ABSTRACT - Purpose - To analyze the main aspects of neuroprotection and excitotoxicity. Discussion - This is a significant theory on the pathophysiology of cerebral ischemia; it is based on the release of excitatory aminoacid (EAA), mainly glutamate. The sequence starts with a decrease of the blood flow and ends in neuronal death. The main stages of this reaction are herein presented and discussed. An in depth study of the effects of the excessive intracellular calcium is undertaken. Neuroprotectors (NP) are a group of drugs that reduce the excitotoxicity, opposing the excessive release of EAA and its intracellular effects. Neuroprotectors represent a rational approach to stroke treatment and offer a number of potential advantages. They prevent or limit ischemia-induced damage. Conclusion - There are many experimental and clinical NP trials. A minimum of 800 trials are currently under study worldwide. The most important NP subgroups are: N-methyl D-aspartate (NMDA) antagonists, gamma-aminobutyric acid (GABA) agonists, amino-hydroxy-methyl-isoxalone propionic acid (AMPA) antagonists, inhibitors of intracellular Ca++ inhibitors of nitric oxide modulation pathway free radicals scavengers, sodium channel antagonists, glutamate release inhibitor, growth factors, hypothermia and potassium channel activators.

KEY WORDS: neuroprotection, excitotoxicity, NMDA antagonists, stroke.

NEUROPROTECTION, EXCITOTOXICITY AND NMDA ANTAGONISTS

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Excitotoxicity is an important and well accepted theory proposed by Olney in 1971 to explain the pathophysiology of brain ischemia. It consists in explaining the basic mechanism of cellular lesion after cerebral ischemia. Excitotoxicity is based on the release of excitatory aminoacid (EAA), mainly glutamate. The sequence starts with a decrease in cerebral blood flow (Fig 1).
Aminoacid glutamate plays an important role in excitotoxicity\(^3,4,6\). A large number of studies have shown an increase in concentration of glutamate after cerebral ischemia. The sequence starts with depletion of energy phosphates, which produces neuronal depolarization due to failure of the ionic pump with the consequent increase in the extracellular potassium concentration. This event leads to a release of glutamate. ATP-dependent re-uptake mechanisms may contribute to glutamate-induced brain injury as well. The duration of the EAA release in human beings is unknown and this time period could determine the precise therapeutic window. The increase of EAA concentrations in experimental models is transient, lasting from 1 to 2 hours\(^7\). In humans this elevation has been observed up to 4 days in patients with prolonged overall post-traumatic ischemic brain damage\(^8\).

The glutamate acts mainly in four receptors, divided in two groups: a- ionotrophic receptors (related to ions action): NMDA, amino-hydroxy-methyl-isoxaline propionic acid (AMPA), kainat (AMPA and kainat are known as non-NMDA receptors); b- methabotrophic.

The NMDA is the most important and the most studied receptor. It is related mainly to \(\text{Ca}^{++}\); the others are related mainly to \(\text{Na}^+\). Activation of the NMDA receptor to a large extent mediates the excitotoxicity and neuronal damage after ischemia; it is the most complex receptor. Besides glutamate, the aminoacid glycine also stimulates this receptor. Glycine is a co-agonist of the receptor-channel complex.

The factor responsible for determining the degree of ischemia in a given area of the brain is the number of NMDA receptors present in that area. The higher the concentration of NMDA in a specific area, for ex. CA1 area of the hipocampus, the more severe the ischemia will be, or vice-versa\(^9\).

Glutamate acts in about 30% of synapses in central nervous system (CNS), it is kept in specific vesicles and it is released in little doses that take over the receptors for 1 or 2 mseg and then are metabolized by specific enzymes\(^8\). The glutamate opens the receptor dependent channel and then a large quantity of \(\text{Ca}^{++}\) enters inside the cell. The excess of \(\text{Ca}^{++}\) is highly toxic for the cells. Under normal conditions the intracellular \(\text{Ca}^{++}\) level is stable and low, around 10000 times lower than the extracellular level. Besides \(\text{Ca}^{++}\), \(\text{Na}^+\) also inflows and this further imbalances the cellular ionic levels. This leads to an increase of cerebral edema. The excess of [\(\text{Ca}^{++}\)] stimulates a sequence of

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\begin{align*}
\text{cessation or decrease of CBF} & \quad \downarrow \\
\downarrow \text{tissue O}_2 & \quad \downarrow \\
\downarrow \text{tissue glucose} & \quad \downarrow \\
\downarrow \text{tissue ATP} & \quad \downarrow & \uparrow \text{anaerobic glycolysis} & \quad \downarrow \\
\downarrow \text{neuronal depolarization} & \quad \downarrow & \uparrow \text{opening of VSCC} & \quad \uparrow \text{release of glutamate} & \quad \uparrow \text{activation of NMDA} & \quad \uparrow \text{activation of non-NMDA} & \quad \downarrow & \downarrow \text{intracellular} & \text{Ca}^{++} \\
\end{align*}
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Fig 1. Excitotoxicity sequence.

