



Myocardial Integrity in Citicoline-Treated Middle Cerebral Artery Occlusion-induced Hypoperfusion in Wistar Rats; the Relationship Between the Insular and Heart

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Abstract

BACKGROUND AND AIM: The study evaluated the histomorphology and histomorphometry of the left ventricle of Wistar rats following middle cerebral artery occlusion (MCAO)-induced cerebral hypoperfusion. These were with a view to providing insight on the effects cerebral hypoperfusion on myocardial integrity.

METHODOLOGY: A total of twenty adult male Wistar rats (200 g -220 g) were used for this study. They were divided into four groups of five rats each. Sham surgery was performed on rats in group 1; MCAO was performed on rats in groups 2-4. Groups 1, 2, and 4 were administered 1ml/kg normal saline intraperitoneal (i. p) while rats in group 3 were treated with 150 mg/kg body weight (i. p) of citicoline daily for 12 weeks respectively. Twenty-four hours after the last administration rats were sacrificed and blood samples were taken for biochemical analysis of brain natriuretic peptide, nitric oxide (NO) and lactate dehydrogenase (LDH) in the serum. The heart and brain were fixed in 10% neutral buffered formalin for histological, histochemical, and immunohistochemical studies. Data were analyzed with one-way analysis of variance (ANOVA) followed by Student Newman-Keuls (SNK) test. Alpha value was set at 0.05.

RESULTS: The result showed increased concentrations of brain natriuretic peptide (0.19 ± 0.08 pg/mL) and, LDH (13.20 ± 0.64 u/l), a decrease in the concentration of nitric oxide (0.12 ± 0.08 mmol/L), reduced collagen and glycogen deposit, distortion of the cross-banding pattern of the myocardium and reduced CnTI immunoreactivity in the MCAO-only group; These perturbations were attenuated in the citicoline-treated group.

CONCLUSION: The study concluded that citicoline ameliorated MCAO-induced neurological perturbations.

Keywords:

Histomorphology, Histomorphometry, middle cerebral artery occlusion, left ventricle

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality globally (Virrane *et al.*, 2021). The clinical management of many disorders of the heart and the brain cannot be viewed in isolation because CVDs, such as stroke, atrial fibrillation, and coronary heart disease (CHD) have been regularly implicated to be connected to neurodegenerative diseases (Sposato *et al.*, 2020). This affiliation may be due to shared risk factors between these diseases, however, there may also be a direct causal link.

Typically, medical specialists tend to have tunnel vision about the organ system that most interests them. Thus, cardiologists often view the heart as the most important organ in the body and frequently discount the input of other systems such as the kidneys and the nervous system. However, recent advances in the understanding of the mechanisms of diverse processes such as cardiac sudden death and digitalis toxicity have implicated the nervous system as an important factor in the pathogenesis of cardiovascular disease (Heron,

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2019). Moreover, therapeutic advances such as the use of (8-receptor blockers to lessen the probability of sudden death for patients recovering from myocardial infarction have forced the cardiologist to expand his horizon to include at least the nerve terminals impinging on the heart (Sposato *et al.*, 2020). Perhaps for similar reasons as the cardiologists, neurologists have not stepped into this void.

In the course of recent years, enormous achievements have been made in the management of cerebrovascular diseases, which relied upon the utilization of clinical models which are needed for a better understanding of the pathogenesis, progression, and mechanism underlying diseases to test therapeutic approaches to reestablish tissue function following injury (Pishak *et al.*, 2006). The Middle Cerebral Artery Occlusion (MCAO), otherwise called focal impediment, is perhaps the most broadly used procedure in animal models (rat) to research the hypoperfusion-induced neurodegenerative disease (Komatsu *et al.*, 2021). Of the neuro-cardiac axis (hippocampus, Insular, Medulla oblongata) of the brain, the insular is perhaps the most regions influenced by MCAO-induced hypoxia (Malpas *et al.*, 2010). The insular cortex has widespread connectivity with other areas of the brain that are known to be involved in autonomic control. Both experimental and clinical evidence strongly suggests a role for the insula in cardiovascular function (Oppenheimer and Cechetto, 2016).

Choline Citicoline (Cytidine 5'-diphosphocholine), a phospholipid made out of cytidine and choline linked together with a diphosphate bond is water-soluble and widely bioavailable (Dávalos and Secades 2012). It is available in its free-base structure or as a sodium salt (Nakazaki *et al.*, 2021). Citicoline was developed in Japan for the therapy of cerebrovascular problems. Citicoline's neuroprotection mechanisms may include preservation of cardiolipin, and sphingomyelin; arachidonic acid content of phosphatidylcholine (PtdCho) and phosphatidylethanolamine; partial restoration of PtdCho levels; and stimulation glutathione synthesis and glutathione reductase activity (Castagna *et al.*, 2021).

The purpose of this research is to investigate the relationship between hypoperfusion-induced neurological conditions and the pathogenesis of cardiac dysfunction.

MATERIALS AND METHODS

Animal Care and Management: A total of twenty (20) adult male Wistar rats weighing between 200 g -220 g were used for this study. They were randomly divided into four (4) groups of five (5) rats each. The animals were acclimatized for two weeks before the experimental period. They were kept up at natural night and day cycles. All rats were freely fed with standard rat chow (CAP Feed Limited) and clean water throughout the period of the experiment. Sham surgery was performed on rats in group 1; MCAO was performed on rats in groups 2-4. Groups 1 and 2 received 1

ml/kg normal saline (i.p.), rats in group 4 were administered 50 mg/kg citicoline (i.p.) while rats in group 3 were treated with 150 mg/kg (i.p.) of citicoline daily for 12 weeks respectively

Blood pressure of Wistar rats was taken before MCAO and then taken, at weekly intervals after MCAO using CONTEC 08A VET sphygmomanometer

Middle Cerebral Artery Occlusion (MCAO): A modified MCAO procedure of Koizumi *et al.*, (1986) by Longa *et al.*, (1989) was adopted. All equipment and surfaces were dabbed with 70% ethanol to prevent infection. The surgical instruments and materials were autoclaved and the surgical procedure was performed under sterile conditions.

The suture was prepared from a 5 cm-long part of a sterile 2/0 nylon monofilament (Ethilon Nylon Suture, Ethicon Inc. Germany). The tip of the suture was blunted before it was used by heating it near a flame. A 20-mm distal segment of the suture was then coated with poly-L-lysine solution (0.1% [wt/vol], in deionized water; Sigma) and dried in a 60°C oven for 1 hour. The diameter of the suture was not changed by the coating process. To obtain intraoperative control on the length of the intraarterially-introduced monofilament, the proximal 22 mm of the suture was marked with a sterile permanent marker in 5, 5, 5, 5, 2 mm distances.

