Review article

Folia Neuropathologica

Mechanisms related to neuron injury and death in cerebral hypoxic ischaemia

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Folia Neuropathol 2011; 49 (2): 79-87

Abstract

Cerebral hypoxic-ischaemic injury is involved in many central nervous system diseases. The mechanisms of neuron injury and death in cerebral hypoxic ischaemia remain unclear. There have been many theories on pathogenesis of neuron injury and death in cerebral hypoxic ischaemia, such as the toxicity of excitatory amino acid, NO, the production of oxygen free radicals, chondriosome injury, complement component, injury of immunological inflammation, matrix metalloproteinase, dopamine, Ca^{2+} overloading, cell apoptosis and so on. The aim of this review is to describe recent observations regarding the mechanisms of neuron injury and death in cerebral hypoxic ischaemia.

Key words: *neuron, hypoxic ischaemia, excitatory amino acid, NO, free radical, cell apoptosis, immunological inflammation, chondriosome, complement component, matrix metalloproteinase, dopamine, calcium.*

Introduction

The brain is an important organ of human beings. Its weight is about 2% of the whole body weight, but the brain consumes up to 25% of all the oxygen the body needs. Most mammalian neurons have a low tolerance to brain anoxia because of severe arterial hypoxia [19,40]. Hypoxic-ischaemic (H-I) brain injury is a major cause of acute mortality and chronic neurological morbidity. The cause of cerebral neuron injury during ischaemic events is an area of major interest to neuroscientists.

It is now well appreciated that a cerebral H-I event related to the depletion of tissue energy reserves is rapidly followed by acidosis, glutamate excitotoxicity, production of oxygen free radicals and oxidative stress, and followed by prolonged periods of delayed cell death or apoptosis, inflammation and so on [13]. The anoxia-induced vulnerability appears to be related to the profound rise of the intracellular concentration of free Ca^{2+} [48]. A large body of evidence suggests that nitric oxide biosynthesis is a key factor in the pathophysiological response of the brain to H-I [33]. And the generation of nitric oxide triggers a cascade of free radical reactions, leading to modifications of cerebral plasticity and increasing blood-brain barrier (BBB) permeability, which may be a contributory factor to the progression of H-I encephalopathy [27,44]. This short review will focus on the mechanisms of neuron injury and death in cerebral H-I.

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The effect of excitatory amino acids

Excitatory amino acids (EAAs) are widely distributed in the mammalian central nervous system and play an important role in excitatory synaptic transmission, but are toxic to neurons. Glutamate (Glu) and aspartate (Asp) are the main EAAs. Under normal conditions, Glu and Asp mainly exist in the vesicle of nerve endings. H-I energy metabolic dysfunction has direct inhibitory effects on the activity of Na^+-K^+-ATPase in the cell membranes, and this induces a higher level of extracellular K^+ concentration. EAAs are released into the extracellular space when depolarization of neurons occurs. When cerebral ischaemia occurs, the inflow of extracellular calcium increases, followed by increased intracellular Ca^{2+}, which might activate phospholipase A2. Phospholipase A2 acts on membrane phospholipid and changes membrane structure. Thus amino acids diffuse outside the cell along a concentration gradient to result in an increase in the efflux of EAAs [39]. Benveniste found that the extracellular contents of Glu and Asp were increased, respectively, eight- and three-fold after a 10-min period of transient complete cerebral ischaemia. The concentration variation of EAA in brain was correlated with the degree of brain damage after acute cerebral ischaemia-reperfusion [25,50].

