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*Review article**

PATHOLOGY OF CEREBRAL ISCHAEMIA

Mechanisms Involved in Neuronal Damage

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Summary: The following features of the ischaemic and postischaemic brain are the focus of interest: development of acidosis, oedema formation, calcium overload, glutamate excitotoxicity, free radical formation and nitric oxide overproduction. The brain is critically dependent on its blood flow for a continuous supply of oxygen and glucose. Energy depletion has fundamental importance in the genesis of subsequent injurious events. Loss of ATP rapidly leads to a massive calcium influx and release of calcium from intracellular compartments. Extracellular concentrations of glutamate are markedly elevated in ischaemic brain tissue. Intracellular Ca^{2+} overload during ischemia has several deleterious consequences including the formation of reactive oxygen species. Nitrogen monoxide (NO) is an important mediator of cellular and molecular events which impacts the pathophysiology of cerebral ischemia. An increase in intracellular Ca^{2+} activates the enzyme NO synthase which catalyzes the synthesis of NO. NO is produced in neurons, glia cells and vascular endothelium in central nervous system. Depending on its origin, its effects are varied. NO is a mediator having both neurotoxic and neuromodulator effects. Neuronal NO is the neurotoxic agent mediating glutamate toxicity and increasing acute ischaemic damage. Vascular NO as a potent vasodilator and an inhibitor of platelet aggregation, may be beneficial in the early stages of focal cerebral ischemia. There is increasing evidence that ischaemic brain injury secondary to arterial occlusion is characterized by acute local inflammation, which involves accumulation of polymorphonuclear neutrophils. Overexpression of inflammatory mediators such as cytokines, chemokines and adhesion molecules promotes recruitment of leukocytes in the ischaemic area.

Key words: cerebral ischemia, glutamate, nitric oxide, free radicals, inflammation

Cerebral ischaemia

Cerebral ischaemia is a common and devastating neurological disorder which is the third leading cause of death in major industrialized countries and also a major cause of long-lasting disability (1 8). Cerebral ischaemia is always of vascular origin and can be divided into haemorrhagic and thromboembolic, with the latter accounting for approximately 90 % of strokes and results from embolic or thrombotic occlusion of the major cerebral arteries, most often the middle cerebral artery (1). Cerebral ischaemia can be classed by topography as global or focal, and by chronology as reversible and irreversible. Focal hypoxia-ischaemia also occurs in such contexts as traumatic insults or cerebral haemorrhages, while global hypoxia-ischaemia occurs in cardiac arrest, near drowning and carbon monoxide poisoning (9).

Occlusion of the middle cerebral artery (MCA) develops an infarct area in MCA territory. Ischaemia due to middle cerebral artery occlusion encompasses a densely ischaemic focus and a less densely ischaemic penumbral zone. A rim of moderate ischaemia surrounds the severely ischaemic area. This is called penumbra which lies between normally perfused brain and infarct area. Penumbra does not exist in global ischaemia. The penumbra defines regions with blood flow below that needed to sustain electrical activity, but above that required to maintain cellular ionic gradients, and that lead in time to infarction (10). In penumbra, pathophysiological mechanisms are dynamic, cell death occurs last and pharmacological approach has been most successful. Penumbra may extend outside (11). Cells in the focus are usually doomed unless reperfusion is quickly instituted. In contrast, although the penumbra contains cells »at risk«, these may remain viable for at least 4 to 8 hours. Cells in the penumbra may be salvaged by reperfusion or by drugs that prevent an extension of infarction into the penumbral zone (12).

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Mechanisms involved in ischaemic brain damage

Many events occurring during and after cerebral ischaemia are well known, but they are not known enough to fully elucidate the mechanisms of brain damage. Factors responsible for the extension of infarction into the penumbral zone include acidosis, oedema formation, acute local inflammation, dissipative ion fluxes, calcium overload, glutamate excitotoxicity, free radical formation, nitric oxide overproduction and programmed cell death (2, 13 16). Central to any discussion of the pathophysiology of ischaemic lesions is energy depletion. Energy failure alone cannot explain the functional damage occurring during the reperfusion phase.

