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SAFE AND ROBUST CEREBRAL ISCHEMIA MODEL: A POSSIBLE WAY TO IMPROVE THERAPEUTIC APPROACH FOR ISCHEMIC STROKE

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Abstract

Cerebral ischemia or Ischemic stroke or is persistent condition as a second leading rate of mortality and disability. Current clinical condition states that there are a few methods of preventive or efficient treatments existing. The aim of the current study is to understand the pathophysiology in order to determine and develop possible treatments that recovers ischemic strokes. Transient global cerebral ischemia is considered to be amongst the interesting pathological stroke conditions due to the degeneration of forebrain and delayed neuronal cell death. **Methods:** Ischemia/Reperfusion was incorporated by Bilateral Common Carotid Artery Occlusion (BACCAO) for 60 minutes followed by reperfusion for 72 hours. Neurological examination was done as per Bederson's et al., after the procedure to confirm the induction of ischemia. **Results:** The results were assessed by histopathological staining such as Hematoxylin & Eosin (H&E) and Cresyl violet which showed Loosely arranged, shrinked cell bodies, increased intercellular spaces, nuclear pyknosis and nuclear fragmentation in motor cortex, striatum and even hippocampus. **Conclusion:** Establishing a mouse model of tGCI through the BCCAO technique has been a general interest in the study field of stroke. Mainly because animals can survive and mortality rate is less compared to other models.

Keywords: tBccao, Cerebral Ischemia, Wistar Rats, Stroke.

INTRODUCTION

Cerebral ischemia/Stroke is a basic neurological deficiency that features to the vascular injury that is believed to be due to the infarction of central nervous system. Ischemic/stroke is the most detected case of disability worldwide. [1] Ischemic stroke occurs when there is thrombotic or embolic event takes place that causes decrease in the blood flow to the brain. Blood flow to the brain gets obstructed within the blood vessel as there happens a dysfunction which generally occurs due to the secondary to atherosclerotic disease, arterial dissection, fibromuscular dysplasia, or inflammatory condition. [2,3] Cerebral circulation needs to be normal and the neurological and psychiatric illnesses are mainly due to the disorders in the cerebral circulation. An unexpected interruption of the blood flow to distinct brain regions may lead to stroke, while a gradual reduction of continuous cerebral blood flow (CBF) weakens the memory processes and pays to the development of dementia. [4]

Preclinical study attempts on neurological conditions scopes to advance the application of favourable results towards the experimentation on clinical studies. However, there are various obstacles in the form of the consistencies in the study

results when using animals and humans. Majority of results with the use of animal models in various cases does not support neuroprotection medicine research in humans. [5]

Stroke incidences has reduced with the mortality rates significantly with the modern specialized treatments and has also provided improvements in the functional outcome of stroke victims. However, the advancement in treatments are not to the level that can be considered to counter the deleterious effects of cerebral ischemia. The present improved form of treatment is thrombolysis with tissue plasminogen activator (tPA). However, thrombolytic tissue plasminogen activator (tPA) is used to treat acute ischemic stroke.

Enormous research is underway to discover alternative treatment options for various medical conditions that otherwise has limited scope with routine treatment methods. Neurological disorders are of major focus since the present treatment options are having limited effect over the condition and thus requires newer approaches. Major concerns over neurological condition requires thorough understanding of the clinical condition and thus researchers are in mainly involved in generating effective preclinical models using variety of species. Presently, there are two rodent models that were developed consisting of either (1) the global cerebral ischemia or focal cerebral ischemia that simulates the clinical condition for up to 80% with thrombotic or embolic stroke that generally occurs in the Middle Cerebral Artery (MCA); or the one that occurs in the intraluminal middle cerebral artery occlusion (MCAO). Both these models are widely accepted and are believed to be relevant preclinical models to support drug discoveries for stroke. MCAO model was first reported by Koizumi et al., in 1986 which was widely accepted to be simple to operate that has enormous success rate. [6]

Two-vessel occlusion (2-VO), also known as transient or permanent, bilateral common carotid artery occlusion, is another model that is most widely used to investigate the mechanisms of neurodegenerative processes. In this paper, we present the surgical procedure for tBCCAO in rats.

It is thus vital to develop most appropriate animal models to mimic the actual clinical scenario for the development of successful treatments. Transient global cerebral ischemia is one of the most interesting pathological conditions in stroke studies because of the observed degeneration of forebrain and delayed neuronal cell death in selective vulnerable regions such as motor cortex, hippocampus, etc.

