**ABSTRACT**

Cerebral ischemia is a neurovascular disease in which blood flow to the brain decreases which leads to glucose and oxygen deprivation in the brain and this finally results in neuronal cell death. On the other hand, it was found that reperfusion augments this damage by increasing availability of oxygen. Cerebral ischemia can be classified into two major categories as focal and global cerebral ischemia. The mechanisms of neural cell damage following cerebral ischemia reperfusion is complex cascade, which ultimately leads to cell apoptosis or necrosis following disturbance in ion distribution, excitotoxicity, oxidative stress, and, mitochondrial dysfunction. The incidence of stroke is higher in postmenopausal women than premenopausal ones and it was found that they suffer from more severe side effects than men in matched age. It was found that estrogen has neuroprotective actions against cerebral ischemia in both male and ovariectomized rats via various mechanisms as antioxidant, anti-inflammatory, nootropic, and glucose transporters enhancing beside activation of survival signal transduction pathway either genomic or non-genomic.

**Keywords:** Cerebral ischemia; Estrogen; Inflammation; Oxidative stress; Postmenopausal women

**INTRODUCTION**

Cerebral ischemia is one of the most leading reasons of mortality worldwide; clinical investigations continue to identify the likely effects of various compounds on cerebral ischemia. Cerebral ischemia, which happens as a result of inadequate blood flow, finally leads to permanent death of the neuronal cell. Stroke is known as the second cause of death and the third cause of disability. The world health organization in 2013 reported that 15 million people suffered from stroke all over the world on an annual basis, and 5 million of them die, while 5 million are debilitated.

One of the main types of stroke is cerebral ischemia. The main mechanisms leading to brain injury in stroke are; ischemia and hemorrhage. In ischemic stroke, which is about 80% of all strokes, reduced or abolished circulating blood, deprives neurons of main substrate, which is divided into embolic in which blood flow is blocked by presence blood clots in arteries; clots travel from heart through the blood stream to brain, or thrombolic in which blood flow is impaired because of fat deposits which cause
blockage on the wall of the blood vessel. Non-trauma intracerebral hemorrhage approximately represents 10 to 15% of all strokes. Intracerebral hemorrhage originates from deep penetrating vessels causing injury to brain tissue by disrupting connecting pathways and causing localized pressure injury.

The main risk factors for ischemic stroke are old age, gender, ethnicity, hypertension, diabetes mellitus, atherosclerosis, atrial fibrillation, tobacco smoking, alcohol consumption, physical inactivity and diet.

These factors can be divided into two categories: (a) factors which cannot be modified such as: age, gender, race, and positive family history of stroke (b) factors which are modifiable including: hypertension, diabetes mellitus, smoking cigarettes, consumption of alcohol, obesity and physical inactivity.

Cerebral ischemia results in decline of energy substrates, such as glucose, and oxygen, an unsubstitutatle electron acceptor which has role in the oxidative phosphorylation cascade, causing a critical decrease in ATP synthesis and generation of reactive oxygen species (ROS). Hence, the continued glycolysis process leads to intracellular acidification and cellular functions are deteriorated.

1. Classification of cerebral ischemia

Brain ischemic injury can be principally classified into global and focal cerebral ischemia.

Global cerebral ischemia happens when cerebral blood flow (CBF) is abolished throughout most or all parts of the brain due to cardiac arrest, resindable severe hypotension, and neonatal asphyxia.

Symptoms following global cerebral ischemia encompass confusion, difficulty in walking, falls, balance problems, non-reactivity to light or abnormal pupil size, memory loss, difficulty in writing or reading, comprehension, talking, dizziness, droopy eyelid, headache, alteration in vision or loss of vision, muscle coordination loss, weakness or numbness, nausea with or without vomiting, paralysis and vision difficulties such as blurriness, double vision, and sudden blindness.

Divergent to global cerebral ischemia, focal ischemia is branded as a decline in blood flow to very specific regions, as in embolic occlusion of the middle cerebral artery (MCA). It also happens due to large-vessel disease, such as thrombotic arterial occlusion, often in setting of arteriosclerosis, or to small-vessel disease, such as occlusion secondary to arteriosclerosis lesions found in hypertension.

There is a distinct variability in the liability of different population of neurons in different brain regions to ischemic brain injury. Global cerebral ischemia, which affects the entire forebrain, generally results in discriminating neuronal damage, thereby only affecting selectively vulnerable neuronal populations. Transient global cerebral ischemia notably affects the small to the medium-size neurons in the corpus striatum and the large pyramidal neurons in the cornu ammonis I (CA1) sector of the hippocampus. Neurons in these brain areas show a notable pattern of cell death, defined as delayed neuronal death (DND). Striatal neurons die 21h; however it proceeds approximately through 72 h for the hippocampal CA1 neurons to die. In severe global cerebral ischemia, extensive neuronal death regardless to regional vulnerability occurs.

The neuronal features that cause selective vulnerability to ischemia, are a high density of excitatory glutamatergic synapses, low antioxidant enzymes, high content of transition metals, and increased expression of pro-apoptotic Bcl-2 associated X (Bax) protein.

On the other hand, in focal cerebral ischemia, the ischemic vascular bed is an area with severe CBF reduction that include an "ischemic core", which represents the most severe injury and a more distal area known as "ischemic penumbra". The ischemic penumbra (sleeping beauty) that surround the infract core suffers milder injury and can be considered as a brain tissue perfused at a level within the thresholds of functional impairment and morphologic integrity, which has the capacity to recover if perfusion is improved (kissed awake). Evidence indicates that, if the blood flow is not restored within hours, the penumbra region becomes part of the core region.

