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MINOCYCLINE IMPROVES CEREBRAL EDEMA AFTER TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS

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Abstract: Background: Ischemic stroke is the third leading reason for disability globally. The aim of this work was to evaluate the neuroprotective effect of minocycline following transient cerebral ischemia induced by occlusion of middle cerebral artery (MCA) in rats.

Methods: 54 male Wister rats 8 weeks old and weighted from 250 to 280 grams were categorized into three main groups each contains 18 rats: Group 1: for Gelatin zymography, Group 2: for Evan's blue and Group 3: for behavioural assessment (n=18). Each group was divided into three subgroups (6 rats each): sham group, ischemia group: through MCA occlusion with reperfusion after one hour and Minocycline treated group: injected with a single dosage (3 mg/kg) into the left jugular vein shortly following reperfusion.

Results: Gelatine zymography test showed that after focal ischemia, matrix metalloproteinases (MMP)-9 and Pro-MMP-2 activity was upregulated compared to sham group. Minocycline treatment reduced the upregulation of MMP-9. Moreover, Minocycline treatment could significantly reduce the blood brain barrier (BBB) disruption as evidenced by the decreased Evans blue staining 1.88 (1.63-2.28) µg/g compared to ischemia group 10.81 (10.15-11.27) µg/g (p=0.023). Cerebral ischemia group showed significant decrease in rotarod 90.3 (81.6-98.13) seconds compare to in

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sham operated group 163.7 (160.3-165.5) seconds (P1<0.001). The treatment with Minocycline caused insignificant increase in the rotarod score 105.1 (98.03-109.7) seconds compared to ischemia group (p=0.160).

Conclusions: Minocycline has neuroprotective effects after cerebral ischemia as evidenced by reduced post-ischemic edema and inhibition of MMP-9 activity with insignificant effect on behavioural test.

Keywords: cerebral edema; evans blue; minocycline; middle cerebral artery occlusion MMP-9.

2020 AMS Subject Classification: 92C50.

1. INTRODUCTION

Ischemic stroke comes in as the 2nd leading reason for mortality and the 3rd leading reason for disability globally [1]. Cerebral ischemia is the condition when blood flow to a specific part of the brain is affected, leading to the death of neurons in that region. Currently, the administration of thrombolytic medicines is the sole medication used to treat such strokes [2]. However, thrombolytic drugs are unable to enhance cognitive and motor impairment in individuals with ischemic stroke [3].

Cerebral ischemia and post-ischemic reperfusion lead to the development of edema and hemorrhagic conversion due to the disruption of the blood-brain barrier (BBB). Cerebral edema is a major factor contributing to the rapid decline in neurological function, and lead to death following ischemia [4], and while other pathways have been suggested, matrix metalloproteinases (MMPs) are regarded as crucial molecules in the breakdown of the basement membrane, resulting in vasogenic-edema [5].

MMPs are zinc-dependent enzymes that participate in several biological processes. Cytokines, growth factors, and redox control the transcriptional level of expression of MMP gene. MMPs are released into the interstitial space or attached to the membrane in an inactive form called zymogen. They are converted into active enzymes by proteolytic processing by other MMPs and plasmin [6]. During an acute cerebral ischemia, the increased production of MMP-2 and MMP-9 is linked to the breaking of the BBB [7]. This breakdown may worsen neuronal injury and lead to hemorrhagic transformation after the administration of recombinant tissue plasminogen activator [8].

Minocycline is a broad-spectrum antibiotic which falls under the category of tetracyclines. It has properties that combat inflammation, oxidation, and cell death [9]. Regarding the effect of these variables on cognitive and motor problems resulting from cerebral ischemia, much research has been conducted on the neuroprotective properties of minocycline against harm produced by ischemic cerebral stroke in animal models [10].

Minocycline's ability to decrease the activities of inducible nitric oxide synthase, COX-2, and MMPs enzymes plays a crucial role in decreasing neuronal damage induced by inflammatory processes following cerebral ischemia [10, 11].

