**IJPSR (2022), Volume 13, Issue 4** **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 June 2021; received in revised form, 03 September 2021; accepted, 06 September 2021; published 01 April 2022

# NEW INSIGHTS ON *IN-VIVO* AND *IN-VITRO* ANIMAL MODELS OF CEREBRAL REPERFUSION INJURY

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**Keywords:**

Cerebral reperfusion injury, *In-vivo* models, *In-vitro* models

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**ABSTRACT:** Re-establishing blood flow in ischemia is essential for rescuing cells from necrosis in any tissue or organ. However, reperfusion post cerebral ischemia may worsen the condition and lead to "cerebral reperfusion injury". In cerebral reperfusion injury, significant changes observed are infarct size, behavioural deficits, hematoma formation, inflammatory mediators, and oxidative stress markers that represent the extent of brain injury. Experimental *in-vivo* and *in-vitro* models mimicking the necessary neurological and pathological process are vital in the research of pathogenesis of cerebral reperfusion injury and the development of credible therapeutic drugs. This review explains the currently used *in-vivo* as well as *in-vitro* models to study cerebral reperfusion injury ranging from animal-based models like middle cerebral artery occlusion model, emboli stroke model, two-vessel occlusion model, four-vessel occlusion model, photochemical stroke model, collagenase induced brain haemorrhage model, autologous whole blood induced haemorrhage model to cell lines. Also, advanced technology likes the synthetic microvasculature model has been discussed. This review also provides contemplative facts to set up authentic and relevant animal models to study cerebral reperfusion injury.

**INTRODUCTION:** Restoration of blood supply or "Reperfusion" is the main aim in the treatment of stroke. Naturally, the process of reperfusion is initiated in around 50-70 % of ischemic stroke patients. Though oxygen rehabilitation has advantageous effect post-reperfusion. However, rapid reperfusion causes harmful effects on brain physiology called "reperfusion injury" [1]. Thrombolysis and embolectomy were considered essential contributors to reperfusion injury [2]. Stroke is the chief cause of morbidity and mortality in humans. In western countries, around 500-800 people for every 1 lac population, whereas in India, 13-33 people per one lac population suffer from stroke every year [3].

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DOI: 10.13040/IJPSR.0975-8232.13(4).1411-27

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(4).1411-27

Worldwide approximately 20.5 million suffer from stroke every year, and about 5.5 million are proven to be fatal. It is estimated that approximately $ 40 billion in the USA is the annual economic impact on stroke. Epidemiological studies reveal stroke as third foremost basis of death in developed

countries. Infarcts are the main reason for 60-80% deaths; parenchymatous haemorrhages affect 5-10% and subarachnoid haemorrhages produce approximately 5-10% of deaths [4]. The mechanisms of reperfusion injury include activation and aggregation of platelets, infiltration of leukocytes, disruption of blood-brain-barrier (BBB), mitochondrial mechanisms and oxidative stress that leads to edema formation produces neurological dysfunctions and neuronal death [1]. Currently, numerous experimental *in-vivo* and *in-vitro* models are used in the research of cerebral reperfusion injury as well as to evaluate effective therapies.

This review was discussed in detail *in-vivo* models like the middle cerebral artery occlusion model, emboli stroke model, global ischemic models, haemorrhagic models, photochemical stroke model. Additionally, *in-vitro* models like the oxygen-glucose deprivation model, chemical hypoxia model, excite toxicity model, and synthetic microvascular model of the blood-brain barrier are also discussed. Although several reviews and book chapters have been published on experimental models of cerebral reperfusion injury, the center point of this review is to give a complete description of currently used models for studying cerebral reperfusion injury along with merits, limitations, and factors that can influence the final result.

## *In-vivo* Rodent Models of Cerebral Ischemia:

**Middle Cerebral Artery Occlusion (MCAO):** This model was first used by Kozuimi *et al.* and is the most widely accepted model that mimics a human stroke [5]. It was further revised with the use of a silicone-coated suture technique to control the premature reperfusion and subarachnoid haemorrhage [6, 7]. MCAO technique splits the external carotid artery and briefly closes the common carotid artery using the thread knot. Then, into the shallow carotid artery trunk, a suture is passed into the internal carotid artery until it reaches the anterior and middle cerebral arteries division. The suture is left at the intersection for a stipulated time and then withdrawn to allow reperfusion. The expected duration of suture occlusion would be 60 min, 90 min, 120 min, and permanent occlusion. The lodging length of the suture can alter infarct size, and this depends upon the age and strain of rats. Suture installed at 15-16 mm of CCA can occlude anterior choroidal and hypothalamic arteries and form small, cortical infracts. Whereas sutures coated with poly-l-lysine or silicone cling firmly with vascular endothelium and form larger infracts. MRI-guided MCAO technique helps in imagining the filament position, haemorrhagic complications and blood flow in intracranial vessels; this could help to improve MCAO modeling.

MCAO primarily affects the region of cortex, striatum, thalamus, and hypothalamus and extends to the hippocampus region. Reports also suggest that MCAO for 30 min restricts the ischemic effect to the striatum region and slowly extends to the surrounding penumbra, increasing the duration of occlusion [8]. The release of inflammatory cytokines worsens the condition of ischemic injury by stimulating stress kinases like P38-MAPK and JNK that trigger mitochondrial apoptotic pathways [9]. Post-ischemic injury activated astrocytes, microglia and neurons cells stimulate large production of cytokines [10]. Cerebral ischemia includes the rise of tumour necrosis factor TNF-α, pro-inflammatory cytokines interleukins IL6, IL-1β leading to brain injury [11].

The main advantage of this model is high reproducibility-a delayed process of cell death in the cortex region resembles close to human stroke. Oxidative injury and inflammatory mediators are the prime targets for studying neuroprotective agents [12]. Histopathological assessment can help study cerebral blood flow patterns during ischemia. As the mortality rate of animals in this model is almost negligible, there would be sufficient data available to conduct statistical analysis and to investigate the changes after focal cerebral ischemia [12]. Limitations with this model are visual confirmation of MCAO cannot be achieved in this model. A specialized technique like MRI guided MCAO method should be used for visual confirmation. Filament dimensions majorly affect reproducibility, and the use of filament has the risk of haemorrhage. The mortality rate is increased with broad strokes of more than 24 h [12]. Jianping Wang *et al.* studied the effect of teriflunomide on brain oedema, infarct volume, neurologic deficits, blood brain barrier permeability neuroinflammation and neurogenesis in mice using MCAO method. Treatment with teriflunomide lowered brain

oedema condition on the 3rd day, reduced infarct volume and showed insignificant neurological deficits on 7th day when compared with vehicle treatment. Teriflunomide improved expression of achaete-scute homolog 1 (Mash1) and increased the number of 5-bromo20-deoxyuridine (BrdU)/ doublecortin (DCX)-positive cells on 7th day of the stroke. Teriflunomide is effective in protecting the disruption of BBB, enhancing neurogenesis and inhibiting neuroinflammation [13]. Kent *et al.* found that treatment with novel carbon nanoparticles in reversible middle cerebral artery occlusion model reduced haemorrhagic score, hemisphere distension, infarct size and improved bederson's score [14]. Qiaoshu Wang *et al.*, from their studies, found that treatment with fluoxetine increased expression of protein HIF-1α expression, VEGF its receptor VEGFR and netrin, and its receptor DDC. Neurological deficits were improved after 4 weeks of treatment. Fluoxetine promotes angiogenesis and recovers from long-term functional deficits after ischemic stroke [15].

