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Astrocytic Response in the Striatum and Corpus Callosum of the Brain of Adult Wistar Rats with Global Ischaemia

Matthew T. Shokunbi[1,2], Funmilayo E. Olopade[1], Omowumi M. Femi-Akinlosotu[1], Olasiji Oladeji[1]

[1]Department of Anatomy, College of Medicine, University of Ibadan, Ibadan, Nigeria
[2]Department of Surgery, College of Medicine, University of Ibadan, Ibadan, Nigeria

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ABSTRACT

Ischaemic stroke occurs when the blood supply to a part of the brain is suddenly interrupted. Astrocytes are known to maintain ionic homeostasis, scavenge free radicals and support neurogenesis. This study examined the astrocytic responses in the corpus callosum and striatum to global ischaemia with functional deficits in adult male rats. The common carotid artery (CCA) in adult rats was occluded temporarily (30 min) or permanently and then kept for 24 h or three days, while a sham operation was done for the control (n=10 per group). Neurobehavioural testing for motor deficits (square grid and pole tests) were carried out before sacrifice. The rats' brains were stained with haematoxylin and eosin, and immunohistochemically with anti-glial fibrillary acidic protein for astrocytes. Quantitative data from behavioural tests were compared using analysis of variance, and significance was set at 0.05. The rats with permanent CCA occlusion had increased latency to turn and return to the base in the pole test, and reduced latency to fall in the square grid test compared to the temporary occlusion and control groups. Sections of the corpus callosum and striatum had increased proliferation and hypertrophy of the astrocytes in the permanent CCA occlusion, which was less obvious in the temporary occlusion group compared to the control. Global ischaemia caused neuronal degeneration and reactive astrogliosis in the corpus callosum and striatum, which were more pronounced in the group with permanent arterial occlusion than those that underwent reperfusion. Early reperfusion is crucial for structural and functional recovery following brain ischaemia in stroke management, and astrocytes play important roles in both the pathogenesis and recovery process.

Keywords: *Common carotid artery, Global ischaemia, Astrocytes, Striatum, Corpus callosum*

INTRODUCTION

Stroke, a sudden loss of brain functions due to disturbance in the cerebral blood supply with symptoms lasting at least 24 h or leading to death, is the second leading cause of death worldwide (Lee et al. 2014a; Kopyta et al. 2015). It is also defined as a loss of neurological function of vascular origin, in which signs and symptoms may occcur suddenly (within seconds) or rapidly (within hours) (Guo et al. 2013). The incidence and mortality rate of stroke increase with age, and the elderly population is rapidly growing in most developed countries. The incidence and mortality rates of stroke increase with age, and with rapidly growing elderly population is in most developed countries, ischaemic stroke is a common societal burden with substantial economic costs (Tsakanova et al. 2015).

Correspondence: Matthew T. Shokunbi, FRCSC; Department of Anatomy, College of Medicine, University of Ibadan, PO Box 200084, Ibadan, Nigeria. Email: temitayoshokunbi@yahoo.com; Phone: +2348022912220; ORCID: 0000-0002-7819-6862

In Nigeria, the prevalence of stroke is 1.14 per 1000 individuals (Wahab et al. 2008). Due to aetiology, stroke can be classified into two major types: ischaemic and haemorrhagic. Ischaemic stroke is caused by an obstruction of cerebral blood flow, possibly due to either a thrombus or embolus. This is a major type of stroke, accounting for 87% of all cases (Campbell et al. 2019). The obstruction of blood flow results in an infarct that encompasses all or part of the territory supplied by the occluded artery. On the other hand, haemorrhagic stroke, which accounts for about 10% of cases, is caused by a vessel rupture that causes the blood to leak into the surrounding nervous tissues. Some risk factors associated with stroke include hypertension, atherosclerosis, diabetes, high blood cholesterol, smoking, physical inactivity, family genetics and age (Boehme et al. 2017).