Thus, interfering with the delay onset of apoptotic pathways in the penumbra allows these neurons of infarcted region to recover and the patient to improve (Figure 1).

The processes of cellular injury and death are notably different in core and penumbral regions. There appear two major modes of cell death that participate in ischemic cell death: necrosis and apoptosis. The idea that brain infarction is a classic example of necrosis has left the place to another approach: apoptosis and necrosis seem like the two poles of continuum of cellular death (aponecrosis) after ischemic stroke. While necrosis is more dominant in the core tissue, penumbral cells die by means of either mode, with apoptosis being more common for cells further away from the core.

Re-establishment of blood supply after stroke can be done through thrombolysis or mechanical recanalization. But, for some patients, reperfusion may aggravate the injury originally occurred by ischemia, causing a so-called "cerebral reperfusion injury". Numerous pathological events are involved in this injury, as leukocyte infiltration, platelet and complement activation, posts ischemic hyperfusion, and breakdown of the blood brain barrier.

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Figure 1. (a) A multiple sequences of events are involved in neurological injury of brain through stroke. Excitotoxicity associates to oxidative stress, and these biochemical alterations cause a mixture of apoptosis, necrosis, and inflammation. The BBB is also affected. Reperfusion is the cause of amplification of oxidative stress. (b) Schematic diagram demonstrate the core (red) and the penumbra (blue) of ischemic lesion.

2. Animal models of cerebral ischemia

Most information about cellular cascades that occur after transient cerebral ischemia derives from the experimentially- induced animal models for cerebral ischemia. Results of the experimental cerebral ischemia models have contributed massively in knowing its pathophysiology. Additionally animal models offer an area for trying novel compounds before the initiation of clinical trials.

2.1. Models of global ischemia

In global cerebral ischemia, there is insufficient CBF to any zone of the brain, which leads to neuronal damage to special prone brain areas. All neural cells would die if global ischemia persisted indefinitely.

Complete global ischemic events include cardiac arrest, aortic occlusion, neck cuff and cephalic artery occlusion. Incomplete global cerebral ischemic events include 2-VO (vessel occlusion), 4-VO, hemorrhage and intra-cranial hypertension.

2.1.1. Complete global ischemia models

1. Neck cuff or neck tourniquet

A neck tourniquet has been used for numerous years to cause global ischemia in rats. But this technique has several obscuring factors such as venous congestion and vagal nerve compression, which lead to variable ischemic results. Neck cuff inflation can also be used in large animals (e.g. dogs) to cause complete ischemia in the whole brain, with the emphasis that the vertebral arteries must be occluded individually because they are enclosed in vertebrae and will not be occluded by neck cuff.

A modification of the neck technique was performed in monkeys in which a reduction in arterial blood pressure to 50 mmHg occurred before neck inflation. A drawback to this technique is that ischemia produced may not be complete because of blood flow via the vertebral and spinal arteries. This neck cuff inflation in addition to hypotension has been used in cats.

2. Cardiac arrest and resuscitation

Ventricular fibrillation is a technique to cause global cerebral ischemia. This technique is estimated to mimic the clinical situation of cardiac arrest, and cardiopulmonary resuscitation (CPR) has been introduced by many researchers after cardiac arrest.

Cardiac arrest can be prompted by injection of potassium chloride, electric shock, thoracic compression, asphyxia and the mechanical obstruction of the ascending aorta while resuscitation is done by artificial ventilation, closed chest cardiac massage, and electrical defibrillation.

This technique is mainly carried out in large animals; however, it is costly and hardworking because the animals need intensive care handling, mainly...
through the first 24-48h after arrest. Moreover, this cardiac arrest/ - cardiopulmonary resuscitation ventricular fibrillation model is considered as a complete ischemia then incomplete ischemia because during the resuscitation part of procedure, pressure of cerebral perfusion and the level of CBF produced are remarkably less than the control level \(^{32}\), despite the attempts to increase perfusion via epinephrine. Although the dog has been used most frequently for the model described above; the pigs and mice have also been utilized often \(^{33}\).

3. Profound systemic hypotension

It can be induced by pharmacological agents, which result in incomplete but almost global cerebral ischemia. The main advantage of this model is the ease of resuscitation of the animal; however possible variability in severity and regional distribution of cerebral ischemia limits the use of this model. A study was done to overcome these drawbacks by combining systemic hypotension and hypoxia, using a standard of five minute of isoelectroencephalography, observed a protective effect of a rapid acting barbiturate on ischemic damage \(^{25}\).

4. Increased intracranial pressure

Cerebral perfusion pressure, or CPP, is the net pressure gradient causing cerebral blood flow to the brain (brain perfusion). It is calculated as the mean arterial pressure (MAP) minus the intracranial pressure (ICP). When the intracranial pressure exceeds more than the systolic arterial pressure, it leads to insufficient cerebral blood supply. It can be utilized in dogs, rabbits and rats. The benefit of this model is its simplicity in managing of the brain temperature by altering the volume of the injected fluid. Though, global cerebral ischemia can easily be performed using this technique; the key pathological alteration is intracranial hypertension not ischemia \(^{34}\).

5. Combination of occlusion of the major arteries

It can be carried out by surgical obstruction of the main arteries in large animals such as dogs, cats and rabbits. The major drawback of this model is the presence of extended intrathoracic or intracranial operation. Care has to be taken while doing the operation as this can lead to myocardial injury \(^{25}\).

