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Neuroprotective Effects of Citicoline on Hippocampal Integrity Following Middle Cerebral Artery Occlusion-Induced Hypoperfusion in Wistar Rats

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ABSTRACT

Citicoline is required for neural efficiency, but there are concerns about its safety profile at prescribed doses in use. This research examined how varying doses of citicoline influenced the integrity of Nissl substance and myelination in the hippocampus in Wistar rats subjected to middle cerebral artery occlusion (MCAO)-induced hypoperfusion. Twenty-five male Wistar rats (200-220 g) were divided into five groups: Sham, MCAO, LDCT (low-dose citicoline), MDCT (medium-dose citicoline), and HDCT (high-dose citicoline) of five rats each. Sham surgery was done on rats in the first group; MCAO was done on rats in other groups. Sham and MCAO were administered normal saline (i.p.), rats in LDCT, MDCT and HDCT were treated with a dose of 50 mg/kg, 100 mg/kg and 150 mg/kg (i.p.) citicoline daily, respectively, for 12 weeks. Twenty-four hours after the last administration, rats were sacrificed and blood samples were taken for biochemical analysis. The hippocampus was harvested and fixed in 10% neutral buffered formalin for histological, histochemical, and immunohistochemical studies. Data were analyzed with one-way ANOVA followed by the Student-Newman-Keuls test. The alpha value was set at $p \leq 0.05$. The results showed a significant increased concentration of brain natriuretic peptide, depleted and shrunken neurons with loss of Nissl substance and damaged myelin in the hippocampus of the MCAO group. These perturbations were attenuated in the citicoline-treated groups in a dose-dependent manner. The study concluded that citicoline demonstrates significant neuroprotective effects against MCAO-induced cerebral hypoperfusion and neuronal damage in Wistar rats.

Keywords

Blood samples, Biochemical analysis, Hippocampus, Histological studies

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INTRODUCTION

Middle cerebral artery occlusion (MCAO) is widely used as an experimental model to simulate ischaemic stroke, leading to reduced blood flow in the brain and consequent neuronal injury (Sokolowski *et al.,* 2023). The hippocampus is perhaps the most affected area in MCAO-induced cerebral hypoperfusion (Neher *et al.*, 2025). MCAO is achieved by the blocking of blood flow into the middle cerebral artery (MCA) with an intraluminal suture (nylon monofilament) inserted through one of the big arteries of the neck (Longa *et al.,* 1989). If performed properly, this method provides reproducible MCA territory infarction (Fluri *et al.,* 2015) and allows for cerebral reperfusion following a temporary blockage by removing the suture, thereby producing lesions of varying severity depending on the duration of occlusion (Komatsu *et al.*, 2021).

Models of occlusion have been reported to show that hypoperfusion can lead to neuronal damage, glial activation, and long-term memory impairments (Durukan and Tatlisumak, 2007). Moreover, measuring parameters like myelination, Nissl body presence, and astrocyte activation helps researchers to understand the extent of damage, monitor disease progression, and evaluate the effectiveness of treatments (Zhang *et al.*, 2006).

Myelin forms the protective sheath around nerve fibres. Myelin damage is a key feature of white matter injury

caused by reduced blood flow, and this damage is associated with cognitive impairments, particularly in processing speed, a core deficit in vascular cognitive impairment (Denecke *et al.,* 2025).

The presence of Nissl bodies, density, and distribution can reflect neuronal integrity, protein synthesis capacity, and overall neuronal health, all of which are crucial for cognitive processes. Nissl body changes, such as their dissolution or dispersion, can be a sign of neuronal dysfunction and may contribute to cognitive decline (Meystre *et al.,* 2024).

Glial cells respond to reduced blood flow by releasing inflammatory mediators, contributing to neurodegeneration and impacting brain function. Glial fibrillary acidic protein (GFAP) is a major component of the astrocyte cytoskeleton and is highly specific to these glial cells. When astrocytes are damaged, as can occur during cerebral hypoperfusion, GFAP is released (Lei *et al*., 2015).