The rats in group 2-4 were anesthetized using 50 mg/kg pentobarbital (i.p.). Paw withdrawal tests were performed to ascertain the depth of anesthesia and animals were restrained in a recumbent position. Under a dissecting microscope, a midline incision was made on the neck and the CCA was located. The suture was inserted 18 to 20 mm from the bifurcation of the CCA. After the intraluminal suture was placed, the neck incision was closed with a skin glue.

After 2 hours of MCAO, the inserted intraluminal suture was carefully removed. The CCA and ICA were then inspected to ensure the return of good pulsations. The neck incision was stitched with a silk suture, and the animals were allowed to survive with free access to food and water. The animals were returned to their cages when they had awakened from anesthesia.

Behavioral Testing: This consists of two tests that have been used previously to evaluate various aspects of neurological function. The postural reflex test, developed by Bederson *et al.* (1986), was used to examine upper body posture while the animal is suspended by the tail while the forelimb placing test, developed by De Ryck *et al.* (1989), was used to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli.

Sham Surgery: Sham surgery was performed on rats in group 1. The rats were restrained in a recumbent position and a midline incision was made on the neck to expose the left carotid artery; a skin glue was placed on the incision for two hours after which it was stitched with a nylon suture. The

neck incision was stitched with a silk suture, and the animals were allowed to survive with free access to food and water. The animals were returned to their cages when they had awakened from anesthesia

Sacrifice, Biochemical, Histological and Immunohistochemical Studies: Twenty-four hours after citicoline and normal saline were last administered, rats were anesthetized using 100 mg/kg body weight of pentobarbital (intra-muscular), blood samples were taken by cardiac puncture, the serum was separated from the blood by centrifugation at 3,000 X g for 10 minutes using a benchtop centrifuge (model R-303 8X15ml Capacity Doctor Centrifuge), Lactate dehydrogenase, serum nitric oxide (NO) and brain natriuretic peptide (BNP), levels were determined using the appropriate assay (BioAssays Systems, Hayward, CA, USA) following the manufacturers' instructions. The rats were transcardially perfused with 0.9% normal saline solution (NS) followed by fixatives (10 % neutral buffered formalin). The brain and heart (left ventricles) were removed weighed, and rapidly and fixed with 10% neutral buffered formalin. Twenty-four hours later, tissues were processed, and prepared for paraffin block. Afterward, tissue blocks were cut transversely using rotary microtome to 5 - 7 μ m of thickness. Sections from the brain (insular) were stained with haematoxylin and eosin for the demonstration of general histoarchitecture while Luxol Fast Blue and Cresyl Violet were used for demonstration of myelin and Nissl body respectively. Sections from left ventricle were separately stained with haematoxylin and eosin for the demonstration of general histoarchitecture while Masson's Trichrome and Periodic Acid-Schiff were used to demonstrate collagen and glycogen respectively. Glial fibrillary acid protein (GFAP) was demonstrated in the hippocampus and insula while cardiac Troponin I (CnTI) was demonstrated in the left ventricle of the heart using immunohistochemical technique.

Haematoxylin and Eosin Staining: Tissues were fixed and processed for paraffin wax embedding. The procedure of Drury and Wallington (1980) was adopted. All tissues were dehydrated through ascending grades of alcohol by immersion for two hours each. Dehydrated tissues were cleared in xylene for two hours. The tissues were infiltrated in two changes of molten paraffin wax at 56 °C in the oven for one hour each and finally embedded in paraffin wax using plastic embedding molds, smeared with glycerin so that paraffin blocked tissues can be separated easily from the molds after embedding. Paraffin blocked tissues were trimmed and mounted on cassettes for sectioning on a rotary microtome. Sections of 5-7 μ m thicknesses were obtained. The sections were spread in warm water bath at 50°C and collected on clean glass slides. The slides were dried on a drying plate at 40 °C overnight to enhance adherence and stored in slide rack for later staining to be done. Haematoxylin and eosin staining procedure by Steven, 1982 was used for this research

Masson's Trichrome: Masson's Trichrome Staining is a histological staining method used for selectively stain collagen, collagen fibers, fibrin, muscles, and erythrocytes. It uses three stains for staining hence the term Trichrome. These are Weigert's Hematoxylin, Biebrich scarlet-acid fuschin solution, and Aniline blue. The staining procedure of Jacques, 1938 was employed for this research

Periodic Acid-Schiff: The Periodic Acid Schiff (PAS) technique is without question the most versatile and widely used technique for the demonstration of carbohydrates or glycoconjugates. This technique is based upon the reactivity of free aldehyde groups within carbohydrates with the Schiff reagent to form a bright red magenta end product. The initial step in the PAS technique is the oxidation of hydroxyl groups attached to adjacent carbon atoms (1, 2 glycols) within the carbohydrate. The PAS technique was carried out according to the modified method as described by McManus (1946) procedure

Glial fibrillary acid protein (GFAP): Sections were stained for glial fibrillary acid protein (GFAP) to demonstrate astrocytic reaction. Study was performed using the Novocastra™ Novolink™ Polymer Detection System (Lieca Biosystems, UK) and appropriate primary antibody for demonstration of astrocytes which is mainly based on staining glial fibrillary acidic protein (GFAP), one of the intermediate filament proteins.

Cardiac Troponin I (CnTI): The demonstration of CnTI, a cardiac and skeletal muscle protein family processes an inhibitory character *I*, is a useful marker in laboratory diagnosis of heart attack (Solaro *et al.*, 1917; Zhang *et al.*, 2012).

Statistical Analysis: GraphPad Prism (Version 8.4.3, Graph Inc. 2020) statistical package was used for data analyses. Data obtained were analyzed using descriptive and inferential statistics. Data was expressed as mean \pm SEM. Statistical differences were examined by one-way analysis of variance (ANOVA), followed by Student Newman-Keuls (SNK) test for multiple comparisons. A significant difference was set at $P < 0.05$.

Photomicrography and image analysis

Photomicrography: Stained sections were loaded into Motic Easy Scan Pro 6 slide digital slide scanner (MOTIC EASY SCAN), scanned images were imported into the Motic digital slide assistant software and photomicrographs were taken at a magnification of 400x.

Image Analysis: Photomicrographs of stained sections were analyzed and processed using image analysis and processing for JAVA (image J), public domain software. Photomicrographs were loaded onto the software, the images were calibrated, stacked, viewed on a montage, and later converted to be viewed as a single image. Basic selections were made on the region of interest using a

rectangular grid, multiple measurements were taken and the mean and standard deviation were recorded; the data was copied into Graphad for further analysis

RESULTS

Relative Weight of The Heart, Brain, and Insula in Sham and Treated Wistar Rats (Table 1): The result showed a significant reduction in body weight, absolute insular weight, and, an increase in brain weight, and relative heart weight in the MCAO-only group (2) and group 4. There was no significant difference in absolute insular weight, relative heart weight, and body weight when the 150 mg/kg citicoline-treated group was compared to group 1 (Sham).