6. Cervical compression

Combination of systemic hypotension with blocking of cervical blood vessels with tourniquet results in ischemic injury in the brain. It appears hard to totally obstruct the vertebral and anterior spinal arteries from an anatomical point. Cervical compression model should be considered as a forebrain ischemia not global cerebral ischemia \(^{25}\).

7. Cephalic artery occlusion

A variety of models have been used that include occlusion of cephalic arteries of the neck and thorax. These models provoke totally ischemia in the renal, splanchnic, and other peripheral circulations. Cerebral blood flow in this model is decreased to near zero throughout the brain, and arterial blood pressure is pharmacologically reduced to less than 80mmHg \(^{35}\), and as in other ischemic models, pathology and behavior outcome can be titrated to the length of time of the ischemic event.

2.1.2. Incomplete global (Forebrain) ischemia models

1. Bilateral common carotid artery occlusion (BCCAO)

The bilateral carotid artery occlusion results in histopathological changes, especially in the forebrain area, both in rats \(^{36, 40}\) and 41, 42. 41, 42.

2. Four- vessel occlusion in the rats

This is the most used model since documented by Pulsinelli et al \(^{39}\). It includes complicated surgical technique. This model is a two-step surgery. First day, occlusion devices are sited round common carotid arteries and thereafter vertebral arteries are electro-cauterized. Both common carotid arteries are occluded 24h later, while rats are awake. However, a drawback may be variation of the ischemia due to the difficulty in confirming the complete electro-cauterized vertebral arteries and the presence of collateral pathways, mostly due to anterior spinal artery.

3. Two -vessel occlusion in rat with hypotension

It is performed by transient occlusion of both carotid arteries with decrease of arterial blood pressure to 40-50mm Hg by phlebotomy. It produces delayed and selective neuronal death, and physiological variables can be easily monitored. The only disadvantage is alteration of these variables as a result of hypotension \(^{44}\).

2.2. Models of focal ischemia

Focal ischemia is carried out by occlusion of a single artery, particularly the internal carotid artery or middle cerebral artery (MCA). It mimics human strokes due to thromboembolism. The target artery can be occluded by many ways namely; mechanical compression, cauterization, injection of a photosensitizing dye (e.g. Rose Bengal) with simultaneous laser irradiation, injection of carbon microsphere, intraluminal occlusion by insertion of a nylon thread or injection of platelet aggregate or small blood clots.

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1. Intraluminal suture middle cerebral artery occlusion (MCAO) model

This model is the most widely used in rat stroke owing to its simplicity and non-invasiveness. A surgical filament is embedded through the internal carotid artery (ICA) until it arrives and occludes the origin of MCA. The method may offer a model of transient ischemia with reperfusion or permanent occlusion depending on whether the suture is kept in artery for 30 min to 2 or 24h. This method does not need craniotomy which causes extra-lesional effects on intracranial pressure and temperature. The intraluminal suture model is suitable for rats and mice. Drawbacks occur such as variation in the temperature of core, injury to adjacent structure, and hemorrhage disturb experimental precision. This model is mainly used in many studies to understand mechanism of neuron injury in case of cerebral I/R and also used to determine protective effect of different compounds against cerebral I/R.

2. Embolic models

They include thromboembolic models and non-clot embolic models

a. Thromboembolic stroke models

These models resemble more closely the pathophysiology of human ischemic stroke and offer potential to test thrombolytic agents, to evaluate ischemic lesions which underwent thrombolysis, and to study combination therapies, such as thrombolytic agents combined with neuroprotective drugs. The most widely utilized method in thromboembolic model is blood clot injection, earlier described in the dog by Hill et al. and later applied to the rat.

Several drawbacks were proper to the early thromboembolic models such as diffuse and uniform infarction in the MCA territory, which is due to microembolism to peripheral blood vessels. Moreover, recanalization spontaneously happens, so it is hard to evaluate thrombolytic therapies. Infarct lesions were variable in size, contralateral strokes occurred often, and ischemia accompanied by many small clots did not resemble typical clinical ischemic stroke. Size (length and diameter) and the biological properties of the blood clot (fibrin-rich) are found to be critical for the significance and reproducibility of the model.

b. Non-clot Embolus models

Many compounds have been utilized to create synthetic emboli, which are inserted into the ICA, mainly in rats. Microsphere embolization is the mainly used model, in which the degree of ischemic damage depends on the number of emboli utilized. The lesions in comparison with intraluminal models need more time to develop (24 h nearly). But, the ischemia is permanent which does not resemble most clinical conditions so limit the applicability of the model.

3. Photochemically Induced Thrombosis

A cortical infarct is induced in this model by systemic administration of a photoactive dye (rose Bengal) with irradiation by a light beam transfer through the intact skull with definite wave length. Oxidative injury to the endothelium occurred due to altered dye, which causes platelet aggregation in the irradiated zone. A drawback of the model is the vasogenic edema and disruption to the blood - brain barrier, which occurred in minutes and that, does not allow penumbra formation. So, this model is considered to be inappropriate for preclinical drug studies.

Nevertheless, a new model solved this drawback and caused ischemia over a long time, and produced a penumbra-like area. In addition to non-invasive and reproducible induced infarction in any cortical area, are noticeable advantages of the photochemical infarct model.