Citicoline, also known as cytidine 5′-diphosphocholine, is a phospholipid consisting of cytidine and choline linked by a diphosphate bond. It is soluble in water and exhibits high bioavailability (Dávalos and Secades, 2011). It is also available commercially in its free-base form or as a sodium salt (Schauss *et al.,* 2009). As an important dietary source of methyl groups, choline is also involved in the biosynthesis of lipids, regulation of metabolic pathways, and detoxification in the body. Citicoline has shown neuroprotective effects, reduced infarct size, and improved neurological outcomes in models of ischaemia (Mankivska *et al*., 2022).

Researchers often presented citicoline's neuroprotective effects on the brain based on the assumption that it is sequentially hydrolysed and dephosphorylated to produce cytidine (or uridine in humans) and choline following injection or ingestion, after which the metabolites enter the brain tissues separately and are used to resynthesise citidine diphosphate-choline-choline, which then exerts neuroprotection intracellularly by supporting biosynthesis of cellular phospholipids. However, dosage and route of administration matter when reporting efficacy and citicoline toxicity (Ramos-Cabre *et al.,* 2011); higher doses of citicoline tend to result in excessive circulation of unhydrolysed citicoline, or perhaps phosphocholine and/or cytidine monophosphate, which are pharmacologically active metabolites of citicoline (Grieb, 2014). At doses of 350 and 1,000 mg/kg/day, a significant increase in serum creatinine, decreases in urine volume, and an increase in blood urea nitrogen have been reported in rats; while a dose of 1,000 mg/kg/day showed a significant increase in total white blood cell and absolute lymphocyte counts as well as an increase in renal tubular mineralisation (Schauss *et al.,* 2009).

This study was specifically designed to investigate the potential role of citicoline, at lower doses, in addressing hippocampal hypoperfusion induced by MCAO. By extending these investigations to focus specifically on hippocampal hypoperfusion in the MCAO model, this study aims to elucidate whether lower doses of citicoline could offer therapeutic benefits in preserving hippocampal function and cognitive abilities under ischaemic conditions.

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MATERIALS AND METHODS

Animal Care and Management

A total of twenty-five rats of the Wistar strain (male) weighing between 200 and 220 g were used for this study. They were kept at natural night and day cycles and acclimatised for two weeks before the experimental period. All rats were fed with standard rat chow (CAP Feed Ltd., Nigeria) and clean water all through the period of the experiment.

The rats were divided into five groups: Sham, MCAO, LDCT (low-dose citicoline), MDCT (medium-dose citicoline) and HDCT (high-dose citicoline), of five rats each. Sham surgery was performed on rats in the sham group; MCAO was performed on rats in all other groups. Sham and MCAO were administered normal saline (i.p.), while rats in LDCT, MDCT and HDCT were daily treated with a dose of 50 mg/kg, 100 mg/kg and 150 mg/kg (i.p.) citicoline, respectively, for 12 weeks.

MCAO

Twenty-four hours after the acclimatisation period, a two-hour MCAO was performed. A modified MCAO procedure of Longa (1989) was adopted. All equipment and surfaces were dabbed with 70% ethanol to prevent infection; all the surgical instruments and materials were autoclaved, and the surgical procedure was performed under sterile conditions.

Suture Preparation and Surgical Procedure

A 5 cm-long segment of sterile 2/0 nylon monofilament (Ethilon Nylon Suture, Ethicon Inc., Germany) was prepared for use as the suture. The tip of the suture was blunted by heating it near a flame. A 20-mm distal portion of the suture was then coated with a solution of poly-l-lysine (0.1%, w/v in deionised water, Sigma) and dried in a 60°C oven for 1 h, without altering the suture's diameter. To provide intraoperative control of monofilament, the proximal 22 mm of the suture was marked with a sterile permanent marker at five points: 5 mm, 5 mm, 5 mm, 5 mm, and 2 mm intervals. Rats were anaesthetised using 50 mg/kg pentobarbital (i.p.). The depth of anaesthesia (moderate) was confirmed by paw withdrawal tests (Interlandi *et al*., 2021), and animals were positioned in a recumbent position. Under a dissecting microscope, a midline incision was made and the common carotid artery (CCA) was exposed. The suture was inserted 18 to 20 mm from the bifurcation of the CCA until it blocked the origin of the MCA, essentially stopping blood flow. Once the intraluminal suture had been placed, the neck incision was sealed with skin glue. After two hours of occlusion, the intraluminal suture was gently withdrawn. The CCA and internal carotid artery (ICA) were examined to ensure normal pulsations had returned. The neck incision was closed using silk sutures, and animals were allowed to recover with free access to food and water. Animals were returned to their cages upon awakening from anaesthesia.

