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Animal Models of Retinal Ischemia

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1. Introduction

Retinal ischemia is a frequent source of irreparable visual impairment and even loss of sight, affecting over a hundred million individuals in the world. It is associated with a wide range of clinical retinal disorders, like ischemic optic neuropathies, obstructive retinopathies, carotid occlusive disorders, diabetic retinopathy and glaucoma. Retinal ischemia occurs when the blood supply to retina is inadequate to meet the metabolic requirements of the retina. If treatment is not given to fix this imbalance, the outcome is irreversible, ischemic and apoptotic cascades resulting in cell death. Appropriate study models, particularly animal models, are necessary for further understanding the etiology, pathology, and evolution of retinal ischemia and also in order to help in the evaluation, development, and improvement of therapeutic strategies. Accordingly, quite a few *in-vivo* and *ex-vivo* mammalian models have been developed to study this syndrome. The rat models of retinal ischemia are frequently used, because the distribution of retinal and choroidal blood supply is quite similar to that in humans.

The retina has been extensively used for the study of pathophysiology of ischemia and mechanism of damage triggered by ischemia and excitotoxicity. Compared to all the other tissues, retina has a higher metabolic rate; any disturbance in blood supply can have an effect on the supply of oxygen and the substrates leading to retinal ischemia. The retina has a dual blood supply. The photoreceptors and most of the outer plexiform layer (OPL) are nourished by choriocapillaries, while the inner retinal layers are nourished by the central retinal artery. The actual effects of retinal ischemia vary, depending on the position of the occlusion. It is clear that occlusion of the retinal artery leads to inner retinal ischemia only, but occlusion of ophthalmic artery leads to global retinal ischemia, as it supplies blood to the central retinal artery as well as choriocapillaries.

2. Retinal architecture

The retina of mammals is a functionally specialised tissue. It is capable of light detection and perception as well as processing and transmission of the information received to the central nervous system. It has two major elements – the neurosensory retina and the pigment epithelium (RPE). During the embryonic development, the RPE and neural development are

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derived from the same layer, i.e. the neuroectoderm, although they are morphologically not similar. Hence, they are considered collectively as "retina".

The retina is made up of three principal layers of nuclei, which are, from internal to external, the thin ganglion cell layer, the inner nuclear layer and the outer nuclear layer (Figure 1). The ganglion cell layer consists of cell bodies of various classes of ganglion cells and amacrine cells. The inner nuclear layer is the layer with nuclei of bipolar cells, amacrine cells, horizontal cells and Muller cells. And the outer layer is the one which contains the nuclei of rod and cone photoreceptors. There are also two plexiform or synaptic layers that are not filled with any cell nuclei. The inner plexiform layer (IPL) lies between the ganglion cells and inner nuclear layer and the outer plexiform layer (OPL) is sandwiched between the outer and inner nuclear layer. These synaptic layers contain axons and dendrites, which aid in early visual processing and also help in adjusting to different light intensities.

The retina contains a rich assortment of cell types including light-sensing photoreceptors. The outermost layer of the cells in the retina is the RPE. It is a simple cuboidal epithelium, containing melanosomes that help in quenching photons that are not absorbed and therefore, minimise light scattering. RPE also has other biological functions like maintenance of choroidal vasculature and blood-retinal barrier. The retinal photoreceptor cells are specialised neurons found in the outer retina. The diversity of inner retinal neurons is really complex. Bipolar cells span from the OPL to the IPL, in which they form synapses with photoreceptor cells and ganglion cells respectively. The nuclei of bipolar cells are found in the inner nuclear layer. Amacrine cells in themselves as a group are considerable in number as well as diversity. These are present in both inner nuclear layer plus the ganglion cell layer where they are involved in relaying impulses (Masland, 1988). Another type of cells that also occur in the retina are the ganglion cells. Ganglion cells have long axons that are pass through the optic nerve (Berson, 2007). Glial cells are also found in the retina. These cells support the retinal microenvironment. These include Muller cells, astrocytes and microglia. Another type of cell found in the ganglion cell layer and optic nerve head are the astrocytes. These cells contribute to the blood retinal barrier (Kaur *et al*., 2008).

3. Retinal blood supply

As mentioned earlier, the cause of retinal ischemia is insufficient supply of blood, which is unable to meet the metabolic demands of the retina. When occlusion occurs in any tissue, anatomy of blood supply plays a significant role. The retina has a higher metabolic rate, even than that of the brain. The retina is a specialised extension of central nervous system and has a complex and dual blood supply, i.e the choroidal and the retinal. The choroid gets the maximum blood supply (around 65-85% of total supply to the eye), whereas the retina gets just 20-30%blood (Henkind, *et al*., 1979). The photoreceptors in the outer nuclear layer and the outer plexiform layer are nourished indirectly from the choriocapillaries; whereas, the inner retinal layers are nourished by branches of central retinal artery, which arises from the ophthalmic artery as the central retinal artery enters the retina, it divides into four main branches. The retinal blood vessels also help in maintaining the blood-retinal barrier. Outer retinal layer ischemia is caused by occlusion in choriocapillaries. On the other hand, complete retinal ischemia and infarction require ophthalmic artery occlusion (Saint-Geniez and D'Amore, 2004).