MATERIALS & METHOD:

Experimental Animals

Wister male rats were used with an age about 10–12 weeks and a weight of 280–320 grams. Rats were fed a commercial rat pellet diet provided by Mass Biotech, Chennai, and were provided with water ad libitum. They were kept at a natural light-and-dark cycle under a temperature of 20–25°C and a humidity of 50 to 60%. 14 animals were divided into 2 groups. They are G1 (Normal-positive control), and G2 (ischemia induced group- negative control). The effect of tBCCAO was examined by using H & E & cresyl violet. The apoptosis is validated by seeing the expression of BAX protein. It was used to understand the changes that occurred in the brain infarct. The mRNA levels of BAX proteins are analyzed by RT-PCR [7]

Ethical approval:

After receiving approval from the Institutional Animal Ethical Committee at Saveetha Dental College, Chennai, India (BRULAC/SDCH/SIMATS/IAEC/08-2022/138), the experiment was carried out. Rat models were subjected to induced stroke (tBCCAO method).

Surgical procedure:

BCCAO Model

The stroke induction was done by transient bilateral common carotid artery occlusion (tBCCAO) model (Figure 1). Rats were anesthetized by injection of 0.3 ml of ketamine and xylazine, followed by the crown of the head shaved and cleaned with 70% alcohol and continuously monitored for spontaneous breathing. Under a stereo microscope (Stemi 2000, Zeiss), a blunt dissection is performed to expose the trachea and retract the muscles to locate the carotid artery. 5. The left common carotid artery (CCA) is carefully dissected from surrounding tissue and the CCA is temporarily occluded by a temporary suture (1) using 3-0 silk suture cut into 20 mm segments. Great care should be exercised not to harm the vagal nerve. The skin incision was done on the ventral aspect of the neck, both the carotid arteries were visualized and occluded by ligating. It was left for 60 minutes. Under aseptic conditions, the incision was sutured, and the animals were placed in a dry cage separately with the assistance of a heat pad. Temperature was monitored periodically [8]. (Figure1)

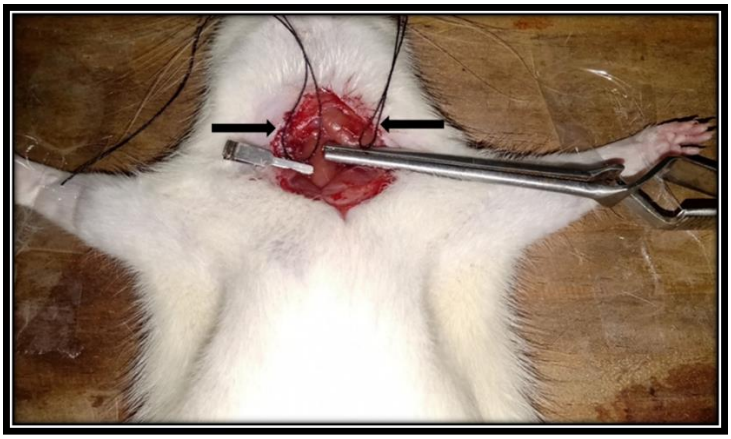

Figure 1 : shows induced tBCCAO

Neurological examination:

Neurological assessment was carried out as discussed by Bederson *et al.* [9] To determine the degree of severity of the injury. Deficiency in the neurological condition was assessed based on scoring as described by Bederson et al., at at 1, 24, 48 and 72 hr post-reperfusion. Efficiency was assessed using six-point scale as described in the table 1 below.(Figure 2 A&B)

Table 1: Neurological Assessment scale, Bederson et al.

Scale	Efficiency condition
0	Normal
1	Mild circling behaviour with or without inconsistent rotation when picked up by the tail, 50% attempts to rotate to the contralateral side
2	Mild consistent circling, >50% attempts to rotate to the contralateral side
3	Consistent strong and immediate circling, the mouse holds a rotation position for more than 1-2 seconds, with its nose almost reaching its tail
4	Severe rotation progressing
5	Comatose or moribund

Figure 1A) Shows tail hanging test 1b) shows cyclic behaviour of stroke induced rat

The brain samples were irrigated with Phosphate Buffered Solution (PBS) and then frozen at -80°C with isopentane. Alternatively, 4% paraformaldehyde can also be used to perfuse the specimens based on the staining that is to be conducted. Coronal brain sections of 17 µm were done using cryostat-cuts a portion of the section was used to obtain a representative slide for cerebral injury.

