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Pre-ischemic treatment with memantine reversed the neurochemical and behavioural parameters but not energy metabolites in middle cerebral artery occluded rats

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ABSTRACT

In the present study, Memantine (MN) an uncompetitive N-methyl-D-aspartate (NMDA) open channel blocker has been investigated for its suitable therapeutic time-window on the basis of its influence on behavioural and biochemical changes in rats subjected to transient focal ischemia. MN (20 mg/kg, ip) was administered at pre, during and post ischemic state and the extent of neuroprotection was compared to ascertain its therapeutic time-window in stroke treatment. Neuroprotective effect was assessed by measuring glutamate, glutamine synthetase, glutathione, Na^+K^+ATPase, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD), lactate and pyruvate levels. Middle cerebral artery occlusion produced neurological deficits, anxiogenic behaviour, histological changes, increased glutamate levels along with depletion of Na^+K^+ATPase, energy stores such as ATP, NAD, lactate, and antioxidant glutathione. MN significantly restored glutamate, glutamine synthetase, Na^+K^+ATPase and lactate levels on preischemic administration. In addition, MN reversed the altered neurological and behavioural paradigms significantly and prevented the neurodegeneration on preischemic treatment. However, it failed to exert any effect on energy metabolite (ATP and NAD) levels irrespective of the treatment phase. Based on the present data, it is summarized that the suitable therapeutic time window of MN is preischemic phase in stroke and it possesses only a subjective role in reversing ischemic brain biochemical alterations preferentially in favor of neuronal homeostasis.

1. Introduction

Cerebral ischemia, one of the leading causes of medical morbidity and mortality in geriatric population often results in irreversible brain damage. Focal impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose. The biochemical alterations following cerebral ischemia starts with depletion of energy phosphates and disruption of ion homeostasis with a consequent increase in the extracellular potassium and glutamate levels rendering over activation of N-methyl-D-aspartate receptors (NMDARs), intracellular Ca^{2+} over load and finally cell death (Arundine and Tymianski, 2004) as time progresses. It will be more appropriate if the therapeutic interventions are based on the bio- and neurochemical status following ischemic-reperfusion (IR).

Various therapeutic strategies are employed in the treatment of stroke, with NMDA blockers gaining greater interest in the recent past. NMDAR antagonists have often failed as therapeutic agents because of their debilitating side effects (Lipton, 2004). Memantine (1-amino-3,5-dimethyladamantane), an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist reduces glutamatergic excitotoxicity. Memantine (MN) has been approved in Europe in 2002 and United States in October 2003 for treatment of moderate to severe Alzheimer's disease. Unlike other NMDA receptor antagonists, memantine possesses fast on/off kinetics, low-moderate affinity and it also blocks the effects of excessive glutamate while preserving the physiologic activation of NMDA receptors (Johnson and Kotermanski, 2006).

Earlier results were paradoxical with NMDA agents administered at different time points of IR (Macleod et al., 2004; Ikonomidou and

Abbreviations: ANOVA, analysis of variance; ATP, adenosine 5′ triphosphate; DNA, deoxyribose nucleic acid; GLAST, glutamate astrocytes transporters; GS, glutamine synthetase; ip, intraperitoneal; IAEC, Institutional Animal Ethical Committee; IR, ischemic-reperfusion; LDH, lactate dehydrogenase; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MN, memantine; NAD, nicotinamide adenine dinucleotide; NMDA, N-methyl D-aspartate; NMDAR, N-methyl D-aspartate receptor; PARP, Poly (ADP-ribose) polymerase; PDH, pyruvate dehydrogenase; SO, sham operated.

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Turski, 2002). In some failed stroke clinical trials, treatments were administered outside the temporal window of efficacy (Labiche and Grotta, 2004) of the drugs. Hence, a study evaluating the correlation of the efficacy of the drugs with different time episodes of ischemia might yield a meaningful result in the treatment of stroke. The present study demonstrates the suitable therapeutic time window of MN and its role on behavioural and biochemical alterations in rats subjected to middle cerebral artery occlusion (MCAO).

2. Materials and methods

2.1. Chemicals

Memantine and L-glutamic acid were purchased from Sigma, US; adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) were purchased from SISCO Research Laboratories, Mumbai, India. 4-0 nylon monofilament Ethicon® was procured locally. All other chemicals, reagents and solvents used were of analytical grade.

2.2. Animals

Male Sprague Dawley rats (290–340 g) were used in this study. Animals were housed in individual polypropylene cages in a well ventilated room (air cycle: 15 per min; 70:30) under an ambient temperature of 23 ± 2 °C and 40–65% relative humidity, with a 12-h light/dark cycle. They were provided with food (Nutrilab Rodent, Tetragon Chemie Pvt Ltd, India) and purified water *ad libitum*. All the animals were acclimatized at least for 7 days to the laboratory conditions prior to experimentation. Guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. Institutional Animal Ethical Committee (IAEC), Sri Ramachandra University, Chennai, India approved the study.

2.3. Surgical procedure

Focal cerebral ischemia was induced by middle cerebral artery occlusion as described by Longa et al. (1989) with minor modifications. Rats were anesthetized with chloral hydrate (350 mg/kg, ip) and right common carotid artery was exposed at 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. 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 20–21 mm until a slight resistance was felt. On achieving occlusion, the filament was held in place with ligature and external incision was sutured temporarily. After 2 h of ischemia the rats were anesthetized, suture was opened, the filament was pulled out and reperfusion in internal carotid artery was ensured visually. Throughout the surgical procedure, body temperature was measured by inserting a thermometric probe into the rectum of rat and it was maintained at 37 ± 0.5 °C using thermostatically controlled heating blanket. Animals were then kept in a cage with a heating lamp, which maintained the cage temperature between 29 ± 1 °C for another 1 h to counteract any possible hypothermic effect. In the sham-operated group, external carotid artery was surgically prepared for insertion of filament, but the filament was not inserted.