Reuptake and absorption by neurocytes is the only way to inactivate Glu in the nervous system. H-I could result in neuron and glia releasing EAAs and reducing the capacity for reabsorption and deactivation [13]. EAA transporters (EAATs) play a major role in this process. The glutamate transporters EAAT3 and EAAT4 are expressed in neurons. They contribute to the cellular uptake of Glu and Asp and thus to the elimination of the excitatory transmitters from the extracellular space. The destruction of the Na^+/K^+ transmembrane gradient leads to the reverse transportation of EAATs in the membrane when cerebral ischaemia occurs. Neurons depolarize because of potassium efflux and energy depletion, by which the Glu diffuses outside the cell along the Na^+ concentration gradient. Glu uptake was inhibited and the release of arachidonic acid was enhanced. And arachidonic acid could significantly inhibit Glu uptake by astrocytes for a long time. Recently Glu transporters have emerged as a potential therapeutic target in a wide range of neurological disorders. There is downregulation of Glu transporters EAAC1 and GLT-1 after rat microsphere embolism and it is associated with extracellular Glu concentration [20]. But there is transient enhanced expression of EAATs in the subcortical white matter early after ischaemia to limit excitotoxicity by means of removing extracellular Glu more efficiently and thus generating a refined Glu environment [2]. The study provides overwhelming evidence that ceftriaxone, a GLT-1 (a major Glu transporter) modulator, caused a significant upregulation of GLT-1 mRNA and protein and induced a significant increase in [^{3}H]-glutamate uptake, which confers neuroprotection in cerebral ischaemia/reperfusion injury [53].

EAA receptors are the primary excitatory neurotransmitter receptors in the central nervous system and are divided into two major categories: ion tropic receptors and metabotropic receptors. Ion tropic receptors contain *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainite receptors. The increase of synaptic tyrosine kinase activity is not only related to the increase in tyrosine phosphorylation of the NMDA receptor and the higher level of basal phosphorylation which would contribute to the increased excitability of the NMDA receptor supporting normal cerebral development, but also renders the brain more vulnerable to H-I damage [18]. The results demonstrated that there was enhanced phosphorylation of the NMDAR subunit, NR1, by PKC and PKA after ischaemia and which may contribute to alterations in NMDA receptor function in the postischaemic brain [8]. The importance of metabotropic Glu receptors in brain ischaemic injury remains uncertain. Glu induces cell death by activating type I metabotropic Glu receptors (mGluRs) [21]. mGluR1 after transient focal ischaemia is involved in the activation of Src and the increase in NADPH oxidase activity that is mediated by PKCδ. mGluR1 antagonist could modify properties of the NMDA receptor, attenuates infarct size, and reduces NADPH oxidase activity and superoxide production after transient focal cerebral ischaemia [36,37]. Metabotropic Glu mGlu5 receptor-mediated serine phosphorylation of NMDA receptor subunit NR1 in the hippocampal CA1 region may be linked to the pathogenesis of cerebral ischaemia and the mGlu5 receptor antagonist could reduce neuron death in this region [49]. But the activation of mGlu4 receptors limits the development of brain damage after permanent or transient focal ischaemia and

plays a protective role [35]. The early cellular swelling occurring during cerebral ischaemia is a result of massive ionic fluxes mediated by EAAs which are released by a Ca^{2+}-dependent exocytotic process from the nerve terminals [23]. Glu increases intracellular Na^+ concentration of neural cells by acting on AMPA receptors on the cell membrane. Meanwhile Cl^- enters cells along the potential difference. The entry of Cl^- and positive ion leads to the influx of a large amount of water that causes acute oedema of neurons. The over-activation of NMDA receptor and excessive stimulation of NMDA receptor can activate another signalling molecule, nNOS, which is mediated by PSD-95 and is crucial for neuronal injury after cerebral ischaemia [55,62]. NMDA or AMPA could worsen the BBB disruption. Any insult increasing the release of EAAs could further aggravate the BBB disruption and brain oedema in the focal cerebral ischaemic period [9].

The experiments showed that the protective effects of EAA inhibitors against cerebral ischaemia are associated with depressing the extracellular levels of amino acid transmitters in brain of rats [57] (Fig. 1).