Acidosis

The brain is critically dependent on its blood flow for a continuous supply of oxygen and glucose. Within minutes of the onset of ischaemia, energy demands exceed the brain capacity to synthesize ATP anaerobically. Energy depletion has fundamental importance in the genesis of subsequent injurious events. Following ischaemia, oxygen and glucose supplies of brain diminish. ATP decreases rapidly. In 2 3 min, lactic acid formation reaches its maximum level. Lactate and unbuffered hydrogen ions accumulate in tissue in proportion to the carbohydrate stores present at the onset of ischaemia. Lactic acidosis exerts its lethal effect in several ways. Mitochondrial respiration is depressed at low pH. This inhibits further ATP formation. Low tissue pH enhances free radical formation and lipid peroxidation (17). *In vitro* acidosis causes delocalization of protein bound iron, and thereby facilitates iron-catalyzed production of oxygen reactive species (18). Low pH can enhance free radical reactions also by shifting the unprotonated form of superoxide radical (O^2) to the protonated form (HO^2) which is more lipid soluble and pro-oxidant (18). In spite of this evidence, it is very difficult to show a correlation between ischaemic acidosis and free radicals because their production is localized into small subcellular compartments. In addition, acidosis worsens the disturbance occurred during ischaemia in calcium ion homeostasis (15).

Oedema formation

In addition to the rapid change in tissue acid-base status, failure of all energy dependent mechanisms, including ion pumps, leads to deterioration of membrane ion gradients, opening of selective and unselective ion channels and equilibration of most intracellular and extracellular ions (anoxic depolarization). As a consequence of anoxic depolarization, potassium ions leave the cell, sodium, chloride and calcium ions enter (11). Cellular accumulation of ions cause formation of cytotoxic oedema. Cellular osmolarity increases. Depending on this, water shifts from extracellular into intracellular compartment. This causes ischaemic neuronal swelling and development of cytotoxic oedema. Brain oedema is one of the major determinants of the survival of stroke patients. Cytotoxic oedema is the first stage of ischaemic oedema and occurs in the first minutes of ischaemia. In addition, increased H^+ ions due to ischaemic acidosis are expelled out of the cells in exchange for Na^+ ions. This worsens cytotoxic oedema. Cytotoxic oedema occurs both in gray and white matter. Blood brain barrier (BBB) is intact. In acute ischaemic stroke, oedema begins as reversible cytotoxic form, later, changes into vasogenic form. Vasogenic oedema follows, secondary to leakiness of the blood brain barrier which is also impaired by energy failure and the hydrostatic force created by the blood pressure. Disruption of BBB leads to extracellular water accumulation and formation of vasogenic oedema. The resultant brain oedema may compress capillaries, thereby further decreasing regional blood flow into the ischaemic zone as well as inhibiting the reperfusion which may follow relief of arterial obstruction or revascularization. Cerebral oedema and increased intracranial pressure affect the perfusion of peripheral areas and cell necrose enlarges (17). Vasogenic oedema develops after 4 6 hours of ischaemia, reaches maximum at 36 72 hours and lasts 7 14 days. If this oedema is severe enough, it causes death in 1/3 of ischaemic lesions and 3/4 of haemorrhagics.

Glutamate neurotoxicity

Extracellular concentrations of glutamate are markedly elevated in ischaemic brain tissue as a consequence of both enhanced release of the amino acid from neurons and its impaired uptake into glia and neurons. Glutamate released from depolarized presynaptic endings activates several postsynaptic receptor /channel complexes which are named according to the preferred agonist (the quisqualate, kainate and NMDA-preferring receptor). Of these, the N-methyl-D-aspartate (NMDA) receptor/channel complex is permeable to calcium ions. Glutamate acts on both N-methyl-D-aspartate (NMDA) and non-NMDA receptors and activates both NMDA and non-NMDA (Kainate and AMPA/Quisqualate) type receptors. Non-NMDA receptors mediate acute neuronal swelling. NMDA receptors are particularly important in mediating subsequent delayed neuronal disintegration. Non-NMDA receptors play a more significant role in global ischaemia and NMDA receptors in focal ischaemia (1).