2.4. Experimental design and drug treatment

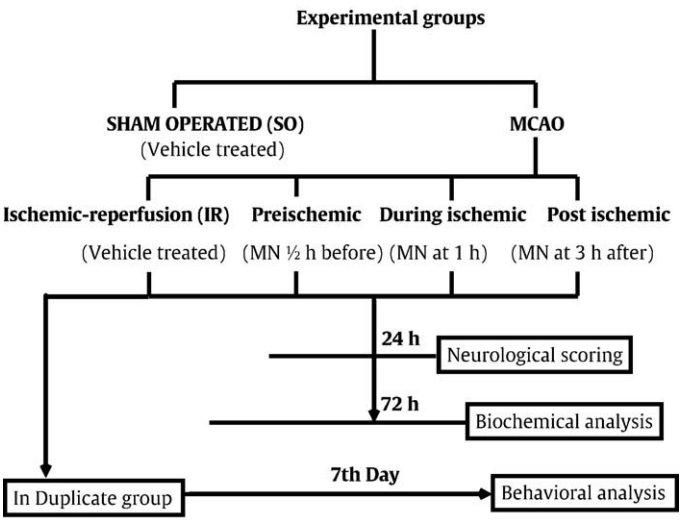
Following the occlusion of MCA with nylon filament the ischemic episode begins. Experimental animals were divided into five groups of 6–9 each. Group 1 served as sham-operated controls (SO) and received 0.9% sterile saline as vehicle. Group 2 animals were subjected to MCAO and received 0.9% sterile saline i.e., vehicle treated (IR) (vehicle was administration in the pre, during and post ischemic phases [$n = 3$/group]; having observed no significant changes in the biochemical and behavioural parameters we have pooled up and presented the data). Group 3 animals were pretreated with MN 30 min before MCAO. Group 4 animals received MN during the ischemic episode i.e., 1 h following MCAO and Group 5 animals were treated with MN 3 h after the onset of reperfusion. MN dose was fixed based on the findings of Block and Schwarz (1996a). MN (20 mg/kg) was dissolved in 0.9% sterile saline and administered intraperitoneally. Behavioural experiments were performed in another set of animals with the same groupings and dose schedule ($n = 6$–7/group) on day 7 following IR, since marked alterations in behaviour were observed only after that period (our unpublished data).

2.5. Steady state experiment

Vehicle or drug treated groups were subjected to neurological deficit scoring after 24 h of IR. Seventy two hours after IR, experimental animals were anesthetized; blood was collected through retro orbital puncture and centrifuged to separate plasma. Earlier reports revealed alterations in the antioxidant, biochemical and neurochemical substances in different brain regions of rats subjected to MCAO. Hence to support the neuroprotective activity of MN the biochemical parameters such as ATP, NAD, lactate, pyruvate, Na^+K^+ATPase and GS along with antioxidants were measured to understand the cell functions and homeostasis. The neurochemical glutamate was measured to elucidate the excitatory activity during the ischemic state. After collection of blood, the animals were euthanized, brains were quickly removed and different brain regions were immediately dissected over an ice-cold plate using the atlas of Paxinos and Watson (1986) as reference. In the duplicate group, behavioural study was performed on the 7th day following IR. Neurobiochemical and behavioural studies were performed by individuals unaware of the treatment schedule to avoid bias.

2.6. Assessment of neurological deficit

Neurological deficits were scored as described by Bederson et al. (1986), with minor modifications 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 pulled by tail; Score 4 — spontaneous circling.



Experimental group and treatment schedule layout

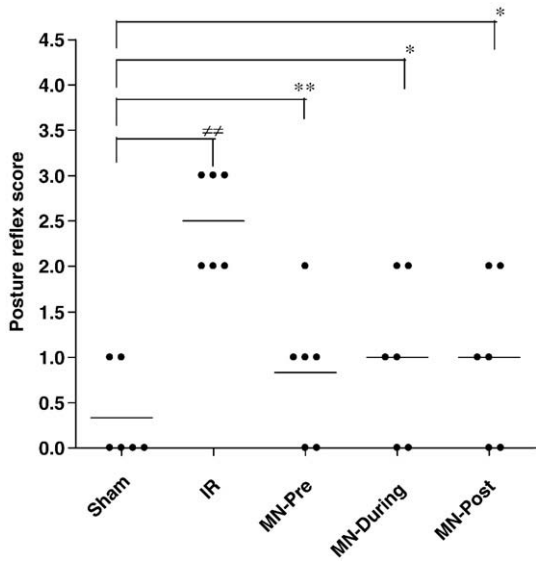


Fig. 1. Effect of memantine on neurological score in MCAO rats. ≠: $p<0.05$ and ≠≠: $p<0.01$ vs. sham-operated group; *: $p<0.05$ and **: $p<0.01$ vs. IR group.

2.7. Neuro and biochemical analysis

2.7.1. Plasma lactate

Plasma lactate was measured according to the kit manufacturer's instructions (Diagnostic kit — Randox, UK).

2.7.2. Pyruvate (Czok and Lamprecht, 1974)

To 500 µl of the plasma or saline, 1.5 ml of 5% tricarboxylic acid was added and centrifuged at 2500 rpm for 10 min. To 500 µl supernatant, 1 ml distilled water and 500 µl of 2,4-dinitro phenyl hydroxide (prepared in 2 N HCl) were added, mixed well and allowed to stand at room temperature for 5 min. To the above mixture, 2.5 ml of 1.5 N sodium hydroxide was added. The intensity of the reddish pink color was measured at 540 nm OD using spectrophotometer (Perkin Elmer, λ 25, USA). The standard calibration curve was plotted using pyruvate in the concentration range of 4–20 µg.

2.7.3. Glutamate content (Babu et al., 2007)

Tissue was homogenized in 0.1 N HCl in 80% ethanol (for every 10 mg tissue/200 µl) and was transferred to polypropylene tubes and centrifuged at 4500 rpm for 20 min at 25 °C. The supernatant was then transferred into micro centrifuge tubes and used at the earliest for glutamate estimation. HPTLC (CAMAG — version 1.3.4, USA) chromatographic condition: silica gel GF_{254} as stationary phase; n-butanol: glacial acetic acid:water (65:15:25 v/v) as mobile phase; applicator: Linomat V; scanner: CAMAG TLC scanner III; developing chamber: twin trough glass chamber (20×10); developing mode: ascending mode (multiple development); detection reagent: 0.2% ninhydrin in acetone; scanning wavelength: 486 nm; experimental condition:25 ± 2 °C temp/RH: 55–65%. Standard solutions of L-glutamic acid were prepared at 20–200 ng for plotting the calibration curve.