Nitric oxide and free radicals

Nitric oxide (NO) is closely correlated with H-I neuron apoptosis since NO biosynthesis is a key factor in the pathophysiological response of the brain to H-I (Fig. 1). But the role of NO in H-I injury is actually far more complex than conceived. A study showed that NO mediated the neurotoxicity of Glu, abnormality of mitochondrial energy metabolism and impairment of antioxidant status, which may account for Glu-mediated neurotoxicity via a mechanism involving NO biosynthesis in rat neurons in primary culture [5]. Superoxide and NO are able to form

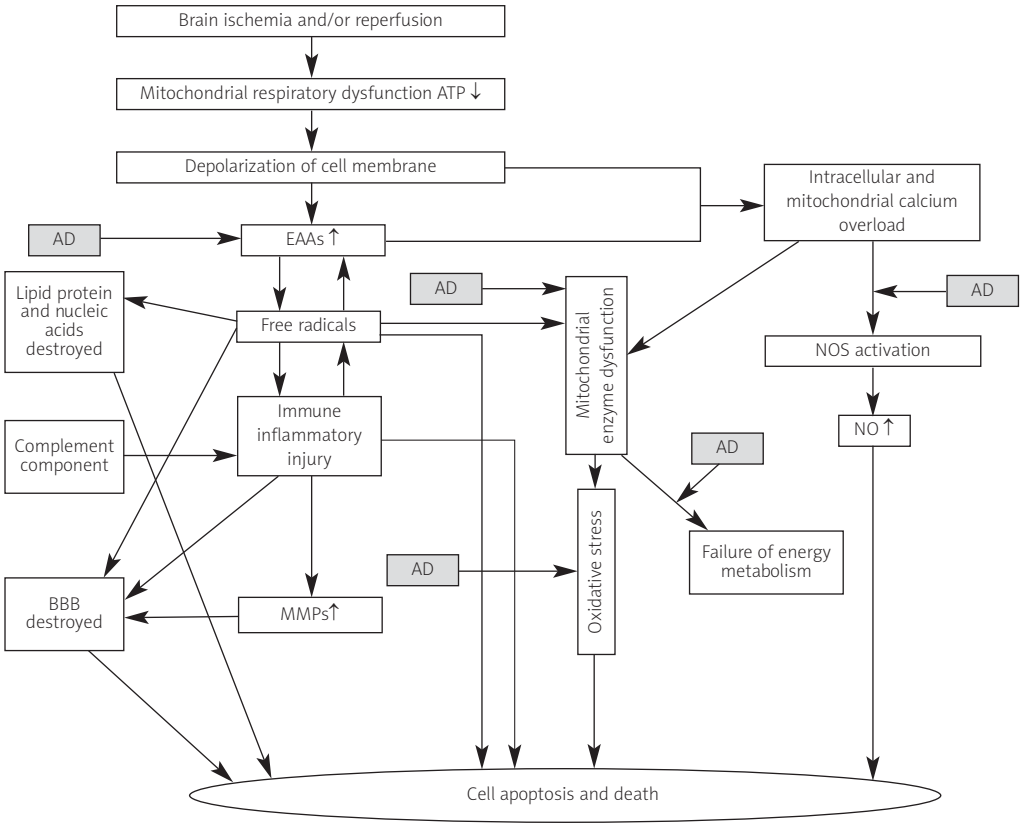

Fig. 1. Pathways leading to cell death in ischaemia-reperfusion.

peroxynitrite, which can be decomposed to produce the powerful and cytotoxic oxidant hydroxyl and nitrogen dioxide. These oxidants are highly diffusible and can easily cross the BBB to exert their destructive action on brain tissue [52]. NO may modulate the balance between glucose consumption through the glycolytic pathway and the pentose phosphate pathway in neurons. This may relate to the mechanisms of neurodegeneration and enhancement of apoptosis due to oxidative and nitrosative stress [4]. The study indicated that activation of the NO/NOS signalling system could trigger amyloid-β (Aβ) production through the beta-site APP-cleaving enzyme 1 (BACE1) pathway during and after acute focal cerebral ischaemia in aged rats. Then, Aβ stimulates reactive oxygen species production and changes mitochondria activity, leading to apoptosis both *in vitro* and *in vivo* [29]. Progressively higher levels of malondialdehyde indicated that free radical causes severe injury in H-I encephalopathy, which is associated with the increased concentration of NO [27].