The sudden reduction in cerebral blood flow restricts the delivery of vital nutrients, oxygen and glucose to nervous tissue, thus causing energy failure, resulting in ionic imbalance and subsequently leading to excitotoxic cell death (Xiong et al. 2004). Excitotoxicity can, in part, be attributed to astrocytes, which fail to clear excess glutamate during ischaemia, promoting excessive neuronal injury and death (Durukan and Tatlisumak 2007). Increased Ca^{2+} levels further activate proteolytic enzymes that degrade cellular cytoskeletal proteins (Aronowski et al. 1997) and profoundly damage cellular integrity. Consequently, cells undergo necrotic cell death forming the irreversibly damaged core of the infarct. Reperfusion of the ischaemic tissue before substantial tissue injury may reverse such neuronal damage (Imran et al. 2021). However, if reperfusion is delayed, its adverse effects overshadow desirable outcomes and exacerbate initial ischaemic damage by causing cerebral reperfusion injury (Mergenthaler et al. 2004).

Astrocytes are a sub-division of glial cells in the central nervous system (CNS), with a characteristic star-shaped cell body and many processes which envelop neuronal synapses. They have transporter proteins and channels which monitor and modulate these neuronal synapses. Astrocytes are the most abundant glial cells in the mammalian brain modulating intracellular calcium. When there is CNS injury or disease, astrocytes respond with various potential changes in gene expression, cellular structure, and function, commonly known as astrogliosis (Sofroniew 2014). A major feature of ischaemia is astrocytic swelling located and forming end feet around capillaries; following an ischaemic insult, the surviving astrocytes adjacent to the injured tissue undergo a process of hypertrophy. Protoplasmic astrocytes are found in all gray matter, while fibrous astrocytes are found throughout all white matter, exhibiting long fibre-like processes morphologically (Sofroniew and Vinters 2010).

Sensorimotor, learning and memory deficits have been reported in experimental models of stroke and clinical patients (Larpthaveesarp and Gonzalez 2017; Hassan and Yarube 2018; Feng et al. 2020; Baba and Yarube 2021). It is, therefore, very important to evaluate these symptoms by neurobehavioural tests so that the pathological mechanisms can be understood and the potential for therapeutic interventions tested. Traditionally, stroke research has often ignored the glial cell populations and has predominantly focused on neurons for effective stroke therapy. With the increasing understanding of the role of glial cells in both CNS health and disease, it has become evident that the neuron-centred perception of protection against ischaemia may not be ideal for effective stroke therapy. Historically, the astrocyte response in ischaemic stroke has been considered detrimental to the injured brain. However, recent findings suggest that targeting astrocytes for repair can successfully treat ischaemic stroke (Chouchane and Costa 2012). Astrocytes have also been shown to attenuate glutamate excitotoxicity in neurons by preserving the expression of glutamate transporters (Mahmoud et al. 2019) and through glutathione-dependent antioxidant mechanisms (Miao et al. 2011). The beneficial role of reactive astrocytes following a stroke can also be attributed to increased infarction in glial fibrillary acid protein (GFAP) null mice compared to wild-type after middle cerebral artery occlusion (Nawashiro et al. 2000). However, the spatial, as well as temporal expression of astrocytes within the brain, determines their various functions (Shen et al. 2021).

The striatum is the largest component and the afferent portion of the basal ganglia, which is necessary for voluntary movement control (Hikosaka et al. 2000). A stroke in this region is believed to have major movement disorders. The corpus callosum is a band of white matter tracts which connects the right and left cerebral hemispheres, integrating and transferring sensory, motor and high-level cognitive information between them, thus known to play a crucial role in refining motor movements and cognitive functions (Goldstein et al. 2022). Determining the spatial arrangement of the reactive astrocytes in different brain regions (corpus callosum and striatum) and the temporal profile of their appearance and severity following stroke may be valuable in exploring their use as a therapeutic target. Therefore, we aimed to determine the motor deficits in rat models of ischaemic stroke by temporarily or permanently occluding the common carotid artery and assessing the neuronal changes and the distribution of protoplasmic and fibrous astrocytes in their striatum and corpus callosum respectively.

