**Abstract:**

A neuroprotective strategy through a combination therapy is always being superior to any other singular therapeutic interventions, as these acts through a multifaceted approach within the brain during focal cerebral ischemia. Therefore, the development of a potential new combination of drug which can bring about desirable improved neuroprotection targeting different pathways against ischemic stroke is necessitated. Numerous past studies have enumerated the neuroprotective roles of minocycline and magnesium administered in single against focal ischemia in animal model, hence we hypothesized that by using magnesium with minocycline in combination would provide additive neuroprotection than either of the agents used alone. To test this hypothesis, we subjected adult male rats to anesthesia and administered them with magnesium and minocycline in single standard dose and then standard doses in combination consequently at 5 minutes after creating focal ischemia by transient middle cerebral artery occlusion with 24 h of reperfusion. The animals were allowed to recover for 5 days before undergoing neurological score after focal ischemia. Following sacrificed, histological and biochemical examination for neuroprotection was carried out and it was observed that a significant greater neuroprotection was rendered by the group administered with a combination dose of minocycline (10 mg/kg) and magnesium (40 mg/kg) in respect to the groups administered the dose in singlet.

**Keywords:** Focal ischemia; Oxidative stress; Minocycline; Magnesium; Neuroprotection
Introduction

The most practiced preventive strategy for the treatment of transient ischemic stroke is anti-platelet therapy which produces clinically considerable benefits. But in the case of acute ischemic stroke, fibrinolytic agents such as tissue plasminogen activator (t-PA) is the only drug that has proven effectiveness in reducing the size of neuronal infarction in humans [1-3]. However, according to present line of approach, treatment of focal ischemia is suboptimal without considering neuroprotective therapy in addition to clot lysing therapy, hence an effective neuroprotective treatment is solicited with an urgent priority [4-7]. With such considerations that several cascade pathways leading to neuronal death are activated in focal cerebral ischemia, drugs in combination is required for effective and efficient neuroprotection [6,7].

Monotherapy however has a restricted approach towards targeting different deleterious mechanisms and has a limiting approach towards neuroprotection. Hence a more effective strategy to target multiple pathways is a combination therapy. Therefore, we designed a combination of minocycline and magnesium for the very first time, which simultaneously acts on distinct pharmacological targets during the progression of ischemic neuronal insult and also improves functional outcome. This combination consists of minocycline, a semisynthetic tetracycline and analogue with bacteriostatic and antiinflammatory property with magnesium, a homeostatic regulator of the pathway involved in the delayed secondary phase of brain injury [8]. The outcome of this pharmacological combination was remarkably effective and efficient in rat model of focal ischemia and provided a significant improvised neuroprotection in comparison to the singlet treatment with minocycline and magnesium studied earlier by different researchers [9,10]. Here in this study we investigate the efficacy of our combination therapy through different biochemical, physiological and behavioral paradigms. We report that this combination therapy confers significantly enhanced neuroprotection than any of the component administered alone.

Experimental procedure

Reagents

Magnesium sulphate (MgSO\(_4\)), minocycline (MIN), Fura 2 AM, 2,3,5-triphenyltetrazolium chloride (TTC) and other chemicals were obtained from Sigma (USA) unless otherwise specified.

Animals

Male Charles Foster rats (6 weeks, 250 ± 10 g) were in-bred at the Central Animal House of Banaras Hindu University (Registration No.- 542/AB/CPCSEA) were used for the experiments. Animals were kept under standard laboratory conditions and maintained with the highest standards of animal care and housing. They were allowed free access to food and water and maintained at 12 h day/night cycle. The approved standard procedures and the institutional animal ethical committee guidelines were followed throughout the experiments.