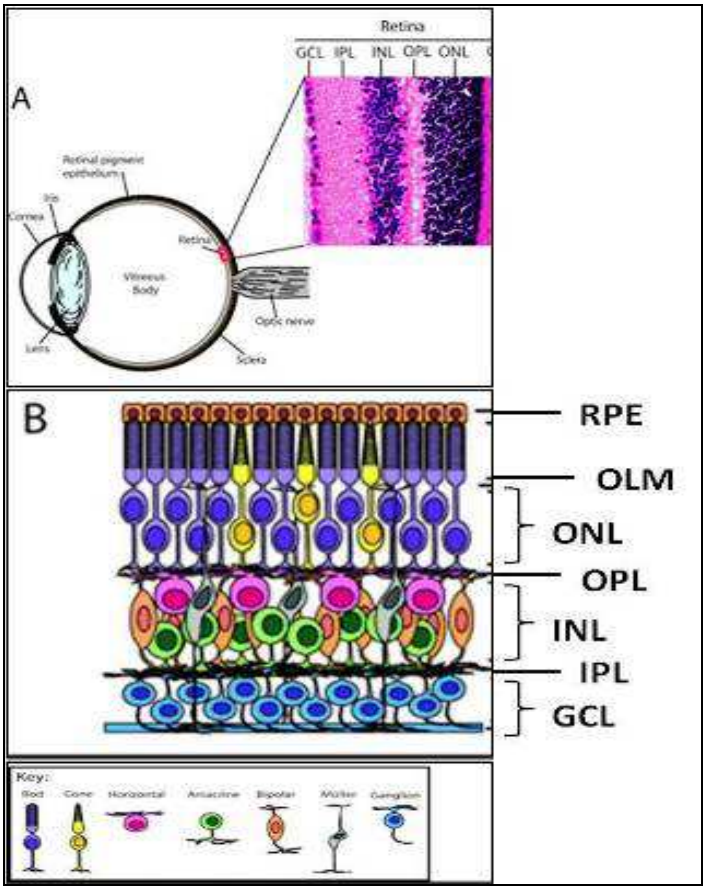

Fig. 1. Different cell layers in retina (where RPE – Retina Pigmented Epithelium, OLM – Outer Limiting Membrane, ONL – Outer Nuclear Layer, OPL – Outer Plexiform Layer, INL – Inner Nuclear Layer, IPL – Inner Plexiform Layer, GCL – Ganglion Cell Layer) *(reproduced and readapted with permission – Poche & Reese, 2009)*

4. Correlation between the brain and the retina

The retina shares many functional, embryological and anatomic characteristics with the brain. In humans, the eye starts developing at about 3 weeks of pregnancy. The eye is mainly derived from three types of embryonic tissue – neuroectoderm forms retina, pigment cell layers and optic nerves, mesoderm leads to cornea, sclera and blood vessels and the ectoderm forms the lens. At 22 days of embryonic stage, a pair of optic vesicles is formed on each side of the forebrain. These vesicles form connections with the developing central nervous system through stalk-like structures. As the development progresses, these stalks become thinner and form optic nerves. Thus, it is a representative of the CNS. The mesoderm in the embryo forms the blood vessels – the hyaloid artery and vein, that nourishes the developing lens, which later on in development transform to the central artery

and the vein. The retina can be visualised directly, thus, it can be used to study stroke. Many retinal conditions are associated with stroke, such as occlusion of middle cerebral artery also leads to retinal ischemia, as can be seen in figure 2, where it shows that the ophthalmic artery originates nearby to the MCA. Thus, the mechanisms that affect the eye and the brain are linked to some extent. But, the examination of retina to predict future stroke incidence is still doubtful.

Retinal neurons and glia show same response to ischemia as the neurons in the other part of the CNS. Retina also has a blood-retinal barrier similar to the blood-brain barrier (Tso & Jampol, 1982). These two also differ in the resistance to the ischemic injury. The retina can survive much longer than the brain. Also, the retina shows geographical difference in sensitivity to ischemia, the outer retinal layers being more prone to the injury than the inner ones.

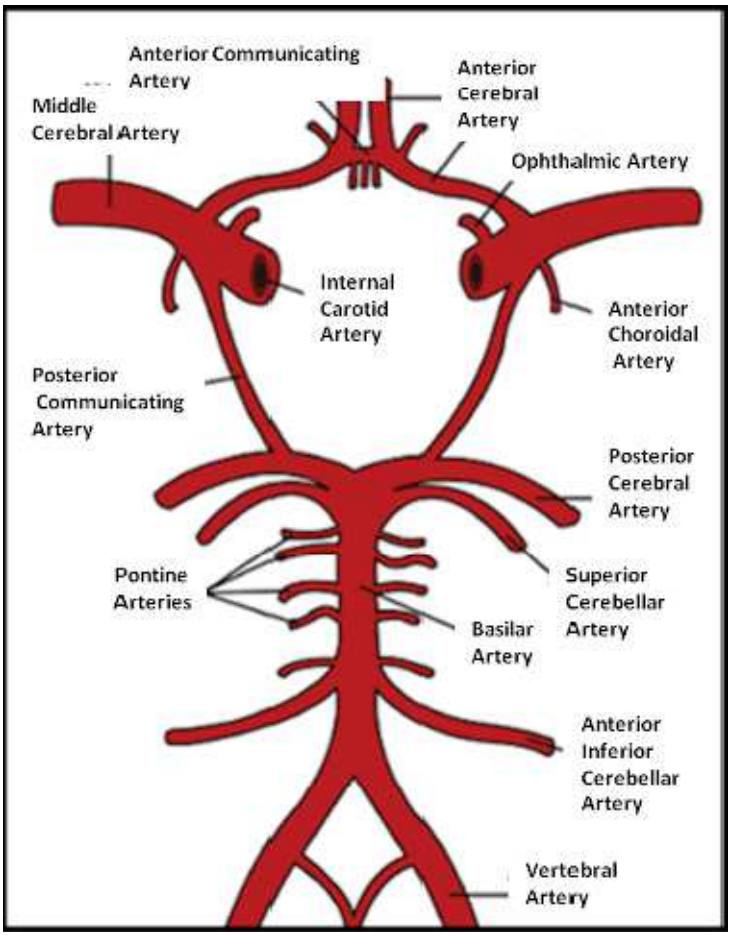

Fig. 2. The figure depicting Circle of Willis showing blood supply to brain and retina (*Reproduced with permission from M. F. Block, 1997)*

5. Susceptibility to retinal ischemia

Various changeable and non-changeable factors are involved in ischemia, such as age, family history, ethnic group, and previous medical history. Kawai *et al* studied the different risk factors related to neuronal injury using an intraocular pressure rat model. The number of retinal ganglion cells (RGCs) keeps on decreasing with age and the residual RGCs become more susceptible to damage. Calorie intake has also been proven to be a factor involved in ischemia. The diet restriction has a neuroprotective effect and thus, leads to lesser damage to RGCs. As seen in the case of glaucoma, pre-existing diabetes is a reason for greater harm to RGCs (Kawai *et al*, 2001).

Genetic background is one of the principal determinants of susceptibility to retinal neovascularisation and breakdown of blood retinal barrier. Vulnerability to ischemia-induced retinal neovascularisation depends on the strain of animal model used. Strain difference in rat model leads to variation in expression of VEGF and thus, causes an increase in permeability and leakage of fluid and plasma proteins, resulting in edema. It has been demonstrated that Brown-Norway rats are more susceptible to Sprague-Dawley rats (Gao *et al*., 2002).