The purpose of this work was to evaluate the neuroprotective effect of minocycline following transient cerebral ischemia that produced by occlusion of MCA in rats.

2. MATERIALS AND METHODS

Experimental Rats: The study was carried on fifty-four male Wister rats 8 weeks old and weighted from 250 to 280 grams. Prior to the commencement of the experiment, the animals had been kept for a duration of one week under a 12-hour light and dark cycle. During this time, they were provided with a nutritionally balanced feed and unrestricted access to water. Each cage had a stocking density of 10 rats. During the trial, the animals were housed in hygienic enclosures and their bedding was replaced on a regular basis. Water was provided to the animals using bottles equipped with synthetic teats that were suspended from the tops of the cages.

Middle cerebral artery occlusion

1) **Presurgical preparations:** Rats were anesthetized by inhalational anesthesia using a mixture of 66% nitrous oxide and 33% oxygen plus 1.5% isoflurane. The area of the throat and left neck region was completely shaved utilizing clippers, extending beyond the intended incision location. The skin was disinfected using Betadine, which was wiped away with a gauze pads. The disinfection process began at the center of the surgical location and extended outward. Next, a sterile gauze pads soaked in 70% ethanol had been utilized for further sterilization. Both steps were repeated for a total of three cycles.

2) **Transient occlusion of middle cerebral artery (tMCAO):** A surgical cut was performed down the centerline of the neck exposing the left CCA. The ECA was tied off, and the ICA was separated close to the bifurcation. A 4.0-sized, 30 mm long, and 0.19 mm diameter monofilament suture with a silicon rubber covered tip was inserted into the ECA and moved through the ICA until it reached the starting point of the MCA. The length of the sutures was usually between 18-20 mm, which necessitated the creation of a kink before inserting it. Upon insertion of a nylon suture of varying length, one may experience a sensation of resistance. If a significant portion of the nylon suture remains outside the arterial, it suggests that the sutures is probably entering the pterygopalatine artery (PPA). In response, we retracted and gently curled the sutures to continue along the ICA, which runs more towards the center. Furthermore, an examination of the source of the PPA might be conducted to enhance the visualization of the route taken by the intraluminal filament. The nylon suture was introduced until encountering resistance at and beyond the 2 cm bent position. The intraluminal sutures has now obstructed the genesis of the MCA. The timer began, and the initiation time of occlusion was recorded (a single hour). The surgical cut was sutured.
3) **Restoration of middle cerebral artery blood flow (reperfusion):** The rats were re-anesthetized just prior to the occlusion period was expected to expire. The sutures were gradually removed after a 1-hour blockage of the MCA, and the rats were then placed back in their cages for a 23-hour period to allow for reperfusion.

Experimental Design: The animals were divided into three main groups each contains 18 rats:

Group 1: Gelatin zymography: in which gelatin zymography was done to measure MMP-9 activity following 24 hours of onset of ischemia, rats had been anesthetized with isoflurane, reperfused transcardiac with cold saline until the returning fluid becomes clear, decapitated, and the middle third of the brain was dissected and divided into right and left hemispheres and immediately immerged in liquid nitrogen then stored at-80°C until extraction of gelatinases.

Brain samples was homogenized in lysis buffer. Following incubation and centrifugation, the liquid above was removed and the gelatin-Sepharose solid was mixed with a solution and centrifuged once more. The activity of gelatinase in the eluant was measured by standard zymography. following separation by electrophoresis, the gel was put in tray of washing buffer and incubated with shaking at room temperature. The gel was put in container with 50 mL of reaction buffer and the container was sealed up. The gel was incubated in the container in incubator at 37°C for 20-40 hrs. 10. After an enzymatic reaction, the gel was put in container with staining solution and incubated for 30-mins period at room temperature to stain protein. The gel was put in container with De-staining solution and incubated for 30minutes – several hours to de-stain (until clear proteolytic bands appeared on the contrasting blue background).