Focal cerebral ischemia

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCA) with modified intraluminal technique [11]. Rats were anesthetized by the administration of a combination of ketamine (75 mg/kg b.w.) and xylazine (10 mg/kg b.w.) and then transferred to the surgical table with a heating pad to maintain a constant body temperature of 37 ± 0.5°C. Rat was placed in a supine position with forelimbs fixed on the table by tape and the fur on the ventral neck was shaved and the skin was cleaned by 0.5% betadine and 75% alcohol. Eye cream was applied to protect corneal drying. Midline incision in the neck was done to expose the left common carotid artery (CCA). Muscle fascia was separated further to expose external carotid artery (ECA) and internal carotid artery (ICA). A 5.0 cm length 3-0 monofilament nylon suture (Ethicon) was introduced into the CA lumen through a small nick and gently pushed from ICA lumen to block the origin point of MCA. Approximately 18-22 mm length of nylon filament was inserted to reach the MCA blockade site from the bifurcation point. The ECA stump was clamped around the intraluminal nylon suture to prevent bleeding.
Reperfusion was done by gently removing the filament after 1 h of ischemia. Animals were allowed to recover from anesthesia and on regaining the righting reflex, were transferred to polypropylene cages in the animal room with temperature maintained at 26 ± 2.5°C with food and water supply in ad libitum. In sham-operated animals, all the procedures were carried on except the insertion of nylon filament.

**Drug treatment and experimental protocol**

Magnesium sulphate and minocycline were dissolved in normal saline and were put to physiological pH before i.p. administration. Magnesium sulphate was administered i.p. in three doses of 5 mg, 10 mg and 15 mg/kg while minocycline dose was kept constant and was administered in 40 mg/kg dose consequently at 5 minutes after creating focal ischemia. A total of 60 animals were divided in six groups consisting of sham (n=6), control (n=6) and magnesium and minocycline treated (n=10) for each dose group. Further, biochemical and molecular studies were undertaken with the effective dose in three more groups viz. sham, vehicle and treated (n=6 in each group).

**Functional outcome assessment**

The neurobehavioral assessment was done 24 h post reperfusion [11]. Briefly, the neurobehavioral deficits was monitored on five point scale with 10 grading scores: a score of 0 indicated no neurologic deficit, a score of 1 meant failure to extend opposite forepaw fully, a score of 2 was assigned when contralateral circling was seen. While the rat which was not able to grip the wire meshes and fell on the contralateral side of brain damage, a score of 3 was assigned. Further when the rats were unable to walk spontaneously and had a depressed level of consciousness was given score of 4. The neurobehavioral scores obtained after testing on each scale were averaged to denote the degree of neurological deficit.

**Evaluation of cerebral infarct volume**

After neurological examination, rat brain was perfused with normal saline by transcardiac perfusion and isolated in chilled conditions. The cerebellum was removed and rest of the brain was immediately transferred at -20°C. Frozen brain was sliced vertically into uniform sections of 2-mm thickness. The brain slices obtained were incubated in TTC (0.5% in 0.1 M PBS) at 37°C for 30 min [12]. TTC stains, viable brain tissue to brick red whereas unstained brain tissue i.e. infarcted portion of tissue remains unstained and appears as white. The infarct area thus obtained was measured and quantified by image analysis software (Image J). Further, infarct volume was calculated by linear integration of the infarct area of each slice multiplied by average thickness of brain section and expressed in mm³.

**Biochemical analysis**

To perform biochemical analysis, we measured nitrite, malondialdehyde, calcium level and antioxidant status at two different time frames following ischemia i.e 20 min for nitrite and calcium while 60 min for MDA and antioxidants in cortex and striatal regions of ipsilateral rat brain. The above brain parts of the model were quickly removed by decapitation after cervical dislocation and homogenized (5:1 v/w) in ice cold 0.1 M phosphate buffer, pH 7.4. The tissue homogenate thus obtained was used for estimation as per protocol given below.