Calcium overload

Calcium ions are among the most powerful intracellular messengers, able to give rise to a great variety of events. Intracellular Ca^{2+} overload during ischaemia is thought to have several deleterious consequences

including: a) beginning of the metabolic cascades, which include activation of phospholipase A_2, attacking cellular membranes, liberating fatty acids (mainly arachidonic acid) and altering membrane permeability and cell function; b) mitochondria accumulates calcium, which uncouples oxidative phosphorylation at a time when ATP production is already reduced; c) alteration of receptor function; d) toxic excitatory amino acid (EAA) release, precipitating neurons in a state of hyperexitability (15,16); e) elevated cytosolic Ca^{+2} activates the neutral protease Calpain I. Activated Calpain I, converts xanthin dehydrogenase into xanthine oxidase by cleaving a peptide bond in xanthine dehydrogenase in endothelium of cerebral blood vessels. Elevated xanthine oxidase activity during reperfusion may contribute to increased oxygen radical formation as a result of the metabolism of xanthine to uric acid; f) elevated cytosolic Ca^{+2} activates ornitin decarboxylase leading to oversynthesis of polyamines (1, 18). This causes activation of polyamine site of NMDA receptor amplifying its response, increases Ca^{+2} influx; g) In the presence of Ca^{+2}, DAG activates protein kinase C. Protein kinase C is a soluble protein and it is inactive catalytically in the absence of DAG. Protein kinase C is activated by Ca^{+2} and DAG which remains in the membrane. The activated kinase then phosphorylates several cellular enzymes and receptors, thereby altering their activity. Protein kinase activation depends on both Ca^{+2} and DAG. This is an indicator of the relationship between the two branches of inozitol-lipid signal pathway. Protein kinase C also phosphorylates NMDA receptor, thereby, enhances NMDA receptor stimulation via reduction of the Mg^{+2} block (1); h) Intracellular Ca^{+2} overload activates nitric oxide synthase (NOS) leading to the formation of NO from arginine (1). NO produces cGMP by activating guanylate cyclase. cGMP has anti-oxidant, anti-aggregant, anti-adhesive, anti-inflammatory and dilating effects; i) Ca^{+2} activates some lytic enzymes (proteases, endonucleases). Endonucleases induce DNA fragmentation, programmed cell death. Proteases degrate protein MAP2 in microfilaments and microtubulis. This correlates with neuronal degeneration.

Reactive oxygen particules

Intracellular Ca^{2+} overload can also set off a cascade of events which may lead to the formation of reactive oxygen species, promoting arachidonic acid metabolism and converting xanthine dehydrogenase into xanthine oxidase. Injury to brain cells may release iron ions that can stimulate free radical reactions. In addition, there is a high concentration of ascorbic acid in the gray and white matter of the central nervous system (CNS). Ascorbate/iron and ascorbate/copper mixtures generate free radicals. One more source of oxygen free radicals is the intramitochondrial electron transfer chain. Free radicals produced in mitochondria may cause point mutations, DNA cross link and DNA strand breaks in mitochondrial genes. Damage to the mitochondrial genome results in impaired respiration, further increasing the possibility of oxygen radical production. The brain and nervous system are prone to radical damage for a number of reasons. Membrane lipids are very rich in polyunsaturated fatty acid side chains. In addition, the brain is poor in catalase activity and has only moderate amounts of superoxide dismutase and glutathione peroxidase (15, 16).

Nitric oxide

Nitrogen monoxide (NO) has recently emerged as an important mediator of cellular and molecular events which impacts the pathophysiology of cerebral ischaemia. An increase in intracellular Ca^{+2} resulting from the activation of voltage-gated Ca^{+2} channels or ligand-gated Ca^{+2} channels or from the mobilization of intracellular Ca^{+2} stores could activate the enzyme NOsynthase (NOS; EC 1.14.13.39) which catalyzes the synthesis of NO from the guanido nitrogen of L-arginine and molecular oxygen. NO is produced in neurons, glia cells and vascular endothelium in CNS. Depending on its origin, its effects are varied. NO is a mediator having both neurotoxic and neuromodulator effects. Neuronal NO is proposed as the neurotoxic agent mediating NMDA toxicity and increasing acute ischaemic damage. It causes cytotoxicity through formation of iron-NO complexes with several enzymes including mitochondrial electron transport chain, oxidation of protein sulfhydryls and DNA nitration. NO may mediate cell death also through formation of the potent oxidant peroxynitrite (ONOO). ONOO decomposes to the hydroxyl radical (OH ō) and nitrogen dioxide radical (NO_2) which is a potent activator of lipid peroxidation. On the other hand, vascular NO as a potent vasodilator and an inhibitor of platelet aggregation, may be beneficial in the early stages of focal cerebral ischaemia. It may facilitate collateral blood flow to the ischaemic territory (15, 16). A third isoform, iNOS, is normally not present in most cells, but its expression is induced in pathological states associated with inflammation. iNOS generates toxic levels of NO, and may contribute to the cytotoxicity induced by inflammation (13). In the brain, iNOS is induced by postischaemic inflammation. After transient or permanent middle cerebral artery (MCA) occlusion in rodents, iNOS messenger RNA has been reported to be upregulated and peaks at 12 48 h after ischaemia (13). iNOS is induced in neutrophils infiltrating the injured brain and in cerebral blood vessels in the ischaemic territory. It has been also reported that postischaemic NO production continues during the recovery phase of ischaemic stroke. The data on iNOS inhibitors along with the data from studies in iNOS-null mice, suggest that NO produced by iNOS is an important factor in ischaemic damage (13).