Group 2: Evan's blue: for evaluation of BBB disruption. following 24 hours of tMCAO, 2% solution of Evans blue dye (Sigma-Aldrich: 4ml/kg in 0.9% normal saline) was injected intravenously via the left jugular vein. After 60 minutes of circulation of dye, rats were euthanized and transcardially perfused with ice-cold saline to remove intravascular dye. Immediately the brain from the skull was removed and placed in the brain matrix. The olfactory bulb and cerebellum were dissected, and then utilizing a clean razor blade, the right non-ischemic hemisphere of the brain was separated from the left ischemic hemispheres along the midline. Left brain hemispheres was weighed rapidly, homogenized in 2.5 mL of phosphate buffered saline, and mixed with 2.5 mL of trichloroacetic acid (60% trichloroacetic acid) and centrifuged at 10,000 g for 20 minutes. The samples allowed it to cool down in a 4 °C refrigerator for 10 minutes. The amount of Evans blue in the supernatant was quantified at an excitation wavelength of 610 nm using a spectrophotometer. Evan's blue concentrations were calculated against a standard curve.

Group 3: Behavioral assessment: in which the rats were subjected to Rotarod test. Rats were trained for 3 days before the day of surgery. Each day three times using MK-670 that includes a rotating rod of 75 mm diameter. The rotational speed of the cylinder was adjusted to 10 revolutions per minute, while the cut-off duration was set to 180 seconds. A trial was ended if the animal fell

off the cylinder. The apparatus automatically recorded the time in seconds until the rat falls to the floor. Rats were tested on the day of surgery and the time that the position of each animal on the rotating rod was documented for 3 trials, with a minimum time interval of 5 minutes between each trial and a maximum trial duration of 180 seconds for each trial. At 24 h post occlusion, rats were tested and the time that the position of each animal on the rotating rod was recorded for 3 trials, with a minimum interval of 5 minutes between each trial and a maximum trial duration of 180 seconds. The score was reported as the average latencies from 3 attempts on the rotating rod.

Each of this groups was divided into 3 subgroups each contain 6 rats:

Sham operated control group (n=6): The animals of this group underwent a midline neck incision, and the CCA and ICA had been separated and exposed. subsequently, the surgical cut was stitched and closed.

Ischemic group (n=6): The animals of this group underwent a MCA occlusion with reperfusion after one hour [12].

Minocycline treated group (n=6): The rats in this group were given a single dosage of minocycline (3 mg/kg, CAS number 13614-98-7, Sigma-Aldrich, Steinheim, Germany)) into the left jugular vein shortly following reperfusion began. Minocycline was solubilized in phosphate buffered saline (PBS) [13].

3. STATISTICAL ANALYSIS

Statistical analysis had been conducted utilising SPSS v27 (IBM©, Chicago, IL, USA). The normality of the data distribution was assessed using the Shapiro-Wilks test and histograms. Quantitative non-parametric data were displayed as median and interquartile range (IQR) and had been analysed by Kruskal-Wallis test with Mann Whitney-test to compare each group. A two tailed P value < 0.05 was considered statistically significant.

4. RESULTS

Gelatine zymography revealed that MMP-9 activity (88 KDa) was absent in the sham operated group. After focal ischemia, MMP-9 activity was upregulated. Minocycline treatment reduced the

upregulation of MMP-9 activity. Pro-MMP-2 (72kDa) was present in the sham operated group, upregulated in ischemia group without change after minocycline treatment as shown in Figure 1.

