Comparison of pre- and post-ischemic treatment of telmisartan and nimodipine combination in experimentally induced cerebral ischemia

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Time dependent intervention plays a crucial role in preventing neurodegeneration after ischemic insult. The intensity of excitotoxicity is greater in the secondary reperfusion phase (2-4 h) compared to the primary occlusion phase (2 h), which could be attributed to secondary elevation of excitatory amino acids (EAA) in cerebral ischemia. In the present study, we tried to assess the neuroprotective effects of telmisartan and nimodipine (TM-NM) combination on the secondary reperfusion phase. The drug treatments were made immediately after reperfusion and their effects were compared with pre-treatment. The neuroprotective effect was studied using middle cerebral artery occlusion (MCAo) transient ischemic model in rats. On the 7th day after reperfusion, the rats were subjected to behavioral studies. The brain was dissected out on the 9th day to measure neurobiochemical alterations and for histopathological observations. The results have shown that TM-NM (5 mg/kg) attenuated the EAA release in different brain regions with partial restoration of energy levels in secondary reperfusion phase. Similarly, it normalized the behavioral alteration and the effect was comparable to pre-ischemic treatment (2.5 mg/kg). Pre-ischemic treatment of TM-NM (2.5 mg/kg) protected the neurons from ischemic reperfusion injury by energy dependent EAA regulation. It can be concluded from the study that, even though the pre- and post-treatment of TM-NM show similar results, the post-ischemic treatment of TM-NM combination is beneficial due to better EAA control. Since hypertension is the primary risk factor for stroke, clinical incidents of stroke in hypertensive patients receiving angiotensin receptor blockers (ARBs) can be further investigated to understand the present study in the clinical situation.

Keywords: Angiotensin, Excitatory amino acids, Focal ischemia, Hypoxia, Neurodegeneration, Stroke

The pathophysiology of cerebral ischemia is multifaceted. Occlusion of major arteries reduce the cerebral blood flow and thereby result in decreased oxygen supply, glucose level, and energy metabolites like adenosine-triphosphate/nicotinamide adenine dinucleotide phosphate (ATP/NADP) in the neurons. Deprivation of micronutrients also increases the intracellular cytosolic calcium level (Ca2+) and releases excitatory amino acids (EAA), toxic free-radicals, and cytokines, which may provoke degeneration of the neurons1,2. These events occur during the primary (2h occlusion) and secondary (reperfusion) phases of transient focal ischemia and are time dependent. Reports indicate elevation of EAA and energy deprivation (up to 72 h) in the brain promotes the neuronal death3. However, results have shown greater neuronal death during the maturation of secondary reperfusion phases at 2-3 h following multiple transient forebrain ischemia4,5. Similarly, significant increase in glutamate, aspartate, and γ-aminobutyric acid (GABA) levels were observed compared to the initial phase (30 min) of transient ischemia in animals5. Even though both the phases exhibit excitotoxicity, the additional excitotoxicity after reperfusion plays an important role in ischemia6.

Currently, in stroke therapeutics thrombolytic agents have been administered within 4 h after ischemic injury to improve cerebral blood flow and to prevent neuronal loss. In pre-clinical studies, pre-treatment with non-hypotensive dose of telmisartan (TM) (5 mg/kg) suppressed cerebral injury in a murine model of transient focal ischemia through blockades of central angiotensin receptor-1 (AT1)7. Voltage dependent Ca2+ channel blocker nimodipine (NM) (5 mg/kg) has exhibited neuroprotection on post-ischemic administration in rats. This effect was attributed to the prevention of Ca2+ dependent EAA release and partial restoration of ATP/NADP levels8. Recently, we have reported that pre-ischemic...
administration of TM (5 mg/kg) and its combination with NM (5 mg/kg) results in neuroprotection. The effect was attributed to attenuation of EAA level by controlling brain cytokines level and partial restoration of energy levels. Hence, in the present study, TM and NM have been employed as an intervention combination to study and compare the beneficial effect of TM-NM combination on pre- and post-ischemic administration. This is because both the drugs are able to control free radical release, calcium levels and EAA. Further, both drugs have also been indicated in the treatment of hypertension as well as stroke. This study will also throw more light on the time dependent treatment effects. The study was carried out using a middle cerebral artery occlusion (MCAo) rat model. The drugs (TM and NM) were administered in the pre- or post-ischemic condition and the effects were compared. The neurochemicals, enzymes, antioxidants, energy metabolites, and behavior parameters were then measured to compare therapeutic efficacy.