Kurozumi K *et al.* reported that mesenchymal stem cells improved functional deficits in MCAO induced stroke model. This activity is attributed to mesenchymal stem cells as they secrete cytokines. Studies confirm the role of the BDNF gene (brain-derived neurotrophic factor) and GDNF gene (glial cell line-derived neurotrophic factor) transfected along with mesenchymal stem cells, improving functional abnormalities by lessening ischemic brain cell damage. Studies reveal that gene-modified cell therapy is the new therapeutic approach in the treatment of ischemic stroke 16. Jun yang *et al.* found that treatment with berberine exerts neuroprotective activity by increasing BDNF, TrkB, and p-Akt expression and also turn down the apoptosis-related proteins after MCAO [17].

**Embolic Stroke Model:** In this model, the rodents will be anesthetized using a subcutaneous injection of a combination of fentanyl (1 mg/kg) and fluanison (3 mg/kg) followed by subcutaneous injection of atropine (0.015 mg) and intraperitoneal injection of diazepam (2.5 mg/kg). If necessary, anaesthesia is continued using fentanyl and fluanison. The femoral artery is catheterized to monitor the blood pressure and collect blood samples to estimate glucose and gases levels. The femoral vein is cannulated for drug administration. Throughout surgical procedure and for the first 30 min after reversal of anesthesia body temperature of the animals need to be maintained 37 ºC by using a heating lamp [18]. A 1 ml syringe has to be filled with thrombin 60 U/ml saline. Once the femoral arterial catheter is fixed required amount of blood needs to be drawn in another syringe. Within 20 s, syringes should be connected using a polyethylene tube, and the suspension containing thrombin solution and blood in a mixture of 1:4 was moved 70 times from one syringe to the other for 3 min.

Then the syringes should be left in a standing position with closed compartments for 30 min until embolization. The external carotid artery (ECA) and thyroid, occipital, and pterygopalatine arteries are ligated. The catheter has to be inserted at the bifurcation and fixed with a ligature. Throughout the procedure, blood flow within the internal carotid artery (ICA) has to be maintained with the normal flow to avoid any injury to the intima. Heparinized saline (5 U/ml) should be continuously flown in the catheter at 0.5 ml/h using an infusion pump to avoid clotting. Thrombus insertion and resultant cell damage can be established by neurological examination, histological changes, and measuring cerebral blood flow (CBF). An eloquent decrease in CBF can be determined after 2 h post thrombin injection. The reduction of CBF after the establishment of thrombus is less when compared to the suture model of MCAO.

A compatible link exists between infarct volume and CBF [18]. Within an hour after thrombin injection, microvessels of the cortex are opened, followed by the opening of cortical vessels after 3 h and by 24 h striatal vessel is cleared. This may be due to thrombolysis. Stability and composition are two essential components of embolic clots that can influence clot dissolution. Recanalization of occluded vessels and dissolution of clots depends upon the concentration of erythrocytes and thrombin in the clots. Fibrinolysis system is less effective in rodents when compared to recombinant tissue plasminogen activator (rtPA) in humans and to overcome this. There is a need for the use of higher doses of rtPA to produce effective thrombolysis in rats. Studies confirm that rtPA administered at 0.9 mg/kg is useful in studying preclinical ischemic diseases [18]. This model has

several returns over other models. As it can produce large vessel thrombosis resembling embolic stroke in humans. Fibrin-containing thrombus is susceptible to thrombolysis by rtPA. This model is highly reproducible and produces anticipated infarct volume within the specified region. Diffusion-weighted imaging technique (DWI), and perfusion weighted imaging technique (PWI) are the advanced techniques employed in observing the development of thrombus and thrombolysis. Accordingly, this model has few limitations as the introduction of emboli intravascular results in multifocal ischemia, and the infarcts formed are of variable size. The high mortality rate is observed in this model; the possible reason would be a brain haemorrhage. Therefore, this model approves a quantitative study of the safety and efficacy of antithrombotic agents.

Wusheng Zhu *et al.* studied the contribution of Nod-like receptor protein 3 (NLRP3), a component of the immune system in the delayed recombinant tissue plasminogen activator (rtPA) induced haemorrhagic transformation and its contribution to neutrophil employment. Delayed rtPA treatment at 4 h post-ischemia activated the expression of NLRP3 contributes in disruption of BBB components, and leads to haemorrhagic transformation. Blocking NLRP3 expression significantly prevented BBB disruption and haemorrhagic transformation. It also improved neurological functions and reduced neutrophil recruitment. Rabbit anti-rat neutrophil serum, like NLRP3 shRNA, reduced hemorrhage score and hemorrhage volumes after rtPA treatment [19].

Rong Jin *et al.* studied the combined effect of AS605240 with a low dose of tPA after ischemic stroke. The studies found that combined therapy of AS605240 and 5 mg/kg tPA at 4 h significantly reduced infarct volume and effectively reduced neurological deficits after 24 h. results suggest that AS605240 might inhibit PI3Kγ and could be a new approach for improving the thrombolytic activity of rtPA in stroke treatment [20]. Guang-xia Ni *et al.* studied the effect of acupuncture in ranging therapeutic window time of rtPA in stroke. Combined treatment of acupuncture and rtPA reduced infarct volume, hemorrhagic transformation, and improved neurological activity. This protective is the result of blocking inhibition of ERK1/2 signal pathway and condensed MMP9 expression [21].

**Global Cerebral Ischemia Model:**

**Two-Vessel Occlusion Model:** In this model, reversible forebrain ischemia will be produced by sealing the carotid artery along with inducing hypotension to decrease the flow of blood to the forebrain region. Blood pressure should accustom to 50 mm Hg by using a specific device [22]. Treatment with phentolamine or trimethaphan can be helpful to produce hypotension [23]. Cerebral blood flow (CBF) measured after 15 min shows that there is a reduced flow to the region of the cerebral cortex, caudoputamen, hippocampus, midbrain, thalamus, and globus pallidus.