Other portions were used to perform staining procedures using Cresyl Violet and Immunohistochemistry. Cresyl Violet stained slides were assessed with Image J Software to evaluate the lesions [10-12]. The output was expressed as percentage of injury (contralateral non-lesioned area] based on the injury volume calculated as pixels. The specimens stored using 4% paraformaldehyde was dissected at 30-50 µm using vibratome and the volume of infarction was measured as described by Han et al. (2009) [13]

Behavioral analysis:

Behavioural analysis was done for motor and sensory impairments**.** Beam walking assay was performed in all animals following specified guidelines to study their balance and locomotion, and adhesive tape removal test was done to evaluate the sensory deficit. [14]

RESULTS

The neuroscore evaluation confirms the success of tBCCAO post-stroke and determines the efficiency of the tBCCAO post-reperfusion. In our study, all rats subjected to the tBCCAo procedure presented at least a moderate consistent circling (neuroscore 2) and the neurological deficits are generally stable up to 72 hr.

Behavioural analysis:

Behavioural analysis showed remarkable difference between normal and stroke induced animal. The stroke induced animals were taken more time to cross the beam, and in adhesive tape removal test as well than the normal rat. The infarct tissue was located with H&E and cresyl violet staining method which showed the presence of infarct in motor cortex, striatum, hippocampus, supra ventricular zone etc. Infarct tissue was showing Loosely arranged, shrinkage of cell body, increased intercellular space, nuclear pyknosis and nuclear fragmentation in motor cortex, striatum and even hippocampus. (Figure-2a & 2b)

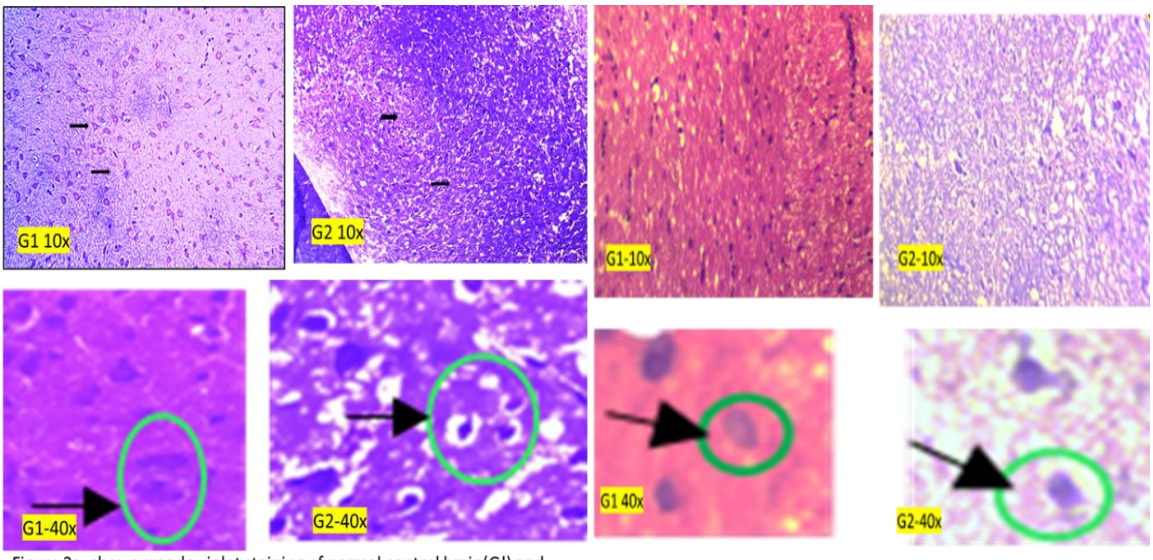

Figure 2a, shows cresyle violet staining of normal control brain(G!) and ischemia induced brain (G2) in 10x & 45x respectively. The black arrow shows the comparison of viable and non viable neurons in G1 & G2.

Figure 2b, shows H & E staining of normal control brain(G!) and ischemia induced brain (G2) in 10x & 45x respectively. The black arrow shows the comparison of viable and non viable neurons in G1 & G2.

DISCUSSION

The current study was designed to analyze the efficacy of Transient BCCAO (tBCCAO) ligation technique could cause cerebral ischemia. The underlying mechanism of this method is reduction/hypoperfusion of blood to the brain tissue leads to formation of infarct in the brain. Alterations of cell population happen in the central nervous system during the pathogenesis of Cerebral ischemia/ ischemic stroke. Morphological changes are quite common in the neurons whereas in the case of axons and cell bodies, they get disintegrated in the system. Disappearance of nucleolus can be seen when the Glial cells and neurons undergo cytoplasmic swelling. Significant changes are appreciable in penumbra with the ischemic neurons showing changes in the Nissl's bodies' disintegration and endoplasmic reticulum. One or the other morphological difference is displayed in the other cells such as glial cells, astrocytes and microglia. [15,16]