MATERIALS AND METHODS

Seventy adult Wistar rats were obtained from Central Animal House of the Faculty of Basic Medical Sciences, University of Ibadan. They were given water and feed *ad libitium*. The rats were equally divided into experimental groups as follows: Group 1: (Control); Group 2 (unilateral common carotid artery occlusion for 30 min, sacrificed after 24 h, CCAO-1D); Group 3 (unilateral common carotid artery occlusion for 30 min, sacrificed after 3 days, CCAO-3D); Group 4 (Sham operation but no common carotid artery occlusion, SCCAO); and group 5 (unilateral permanent common carotid artery occlusion, no reperfusion, sacrificed after 3 days, PCCAO)
All the procedures on animal experiments conformed to the animal research: reporting of *in vivo* experiments (ARRIVE) guidelines and were conducted in accordance with the NIH Guide for the Use and Care of Laboratory Animals (NRC 2011)

Induction of Stroke
The rats were anaesthetized with intraperitoneal injection of ketamine/xylazine at 60 mg/kg/ 10 mg/kg, and unilateral global ischaemic stroke was induced. It began by excision of the skin over the cervical region of the rat: An incision of 1-2 cm was made. Thereafter, by careful blunt dissection, the connective tissue in this region was removed to expose the sternocleidomastoid muscle, which was in turn, reflected to reveal the common carotid artery lying in close apposition to the vagus nerve. The artery was subsequently picked and ligated with the aid of 2.0 silk suture for 30 min, and then the suture removed (for reperfusion) in the CCAO-1D and CCAO-3D groups. In the PCCAO group, the artery was permanently ligated (no reperfusion), while the rats with sham operation equally underwent this procedure but the arteries were not ligated (Lee et al. 2014b). The control rats did not undergo any procedure at all. All the rats were monitored for an hour and subsequently returned to their cages.

Neurobehavioural Testing
All the rats were taken through neurobehavioural tests to assess motor function and muscular strength with the pole and square grid tests respectively, as explained below.

Pole Test: This is a test of motor coordination, for striatal and cerebellar function (Matsuura et al. 1997). Rats were held (head-up) against a vertically placed 50 cm long pole (close to the top-most portion of it) that had adhesive paper wrapped round for effective traction. The time (in seconds) taken by each rat to turn head downwards (latency to turn), and descend to the base of the pole (latency to descend) was recorded in five trials (for each rat). A maximum time of 60 sec was allowed for each test.

Square Grid Test: This is a test of muscular strength in the fore and hind limbs in rodents. In this test, the rats were each placed on a square shaped metal mesh surrounded with a wooden handle around it. Once they had gripped the mesh, it was inverted causing the rats to hold on in an upside down position. The amount of time (in seconds) spent by each of the rats before losing grip and falling (latency to fall) unto a cushion of beddings about 1 m below was recorded in three trials for each animal (Mann and Chesselet 2015).

Animal Sacrifice and Dissection
The rats were euthanized 24 h or 3 days post induction of stroke. Each rat was anaesthetized the second time and had transcardial perfusion with 10% neutral buffered formaldehyde, according to the method of Mustapha et al. (2014). Their brains were removed and post-fixed for 48 h in the same solution.

Histology
Coronal sections of paraffin embedded brain sections of 5 µm thickness were mounted on Leica X-tra Adhesive pre-cleaned glass slides, rehydrated and stained with haematoxylin and eosin (to demonstrate brain histoarchitecture), and cover-slipped in distyrene, plasticizer and xylene mixture (DPX, BDH Chemicals Ltd, England). The slides were then examined with a Leica DM 750 light microscope (Leica Microsystems, Heerbrugg, Switzerland) and representative photomicrographs of the corpus callosum and striatum were taken.

Immunohistochemical Staining
Immunohistochemical staining was performed to illustrate the astrocytes following the protocol described by Gilbert et al. (2020). Briefly, paraffinized brain sections of 5 µm thickness on glass slides were heated in an oven at 65ºC for an hour, deparaffinized in xylene, hydrated in decreasing concentrations of ethanol, followed by distilled water and a phosphate buffer (PBS) wash for minutes on a shaker. To achieve antigen retrieval, the slides were immersed in a staining dish containing citric buffer, microwaved for 20 min (4 × 5 min cycles) at 400 W, and subsequently washed with PBS. Endogenous peroxidase was inactivated by incubating the tissue with 3% hydrogen peroxide for 30 min, then primary antibody, anti-glial fibrillary acid protein (rabbit polyclonal anti-GFAP, Dako, 1:500 dilution) was applied to the sections and incubated overnight in a humidified chamber at 4ºC. Secondary HRP-conjugated anti-rabbit antibody diluted in the blocking solution at 1:1,000 was added to each section and incubated for an hour at room temperature (30ºC). Slides were then washed three times with PBS (3 min each on a shaker) and incubated with freshly prepared 3,3'Diaminobenzidine (DAB) substrate. They were thereafter counterstained with haematoxylin, dehy-

drated in ascending concentrations of ethanol, cleared in two changes of xylene and coverslipped with DPX.