It is seen that a certain percentage of cell death that occurs in transient cerebral and retinal ischemia occurs by means of apoptosis. An apoptosis cascade involves both pro-apoptotic and anti-apoptotic genes. One of these is p53, which is a DNA-binding transcription factor involved in DNA damage and repair. Ischemia leads to increase in expression of p53. The p53 causes selective vulnerability of inner retina to transient ischemia. Also, it is observed that transgenic mice lacking in p53 are resistant to excitotoxicity. Mice heterozygous for null mutation in p53 gene are resistant to retinal ischemia. From the above discussion, it is safe to say that p53 can be one more target for therapeutic strategy for retinal ischemia (Zhang *et al*., 2005).

6. Pathophysiology of retinal ischemia

Retina has a really high metabolic rate. Glucose and oxygen deprivation can harm the whole retina, but all cells are not equally vulnerable. The loss of cells due to ischemia is irregular. The retinal cells that lie near the blood vessels are exposed to an environment rich in oxygen and thus, are more prone to the ischemic damage. But, another thing to consider is that these cells are the first one to be cured on reperfusion. Temporary interruption of blood circulation prevents the exchange of metabolic substrates and products, affecting cells in retina. However, in addition, there are many indirect effects which are sustained even after restoration of blood supply. These effects may be systemic, such as respiratory or vasomotor centre failure or the outcome can also be localised, i.e. impaired reperfusion, edema or breakdown of blood-brain barrier. Thus, evidently, these effects are so complicated that it is hard to identify the order of events leading to damage.

Retinal ischemia causes a number of morphological and functional changes. These changes are a product of combined and inter-related pathophysiological pathways – leading to imbalance in ion transport, changes in neurotransmitter levels, neuronal depolarisation, oxidative stress, and energy failure. The occurrence of ischemia leads to a complex cascade of response to energy failure and ATP depletion which eventually causes cell death. Studies have shown that substrate deprivation is the less damaging as compared to oxygen deprivation, which reduces protein synthesis. The irreversible damage increases with an increase in the duration of oxygen deprivation. The cells which are the most affected are the

photoreceptor cells as these have the maximum oxidative metabolic rates. But when both substrate and oxygen deprivation are combined, a reduction in the ATP synthesis is observed. This leads to energy metabolism failure. The process can be explained as follows - decrease in ATP levels disrupt the Na^+/K^+ ATPase transporter, leading to disruption of membrane potential and ion gradients, preventing the repolarisation of axons and synaptic membranes. There are many studies which testify to the discharge of various neurotransmitters, e.g. GABA, glycine, dopamine, acetylcholine, after the occurrence of ischemia. During the ischemia the receptors for different neurotransmitters, present on the retina, are opened in response to the elevated levels of their ligands, e.g. GABA, glycine, extracellularly. Under normal circumstances, neurotransmitter levels are low extracellularly. Glutamate is recognized as the major excitatory retinal neurotransmitter. It is released by photoreceptor bipolar cells and the ganglion cells. During retinal ischemia glutamate gets accumulated in the extracellular space (Louzada-Junior *et al*., 1992). Lucas and Newhouse showed the occurrence of glutamate excitotoxicity in ischemia. Neurons in inner retina and ganglion cells are more susceptible to ischemia due to the incidence of high levels of glutamate receptors. Glutamate causes neurotoxicity by several different mechanisms, i.e., increase in Ca^{2+} ion levels, Na^+ influx, which depolarises the plasma membranes. There are a number of receptors which can activate glutamate neurotoxicity. These can be NMDA as well as non-NMDA based receptors (Lucas & Newhouse, 1957). In 1992, Osborne stated the role of NMDA – based excitotoxicity in retinal ischemia. NMDA receptors are Ca^{2+} permeable, and thus, increase in glutamate levels raises the Ca^{2+} ion levels intracellularly. Excess glutamate causes Na^+ influx, which is followed by Cl^- influx, which cannot be countered by outward efflux as the membranes are impermeable to most intracellular anions. Therefore, transport of cations and Cl^- ions increases the intracellular osmolarity, causing osmotic shock, edema and cell lysis and death (Osborne *et al*., 1992). The voltage gated Ca^{2+} channels are also opened, leading to rise in intracellular levels of Ca^{2+} ions, which inhibit the mitochondrial metabolism. Another group also validated the involvement of NMDA as well as non-NMDA based receptors in retinal degeneration. Romano *et al*., in an *in-vitro* model of ischemia in the retina from a chick embryo reported by using the blockers of both NMDA and non-NMDA receptors that the damage to the retina is due to overexcitation of the receptors (Romano *et al*., 1998). Ueda *et al* also showed that the NMDA caused damage to blood vessels in the retina. They created a rat model by injecting NMDA into the eye, which led to loss of retinal ganglion cells and thinning of the inner plexiform layer, thus damaging the inner retinal layers and also led to loss of endothelial cells in the blood vessels in the retina (Ueda *et al*., 2010).

Reperfusion, i.e. restoration of blood supply after the ischemic injury, can also lead to cell damage. Oxygen restoration to the deprived tissue can add up to the injury caused by ischemia (Jennings *et al*., 1960). Also, it has been shown that LDH, marker of cell death increases after oxygen restoration (Sims *et al*., 1992).

7. *In-vitro* models of retinal ischemia

Most of the cell culture models for ischemia utilise primary nerve cell cultures, that are exposed to insults associated with ischemia *in-vivo*, e.g., glutamate neurotoxicity, glucose and oxygen deprivation. One of the approach involved chemical ischemia in immortalised rat retinal ganglion cell line (RGC-5). They used iodoacetic acid (IAA), a known inhibitor of enzyme glyceraldehyde 3-phosphate dehydrogenase. This IAA treatment induces changes

seen in retinal ischemia, such as disturbance in membrane potential, ATP loss and reactive oxygen species generation (Malur *et al*., 2008). Another way of inducing ischemic-like changes *in-vitro* is to incubate retinal pigmented epithelium (RPE) cells with oligomycin (an ATP synthase inhibitor) and sodium cyanide (inhibits cytochrome-c oxidase), along with IAA (Palmero *et al*., 2000). In-vitro model can also be induced by glucose/oxygen deprivation.