Free radicals are produced during ischaemia, which can strengthen activity of lipid peroxidation, induce lesions of the cell and cellular barrier, and further result in necrosis or apoptosis of neurons. Free radicals can reduce the vasoconstrictor response to arterial hypocapnia, damage the vascular endothelial cells, increase the BBB permeability, interfere with and inhibit protein synthesis, and damage the structure of DNA [46]. Endogenous neurotrophin-3 (NT-3) enhanced neuronal injury by increasing oxygen radical mediated cell death, accelerated the dissolution of neurons by promoting the release of EAAs, cracked the cytolysosome to result in the release of numerous lysosomes, and damaged mitochondria to result in energy dyspoiesis [3].

Two days after hypoxia-reoxygenation, nNOS and iNOS expression remained high. The study supports the intriguing possibility that induction of iNOS and nNOS after brain hypoxic insult would enhance the susceptibility of brain to a subsequent excitotoxic insult [14]. Edaravone, a free radical scavenger, had a novel neuroprotective mechanism in cerebral infarction by abrogating the release of high-mobility group box-1 in neuronal cells [24].

Mitochondrial dysfunction

Mitochondria are the cell's energy converter. Cerebral H-I damage induced intracellular Ca^{2+} overload and generation of great amounts of free radicals. As the subcellular target, mitochondria were injured. Mitochondrial respiratory dysfunction results in decrease of ATP synthesis, thus affecting the energy supply to brain cells and leading to mtDNA disorder. The animal experiments indicated that there was decreased expression of mtDNA in CA1 pyramidal neurons during initial H-I. Meanwhile, there was decreased expression of mtRNA and mtDNA encoded protein in the period of reperfusion. All of these factors seriously affected the function of the respiratory chain, leading to energy exhaustion and metabolism level decrease of neurons, which was the major cause of neuron delayed death [17]. After cerebral ischaemia, mitochondria overproduce reactive oxygen species (ROS), which activate various molecular signalling pathways. Apoptosis-related signals return to mitochondria, and then mitochondria induce cell death through the release of pro-apoptotic proteins such as cytochrome c or apoptosis-inducing factor [38]. But Yin *et al*. identified that, for the first time, increased mitochondrial mass would clearly improve the overall oxidative function and energy state of the H-I brain, which may be an endogenous neuroprotective response against H-I injury [59] (Fig. 2).

Complement component

Permeability of the BBB was increased after ischaemia-reperfusion injury, resulting in macromolecule complement component in blood permeating into brain tissue. And astrocytes in brain possess the potential ability of complement synthesis. Astrocytes can synthesize complete complement under stimulation of cytokine and regulate the synthesis of complete complement when the brain is injured by H-I. Complement activation is a pathological mechanism of injury in the post-H-I neonatal brain (Fig. 1).

A role of complements in ischaemia-reperfusion injury was first described by Ward [43]. C3a and C5a could induce histamine release from inflammatory cells that resulted in a further increase of vascular permeability. C5a stimulated vascular smooth muscle to lead to exaggeration of brain ischaemia. Furthermore, complements, such as leukocyte chemotactic factor (LCF), attract leukocyte accumulation and make the inflammatory reaction more severe. Complement activation results in the production of inflammatory C3a and C5a, the opsonization of cells