Máté Marosi *et al.* studied the effect of oxaloacetate on impaired long-term potentiation induced using a two-vessel occlusion ischemic model in rats. Administration of oxaloacetic acid within 30 min of reperfusion effectively prevented long-term potentiation impairment caused by ischemia. This effect of oxaloacetic acid is because of its blood glutamate scavenging property. Oxaloacetic acid also effectively improves neurological condition after ischemic induced mitochondrial dysfunction [24]. However, there are variable differences in the CBF in the ischemic region. Cerebral reperfusion injury induces neurobehavioral deterioration in experimental animals. Studies suggest that reperfusion injury and global cerebral ischemia have similar behavioural alterations and very close clinical signs in stroke patients [25].

Experimental animals subjected to cerebral reperfusion injury showed eloquent damage to cognitive abilities and motor coordination. Reperfusion injury depletes glutathione and anti-oxidants enzymes in the brain's hippocampal, striatum, and cortical regions. Depletion of energy and oxygen triggers oxidative stress in the brain. Auto oxidation of xanthine oxidase and NADPH oxidases, neurotransmitters, and mitochondrial respiratory chain generate several oxygen free radicals and their derivatives [25]. Wen-li *et al.* studies revealed that etidronate, a potential neuroprotective agent, restored working memory deficits. This effect was attributed to its ability to inhibit oxidative stress and increase synaptic

neurotransmission [26]. Olubukola Benedicta Ojo *et al.* reported post-treatment with kolaviron scaled-down enhanced anti-oxidant enzymes in hippocampal, cortex, striatum, and weakened pro-oxidants that enfeeblements post-ischemic oxidative stress [27]. Reperfusion injury increases brain water content due to the increase of inflammatory mediators like MPO and NO and ionic place leading edema in the site on an injury. The two-vessel occlusion model helps in understanding changes in the selective structures like pyramidal neurons of the hippocampus, neocortex, and caudoputamen. Histopathological reports from studies confirm that the two-vessel occlusion model is associated with neuronal injury to hippocampal pyramidal cells within 2 min.

In contrast, injury to caudoputamen happens with 8 min of ischemia and injury to the neocortex takes place in 4 min of ischemia [28]. Zhanyong Li *et al.* found that exogenous $H_2S$ undoubtedly improved memory deficits and spatial learning induced by brain ischemia by inhibiting edema around pyramidal neurons and boost the expression of growth-associated protein -43 (GAP-43) in hippocampal region of the brain. Reactive oxygen trigger neuroinflammation that articulates expression of interferon regulator factor 1, STAT3, NF-κB and hypoxia-inducible factor -127. Hypoxia-inducible factor-1α (HIF-1α) is reported to be protective in focal ischemia. Dachun Zhou *et al.* reported that 2-methoxyestradiol, a natural metabolite obtained from estrogen and inhibitor of HIF-1α, did significantly inhibit the levels of hypoxia-inducible factor-1α but did not produce positive results

in the treatment but rather worsened the condition [29]. Homeostasis dysfunction disrupts ionic pumps like Na + -K + -ATPase. Na + -K + ionic pumpimpaired function leads to increased intracellular Na+ concentration galvanized by a strong electrochemical gradient. Douglas E. McBean *et al.* reported that lifarizine, a derivative of diphenyl piperazine, selectively blocks the inactivated sodium channels and decreases the overactivity of the ischemic neuron [30]. Orsu Prabhakar *et al.* studied the protective effect of naringin in ischemic reperfusion using a two-vessel occlusion model. The study found that increased levels of inflammatory mediators and infiltrating leukocytes play an important role in reperfusion injury. Treatment with naringin reduced infiltrating leukocytes, inflammatory mediators like Myeloperoxidase, Tumour necrosis factor-α, IL-6, and increased IL-10 Interleukins [31]. Orsu Prabhakar *et al.* found that resveratrol reduced inflammatory mediators and oxidative stress markers like Myeloperoxidase, Tumour necrosis factor-α, IL-6, malondialdehyde and increased levels of anti-inflammatory and anti-oxidants parameters like IL-10 (Interleukins), superoxide dismutase, and catalase [32]. The two-vessel occlusion model helps study energy metabolism in ischemia, neurotransmitter metabolism, phospholipids, histopathology, and the effects of cerebral hypothermia [33]. This model helps study long-term recovery studies as it reverses the condition of ischemia [34]. This model is highly reproducible as it produces more than 90 % of ischemic damage that almost mimics ischemic stroke in humans [35].

Two vessel occlusion model helps in producing consistent brain injury. The regions such as the CA1 region, caudate-putamen, hippocampus, and the neocortex area are the locations of brain injury in rats. This model will help achieve a good outcome when the blood pressure is regulated correctly at a level of 50 mm Hg without any fluctuations. It also helps in inducing efficient forebrain ischemia, ease of cerebral recirculation, low experiment failure rate. The limitations associated with two-vessel occlusion are the use of anaesthesia and induction of hypotension; the use of these can affect the resulting outcome. In this model, behavioural changes cannot be assessed immediately after occlusion. The time frame would be around 10 min beyond which permanent brain damage occurs. A high mortality rate is observed in this model as maximum animals would die just after 15 min of completion of the procedure. In this model, few experimental animals may develop post-ischemic seizures, which may be the reason for their death.

**The Four-vessel Occlusion Model:** This model is widely used in producing forebrain ischemia with significant, reproducible neuropathological results. The four-vessel occlusion method is followed in two stages. In the first stage, rats are anesthetized, and the arterial clasp is fixed around the carotid artery and objectified by an incision at the ventral

midline of the neck [36]. Alar foramina of the first cervical vertebrae are identified by dorsal incision. An electrocautery needle is passed through foramina to electro coagulate vertebral arteries [37]. In the recent studies, slight technical modification is recommended that the rat head should be held with a stereotactic ear bar and tilted around 30 o horizontal and spine should be extended using tension on the rat tail so that the alar foramina are brought to the plane and helps in improving visualization. In the second stage, forebrain ischemia is produced after 24 h of the first stage by tightening the carotid clasps for a moment, and later these clasps are removed for reperfusion. Dan Lu *et al.* found that the survival rate of experimental animals depends on the duration of ischemia. Their study found that ischemic for 10 min, 20 min, and 30 min reduced the survival rates of animals by 93%, 90%, and 70%, respectively. It was also found that animals subjected to ischemia above 20 min show seizure activity [37].

Laboratories have reported variability among different strains of rats that show different grades of ischemic effect. Sprague- Dawley rats do show respiratory failure. Around 50-60% of animals exhibit coma that helps us to understand the extent of the ischemic effect in the strain. Whereas, in wistar rats, animals suffer respiratory failure after the first stage of the procedure, and around two-third of animals die during the interval of the first and second stages. Histopathological studies confirm that 10-20 min of ischemia would be sufficient to produce ischemic cell changes in the region of the hemispheres and hippocampus. Four vessel occlusion models do not bring significant changes in the neocortical region, even with 30 min of ischemia. Behavioural changes observed in this model confirm that there would be permanent damage to the memory of rats, which reflects the hippocampal injury [38].