Sham surgery is similar to the MCAO surgery, with the difference being that there was no occlusion of MCA.

Behavioural Testing

The radial arm maze (RAM) test, developed by Olton and Samelson (1976), was used to assess spatial learning and memory. The RAM is made up of eight equally spaced, rectangular arms with openings at the top extending from a central octagonal platform: A small cavity in which a pellet was placed at the end of each arm. Illuminated visual cues, including different objects like candle stands and picture frames presenting different geometrical patterns, were placed next to the maze as distal cues. The rats were habituated to the experimental setup for 3 to 5 days. Pellets were placed all over the maze while rats were allowed to move freely within the maze for around 30 min per day. The RAM test was carried out on day 28 post-MCAO. Rats were food deprived for 8 h before testing, and the observer was blinded regarding treatment group assignment. A pellet was placed by the end of each of the eight arms of the maze, the rats were placed onto the central platform of the maze (one at a time), and the time taken to complete the task was analyzed to assess spatial learning and memory**.**

Sacrifice, Histological and Immunohistochemical Studies

Twenty-four hours after the last administration of citicoline and normal saline, rats were anaesthetised with 100 mg/kg (i.p.) pentobarbital (Tsubokura *et al.,* 2016). Blood samples were collected via cardiac puncture for biochemical investigations, and the rats were then perfused transcardially with 0.9% normal saline solution followed by 10% neutral buffered formalin fixative. The hippocampus was carefully removed and post-fixed in 10% neutral buffered formalin. After 24 h, the tissues were processed and prepared for paraffin embedding. Tissue blocks were then sectioned transversely to a thickness of 5 µm using a rotary microtome. The sections were stained with haematoxylin and eosin to visualise the general histoarchitecture of the tissue. Luxol fast blue staining was used to highlight myelin, while cresyl violet staining was employed to demonstrate Nissl bodies. Additionally, immunostaining for GFAP was performed to examine the presence of astrocytic activation in the hippocampus.

Biochemical Analysis

At the conclusion of the experiment, the obtained blood samples were centrifuged at 3,000 x g for 10 min to separate the serum (Allison *et al.*, 2020). Brain natriuretic peptide (BNP) levels in the serum were measured using a suitable assay kit (BioAssays Systems, Hayward, CA, USA) according to the manufacturer's instructions.

Photomicrography and Image Analysis

Stained sections were loaded into the Motic Easy Scan Pro 6-slide digital slide scanner, scanned images were imported into the Motic digital slide assistant software, and photomicrographs were taken at various magnifications.

Photomicrographs of stained sections were analyzed and processed using image analysis and processing for JAVA (imagej, version 1.54p, Rasband, 2024).

Statistical Analysis

Statistical analyses involved conducting one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test for multiple comparisons to assess differences. Data analyses were performed using GraphPad Prism (version 8.4.3, GraphPad Software Inc., 2020). Descriptive and inferential statistics were employed for data interpretation, with significance set at $p < 0.05$. Results are presented as mean ± SEM (standard error of the mean).

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RESULTS

Neurobehavioural Studies

There was a significant increase in the time taken to locate the baited arms in the MCAO, LDCT, and MDCT groups when compared to the Sham and HDCT groups (Fig. 1).

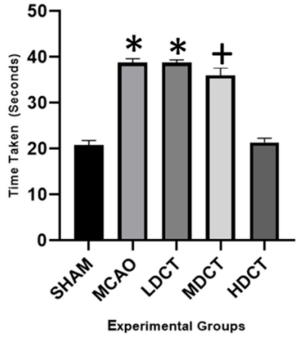

Fig. 1: Effects of citicoline on MCAO-induced changes on the radial arm maze test in experimental rats (n=5). Radial arm test, 5 trials. Day 28, * = significant difference when compared to SHAM, + = significant difference when compared to the MCAO group. Values are expressed as mean ± SEM. SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

Concentration of Brain Natriuretic Peptide

There was a significant increase in the concentration of serum brain natriuretic peptide in the MCAO, LDCT, and MDCT groups when compared to the Sham and HDCT groups (Fig. 2).