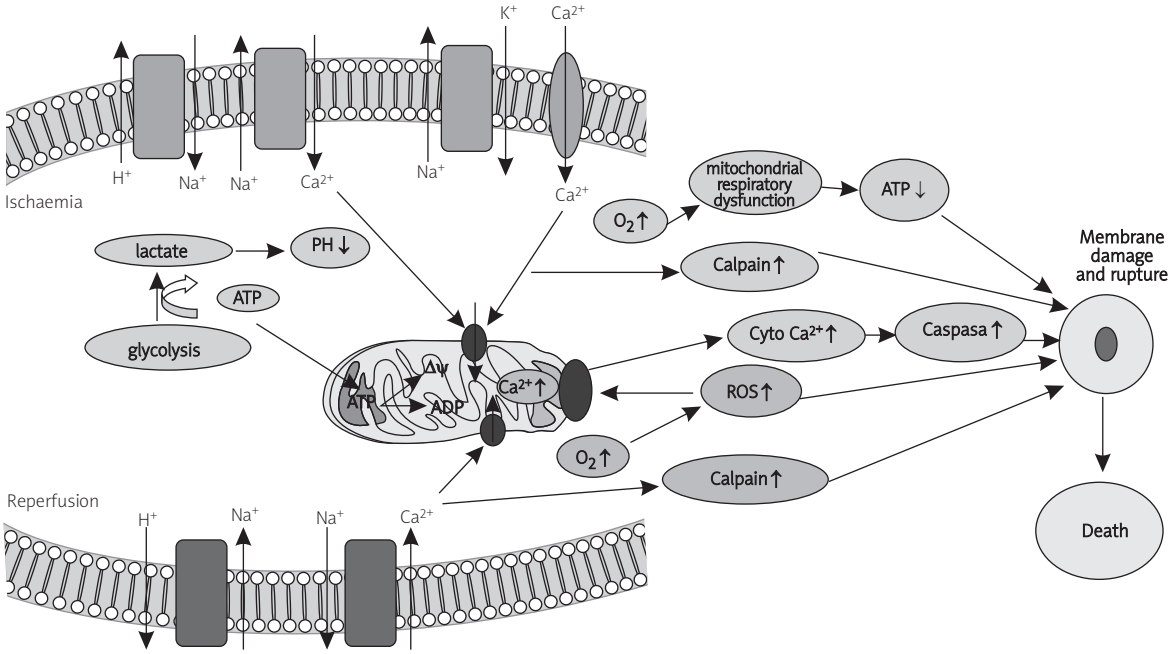

Fig. 2. Mitochondrial dysfunction and calcium leading to cell death in ischaemia-reperfusion.

with component C3b and iC3b for recognition and phagocytosis by macrophages, and the formation of lethal membrane attack complexes (MAC) (C5b-9) on target cell membranes. All these effects finally cause oedema and death of neurons [1], which demonstrated that complements are acutely activated in H-I brain. Another study showed that complement component C1q can exacerbate cerebral H-I injury by potentiating the severity of mitochondria-mediated oxidative stress [51]. The complement depletion reduces H-I-induced complement activation and injury [10].

Inflammatory and immune mechanisms

The central nervous system produces inflammatory responses to many injuries. Acute inflammation plays a key role in secondary brain injury induced by H-I (Fig. 1). The release of oxygen free radicals, inflammatory cytokines, chemotactic factors and the upregulation of leucocyte adhesion molecule expression are involved in the event of cerebral ischaemia-reperfusion injury. Thus, local leukocyte accumulation and the increased secretion of cytokines appeared in cerebral ischaemia-reperfusion injury in the acute stage [54]. Inflammation and immune response involving the adherence, accumulation and infiltration of leucocytes produced much proteolytic enzymes, oxygen free radicals and other factors, causing the destruction of capillary endothelium and basal membrane, and increasing the permeability of the BBB [11]. Compared with controls, there was a significant increase in the proinflammatory cytokine nuclear factor kB (NFkB) with concomitant upregulation of cell adhesion molecules. Therefore, NFkB plays an important role in hypoxia-induced transvascular leakage and cerebral oedema in brain of rats [42]. Mice with NOX2 subunit gp91 (phox) knockout (gp91 KO) exhibited less severe post-ischaemic inflammation, demonstrating that NADPH oxidase is involved in post-ischaemic neuroinflammation, as evidenced by reduced microglial activation and decreased upregulation of inflammation mediators, including IL-1, TNF, iNOS, CC-chemokine ligand 2, and CC-chemokine ligand 3 [7].