Materials and Methods

Animals
Male Sprague Dawley (SD) rats (250–300 g, 6 months old) used in this study were supplied from the central animal house facilities, PSG Institute of Medical Sciences and Research, Coimbatore, India. The animals were housed in individual polypropylene cages in a well-ventilated room under an ambient temperature of 25±2°C and 55% relative humidity (RH), with a 12 h light/dark artificial photoperiod. They were provided with standard animal food and purified water ad libitum. All the experimental animals were acclimatized at least for 7 days to the laboratory conditions prior to experimentation. Guidelines pertaining to the “Guide for the Care and Use of Laboratory Animals” (ICMR) were strictly followed throughout the study. Institutional Animal Ethical Committee (IAEC) approved the ethical aspects of the study (proposal authorisation number-158/PO/BC/99/CPCSEA/168).

Experimental design and drug treatments
The rats (each group, n=9) were divided into five groups: 1\textsuperscript{st} and 2\textsuperscript{nd} groups were sham-operated (SO) (Gr. I) and ischemic reperfusion (IR) (Gr. II) groups received only 0.3 % carboxy methyl cellulose (CMC), respectively. The group (Gr. III) received pre-treatment of TM-NM (TM and NM @ 2.5 mg/kg) combination. The 4\textsuperscript{th} and 5\textsuperscript{th} groups received post-treatment of TM-NM @ 2.5 (Gr. IV) and 5 mg/kg (Gr. V), respectively. The drugs were suspended in 0.3% CMC and administered 30 min prior to the surgery in the pre-ischemic treatment. The drugs were administered immediately after the ischemic reperfusion (IR) in case of post-ischemic treatment. The same treatment was continued once daily in the morning hours for all the groups until the study was completed (Fig. 1).

Surgical procedure

Rat focal ischemic model
Focal cerebral ischemia was induced in rats by middle cerebral artery (MCA) occlusion surgical model as described by Babu & Ramanathan.

Neurobehavioural paradigms

Neurological deficit
Neurological deficit was assessed after 24 h of IR using the scoring pattern: 0, no neurological deficit; 1, failure to extend left forepaw fully; 2, circling behaviour; 3, falling to the left; 4, no spontaneous walking with a depression; and 5, death.

Actophotometer
Locomotor activity (horizontal activity) was measured by placing animal for 5 min in actophotometer.

Open field test
The Open field apparatus was made of plywood and consisted of a floor (96×96 cm) with high walls. The entire apparatus was painted black except for 6 mm thick white lines which divided the floor into 16 squares. Each animal was placed at one corner of the apparatus up to 5 min, the parameters observed were ambulation (number of squares crossed), total period of immobility (in seconds), rearing, and grooming.

Rotarod
The experimental animals were placed 5 min on a rotating rod apparatus at a speed of 20 rpm and the time spent by animals on the rotating rod was recorded to assess the muscle co-ordination and grip strength of the animal.
Neurotransmitters and neurobiochemicals analysis

After the behavioural studies, euthanasia was performed on these animals with excess of anesthesia and their brains were isolated. Immediately the brain was micro-dissected into cortex, striatum and hippocampus to analyze the neurotransmitters (n=3) and neurobiochemicals (n=3) alterations with respect to ischemia and drug treatment. The brain samples were then stored at −80°C until analysis.

Neurotransmitters estimation
Glutamate, Aspartate and GABA

Brain samples were homogenized in 0.1N HCl in 80% ethanol (for every 10 mg tissue/200 μL) and were 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 immediately for neurotransmitter estimation by HPTLC as described by Babu & Ramanathan.

Neurobiochemicals analysis

Brain homogenate was prepared with 10% ice cold KCl (10 mg of brain tissue in 100 μl of KCl) for neurobiochemical estimation. Adenosine triphosphate (ATP), Na\(^+\)K\(^+\)ATPase, reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARs), and nitric oxide (NO) levels were estimated in different regions of brain homogenate using standard protocol as followed by Justin et al.

Protein estimation

Protein content in the different brain tissues was estimated by the method of Lowry et al.

Histopathological studies
Cresyl violet staining

On the 9th day after IR, three rats per group were sacrificed following chloral hydrate anaesthesia. Their brains were dissected out, quickly fixed in 10% formalin, and 5 μm thin sections were taken. The sections were processed and stained with 0.1% cresyl violet stain. The stained hippocampus regions was observed under a binocular light microscope (10X) and photographed to assess the extent of neurodegeneration.