Several complex processes like oxidative stress, inflammation, reduced blood circulation, and apoptosis occurring during reperfusion injury contribute permanent damage to neurons. Reactive oxygen species (ROS) is the prime reason for reperfusion injury. Mitochondria are an essential source of generating ROS as the electron transport chain happens within the mitochondria. Ischemia contributes to mitochondrial dysfunction and increases ROS synthesis such as hydrogen peroxide, and superoxide, this rise in ROS is due to the reintroduction of oxygen during reperfusion [38]. Increased ROS devotes cell membrane destruction and raises lipid peroxidation.

Sung Wook Kim *et al.* investigated the effect of paricalcitol using the transient global cerebral ischemia model. They found no significant differences among the groups in correlation to motor coordination, memory function, and survival rate. Animals treated with paricalcitol expressed improved neurological function, and neurodegeneration was also found to be less when compared to control-treated animals [39]. The hippocampal CA1 region is the brain's most sensitive region, and neuronal death in the hippocampal CA1 region is evident following ischemic insult. Zhang Y *et al.* studies found that treatment with the effect of Rhodiola side significantly reduced destruction of hippocampal neurons in CA1 region, improved expression of proteins Bcl-2/Bax, and reduced p53 levels [40]. Rongli Yang *et al.* studied the role of Necrostatin-1(Nec-1) using global cerebral ischemia and found that reperfusion injury endorses RIP3 activation initiated by the interaction of RIP3 with RIP1. Reports suggested that Nec-1 inhibited activation of RIP3 [41]. The main advantage of the four-vessel occlusion model is that seizure incidence is low compared with the two-vessel occlusion model. This model's significant targets of brain regions are the striatum, the paramedian, hippocampal, and posterior neocortex [37]. The use of anesthesia is very much limited using this model. The limitation of this model is the high mortality rate, and significant induction is restricted to wistar rats [42]. The cell death mechanism is still unclear as ambiguity exists between necrosis and apoptosis.

**Photochemical Stroke Model:** In this model, rats weighing around 350 g were selected and anesthetized using halothane and were continued with 1.5-2% halothane, 70% nitrous oxide, and oxygen. The scalp covering the left hemisphere was exposed, and the focal infarct was introduced in rats using the photochemical method. The photosensitive dye rose bengal (1mg in 0.133 ml vehicle/100 g body wt) was administered using intravenous injection, and the rat was restrained using a stereotactic frame. Light emerging from the

xenon arc lamp at 560 nm was focused on the skull. The light was focused for a period of 120 sec, and the intensity of light used was 0.58 W/cm2. Light penetrating the brain interacts with intravascular photosensitive dye resulted in the production of oxygen free radicals that are highly reactive within the blood vessels. These free radicals caused injury to the endothelial cells and initiated the process of platelet aggregation. Once the procedure was completed, animals were placed back into cages. After 7 days, animals were sacrificed for histopathological evaluation and measuring infarct size.

This model is mainly used to study blood flow in the cerebral region and to quantity infarct size [43]. Nai-Wei Liu *et al.* improved the characterization of the model by using 18F-2-deoxyglucose positron emission tomography, fluorescence, bioluminescence imaging system, and magnetic resonance imaging. Investigators used hematoxylin-eosin staining, triphenyl tetrazolium chloride (TTC), immunohistochemistry examinations of glial fibrillary acidic protein, α-smooth muscle actin and CD 68, NeuN, von Wille brand factor in infarct area [43].

Takashi Masuko *et al.* found inhibitors of oxidizing polyamine enzymes, N1 -acetylpolyamine oxidase (PAOX), and spermine oxidase (SMOX) as targets for stroke treatment. N1 -Nonyl-1, 4-diaminobutane (C9-4) a potential inhibitor of oxidizing polyamine enzymes, significantly reduced infarct volume and was effective for a period of 12 h of postischemic [44]. Peng Wang *et al.* studied the effect of Sestrin2 (Sesn2) a stress response protein on cerebral ischemia damage. Activated Nrf2, HO-1 signal pathway up-regulated VEGF and improve angiogenesis. Reports also suggest that overexpression of Sesn2 reduced cerebral infarct volume and improved neurological deficits [45]. Following stroke induction, blood-brain leakage, cytotoxic and vasogenic edema appear and gradually vitiate with time. Vasogenic edema is associated with blood-brain barrier damage. Decreased diffusion of water indicates the initiation of vasogenic edema and infarct formation. The pattern of human stroke is different as cytotoxic edema is formed first and followed by vasogenic edema [46]. Jens Minnerup *et al.* revealed that intracarotid infusion of human bone marrow cells after 3 days of induction of stroke have no significant changes in neurological deficits [47]. Boruhou *et al.* studied the effect of exogenous Neural Stem Cells (NSC) transplantation in Photo thrombotic ischemia stroke in mice. NSC restored all brain functions, and this was evident with their performance in behavioural tests. Histopathological studies also confirmed that treatment with NSC showed a decline in brain cell damage caused by an ischemic stroke [48]. This model has several advantages as it is a minimally invasive technique and reproduces cortical photothrombotic in mice and rats. The mortality rate is very low. Ischemic damage can be controlled by simply altering the duration and intensity of light and the dye's plasma concentration. The major limitation with this model is ischemic damage depending on vasogenic and cytotoxic edema formation that differs from human stroke patterns. The end-arterial occlusive nature slows down flow to the region of injury. Antithrombotic agents cannot be studied using this technique.

**Collagenase Induced Brain Haemorrhage Model:** In this model, Sprague-Dawley rats are used. Animals were anesthetized using 50 mg/kg of pentobarbital. Then, they were placed in stereotactic equipment, and a 23-gauge needle was used to implant the caudate nucleus. Rats were infused with 2 µl of saline containing (Type VII or Type XI collagenase) for 9 min. After completing the process of infusion, the needle was removed, and the wound was sutured. Rats were allowed for recovery then sacrificed using an intracardiac injection of KCl. The brains of the rats were removed and kept in phosphate-buffered formalin for 24 h. Later, brains were sliced, and histopathological changes were studied [49].

Reports confirm the role of oxidative stress in intracerebral haemorrhage (ICH). ICH increases the generation of free radicals; however, there is a marked reduction of antioxidant enzymes glutathione peroxidase, superoxide dismutase (SOD) and catalase [50]. Rong-Xia Xie *et al* studied the effect of Carnosine, a dipeptide (β alanyl histidine), using rat ICH model by collagenase caudatum infusion. Reports found that Carnosine inhibited oxidative damage by decreasing elevated levels of MDA, ROS, 8-OHDG and 3-NT levels of DNA, lipids, and proteins. It also restored SOD and

GSH-Px activity [51]. Collagenase induction activates calcium-dependent nitric oxide synthesis and increases nitric oxide levels. This increased nitric oxide level causes nerve terminal damage which impacts cognitive functions. Reports suggest that oxidative stress followed by intra cerebrovascular administration of collagenase is the prime reason for neurological deficits like depression, pain and dementia50. Functional and movement deficits are most prevalent in haemorrhagic and ischemic stroke. The underlying mechanism involves damage of striatal neurons that act as relay centers for basal ganglia and substantia nigra.

