapone, an inhibitor of 11β-hydroxylase, may

have reduced glucocorticoid synthesis and raised the levels of DOC providing greater availability of DOC for metabolic conversion to the $GABA_A$ receptor modulating neurosteroids and THDOC. Neurosteroids may have further attenuated generation of ROS in mitochondria and decrease the oxidative stress [66]. This contention is further supported by the observation that finasteride, an inhibitor of 5α-reductase, has attenuated metyrapone and ischemic postconditioning induced decrease in brain TBARS, may be due to the fact that finasteride reduces the neurosteroidogenesis [64].

Ischemia-reperfusion is accompanied by impairment to oxidative reductive reactions of cell energy metabolism, which involve a number of key enzymes, including lactate dehydrogenase (LDH) [67]. Hippocampus plays an important role in the formation of memories and is involved in the detection of novel events, places and stimuli [68]. Ischemic postconditioning after global cerebral ischemia exerts protection to the vulnerable hippocampal CA1 region of the brain [69]. Ischemic postconditioning protects neurons against ischemia/reperfusion injury by increasing their LDH (lactate dehydrogenase) activity. Therefore, ischemic postconditioning induced improvement of memory noted in the present study may be due to its neuroprotective effects in hippocampal neurons exerted via neurosteroids. Neurosteroids exhibit neuroprotective effects in both central and peripheral nervous system [29]. Excitability of central nervous system is rapidly altered by neurosteroids through modulating neurotransmitter-gated ion channels such as $GABA_A$ and NMDA receptor [65]. Neurosteroids promote learning and memory by modulating synaptic functions in the hippocampus [70]. 11β-hydroxylase inhibitor, metyrapone and ischemic postconditioning has attenuated ischemia-reperfusion-induced impairment in memory. In the present study, metyrapone enhanced the neuroprotective effect of ischemic postconditioning. This may be due to the fact that metyrapone inhibited the synthesis of corticosterone and enhanced the synthesis of neurosteroids and provided neuroprotection in hippocampus. This contention is further supported by the observation that finasteride, an inhibitor of 5α-reductase, has prevented metyrapone and ischemic postconditioning induced improvement of memory.

Ischemia-reperfusion injury increases the Ca^{+2} influx, due to which ROS production increases and infarction occurs due to cell death. Ischemic postconditioning reduces neuronal cell death induced by global cerebral ischemia. This protective mechanism of postconditioning against global ischemic injury may involve reducing neuronal death by improving the disturbance in CBF [71]. Cerebral ischemia can induce considerable corticosterone secretion, which can cause the brain damage [72]. In the present study, metyrapone and ischemic postconditioning significantly attenuate the increase in cerebral infarct size induced by global cerebral ischemia. Metyrapone reduced the brain damage induced by global cerebral ischemia may be by suppressing the increased levels of corticosterone. Metyrapone enhanced the neuroprotective effect of ischemic postconditioning and attenuated the increase in cerebral infarct size induced by global cerebral ischemia. This may be due to the fact that metyrapone and ischemic postconditioning reduce brain damage induced by global cerebral ischemia may be by suppressing the increased level of corticosteroids and hence reducing the brain damage. This contention is further supported by the observation that finasteride, an inhibitor of 5α-reductase, has prevented metyrapone and ischemic postconditioning induced improvement in cerebral infarct size.

Brain tissue infarction as a result of oxygen free radicals has been noted to decrease motor performance and motor co-ordination [73]. Ischemic postconditioning attenuates ROS production and neuronal damage and hence improves the motor performance [19] in the cerebellum. In the present study, metyrapone and ischemic postconditioning improved the impairment of motor coordination and motor performance induced by ischemia-reperfusion injury, may be due to increase in synthesis of neurosteroids. Metyrapone enhanced the neuroprotective effect of ischemic postconditioning, may be due to increase in the synthesis of neurosteroids and the consequent prevention of generation of reactive oxygen species leading to an improvement of motor performance. This contention is further supported by observation that finasteride, an inhibitor of 5α-reductase, has attenuated the neuroprotective effect of metyrapone and ischemic postconditioning on motor performance due to the fact that finasteride inhibited the production of neurosteroids.