These in-vitro models can be used for testing and identifying novel neuroprotective compounds. Many herbal products are nowadays being used, the Chinese herbal medicines, in a large number of disorders, e.g. coronary heart disease, cardiovascular disease and traumatic wounds. Romano *et al* examined the neuroprotective activity of an extract from Chinese safflower (*Cartnamus tinchoris*, Honghua). An *in-vitro* model of retinal ischemia was made from chick embryo retina. The ischemia was generated by removing glucose from the media and growing the culture in nitrogen atmosphere. It was seen that Honghua protected the retina from the effects of toxins like NMDA and also from the ischemic conditions (Romano, *et al*., 1993).

Another herbal extract that has been used in Korea and China are derived from the shrub, *Thuja orientalis*. It has been shown to be effective in disorders such as, gout, diarrhoea and rheumatism. It was demonstrated by Jung *et al* in the transformed retinal ganglion cell line (RGC-5) *in vitro* that the extract of *Thuja orientalis* has anti-oxidant properties. In this study, RGC-5 cells were exposed to H_2O_2 to create oxidative stress. The major component found in this extract with anti-oxidant properties is the isoquercitrin, which can be in future used for treating glaucoma, but requires further investigation (Jung, *et al*., 2010). The roots of another plant, *Scutellaria baicalensis*, are also used in China. It contains three flavnoids – wogonin, baicalin, baicalein. These flavenoids are natural free-radical scavengers. Out of the three flavenoids found, baicalin has been shown to have neuroprotective action, but the mode of its action is unknown. The role of baicalin was observed in *in vitro* model in RGC-5 cell line, where it reduces the damage caused by reactive species and apoptosis (Jung *et al*., 2008). Matteucci *et al* have also tested the curcumin, the phenolic extract obtained from *Curcuma longa* in primary retinal cell cultures. It showed protective effect for both the retinal as well as hippocampal neurons from NMDA excitotoxicity. The mode of action of curcumin may be through the increase in production of NMDA receptor subunits (Matteucci, *et al*., 2011). But these *in-vitro* models have some limitations of their own, such as getting sufficient quantities of cells and obtaining reproducible and comparable data. Also, the *in vitro* models do not correlate much to the *in vivo* conditions as these do not provide apt physiological environment and are based on chemical interactions.

8. Need for animal models

Animal models have been a mainstay of basic and applied research. Animal testing has been used since second century where early writings by Greeks discuss its use. Dissection and experimentation has been used to get knowledge about anatomy and physiology in humans. The use of animal models has allowed the fast progression of scientific discovery. Animal models have had a central place in medical research, in developing new therapeutic strategies for treating human diseases as well as in preclinical trials. The aim of using animal models is to achieve better understanding of pathways involved in the disease without causing any harm to the human being. A number of animal models are available for studying the mechanisms of retinal ischemia. The vascular supply and pathways involved

in retinal ischemia in animal models must be better understood for developing new therapies for human disorders.
In-vitro models have always been used to get insight of biochemical and molecular events caused by ischemia; but animal models are essential in understanding pathophysiology of retinal ischemia. In all animal models of retinal ischemia, the retinal circulation is obstructed to study the balance between energy supply and demands, vascular and neuronal changes. Different species such as monkeys, rabbits, rodents, cats, dogs have been used as animal models. In choosing an animal model, several factors are considered like anatomy of vascular circulation and retina, relevance to humans and also the availability of the animals.

9. Desirable characteristics in animal models

Animal models are used to understand the mechanism behind a particular disease and also to discover new as well as validate the already therapeutics for it. Animal models can either be spontaneous or induced and can be developed in any species which fits best for the disease to be studied and the purpose of our study.
The model should show up same characteristics and symptoms as seen in human disorders. The model should have common features with the humans, such as anatomy, vascular system and retina in case of animal models for retinal ischemia. But at the same time these animals should be easy to manipulate, i.e. they should have small size, high reproducibility and easy for genetic manipulation. For testing the therapeutics, the animal models being used should mimic the humans and give same response as desired in the humans, so that the humans will show similar response. Other desirable characteristics include low cost, easy availability, easy to handle and breed and less prone to infections or diseases other than the desired one.

10. Animal models of retina ischemia

10.1 Raising the intraocular pressure

It is the most widely used method to study the mechanisms involved in retinal ischemia. Peachey *et al*. demonstrated that the high intraocular pressure (IOP) induced retinal ischemia model has been established as an important model system. IOP model creates ischemia by elevating and maintaining the intraocular pressure above the systemic arterial pressure (Peachey *et al*., 1993). Flower *et al* described the retinal ischemia model in cats. The IOP was raised to 110mm Hg by cannulation of anterior chamber with a 26-gauge needle, which is connected through nylon tubing to an elevated container with normal saline. This increase in intraocular pressure blocks the retinal blood circulation and thus, leads to ischemia (Flower *et al*., 1971). Following a similar procedure, a model of pressure-induced retinal ischemia/reperfusion injury was established in rats (Buchi *et al*., 1991).
It has been used to study changes in protein expression, excitotoxicity and alteration in membrane properties in various different models. IOP animal models have also been used to study changes in serum antibody reactivities after ischemia. Joachim *et al*. created IOP model by raising the pressure to 130mm Hg for an hour to check the antibody response to ischemia/reperfusion injury (Joachim, *et al*., 2011). On the other hand, Hirelinger *et al*. investigated the involvement of ion imbalance and role of Muller cells in degeneration of retina in a mouse IOP model (Hirelinger, *et al*., 2010).

Baicalin, a flavenoid found in roots of *Scutellaria biacalensis*, when was administered in rats intraperitoneally showed protection from the retinal ischemia'reperfusion injury, as was seen *in vitro.* The baicalin was given before subjecting the animals to raised intraocular pressure of 120mm Hg for 50 minutes (Jung, *et al*., 2008).
The principle limitation of this model include that the raised intraocular pressure itself can contribute to the resulting retinal damage. Thus, this model is complicated by a combination of ischemic as well as pressure-induced injury.