**Measurement of nitrite**

Nitrite and nitrate are generally the markers for NO production in a damaged cell or tissue. The nitrite levels were estimated in affected brain regions using Griess reaction [13]. Briefly, the tissue homogenate was centrifuged at 1580 g for 15 min at 4°C and the supernatant thus obtained was deproteinized by mixing with an equal amount of 4% sulfosalicylic acid. Further 350 µl of this reaction mixture was made to react with 350 µl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphtyl) ethylenediamine in distilled water) and incubated for 10 min in dark at room temperature. The absorbance of the assay samples was measured at 540 nm using spectrophotometer (Perkin Elmer, Germany). Nitrite concentrations were calculated using a calibration curve prepared from sodium nitrite and expressed as pmole/mg of protein in tissue homogenate.
**Estimation of malondialdehyde (MDA)**

The MDA is a by-product of lipid peroxidation and is a biomarker of membrane damage. It was determined based on its reaction with thiobarbituric acid (TBA). Briefly, the tissue homogenate (500 µl), 30% trichloroacetic acid (300 µl), 5M HCl (150 µl) and 2% (w/v) TBA in 0.5M NaOH (300 µl) were thoroughly mixed up. After each addition, the total volume was made 3 ml with distilled water. The above mixture was heated in a water bath at 80°C for 20 min and centrifuged at 4000 Xg for 10 min. The resultant pink chromogen formed was measured at 532 nm using spectrophotometer. MDA concentration in brain was determined using standard curve and values expressed as nmole/mg tissue protein in homogenate.

**Measurement of Ca²⁺**

Measurement of [Ca²⁺] in tissue homogenate of cortex and striatum was performed at 37°C under continuous stirring using Varian Fluorescence Spectrophotometer equipped with a water jacketed cuvette holder and magnetic stirring system. Dual wavelength excitation at 340 and 380 nM (the 340/380 ratio) were used and the resting fluorescence was measured at 510 nM.

**Determination of antioxidant status**

The antioxidant status of tissue homogenate of cortex and striatum was determined fluorimetrically by the method of Bruchill et al. [14]. The glutathione peroxidase and glutathione reductase activities were determined in tissue using glutathione peroxidase and glutathione reductase assay kits as per manufacturer’s instructions.

**Protein estimation**

Protein content was estimated by the method of Bradford using bovine serum albumin as standard [15].

**Data analysis**

Experiments were carried out on five different set and the results were represented as mean ± S.D. Statistical significance was analyzed using one-way ANOVA followed by Tukey’s post hoc test. Differences of P ≤ 0.01 and P ≤ 0.05 were considered significant.

**Result**

**Effect of combination therapy of magnesium and minocycline on neurological deficit and cerebral infarct volume**

We optimized the minimum effective neuroprotective dose of magnesium by considering constant dose of minocycline i.e. 40 mg/kg on the basis of reduction in neurological deficit and cerebral infarct volume of rats subjected to 1/24 h of I/R injury [16]. Neurological deficit was analyzed on the basis of neurological scores obtained 1/24 h of I/R injury in all experimental groups. The vehicle treated group of rats shows significantly higher neurological deficit as compared to sham group of animals while significant (P ≤ 0.01) improvement in neurological deficit score was found at 10 mg/kg. of magnesium and 40 mg/kg. of minocycline treated rats as compared to vehicle treated group (Fig. 1). In our studies, 1/24 h I/R injury consistently produced marked infarcts in both cortical and subcortical ipsilateral regions of rat brain as evidenced in TTC stained coronal brain sections (Fig. 2b). The mean of infarct volume was found to be 242.43 ± 21.30 mm³ in vehicle treated control rats whereas treatment with magnesium at 5, 10, 15 mg/kg i.p. doses with 40 mg/kg of minocycline produced marked reduction in infarct volume, ranging from 187.63 ± 39.30, 71.87 ± 27.20, 147.37 ± 30.40 mm³, respectively (Fig. 3). Irrespective of the infarct volume obtained with a combination dose, the mean of infarct volume ranging from 190.67 ± 7.75, 80.72 ± 9.88 and 100.74 ± 5.09 mm³ was observed with singlet treatment of 5, 10, 15 mg/kg i.p. doses of magnesium and 140.83 ± 4.14 mm³ with 40 mg/kg of minocycline separately (Fig 2a) with a significance of P ≤ 0.01. Thus 10 mg/kg magnesium with 40 mg/kg of minocycline i.p. showed significant improvement in neurological deficit and infarct size, therefore such a combination dose was selected as an optimum dose for further studies.
Effect of combination therapy of magnesium and minocycline on brain nitrite levels