Mean Arterial Blood Pressure of Sham and Treated Wistar rats (Figure 1): There was a significant increase in the mean arterial blood pressure (MAP) of rats in MCAO-only (group 2) and 50 mg/kg citicoline-treated (group 4) groups when compared to the control group. However, there was no difference in MAP of Wistar rats treated with 150 mg/kg and those in the control group (1).

Hematoxylin and Eosin (H&E) staining for demonstration of general histoarchitecture of Insular in Sham and Treated Wistar rats (Figure 2): H&E staining showed normal neurons and regular arrangement of the neuronal layer in the granular insular of sham and 150 mg/kg citicoline-treated (3) groups; groups 2 (MCAO-only) and 3 showed red neurons, shrunken dark neuronal cells, and pyknotic neurons.

Glial Fibrillary Acid Protein (GFAP) Immunocytochemistry of Sham and Treated Wistar Rats (Figure 3): GFAP showed astrogliosis in the MCAO-only and 50 mg/kg citicoline-treated groups; however, there were no astrocytic reactions in the Sham and 150 mg/kg citicoline-treated groups.

Cresyl Violet Staining for demonstration of Nissl-positive neurons in Sham and Treated Wistar rats (Figure 4): Image J quantification of Cresyl violet stain showed an increase in the quantity of Nissl-positive neurons in the sham and 150 mg/kg citicoline-treated groups; with the MCAO-only and 50 mg/kg citicoline-treated groups showing few Nissl-positive neurons.

Luxol Fast BLUE (LFB) staining for Demonstration of Myelin volume in Sham and Treated Wistar rats (Figure 5): Image j quantification of LFB staining showed myelin deposit in the Sham and 150 mg/kg citicoline-treated groups while the MCAO-only and group 4 showed depletion of myelin.

H&E Staining of the Left Ventricle of Sham and Treated Wistar rat (Figure 6): H&E staining of the left ventricle showed well-arranged, regularly branched myocardium in groups 1 and 3 while groups 2 and 4 showed irregularly branched degenerated myocardium.

Masson's Trichrome for Demonstration of collagen in The Left Ventricle of Wistar rats (Figure 7): Quantification of Masson's trichrome-stained sections showed increased collagen deposit in the myocardium of the left ventricle in groups 2 and 4 when compared with group 1 (sham). There was no significant difference in glycogen volume when group 3 was compared with the sham group.

Periodic Acid Schiff (PAS) for the Demonstration of Glycogen in The Left Ventricle of Wistar rats (Figure 8): Quantification of PAS-stained sections showed increased glycogen deposit in the myocardium of the left ventricle in groups 2 and 4 when compared with group 1 (sham). There was no significant difference in glycogen volume when group 3 was compared with the sham group.

Cardiac Troponin I Demonstration in The Left Ventricle of Sham and Treated Wistar rats (Figure 9): Quantification of troponin shows loss of troponin in group 2, reduced troponin in group 4 and increased troponin reactivity in groups 1 and 3.

Concentration of Serum Nitric Oxide (NO) of Sham and Treated Wistar rats (Figure 10): Serum nitric oxide was higher in groups 2 and 4, but lower in groups 1 and 3.

Serum concentration of Lactate Dehydrogenase (LDH) of Sham and treated Wistar rats (Figure 11): Serum LDH was higher in groups 2 and 4 compared to Sham group (group 1); there was no significant difference when LDH concentration in group 3 was compared to group 1.

Concentration of Serum Brain Natriuretic Peptide (BNP) of Sham and treated Wistar rats (Figure 12): Serum BNP was higher in groups 2 and 4 compared to Sham group (group 1); there was no significant difference when BNP concentration in group 3 was compared to group 1.

Table 1: Effects of MCAO-induced hypoperfusion on the relative weight of the heart, brain, and insula in sham and treated rats (n = 4). Values are expressed as relative heart weight Mean \pm SEM. *= statistical significance.

Groups	AHW(g)	RHW(g)	BrW (g)	RBW	IWAS(g)	AIW (g)	BWAS (g)
1	0.60 \pm 0.04	0.55 \pm 0.01	1.39 \pm 0.02	1.09 \pm 0.03	0.21 \pm 0.08	0.14 \pm 0.04	198 \pm 1.9
2	0.84 \pm 0.05	0.71 \pm 0.05	1.59 \pm 0.01	1.20 \pm 0.04	0.15 \pm 0.02*	0.07 \pm 0.03*	193 \pm 1.7*
3	0.62 \pm 0.06	0.59 \pm 0.08	1.41 \pm 0.02	1.10 \pm 0.02	0.19 \pm 0.02	0.09 \pm 0.01	199 \pm 1.1
4	0.78 \pm 0.06	0.67 \pm 0.06	1.50 \pm 0.02	1.19 \pm 0.04	0.16 \pm 0.01	0.07 \pm 0.01	194 \pm 2.1

RHW= relative heart weight ($RHW=HW\div BWAS\times 100$); AHW= Absolute heart weight; BWAS=body weight at sacrifice; BrW= brain weight; RBW= relative brain weight ($RBW=BrW\div BWAS\times 100$); IW= Insular weight; AIW = Absolute Insular weight ($AIW = IW \div (BWAS \times 100)$); IWAS = Insular weight at sacrifice; $P > 0.005$; 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline

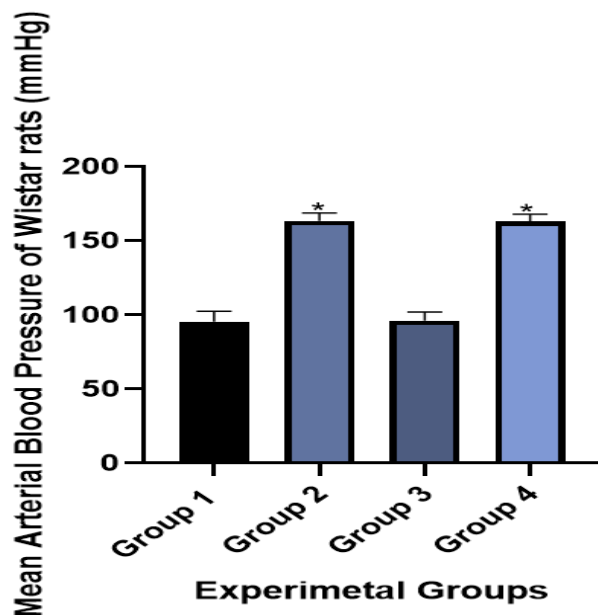


Figure 1: The mean arterial blood pressure of control and treated Wistar rats. Values are expressed as Mean \pm SEM. *= statistical significance. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