Statistical analysis

Data were expressed as mean ± SE. Statistical significance between the groups in behavioural parameters and neurobiochemicals were analyzed by one way ANOVA followed by Dunnett multiple comparison tests. The statistical significance in neurochemical changes was analysed by two-way ANOVA followed by Bonferroni test to compare the treatment effect across the regions using GraphPad Prism, 4.03 (San Diego, US). Neurological scores were analysed by Mann–Whitney U test. Probability levels less than 0.05 were fixed as the criterion for statistical significance.

Results

Effect of TM-NM combination on neurobehavioural paradigms in ischemic rats

Neurological deficit

Induction of ischemia by MCA occlusion resulted in muscle coordination difficulty as observed by the significant increase in the neurological score (P <0.001) in comparison to SO rats. The pre- (Gr. III) (P <0.001) and post-treatment (P <0.05) of TM-NM (Gr. IV & V) combination significantly improved the neurological function as noted by decrease in the neurological score compared to IR rats. Post-treatment of TM and NM has shown dose dependent effect. The pre- and post-treatment groups behaved similarly in this parameter (Fig. 2A).

Locomotor activity

Ambulatory behaviour was significantly reduced in IR (P <0.001) rats compared to SO rats (Fig. 3). The pre- (P <0.001) and post-treatment of TM-NM (Gr-V)
(P <0.01) significantly increased the ambulation and behaved similarly. Post-treatment of TM-NM (Gr. IV) did not alter the locomotor activity in IR rats in comparison to pre-treatment group (Gr. III) (Fig. 2B).

**Open field behaviour**

Vehicle treated ischemic rats exhibited anxiogenic behaviour as evidenced by decrease in the ambulatory (P <0.001), rearing (P <0.001) and grooming (P <0.001) behaviour along with increased immobility period (P <0.001) compared to SO (Gr. I) rats. The pre- (Gr. III) and post- treatment of TM-NM (Gr. V) in IR rats increased the ambulatory (P <0.05), rearing (P <0.01), grooming (P <0.05) behaviour and decreased the immobility period (P <0.01). Post-treatment of TM-NM (Gr. IV) failed to alter the exploratory behaviour in open field test. The behavioral effects of rat pre- and post treated rats with TM-NM (Gr. V) are comparable and no significant difference was noted (Table 1).

**Rota rod**

Ischemic reperfused vehicle treated rats have spent less time (P <0.001) in the rotating rod as compared to SO rats. Pre-treatment of TM-NM (Gr. III) improved the muscle grip in IR rats (P <0.001) as noted by increased time spent in rotating rod. Similar effect was observed with post-treatment of TM-NM (Gr. V) (P <0.001) in comparison to IR rats. Post-treatment of TM-NM (Gr. IV) failed to produce any significant effect in this study (Fig. 2C).

**Effect of TM-NM combination on neurotransmitters in ischemic rats**

**Glutamate**

Occlusion of MCA in rats significantly increased the glutamate levels (F(2,75)=13.65; P <0.0001) in cortex, striatum, and hippocampus regions of brain indicating that induction of ischemia and elevated differently the glutamate levels among the regions studied (Table 2). The interaction effect between regions and treatment groups have shown significant difference (F(8,75)=3.980; P <0.001). The treatment of TM and NM combination have exhibited remarkable reduction in glutamate level in ischemic brain regions (F(4,75)=61.63; P <0.0001). In comparison to vehicle treated IR rats, rats pre-treated with TM-NM (Gr. III) significantly decreased the cortex (P <0.01) and hippocampus (P <0.001) glutamate levels. Similarly, post-treatment of TM-NM (Gr. V) decreased the glutamate levels in all three regions (P <0.001) tested. Post-treatment of TM-NM (Gr. IV) (P <0.01) decreased the striatal glutamate levels. However, striatal glutamate levels reduction in IR rats were better with post-treatment of TM-NM (Gr. V) (P <0.01) than the pre-treated rats.