During cerebral ischaemia-reperfusion, astrocytes and gitter cells could secrete many cytokines, such as TNF, IL-1, IL-2, IL-8 and so on [47]. TNF is a kind of multi-functional proinflammatory cytokine. It aggravated the injury of cerebral ischaemia-reperfusion through promoting coagulation, increasing endothelial cell permeability and inducing adhesion molecule

expression. TNFα and IL-6 may destroy brain cholesterol homeostasis by insulting oligodendrocytes, which might be important in the molecular pathology of H-I white matter injury [61]. IL-1 and adhesion molecules facilitate the adherence of leucocytes and endothelial cells, which promotes the inflammatory reaction.

Hydrolysis of matrix metalloproteinase

Leukocytes release many toxic products and destructive proteinases in the process of activation and adhesion. Among them, matrix metalloproteinases (MMPs) possess very strong destructiveness to the vascular basement membrane, increase blood vessel permeability, and induce cerebral oedema [45] (Fig. 1). MMP-2 and MMP-9 are crucial for the degradation of various components of the extracellular matrix and the basement membrane, and can hydrolyze type IV and type V collagen, fibronectin, elastin and metamorphic matrix collagen to aggravate the vasogenic brain oedema [12]. Previous studies have proved that basement membrane as the second barrier is important to maintain the integrity of the BBB. After cerebral ischaemia-reperfusion injury, TNF induces the expression of C-jun and C-fos proto-oncogene through a series of transcription factors and facilitates MMP gene transcription. MMPs promote the degradation of extracellular matrix and basement membrane to result in brain oedema [34]. During H-I, abnormal expression and activation of MMP result in the opening of the BBB, prevent normal cell signalling, and eventually lead to cell death. Neuroprotection after inhibition of MMP (MMP-2 and -9) activation has been previously demonstrated in the adult brain after focal cerebral acute and chronic ischaemia in both mouse and rat [15,28]. The data showed that there was increased activity of MMP-2 and -9 in the ischaemic neuronal nuclei at 3 h and it significantly attenuated ischaemia-induced PARP-1 cleavage, degradation of XRCC1 and elevation of oxidized DNA. This suggested that intranuclear MMP activity cleaves PARP-1 and XRCC1, and interferes with oxidative DNA repair. This novel role for MMPs could contribute to neuronal apoptosis in ischaemic injuries [58].

Toxicity of dopamine

Dopamine (DA) is distributed mainly in the nigrostriatal system, presents in vesicles when it is synthesized, and is released by exocytosis during neuronal excitation (Fig. 1). DA has two forms, calcium-dependent and calcium-independent, when it is released in cerebral ischaemia-reperfusion injury. Intracellular calcium overload caused by many factors can promote the release of DA after ischaemia. Meanwhile, there is a decrease of Na^+-K^+-ATPase activity and a decline in the levels of cellular Na^+ due to energy exhaustion, which promotes reverse DA transport in a Ca^{2+}-independent way and participates in the release of DA. DA and its metabolites all induce neuronal damage. The toxicity of DA mainly includes: DA own toxicity, the toxicity of its metabolites, increasing the toxicity of EAAs, inducing neuronal apoptosis and so on [6]. Yoshimoto *et al*. [60] examined the effects of stimulations of ischaemia and/or potassium on the release of DA and serotonin (5-HT) in the nucleus accumbens (ACC) of anaesthetized rats and found after ischaemia for 10 min increased DA and 5-HT release in the ACC 200-fold and 15-fold in the first experiment, respectively. This research suggested different brain vulnerability in the dopaminergic and serotonergic neurons in the same area of the ACC. And there are higher levels of DA and its metabolites in the extracellular fluid of the striatum in acute cerebral ischaemia-reperfusion injury. Electroacupuncture can decrease the accumulation of DA and its metabolites, which may contribute to its effect in protecting the brain from ischaemia-reperfusion injury [56,60].