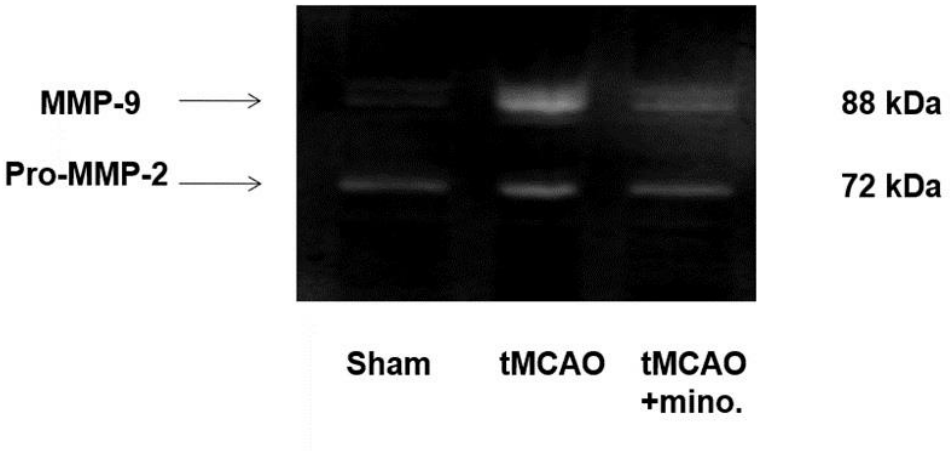

Figure 1: Minocycline treatment reduced the upregulation of MMP-9 activity. Pro-MMP-2 (72kDa) was present in the sham operated group, upregulated in ischemia group without change after minocycline treatment

Evans blue dye extravasation showed a significant increase 10.81 (10.15-11.27) µg/g in the ischemia group compared to 1.16 (1.09-1.22) µg/g in sham group (P<**0.001)**.

Moreover, the Minocycline treatment significantly reduced the amount of extravasated dye in brain tissue to 1.88 (1.63-2.28) compared to ischemia group (p=**0.023). (Table 1)**

Table 1: Evans blue dye in all studied groups

	Sham group (n=6)	**Ischemia group (n=6)**	**Minocycline treated group (n=6)**	**P**	**Post Hoc**
Evans	1.16 (1.09-1.22)	10.81 (10.15-11.27)	1.88 (1.63-2.28)	**0.001***	**P1<0.001*** P2=0.194 **P3=0.023***

*: Significant as P value≤0.05. Data are presented as median (IQR). P1: P value between Sham group and Ischemia group, P2: P value between sham group and Minocycline treated group, P3: P value between Ischemia group and Minocycline treated group.

Top view showed that no dye extravasation occurred in the sham-operated group (A). Marked bluish discolouration of whole left cerebral hemisphere in ischemia group indicating marked

extravasation of the dye (B). Mild bluish discolouration of the left hemisphere in minocycline treated group (C). Coronal sections showed that no dye extravasation occurred in the sham-operated group (D), marked bluish discolouration of most of left cerebral hemisphere in ischemia group indicating marked extravasation of the dye (E) and mild bluish discolouration of the left hemisphere in minocycline treated groups (F) as shown in Figure 2.