10.2 Cerebral artery occlusion

Acute thrombotic/embolic stroke in humans are often associated with temporary diminishment (amaurois fugax) or even permanent loss of vision. Middle cerebral artery occlusion (MCAO) is a purely vascular model of retinal ischemia that reproduces transient human vision loss. MCAO in rodents is one of the most widely used experimental paradigms to induce focal cerebral ischemia. This model occludes arterial blood flow intraluminally and allows reperfusion by removing the inserted filament. The ophthalmic artery that mainly supplies the inner retina originates from the internal carotid artery proximal to the origin of the middle cerebral artery. Therefore, it is expected that MCAO simultaneously obstructs blood flow in the ipsilateral retina. Block *et al* demonstrated the first evidence of retinal ischemia by MCAO in rats (Block *et al*., 1992). Steele *et al* have shown for the first time that MCAO simultaneously obstructs blood flow in ipsilateral retina in mice (Steele *et al*., 2008).
Kaja *et al* indicates that the MCAO/ reperfusion model is more appropriate than other retinal ischemia models such as high intraocular pressure or optic nerve ligation models to study the cellular and molecular changes in retina after stroke (Kaja *et al*., 2003). This model is non-invasive with respect to the eye and does not induce blood-retina barrier disruption or mechanical damage to retina. The model is reproducible and easily reversible and involves vascular structure of the entire eye. Therefore, MCAO is a more relevant model for studying changes and testing the efficacy of therapeutic strategies for retinal ischemia.
This model has been used to validate the effect of a various herbs of Chinese origin. The extracts from wolfberries *(Lycium barbarum,* Gougizi*)* mostly consist of polysaccharides and are believed to be good for the eye. It has also been shown previously in many studies that these extracts have protective effect against liver damage, ageing and oxidation (Ha, *et al*., 2005, Li, *et al*., 2007, Yu, *et al*., 2007).The group created a model by occluding the internal carotid artery in rats. The extracts were given orally for 1 week before the occlusion. This study showed that this pre-treatment with the extracts protected the retina from various conditions linked with retinal ischemia, such as, neuronal death, apoptosis, glial cell activation and blood-retinal barrier disruption (Li, *et al*., 2011).

10.3 Chronic carotid ligation

In two-vessel occlusion model, the occlusion is permanent and long-lasting and reperfusion does not occur. This model reduces the cortical/hippocampal blood flow to 25-50% of the normal levels in 2.5 hours post occlusion (Yamamoto *et al*., 2006). The blood flow in the retina may be more severely reduced than in the brain. Davidson *et al* have also shown that the two-vessel occlusion, i.e. the bilateral carotid artery occlusion causes an early loss of the pupillary reflex in 50% of the animals (Davidson *et al*., 2000). The 2-VO model mimics the ocular pathology of human carotid artery disease. The internal carotid artery (ICA) which

begins at the bifurcation of the common carotid artery (CCA) provides the major blood supply to brain. It also provides the blood supply to the eye through the ophthalmic artery.
Many studies have shown that bilateral common carotid artery occlusion in rat causes functional impairment of retina (Block *et al*., 1992). The electroretinogram have also demonstrated that b-wave amplitude representing bipolar and Muller cell activity in response to light exposure is decreased 7 days after the onset of 2VO (Barnett and Osborne, 1995). These functional changes are accompanied by structural damage. In animals that lost their pupillary reflex, the total retinal thickness decreased from approximately 120 µm to around 87 µm. The most affected layers being the synaptic zones in inner plexiform as well as the outer plexiform layer (Lavinsky *et al*., 2006).
In this model, occlusion of both CCAs cuts off blood supply to the retina, however, some retinal perfusion is maintained by retrograde blood flow to ophthalmic artery through the Circle of Willis. The degree of retinal damage also varies greatly within the same experiment, due to heterogeneity in tolerance towards ischemia in individual animal.

10.4 Photocoagulation of retinal vessels

There are only a few studies at present that report the ischemia of less than 5 minutes, which is often observed during the ocular surgeries. The faults that are present in other methods, such as invasiveness and inflammation in raising the intraocular pressure or ligation of optic vessels that causes changes in retina unrelated to ischemia, can be reduced by direct laser exposure of the main retinal vessels.
Kalamkarov *et al.* induced the characteristics of retinal ischemia in rats by direct laser coagulation of blood vessels using argon laser (Kalamkarov *et al*., 2000). Selective occlusion of vessels using laser permits creation of local and extensive retinal ischemia by choosing various retinal vessels and by modifying the exposure dose. In this technique, Rose Bengal, an iodinated photosensitive dye is injected intravenously through tail vein. The eyes are then exposed to 7 minutes of intense light (550nm, which is the absorption peak for this dye). Retinal vein occlusion was simulated in non-human primate model, i.e. cynomolgus monkey (*Maccaca fascicularis*). Dye yellow (577nm) laser light was used to occlude all branch retinal veins in the eye (Miller *et al*., 1994). Photodynamic thrombosis with green argon laser light and Rose Bengal dye was also used to create retinal ischemia model in pigs by occluding the retinal veins.
Laser coagulated Sprague Dawley rats were used to validate the effect of Hongua *in vivo*. In this model, Honghua extract was injected intravitreally, before they were subjected to Rose Bengal dye and laser (550nm). It is hypothesised that as the major component in the Honghua extract is glucose, the neuroprotective effect of the same could be due to availability of energy source after ischemia (Romano, *et al*., 1993). Another Chinese herbal medicine – Fufang Xuehuantong capsule was also studied by Yuan *et al*. They created a rat model for retinal vein occlusion by using laser photocoagulation and then validated the therapeutic benefits Chinese herbal medicine by quantitating the expression of various growth factors in the animal model (Yuan *et al*., 2011).

10.5 Central retinal artery occlusion

In humans, CRAO results in severe retinal ischemia, resulting in irreversible damage within hours. A minimal invasive model of transient retinal ischemia was introduced by Dangeliene *et al*., which involves photothrombotic central retinal artery occlusion (CRAO)

using intravenous injection of Rose Bengal and green laser irradiation of CRA in rats (Dangeliene *et al*., 2000). Rose Bengal is a photosensitive dye that releases oxygen free radicals when irradiated by the laser. This active oxygen results in intraluminal thrombus formation and thus, occlusion of CRA. Another retinal ischemia-reperfusion model through the occlusion of central retinal artery involves placing a suture behind the eye globe, including the CRA and ciliary artery. Both ends of the suture were then passed through a small plastic tube and ischemia is caused by pressing the tube against the artery. Prasad *et al* used the central retinal artery ligation model in rats to compare between different occlusion times as well as different time-period of reperfusion. They studied gene expression of various transcription-related genes after 30 and 90 minutes of occlusion and at 3 hours and 12 hours of reperfusion (Prasad *et al*., 2010).