CONCLUSION

On the basis of above discussion it may be concluded that neurosteroids may be involved in neuroprotective mechanism of ischemic postconditioning. The neuroprotection through ischemic postconditioning and metyrapone may be due to reduced glucocorticoid synthesis and raised the levels of DOC providing greater availability of DOC for metabolic conversion to the $GABA_A$ receptor modulating neurosteroids and THDOC. This increase in neurosteroids may be responsible for decrease in infarct size, improvement in motor performance and short term

memory. Nevertheless, further studies are required to elucidate the involvement of neurosteroids in the neuroprotective effects of ischemic postconditioning.

REFERENCES

[1] MC Shamy; CS Jaigobin, *Neurology*, **2013**, 81, 1130–1133.
[2] C Werner; K Engelhard, *Brit. J. Anaesthesia*, **(2007),** 99(1), 4-9.
[3] L Tiegang; N Wang; M Zhao, *J. Biomedicine and Biotechnology*, **(2012)**, 1-8.
[4] C Morin; S Thibierge; M Perrigot, *J. mind and behavior*, **(2001)**, 22 (3), 69-90.
[5] SM Cain; TP Snutch, *Biofactors*, **(2011)**, 37, 197–205.
[6] T Yamashima, *Prog. Neurobiol*, **(2000)**, 62, 273–295.
[7] J Castillo; R Rama; A Davalos, *Stroke*, **(2000)**, 31, 852-857.
[8] JB Redell; J Zhao; PK Dash, *J. Neuroscience res.* **(2007)**, 85(9), 1980-1988.
[9] V Adam-vizi; AA Starkov, **(2010)**, *J Alzheimers Dis*. 20, S413–S426.
[10] N Vanlangenakkar; TV Berghe; DV Krysko; N Festjens; P Vandenabeele, *Curr. Mol. Medicine*, **(2008)**, 8, 207-220.
[11] O Herkert; I Diebold; RP Brandes; J Hess; R Busse; A Gorlach, *Circulation*, **(2002)**, 105, 2030– 2036.
[12] J Vinten-Johensen; Z Zhao; R Jiang; AJ Zatta; GP Dobson, *J Appl Physiol*, **(2007)**, 103, 1441- 1448.
[13] C Szabo, *Toxicology Letters*, **(2003),** 140-141, 105-112.
[14] JL Zweier; MA Hassan Talukder, *Cardiovas. Res*, **(2006)**, 70, 181-190.
[15] NR Sims; MF Anderson, *Neurochemistry International*, **(2002),** 40(6), 511-526.
[16] T Hirsch; SA Susin; I Marzo; P Marchetti; N Zamzami; G Kroemer, *Cell biology and toxicology*, **(1998),** 14, 141-145.
[17] G Fiskum, *J Neurotrauma*, **(2000),** 17, 843-855.
[18] U Dirnagl; RP Simon; JM Hallenbeck, *Trends Neurosci*, **(2003)**, 26, 248–254.
[19] H Zhao; C Ren; C Chen; J Shen, *Curr. Drug Targets*, **(2012)**, 13 (2), 173-187.
[20] X Gao; H Zhang; T Takahashi; J Hsieh; J Liao; GK Steinberg; H Zhao, *J Neurochem*, **(2008)**, 105, 943–955.