Statistical Analysis

Quantitative data from the neurobehavioural tests were expressed as means ± SEM following the D'Agostino-Pearson normality test. The means were compared with the analysis of variance, followed by Tukey's post hoc test, using Graphpad prism version 7.0 (SanDiego, California, USA). Statistical significance was set at $p<0.05$, and the confidence interval calculated at 95% level.

RESULTS

Neurobehavioural Tests

In the pole test, the latencies to turn, and to get to the base were significantly higher in the CCAO group, compared to the control ($p<0.0001$, $F=636.1$; $p<0.0001$, $F=312.1$ respectively). The latencies to turn, and to get to the base were significantly higher in the PCCAO group compared to the CCAO-1D, CCAO-3D and SCCAO groups (Fig. 1A and B). This means that the common carotid occluded groups spent a longer time to orient themselves downwards (latency to turn) as well as to climb down the pole (Latency to get to the base), thus showing an impairment in motor coordination relating to function of the corpus striatum and cerebellum. This impairment was worse in the group with permanent arterial occlusion than in the other common carotid occlusion groups.

In the square grid test, the rats in the CCAO group had a significantly shorter latency to fall than the control ($p<0.0001$, $F=91.29$). The PCCAO group had the shortest latency to fall, which was significant compared to the CCAO-1D, CCAO-3D and SCCAO groups (Fig 1C). This means that the common carotid occluded groups spent a shorter time hanging on the square grid than the controls, thus showing they have reduced muscular strength. This impaired muscular strength was worse in the group with permanent arterial occlusion than in the other common carotid occlusion groups.

Histology

Histological examination of the striatum in the control and SCCAO groups revealed healthy medium spiny neuron cells interspersed with bundles of white matter coursing through it. However, there were a number of shrunken (pyknotic) cells with nuclear condensation and peri-cellular vacuolations in the CCAO groups. This was more pronounced with rats in the CCAO-3D and PCCAO groups (Fig. 2). The corpus callosum of the control and SCCAO groups were well delineated and compact compared to the CCAO group, which the axonal fibres within the corpus callosum were loosely packed (Fig. 3). In the CCAO group, these axonal fibres appeared stretched with fewer cells and enlarged extracellular spaces/ vacuolations compared to the compact appearance in the control.