Intracellular calcium overload and cell apoptosis

Under normal conditions, intracellular calcium is mainly stored in mitochondria and sarcoplasmic reticulum. In acute cerebral ischaemia-reperfusion, it has been found that there was abnormality of Na^+/Ca^{2+} exchange, which is mainly due to metabolic acidosis. Furthermore, a lot of free radicals damage biofilm, leading to significantly increased permeability of the membrane and mitochondrial dysfunction, thus resulting in intracellular calcium overload [41]. It is well known that there is a Ca^{2+} overload in destined death neurons in the period of ischaemia and immediate reperfusion. Under these conditions, the mitochondrial Ca^{2+} pump was stimulated to take up Ca^{2+}, and excessive Ca^{2+} binds with mixtures containing phosphatidate to form insoluble calcium acid phosphate that could interfere with mitochondrial oxidative phosphorization and reduce the production of ATP. On the other hand,

the free Ca^{2+} increase in cells can activate many Ca^{2+}-dependent degrading enzymes to result in matrix degradation and cell injury. Meanwhile, the elevation of cytoplasmic Ca^{2+} impairs the encoding of action potentials and the dynamics of sodium channels and function in GABAergic neurons to lead to neural excitotoxicity [22].

Ca^{2+} overload triggers the elevation of superoxide radicals and other oxygen radicals. But Ca^{2+} overload in the period of ischaemia and immediate reperfusion is only a trigger. The later persistent downregulation of L-type calcium channels may be one of the executors of delayed neuronal death [30] (Fig. 2).

Apoptosis is an important way of neuronal death after cerebral ischaemia-reperfusion injury, especially delayed neuron death. Neuronal apoptosis is related to ischaemia type, severity and the time of reperfusion. Immediate early gene is a class of rapid and transient expression and takes part in intercellular signal transmission, growth, differentiation and damage repair, such as c-fos, c-jun, krox-24, jun-B, jun-D and so on. After 5 min of ischaemia and 2 h of reperfusion, the expression of c-fos, c-jun, and krox-24 was higher in the hippocampal CA1 region, which suggested that they may relate to cell apoptosis in these positions [26].

Caspases are the major executioners of apoptosis. Zhu *et al*. found that the total level of Bcl-2 decreased after H-I in vivo and after ionophore challenge in cultured human neuroblastoma (IMR-32) cells in vitro, which suggested cross-talk between excitotoxicity and apoptosis [63]. Cerebral ischaemia significantly upregulated the expression of Fas, FasL and caspase-3, which induced neurocyte apoptosis. Moreover, IL-10 treatment significantly inhibited neurocyte apoptosis and the mechanisms seemed related to inhibition of the expression of proapoptotic gene FasL and caspase-3 [31]. Increased levels of ROS are a major cause of tissue injury after cerebral ischaemia, in which ROS can react with cellular macromolecules through oxidation and cause the cells to undergo necrosis or apoptosis [32]. A flux enhanced autophagy may be related to apoptosis since some neurons presenting a high level of autophagy also expressed apoptotic features. These results suggest that the role of enhanced autophagy in delayed neuronal death after severe H-I is differentially linked to apoptosis according to the cerebral region [16].

Conclusions

H-I is a complicated pathological process which involves primary injury during the ischaemic period and secondary injury during the reperfusion stage. Its initial factor is cerebral H-I, but the inducing damage after reperfusion consists of many factors. These may be reciprocal causation or influence each other, and finally cause brain oedema and neuronal injury, apoptosis, and necrosis. So far, the majority of studies on cerebral H-I injury come from animal experiments, and some are even from neonatal brain. However, although age-dependent differences do exist, these experimental data provided important enlightenment and reference for clinical therapy, and the theoretical basis for clinical study.

Acknowledgment

Supported by Natural Science Foundation of China (81070957), Natural Science Foundation of Shanxi (2008011082-1).

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