Brain herniation initiated by ischemia and increased intracranial pressure guide to focal neurological deficits. Damage of a particular brain region leads to loss of physical activities associated with that region of the brain. Collagenase administration significantly affects neurological, and motor functions and these defects are results of oxidative inflammatory cerebral injury, brain herniation, and caudate vascular lesions [50]. Marc Del Bigio *et al.* assessed the motor activity and cognitive functioning post-treatment with fucoidan. Reports suggest that fucoidan successfully restored motor and cognitive activity [52].

Studies have reported microglia as an essential component associated with Blood-brain damage, delayed neuronal loss, inflammation, and hematoma resolution. Microglial cells release important mediators like chemokines and cytokines associated with neurological damage and neuroinflammation. However, the death of glial cells terminates neuronal function and impacts motor recovery. A rise in cortical microglial cells is considered as an acknowledgment of secondary response to ICH which affects sensorimotor recovery. Thus, microglial changes establish perceptivity of cerebral cortex to ICH and could be the center point for future treatment strategies [53]. Early hematoma is the sign associated with neurological deterioration after intracerebral haemorrhage. N. Kawai *et al.* studied the effect of recombinant factor VIIa 2 h after injection of collagenase; there was an accumulation of blood in the region of the striatum and slowly extended to the region of the thalamus by 24 h. Thus, the administration of recombinant factor VIIa immediately after collagenase injection would help in reducing the average hematoma volume and frequency of hematoma formation 54. Arne Lauer *et al.* conducted a comparative study among direct thrombin inhibitor dabigatran etexilate (DE) over anticoagulant warfarin. Reports suggest that intracerebral bleeding was severe during warfarin treatment, along with severe hematoma expansion. Treatment with dabigatran etexilate did not exaggerate the ongoing process of intracerebral bleeding neither increased hematoma growth [55].

Collagenase-induced cell death is because of the activation of essential factor TNF-α released from neutrophils and macrophages. Reports have confirmed the role of TNF-α, its correlation with hematoma formation, edema formation, and release of interleukins, matrix metallopeptidase (MMPs) [56]. Yan Zhang *et al.* reported that stereotactic injection of edaravone is more effective in hemorrhagic lesions, as it encourages anti-inflammatory response and curbs pro-inflammatory response. Edaravone treatment effectively hindered neuronal apoptosis, protected damage of BBB, restored neuronal functions, and lessened cerebral edema formation [57].

P. Plema *et al.* reported that treatment with dexamethasone restored functional deficits, reduced hematoma volume, decreased filtration of neutrophils, astrocytes into a hematoma58. Collagenase induced haemorrhagic brain model is highly reproducible and has many similarities with the injury pattern in humans. Hematoma formation can be witnessed after 10 min of administration of collagenase. The lateral striatum, medial striatum, and corpus callosum are the most affected regions of the brain. Significant neurological deficits can be replicated using this model. Hematoma volume formed is low compared to other ICH models, and high mortality rates are the main limitations of this model.

**Autologous Whole Blood Induced Haemorrhage Model:** In this model, the animal was anesthetized using pentobarbitone injection (50 mg/kg). The animal was fixed on a stereotactic frame, a scalp incision was made to drill a hole of 0.02 mm forward to the coronal suture and 3 mm adjacent to the midline. A 25-gauge needle was introduced into the cerebral cortex region on the surface of the skull. The rat received 50 -µl of whole autologous

blood for the duration of 5 min. after completing the process of infusion, the needle was kept at the same place for a period of 3 min and later removed. The hole drilled in the skull was closed using bone wax, and the scalp wound was sutured [59]. Studies demonstrated that ICH persuaded brain injury because of hematoma expansion and secondary brain injury (SBI). Exploring mechanisms to lessen SBI is the new target of researchers. The mechanisms involved in SBI after ICH are associated with apoptosis, hematoma formation, increased ROS, excitatory amino acid toxicity, inflammatory mediator neutrophils, macrophages, and activated microglial cells in brain tissues.

Brain oedema is an essential pathological condition developed after ICH. Mechanism like destruction of the BBB, thrombin formation, hydrostatic pressure generated during blood clot formation, reperfusion injury, coagulation cascade, the toxic effect of haemoglobin contributes to brain edema formation. Takehiro Nakamura, *et al.* found that treatment with edaravone after 2 h of ICH successfully ameliorated the formation of brain edema and reduced impact on neurological activity [60]. Takehiro Nakamura, MD studied the effect of nafamostat mesylate (FUT), a serine protease inhibitor, on brain injury and edema formation using the intracerebral haemorrhage model in rats. FUT was injected intraperitoneally after 6 h of intracerebral haemorrhage.

Treatment with FUT after 6 h of ICH reduced brain water content in the region of basal ganglia and inhibited 8-hydroxyl-2-deoxyguanosine changes [61].ICH activates microglia cells, macrophages, vascular endothelial cells and increases the expression of MMP-9. MMP-9 is the prime reason for the disruption of BBB and contributor of brain oedema formation.

Previous studies have reported that macrophages block small arteries and decrease cerebral perfusion, leading to BBB disruption. TNF-α, IL1β, are also associated with SBI. Zhong Wang *et al.* reported that melatonin significantly abridged inflammatory cytokine TNF-α and IL1βlevels and reduced movement of these mediators into brain tissues [62]. ICH-induced brain damage expresses NOX-1 and NOX-2. Zhong Wang *et al.* found that expression of NOX-1 and NOX-2 after ICH could be done using melatonin [62]. Oxidative stress is an essential factor involved in edema and SBI after ICH in the brain. A substantial number of Reactive oxygen gets activated following ICH. Activated ROS leads to oxidative damage and lipid oxidation. Heme oxygenase-1 is an important anti-oxidant that protects cells and tissues from oxidative damage. T. Nakamura *et al.* studied the effects of endogenous and exogenous estrogen in male and female rats and found that treatment of oestradiol after 2 h of ICH to male rats restored neurological deficits, reduced edema formation, and increased Heme oxygenase-1 when compared to female rats ICH [63].

Mitochondrial dysfunction can activate several intracellular signaling pathways like apoptosis and oxidative stress. Studies establish a direct relation between neuronal apoptosis and mitochondrial damage. Jing Huang and Qiang Jiang reported treatment with dexmedetomidine inhibited expressions of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) and mitochondrial impaired function [64].