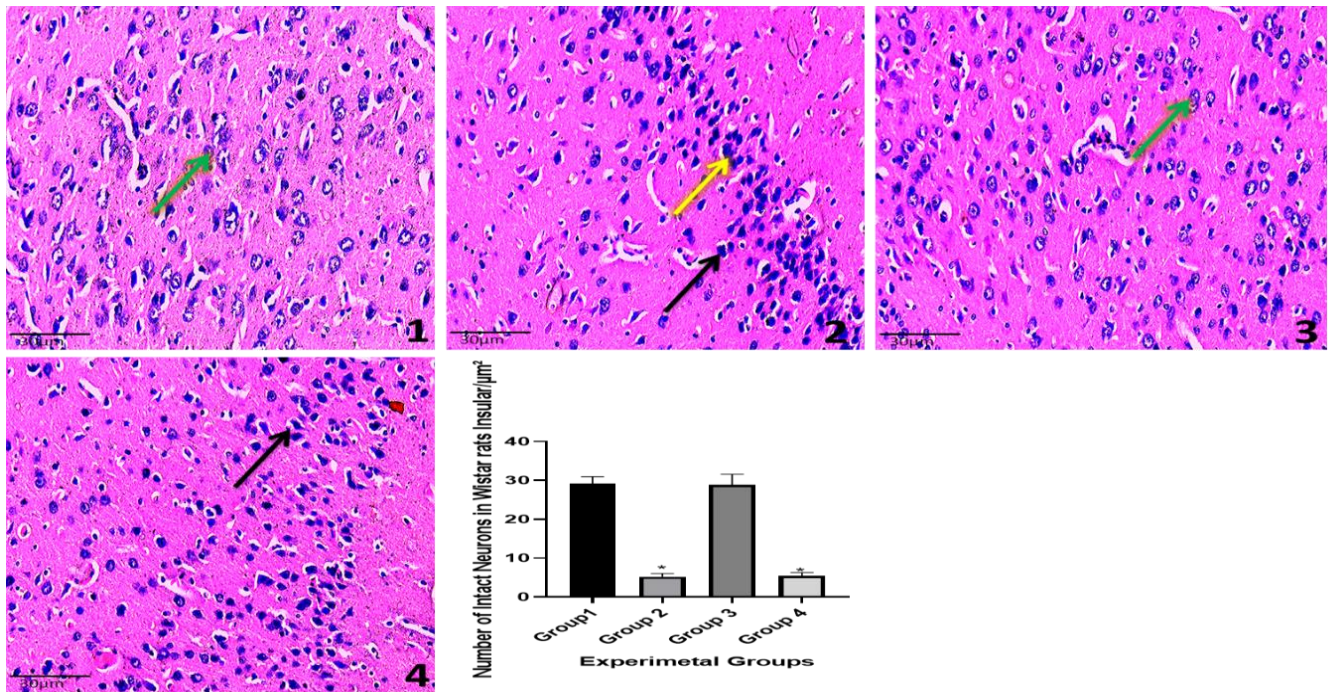


Figure 2: Photomicrographs of rostral insular (GI-granular insula) of control and treated rats. Showing: (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Green arrow –normal neurons, red arrow—red neuron, black arrow - shrunken dark neuronal cells, yellow arrow – pyknotic neurons (H&E; Scale bar = 30μm). Graph – Image J quantification of normal/intact neurons in insular of control and treated groups. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

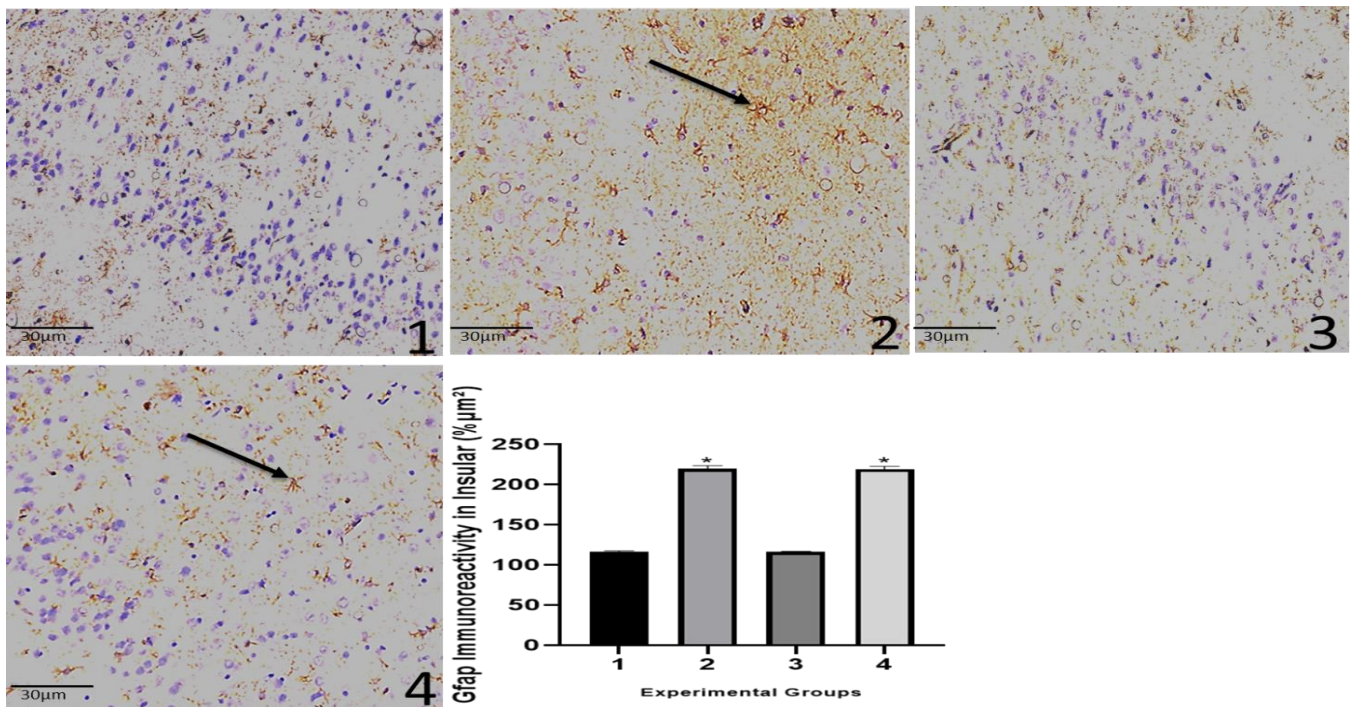


Figure 3: Photomicrographs of the Insular of control and treated Wistar rats showing GFAP Immunoreactivity. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Black arrow – astrocytic cells (GFAP; Scale bar = 30μm). Graph – Image J quantification of GFAP immunoreactivity in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