**Aspartate**

Elevation of aspartate levels in cortex, striatum, and hippocampus regions was observed after occlusion of MCA in rats in comparison to vehicle...
Table 2—The effect of TM-NM combination on glutamate, aspartate and GABA levels in middle cerebral artery occluded rats

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Glutamate (µ moles/g tissue) [%Δ]</th>
<th>Aspartate (µ moles/g tissue) [%Δ]</th>
<th>GABA (µ moles/g tissue) [%Δ]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex (µ moles/g tissue)</td>
<td>Striatium (µ moles/g tissue)</td>
<td>Hippocampus (µ moles/g tissue)</td>
</tr>
<tr>
<td>SO</td>
<td>4.65±0.37</td>
<td>6.25±0.34</td>
<td>1.58±0.17</td>
</tr>
<tr>
<td>IR</td>
<td>15.60±1.50</td>
<td>14.88±0.97</td>
<td>12.64±0.87</td>
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<td></td>
<td>[235.48]</td>
<td>[138.08]</td>
<td>[700]</td>
</tr>
<tr>
<td>TM2.5+NM2.5e</td>
<td>6.83±0.53e</td>
<td>12.37±0.75</td>
<td>5.97±0.98e</td>
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<td>[56.21]</td>
<td>[16.86]</td>
<td>[52.76]</td>
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<tr>
<td>TM5+NM5f</td>
<td>11.39±1.40</td>
<td>9.51±0.55f</td>
<td>9.32±1.16</td>
</tr>
<tr>
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<td>[26.98]</td>
<td>[36.08]</td>
<td>[26.26]</td>
</tr>
<tr>
<td>TM5+NM5</td>
<td>5.49±0.73e</td>
<td>6.93±0.50e</td>
<td>6.27±0.62e</td>
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<tr>
<td></td>
<td>[64.80]</td>
<td>[53.42]</td>
<td>[50.39]</td>
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</tr>
</tbody>
</table>

[Values are expressed in mean ± SE. Superscript b and c denotes statistical significance versus SO group at P < 0.01 and P < 0.001; e, f and g vs. IR group at P < 0.05, P < 0.01 and P < 0.001; x and y versus TM-NM (2.5) pre-treatment group at P < 0.05 and P < 0.01, respectively. Values in parentheses [%Δ] indicate percentage difference compared between SO and IR group; treatment groups against IR group; ↑: increase; ↓: decrease; @: pre-treatment; $: post-treatment.]

treated SO rats. But there is no significant (F(2,75)=2.452; P > 0.05) difference between the regions with respect to aspartate levels in ischemic rats. Similarly, the interaction between regions and treatment also exhibited no significant (F(8,75)=1.788; P > 0.05) difference. However, the groups analysis, treatment with TM and its combination with NM have shown significant (F(4,75)=68.66; P < 0.0001) reduction in the aspartate levels in different brain regions of ischemic rats. Pre-treatment of TM-NM (Gr. III) significantly attenuated the aspartate elevation in cortex (P < 0.001), striatum (P < 0.05), and hippocampus (P < 0.01) regions compared to the vehicle treated IR rats. Similarly, post-treatment of TM-NM (Gr. V) exhibited decrease in aspartate level (P < 0.001) in all three regions compared to the vehicle treated IR rats. Whereas, post-treatment of TM-NM (Gr. IV) produced the effect only in hippocampus region (P < 0.05), aspartate levels in other regions remain unaltered. Compared to the pre-treatment (Gr. III), rats post-treated with TM-NM (Gr. V) showed significantly reduced aspartate levels at striatum (P < 0.05) and hippocampus (P < 0.01) regions.

GABA

Induction of ischemia elevated the GABA levels (F(2,75)=3.710; P < 0.05) in cortex, striatum, and hippocampus regions. GABA levels in different regions and their interaction with treatment have not shown significant (F(8,75)=2.008; P > 0.05) changes. The GABA levels were significantly reduced in the above regions with treatment of TM and NM combination (F(4,75)=50.80; P < 0.0001) in ischemic rats. The pre-treatment of TM-NM (Gr. III) significantly decreased the hippocampus GABA levels (P < 0.05) only. Post-treatment of TM-NM (Gr. V) significantly decreased the GABA elevation in cortex (P < 0.001), striatum (P < 0.001), and hippocampus (P < 0.01) regions. Similarly, decreased GABA levels in cortex (P < 0.05) and striatum (P < 0.01) regions were observed with post-treatment of TM-NM (Gr. IV). Compared to the pre-treatment (Gr. III), post-treatment of TM-NM (Gr. IV) (P < 0.05) and TM-NM (Gr. V) (P < 0.01) combination significantly further reduced the striatal GABA levels.