4. Endothelin-induced MCAO

Endothelin-1 (ET-1) is a natural peptide, which causes vasoconstriction and many models utilize this as a mediator to cause MCA stroke. Invasive methods have been greatly substituted with stereotactic intracerebral administration of ET-1 next to the MCA, which prevent complications of surgery.

When ET-1 is injected to the MCA there is obvious reduction in the cerebral blood flow (CBF) in its area, causing an ischemic lesion similar to the direct MCAO. This model may be beneficial in restorative drug researches. Remarkably, after a period (~20 minutes) of intensive CBF decrease, the return of blood flow to normal is slow and progressive depending on the dose of ET-1. This is a disadvantage due to variability unless the dose is cautiously standardized in the model.

5. Surgical MCAO (Mechanical or electrical arterial occlusion with craniotomy)

This involves direct surgical occlusion of MCA and models combining MCA and other vessel occlusions. Surgical focal cerebral models are invasive, and they need craniotomy which exposes the brain to the atmosphere and affects intracranial pressure and blood brain barrier (BBB) function in addition damage to the surrounding autonomic nerves caused by clipping or cauteterization of the MCA.

The rat is the most widely used species to undergo stroke with surgical technique. Among several approaches, the orbital route has been found less traumatic. While electro-cauteterization of portion of MCA results in permanent occlusion, the use of microclips and ligature snare allows reperfusion.
Tandem vessel occlusion techniques involve electro-cauterization of the distal MCA on the surface of the brain, and unilateral 69 or bilateral common carotid artery occlusion 70 (three vessel occlusion model).

3. Invitro models for stroke

3.1. Chemical and enzymatic methods

In this method the researchers used chemical substances such as rotenone, antimycin and sodium azide to inhibit the electron transport chain. The chemical and enzymatic methods may be suitable for high throughput (HT), owing to ease of application and rapid response, but this is at the expense of relevance (for example chemical hypoxia produces more free radical than anoxia)71.

3.2. Oxygen–glucose deprivation (OGD)

Ischemia-like conditions can be mediated by replacing the normal O2/CO2 equilibrated medium with N2/CO2 equilibrated medium and keeping cells in a hypoxic chamber. Which can be incorporated with glucose deprivation, many in vitro studies have demonstrated hypoxia alone leads to dramatic alterations in endothelial cell (EC) actin cytoskeleton and tight junction protein localization in BBB models. However, the physiological relevance of anoxia without glucose depletion is most significant to carbon monoxide poisoning, thus in the modeling of stroke, OGD is most appropriate. GD alone is rarely utilized to model stroke and over 4h does not considerably influence survival of rat cortical neurons in culture. However, in organotypic mouse hippocampus cultures, GD alone for 2.5h caused a significant CA1 cellular injury. This can be explained by the relative sensitivity of CA-1 neurons to ischemia. In OGD reperfusion experiments, neuronal injury occurs over several hours, instead of returning to standard culture conditions with similar observations as in vivo I/R injury71. In vitro OGD-reoxygenation cell culture model is considered as an excellent preparation to examine the cellular mechanisms mediating ischemia-reperfusion injury and/or cytoprotection72.

3- Excitotoxicity

In vitro models permit study of excitotoxicity in isolation of ischemic damage, through application of glutamate receptor agonists such as N-Methyl-D-aspartate (NMDA) or glutamate73.

4. Mechanisms of brain injury following cerebral ischemia and reperfusion

The mechanisms underlying ischemia reperfusion (I/R)- induced neuronal death implicate a complex interplay of myriad pathways. The primary cause of cell damage in cerebral is the reduction of CBF. The secondary cause is the triggering of several destructive mechanisms, such as edema, energy failure, glutamate receptor-mediated excitatory, oxidative/nitrosative stress, release of inflammatory mediators, calcium overload, as well as up-regulation of apoptotic genes73.

Although restoring circulation is a mandatory therapeutic tactic to revive ischemic tissue and prevent stroke related disability, it also and ironically, initiates a cascade of events that may lead to additional cell injury, known as reperfusion injury 74.

Reperfusion injury is a type of brain damage caused by reoxygenation of energy-deprived cells resulting in progression of vasogenic edema, generation of reactive oxygen species (ROS), and inflammatory response 3. (Figure 1 a)

4.1. Energy failure and ionic changes

Brain is mainly reliant on the continuous steady flow of glucose and oxygen to carry out oxidative phosphorylation for energy production because it has no stores of energy and depletion of its ATP happens in 3-5 minutes only 76,77,78.

The first result of reduction in CBF is the loss of substrates, mainly oxygen and glucose, which cause buildup of lactate via anaerobic glycolysis. Tissue acidosis may promote free radical formation, interrupting intracellular synthesis of protein and worsen injury of the ischemic brain 79,80, 81.

Na+/K+-ATPase, and Ca2+/H-ATPase pumps are perturbed due to energy loss; in addition to reversal of the flow of Na+-Ca2+ transporter. This is followed by dyshomeostasis of ions (Na+, Ca2+, Cl- ions are increased intracellularly and K+ ions are increased extracellularly) which leads to cytotoxic edema and causes events activated by increase of intracellular Ca2+ ions82 (Figure 2).

4.2. Excitotoxicity

Excitatory synaptic transmission is critical for normal information processing and is facilitated by glutamate through the stimulation of different receptors. Glutamate can interact with at least five receptors: low and high affinity kainite, N-methyl-D-aspartate (NMDA), and alpha-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) that are ligand gated ionotropic receptors and quisqualate receptor, which is a G-protein-linked metabotropic receptor 84, 85.