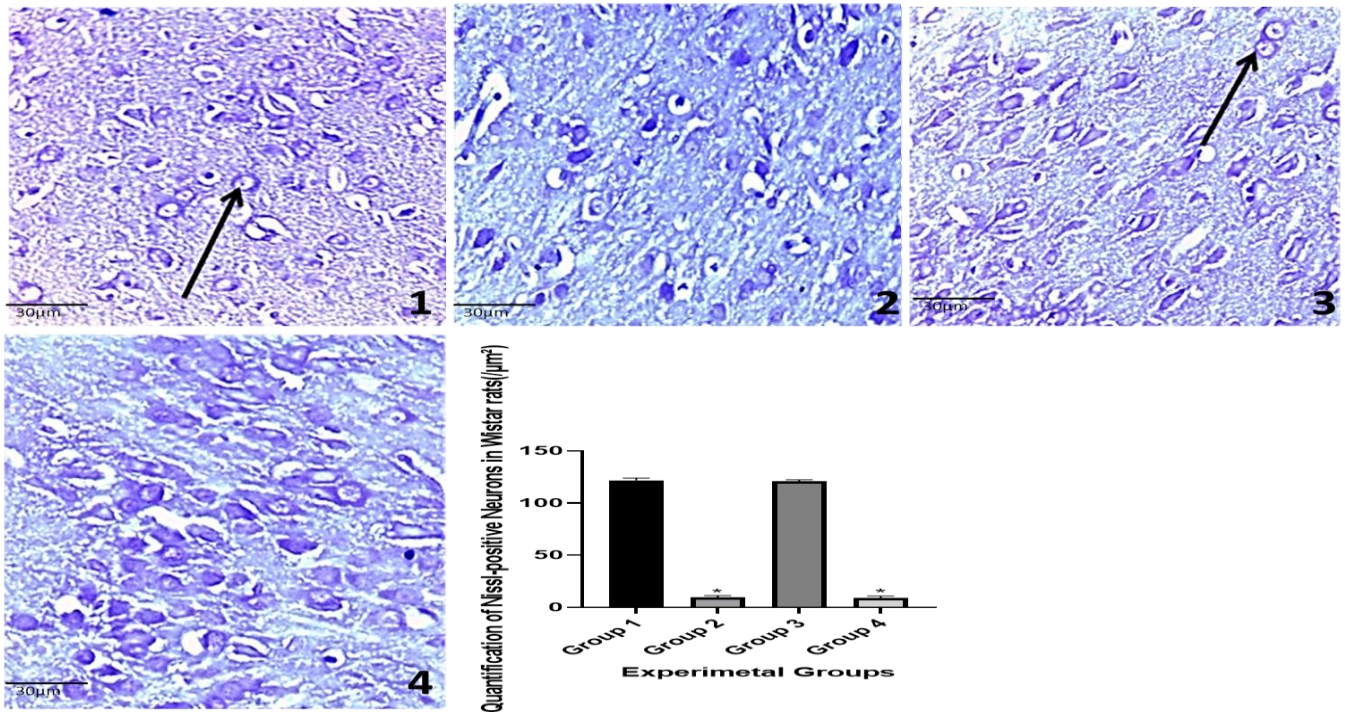


Figure 4: Nissl-positive neurons of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Black arrow – Nissl-positive neurons (Cresyl Violet; Scale bar = 30μm). Graph – Image J quantification of Nisil-positive neurons in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

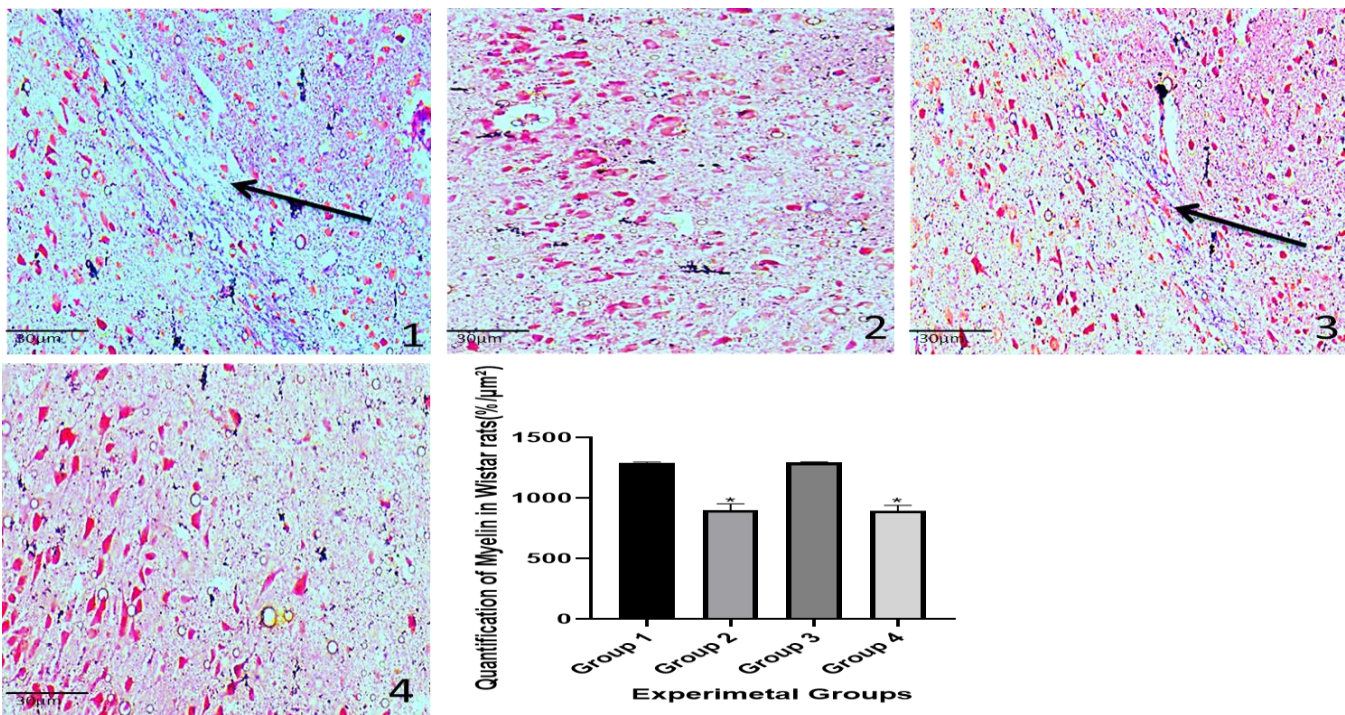


Figure 5: Myelin volume of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Black arrow – myelin deposit (LFB X400). Scale bar 30μm. Graph – Image J quantification of myelin volume in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50mg/kg citicoline.