Effect of TM-NM combination on energy metabolites and neurobiochemicals in ischemic rats

**ATP**

The ATP levels were significantly reduced (F(2,75)=3.231; P < 0.05) in different brain regions after ischemic induction. The interaction study has shown no significant (F(8,75)=0.8920; P > 0.05) effect between different regions and treatment groups. TM-NM combination treatment significantly restored the ATP levels in cortex, striatum, and hippocampus regions of ischemic rats (F(4,75)=95.26; p < 0.0001). Pre-treatment of TM-NM (Gr. III) increased the ATP levels (P < 0.01) in cortex, striatum, and hippocampus regions compared to the vehicle treated IR rats. Post-treatment of TM-NM (Gr. V) improved the ATP levels in cortex, striatum (P < 0.05) and hippocampus (P < 0.01) regions. Post-treatment of TM-NM (Gr. IV) did not alter the ATP levels in all the brain regions. In comparison to post-treatment, the ATP restoration was significantly higher with pre-treatment of TM-NM (Gr. III) (Table 3).
Table 3: The effect of TM-NM combination on ATP and Na⁺K⁺ATPase levels in middle cerebral artery occluded rats

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>ATP (µ moles/g tissue) [Δ%]</th>
<th>Na⁺K⁺ATPase (nano moles of inorganic phosphorus liberated/min/mg protein) [Δ%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Striatum</td>
</tr>
<tr>
<td>SO</td>
<td>10.78±0.89</td>
<td>12.59±0.53</td>
</tr>
<tr>
<td>IR</td>
<td>2.35±0.68</td>
<td>2.68±0.34</td>
</tr>
<tr>
<td></td>
<td>[89.63]</td>
<td>[89.41]</td>
</tr>
<tr>
<td>TM2.5+NM2.5[8]</td>
<td>6.62±0.56</td>
<td>7.09±0.43</td>
</tr>
<tr>
<td></td>
<td>[181.70]</td>
<td>[164.55]</td>
</tr>
<tr>
<td>TM5+NM5[8]</td>
<td>4.95±0.70</td>
<td>4.23±0.87</td>
</tr>
<tr>
<td></td>
<td>[110.63]</td>
<td>[57.83]</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SE. Superscript c denotes statistical significance versus SO group at P < 0.001; e, f and g vs. IR group at P < 0.05, P < 0.01 and P < 0.001. Values in parentheses [%Δ] indicate percentage difference compared between SO and IR group; treatment groups against IR group; †: increase; ‡: decrease; @: pre-treatment; $: post-treatment.

Table 4: The effect of TM-NM combination on GSH, TBARs and NO levels in middle cerebral artery occluded rats

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Reduced Glutathione (GSH) (nano moles /g tissue) [Δ%]</th>
<th>TBARs (nano moles /mg protein) [Δ%]</th>
<th>NO₂/NO₃ (nano moles /mg protein) [Δ%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Striatum</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>SO</td>
<td>5.21±0.41</td>
<td>5.01±0.34</td>
<td>7.54±0.72</td>
</tr>
<tr>
<td>IR</td>
<td>0.54±0.55</td>
<td>0.79±0.14</td>
<td>1.06±0.07</td>
</tr>
<tr>
<td></td>
<td>[89.63]</td>
<td>[84.23]</td>
<td>[85.94]</td>
</tr>
<tr>
<td>TM2.5+NM2.5[8]</td>
<td>3.60±0.34</td>
<td>3.23±0.38</td>
<td>2.41±0.30</td>
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<tr>
<td></td>
<td>[566.67]</td>
<td>[308.86]</td>
<td>[127.35]</td>
</tr>
<tr>
<td>TM5+NM5[8]</td>
<td>1.16±0.18</td>
<td>2.09±0.21</td>
<td>1.86±0.41</td>
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<tr>
<td></td>
<td>[114.81]</td>
<td>[164.55]</td>
<td>[75.47]</td>
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</table>

Values are expressed in mean ± SE. Superscript b and c denotes statistical significance versus SO group at P < 0.01 and P < 0.001; e, f and g vs. IR group at P < 0.05, P < 0.01 and P < 0.001. Values in square parentheses [%Δ] indicate percentage difference compared between SO and IR group; treatment groups against IR group; †: increase; ‡: decrease; @: pre-treatment; $: post-treatment.