10.6 Endothelin administration

Transient obstruction of central retinal artery can also be obtained by injecting vasoconstrictive drug. This method is less invasive, simple and does not require any special equipment. Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced by the vascular endothelial cells. Endothelin is found naturally in various tissues and is involved in a variety of biological activities. It has been linked with pathophysiology of various human disorders, e.g. cardiovascular, renal and ocular. Endothelin-1 can cause apoptosis of neurons in the CNS (Syed, *et al*., 2006). Endothelin-1 causes cell death through mechanism involving free-radicals (Oku, *et al*., 2008). Sugiyama *et al* have shown the association of endothelin with glaucoma. The authors investigated the effect of ET-1 on rabbit eye and observed that the intravenous as well as intravitreal administration of ET-1 reduces both the intraocular pressure and the blood flow in the optic nerve (Sugiyama, *et al*., 1995). Granstam *et al* demonstrated similar effects of endothelin in a cat model (Granstam, *et al*., 1992). Masuzawa *et al* have shown that a high dose of ET-1 when injected under the conjunctiva obstructs the central retinal artery without any damage to other tissues. Endothelin-1 causes retinal ganglion cell loss and activates glial cells (Masuzawa *et al*., 2006). It has also been seen that the intravitreal administration of ET-1 affects the retinal arteries directly. A dose of 10^{-7} M ET-1 led to decrease in the diameter by 17% (Bursell, *et al*., 1995).

Endothelin-1 also causes constriction of arteries *in-vitro*. Yu *et al* showed that ET-1 dose causes constriction even in cryopreserved human retinal arterioles. But the relation between the dose and the related activity is still not known (Yu, *et al*., 1998).

Thus, in this method there is no problem regarding inflammation or infection. But, like any other method being used, this method too is not completely free from drawbacks. The dose of endothelin-1 used is quite high, which may pass into the systemic circulation and exert some undesired effects in other tissues.

11. Current and potential therapeutic strategies for retinal ischemia

Many strategies have been used but have not been successful or have shown various limitations and are at experimental stage. Current treatments available for retinal ischemia include intravitreal or retinal vein administration of tissue-plasminogen activator (t-PA), hemodilution, pan-retinal laser photocoagulation or anti-VEGF antibodies or medication (Lucentis or Avastin). Occlusion of retinal vessels or retinal ischemia leads to retinal neovascularisation. Laser photocoagulation is used to decrease the neovascularisation in retina and thus, the oxygen demand. The decrease in overall oxygen requirement will stop

ischemia and hence, further damage to the eye. Ischemic conditions also cause up-regulation of expression of angiogenic factors, such as vascular endothelial growth factor or VEGF. VEGF is involved in angiogenesis and causes abnormal vessels growth or neovascularisation. To reduce the damage of ischemia, oxygen supply to retina has to be improved. Therefore, anti-VEGF drugs can be directly injected in the eye, e.g. the drugs that have been most tested in animal models include, bevacizumab, ranibizumab, pegaptanib sodium. Corticosteroids, such as, dexamethasone, are also under experimentation, as these inhibit VEGF and inflammatory factors (Lattanzio, *et al*., 2011). Another technique that holds promise is the use of anti-VEGF antibodies. Neutralising anti-VEGF monoclonal antibodies have been demonstrated to block neovascularisation when administered in a primate model of laser-induced retinal ischemia. Aiello *et al* showed the in-vivo inhibition of VEGF with the help of VEGF- neutralising proteins-containing extracellular domain of human (Flt) or mouse (Flk) VEGF receptors attached to IgG. These chimeric proteins showed 100% reduction in neovascularisation with human Flt and 95% with murine Flk domains. The suppression of VEGF was dose-dependent (Aeillo *et al*., 1995).

There are many other drugs and chemical compounds which have been tested in animal models of retinal ischemia with positive results and hope for future therapeutics. Cao *et al* in 1994 provided the evidence for the neuroprotective effect of NMDA antagonists in retinal ischemia. They showed that the NMDA receptor antagonist, dextromethorphan has a protective effect after retinal ischemia. However, it is not still clear whether dextromethorphan works via NMDA receptors. Other NMDA antagonists, such as MK-801 or memantine also protect from retinal ischemia (Lam *et al*., 1997 and Osborne, 1999). Blockers of voltage-gated Ca^{2+} channels, e.g. nifediprine and betaxolol also decrease neurotoxicity by reducing Ca^{2+} ions influx (Melena *et al*., 1999).

Another approach can be the use of the free- radical scavengers. Free-radicals play an important role in the damage caused by retina ischemia. Free-radicals are formed when reduced compounds, which accumulate during ischemia, are reoxidised during reperfusion. This free radical burst causes oxidative stress (Gilgun Sherki, 2002). Intravenous injection of SOD reduces the development of edema in rat model. SOD or superoxide dismutase is a well-known scavenger of superoxide radicals. Another compound that can be used is dimethylthiourea or DMTU, which is a synthetic compound that traps $OH^{\cdot}$, H_2O_2 and other free radicals. Intravitreal injections of both SOD and DMTU have been shown to lead to recovery in IOP-induced ischemia rat model. DMTU (75µg/eye) resulted in 40% functional recovery when assessed through electroretinogram. SOD, on the other hand, leads to 99% functional recovery on post-treatment and 81% on pre-treatment. Thus, all these chemical compounds and drugs that decrease or reverse the cause of ischemia can be helpful in reducing the damage to some extent.

RNA interference (RNAi) is a natural phenomenon in mammals, which is involved in silencing of gene expression. It involves a double-stranded RNA which cleaves any RNA complimentary to it. RNAi has been proposed to be used in therapeutics by downregulating the expression of specific genes. Reich *et al* used the technique of RNA interference in retinal cells *in vitro*, as well as *in vivo* in the mouse retina. They used this technique to downregulate the VEGF expression, which is known to be upregulated in retinal ischemia (Reich *et al*., 2003). RNAi can be further investigated and tested for other genes, e.g. cytokines that are upregulated in the pathophysiology of retinal ischemia.

Stem cell therapy is a promising technique for tissue repair and regeneration. Advances in the field of stem cells have lead to their use in treatment of various disorders (Lenka &

Anand, 2010, Rajarathna, 2009). Stem cells basically are unspecialised cells which are capable of self – renewal and under specific defined environment these cells can form functionally specialised cells. The stem cells can either be obtained from early embryos or certain tissues in adults, such as umbilical cord and peripheral blood, bone marrow. They work through either replacing damaged cells or through the factors released by them. Stem cells have been used in various vascular neurodegenerative diseases and most of the ocular disorders involve problem in either of the two. Eye is an accessible organ and with large number of animal models available, the use of stem cells poses a promise for preserving functionality (Cogliati & Swaroop, 2009).