As we know that calcium dependent activation of neuronal nitric oxide synthase (nNOS) has always been associated with early rise in post ischemic nitrite levels in rat brain. Therefore, nitrite levels were measured at 20 min post ischemia in ipsilateral cortex and striatal regions of rat brain. In both brain loci the nitrite levels were found to be significantly higher ($P \leq 0.05$) in vehicle treated as compared to sham group of animal while pretreatment with magnesium and minocycline (10 mg/kg and 40 mg/kg i.p.) significantly ($P \leq 0.05$) attenuated the increase in nitrite levels in both striatal and cortical regions of ischemic rat brain. (Fig. 4)

Effect of combination therapy of magnesium and minocycline on brain MDA levels

Malondialdehyde (MDA) is a marker of lipid peroxidation, therefore the MDA levels were measured post 60 min of ischemia in cortex and striatal regions of rat brain. MDA levels in brain were significantly ($P \leq 0.05$) increased in vehicle treated rats compared to sham rats. The MDA levels in vehicle treated rats were found to be 3.46 ± 0.49 and 3.67 ± 0.63 nmol/mg proteins whereas in magnesium and minocycline (10 mg/kg and 40 mg/kg, respectively) i.p. treated rats were 2.26 ± 0.42 and 2.49 ± 0.75 nmol/mg proteins, in cortical and striatal regions, respectively (Fig. 5). Thus magnesium and minocycline treatment resulted in significant ($P \leq 0.05$) reduction in post ischemic brain MDA levels.
Figure 2 2,3,5-triphenyltetrazolium chloride (TTC) stained 2-mm coronal rat brain sections a) with individual drug (singlet) b) with drug treated brain sections of sham, vehicle and a drug combination, with 1/24 h I/R injury. Effect of different doses of magnesium (MAG) 5, 10, 15 mg/kg with minocycline (MIN) 40 mg/kg are represented.

Figure 3 Effect of a combination of magnesium (MAG) and 40 mg/kg of minocycline (MIN) administered to rats on cerebral infarct volume following 1/24 h I/R injury (** significant versus vehicle)
Interestingly, treatment of magnesium and minocycline (10 mg/kg and 40 mg/kg, respectively) inhibited the focal ischemia mediated increase in Ca$^{2+}$ in cortex and striatum. The significant inhibition of calcium was observed in magnesium and minocycline treated group due to the calcium channel blocking capacity of magnesium ($P \leq 0.05$). This underlies the specificity of combination therapy in reducing the Ca$^{2+}$ mediated excitotoxicity (Fig. 6).

**Effect of combination therapy of magnesium and minocycline on brain Ca$^{2+}$ level**

The changes in antioxidant levels during focal ischemia are shown in Table 1. There was a significant decrease in GSH levels in the cortex and striatum as compared to vehicle (data not shown); however, in the presence of magnesium and minocycline in combination higher levels of glutathione (GSH) were observed. Further, there was a significant decrease in glutathione peroxidase (GPx) and glutathione reductase (GR) activities in vehicle, while treatment with magnesium and minocycline restored
both GPx and GR levels towards the control values.

Discussion

Neuronal insult following cerebral ischemia results from the overload of calcium within neurons which is due to an activation of N-methyl-D-aspartate (NMDA) receptors, followed by oxidative stress, inflammation and apoptosis, and subsequently leading to a sequel of biochemical events and ischemic insult. The elevated calcium level inside the neurons activates a group of calcium dependent enzymes like neuronal nitric oxide synthase (nNOS), calpain, phospholipase, xanthine oxidase, ligase and DNases which are involved in generating free radicals and catabolism of proteins, phospholipids and nucleic acids [17]. Following reperfusion after ischemic insult there is further mass generation of free radicals [18]. These altered pathological inputs cause membrane alterations and breakdown leading to severe cellular insult.