Histological and Immunohistochemical Examinations
Haematoxylin and Eosin (H&E) Staining for Demonstration of General Histoarchitecture of Hippocampus in Sham and Treated Wistar Rats

H&E staining of the Ca1 region of the hippocampus showed a distinct arrangement of a well-organised layer of compact cells, healthy neurons with pale and round nuclei, well-defined nuclear boundaries and prominent nucleoli, with a few interneurons in the sham and HDCT groups; the Ca1 region of the hippocampus in the MCAO group showed shrunken dark neuronal cells and

pyknotic neurons. LDCT showed fewer intact neurons with some degenerating neurons while MDCT showed some degenerated neurons interspersed with intact neurons (Fig. 3a).

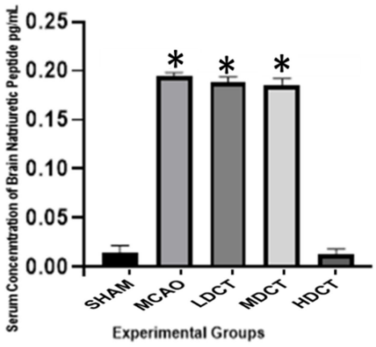

Fig. 2: Effects of citicoline on MCAO-induced changes on serum brain natriuretic peptide in experimental rats (n=5). * = significant difference when compared to SHAM. Values are expressed as mean ± SEM. SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline

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ImageJ quantification of the number of intact and degenerated hippocampal neurons showed that the quantity of normal neurons is significantly high in sham and HDCT groups when compared to the MCAO, LDCT and MDCT groups (Fig. 3b).

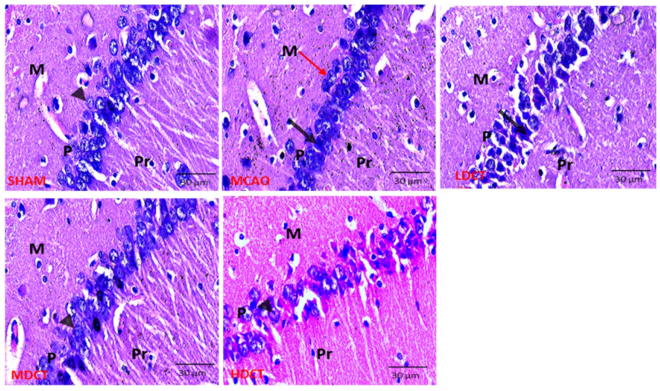

Fig. 3a: Photomicrographs of hippocampal sections of treated and control Wistar rats showing SHAM – normal three layers including the pyramidal layer (P) that contains about 4-6 layers of intact pyramidal neurons (arrowhead), the molecular layer (M) and the polymorphic layer (Pr), which contain abundant glial cells. MCAO – Almost no distinctive layers with pyknotic neurons (red arrow) and shrunken degenerating neurons with dark nuclei (black arrow). LDCT-Reduced number of pyramidal cell layers (P) and reduced number of atypically morphologic cells compared to the MCAO group. MDCT – degenerating neuron with dark nuclei (black arrow). HDCT – mostly normal and recovering hippocampal neurons (arrowhead) with few degenerated neurons. Scale bar – 30 µm, H&E staining. SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

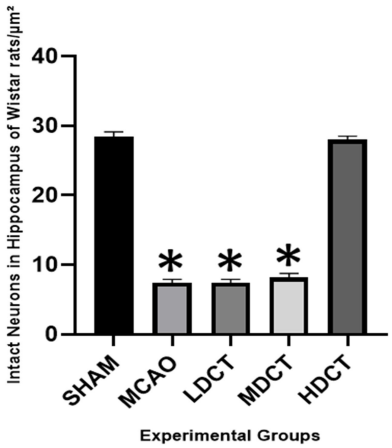

Fig. 3b: ImageJ analysis showing that the numbers of intact neurons were significantly lower in the MCAO group, LDCT, and MDCT group. However, intact neurons are higher in the SHAM group and HDCT groups. *= significant difference when compared with SHAM (P - 0.9835). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

Luxol Fast Blue Stain for Demonstration of Myelin

Luxol fast blue staining of the Ca1 region of the hippocampus showed a normal distribution pattern and myelin volume in the Sham and HDCT when compared with other groups. There was depletion of myelin in MCAO, LDCT and MDCT groups. ImageJ analysis showed that there is a reduction in myelin in the MCAO, LDCT and MDCT groups (Fig. 4a and 4b).