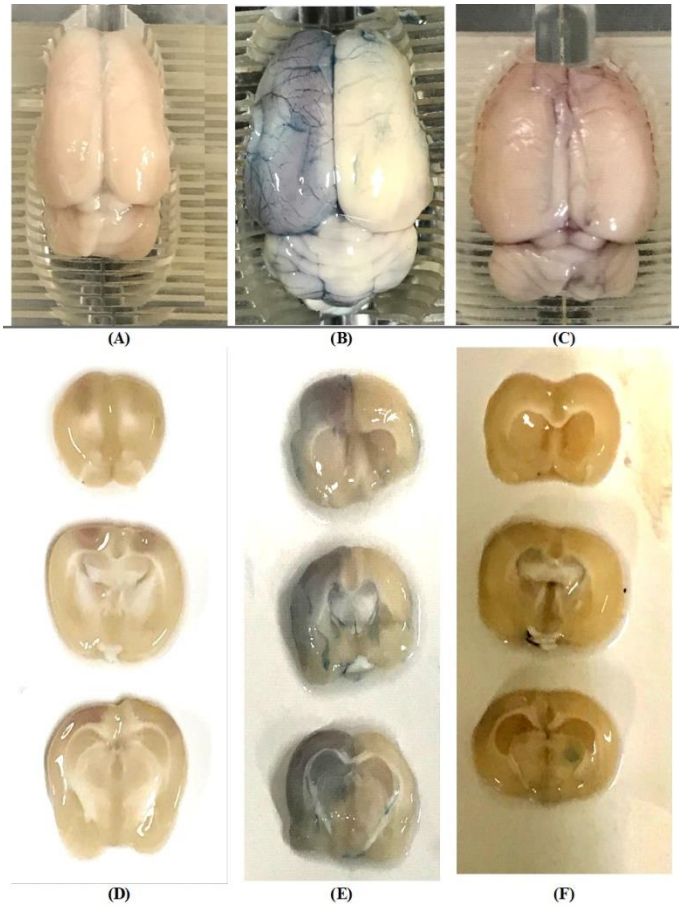

Figure 2: Top view showed that no dye extravasation occurred in the sham-operated group (A). Marked bluish discolouration of whole left cerebral hemisphere in ischemia group indicating marked extravasation of the dye (B). Mild bluish discolouration of the left hemisphere in minocycline treated group (C). Coronal sections showed that no dye extravasation occurred in the sham-operated group (D), marked bluish discolouration of most of left cerebral hemisphere in ischemia group indicating marked extravasation of the dye (E) and mild bluish discolouration of the left hemisphere in minocycline treated groups (F)

Rotaroad score significantly decreased in ischemia group 90.3 (81.6-98.13) seconds compare to in sham operated group 163.7 (160.3-165.5) seconds (P1<0.001). The treatment with Minocycline caused an insignificant increase in the rotaroad score 105.1 (98.03-109.7) seconds compared to ischemia group (p=0.160) as shown in Table 2

Table 2: Roataroad score in all studied groups

	Sham group (n=6)	**Ischemia group (n=6)**	**Minocycline treated group (n=6)**	**P**	**Post Hoc**
Roataroad	163.7 (160.3-165.5)	90.3 (81.6-98.13)	105.1 (98.03-109.7)	**0.001***	**P1<0.001*** **P2=0.027*** P3=0.160

*: Significant as P value≤0.05. Data are presented as median (IQR). P1: P value between Sham group and Ischemia group, P2: P value between sham group and Minocycline treated group, P3: P value between Ischemia group and Minocycline treated group.

DISCUSSION

The major findings in the current work showed that minocycline could significantly reduce the BBB disruption as evidenced by the decreased Evans blue staining and decreased MMP-9 levels in the Minocycline managed group contrasted to the ischemia group with no significant improvement in the behavioural test measured by Roataroad.

The disruption of the BBB in localized cerebral ischemia, which happens quickly after blood flow is restored, seems to be linked to a significant increase in blood flow in response to the removal of arterial blockage in regions that were previously deprived of flow [14]. Another potential mechanism of reperfusion damage involves the reintroduction of oxygen following a period of reduced blood flow. Oxygen-derived free radicals may induce lipid peroxidation in cellular membranes, leading to the dysfunction of membrane ATPase, disruption of ion balance, and modification of free fatty acids [15]. Previous studies have reported that the BBB can become permeable after reperfusion following global ischemia. This phenomenon has been linked to the

disruption of autoregulation in acidotic and dilated blood vessels. When these vessels are exposed to massive flow and intraluminal pressure throughout re-circulation, the tight junctions between cells may widen and pinocytosis may be induced [16].

The integrity of the BBB is thought to be influenced by both the magnitude of cerebral flow throughout ischemia and the length of the ischemic event [17].

In the current work, the ischemia group revealed significantly higher MMP-9 levels indicating a significant rise in the BBB disruption. Consistent with our research results, Yang et al. [18] showed that in the brain occluded rats the MMP-9 increased significantly compared to the sham group. Moreover, Fujimoto et al. [19] affirmed upregulated MMP-9 expressions in Wild Type mice after cerebral ischemia. The increase in MMP-9 activity after ischemia is consistent with previous studies [20, 21].

Justicia et al. [20] reported that the expression of MMP-9 significantly increased in the brain tissues after ischemia/reperfusion. Moreover, Yu et al. [22] showed that the in situ zymography showed that gelatinolytic activity through MMP-9 activity increased after SCI in the wild type rats. Gidday et al. [21] indicated that endothelial-adherent or infiltrated leukocytes are the main cells that have the responsibility of the alteration in brain tissue expression of MMP-9, which is determined 24 hours following tMCAO, Additionally, it has been shown that post-ischemic vasogenic edema, indicated by increased cerebrovascular permeability to Evans blue, is frequently seen in rodents following transient MCAO and occurs at the same time as the rise in pro- and active MMP-9 expression.