Barbhuiya et al. (2015) argued that rats striatum damage could be induced by tBCCAO with 15 minutes ligation and 4320 minutes reperfusion. However, the study was not successful in measuring the ischemic volume of the rats' brain. The fresh brain sections stained with TTC were examined for observing the induction intensity of global ischemia. Damage to the Hippocampus occurred after tBCCAO. Handayani et al. (2018) reported that the damage to hippocampus occurred in rats that underwent ischemia induction for at least five minutes and reperfusion happened post 24 hours of induction. TTC staining confirmed the ischemic areas in the hippocampus. Furthermore, hippocampus damage in 494 Transient Bilateral Common Carotid Artery Occlusion (tBCCAO) of Rats as a Model of Global Cerebral Ischemia rats were induced with 5 minutes ligation and 168 hours reperfusion. Iwasaki et al (1995) investigated the effects of ischemic/reperfusion injury on the number of CA1 hippocampus neurons of different rat strains. [17]. In this present study hypoperfusion (tBCCAO) was done for 60 minutes followed by 72 hrs of reperfusion which showed infarct formation in motor cortex, hippocampus, subventricular zone, striatum etc. But the mortality rate was less compare to MCAO models reported by other researchers.

TTC staining is one of the widely used method to macroscopically evaluate ischemia. It is a simple technique that can be completed in less than 60 minutes without using microscopic observations. The principle of this staining is based on the nature of TTC which is easily oxidized by the mitochondrial dehydrogenase enzyme (Lactate dehydrogenase / LDH). This oxidation produces formazan compounds. [6]

In Cerebral ischemia, the energy deficiency is a known reason to cause various changes such as the mitochondrial dysfunction and the damage caused during oxidative stress. Neuronal cell depolarization and glutamate release is triggered due to oxygen and glucose deficiency [18]. Certain signalling pathways that get triggered in ischemic stroke includes: Phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway wherein synaptic stimulation results in NMDARs that activates the pro-survival PI3K/Akt signalling pathway, exerting a neuroprotective effect. NMDAR activation at the synaptic junction and Ca2+ influx activates the RAS/extracellular signal-regulated kinase (ERK) signalling pathway and nuclear Ca2+/ calmodulin dependent protein kinases, which subsequently activates and phosphorylates CREB. [18-20] The evaluation of BCCAO model was done by using Cresyl Violet staining (Figure 2a) and Haematoxylin & Eosin(2b). Lesions were relatively consistent as displayed by the staining techniques. However, around 30% of rats showed injuries prominent in the hippocampus. It is noteworthy that the stored specimens can be used even after 7 days to stain using cresyl violet. Though studies have shown some inconsistencies in the brain infarcts, it is common due to the variabilities in the study design that is dependent on the monofilaments used, the anaesthesia, the thickness of brain sections, the choice of mouse/rat strain, or the staining used [6].

Cresyl Violet (CV) staining depends on the way that from the beginning in a space of localized necrosis under light microscopy, the earliest neuronal modification after ischemia is miniature vacuolation of the cytoplasm. This correlates with the findings of swollen mitochondria, an increase in the density of ribosomes and the cytoplasmic matrix, dilatation of the endoplasmic reticulum, and swollen astrocytic processes that surrounds the damaged neurons. CV stains the cytoplasm of healthy neurons to blue and the infarct regions of the segment contain less flawless cells than the typical cerebrum. In a normal area of the brain, the CV staining is dark blue, while in the infarct area, it is light blue [21].

CONCLUSION

The result of current study shows that evaluation of ischemia induced by transient cerebral hypoperfusion and reperfusion. Most researchers concur that the model's cerebral hypoperfusion is moderate, and that the ensuing neuronal damage is comparatively less. The absence of experimental data might be explained by the fact that failed attempts have not been reported or because the need for such data has not been very high, but the potential relevance of oxidative stress and infarct tissue formation in tBCCAO -induced model for human cerebral hypoperfusion makes it a desirable area of study. In general, cerebral hypoperfusion's causal involvement in neurodegenerative illnesses has become clearer thanks to the BCCAO rat model. Because mortality rate is also very less. Lastly, the tBCCAO model has now been thoroughly described and appears to be quite appropriate for evaluating potential of any neuroprotective and neuro restorative drug in cerebral ischemia or stroke. medicines.

Use of AI Tools Declaration

The authors declare they have not used artificial intelligence tools in the creation of this article.

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Conflicts of Interest

The authors of this manuscript declare that there are no financial interests associated with this publication and disclose no conflicts of interest exist.

Author's Contributions

Suganitha B: Conceptualization, methodology, study preparation and conducting, original draft preparation.

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