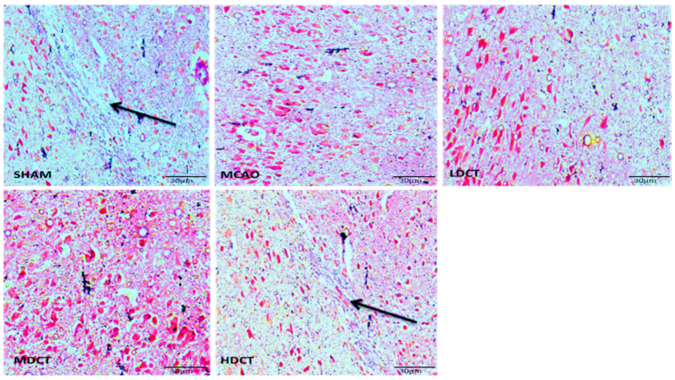

Fig. 4a: Luxol fast blue staining of the brain is shown. SHAM and HDCT-normal distribution pattern and volume of myelin (black arrow). MCAO, LDCT and MDCT – reduction in myelin volume (Fig. 4a). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

Cresyl Violet Staining for Demonstration of Nissl-Positive Neurons in Sham and Treated Wistar Rats

Cresyl violet stain showed Nissl bodies that appear as purple, granular clumps within the cytoplasm of neurons, contrasting with the blue-stained nuclei. ImageJ quantification of cresyl violet stain of the hippocampus showed an increase in the quantity of Nissl-positive neurons in the sham and HDCT, with the MCAO and LDCT groups showing few Nissl-positive neurons (Fig. 5a and 5b).

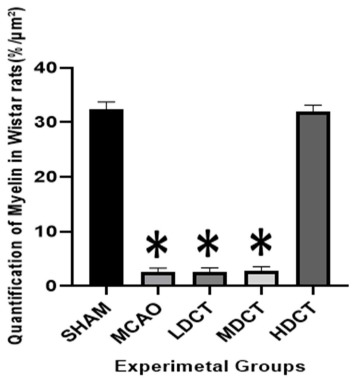

Fig. 4b: ImageJ analysis showed that myelin volume was significantly higher in SHAM and HDCT groups. Myelin volume was lower in MCAO and LDC. * = significant difference when compared with SHAM (P - 0.9950). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

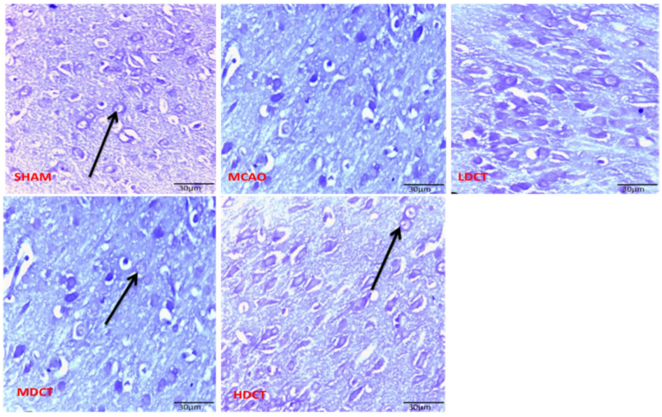

Fig. 5a: Photomicrographs of the hippocampus of control and treated rats. Showing: Black arrow = Nissl body (cresyl violet stain scale bar – 30 µm). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

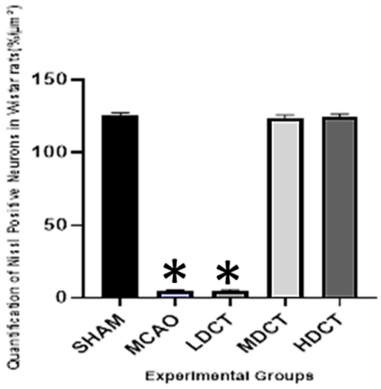

Fig. 5b: ImageJ analysis showing a significant reduction in Nissl-positive neurons in the MCAO group and LDCT group. * = significant difference when compared with the SHAM group (P - 0.9864). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

GFAP Expression

GFAP staining showed intense expression in the MCAO and LDCT groups. There was mild protein expression in the Sham, MDCT and HDCT groups (Fig. 6a).