2.7.4. GS activity (Sadasivam and Manickam, 2003)

Glutamine synthetase was assayed by taking 0.5 ml of Tris HCl buffer (20 mM, pH 8.0) followed by the addition of 25 µl of 0.2 M glutamate, 25 µl of 100 mM ammonia, along with 50 µl of 3 mM $MgCl_2$ and 100 µl of 1 mM ATP. The reaction mixture was pre-incubated at 37 °C for 5 min. Then 100 µl of 10% homogenate was added to the test alone and further incubated at 37 °C for 15 min. The reaction was immediately arrested by the addition of 500 µl of 10% TCA. The control reaction rate was correspondingly assessed by adding 100 µl of 10% homogenate only after arresting the reaction. The precipitate was removed by centrifugation at 3500 rpm for 10 min. To 200 µl of the supernatant, 925 µl of distilled water, 125 µl of ammonium molybdate and 50 µl of ANSA (1-amino-2-napthol-4-sulphonic acid) were added and incubated for 10 min at 37 °C. The intensity of blue color was read at 640 nm using 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. Standard calibration was plotted using potassium dihydrogen phosphate in the concentration range of 1.6–8.0 µg.

2.7.5. Na^+K^+ATPase (Sovoboda and Mossinger, 1981)

Na^+K^+ATPase was assayed by taking 250 µl of Tris HCl (184 mM; pH 7.5) buffer followed by the addition of 50 µl of 600 mM NaCl, 50 µl of 50 mM KCl, along with 50 µl of 1 mM Na.EDTA, and 50 µl of 80 mM ATP. The reaction mixture was pre-incubated at 37 °C for 10 min. Then 25 µl of 10% homogenate was added to the test alone and further incubated at 37 °C for 1 h. The reaction was immediately arrested by the addition of 10% TCA. The control reaction was correspondingly performed by adding 25 µl of 10% homogenate only after arresting the reaction. The precipitate was removed by centrifugation at 3500 rpm for 10 min. From the 50 µl of the supernatant, the liberated inorganic phosphorus was measured as mentioned in GS estimation method.

2.7.6. Glutathione (Jollow et al., 1974)

0.25 ml of tissue homogenate was added to equal volume of ice cold 5% TCA. The precipitate was removed by centrifugation at 4000 rpm for 10 min. To 1 ml aliquot of supernatant, 0.25 ml of 0.2M phosphate buffer (pH 8.0) and 0.5 ml of DTNB (0.6 mM in 0.2M phosphate buffer, pH 8.0) were added and mixed well. The absorbance was read at 412 nm using spectrophotometer (Perkin Elmer, λ 25, USA). Values were expressed in nano moles/g tissue.

Table 1
Effect of memantine on L-glutamate level in various brain regions following MCAO.

Treatment	Glutamate (µmol/g wet tissue)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	3.02 ± 0.18	2.87 ± 0.26	2.68 ± 0.2	2.98 ± 0.33	3.18 ± 0.21	2.78 ± 0.19
IR control ($n=9$)	8.64 ± 0.32 $^{\neq\neq}$ [186.1 ↑]	4.28 ± 0.32	8.87 ± 0.91 $^{\neq\neq}$ [230.97 ↑]	3.72 ± 0.11	7.33 ± 0.31 $^{\neq\neq}$ [130.50 ↑]	4.96 ± 0.54
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	4.98 ± 0.41** [42.36 ↓]	3.09 ± 0.18	6.02 ± 0.19 ** [32.13 ↓]	3.43 ± 0.33	5.95 ± 0.23** [18.83 ↓]	4.16 ± 1.03
During ischemic ($n=8$)	7.48 ± 0.64 [13.43↓]	3.84 ± 0.48	7.10 ± 0.28 [19.96 ↓]	3.11 ± 0.43	6.21 ± 0.16* [15.28 ↓]	4.75 ± 0.42
Post ischemic ($n=9$)	6.84 ± 0.6 [20.83 ↓]	3.56 ± 0.43	7.2 ± 0.46 [18.83 ↓]	3.51 ± 0.57	6.78 ± 0.33 [7.50 ↓]	4.28 ± 0.33

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

Table 2
Effect of memantine on glutamine synthetase activity in various brain regions following MCAO.

Treatment	Glutamine synthetase activity (nano moles of inorganic Phosphorus liberated/min/mg protein)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	1.09 ± 0.07	0.98 ± 0.06	1.14 ± 0.24	1.32 ± 0.35	1.54 ± 0.43	1.42 ± 0.39
IR control ($n=9$)	4.28 ± 0.33$^{\neq\neq}$ [292.66 ↑]	1.9 ± 0.15	5.01 ± 0.20$^{\neq\neq}$ [339.47 ↑]	2.15 ± 0.17	5.01 ± 0.26$^{\neq\neq}$ [225.33 ↑]	2.09 ± 0.21
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	2.19 ± 0.20** [48.83 ↓]	1.53 ± 0.22	2.17 ± 0.38** [56.69 ↓]	1.41 ± 0.05	2.15 ± 0.29** [57.09 ↓]	1.67 ± 0.14
During ischemic ($n=8$)	4.19 ± 0.15 [2.10↓]	1.35 ± 0.37	2.4 ± 0.32** [52.10 ↓]	1.72 ± 0.06	2.69 ± 0.37** [46.31 ↓]	1.81 ± 0.14
Post ischemic ($n=9$)	4.1 ± 0.56 [4.21 ↓]	1.12 ± 0.3	4.1 ± 0.35 [18.16 ↓]	2.09 ± 0.27	4.55 ± 0.13 [9.18 ↓]	1.85 ± 0.1

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

2.7.7. ATP and NAD content (Zhan and Yang, 2006)

Aliquots of the homogenates were sonicated immediately in an ice-cold perchloric acid (0.1 N) to inactivate ATPases. After centrifugation (14,000 *g*, 4 °C, 5 min), supernatants containing ATP were neutralized with 1 N NaOH and stored at −80 °C until analysis. ATP/NAD levels in supernatants were quantified using a reverse-phase HPLC (Perkin Elmer). RP-HPLC determination was performed on a reversed-phase Hypersil C18 (4.6 mm × 250 mm, 5 µ) column (Elite, Dalian, China) attached to two LC-10ATvp pumps (Shimadzu, Kyoto, Japan), equipped with UV–Vis detector. The mobile phase was 100 mM KH_2PO4–K_2HPO4 buffer solution (pH 6.0), the flow rate 1.2 ml min^{-1}, the column temperature 25 °C and the detection wavelength 254 nm. Reference solutions of ATP and NAD were prepared according to dissolving standards (Sigma, St. Louis, MO, USA).