Minocycline has demonstrated anti-apoptotic, anti-inflammatory, and neuroprotective properties, and the ability to inhibit MMPs, in several models of ischemic stroke and neurodegenerative disorders. Minocycline is an appropriate choice for stroke therapy due to its high ability to penetrate the BBB and its excellent safety profile [23, 24]. Previous research on minocycline in animal models has focused on examining its neuroprotective properties specifically throughout the acute and subacute stages [24].

Our findings revealed that, in the treated rats with minocycline the MMP-9 significantly reduced contrasted to the ischemia group with no significant difference contrasted to the sham group. Supporting our results, Previous studies revealed that low dosages of intravenous minocycline are neuroprotective following MCAO/R in the rats and these low dosages appears to be selective for MMP-9 [25-27]. This study showed that minocycline treated group had lower MMP-9 activity than ischemic group.

Machado et al., [26] provided evidence that intraperitoneal administration of minocycline may effectively reduce the enzymatic function of MMPs during experimental ischemic stroke. Minocycline's neuroprotective effect in stroke is likely due to its ability to suppress MMP activity. In addition, they reported that minocycline had a significant inhibitory impact on MMP-9 at very low doses in an in vitro setting.

According to the present study, the Evans blue increased significantly in the ischemia group in comparison with the sham group. In line with our results, the results from Fujimoto et al. [19] showed increased Evans Blue extravasation in the ischemic group contrasted to sham group.

The increase in the Evans blue dye in ischemia group is an indicator of increased BBB permeability following stroke that was attributed to induction of proinflammatory molecules including HIF-1α and vascular endothelial growth factor (VEGF) [18].

In addition, the treatment with minocycline significantly decreased the Evans blue staining compared to the ischemic group. In accordance with our results, minocycline was proved also to decrease edema after ischemic stroke [13, 27].

Minocycline is a very effective inhibitor of inflammatory reactions that occurs early following cerebral stroke [28]. Microglia, the immune cells that reside in the brain, become activated when there is an injury and coordinate the brain's inflammatory reaction [29]. Experimental and clinical investigations have focused on neuroinflammation following localized cerebral ischemia by targeting activated microglia [30]. Recent research indicates that administering minocycline for a

duration of five to seven days following reperfusion substantially decreased the activity of microglial cells, leading to enhanced motor function throughout stroke rehabilitation [31, 32].

Rotaroad performance results of the current study revealed that the motor performance of rats was significantly reduced in ischemia group contrasted to sham operated group. However, minocycline did not improve the motor performance of rats.

Previous study by Yang et al. [33] showed that there was a significant decrease in motor performance in cerebral ischemic rats. Moreover, Melo et al. [34] showed that the induction of cerebral ischemia via occlusion of the middle cerebral artery could significantly decrease the rats motor performance.

It is well known in previous studies that minocycline improved the motor performance of rats after ischemia model [35, 36]. However, the non improvement of motor activities of rat's minocycline therapy in this study may be attributed to the time point of the study (scarification after 24 hours from the onset of ischemia) which is not enough to show the motor improvement of rats.

The study has some limitations that includes the relatively small sample size and limited time of therapy for acute stage in ischemic stroke. Further studies with longer duration and different doses of minocycline are required to ensure our results and estimate the effect on the motor performance after ischemia.

CONCLUSIONS

Minocycline has neuroprotective effects after cerebral ischemia as evidenced by decreased post ischemic edema and inhibition of MMP-9 activity with no significant effect on behavioural test.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The rats experimental protocol utilized in this work was approved by the Laboratory Animal Medical Ethical Committees at Faculty of Medicine, Tanta University, Egypt (approval code 36264PR450/12/23). The consent to participate is not applicable to this manuscript.

AVAILABILITY OF DATA AND MATERIAL

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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