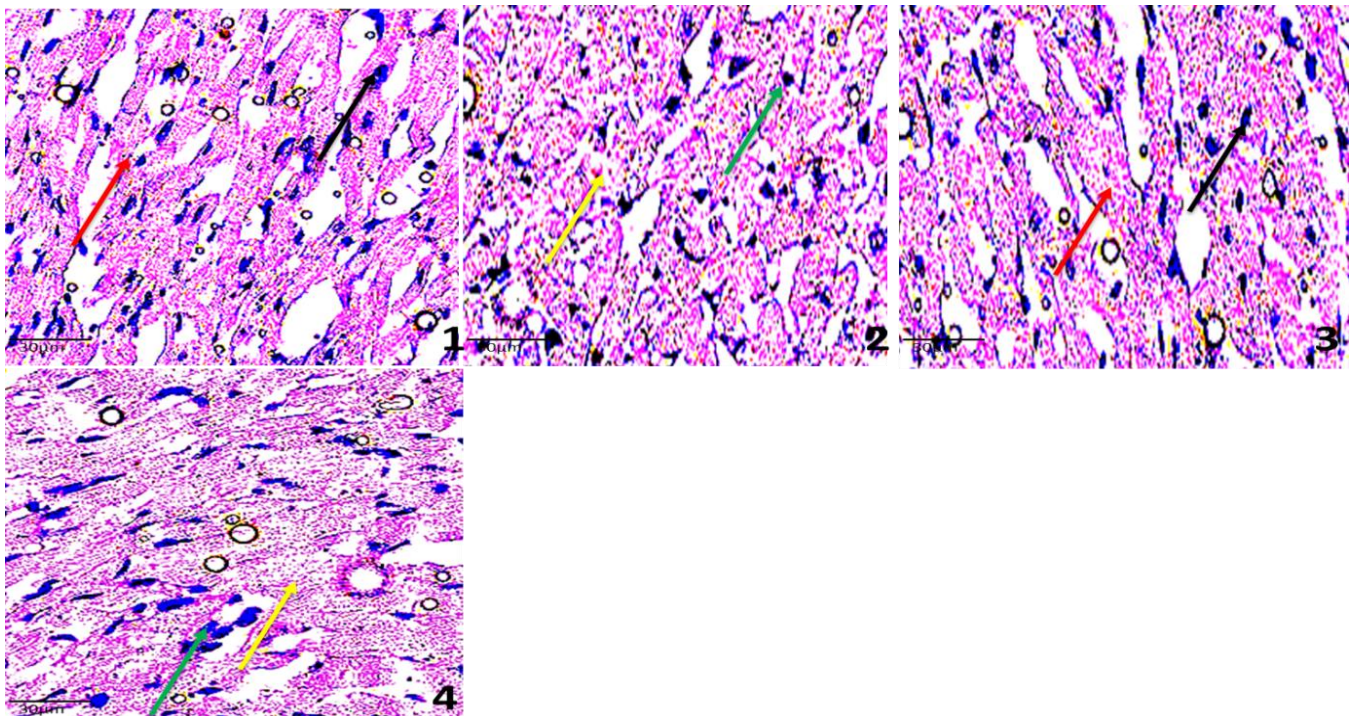


Figure 6: Photomicrographs of the left ventricle of treated and control rats. Black arrow – Nucleus, Red arrow-regularly branched myocardium, Green arrow- Degenerated/inflamed myocardium, Yellow arrow- irregularly branched myocardium (H&E X1000). Scale bar 30µm. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

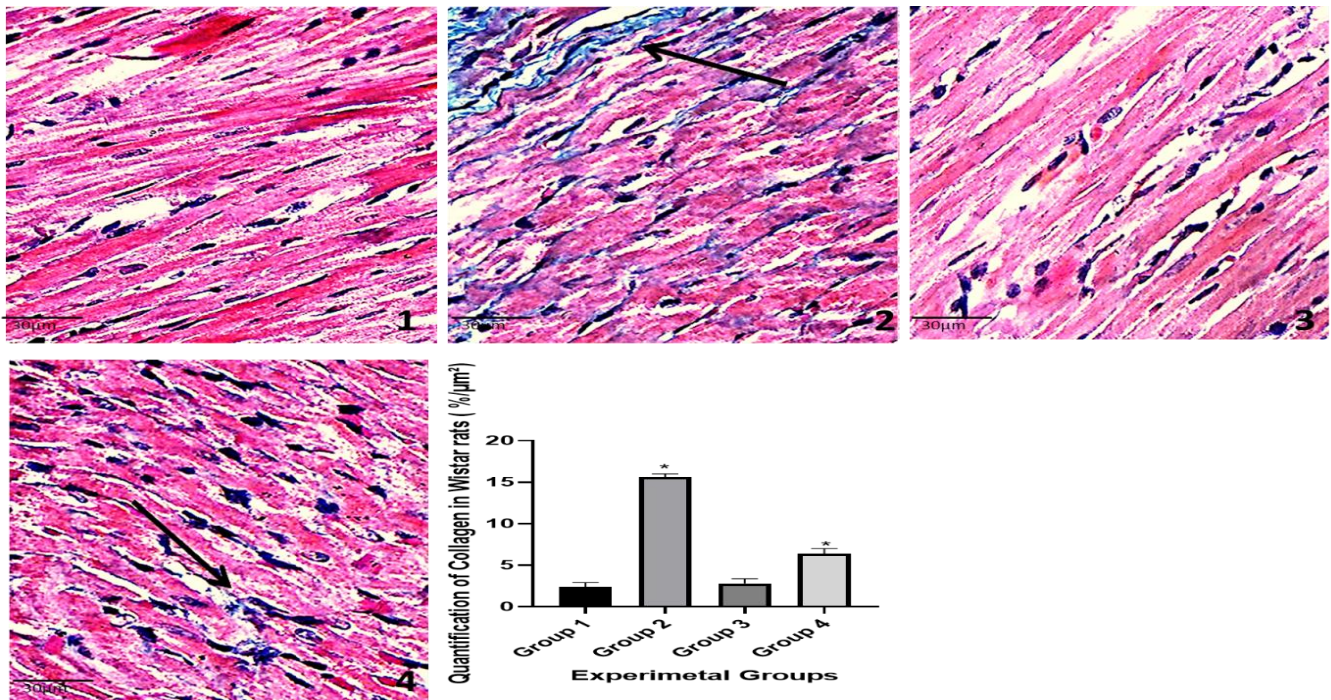


Figure 7: Effects of MCAO-induced hypoperfusion on collagen volume in left ventricle of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Black arrow – collagen (Masson's Trichome X400). Scale bar 30µm. Graph – Image J quantification of collagen volume in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