2.7.8. Protein

The protein content in the brain homogenate was estimated by Lowry et al.'s (1951) method.

2.8. Assessment of open-field exploratory behaviour

Behavioural alterations in experimental groups were assessed by open-field exploratory test (Lister, 1990). Open field apparatus made of plywood, consisted of a floor (96 × 96 cm) with high walls. The entire apparatus was painted black except for 6-mm-thick white lines that divided the floor into 16 squares. Each animal was placed at one corner of the apparatus and for the next 5 min it was observed for ambulation (number of squares crossed), total period of immobility (in seconds), rearing, grooming and number of fecal pellets excreted.

2.9. Histopathological examination

After 72 h of IR, the experimental animals were euthanized. The brains were dissected out quickly, fixed in 10% formalin and 5-µm thick sections were taken. The sections were processed and stained in 0.1% cresyl violet stain. The stained sections were observed under a binocular light microscope and photographed. Quantitative scoring of histopathological examination was performed according to Block and Schwarz (1996b) method with slight modifications. Scoring of brain ischemic injury in the sampled regions was done by assessing the histological picture as follows: 0–10% = 1 (no morphological signs of damage and few dark stained cells); 11–30% = 2 (meagre edema or eosinophilic or dark neurons or dark/shrunk cells); 31–50% = 3 (moderate number of dark staining cell bodies) and 51–70% = 4 (clearly infarctive foci, severe lesions with stellate neurons, cell swelling) 71–100% = 5 (almost all cells with a necrotic appearance). A total histological score of the brain area was calculated by adding all the regional scores and then expressed based on their respective percentage of damage.

2.10. Data analysis

Statistical analysis was performed using GraphPad Prism, 4.03 (San Diego, US). Data were expressed as mean ± SE. Mean differences in neurochemical, biochemical and behavioural data were analyzed by one way ANOVA with Tukey's multiple comparison post hoc. Neurological scores (median value) were analyzed by Mann–Whitney *U* test. *p*-value < 0.05 was fixed as the statistical significance criterion.

3. Results

In the present study, the suitable therapeutic time window of memantine was investigated based on its influence on biochemical and behavioural parameters in rats subjected to transient focal ischemia.

3.1. Effect of MN on neurological deficit

Effect of MN on neurological deficit administered at different phases of IR is shown in Fig. 1. Induction of cerebral ischemia engendered spontaneous movements, contralateral circling and decreased balance to lateral push in IR rats in comparison to SO rats ($p<0.01$). Preischemic

Table 3
Effect of memantine on Na^+K^+ATPase activity in various brain regions following MCAO.

Treatment	Na^+K^+ATPase activity (nano moles of inorganic Phosphorus liberated/min/mg protein)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	302.52 ± 19.81	311.90 ± 21.57	354.28 ± 19.28	367.45 ± 21.57	388.14 ± 21.79	375.29 ± 23.89
IR control ($n=9$)	99.51 ± 9.34$^{\neq\neq}$ [67.11 ↓]	226.42 ± 30.85	101.73 ± 20.94$^{\neq\neq}$ [71.29 ↓]	252.08 ± 23.012$^{\neq\neq}$	89.70 ± 7.46$^{\neq\neq}$ [76.89 ↓]	227.28 ± 12.34$^{\neq\neq}$
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	201.55 ± 11.97** [102.54 ↑]	267.17 ± 20.46	185.46 ± 12.52** [82.31 ↑]	254.12 ± 8.56	177.53 ± 14.18** [97.92 ↑]	249.30 ± 11.53
During ischemic ($n=8$)	127.7 ± 7.01 [28.32 ↑]	225.39 ± 11.76	186.28 ± 15.33** [83.11 ↑]	264.07 ± 17.46	160.19 ± 7.68* [78.58 ↑]	223.20 ± 18.61
Post ischemic ($n=9$)	139.99 ± 11.24 [40.68 ↑]	217.92 ± 41.54	157.71 ± 7.32 [55.03 ↑]	231.64 ± 10.78	102.46 ± 17.44 [14.22 ↑]	257.14 ± 22.96

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

Table 4
Effect of memantine on ATP level in various brain regions following MCAO.

Treatment	ATP (μmol/g tissue)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	6.90 ± 0.78	6.35 ± 0.49	4.5 ± 0.31	4.93 ± 0.37	7.1 ± 0.45	6.92 ± 0.53
IR control ($n=9$)	1.61 ± 0.21$^{\neq\neq}$ [76.67 ↓]	3.02 ± 0.28$^{\neq\neq}$	1.38 ± 0.33$^{\neq\neq}$ [69.33↓]	3.2 ± 0.42$^{\neq}$	1.57 ± 0.12$^{\neq\neq}$ [77.89↓]	2.53 ± 0.22$^{\neq\neq}$
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	2.09 ± 0.27 [29.81 ↑]	3.89 ± 0.42	2.38 ± 0.52 [72.46 ↑]	3. 84 ± 0.61	2.52 ± 0.33 [60.51 ↑]	3.62 ± 0.37
During ischemic ($n=8$)	2.20 ± 0.14 [36.65 ↑]	3.26 ± 0.163	2.15 ± 0.18 [55.8 ↑]	3.65 ± 0.26	1.46 ± 0.13 [7.01 ↓]	2.23 ± 0.15
Post ischemic ($n=9$)	2.10 ± 0.34 [30.43 ↑]	3.22 ± 0.17	2.46 ± 0.15 [78.26 ↑]	4. 09 ± 0.21	2.24 ± 0.19 [42.68 ↑]	3.16 ± 0.29

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

administration of MN ameliorated the neurological deficits more significantly ($p<0.01$) than during or postischemic conditions ($p<0.05$).

3.2. Effect of MN on brain glutamate level

A significant elevation in glutamate level was observed in the ipsilateral cortex [F (4,35) = 18.50, $p<0.01$], striatum [F (4,35) = 15.54, $p<0.01$] and hippocampus [F (4,35) = 29.57, $p<0.01$] of IR rats in comparison to SO rats. Preischemic administration of MN significantly ($p<0.01$) decreased the glutamate levels in all the regions in comparison to vehicle treated IR rats (Table 1) while MN failed to control glutamate elevation during and postischemic administration. Furthermore, a non-significant increase in glutamate level was observed in contralateral tissues of IR group rats in comparison to SO rats. MN did not exert any effect on glutamate levels of contralateral tissues.