Inflammation in cerebral ischaemia

There is increasing evidence that ischaemic brain injury secondary to arterial occlusion is characterized by acute local inflammation, which involves accumulation of polymorphonuclear neutrophils (PMN). An inflammatory reaction is a common response of the brain parenchyma to various forms of insult. It is characterized by the infiltration of leukocytes, which are mainly polymorphonuclear leukocytes and monocytes/macrophages (5, 7, 19). Overexpression of inflammatory mediators such as cytokines, chemokines and adhesion molecules promotes recruitment of leukocytes in the ischaemic area. Leukocytes have deleterious effects in brain ischaemia and play the key role in the progression from ischaemia to irreversible injury. Human data regarding inflammation in stroke are scarce. The inflammatory response of ischaemic brain parenchyma has been more thoroughly explored in animal models. In animal models of cerebral ischaemia, accumulation of PMN has been detected within the first 12 hours after induction of ischaemia (5, 7, 20). During reperfusion after acute ischaemia, polymorphonuclear neutrophils (PMN) are believed to exacerbate tissue damage by both physical obstruction of vessels and release of oxygen radicals, proinflammatory cytokines, and cytolytic enzymes (5, 21). There are a number of mechanisms by which leukocytes may produce deleterious effects on ischaemic parenchyma (5, 7, 21). It has been proposed that leukocytes obstruct the microvessels and contribute toward the so-called »no-reflow« phenomenon (7, 22). This indicates the lack of complete recovery of cerebral blood flow in the ischaemic area after reperfusion (7, 23 25). Other detrimental effects of leukocytes during ischaemia may be due to the release of vasoconstrictive mediators, such as thromboxane A_2, endothelin-1, and prostaglandin H_2; an alteration in cerebral artery vasoreactivity; the release of cytotoxic enzymes; free oxygen radicals; NO; and products of the phospholipid cascade (5, 7, 21, 26). It is believed that the release of proteolytic enzymes such as elastase might damage endothelial cell membranes and the basal lamina, alter the blood-brain barrier, and contribute to the formation of post-ischaemic oedema. In addition, loss of the integrity of the endothelial cell-basal lamina lining might facilitate the escape of red blood cells and the haemorrhagic transformation of a brain infarct (7, 27 29).

Recent studies have provided evidence that expression of the inflammation-related enzymes, nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 are critical mechanisms by which inflammatory cells influence the progression of cerebral ischaemic damage (13). COX is a rate limiting enzyme in the synthesis of prostaglandins and tromboxanes. Two isoforms have been described: COX-1 and COX-2. COX-1 is involved in normal cellular function. COX-2 is normally expressed at low levels in neurons. COX-2 is upregulated in response to mitogens, inflammatory mediators and hormones. In inflammation, it contributes to tissue damage through the production of reactive oxygen species and toxic prostanoids. Superoxide produced by COX-2 reacts with NO to form the powerful oxidant peroxynitrite (13). There is evidence that COX-2 participates in cerebral ischaemia (13). It was shown that COX-2 messenger RNA and protein expression are upregulated 12 24 h after cerebral ischaemia in rodents. COX-2 expression in rodents has been observed in neurons at the periphery of the infarct, in vascular cells, and in microglia (3, 13). It was reported that administration of a selective COX-2 inhibitor, 6 h after ischaemia reduced infarct volume in a model of focal cerebral ischaemia in rats (3,13).