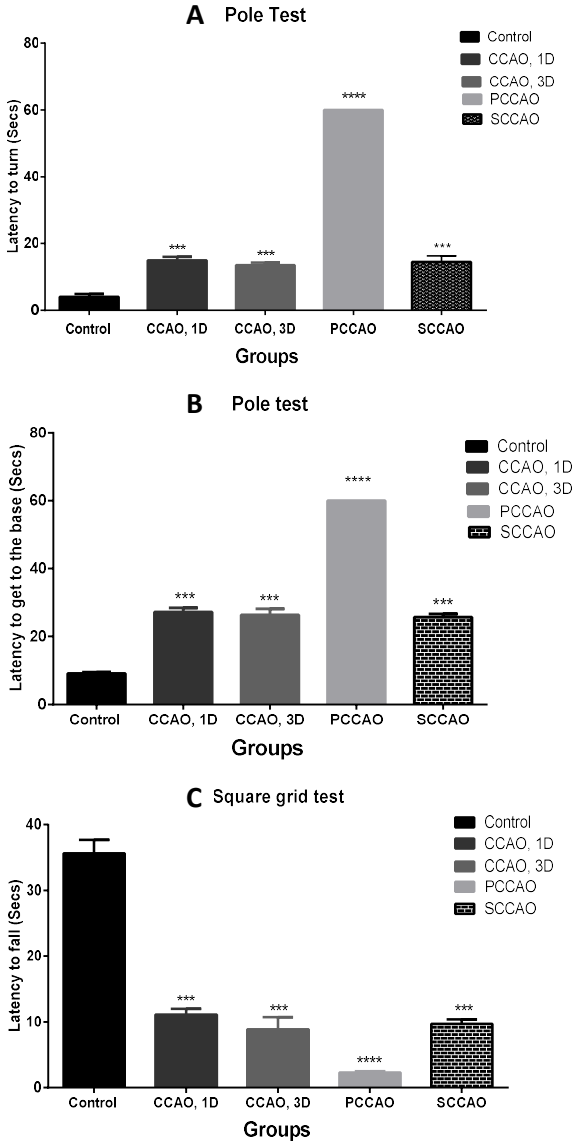

Fig. 1: Neurobehaviour in the pole and square grid tests. The latencies to turn (A), and to get to the base (B) in the pole test; and latency to fall (C) in the square grid test ***p<0.001, ****p<0.0001 compared to control

Glial Fibrillary Acidic Protein (GFAP) Immunohistochemistry

Immunohistochemical reactivity for GFAP was to assess the astrocytes in the striatum and corpus callosum of the rats. The GFAP+ stained cells (astrocytes) in the striatum of control rats had small cell bodies with fine processes. However, sections of the striatum in the temporary CCAO rats revealed reactive astrogliosis observed as astrocytes with enlarged

cell bodies and thickened cell processes, in the rats sacrificed after 24hrs and 3days and the PCCAO rats, with increasing intensity (Fig. 4). Similarly, astrocytes were seen as scattered cells with fine processes in the corpus callosum of the control group, while more prominent astrocytes having bigger cell bodies and hypertrophied processes were observed in the corpus callosum with increasing intensity in the CCAO-1D, CCAO-3D and PCCAO groups (Fig. 5).

DISCUSSION

This study shows that transient unilateral common carotid artery occlusion caused minimal function deficits in motor coordination and muscular strength, as seen in the pole and square grid tests, comparable with sham-operated rats. However, they were more pronounced in rats with permanent occlusion compared to the controls. There were, however, pyknotic neurons with pericellular vacuolations and reactive astrogliosis in the striatum and corpus callosum with graduated intensities in the temporary and permanently occluded rats.

The neurobehavioural tests, i.e., pole and square grid tests were deployed to examine muscular and functional deficits in the forelimbs and hind limbs following brain ischaemia. The functional motor deficits observed in the temporarily occluded groups and then reperfused for 1 or 3 days (CCAO-1D and CCAO-3D) were found to be at the same level as the sham-operated rats, giving the impression that the deficit is actually due to the procedure and not the brain ischaemia. However, there was a definite difference between these groups and the permanently occluded rats, showing that the effect was worse when there was no reperfusion. Similarly reported was no difference in neurological scores between sham-operated rats and those subjected to mild ischaemia (i.e. 30 min of middle cerebral artery occlusion). However, when a more sensitive rotarod test was performed, it showed a difference between them (Zhang et al. 2000). This may explain why no difference was observed between the sham-operated rats and those sacrificed 1 and 3 days post-temporary occlusion.

The pathological features in the CCAO groups, including pyknotic neurons with perineuronal vacuolations, are typical for ischaemic brain injury. Unlike what was found in the functional tests, these pathological features were observed in all the occluded groups but not in the sham-operated, which did not differ from the controls. Previous studies of post-ischaemia have also reported cellular shrinkage and selective neuronal loss when examined 24 h and 14 days post-stroke, respectively (Hughes et al. 2010; Dang et al. 2011). However, direct neuronal loss was not observed since this study was relatively short-termed.