3.3. Effect of MN on brain glutamine synthetase (GS) activity

Occlusion of MCA increased GS activity in the ipsilateral cortex [F (4,35) = 15.38, $p<0.01$], striatum [F (4,35) = 24.30, $p<0.01$] and hippocampus [F (4,35) = 26.40, $p<0.01$] of IR rats in comparison to SO rats. Preischemic administration of MN attenuated GS activity significantly ($p<0.01$) in all the brain structures studied in comparison to vehicle treated IR group. MN treatment during ischemic condition decreased GS activity in striatal and hippocampal regions. However, post ischemic administration of MN failed to decrease GS activity (Table 2). A non-significant increase in GS activity was observed in contralateral tissues in IR group in comparison to SO group.

3.4. Effect of MN on Na^+K^+ATPase activity

Induction of cerebral ischemia resulted in decreased Na^+K^+ATPase activity in cortex [F (4,35) = 41.42, $p<0.01$], striatum [F (4,35) = 30.78, $p<0.01$] and hippocampus [F (4,35) = 61.70, $p<0.01$] of IR group in comparison to SO group. Preischemic administration of MN significantly ($p<0.01$) increased Na^+K^+ATPase activity in the ischemic tissues of different brain regions in comparison to vehicle treated IR group. Administration of MN during ischemia increased Na^+K^+ATPase activity in striatal and hippocampal regions. Similarly, increased Na^+K^+ATPase activity was observed in hippocampal tissues of the post ischemic MN treated group (Table 3). No significant change in contralateral Na^+K^+ ATPase activity was observed between the vehicle and treated groups.

3.5. Effect of MN on brain ATP content

The effect of MN on brain ATP content in the experimental groups is represented in Table 4. In comparison to SO group, ATP content was found to be decreased in the ipsilateral cortex [F (4,35) = 31.06, $p<0.01$], striatum [F (4,35) = 10.83, $p<0.01$] and hippocampus [F (4,25) = 10.83, $p<0.01$] of IR group. Administration of MN in pre or during or postischemic state failed to increase the ATP content. A non-significant decrease in ATP content was also observed in the contralateral region of all the MCAO groups in comparison to the SO group.

3.6. Effect of MN on brain NAD content

Middle cerebral artery occlusion resulted in significant ($p<0.01$) decrease in NAD content in ischemic cortex [F (4,35) = 10.68, $p<0.01$], striatum [F (4,35) = 15.50, $p<0.01$] and hippocampus [F (4,35) = 32.64, $p<0.01$] of IR rats in comparison to SO rats. Administration of MN, irrespective of treatment phases, failed to increase NAD level in ischemic brains. A non-significant decline in NAD content was observed in the contralateral region of all MCAO rats (Table 5).

3.7. Effect of MN on brain GSH content

Effect of MN on brain GSH content in MCAO rats is shown in Table 6. A significant decrease in ipsilateral cortex [F (4,35) = 13.47, $p<0.01$],

Table 5
Effect of memantine on NAD content in various brain regions following MCAO.

Treatment	NAD^+ (μmol/g tissue)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	402.36 ± 32.70	395.78 ± 28.91	424.70 ± 54.70	412.35 ± 21.79	480.35 ± 32.57	432.16 ± 31.79
IR control ($n=9$)	174.62 ± 15.35$^{\neq\neq}$ [56.60 ↓]	293.54 ± 20.56	151.25 ± 18.77$^{\neq\neq}$ [64.39 ↓]	351.42 ± 10.50	129.06 ± 12.41$^{\neq\neq}$ [73.13↑]	357.67 ± 23.03
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	252.78 ± 22.05 [44.76 ↑]	329.11 ± 31.66	224.22 ± 20.95 [48.25↑]	363.91 ± 19.05	147.94 ± 25.45 [14.63↑]	375.09 ± 23.64
During ischemic ($n=8$)	222.73± 24.94 [27.55 ↑]	309.13 ± 19.39	163.46 ± 24.12 [8.07 ↑]	292.70 ± 13.99	117.92 ± 17.46 [8.63 ↓]	308.42 ± 36.14
Post ischemic ($n=9$)	211.30 ± 28.60 [21.01 ↑]	290.96 ± 27.2	199.22 ± 12.00 [31.72 ↑]	296.63 ± 25.72	200.63 ± 29.48 [55.46 ↑]	335.47 ± 22.64

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

Table 6
Effect of memantine on GSH content in various brain regions following MCAO.

Treatment	GSH (nano moles/g tissue)					
	Cortex		Striatum		Hippocampus	
	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral	Ipsilateral [% Δ]	Contralateral
Sham operated ($n=6$)	4.01 ± 0.60	3.98 ± 0.37	4.87 ± 0.52	4.56 ± 0.65	4.25 ± 0.32	4.38 ± 0.19
IR control ($n=9$)	$0.85 \pm 0.10^{\neq\neq}$ [78.80% ↓]	3.31 ± 0.07	$0.96 \pm 0.18^{\neq\neq}$ [80.29%↓]	$3.32 \pm 0.21^{\neq}$	$0.76 \pm 0.11^{\neq\neq}$ [82.12% ↓]	3.66 ± 0.15
Memantine (20 mg/kg, ip)						
Preischemic ($n=8$)	2.97 ± 0.20** [71.38% ↑]	3.19 ± 0.30	2.82 ± 0.28** [65.96%↑]	2.83 ± 0.17	2.61 ± 0.23* [70.88%↑]	2.92 ± 0.21
During ischemic ($n=8$)	1.94 ± 0.43 [56.19% ↑]	3.29 ± 0.26	1.88 ± 0.20 [48.94% ↑]	2.65 ± 0.26	1.84 ± 0.15 [58.70% ↑]	2.95 ± 0.34
Post ischemic ($n=9$)	1.58 ± 0.26 [46.20% ↑]	3.90 ± 0.34	1.54 ± 0.14 [37.66% ↑]	2.80 ± 0.11	1.37 ± 0.44 [44.53%↑]	2.93 ± 0.16

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

striatal [F (4,35) = 30.47, $p<0.01$] and hippocampal [F (4,35) = 20.17, $p<0.01$] glutathione content was observed in IR rats in comparison to SO group. Preischemic administration of MN increased glutathione content in ischemic cortex ($p<0.01$), striatum ($p<0.01$) and hippocampus ($p<0.05$) significantly in comparison to contralateral tissues. During and post ischemic administration, MN failed to increase the glutathione content.