Numerous research are being conducted in search of a neuroprotective pharmacological composition for cerebral ischemia but none of them could be translated as a promising outcome for an efficient therapy. Eminent researchers and clinicians participated in Stroke Therapy Academic Industrial Roundtable (STAIR) unanimously suggested that due to complex and multifactorial pathology of cerebral ischemia agents with mixed pharmacology or sequential staging of therapy by a combination of different mechanistic approaches might hold good for stroke therapeutics [19]. Thus keeping in mind the above observations, an effort has been made to study the neuroprotective efficacy of minocycline and magnesium in combination against neuronal infarction, oxidative stress, calcium mediated excitotoxicity, lipid peroxidation and also towards the functional outcome following cerebral ischemia/reperfusion injury.

Tetracycline family especially minocycline has exhibited neuroprotection and positive therapeutic outcome against certain biological processes such as inflammation, apoptosis and oxidative insult, but the cellular and molecular mechanism operating behind this is yet to be explored more vividly. However, past studies have revealed that its anti inflammatory action [20] is due to the modulation of the microglial activity, immune cell activation and subsequent release of cytokines, chemokines, lipid mediators of inflammation, matrix metalloproteinase and nitric oxide (NO) release [21]. Microglial, astrocytic, neutrophilic and macrophagic

Figure 6 Effect of a combination of 10 mg/kg magnesium (MAG) and 40 mg/kg of minocycline (MIN) administered to rats on calcium levels in cortical and striatal regions of rat brain following post 20 min of ischemia (** significant versus vehicle, ## versus sham)
products of pro-inflammatory cytokines such as TNF-α, IL-β and IL-6 production which are the contributors towards inflammation and subsequent immune response are reported to be augmented by minocycline [21]. Minocycline also has a significant role in reducing the proliferation/activation of resting microglial cells as evidenced by CD 11b/OX 42, MAC-2 or isolectin B4 staining in hypoxic-ischemic brain injury model and 6-hydroxydopamine induced mouse model respectively [22, 23]. Minocycline has been reported to up-regulate expression of anti-apoptotic protein Bcl-2 [24] and also reduces the cleavage and thus the activation of a pro-apoptotic protein of the Bcl-2 family and prohibits the formation of activated caspase preferably 3,8 and 9 [25]. Previous study also showed that 5-lipoxygenase (5-LOX) expression increased at the region of proliferated astrocytes after ischemic insult [26]. Recently it has also been reported that minocycline protects PC12 cells against ischemia like injury or NMDA induced excitotoxicity in vitro. This consequence is due to inhibition of 5-LOX translocation to the nuclear membrane process incorporating 5-LOX activation [27,28]. It has also been reported that minocycline reduces acute brain injury and inflammation after focal cerebral ischemia and this is due to the 5-LOX expression inhibition and activation [29]. Minocycline has proved to decrease apoptotic neuronal cell death observed under various experimental models of neurodegenerative diseases including cerebral ischemia [29,21] and also increase the phosphorylation of eukaryotic initiation factor 2-α (eIF2α) in neuronal cells [29]. A recent study elucidated that minocycline protects against beta-amyloid (Aβ)-25-35 induced alterations of the somatostatin signaling pathway in the temporal cortex of Aβ-25-35 infused rats [30]. With reference to the past studies it has been shown that minocycline increases neuronal survival in mixed spinal cord culture treated with glutamate, kinite or N-methyl-D-aspartate. This action is a result of reduction in microglial activation through a p38 MAPK-dependent mechanism [31]. Another study showed that minocycline reduces NO-induced death of rat cerebellar granule neurons, which correlates with the reduction in p38 MAPK activities [32].