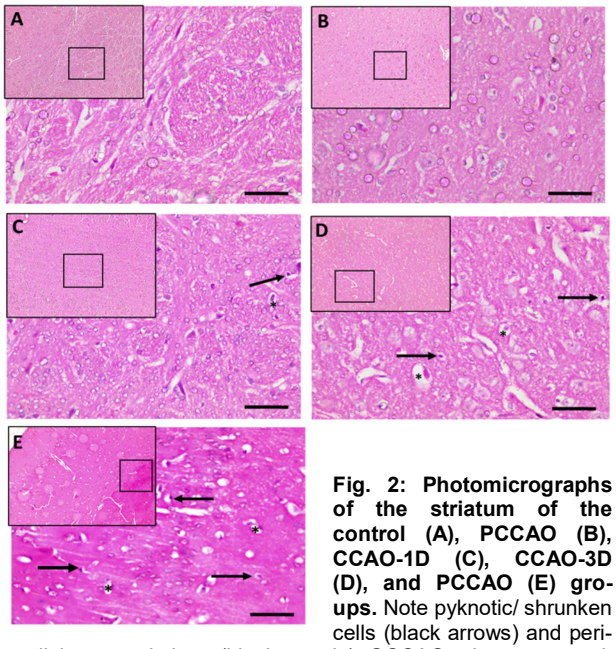

Fig. 2: Photomicrographs of the striatum of the control (A), PCCAO (B), CCAO-1D (C), CCAO-3D (D), and PCCAO (E) groups. Note pyknotic/ shrunken cells (black arrows) and pericellular vacuolations (black asterix). SCCAO- sham operated; CCAO-1D- temporary common carotid artery occlusion and sacrificed after 24 h; CCAO-3D- temporary common carotid artery occlusion and sacrificed after 3 days; PCCAO- permanent common carotid artery occlusion with no reperfusion. ×400; insert: ×100, H&E stain, Scale bar: 25 µm

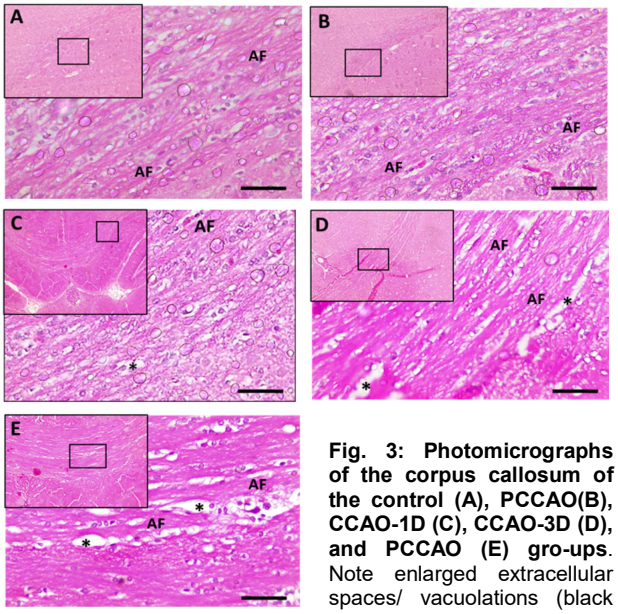

Fig. 3: Photomicrographs of the corpus callosum of the control (A), PCCAO(B), CCAO-1D (C), CCAO-3D (D), and PCCAO (E) gro-ups. Note enlarged extracellular spaces/ vacuolations (black asterix) and axonal fibres (AF) in the corpus callosum. SCCAO- sham operated; CCAO-1D- temporary common carotid artery occlusion and sacrificed after 24 h; CCAO-3D- temporary common carotid artery occlusion and sacrificed after 3 days; PCCAO- permanent common carotid artery occlusion with no reperfusion. ×400; insert: ×100, H&E stain, Scale bar: 25 µm