Kainite and AMPA receptors seem to be linked to the same type of ion channel that causes Na+, K+ and H+ influx and depolarizes the membrane. Membrane depolarization results in Ca2+ entry through voltage sensitive calcium channels, as well as opening of NMDA receptors, which also causes the entry of Ca2+, Na+ and K+86.

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The activation of glutamate receptors is a common physiological mechanism in which Ca\(^{2+}\) increase is terminated by the reuptake of excess glutamate. However, excessive stockpile of glutamate can be observed during ischemia \(^{87}\). This is due to release of glutamate by cellular depolarization \(^{86}\) and in response to Ca\(^{2+}\)-mediated stimulation of presynaptic terminals \(^{88}\). In addition, glutamate release is coupled with the inhibition of its ATP-dependent reuptake by astrocytes and presynaptic neurons \(^{89}\). The reuptake of glutamate is mediated by five types of Na\(^{+}\) dependent glutamate receptors, two of them located on astrocytes and the other three are neuronal \(^{90, 91}\). In addition, severe ischemia can reverse Na\(^{+}\)-glutamate transporters on astrocytes leading to leakage of glutamate and exaggerating the damage \(^{89, 92}\).

The prolonged availability of extracellular glutamate leads to excessive activation of its receptors with the sustained abnormal elevation on intracellular Ca\(^{2+}\), which in turn aggravates a neurotoxic cascade that leads to ischemic brain damage \(^{93, 94}\). Furthermore, it has been reported that glutamate-induced neuronal cell death is accompanied with apoptosis, as evidenced by characteristic DNA fragmentation, alterations in morphology, stimulation of calpain (cysteine protease), and cysteinyl aspartate-specific protease-3 (caspase-3). In addition apoptosis inducing factor (AIF) are up-regulated and / or translocate from mitochondria to cytoplasm and nuclei. Thus, it is suggested that glutamate, at higher concentrations, may induce apoptosis, possibly by executing caspase-dependent and caspase-independent mechanisms \(^{85}\).

4.3. Disturbance of calcium hemostasis

Tymianski et al. \(^{96}\) had reported that Ca\(^{2+}\) is modulated by the balance of several processes: influx and efflux across the cell membrane, release and uptake from intracellular stores and binding to intracellular proteins. Influx is by voltage-gated or ligand-gated Ca\(^{2+}\)channels. Ca\(^{2+}\) is extruded from neurons by Ca\(^{2+}\)-ATPase and Na\(^{+}\)/Ca\(^{2+}\) exchange. Release from intracellular stores is also managed by channels which are opened by inositol triphosphate(IP\(_3\)) and possibly by Ca\(^{2+}\) itself, whereas re-uptake occurs into stores by Ca\(^{2+}\)-ATPase\(^{97}\).

Brain ischemia/reperfusion disturbs this system at many levels \(^{98}\). ATP reduction through ischemia leads to depolarization of neurons, which opens voltage-gated Ca\(^{2+}\) and Na\(^{+}\) channels. Neuronal depolarization also results in release of glutamate, which opens ligand-gated Ca\(^{2+}\) (NMDA) and Na\(^{+}\) (AMPA) influx. The effect is Ca\(^{2+}\) and Na\(^{+}\) influx \(^{86, 99}\). Depolarization together with dissipation of the Na\(^{+}\) gradient results in reversal of the Na\(^{+}\)/Ca\(^{2+}\) exchanger and further Ca\(^{2+}\) influx \(^{100}\).

Moreover, glutamate can react with metabotropic receptors, which results in activation of phospholipase C to yield inositol triphosphate (IP\(_3\)) that binds to its receptor on the endoplasmic reticulum. This prompts more release of Ca\(^{2+}\) from intracellular stores into the cytosol; thus, there will be a sustained increase in Ca\(^{2+}\) because there is no ATP to drive sequestration or efflux \(^{101}\).

Intracellular Ca\(^{2+}\) overload has a main role in post-ischemic neuronal injury\(^{82}\). This can be done through the stimulation of Ca\(^{2+}\)-dependent enzymes, such as proteases, phospholipase, endonucleases, protein kinases, and nitric oxide synthases \(^{102}\). Ca\(^{2+}\)-dependent activation of caplains leads to the breakdown of vital proteins, such as microtubule-associated proteins (MAP-2) and spectrin to disturb neuronal cytoskeleton \(^{103}\). It also has a role in the transformation of xanthine dehydrogenase to xanthine oxidase (XO), which causes metabolism of xanthine to its ROS, O\(_2\)\(^{-}\) \(^{104}\). Additionally, Ca\(^{2+}\)-dependent activation of caplains causes breakdown of Bcl-2 interacting domain death agonist (Bid), a pro-apoptotic member of B cell lymphoma-2 (Bcl-2) family to truncated Bid (tBid) \(^{105}\), which targets the outer mitochondrial membrane, aids release of the cytochrome C, and encourages neuronal injury \(^{19}\).

4.4. Oxidative stress

It was found that oxidative stress is a main mechanism of cerebral I/R damage \(^{104}\). Reestablishing oxygenated blood flow to the ischemic tissue is important for restoring aerobic ATP synthesis; it also leads to release of ROS \(^{82}\).

The brain is highly susceptible organ to oxidative stress damage because it extensively continues polyunsaturated fatty acids and regions of the brain have elevated contents of iron, which can contribute in the increased liberation of ROS \(^{106}\). Besides, the brain features, which are high metabolic activity, low antioxidant capacity \(^{104}\), low repair mechanism activity and non-replicating nature of neuronal cells \(^{107}\).