[21] JJ Lee; L Li; HH Jung; Z Zuo, *Anesthesiology*, **(2008)**, 108, 1055–1062.
[22] N Noshita; T Sugawara; T Hayashi; A Lewen; G Omar; PH Chan, *J Neurosci*, **(2002)**, 22 (18), 7923–7930.
[23] T Shimohata; H Zhao; GK Steinberg, *Stroke*, **(2007)**, 38, 375–380.
[24] J Chen; T Nagayama; K Jin; RA Stetler; RL Zhu; SH Graham; RP Simon, *J Neurosci*, **(1998)**, 18(13), 4914–4928.
[25] Onkosullama Ve Ardkosullamanin Noroprotektif Etkileri, *Turkish Neurosurgery*, **(2009)**, 19 (4), 406-412.
[26] RA Kloner; SH Rezkalla, *Cardiovas. Res*, **(2006)**, 70, 297-307.
[27] EE Baulieu, *Psychoneuroendocrinology*, **(1998)**, 23, 963-987.
[28] K Wojtal; MK Trojkar; SJ Czuczwar, *Pharmacol. Rep*, **(2006)**, 58, 335-340.
[29] KK Borowicz; B Piscorska; M Banach; SJ Czuczwar, *Front. in Endocrinology*, **(2011)**, 2, 1-10.
[29] AA Alangari, *Ann Throc Med*, **(2010)**, 5(3), 133-139.
[30] DS Reddy; MA Rogawski, *The J. Neurosci*, **(2002)**, 42, 3795–3805.
[31] N Himori; H Watanabe; N Akaike; M Kurasawa; J Itoh; Y Tanaka, *J. Pharmacol. and Toxicological Methods*, **(1990)**, 23, 311-327.
[32] S Kaur; AK Rehni; N Singh; AS Jaggi, *The pharmaceutical society of Japan*, **(2009)**, 129(4), 435-443.
[33] T Brott; JR Marler; T Tomstick; WG Barsen; J Biller; R Eberle; M Wlker, *J. Acute heart associ*, **(1989),** 20, 871-875.
[34] K Yagi, Assay for serum lipid peroxide level and its clinical significance. In: *Lipid Peroxides in Biology and Medicine, K. Yagi (ed.) Academic Press*. New York, **1982**; pp. 223-242.
[35] OH Lowry; NJ Rosebrough; AL Far; RJ Randall, *J. Biol. Chem*, **1951**, 193, 265-275.
[36] NW Dunham; TS Miya, Journal of the American Pharmaceutical Association, 1957, 46(3), 208-209.
[37] AK Rehni; TG Singh; AS Jaggi; N Singh, *Pharmacol. Rep*, **(2008)**, 60, 904–913.
[38] SL Handley; S Mithani, Naunyn-Schmiedeberg's Archives of Pharmacology, 1984, 327, 1-5.
[39] M Alonsode de Lecinana; E Diez-Tejedor; F Cearcellar; JM Roda, *Cerebrovas. Dis*, **(2001)**, 11(1), 20–30.
[40] R Gupta R; M Singh; A Sharma, *Pharmacol. Res*, **(2003)**, 48, 209–215.
[41] R Shri; KS Bora, *Fitoterapia*, **(2008)**, 79, 86–96.
[42] CE Cross; B Halliwill; ET Borish; WA Pryor; BN Ames; RL Saul; JM McCord; D Harman, *Annals of Internal Medicine*, **(1987)**, 107, 526-545.
[43] MY-T Globus; O Alonsa; WD Dietrich; R Busto; MD Ginsberg, *J. Neurochemistry*, **(1995)**, 65, 1704-1711.