3.8. Effect of MN on plasma lactate level in MCAO rats

MCAO provoked a significant ($p<0.01$) elevation in plasma lactate level in IR group when compared to SO group [F (4,35) = 21.59, $p<0.01$]. Administration of MN at pre ($p<0.01$) and post ($p<0.05$) ischemic phase significantly decreased the lactate level but failed to control it during IR (Fig. 2).

3.9. Effect of MN on plasma pyruvate level in MCAO rats

A non-significant decrease in plasma pyruvate level was observed in IR group in comparison to SO group [F (4,35) = 15.50, $p>0.05$]. MN did not show any effect on pyruvate levels in MCAO rats (Fig. 3).

3.10. Effect of MN on open field exploratory test in MCAO rats

In open-field exploratory test, IR group rats expressed anxiety as evidenced by significant decrease in ambulation ($p<0.05$) [F (4,35) = 5.72, $p<0.01$] with increased immobility period ($p<0.01$) [F (4,35) = 11.73, $p<0.01$] in comparison to SO rats. Pre and post-ischemic administration of MN significantly ($p<0.01$) reversed the ambulatory and freezing behaviour in MCAO rats. Administration of MN during ischemic phase attenuated the freezing behaviour significantly ($p<0.01$) but failed to reverse the ambulatory behaviour. However, there were no significant differences in grooming, rearing and fecal pellet counts between any of the experimental groups (Table 7).

3.11. Histopathological examination

Fig. 4(a, b, c, d, e) illustrates the representative photographs of experimental rats' brain (striatal region). Occlusion of middle cerebral artery for 2 h and reperfusion for a period of 70 h produced severe neuronal damage in the vehicle treated IR group in comparison to SO group. In IR group, the rat brain (striatal region) showed almost 70% of neuronal damage characterized by dark-staining cell bodies and high tendency for neuronal shrinkage with a moderately eosinophilic perikaryal cytoplasm. In addition, neurons were irregularly stellate in shape. The foci of neuronal injury present throughout the brain are suggestive of ischemic damage. In the experimental group pre-treated with memantine (20 mg/kg) the striatal regions revealed scanty irregular shape or stellate neurons and almost 25–30% of neuronal damage only. Experimental group treated with memantine during ischemic episode showed moderate necrosis and cell swelling with loss of cytoarchitecture (50–55%). Post ischemic treatment group also revealed a moderate tendency of protection and the percentage of damage was found to be around 40–45% i.e. characterized by a

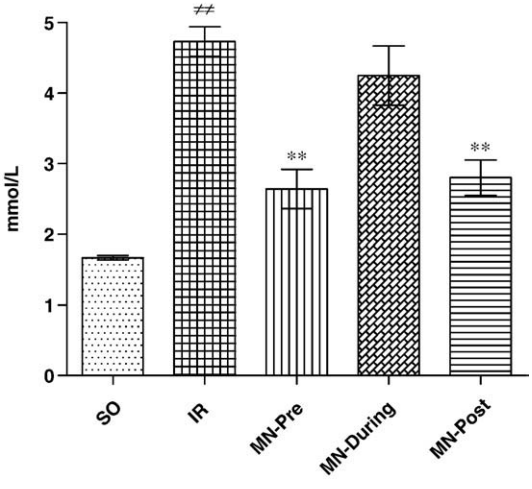


Fig. 2. Effect of memantine on plasma lactate level in MCAO rats. ≠: $p<0.05$ and ≠≠: $p<0.01$ vs. sham-operated group; *: $p<0.05$ and **: $p<0.01$ vs. IR group.

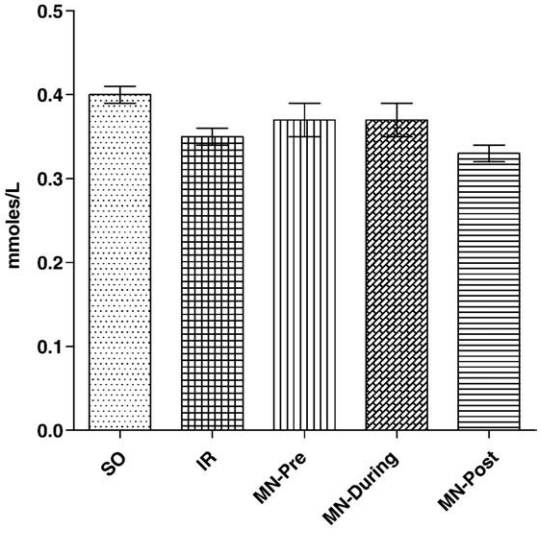


Fig. 3. Effect of memantine on plasma pyruvate level in MCAO rats. ≠: $p<0.05$ and ≠≠: $p<0.01$ vs. sham-operated group; *: $p<0.05$ and **: $p<0.01$ vs. IR group.

Table 7
Effect of memantine on open field exploratory behaviour following MCAO.

Treatment	Ambulation (numbers)	Immobility (s)	Rearing (numbers)	Grooming	Fecal pellets
Sham operated ($n=6$)	60.23 ± 4.79	81.89 ± 7.26	19.80 ± 2.90	14.27 ± 0.98	1.02 ± 0.12
IR control ($n=7$)	$27.83 \pm 4.29^{\neq}$	$182.83 \pm 26.56^{\neq\neq}$	14.00 ± 0.58	11.17 ± 1.50	2.00 ± 0.37
Memantine (20 mg/kg, ip)					
Preischemic ($n=6$)	66.50 ± 5.87**	65.50 ± 5.38**	17.50 ± 1.88	17.50 ± 2.67	1.60 ± 0.22
During ischemic ($n=7$)	52.50 ± 5.16	75.00 ± 6.48**	15.17 ± 1.85	15.17 ± 1.94	1.33 ± 0.21
Post ischemic ($n=6$)	59.67 ± 10.94*	122.17 ± 8.88*	19.33 ± 2.93	17.17 ± 3.15	1.67 ± 0.21

• Values expressed in mean ± SE; significance with Tukey's test following one way ANOVA is indicated as $^{\neq}p<0.05$ and $^{\neq\neq}p<0.01$ vs. sham-operated group or $^{*}p<0.05$ and $^{**}p<0.01$ vs. IR group.
• Values in square parentheses [%Δ] — percentage difference compared to sham operated or IR group; IR group was compared with sham operated group; treatment groups were compared with IR group; ↑ — increase; ↓ — decrease.

moderate neuronal shrinkage with irregular stellate cells. The percentages (based on scoring grade) of neuronal damage are represented graphically in Fig. 5.