Na⁺K⁺ATPase

Occlusion of MCA in rats significantly depleted the Na⁺K⁺ATPase levels (F(4,75)=29.75; P < 0.0001) in cortex, striatum, and hippocampus regions. Drug treated rats have shown significant (F(4,75)=3.954; P < 0.001) effect indicating relationship between these parameters. The Na⁺K⁺ATPase levels were significantly restored with TM and NM combination treatment in all the regions of the ischemic rats (F(4,75)=184.5; P < 0.0001). Pre-treatment of TM-NM (Gr. III) significantly elevated the Na⁺K⁺ATPase levels in cortex (P < 0.001), striatum (P < 0.01), and hippocampus (P < 0.05) regions compared to IR rats. Also, those rats that received TM-NM (Gr. V) treatment after ischemic induction, showed attenuated ischemic effects and elevated hippocampus (P < 0.05) and striatum (P < 0.05) enzyme levels compared to IR rats. Pre-treatment had better restoration of Na⁺K⁺ATPase levels than post-treatment (Table 3).

GSH

In ischemic condition, GSH levels were found depleted in cortex, striatum, and hippocampus regions of the brain. The interaction study between regions and treatment groups showed no significance (F(4,75)=0.4760; P > 0.05). Treatment of TM with NM exhibited significant elevation in GSH levels (F(4,75)=9.186; P < 0.0001) in different brain regions. Pre-treatment of TM-NM (Gr. III) significantly elevated the cortex (P < 0.01) and striatum (P < 0.01) GSH levels compared to the vehicle treated IR rats. Group V rats that received TM-NM post treatment showed significantly elevated GSH levels in cortex (P < 0.01), striatum (P < 0.001), and hippocampus (P < 0.01) regions compared to vehicle treated IR rats. The pre- and post-ischemic treatment groups behaved similarly with regard to this parameter (Table 4).
TBARs
The TBARs level were significantly increased after ischemic insult in different regions (F(2,75)=36.68; 
P<0.0001). No significant interaction between different regions and treatment groups (F(8,75)=1.851; 
P>0.05) was observed. Treatment with TM-NM combination in ischemic condition has shown significant reduction in TBARs levels in all three regions (F(4,75)=33.91; 
P<0.0001). Pre- (Gr. III) and post-treatment of TM-NM (Gr. V) significantly decreased the TBARs levels (P<0.05) in cortex and striatum regions compared to the vehicle treated IR rats. Hippocampus TBAR levels remained unaltered with these treatments. Post-treatment of TM-NM (Gr. IV) could decrease the TBARs level only in striatum region. The pre- (Gr. III) and post-treatment effects of TM-NM (Groups IV & V) on TBAR level were found to be similar (Table 4).

NO
Occlusion of middle cerebral artery followed by reperfusion injury has shown significant elevation of NO level (F(2,75)=10.78; 
P<0.0001) in cortex, stratum, and hippocampus regions. No significant interaction was observed between the regions and treatment groups (F(8,75)=1.190; 
P>0.05). NO levels were significantly reduced at different regions with TM and NM treatment (F(4,75)=20.32; 
P<0.0001). In comparison to IR rats, NO levels in hippocampus region were significantly reduced with pre-treatment of TM-NM (Gr. III) (P<0.05) and post-treatment of TM-NM (Gr. V) (P<0.01). Post treatment of TM-NM (Gr. IV) failed to alter the NO levels in all the regions studied (Table 4).

Histopathology
Cresyl violet staining
Hippocampus region of SO group (Fig. 3A) has shown more viable normal neuronal cells and no significant changes in their morphology was observed. IR group has shown loss of neuronal cells, disturbed morphology, with dark stained neurons in comparison to SO rats (Fig. 3B). Pre-ischemic treatment with TM-NM (Gr. III) has shown protection of neurons from ischemic neurodegeneration (Fig. 3C). Post-ischemic treatment of TM-NM (Gr. IV) has shown less number of protected neurons (Fig. 3D), whereas TM-NM (Gr. V) shows protected neuronal cells with regeneration of neuronal structures compared to pre-ischemic treatment groups (Fig. 3E).