Adult bone marrow contains hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs), which can differentiate into various cell types of myeloid and endothelial lineages. Bone-marrow contains stem cells which can either differentiate into Lin+ (hematopoietic lineage) or Lin- (non-hematopoietic lineage). Lin- population contains progenitor cells that differentiate into vascular endothelial cells, i.e. the endothelial progenitor cells (EPCs). Many preclinical and clinical studies have shown bone-marrow derived cells contribute to neoangiogenesis during wound healing, retinal ischemia, myocardial infarction, neonatal growth and tumor growth. Lin- population have been shown to express neuronal markers after transplantation in brain or retina in various mouse models. Bone-marrow cells differentiate into neuronal cells, astrocytes *in-vitro* and also *in-vivo* when injected intravenously into brain of mouse model (Mezey, *et al*., 2000). Also, it has been shown that BMCs can differentiate into retinal neural cells *in-vivo* (Woodbury, *et al*., 2000). Lin- bone-marrow stem cells when injected intravitreally into photocoagulated retina of a mouse model, migrated to astrocytes and formed retinal vessels. Ischemic conditions release cytokines that recruit EPCs to the site. Ischemia results in up-regulation of angiogenic factor, vascular endothelial growth factor or VEGF-A, which has its receptors Flk-1 and Flt-1 on EPCs, HSCs and HPCs, thus leading to their migration to the site (Kalka, *et al*., 2000). But their importance in clinics is still unknown as the success depends on their functional incorporation.

Mesenchymal stem cells (MSCs) also found in bone marrow and other tissues such as cord blood, peripheral blood, fallopian tube, and fetal liver and lung have been used in over a range of different clinical trials (US NIH clinical trial database – www.clinicaltrials.gov), including those in fractures, diabetes, heart and liver disease and neurological disorders. MSCs have the potential to differentiate into neurons, especially retinal neurons. These cells also secrete molecules that modify the environment for the surrounding cells. MSCs for instance, express a number of neuroprotective factors, such as BDNF, CNTF, IGF, bFGF and NGF, which protect the injured retina. MSCs have another remarkable property, i.e. the homing potential; they can migrate to pathological areas (Prabhakar, *et al*., 2010). They can migrate from blood circulation to brain, spinal cord, and eye (Kan, *et al*., 2005). Thus, MSCs have shown neuroprotection in various neurodegenerative models, but clinical translation is still questionable.

Another source of stem cell therapy is the embryonic stem cells (ESCs), obtained from the inner cell mass of blastocyst. ESCs can differentiate into various cell types, such as hematopoietic cells, astrocytes, hepatocytes, glial cells, neurons. Wei *et al*., 2005, showed that the transplantation of human embryonic cells in MCAO stroke model led to structural as well as functional recovery. Transplanted ESCs differentiated into neurons, astrocytes, oligodendrocytes and endothelial cells. These ESC – derived endothelial cells can form vascular-like structures *in-vivo* as well as *in-vitro*, thus induce angiogenesis (Levenberg, *et*

al., 2002). Embryonic stem cells have issue of ethical restrictions as well as immune rejection. Thus, alternate source of stem cells was identified from non-pluripotent cells. Every nucleated cell in an individual has identical genome, except the gametes. Different cell types are identified on the basis of the genes that are expressed. In 2006, four transcription factors – *Oct 3/4, Sox-2, Klf-4* and *c-myc*, that are capable of reprogramming DNA were identified (Takahashi & Yamanaka, 2006). Forced expression of these specific genes in a non-pluripotent cell lead to a pluripotent stem cell, known as induced pluripotent stem cell (iPC). Human iPCs have been derived successfully from patients with neurological disorders – Parkinson's disease, muscular dystrophy, Huntington's (Park *et al*., 2008). iPC from the skin cells of an amylotrophic lateral sclerosis (ALS) patient have been differentiated into motor neurons (Dimos *et al*., 2008). Takahashi group for the first time generated photoreceptor cells from the embryonic stem cells (Takahashi & Yamanaka, 2006). The same method has been used to create human photoreceptor and retinal pigmented epithelium phenotype (Hirami *et al*., 2009). Human neuronal cells can also be generated from iPC (Karumbayaram *et al*., 2009). Human umbilical cord blood is a well known source of hematopoietic stem cells and has been used in various disorders. Umbilical cord blood contains higher percentage of hematopoietic stem cells than the bone marrow and also poses a lesser risk of immune rejection. The cells from cord blood have the potential to form retinal neuronal cells.