4. Discussion

MCA occluded rats demonstrated neurological deficit and anxiogenic behaviour. There was a significant increase in glutamate levels with simultaneous depletion of Na^+K^+ATPase, energy metabolites (ATP, NAD), lactate, antioxidant-glutathione content along with degenerative neurons indicating excitotoxicity, cell membrane damage and oxidative stress. In cerebral ischemia, neurons and glia in the ipsilateral region are lethally damaged by several events such as peri-infarct depolarization, increased levels of reactive oxygen species, delayed postischemic inflammation and apoptosis (Hata et al., 2000). Further, biochemical alterations in contralateral region have also been reported (Thiyagarajan and Sharma, 2004; Serteser et al., 2002).

Glutamate levels peaked [~200%] at 72 h following IR and then reached near basal levels approximately on the 7th post operative day (our unpublished baseline data). Based on this observation, we decided to terminate the experiment at 72 h following IR. In the present study, MN showed consistent neuroprotection and functional recovery when administered in the pre-ischemic state. In earlier studies, pre-ischemic therapeutic approach showed satisfactory neuroprotective effect (Culmsee et al., 2004; Yanagisawa et al., 2008) and our report is also consistent with their findings. However, MN did not show any effect on contralateral tissue biochemistry, which might be due to the verity that MN preferentially binds to the hyperactivated channels. In MCAO rats, glutamate level was found to progressively increase till 72 h following ischemia (our basal unpublished data) and MN was expected to counteract this elevation. However it failed to do so in the during and post ischemic phases. Histopathological studies also revealed the same and hence it can be stated that the neuroprotective activity of MN is better with pre-ischemic administration. It can be argued that during ischemic condition, the compromised blood flow would have retarded MN to reach the ischemic site. Further in the post ischemic state, due to continuous and strong activation of NMDARs by glutamate, memantine, like Mg^{2+}, might have unbounded NMDAR due to voltage-dependency and fast unblocking kinetics (Kornhuber and Weller, 1997; Lipton, 2006). It is also possible that, the glutamate release following ischemia may involve different mechanisms i.e. initial phase release may be neuronal and later phase might be mediated through altered glial transporter (Dawson et al., 2000).

Increased glutamate level was paralleled by increased GS activity in different brain regions of MCAO rats. GS is essential for the clearance of extra cellular glutamate which is confined within astrocytes (Cooper et al., 1985). Pre-ischemic administration of MN decreased GS activity significantly in the ipsilateral structures (cortex: 48.83%, striatum: 56.69% and hippocampus: 57.09%). However, it failed to control GS activity in during and post ischemic phases which could be corroborated to its failure to control glutamate levels. Synaptic clearance of glutamate depends on glial bound Na^+K^+ATPase activity, which is very susceptible to the attack of free radicals induced by excessive glutamate accumulation (Nagafuji et al., 1992). Though MN increased Na^+K^+ATPase activity at different phases of IR, the pre-ischemic treatment was found to be more effective. These results clearly show that the neurochemical alterations (glutamate elevation) begin to increase gradually following the onset of ischemia and massively and immediately after reperfusion, which could not be controlled by MN administration. Hence, the control of excessive glutamate is one of the crucial factors to have better neuroprotection.

Depletion of ATP, NAD and antioxidant-glutathione content with simultaneous increase in plasma lactate level in IR rats reflects altered energy metabolism between neurons and glia. ATP is necessary for passable functioning of cells including cellular active transport, protein biosynthesis and preservation of cellular integrity. In astrocytes, glucose is metabolized to lactate by lactate dehydrogenase (LDH) or to pyruvate by pyruvate dehydrogenase (PDH) which further yields ATP. Cerebral ischemia decreases pyruvate dehydrogenase PDH activity (Niitsu et al., 1999) and hence ATP, which supports our present findings. In the setting of ischemia, LDH activity overwhelms to elevate lactate, which acts as cellular fuel during energy crisis (Benarroch, 2005). Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme, has been proposed to be a possible factor contributing to secondary energy failure following transient ischemia (Endres et al., 1997). During energy crisis, excessive activation of PARP depletes NAD, an important co-enzyme in energy production and this PARP-induced depletion of NAD pools aggravates the energy crisis resulting in DNA damage. Depletion of NAD (level) in ischemic brains in the present study may be due to PARP hyperactivation. Further, MN failed or only partially increased ATP and NAD levels. Memantine has been tested in animals against primary insults dependent on mitochondrial impairment and energy depletion and it has shown protection from inhibition of mitochondrial function (Schulz et al., 1996). One important function of glutamate in astrocytes is glutathione synthesis (Dringen 2000). Impaired astrocyte function would have decreased glutamate uptake and hence decreased the glutathione content in vehicle treated MCAO rats. Preischemic MN administration increased the glutathione levels and this demonstrates the significance of early control of glutamate level in upholding astrocyte function. No significant alteration in pyruvate levels was observed between the experimental groups. The increased plasma lactate level without any change in pyruvate level tends to confirm the view that anaerobic glycolysis was increased and also that the increase in lactate level may not be due to total increase in glycolysis. The ATP and NAD^+ data of the present study clearly indicate that MN possesses no or only lesser role in energy metabolism in cerebral stroke.