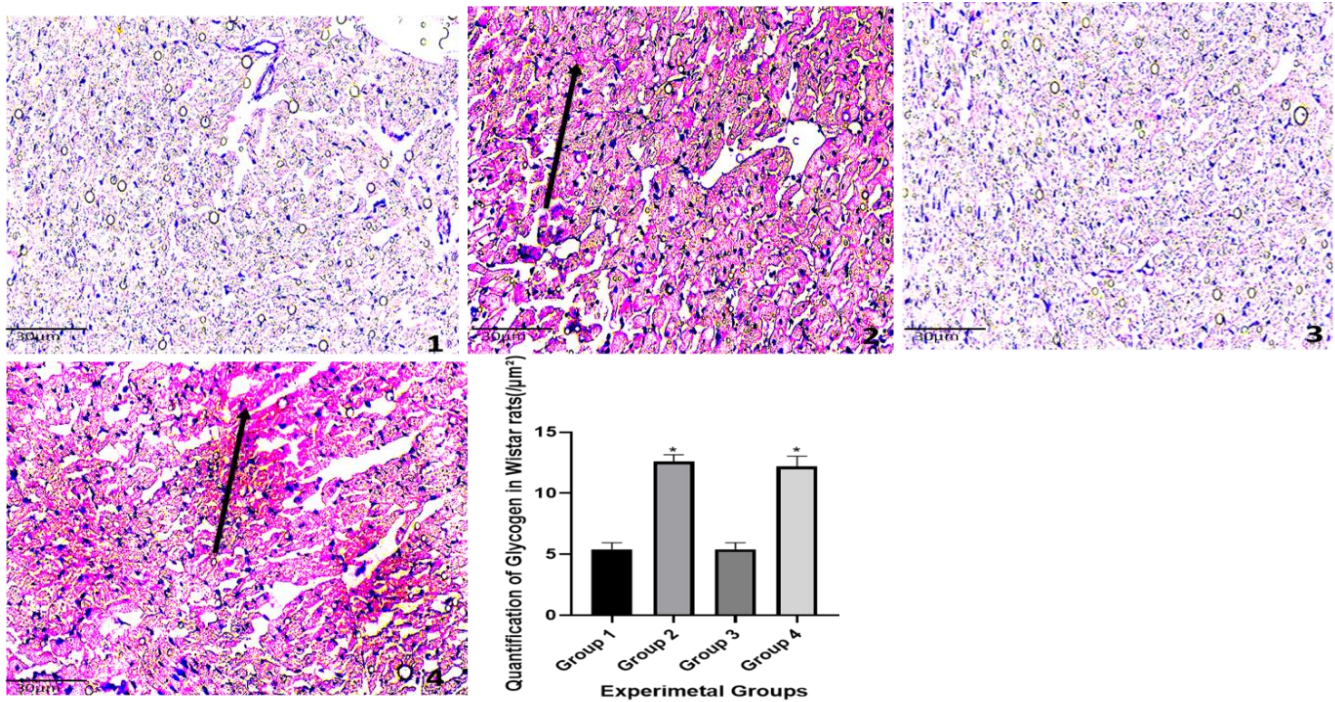


Figure 8: Glycogen volume in the left ventricle of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Black arrow – glycogen (Periodic acid Stain X400). Scale bar 30µm. Graph – Image J quantification of glycogen volume in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

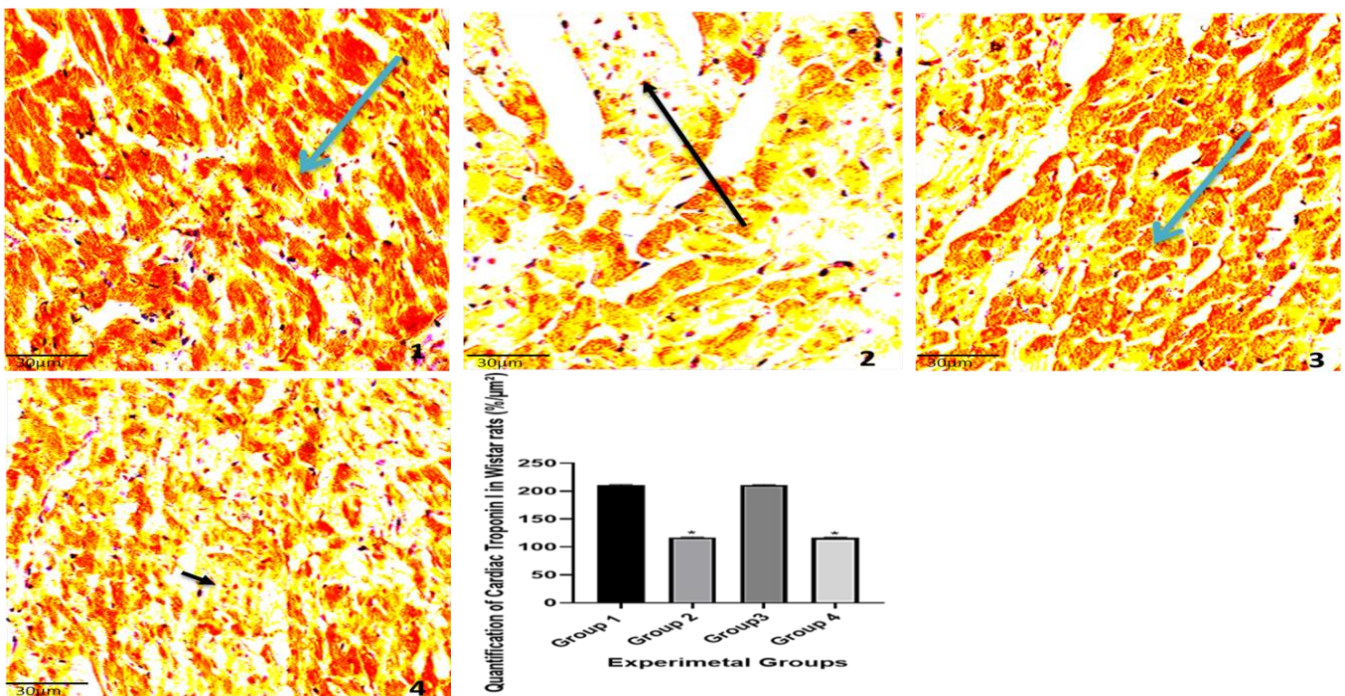


Figure 9: Cardiac Troponin I in left ventricle of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. Blue arrow – Increased Troponin I reactivity, black arrow- reduced troponin reactivity, black short arrow-moderate troponin reactivity. (CnTI X400). Scale bar 30µm. Graph – Image J quantification of Cardiac Troponin I volume in control and treated Wistar rats. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

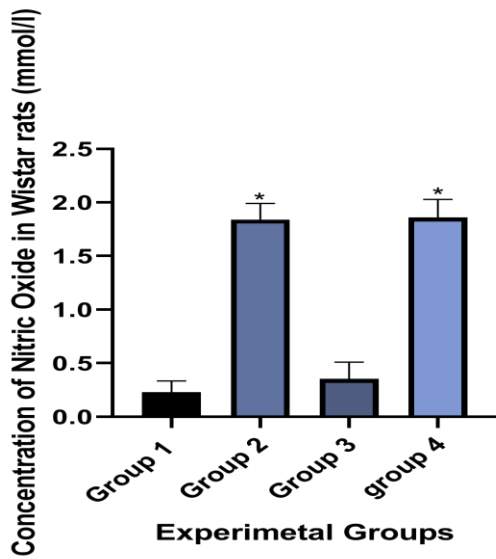


Figure 10: Serum nitric oxide concentration of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