Takehiro Nakamura, *et al.* studied the effect of Deferoxamine, an iron chelator, on brain edema and neurological deficits. 8-hydroxyl-2-deoxyguanosine (8-OHdG), key representative to check the oxidative damage, using immunohistochemical analysis followed by a western blot test to estimate the amount of redox effector factor-1 and apurinic/apyrimidinic endonuclease (Ref-1/APE) to assess oxidative damage. Treatment with Deferoxamine inherited brain edema formation, restored all neurological deficits, and prevented intracerebral haemorrhage induced changes [65].

There are several advantages of this model. High reproducibility is a crucial advantage in this model. Hematoma volume formed is higher when compared with other ICH models. The regions of the brain-damaged after induction of ICH are the medial striatum and corpus callosum.

Neurological deficits developed are comparable with that of human stroke. The limitation in this model is that there is no significant loss of neurons in the striatum region. Animal recovers faster from neurological deficits, which limit the long-term study of new drugs.

**TABLE 1: COMPARISION BETWEEN *IN VIVO* CEREBRAL ISCHEMIC MODELS**

| Model | The affected region of the brain | Mortality Rate | Significance | Key limitation |
|---|---|---|---|---|
| MCAO (middle cerebral arteryocclusion ) | Cortex | + | Formation of cerebral oedema, which resembles a human stroke | Dimensions of filament affect final results |
| Emboli Stroke Model | Cortical and subcortical regions of the brain | +++ | Neurobehavioral deficits are similar to that of humans | Brain haemorrhage |
| Two vessel occlusion model | CA1 region, caudate-putamen, hippocampus, and the region of neocortex | ++ | Useful in studying long term recovery studies | Postischemic seizures |
| Four-Vessel Occlusion Model | The striatum, paramedian, hippocampal, and posterior neocortex | +++ | Neuronal damage produced is similar to that in humans | The cell death mechanism is still unclear whether it is necrosis or apoptosis |
| Focal thrombotic stroke model | Cortical and subcortical regions | + | Easy measurement of cerebral infracts | Formation of vasogenic edema |
| Collagenase induced brain hemorrhage model | lateral striatum, medial striatum and corpus callosum | +++ | Assess long-term functional outcomes. | Neurotoxicity of collagenase |
| Autologous whole blood induced hemorrhage model | Medial striatum and corpus callosum | +++ | Produces consistent hemorrhage volume | Evaluation of microvascular breakdown cannot be done |

Mortality rate: "+" Low, "++" Medium, "+++" High

## *In-vitro* Models of Cerebral Ischemia:

**Oxygen Glucose Deprivation *In-vitro* Model:** *In-vitro* focal ischemic model desires a freshly prepared sliced rat brain. Male Sprague Dawley rats remained anesthetized and beheaded following immediate brain isolation and stored in artificial cerebrospinal fluid (aCSF) at 2-3 ºC. The brains were sliced coronally into 400 µm thickness while they were submerged in ice-cold aCSF. The brain slices were generally incubated for 1 hr at room temperature before the experiment. A single slice was taken into an experimental apparatus and observed under a microscope. Sliced brain tissue was super fused using aCSF at 3 mL/min and maintained at 34 ± 1 ºC using a thermostat. After an interval of 30 min, drugs were added to the bath till the end of the experiment. To mimic focal ischemia in this method, the OGD medium has to be applied at a specific region of the cortex.

The composition of the medium is slightly variable from that of aCSF solution where 10 mM d-mannitol was used instead of glucose and a combination of 95% $N_2$ and 5% $CO_{2\ was}$ used instead of oxygen. A microperfusion infusion pump attached to the micromanipulator is used to distribute CSF and OGD solutions. A heating element has to be connected at the end of the nozzle so that focally applied media is warmed when delivered on to the brain. The rate of focal solution application has to be at 100 be 100 L/min as this prevents the mixing of contents. Micro perfusion of stain helps in identifying the area of the focal solution. Slices were incubated in 2% 2, 3, 5-triphenol tetrazolium chloride (TTC) for 10 min at 37 ºC followed by focal solution application. Staining slices using TTC helps identify healthy tissue as they are stained red in color, whereas dead tissue is white in color. After staining, slices have to be fixed using 10% formalin and stored for future studies. The merits of the OGD model are the ability to mimic focal ischemia. The development of focal infarcts within the brain slice helps study ischemic reperfusion injury. The major limitation with this model is it does not mimic the events that happen in the region of penumbra where oxygen and glucose are supplied even after induction of stroke [66].

Huaqiu Zhang *et al.* studied the role effect of resveratrol in rat hippocampal brain slice using oxygen and glucose deprivation. They found that resveratrol inhibited neuronal damage by decreasing the increased NMDA receptor-mediated neuronal activity excitatory postsynaptic potential currents by stimulating. K+ channel [67]. Jibiao WU et al. studied the role paeonol on rat hippocampal neurons against oxygen-glucose deprivation injury

and found that paeonol protected the neurons by decreasing the morphological changes and improving cell survival abilities [68]. X Q Wang *et.al.;* studied the effect of quercetin in oligodendrocyte precursor cells (OPCs) deprived of oxygen and glucose from the studies; they concluded that quercetin activated PI3K/Akt signaling pathway that prevented the damage of oligodendrocyte precursor cells [69]. Q Wang *et al.,* found that dendrobium alkaloids down-regulated mRNA expression of caspase 12, caspase 3 and decreased $Ca_2$+ influx, and increased MMP levels in rat cortical neurons [70].

**Excitotoxicity *In-vitro* Model:** 15 days old mice should be selected to prepare cultured cortical neurons [71]. After separating, meninges cortices should be stored in a cold solution containing 30 mM glucose and phosphate buffer. The cell dissociation for cortices of each embryo has to be performed individually. Then the cortices are incubated at 37 ºC for 7 min using 2.5% trypsin and rinsed thoroughly three times using phosphate buffer. The neurons were transferred into 24 well plates. Cultures are incubated in an atmosphere containing 95 % $O_2$ and 5 % $CO_2$, and then on every 4th day, 50 % of the medium needs to be adjusted with the fresh neurobasal medium. After 5-7 days, growth of non-neuronal cells should be hindered using 5 µM uridine and 5 µM deoxyuridine.