In the current experiment, IR rats have shown increased levels of glutamate in regions like cortex, striatum and hippocampus. As increased glutamate level in the brain is associated with exploratory and anxiogenic behaviour (Swanson et al., 2005), open field exploratory test was performed to assess the same in IR rats to see how it was affected by MN treatment. IR rats exhibited decreased ambulation,

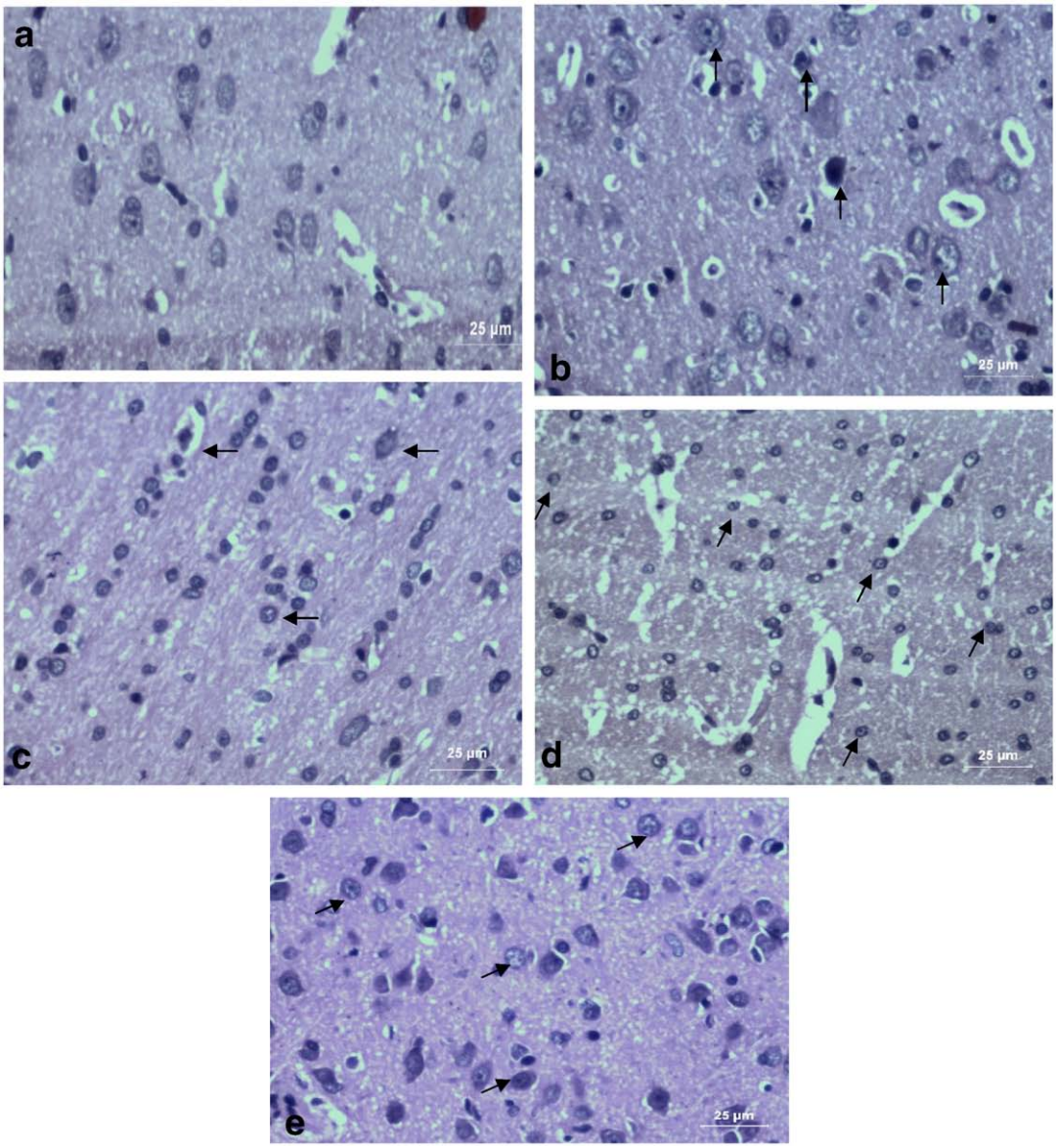


Fig. 4. Represents the cresyl violet staining of striatal region of ischemic rats after 70 h reperfusion. a Sham operated animals showing intact striatal region. b Photographs showing the striatal neurons of vehicle treated ischemic reperfusion group. Arrows indicate degenerating neurons, darkly stained cell bodies, shrunk eosinophilic perikaryal cytoplasm, irregularly stellate in shape. c – represents photograph showing the striatal neurons of MN – pre treated group, reduced number of dark-staining cell bodies and less number of irregularly shape of stellate neurons indicate neuroprotective effect of MN on pre-ischemic treatment. d and e represents the photograph of striatal neurons of rats received MN — during and post ischemic period respectively. Reduced anomaly in neuronal density, necrosis, cell swelling and loss of cytoarchitecture were observed. Intact neurons in the core of the lesions were moderate. The tendency of cells that underwent necrosis and swellings were moderate. Irregular stellate neurons were scanty and MN produced moderate neuroprotective activity.

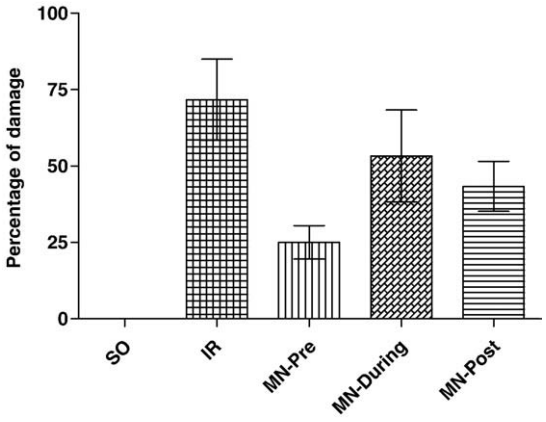


Fig. 5. The percentage (based on scoring grade) of neuronal damage in experimental groups.

rearing and grooming behaviour with increased immobility period in comparison to SO rats. Decreased ambulation and rearing behaviour reflects anxiogenic behaviour in ischemic rats (Yanpallewar et al., 2004). Ramanathan et al. (2007) had demonstrated that administration of exogenous glutamate to the experimental animals precipitates depression and anxiety. MN reversed the observed altered behavioural paradigm in IR rats which could be attributed to the normalisation of increased glutamate levels and its neuroprotective effects.

From the foregoing discussion it is clear that in the current animal model used, prevention of accumulation of extracellular glutamate is the very first step to be considered to prevent ischemia induced behavioural and biochemical abnormalities. In the present study, MN reversed the excessive glutamate levels better than other markers observed following IR and hence it can be concluded that the best therapeutic time window of MN administration is the preischemic phase. MN only partially restored the level of energy metabolites irrespective of the treatment phase. This shows that MN may possess

only a subjective role in reversing ischemic brain biochemical alterations, preferentially in favor of neuronal homeostasis.

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