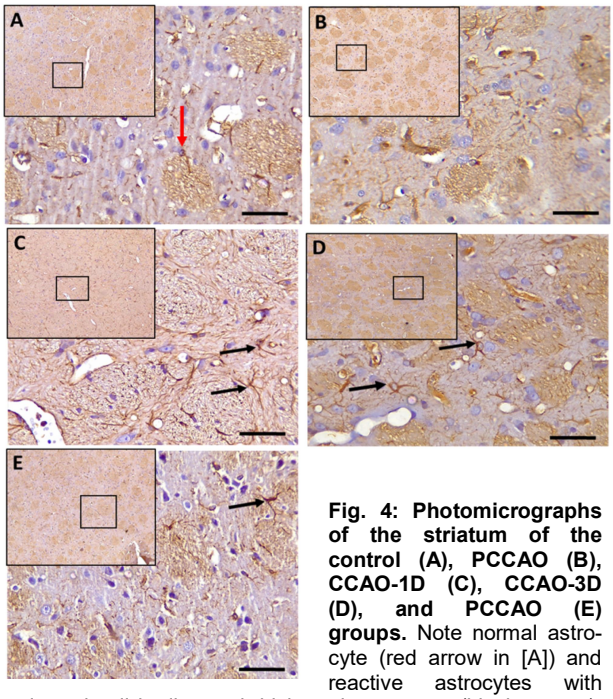

Fig. 4: Photomicrographs of the striatum of the control (A), PCCAO (B), CCAO-1D (C), CCAO-3D (D), and PCCAO (E) groups. Note normal astrocyte (red arrow in [A]) and reactive astrocytes with enlarged cell bodies and thickened processes (black arrows). SCCAO-sham operated; CCAO-1D- temporary common carotid artery occlusion and sacrificed after 24 h; CCAO-3D- temporary common carotid artery occlusion and sacrificed after 3 days; PCCAO- permanent common carotid artery occlusion with no reperfusion. ×400; insert: ×100, GFAP immunostaining, Scale bar: 25 µm

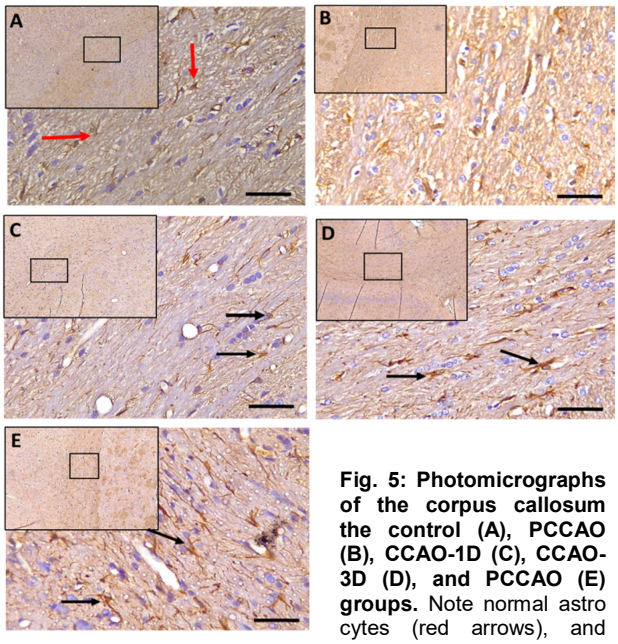

Fig. 5: Photomicrographs of the corpus callosum the control (A), PCCAO (B), CCAO-1D (C), CCAO-3D (D), and PCCAO (E) groups. Note normal astro cytes (red arrows), and reactive astrocytes (black arrows). SCCAO- sham operated; CCAO-1D- temporary common carotid artery occlusion and sacrificed after 24 h; CCAO-3D- temporary common carotid artery occlusion and sacrificed after 3 days; PCCAO- permanent common carotid artery occlusion with no reperfusion. ×400; insert: ×100, GFAP immunostaining, Scale bar: 25 µm