Under normal conditions, mitochondria utilize oxygen to produce ATP via oxidative phosphorylation. Resulting in release of small amount of superoxide anion (O\(_2\)) and hydrogen peroxide (H\(_2\)O\(_2\)) which are safely metabolized to H\(_2\)O through superoxide dismutase (SOD), catalase (CAT), and the peroxidase \(^{108}\).

In case of ischemia, lactate builds up which leads to tissue acidosis. Tissue acidosis induces pro-oxidant and harmful alterations in neural cells such as the deactivation of antioxidant mechanisms, oxidant iron release from proteins \(^{109}\), and toxicity of glutamate increase \(^{110}\). Other sources to ROS in ischemia are the enzyme XO and depolarization of mitochondria \(^{111}\).

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After restoring supply of oxygen, the generation of ROS by mitochondrial dysfunction elevates dramatically\textsuperscript{112}.

Harmful ROS generated during I/R injury are unpaired electron molecules, for instance superoxide anion (O$_2^-$), hydroxyl radical and peroxynitrite (ONOO$^-$), in addition to non-radical molecules such as hydrogen peroxide (H$_2$O$_2$). They are chemically unstable and reactive molecules distorting tissue of neuronal cells accompanied with secondary cell death\textsuperscript{113}.

Severe production of ROS can lead to cellular injury and consequently death of cell, because ROS may oxidize critical components of cell, like lipids, proteins and DNA\textsuperscript{114}.

Lipid peroxidation produces lipid hydroperoxides that are consequently degraded into many radical species and aldehydic yields in reactions that frequently contain transition metal as catalyst\textsuperscript{115}. After I/R injury, it has been reported that aldehydic yields, as 4-hydroxynoneal and malonaldehyde (MDA) are built-up as final yields of lipid peroxidation\textsuperscript{116}, which covalently alters membrane transporters such as Na$^+$/K$^+$ ATPase, glucose transporter and glutamate transporter, thus hindering their function\textsuperscript{117}.

Additionally, proteins can be targets of oxidative alteration leading to cross-linking, fragmentation, and aggregation of protein\textsuperscript{118}. Oxidation of protein can disturb receptors, function of enzymes, and signal transduction pathway\textsuperscript{119}.

Attack of DNA by ROS leads to DNA strand breakage, mutations, and changes in transcription and translation processes\textsuperscript{120}. DNA strands break can cause stimulation of poly ADP-ribose polymerase (PARP), which changes gene expression, DNA replication, and may initiate apoptosis\textsuperscript{121}. Overstimulation of PARP causes deprivation of NAD$^+$ and ATP pools, finally leading to cell death by necrosis due to loss stores of energy\textsuperscript{122}.

Confirmations recommend that increased production of ROS not only initiate the damage of cell membrane and other structures of cell by inducing lipid peroxidation and changing membrane phospholipid, but also cause mitochondrial dysfunction\textsuperscript{123}. This happens by opening of the proton permeability transition pore (PTP), which collapses the proton motive force needed for oxidative phosphorylation and ATP production. Subsequently, this causes the release of mitochondrial pro-death proteins, such as cytochrome c, which triggers apoptotic signaling pathways\textsuperscript{124}.

Many pro-oxidant enzymes have a role in the generation of ROS, as nitric oxide synthetase (NOS), cyclooxygenase (COX), xanthine oxidase (XO), nicotinamide adenine dinucleotide phosphate (NADH) oxidase (NOX) and myeloperoxidase (MPO)\textsuperscript{125}.

4.4.6. Neuroinflammation

The inflammatory responses of brain following reperfusion are characterized by a rapid activation of resident cells (activated microglia/macrophages, astrocytes), production of pro-inflammatory mediators.
and infiltration of leucocytes in the ischemic brain region and in stroke patients.

Activated microglia and astrocytes cause neuronal damage by the activation of NF-κB, which is a redox-sensitive transcription factor that causes critical inflammatory cascades following I/R. These cascades involve the expression battery of downstream pro-inflammatory enzymes, cytokines and cell adhesion molecules (CAMs).

NF-κBIs mainly presented as a heterodimer of p50 (NF-κB1) and p65 (Re1A) subunits. In resting cells, the inhibitor NF-κB proteins (IκBs), containing IκBa and IκBβ, maintain the inactive NF-κB dimmers into cytosol. But, when stimulation occurs, IκB kinase (IKK) phosphorylates IκB proteins, leading to their ubiquitination and proteosomal degradation. This cascade frees and stimulates NF-κB to translocate into nucleus and reacts with promoter region of specific genes, triggering their transcription. NF-κB can be stimulated by many factors known to be induced after I/R, such as glutamate, increased intracellular calcium, ROS, and inflammatory cytokines.

During I/R, the activated NF-κB reacts with specific DNA sequences called κB sites; enhancer domains exist in pro-inflammatory genes, triggering their transcription. NF-κB can be stimulated by many factors known to be induced after I/R, such as glutamate, increased intracellular calcium, ROS, and inflammatory cytokines.