ImageJ analysis showed that GFAP expression was higher in MCAO, LDCT and MDCT when compared to the Sham (Fig. 6b).

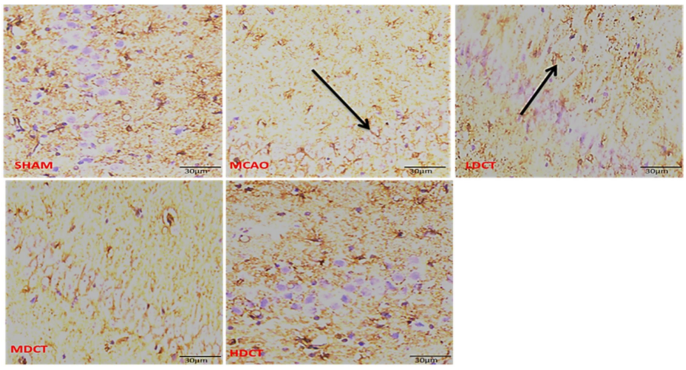

Fig. 6a: Photomicrographs of GFAP staining of hippocampus (CA1) showing SHAM, MDCT and HDCT – no expression of fibrillary protein; MCAO – intense fibrillary protein expression; and LDCT – mild fibrillary protein expression. Scale bar – 30 µm, GFAP stain. SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

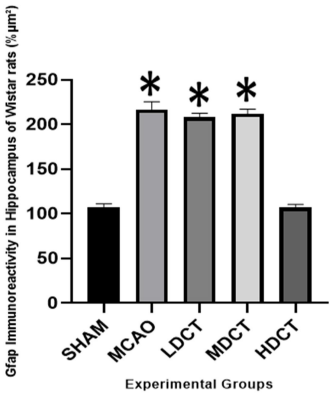

Fig. 6b: ImageJ analysis showed that GFAP expression was higher and the same in MCAO and LDCT. * = significant difference when compared with SHAM (P - 0.9999). SHAM = control, MCAO = middle cerebral artery occlusion, LDCT = low-dose citicoline, MDCT = medium-dose citicoline, HDCT = high-dose citicoline.

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DISCUSSION

This research explored the potential therapeutic benefits of citicoline in addressing hippocampal damage and cognitive deficits caused by MCAO in Wistar rats.

The RAM behavioural test apparatus was used to assess both working memory (remembering which arms have been visited within a trial) and reference memory (remembering which arms are consistently baited) (Levin, 2015). On experimental day 28, the result showed an increase in time taken to locate the baited arm only in the

MCAO and LDCT groups. This may be attributed to hypoperfusion-induced neural damage, which can disrupt the efficient transmission of nerve impulses and contribute to neurological dysfunction (Heimfarth *et al.,* 2022), ultimately leading to an impaired performance in the RAM test, an important marker of cognitive deficits (Levin, 2015).

The elevated serum levels of BNP observed in the MCAO-only group provided significant insight into the extent and severity of neuronal damage. BNP is a peptide hormone predominantly secreted by the ventricles of the heart in response to excessive stretching of heart muscle cells. Although its primary role is in cardiovascular homeostasis, its elevation in the context of cerebral ischaemia is indicative of neuronal stress and injury (Woodard and Rosado, 2008; Krylatov *et al.,* 2021; Truter, 2021).

Rats in the MCAO group exhibited significant hippocampal damage. These observations are consistent with previous research indicating that during ischaemia-reperfusion injury is significant; MCAO causes an overproduction of reactive oxygen species (ROS), leading to oxidative stress and cell damage in the hippocampus, especially in the CA1 region (Aboutaleb *et al.,* 2016). This disruption often results in diminished antioxidant levels in damaged neurons, promoting the formation of ROS. These ROS cause ribosome detachment, deoxyribonucleic acid (DNA) damage, lipid peroxidation, and extensive neuronal injury (Stephenson *et al.,* 2018).