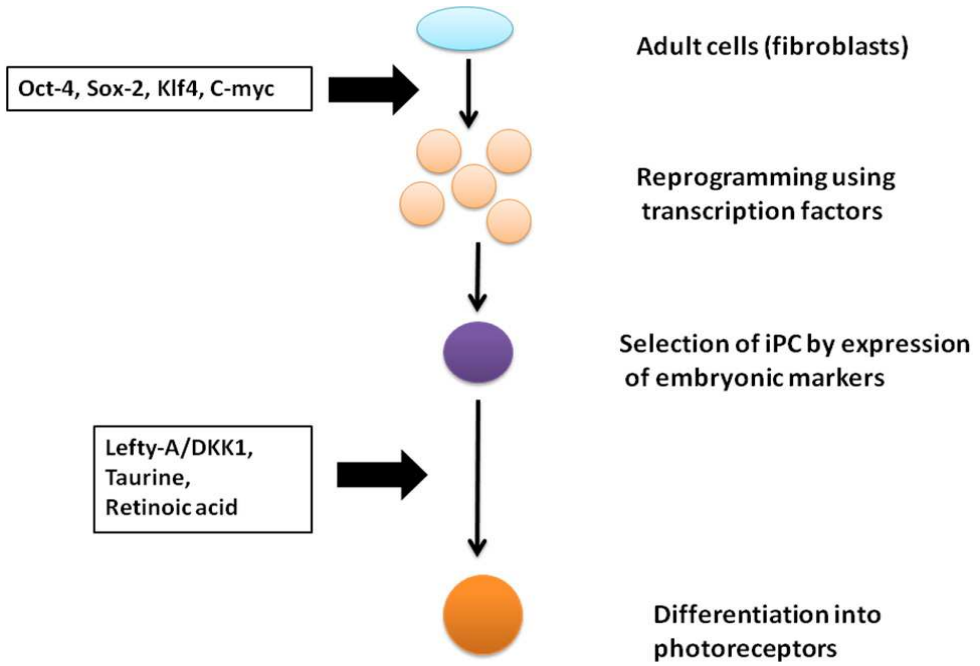

Fig. 3. Formation of iPC from adult somatic cell

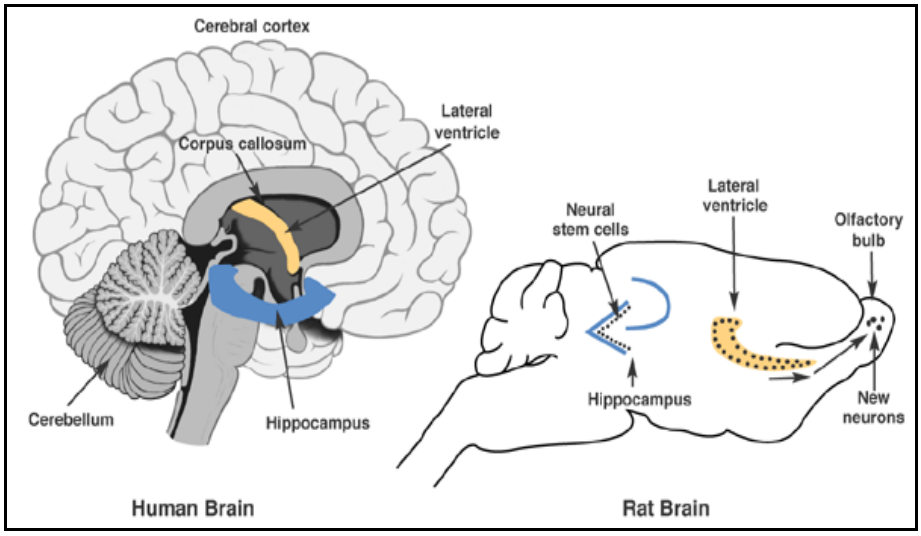

Fig. 4. Different sources of neural stem cells (NSCs) in human and rat brain *(Reproduced with permission http://pubs.niaaa.nih.gov/publications/arh27-2/197-204.htm)*

The mammalian central nervous system was considered is a non-renewable tissue. But studies have demonstrated that neural stem cells (NSCs) do exist not only in developing CNS, but also in adult nervous system of all mammals and are capable of differentiating into neurons, astrocytes and oligodendrocytes. Neural stem or progenitor cells can be isolated from various parts of CNS, such as hippocampus, subventricular zone, spinal cord and ependyma. Palmer *et al* for the first time isolated NSCs from the hippocampus of an adult rat. These hippocampus-derived NSCs have an ability to migrate and differentiate into neuronal lineage in injured retina, but are unable to form retina-specific cells (Nishida *et al.*, 2000). Embryonic retina derived neural progenitors can differentiate into photoreceptors *in-vitro* (Ahmad *et al.*, 2004). NSCs have an advantage over the embryonic stem cells with respect to their clinical translation, i.e. NSC can be expanded through numerous passages in-vitro and can be easily manipulated (English & Anand, 2010) . But, further studies are required to derive retinal neurons from the NSCs.

But there are some limitations and barriers for stem cell transplantation in retina, as retina shows poor cell integration. Like any other part in the central nervous system, the retina too is rigid to cell migration. Thus, most of the cells that are transplanted do not reach the retina. It has been shown that only 1% of intraocularly transplanted cells reach the retina (Johnson, *et al.*, 2010).The stem cell therapy holds a promising future in retinal disorders, but the problems need to be dealt with before clinical translation.

Another therapeutic approach that shows a promising future is the concept of personalised therapy, where the candidate genes linked with various eye disorders can be identified and the genetic make-up of an individual can be used for disease prediction and treatment.

12. Conclusion

Retinal ischemia is a common cause for visual impairment and vision loss. It is a condition related with many different human disorders, such as diabetic retinopathy, glaucoma, and it

occurs when the blood supply is not sufficient to meet the demand. The retina and brain share common development pathway. Retina, like CNS, originates from ectoderm, however, it can be non-invasively studied. Also, as the retinal blood vessels share many features with the cerebral blood vessels, the investigations can be extrapolated to brain pathology. Thus, studying the retina, through the retinal ischemia models can help in understanding the mechanism and pathophysiology of stroke as well as in validating potential therapeutics. But all the methods discussed here have their own strengths and limitations. Out of all the above mentioned animal models, pressure elevation model is commonly used as it is easily reproducible and it mimics many human disorders – central retinal artery occlusion (CRAO), glaucoma, occlusion of ophthalmic artery. For example, some methods require penetration of a needle through the cornea, and must be fixed in the anterior chamber for one hour. The invasiveness can lead to inflammation and other damages. Similarly, unilateral and bilateral occlusion of carotid artery requires specialised skills in vascular surgery. It may cause incomplete ischemia and all of these procedures alter blood flow to the brain. Likewise, photocoagulation is simple but has many disadvantages including variable degrees of exposure and hence, variable damage. Besides, the ischemic damage caused is permanent because of which reperfusion cannot be studied. The choice of animal models for pre-clinical testing, therefore, depends on the research questions which have been raised.

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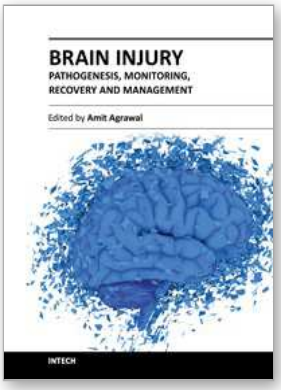

Brain Injury - Pathogenesis, Monitoring, Recovery and Management

Edited by Prof. Amit Agrawal

ISBN 978-953-51-0265-6
Hard cover, 522 pages
Publisher InTech
Published online 16, December, 2011
Published in print edition December, 2011

The present two volume book "Brain Injury" is distinctive in its presentation and includes a wealth of updated information on many aspects in the field of brain injury. The Book is devoted to the pathogenesis of brain injury, concepts in cerebral blood flow and metabolism, investigative approaches and monitoring of brain injured, different protective mechanisms and recovery and management approach to these individuals, functional and endocrine aspects of brain injuries, approaches to rehabilitation of brain injured and preventive aspects of traumatic brain injuries. The collective contribution from experts in brain injury research area would be successfully conveyed to the readers and readers will find this book to be a valuable guide to further develop their understanding about brain injury.

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