Discussion
During cerebral ischemia, excess accumulation followed by uptake of glutamate by neurons exhibit excitotoxicity via Ca2+-dependent N-methyl-D-aspartate (NMDA) receptor signaling, leading to neuronal injury and cell death16. Earlier studies have shown that, less significant increase of glutamate, aspartate, GABA levels, 30 min after occlusion followed by significant elevation after 2-4 h of reperfusion. Since the secondary reperfusion phase plays a crucial role in ischemia pathology, in the present study TM-NM combination was administered in this phase (post-ischemic) to assess the intensity of neuroprotection and the effect was compared with pre-ischemic administration. The results indicated that low dose of post-ischemic TM & NM administration have shown better effects, compared to pre-ischemic treatment, only in the reduction of striatal GABA levels. Similarly at higher dose, TM-NM have shown good reduction of striatal EAA levels while all other parameters behaved similar to pre-ischemic group. The experimental observation clearly shows that low dose of pre-ischemic treatment is comparable to higher dose of TM-NM post-ischemic treatment.

In addition, pre-ischemic treatment has exhibited better regulation of energy imbalance by restoration of enzymes levels. Earlier studies have shown that pre-ischemic administration of TM7 exhibited neuroprotection by ameliorating pro-inflammatory cytokine release during cerebral ischemia and was potentiated by NM8. This effect may be attributed to anti-inflammatory property, since both the drugs have proven brain cytokines ameliorating properties in in vitro models17,18. Further, NM potentiating effect may be due to prevention of the Ca2+-dependent NMDA receptor mediated glutamate excitotoxicity in neurons8. It can also be stated that drugs upon pre-ischemic administration controlled the ischemic damage by regulating the release of EAA through its pre-synaptic heteroreceptors. The drugs might have occupied the pre-synaptic receptor to control the neurotransmitters release. Hence, we predict that pre-ischemic TM-NM treatment would have regulated glutaminergic NMDA or AMDA receptor on EAA release. Earlier studies have also shown decreased ambulation and rearing behavior in open field test, reflecting anxiogenic behaviour in ischemic rats19. Pre- and post-ischemic administration of TM-NM in ischemic rats exhibited normal behavior which indicates that TM-NM combination regulated...
EAA release, which was clearly reflected in the neurotransmitter levels measurement in different brain regions \(^{20}\).

The observed energy failure was attributed to depletion of ATP in brain tissues and this depletion of ATP was observed more than 6 h after reperfusion \(^{21}\). The drug treatment which can restore the ATP levels within this secondary energy failure could be an effective therapy in cerebral ischemia. In the present study, ATP depletion was restored with pre- as well as post-ischemic treatment of TM-NM. The recovery of ATP in ischemia may be due to central vasodilatation of NM and prevention of Ca\(^{2+}\) overload induced mitochondrial dysfunction \(^{22}\). Depletion of ATP during ischemia will induce depolarization in GABAergic neurons to open voltage dependent Ca\(^{2+}\) channels of pre-synaptic terminals \(^{23}\) to increase the GABA release during ischemia. Previous studies have reported secondary elevation of GABA at 2-3 h after reperfusion \(^{5}\). This effect was being observed in secondary phase, the NM combination would have attenuated the Ca\(^{2+}\)-dependent GABA release in the synapse effectively upon post-treatment than pretreatment \(^{23}\). The decreased Na\(^+\)K\(^+\)ATPase and GSH levels were restored by pre- and post-ischemic treatment of TM-NM in all the regions. This effect may be mediated by antioxidant and anti-inflammatory activities of both the drugs \(^{24},^{25}\). It is further made evident by decreased TBARS and NO levels. The NO scavenging property of the above combination could attenuate the NO dependent cytokines mediated glutamate release because recent studies have shown that cytokines enhance glutamate release by reducing its uptake into astrocytes and this enhancement is mediated by NO \(^{26}\).

In the present study, most of the parameters have shown similar results between pre- and post-ischemic administration of TM-NM. Interestingly, glutamate levels in striatum and aspartate levels in striatum and hippocampus regions were significantly reduced with post-ischemic administration of TM-NM (Gr. V) compared to the pre-ischemic treatment. The higher dose was required to antagonize the excess excitatory amino acids (EAA) release in reperfusion phase where the neuronal damage by excitotoxicity is more \(^{4},^{5}\). Hence, it can be concluded that pre-ischemic treatment of TM-NM has protected the neurons from ischemic reperfusion injury by energy dependent EAA regulation. Similarly, this combination exhibited neuroprotection in reperfusion phase and regulated EAA with partial restoration of energy levels after ischemia. The post-ischemic treatment of TM-NM combination is beneficial due to better EAA control. Since hypertension is the primary risk factor for stroke, clinical incidents of stroke in hypertensive patients receiving angiotensin receptor blockers (ARBs) can be further investigated to understand the present study in the clinical situation.

References

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