5. Estrogen and stroke

Estrogen receptor-alpha (ER-α) causes neuroprotective effect against cerebral ischemia

Estrogen exerts most of its actions in the body by interaction with two estrogen receptors: estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β). The structures of two receptors have remarkable homology, but have different function, localization and expression pattern in the brain. The receptors consist of seven domains, react with E2 in a high affinity, and they dimerize and use the estrogen response elements in the same way. But many differences present themselves between ER-α and ER-β, like it has been found that they have a different binding domains and each one is expressed by a different gene. They also differentially signal at the activation function-1 (AF-1) site in the existence of E2, where E2 stimulates transcription at ER-α but it prevents transcription at ER-β, respectively.

ER-α, and ER-β are found in the nucleus of cells, but extra-nuclear presence also has been confirmed in the cytoplasm and membrane of cells and neuronal cells. Both receptors are involved in genomic signaling and non-genomic signaling in cells. Another difference between them is tissue distribution of receptors, ER-α is expressed in breast, uterus, ovary and brain, while ER-β is found in lungs, kidney, heart, bone, endothelial cells and brain.

Most studies propose that ER-α has the chief role in causing neuroprotection induced by E2. It was found that the neuroprotection of E2 that has been shown against PCI, is lost in ER-α KO mice, but maintained in ER-β KO mice. Moreover, studies of antisense knockdown confirmed the main role of ER-α, but not ER-β in causing neuroprotection by E2 in the hippocampal (CA1) region in rats after global cerebral ischemia. Also, administration of selective agonist of ER-α, propyl pyrazoletriol (PPT) has also been documented to cause neuroprotective effect in the hippocampal (CA1) region after GCI. E2 causes its neuroprotection through many effects on multiple

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cell types in the brain, including neurons, microglia, astrocytes, and endothelial cells. Other studies suggest that ER-β may have a neuroprotective effect in certain conditions. For example administration of a selective agonist of ER-β such as WAY 200070-3, has been reported to cause neuroprotective effect in the hippocampal (CA1) region of rats after GCI, and another one found that the agonist of ER-β receptor , as DPN, decreased global cerebral ischemia injury in the hippocampal (CA1) region of mouse by 55%. Moreover, the phytoestrogen, genistein, has also been reported to exert neuroprotective effect in the hippocampal region against GCI, and this effect was inhibited by treatment with specific antagonist of ER-β receptor. These studies propose that exogenous stimulation of ER-β can cause neuroprotective action against cerebral ischemia.

5.2. Estrogen has neuroprotection due to numerous mechanisms

5.2.1. Estrogen and control of ROS and oxidative stress

ROS, especially superoxide, have been involved to play a main role in the neurons death after cerebral ischemia. The precursor of most ROS is the superoxide anion radical (O$_2^-$) containing the extremely toxic and damaging hydroxyl ion and peroxynitrite. It is documented that after either permanent or transient FCI, ROS are markedly increased in the cortex and other regions in brain. It has been reported that the ROS have shown a steady increase in the cortex during a 3h measurement post permanent cerebral ischemia. It was also found that ovariectomized female rats exposed to permanent cerebral ischemia caused an increase in the generation of superoxide anion in cortex from 1 to 3 h. Additionally, O$_2^-$ generation was elevated rapidly in the hippocampus(CA1) region after GCI in either male or female rats, with an increase starting early 30 min following reperfusion and peak noticed at 3h following reperfusion. On contrary, it was observed that E2 treatment decreased the increase of O$_2^-$ levels in the hippocampus(CA1) region after cerebral ischemia, which related to its neuroprotection. More studies reported that the E2 reduction of O$_2^-$ levels was correlated with a severe decrease of oxidative stress injury in the hippocampus (CA1) region after 24h of cerebral ischemia. The neuroprotective effect of estrogen against ROS and oxidative stress owes to direct and indirect antioxidant property.

5.2.2. Estrogen decreases NADPH oxidase activity

In vitro studies have proposed that there are three mechanisms for producing ROS in neurons of hippocampus and cortex through hypoxia/reoxygenation. The studies reported that the mitochondria liberate the initial burst of ROS during hypoxia, then xanthine oxidase (XO) through the delayed phase, and finally NADPH oxidase-produced ROS production during reperfusion.

E2 has protective effects on mitochondria to reserve mitochondrial function. These effects contain regulation/preservation of ATP production, ROS generation, apoptotic factors of mitochondria, and antioxidant effects.

It was noticed that NADPH oxidase activity, is rapidly increased in the hippocampus (CA1) region after GCI in ovariectomized rats, which peaked 3h after reperfusion, while E2 treatment significantly decreased elevation of NADPH oxidase activity in the hippocampus(CA1) region after cerebral ischemia, which is associated with suppression levels of O$_2^-$.

E2 prevented GTPase, Rac1, in an Akt-dependent manner activation after cerebral ischemia, which is essential for NADPH oxidase initiation.

5.2.3. Anti-Inflammatory Effects of estrogen

Anti-inflammatory functions of estrogens have been reported in a various studies, and are mainly taken as essential mechanisms for estrogens’ neuroprotection in stroke. Estrogens have been observed to cause a lot of anti-inflammatory effects for example, decreasing leukocyte adhesion attenuating pro-inflammatory cytokine generations, preventing monocyte activation and changing activation pattern of the microglial.

5.2.4. Estrogen (E2) neuroprotection is mediated via extranuclear receptor signaling

It has been mostly believed that E2neuroprotection in the brain occurs mainly by genomic signaling pathway which is mediated by nuclear ER, through binding E2 with nuclear ER and controlling transcription of many genes that cause neuroprotective effects. For example, E2 has been observed to augment expression of the anti-apoptotic gene, as bcl-2, in the ischemic penumbra following FCI and GCI. E2 also increases bcl-2in rat neurons of hippocampus in vitro, while it prevents production of pro-apoptotic BAD (bcl-2-antagonist of cell death) and prevents production of the anti-apoptotic pro-survival factor, surviving after GCI in the hippocampus CA1 region, which causes survival of neurons. Expression of brain derived neurotrophic factor (BDNF) in the brain is enhanced by E2, which has been considered as a neuroprotective factor and essential for synaptic learning, plasticity and memory.