Our study recorded an increase in the quantity of Nissl-positive neurons in the sham and HDCT; with the MCAO and LDCT groups showing few Nissl-positive neurons which could be an indicator of neuronal damage or dysfunction triggered by ischaemic injury that has led to the breakdown and dispersal of Nissl bodies (Unal-Cevik *et al.,* 2004). The hippocampus in the MCAO group also showed increased expression of glial fibrillary acidic protein (GFAP), a protein that plays a crucial role in maintaining astrocyte structure and function, including cell communication and blood-brain barrier maintenance. GFAP expression is upregulated in response to neural damage, a process known as gliosis (Zhang *et al.,* 2017). These findings suggest that after MCAO, reperfusion exacerbated the cellular damage already caused by ischaemia (Ikhlas and Atherton, 2023). In the MCAO group, Luxol fast blue staining of the hippocampus also showed demyelination, indicating neurodegeneration (Karimi *et al.,* 2017). This present study recorded demyelination (in the MCAO group), which is a common consequence of ischaemia. With a restriction of blood supply to tissues, there is energy depletion that can cause breakdown of myelin sheaths (Cheng *et al.,* 2024).

Citicoline treatment ameliorated all the observed perturbations in the hippocampus of the MCAO-only group in a dose-dependent manner. Improved performance in the RAM test as observed in the HDCT group can be attributed to citicoline's efficiency in restoring neural cytoplasmic membranes and myelination (Ramos *et al*., 2011).

Treatment with citicoline significantly mitigated hippocampal pathological changes in a dose-dependent manner, with the greatest protective effect observed at a dosage of 150 mg/kg. Citicoline's neuroprotective properties, as observed in the HDCT group, are likely due to its ability to counteract reperfusion injury-induced neuronal damage (Farshad *et al.,* 2020). It enhances glutathione synthesis, a crucial antioxidant, and facilitates the incorporation of free fatty acids (FFA) into phosphatidylcholine, thereby reducing the levels of arachidonic acid (AA), a key contributor to oxidative damage in neurons (Alvarez-Sabín and Román, 2011; Aminzadeh and Salarinejad, 2018).

Although citicoline does not directly affect brain natriuretic peptide, the downregulation of serum brain natriuretic peptide in the HDCT group indicated that citicoline promoted neuronal repair, contributing to the overall neuronal health in that group.

The downregulation of GFAP expression in the HDCT group indicated that citicoline has the potential to mitigate astrocyte activation and neuroinflammation. Additionally, restoration of Nissl bodies as observed in the citicoline-treated group at 150 mg/kg supports the claim that citicoline promotes the formation of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, essential components of neural cytoplasmic membranes; This process has been documented to enhance the incorporation of FFA, including AA, into phosphatidylcholine and other major phospholipids, thereby mitigating oxidative damage (Rao *et al.,* 2000).

Citicoline significantly reduced demyelination; this can be attributed to citicoline's role in supplying abundant choline for phosphatidylcholine synthesis. Phosphatidylcholine is crucial for the maintenance of myelin, which consists of 70%-80% fat, with phosphatidylcholine making up 45% (Jasielski *et al.,* 2020). .

Conclusion

In conclusion, citicoline demonstrates significant neuroprotective effects against MCAO-induced cerebral hypoperfusion and neuronal damage in Wistar rats. Citicoline exhibited neuronal repair, myelin restoration and Nissl repair, as well as improved RAM mance. These findings suggest that citicoline holds promise as a therapeutic agent for reducing brain damage and cognitive impairment associated with ischaemic stroke. Further research is needed to elucidate the underlying molecular mechanisms of citicoline's metabolism to explore the clinical applications of citicoline in stroke treatment and its implications for other organs such as the liver and kidneys.

DECLARATION

Acknowledgement

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Grants and Financial Support
Not Applicable.

Conflict of Interest
None declared.

Ethical Approval
Ethical approval for this research (IPH/OAU/1737) was obtained from the Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo University, Ife.

Authors' Contribution
BIO: Conceptualisation, collection of data, data analysis, methodology, investigation, resources, original draft preparation. KOA: Supervision, Visualisation, Validation, Reviewing and Editing. TOS: Reviewing and Editing.

Consent to Participate and Publish Data
Not Applicable.

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