The other pharmacological component of choice is magnesium as it plays an important role in homeostatic regulation of the pathway involved in the delayed secondary phase of brain injury [8] Magnesium being a non competitive inhibitor of the NMDA receptor [33,34] during normal physiological process and hence play an important role in regulating calcium influx [35]. During post ischemic condition there is massive depletion of magnesium and its homeostasis control of the NMDA receptor is lost which leads to massive influx of calcium contributing to neuronal degeneration and cell death. Magnesium deficiency is associated with increased oxidative stress in rats through a reduction in plasma antioxidant and increased lipid peroxidation, possibly due to increased susceptibility of body organs to free radical injury [96]. Molecular mechanisms have been studied on the efficacy of magnesium in attenuating the neurological damage in post ischemic condition. The tumour suppressor gene p53 encodes the cellular tumour antigen p53 and is a regulator of neuronal apoptosis. Up-regulation of p53 mRNA was observed in cortex, thalamus and hippocampus after ischemic insult in rats [37]. Treatment with magnesium reduces the up-regulation of p53 gene and apoptosis in rats with brain injury. Also, magnesium has an important role in down-regulating aquaporin-4 channels and hence attenuating brain edema [38].

Our studies involved, use of focal cerebral ischemia rat model and analysis of downstream calcium dependent survival and death mechanisms in striatal and cortical regions of ischemic brain. The reason behind selecting these regions lie in the observation that following MCA occlusion cells in the striatum die immediately [39,40]. It is expected from the hypothesized combination that cell death in these areas should be slow or prevented. Treatment with magnesium and minocycline in combination offered significant neuroprotection at a combination dose of 10 mg/kg magnesium plus 40 mg/kg minocycline was found to be optimal in providing neuroprotection as exhibited by the reduction in neurological deficit score and cerebral infarct volume. The neurological deficit arises due to damage in the region of MCA occlusion i.e. caudate putamen and cortex that control motor function and could be well correlated
to cerebral damage mapped out as infarct. Therefore, it seems that magnesium and minocycline combination is modulating the mechanism of neuronal damage and thus helping in reducing the progression of neuronal infarction. Further the inhibitory effect of magnesium and minocycline combination on calcium mediated rises in NO level also justifies the neuroprotective profile following ischemic insult. An abrupt rise in intraneuronal calcium triggers calcium calmodulin dependent kinase and protein kinase C regulated neuronal nitric oxide synthase (nNOS). Activation of nNOS generates nitric oxide (NO) which is a physiological mediator of vasodilation by elevating level of cGMP in vascular smooth muscles. However at high concentrations during cerebral ischemia, NO causes neuronal insult by combining with the superoxide anion to form peroxynitrite anion, which further degrades to give rise to toxic hydroxyl and nitrogen dioxide radicals [41].

In our work NO produced following focal ischemia/reperfusion injury was quantified by measuring nitrite which is a stable end product of NO [42]. A rise in the nitrite level is being evident in the region of ischemic insult of brain after 10 min following cerebral ischemia and declined to basal levels after 60 min [43]. A significant decrease in NO levels after administration of minocycline and magnesium in combination could be associated with the decrease in calcium influx leading to neuroprotection. Furthermore, we have looked into the status of lipid peroxidation, which is evident from increased MDA levels in the ischemic rat brain immediately to post reperfusion [44]. MDA is a biomarker for lipid peroxidation and its quantification elucidates the extent of neuronal membrane damage. The lipid peroxidation cascade in a post ischemic brain sample is a result of combined action of NO and free radicals generated due to the activation of nNOS and phospholipase A2 [45]. The result shown in Table 1 is in accordance with the past findings. Our result justified the antioxidative property of minocycline and also elucidated that minocycline did not loose its antioxidative property even being in combination with magnesium as reported by Morimoto et al. [46]. The combination of minocycline and magnesium mediated decreases in MDA level suggesting the inhibition of calcium influx involved in activation of nNOS and phospholipase A2 as well.

**Conclusion**

The outcome of present study provides significant evidences that minocycline and magnesium in combination provides enhanced neuroprotective effects and confers neuroprotection in middle cerebral artery occluded rat model of focal cerebral ischemia. Further post ischemic neuroprotective effect is also been seen with this combination and thus may be a curative potential in clinical settings. Nevertheless, our present study suggests that minocycline and magnesium in combination could be clinically exploited along with other anti-stroke therapeutics for effective treatment of patient of human stroke. In future, we will try to elucidate in more details by carrying out experimentation with this pharmacological combination with neuronal cells *in vitro*.

**References**