[44] K Baker; CB Marcus; K Hirffman; H Kruk; B Malfory; SR Doctrow, *J. P'cological and Exp. Ther*, **(1998)**, 284, 215-221.
[45] B Halliwell; J Gutteridge, *J. Biochem*, **(1984)**, 19, 1-14.
[46] BK Siesjo, *J. Neurosurgery*, **(1992)**, 77, 337-354.
[47] M Dib; C Garrel; A Favier; V Robin; C Desnuelle, *J. Neurology*, **(2002)**, 249-367.
[48] K Kumari; KT Augusti, *Indian J Exp Biol*, **(2002)**, 40, 1005–1009.
[49] FY Yonemori; T Yamaguchi, H Yamada; A Tamura, *J. cerebral blood flow and metabolism*, **(1998)**, 18, 1099-1106.
[50] L Zhang; T Schallert; ZG Zhang; Q Jiang; P Arniego; Q Li; M Lu; M Chopp, *J. Neurosci. Methods*, **(2002)**, 117, 207-214.
[51] BH Dobkin, *Clin. Geriat. Med*, **(1991)**, 7, 507-523.
[52] J Burda J; V Danielisova; M Nemethova; M Gottlieb; M Matiasova; I Domorakova; E Mechirova; M Ferikova; M Salinas; R Burda, *Cell Mol Neurobiol*, **(2006)**, 26, 1139–1149.
[53] ZQ Zhao; JV Johensen, *Cardiovascular Research*, **(2006)**, 70, 200 – 211.
[54] M Ovize; GF Baxter; FD Lisa FD; P Ferdinandy; DG Darado; DJ ff; G Heusch; J Vintrn-Johansen; DM Yellon; R Schulz, *Cardiovascular research advances*, **(2010)**, 1-76.
[55] PH Chan, *Stroke*, **(1996),** 27, 1124–1129.
[56] J Shen; J Zhou; Y Peng; Q Xia, *The FASEB Journal*, **(2009)**, 23.
[57] DJ Hausenloy, DM Yellon, *Heart Fail Rev*, **(2007)**, 12(3-4), 217-34.
[58] GM Rune; M Frotscher, *Neurosci*, **(2005),** 136, 833–842.
[59] RL Roof; SW Hoffman; DG Stein, *Mol. Chem. Neuropathol*, **(1997)**, 31, 1–11.
[60] M Leskiewicz; D Jantas; B Budziszewska; W Lason, *J. Physiol. Pharmacol*, **(2008)**, 59, 457–475.
[61] CA Frye, *Brain Res*, **(1995)**, 696,113–120.
[62] A Yoo; J Harris; B Dubrovsky, *Exp Neurol*, **(1996)**, 137, 151–156.
[63] JJ Lambert; D Belelli; DR Peden; AW Vardy; JA Peters, *Progress in Neurobiology*, **(2003)**, 71, 67–80.
[64] RM Kaminski; MA Rogawski, *Neuropharmacology*, **(2011)**, 61(1-2), 133–137.
[65] DS Reddy; K Jian, *J. Pharmacol. Exp. Ther*, **(2010)**, 334, 1031–1041.
[66] N Sharma; NK Singh, OP Singh; V Pandey; PK Verma, *J. Anim. Sci*, **(2011)**, 24(4), 479 – 484.
[67] GA Donnan; Zapf P; AE Doyle; P.F. Bladin, *Stroke*, **(1983),** 14, 266-269.
[68] M VanElzakker; RD Fevurly; T Breindel; RL Spencer, *Learn Mem*, **(2008)**, 15(12), 899–908.
[69] C Zhou; J Tu; Q Zhang; D Lu; Y zhu; W Zhang; F Yang; DW Brann; R Wang, *Neurochem Int*. **(2011)**, 59, 749-758.
[70] M Tanaka; M Sokabe, *Neuropharmacology*, **(2012)**, 62(7), 2373-2387.
[71] HJ Krugers; S Maslam; J Korf; M Joe¨ls, *Stroke*, **(2000)**, 31, 1162- 1172.
[72] W Jing, J Shen; Q Gao; Z Ye; S Yang; H Liang; LC Bruce; B Luo; Q Xia, *Stroke*, **(2008)**, 1-9.
[73] JS Leinonen; JP Ahonen; K Lonnrot; M Jehkonen; P Dastidar; G Molnar; H Alho, *Stroke*, **(2000)**, 31, 33–39.