Astrocytes' primary and secondary proximal processes contain GFAP, which emerge from the soma giving fine branches and forming a sponge-like network of ultrathin and connected filaments intermingled with neurons (Papouin et al. 2017). GFAP is an intermediate filament protein (others in this family include nestin and vimentin, which are also upregulated in reactive astrocytes) that is essential for astrogliosis and scar formation but is not essential for normal astrocytic function (Magaki et al. 2018). Astrocytes are noted for converting glucose in the brain into lactate, which is subsequently transported to neurons for energy and Ca^{2+} signalling, which modulates synaptic transmission (Guerra-Gomes et al. 2018). Astrocytes are of significant interest in stroke because they are key players in its pathogenesis and recovery following insults (Moulson et al. 2021). GFAP levels are a novel and complementary biomarker to predict functional outcomes in acute ischaemic stroke, which is proportional to the volume of the damaged brain (Liu and Geng 2018). The haemostatic role of astrocytes, for example, ion transport and blood-brain barrier system, cannot be overemphasized in ischaemic injury. They perform this role by uptake ions through transporters and channels via gap junctions. They thereby respond to the release of neurotransmitters by increasing their intracellular calcium levels and controlling neuronal excitability (Ota et al. 2013). Examination of the GFAP immunoreactivity in the ischaemic rat brains 24 h and three days post-occlusion and in permanently occluded animals revealed reactive astrogliosis, which is in tandem with previous studies of ischaemic insults (Barreto et al. 2011; Li et al. 2014). The astrocytes in the injured brain tissue undergo a process of hypertrophy and, to a lesser extent, proliferation referred to as reactive astrocytosis, thus, leading to increased immmunoreactivity. The glial cytoplasmic processes of the astrocytes create a meshwork around the area of necrosis, forming the "glial scar", a toxic environment for the myelination of neurons. The precise significance of this response to CNS injury is uncertain but likely reflects changes aimed at restoring the composition of the external environment and stimulating reparative processes (Norenberg 2005). It is a known fact that astrocytes are more resistant to anoxic-ischaemic injury and oxygen-glucose deprivation than neurons (Gürer et al. 2009). The proliferation of astrocytes normally occurs after CNS injury and has been reported in ischaemic injuries (Ding 2014). Two different areas of the brain (i.e. corpus callosum and striatum) with different types of astrocytes (i.e. fibrous and protoplasmic) were studied here, and we found a similar reaction to stroke over time. This suggests that despite their differences in function and location, they are similarly affected by ischaemia. GFAP has been implicated in the regulation of blood flow in a stroke model (Brenner 2014), which has been previously stated by Li et al. (2008) and Nawashiro et al. (2000).

It was later observed that transient carotid artery occlusion alone produced a decrease in local cerebral blood flow in the GFAP null mice compared to the wild type and increased the intracranial pressure during reperfusion (Nawashiro et al. 2000); these have been attributed to astrocytic swelling reported during ischaemia (Brenner 2014). It is also possible that astrogliosis observed in the present study could have been due to the alteration between the association of astrocytic endfeet with the vasculature, causing both structural support and astrocytic signalling to be defective. This aligns with increased calcium in astrocytic endfeet being critical for astrocyte-vascular signalling (Carmignoto et al. 2010). Moreover, there could have been a reduction in the ability of the blood-brain barrier to induce permeability in this stroke model (Pekny et al. 1998).

There is evidence that GFAP might be useful as a biofluid-based marker for some neurological conditions (Yang and Wang 2015). A concept also suggests that brain injury causes the release of GFAP from injured astrocytes to the interstitial fluid/extracellular fluid, where they equilibrate into the subarachnoid CSF compartment, then release to the circulating blood by direct venous drainage through the lymphatic pathway (Plog et. al. 2015). The elevated GFAP expression reflects modifications of the functional activity of astrocytes related to damage in the nerve tissue, metabolic abnormalities, and the development of neurodegenerative states. The strength of our study lies in the fact that we have been able to describe both the functional and morphological alterations in the striatum and corpus callosum of transiently and permanent occlusion of the common carotid artery model using a temporal profile.

However, it is important to elucidate the mechanism behind the astrocytic reactions in ischaemic rodent models to advance the search for effective management of this menace that is ravaging the world. In stroke management, there may be a light at the end of the tunnel for the therapeutic potentials around glial cells, such as the astrocytes.

Conclusion

In conclusion, brain ischaemia induced impaired motor coordination and muscular strength, accompanied by neuronal pyknosis, peri-cellular vacuolations and reactive astrogliosis in the striatum and corpus callosum of Wistar rats, and these were more pronounced in the group with permanent arterial occlusion than those that underwent reperfusion. Early reperfusion is crucial for structural and functional recovery following brain ischaemia in stroke management, and astrocytes play important roles in the the pathogenesis and recovery processes.

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Conflict of Interest

None declared.

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Authors' Contribution

TS: Conceptualization, supervision and final approval of manuscript. FO: Supervision, statistical analysis and final draft of manuscript. OFA: Supervision, statistical analysis and first draft of manuscript. OO: Performed the bench work of the study.

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