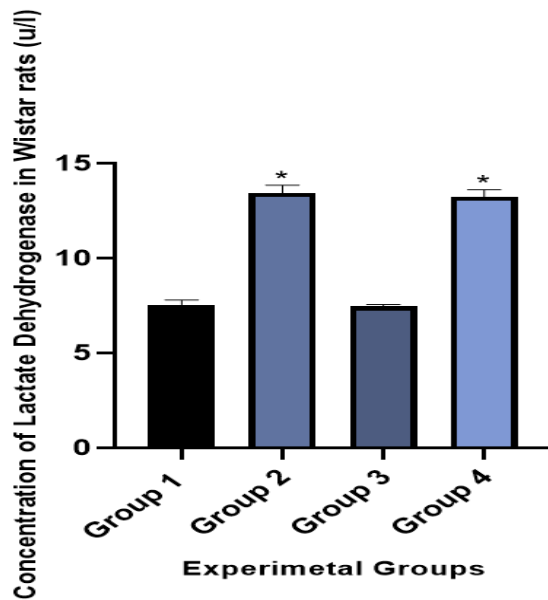


Figure 11: Serum concentration of LDH of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

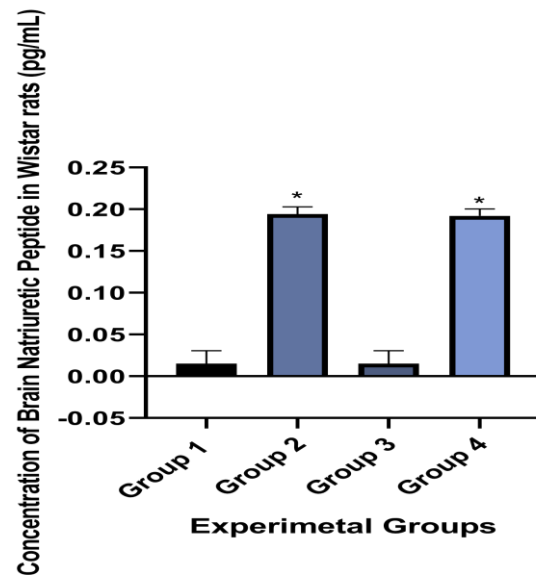


Figure 12: Concentration of serum BNP of treated and control rats. (n = 4). * = statistical significance. Values are expressed as Mean ± SEM. 1- Sham surgery, 2- middle cerebral artery occlusion, 3- middle cerebral artery occlusion and 150 mg/kg citicoline, 4-middle cerebral artery occlusion and 50 mg/kg citicoline.

DISCUSSION

In the MCAO only and 50 mg/kg citicoline – treated groups, derangement of neuronal layer, red neurons, loss of neurons, shrunken neuron, loss of myelin, reduction in Nissl-positive neurons, and astrocytic neurons were observed in insula. This in support of a research by Stepheson et al that stated that cerebral hypoperfusion resulted into imbalance oxygen/antioxidant ratio after reperfusion causing formation of ROS which led to detachment of ribosome, deoxyribonucleic acid (DNA) damage, lipid peroxidation and neuronal oxidative damage (Stephenson *et al.*, 2018).

The role of the insular in cardiovascular function has been suggested by experimental evidence (Nagai *et al.*, 2020). Damage to certain areas of the brain is associated with cardiac damage including the insular cortex (Oppenheimer and Cechetto, 2016), the medulla oblongata among many others are termed the “neurocardiac axis” (Shivkumar *et al.*, 2016); also, cerebral hypoperfusion involving the insular cortex has been reported to have effects on mean arterial blood pressure (Nagai *et al.*, 2020). Moreover, increase in sympathetic activity has been reported to result in cardiovascular disease (Malpas *et al.*, 2010).

In our study, we observed derangement of normal branching patterns of myocardial fibers, myocardial degeneration, fibrosis (kong *et al.*, 2014), increased glycogen deposit (Milutinović and Zorc-Pleskovič, 2012) increased mean arterial blood pressure, and loss of Cardiac troponin I (Toyo-Oka and Ross, 1981) in the MCAO-only and 50 mg/kg citicoline-treated groups; MCAO-induced I/R, have been

documented to cause sympathetic deregulation that could result in adrenaline release and hyperadrenergic stimulation which could lead to up-regulation of CO (cardiac output), HR (heart rate) and mean arterial pressure (MAP) (Emily *et al.*, 2021).

Loss of troponin I as recorded by our study in MCAO-only and group 4 suggest a disrupted autonomic function after all, MCAO decreases perfusion (Emily *et al.*, 2021) which could have caused an increased catecholamine response (Butcher *et al.*, 1993); increased catecholamine response has been suggested to cause deregulation of contraction and relaxation of cardiac myocytes which results into loss of cytoplasm cardiac regulatory proteins in the myocytes and subsequently into abnormal muscle contraction and relaxation (Salamah *et al.*, 2021).; Moreover, the most common cause of injury to the cardiac muscle is oxygen supply and demand mismatch and any condition that causes damage to cardiac muscle contraction can affect troponin (Salamah *et al.*, 2021). Sustained MAP as a result of cerebral hypoperfusion as evidenced by an increased glycogen deposit in the MCAO-only group (2) suggests the increase in contractility of cardiac muscle; the increase in contractility of cardiac muscle has been documented to initiate conversion of glucose to make more energy available for cardiac function and research has established that excessive availability of glucose causes channeling of glucose moieties towards glycogenesis.

The perturbations that were evident in the insular of MCAO-only and 50 mg/kg citicoline-treated groups were attenuated by citicoline at a dose of 150 mg/kg; Citicoline is a neuroprotectant and neurorestorative agent that is used as a trial treatment of hypoperfusion-induced cerebral damage (Dávalos *et al.*, 2012). Citicoline has the potential to improve endogenous brain plasticity and repair which may reduce acute brain damage and improve functional recovery in animal models of cerebral ischemia (Overgaard, 2014), even when it is administered several hours after the ischemic event. Moreover, it has been demonstrated that citicoline affects different levels of the ischemic cascade, and a series of brain repairs have been documented (Fatima *et al.*, 2020).

We discovered an increase in BNP (a cardiac hormone produced by ventricular myocytes, that plays an important role in the regulation of blood pressure and fluid volume) (Atas *et al.*, 2015), increased concentration of LDH, and reduced concentration of NO in serum of Wistar rats in MCAO-only and 50 mg/kg citicoline-treated groups. This suggests that cardiovascular may have received feedback (hyperadrenergic stimulation) which probably stimulated left ventricular compensatory enlargement to maintain pumping action; upregulation of pumping action and increased energy demand could have activated anaerobic metabolism, increased concentration of BNP, LDH, and reduction of NO concentration (all of which are serum markers of myocardial damage) (Waziri *et al.*, 2023).

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