The Cultures are ready to carry in vitro experiments after 14 and 21 days. Cortical cultures should be treated with NMDA using different concentrations ranging between 50 to 300 µM in attendance of 10 µM glycine for 10 min at 37 ºC in $CO_2$ containing incubator. After 10 min, cultures should be washed using a serum-free neurobasal solution before those dishes are placed back into the incubator for a period of 24 h. NR2B selective antagonist 10 µM ifenprodil and NR2A selective antagonist, 50 nM NVP-AAM077, are used to block NMDA receptor subtypes. Total neurons dead after one-day treatment of NMDA can be done by counting cells stained with trypan blue and measuring lactate dehydrogenase in the medium. A cytotoxicity testing kit and spectrophotometric plate reader is used to estimate lactate dehydrogenase activity. Lactate dehydrogenase estimated value had to be deducted from the experiment value to find experimentally induced cell death. Cells collected from DIV21 cultures should be reaped with buffer containing 150 mM NaCl, proteinase inhibitor, and 25 mM Hepes at 7.4pH. Cell nuclei separate by 1000 × g for 5 min and 25000 × g centrifugation for 30 min. Proteins are then transferred onto the nitrocellulose membrane, where they are immunoreacted with antibodies NR2B and synapsin. Amersham ECL Western blotting testing kit, anti-mouse antibodies, and anti-rabbit antibodies are used to observe Protein bands. Immunoblots are scanned, and signal quantification can be done using NR2A and NR2B signal intensities that are equalized to the synapsin signal intensities. To activate somatic currents, L-glutamate (1 mM) application is required along with soma patches at -60 mV. 5 µM NBQX, 10 µM glycine, and 0 mM MgCl2 are helpful in the determination of NMDA currents, whereas 50 µM D-AP5 (Tocris) and 1 mM MgCl2 are used to record AMPA current. A selective antagonist is used to rule out the role of NR2-type NMDARs to somatic NMDA currents.

Excitatory postsynaptic potential in AMPA and MNDA components were registered at -70 mV in aCSF containing 0 mM $MgCl_2$, 10 µM glycine, 10 µM bicuculline methiodide and 1 µM tetrodotoxin. 1 mM $MgCl_2$ and 50 µM D-AP5 should be added to record AMPA miniature EPSCs. Advantages of this model it exactly mimics the conditions of ischemic reperfusion injury in brains. The most widely used *in vitro* model and neuronal death can be assessed in this model. Limitations with this model is tissue damage and the need for specific cellular platforms in the experiment. Shaida A. Andrab et al. found that iduna, binds effectively with PAR polymer binding protein and interrupts the cell death mechanism [72].

De Maw Chuang *et al.* reported lithium as a potent protective agent effectively down streaming signaling and interrupted NMDA receptor-mediated calcium influx. They also explained GSK3 as a new target for lithium to exhibit neuroprotective action [73]. Christiane Volbracht et al. studied the protective role of memantine in NMDA-induced toxicity in cerebellar granule cells and organotypic hippocampal slices. It was observed that depletion of energy and dysfunction of mitochondria contributes to the activation of

NMDA-R and also a major reason for the development of excitotoxicity. From the results, it was learned that memantine at very low concentrations protected cells against NMDA-induced excitotoxicity, and this action of memantine was associated with its ability to block the NMDA receptor [74]. A. Shabbir et al. study the effect of methoxyflurane on motor neurons against excitotoxicity. It was found that methoxyflurane delayed the process of cell death, which is activated by excitotoxicity and the effect of methoxyflurane is also effective during the 1-2 h [75].

**Chemical Hypoxia *In-vitro* Model:** In this model, neuroblastoma cell lines, glucose-free DMEM cell culture media, and dialyzed FBS are used 76. Antimycin A is the agent used for the induction of chemical hypoxia. The neuroblastoma cells are cultured in 10% bovine serum in 5% $CO_2$ incubator maintained at 37 ºC. Once we observe that cells are starting to merge, the culture media is replaced with chemical hypoxic that induces 10 uM of antimycin A and FBS in glucose-free DMEM. Following chemical hypoxia, after 2 h we can check for the cell survivability using a cell viability analyzer. Reactive oxygen can be articulated using 10 uM of DCFDA for 5 min. IX-70 fluorescence microscope is used to observe these ROS expressions.

There are several advantages in this model as it has high relevance to *in-vivo* stroke events. This model is cost-effective and can record responses rapidly. This method is more suited for high throughput screening assays. Limitations with this model are the production of high levels of ROS and cellular dang during the experimental procedure. Li-Ting Wang *et al*., from their studies, reported the role of AMP kinase AMPK induced autophagy as a key factor in protecting renal proximal tubular cell death in an *in-vitro* Ischemic reperfusion injury [77]. J. Aghazadeh-Attar *et al.* reported that hypoxia-induced using Antimycin A and 2-deoxy glucose would up-regulate the formation of hypoxia-inducible factor-1α and also increase the levels of ROS and NO with a significant reduction in the levels ATP in Neuro-2A cells. Treatment with allopurinol reversed the changes, and this effect was attributed to its regulation of hypoxia-inducible factor-1 an expression [78]. Anna Leichsenring *et al.* studied anoxic depolarization induced by oxygen/glucose-deprived medium having antimycin and sodium iodoacetate and involvement of P2X7 receptors. The results show that neither non-selective nor selective P2X7 receptor antagonists have a significant role in anoxic depolarization [79].

***In-vitro* Synthetic Microvasculature Model of the Blood-Brain Barrier (SyM-BBB):** Eternalized rat brain endothelial cell lines (RBE4 cells) are cultured in Ham's F-10 media and Eagle's Minimum Essential Medium in the ratio of 1:1 along with 2 mM L-glutamine and G418 300 µg/ml 1% Pen/Strep and 10% FBS. Cells are incubated in 95% humidity and 5% $CO_2$ at a temperature of 37 °C till they are confluent. To the apical side of SyM-BBB, fibronectin (100 µg/ml) is injected and incubated and regular room temperature for a period of 2 h. Media free of serum is infused into the chamber and incubated for 30 min at 37 °C.

RBE4 cells at a concentration of 5 × 106 /ml should be infused into the basal chamber and left to get attached for a period of 2 h. Later, cell media should be injected into culture containing endothelial cells under fluidic shear of 0.1 µl/min for a duration of 24h to 48 h. Astrocyte culture medium containing 10% inactivated horse serum, 100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml Fungizone mixed with RBE4 medium of equal volume. Then the mixed medium was transferred to the basolateral chamber for a period of 48 h to form tight junctions. Ethidium Homodimer-1 and Calcein AM are injected into the apical chamber to check active metabolizing members and healthy cells [80].

REB4 cells are harvested using trypsin, washed with buffer, Radio Immuno Precipitation Assay, and phosphate-buffered saline (PBS). Later a mixture of protease inhibitors was supplemented to the cells before sonication. A protein assay was performed using bicinchoninic acid to find the protein concentration in cell homogenates. Cells are then transferred to 10% SDS and polyacrylamide gels to separate the proteins. Separated proteins are then loaded to a polyvinylidene fluoride (PVDF) membrane. Later membranes are incubated using blocking solution; subsequently, membranes are incubated overnight at 4 °C along with P-glycoprotein (P-gp) claudin-1

and zonula occludens[-1]. Later the membranes are washed thrice and incubated with secondary antibody for a period of 2 h at room temperature. Chemilumine science signal normalized with β-actin expression is used in the final detection process. Efflux activity in SyM-BBB can be investigated using Rhodamine 123 in REB4 cultured cells. Verapamil, a potent inhibitor of P-Glycoprotein, is used to block the efflux activity in the cultured cells completely. Rhodamine 123 at a concentration of 150 ng/ml is equilibrated with SyM-BBB for a period of 20 min at a temperature of 37 °C. Cells are washed using phosphate buffer saline mixed 0.1% Bovine Serum Albumin to check rhodamine permeability.