Cytokines and cerebral ischaemia

It is believed that cytokines play a key role in the entry of leukocytes into the ischaemic area (2 8). Cytokines are low-molecular-weight glycoproteins that act as intercellular messengers and mediate and regulate immune and inflammatory responses (5, 7). They are produced by activated macrophages, monocytes, lymphocytes, endothelial cells, fibroblasts, platelets, and many other cell types (7, 30). Cytokines act at very low concentrations on specific target-cell receptors, whose expression is modulated by the cytokines themselves. Binding of cytokines to receptors activates intracellular second messenger systems and several protein kinases and phosphatases. These enzymes trigger the expression of a number of proinflammatory genes by inducing the synthesis of transcription factors including nuclear factor-κB, hypoxia inducible factor-1, interferon regulatory factor-1 and Stat3 (2, 31 34). Cytokines are considered to be the principal mediators of immunologic and inflammatory responses (7). During cerebral ischaemia cytokines can attract leukocytes and stimulate the synthesis of adhesion molecules in leukocytes, endothelial cells, and other cells, thus promoting the inflammatory response of damaged cerebral tissue. They can facilitate thrombogenesis by increasing levels of plasminogen-activating inhibitor-1, tissue factor, and platelet-activating factor and by inhibiting tissue plasminogen activator and protein S (7, 35 37). Increased production of several cytokines has been demonstrated intrathecally in patients with acute ischaemic stroke (5, 38 40). Increased synthesis of cytokines in acute stroke is, however, not restricted to the CNS but can also be detected systemically (5, 8, 41, 42).

Chemokines in cerebral ischaemia

Chemokines constitute a subgroup of the cytokine family, which may play a pivotal role in the attraction and accumulation of leukocytes through the parenchyma and toward the ischaemic area (5, 7,

43 46). Chemokines are low-molecular-weight molecules with chemotactic activities on selective leukocyte subpopulations (7, 8). The number of discovered chemokines is continuously growing, and so far, more than 20 members of this cytokine family have been identified (7). They are characterized by the presence, as a common structural pattern, of four cysteine residues. They are divided into two main subfamilies (α- or C-X-C subfamily and β- or C-C subfamily) according to the presence or absence of an amino acid between the residues of the two most amino-proximal cysteines (7, 47). Members of the IL-8 family belong to α-chemokines, while monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and MIP-1β belong to the C-C chemokines. The structural distinction is reflected *in vitro* by a peculiar effect on different cell types: C-X-C chemokines tend to attract polymorphonuclear leukocytes (PMNL), primarily neutrophils, whereas C-C chemokines preferentially act on monocytes/macrophages (7, 8). Each chemokine family binds to specific receptors formed by seven transmembrane domains that activate G proteins and subsequently an intracellular kinase cascade (7, 48). Chemokine production and secretion are stimulated by a number of compounds, such as bacterial lipopolysaccharide, IL-1 α, IL-1 β, and TNF-α (7, 47).

Cell adhesion molecules in cerebral ischaemia

The inflammatory process initiated by locally produced proinflammatory cytokines induce or enhance the expression of several adhesion molecules (7, 8, 41, 45). The adhesion of leukocytes to the endothelial surface and their subsequent migration from the microvessels into the brain parenchyma are mediated by a variety of molecules located on the surface of both leukocytes and endothelial cells (7). Adhesion molecules are divided into four main families: integrins, the immunoglobulin superfamily, cadherins, and selectins (49). Under normal conditions, there is little or no cell-surface expression of adhesion molecules (50). Inflammatory processes, such as cerebral ischaemia induce their expression with the upregulation mediated by cytokines (7). They are glycoprotein in nature and are the anchors that mediate the attachment of leukocytes (51).

Therapeutic approach

Heavy studies are being performed to develop drugs that will prevent neurodegeneration following acute ischaemic stroke (15, 16, 52 55). For this purpose, animal models have been produced that mimic the neuropathological consequences of stroke. During the past 10 15 years of stroke research, reproducible techniques for the induction of focal and global ischaemia have been developed. These models have several advantages and disadvantages. Reversible or irreversible focal ischaemia models like stroke in humans are useful for studies of molecular mechanisms of stroke and also for the development of neuroprotective drugs. The advantages of using rats for stroke study include the similarity of their intracranial circulation to that of man and the relatively low animal cost which is important for large scale studies for statistical analysis. They provide the exact time of the onset of ischaemia and the possibility to test new drugs.