Besides genomic signaling, there is growing confirmation that rapid non-genomic signaling through

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membrane localized extranuclear ER may possibly have a role in causing E2 neuroprotection in the brain. Many studies have reported that the rapid stimulation of extracellular signal-regulated kinases (ERKs) by E2 is important for its neuroprotective action, since treatment with MEK inhibitor inhibits E2 neuroprotective effects in neurons in vitro. Also, E2-promoted ERK stimulation in the CA1 region following GCI, which is important for its neuroprotection since administration of MEK inhibitor prevents induction of activation of ERK by E2 and neuroprotection of the hippocampus.

Similarly, the role of the pro-survival serine kinase Akt in E2 neuroprotection has been involved, as E2 causes rapid Akt activation in neurons of cortex in vitro, and in the hippocampus (CA1) region in vivo after GCI, while administration of PI3K inhibitor decreases the neuroprotection of E2 in vitro and in vivo. Furthermore, it was found that E2 decrease the rapid stimulation of the pro-apoptotic signaling kinase, JNK in the hippocampus(CA1) region following GCI. All together, these findings recommend that E2 led to rapid non-genomic signaling, which may have a main role in E2 neuroprotection.

After binding of E2 to ER, a macromolecular signaling complex is composed with the IGF-I receptor in the plasma membrane; the complex recruits and stimulates downstream kinases as ERK/MAPK, which leads to phosphorylation and activation of nuclear transcription factors like CREB, that regulates target genes important for neuron survival. CREB is involved in neuronal survival by increase expression of BDNF and the anti-apoptotic protein Bcl-2, which stabilizes the integrity of the mitochondrial outer membrane and inhibits the caspase death cascade, permitting neurons to survive. E2 maintains ERK and CREB activation which opposes ischemia by inducing down-regulation of Bcl-2. Besides that it was found that estradiol binding to cytoplasmic receptors causes recruitment of phosphatidylinositol 3-kinase (PI3K) to the plasma membrane and phosphorylation of phosphatidylinositol. Activated PI3K-p110 catalyzes the phosphorylation of membrane phosphatidylinositol-4,5-bisphosphate (PIP2) to yield phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 and its phospholipid phosphatase product, phosphatidylinositol-3,4-bisphosphate, accumulate in the membrane, creating docking sites for Akt. Akt becomes active by phosphorylation on both Thr308 and Ser473, which is catalyzed mainly by phosphatidylinositol-dependent kinase (PDK). Activated PI3K/Akt induces cell survival by phosphorylating and inactivating various pro-apoptotic proteins to deliver the protective signal. Inhibition of Rac1 activation by Aktis down-regulated through autophosphorylation of mixed-lineage kinase 3 (MLK3) and the activation of c-Jun N-terminal kinase 3 (JNK3), which, sequentially, regulates the activation of c-Jun and Fas-ligand (Fas-L) expression. On the other hand, inhibition of JNK3 attenuates the serine phosphorylation of 14-3-3, increases the 14-3-3–Bax complex in the cytosol, and subsequently inhibits Bax.

**Figure 3.** Summary diagram depicting the neuro-protective mechanisms of E2 via nuclear and extra-nuclear signaling pathways.
translocation to the mitochondria and the release of cytochrome c (Cyt c)\(^{193,194,195}\).

We can conclude that either genomic or non-genomic pathway of E2 finally lead to increase stabilization of mitochondria outer membrane by increasing expression of antiapoptotic genes such as Bcl-2 that consequently led to decrease production of ROS, which is the chief step in apoptosis of neuron cell by oxidation of all vital cell component as previously described in oxidative stress\(^{174,192}\).

5.2.5. Estrogen action on proliferation, synaptogenesis and regeneration of neuronal cell

The possibly estrogenic neuroprotection on neuronal cells proliferation, synaptogenesis, synaptic connectivity and regeneration is mediated via E2 by controlling expression of many growth factor genes through ERs’ combination with ERE in gene promoters. The factors affected by this mechanism are vascular endothelial growth factor (VEGF), TGF-\(\alpha\), BDNF, and NGF\(^{196}\).

5.2.6. Effect of estrogen on glucose

Estrogens enhance expression of glucose transporter subunits. So, they facilitate glucose transport into neuronal cells. Additionally, they increase expression of glycolytic enzymes such as hexokinase, phosphofructokinase, phosphoglycerate kinase, and components of pyruvate dehydrogenase complex\(^{173}\).

CONCLUSION

Cerebral I/R is a dangerous neurovascular disease that leads to high rate of mortality worldwide. Various mechanisms are implicated in its pathogenesis such as oxidative stress, inflammation, excitotoxicity, mitochondrial dysfunction and ion disturbances. Beside that it was found that the incidence of stroke in postmenopausal women is higher and accompanied with more severe harmful effects than premenopausal ones. We showed here the different studies that have reported that estradiol has neuroprotective effect via multiple mechanisms against cerebral ischemia in both male and ovariectomized rats.

Conflict of Interest

The authors declare that they don’t have any kind of interest.

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