Later the dye is allowed to efflux for 2 h at 37 °C so that efflux rates can be calculated. The advantage of the model is that it provides an artificial environment with identical microcirculatory vasculature comparable to that of humans. Maintains exact physiological flow properties and shear stress. Oxygen permeability helps in maintaining in situ cell culture for a longer duration. The model helps understand the extent of BBB damage. Real-time monitoring is possible and would help to speeds up the research and development. The limitation in this model is the simultaneous observation of neuronal and vascular sides of BBB is not possible, and the model is very expensive.

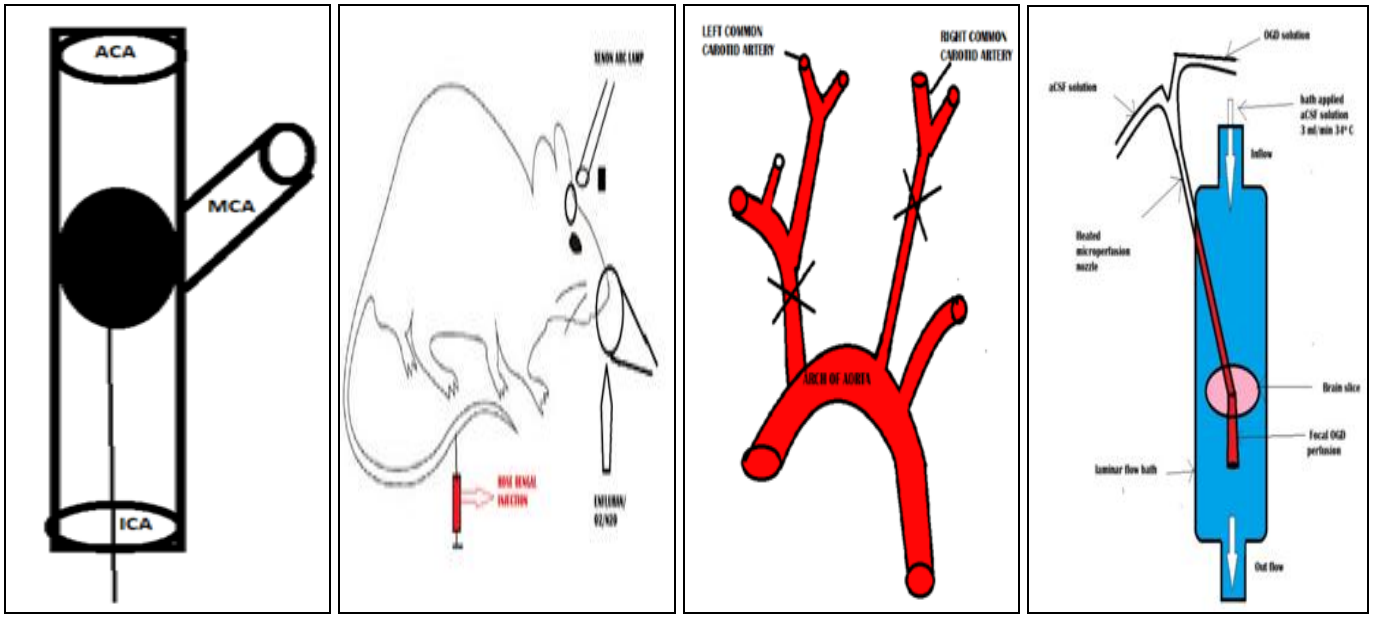


**FIG. 1: (A) MIDDLE CEREBRAL ARTERY OCCLUSION MODEL (B) PHOTOCHEMICAL MODEL (C) TWO-VESSEL OCCLUSION MODEL (D) OXYGEN-GLUCOSE DEPRIVATION MODEL**

**TABLE 2: COMPARISON BETWEEN *IN-VITRO* CEREBRAL ISCHEMIC RAT MODELS**

| Model | Relevance with *in-vivo* model | Cellular platforms used | Technical complexity | Key limitation |
|---|---|---|---|---|
| Oxygen Glucose Deprivation Invitro Model | Very high | Brain slice, Organotypic cell culture Primary cells, Cell lines, Embryonic stem cells, induced pluripotent stem cells | ++ | Tissue damage during the procedure |
| Excitotoxicity Model | Very high | Brain slice, Organotypic cell culture Primary cells, Cell lines, Embryonic stem cells induced pluripotent stem cells | ++ | Tissue damage during the procedure |
| Chemical Hypoxia Model | Very high | Brain slice, Organotypic cell culture Primary cells, Cell lines, Embryonic stem cells induced pluripotent stem cells | ++ | Generation of reactive oxygen species |
| Synthetic Microvascular Model of Blood-Brain Barrier | Very high | Primary cells, Cell lines, Embryonic stem cells induced pluripotent stem cells | +++ | Very expensive |

Technical complexity: "+" Low, "++" Medium, "+++" High

**CONCLUSION:** The application of *in-vivo* and *in-vitro* models in studying neuroprotective agents has been grilled as they fail to predict drug efficiency in preclinical studies. In this review, we have explained in detail few *in-vivo* and *in-vitro* models of cerebral ischemic injury and highlighted the uses of each model. Based on investigator experimental interest, suitable *in-vitro* or *in-vivo* models can be selected. Besides the regular *in-vitro* models, the advanced *in-vitro* model like synthetic microvasculature would help understand the molecular mechanism and pathophysiology of ischemic injury. Each *in-vitro* model has its own functionality and complexing and should be selected based upon its applications. *In-vivo* model has lesser variations to mimic the pathological condition of ischemic reperfusion injury. *In-vivo* models are helpful in studying the long-term effects of drugs.

**ACKNOWLEDGEMENT:** The authors express sincere thanks to the Management and Principal of GITAM Institute of Pharmacy, GITAM Deemed to be University, Visakhapatnam, Andhra Pradesh, for continuous support.

**CONFLICTS OF INTEREST:** The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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**How to cite this article:**
Sreehari Y and Prabhakar O: New insights on in-vivo and *in-vitro* animal models of cerebral reperfusion injury. Int J Pharm Sci & Res 2022; 13(4): 1411-27. doi: 10.13040/IJPSR.0975-8232.13(4).1411-27.