Even though a large number of different compounds have been proven to reduce the size of brain infarct in animal studies, these drugs caused disappointing results in stroke patients. The reasons for the unsuccessful clinical trials have been either the toxic side effects, which have overridden the neuroprotective potential of the compounds demonstrated in animals, or a limited time window for human therapy. Compounds with no or tolerable side effects combined with a protective potential when administered several hours after ischaemic insult are under heavy research (2, 6, 7). Currently, the only treatment of patients with acute ischaemic stroke is thrombolysis and restoration of blood flow (3, 6, 7). Only a fraction of stroke patients benefits from this therapy. Therapeutic recanalization of an occluded cerebral artery is a risky option that can be applied only in the case of selected patients. The main limitation of cerebral thrombolysis is the narrow, 3-hour therapeutic »window« during which the thrombolytic agent has to be administered to be effective. Beyond this time limit, its effectiveness is neutralized by the high risk of cerebral hemorrhage (7). In acute stroke, only a small fraction of patients benefit from intravenous administration of recombinant tissue plasminogen activator, which is the only drug with proven effectiveness in reducing the size of infarct in humans (6).

The rationale for the use of a number of other pharmacological treatments for acute stroke is based on recent advances in the pathophysiology of brain ischaemia. One of the most rapidly expanding and promising areas of which is the role of inflammation in stroke (2, 4, 6, 7). Factors that influence the recruitment of PMN could be new therapeutic targets in acute stroke (5). Drugs capable of interfering with inflammation related mechanisms have given encouraging results in experimental stroke models in animals. Possible future pharmacological treatments could be based on the inhibition of proinflammatory mediators, prevention of adhesion between the leukocytes and endothelial cells, controlling the specific transduction pathway signals following cytokine production and promoting neovascularization. Strategies that block the activity of inflammation-induced enzymes, such as iNOS and COX-2 should also be investigated.

PATOLOGIJA CEREBRALNE ISHEMIJE

Mehanizmi uključeni u oštećenje neurona

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Kratak sadržaj: Interesovanje je usredsređeno na sledeće karakteristike ishemijskog i posthemijskog oštećenja mozga: razvoj acidoze, stvaranje edema, opterećenja kalcijumom, eksitoksičnost glutamata, stvaranje slobodnih radikala i veliku proizvodnju nitričnog oksida. Mozak kritično zavisi od protoka krvi u njemu u pogledu kontinuiranog snabdevanja kiseonikom i glukozom. Deplecija energije je od fundamentalne važnosti u genezi kasnije nastalih oštećenja. Gubitak ATP brzo dovodi do masivnog priliva kalcijuma i oslobađa kalcijum iz intracelularnih delova. Ekstracelularne koncentracije glutamata su značajno povećane u ishemijskom moždanom tkivu. Intracelularna preopterećenost sa Ca^{2+} za vreme ishemije ima nekoliko štetnih posledica uključujući stvaranje reaktivnih vrsta kiseonika. Azot monoksid (NO) je važan medijator celularnih i molekularnih događanja koja utiču na patofiziologiju cerebralne ishemije. Povećanje intracelularnog Ca^{2+} aktivira sintezu enzima NO sintaze koja katalizuje sintezu NO. NO se prozivodi u neuronima glia ćelija u vaskularnom endotelijumu centranog nervnog sistema. Neuronalni NO, kao potentan vazodilatator i inhibitor agregacije pločica, može biti koristan u ranim stanjima fokalne cerebralne ishemije. Postoji mnogo dokaza da se u ishemijskom oštećenju mozga posle okluzije arterija javlja karakteristično akutno lokalno zapaljenje sa akumulacijom polimorfno nuklearnih neutrofila. Veliko ispoljavanje inflamatornih medijatora, kao što su citokini, hemokini i adezioni molekuli, pomaže u regrutovanju leukocita u ishemičnom delu mozga.

Ključne reči: cerebralna ishemija, glutamat, azot oksid, slobodni radikali, zapaljenje

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