\(\text{CBF, cerebral blood flow; VSCC, voltage sensitive calcium channel; NMDA, receptor n-methyl d-aspartate.}\)
toxic enzymatic reactions and leads to cell death. In short, the main enzymatic reactions can be described as:

1. Damage of oxidative phosphorylation, lowering function and energy production by the mitochondria.
2. Activation of phospholipases that act on the phospholipids and contributes to the destruction of neuronal membranes and brain vascular endothelium, through a mechanism called enzymatic lipid peroxidation (the non enzymatic lipid peroxidation is of another kind performed by free radicals). The excess of arachidonic acid will lead to a biochemical sequential process, called arachidonic cascade with a final production of prostaglandins (thromboxane A₂ and prostacyclin) and leucotriens, mainly thromboxane, which has a vasoconstrictive property.
3. Activation of proteases that play an important role in the production of free radicals. Ca²⁺ activates the protease, which on its turn changes dehydrogenase xanthine into oxidase xanthine, an important enzyme in the production of free radicals, like superoxide and -hydroxyl. The brain is particularly sensitive to the action of free radicals because it lacks normal scavengers and contains a large quantity of iron, an important coenzyme in this reaction. The free radical acts in the membrane phospholipids breaks the membranes and destroys the cell.
4. Activation of nitric oxide synthase (NOS). Ca²⁺ stimulates NOS and consequently increases the nitric oxide (NO) concentration. Over/production of NO from excessive or inappropriate stimulation of nNOS seems to mediate a major component of excitotoxicity damage. NO is one of the factors that mediates the excitotoxicity, producing free radicals. NO binds with superoxide to produce peroxinitrite, which in water solution spontaneously changes into the hydroxyl radical. This free radical unchains the lipid peroxidation. The NO has also played a role in the liberation of glutamate responsible for the fact that the NO formed leaves the neuronal cell, goes to the pre-synaptic space and releases glutamate inside the pre-synaptic cells. There are different kinds of NO, according to the specific form of NOS. There are three important isoforms of NOS: nNOS (type I), iNOS (type I), eNOS (type III). Pharmacological and genetic approaches have significantly improved our knowledge regarding the role of NO and the different NOS isoforms in cerebral ischemia. The iNOS is not detectable in healthy tissue; under pathological conditions iNOS can be observed in most tissues, including neurons, astrocytes and endothelial cells. It is detectable 12 hours after onset of ischemia and has its peak 48 hours later.
5. Other important action of the excess of Ca²⁺ inside the neuronal cells, is its role in protein kinase C (PKC). In a normal situation PKC remains inactive in the cytosol but in the presence of excessive Ca²⁺ it goes to the neuronal membrane and it is activated by diacylglycerol and phosphatidylserin, then produces protein phosphorylation responsible for mechanisms of extrusion of Ca²⁺. PKC acts also by rising the post synaptic sensibility to glutamate.

**NMBA Antagonists and Neuroprotection**

Neuroprotectors (NP) comprise a new group of drugs that reduce excitotoxicity, resisting the excess release of AAE and its intracellular effects. Neuroprotectors represent a rational approach to stroke treatment and offer a number of potential advantages. They prevent or limit ischemia-induced damage, could reduce infarction size and mortality and the functional outcome can be improved. Moreover, because neuroprotective treatment is not expected to affect bleeding, it could be used in all patients with suspected stroke, without the need of confirming the diagnosis of ischemic stroke.
Although the prospects for the use of NP have been promissory, there is no convincing clinical evidence that any neuroprotective drug is effective in either reducing size of infarction or improving the overall outcome.

Neuroprotection intends to interrupt the pathological cascade that occurs during the ischemic process. It can also limit or stop the progressive depression, other important mechanism of aggression in ischemia. There are important limitations for the reasonable use of these drugs: short time (therapeutic window) necessary to introduce the drug; treatment should be started a few hours after the onset of symptoms; difficulty to reach the ischemic site; strong side-effects, mainly psychotic, renal, blood pressure disorders, vacuolization; trials have failure to demonstrate any effectiveness of these agents.

There are many trials studying these sequences. We can analyze the main items based on neuroprotective action:

a. NMDA antagonists - The NMDA receptor is a complex structure, with binding sites for divalent cations, polyamines and glycine, in addition to the ligand-binding site. Glutamate is the main agonist; glycine and polyamide are co-agonists of the NMDA receptor-channel complex. Antagonists binding to each of these sites have been developed and shown to have neuroprotective effects in animal models.

Competitive and non-competitive are the two kinds of NMDA antagonists. The competitive ones compete directly against the glutamate; weakly cross the hemato-encephalic barrier, but have high specificity and potency. The non-competitive ones act in the membrane of specific NMDA places and prevent the Ca²⁺ influx. These agents seem to cause similar adverse effects regardless of their pharmacological action. Low doses are associated with altered sensory perception, dysphoria, nystagmus and hypotension, while higher doses may cause psychological adverse events such as excitement, paranoia and hallucinations; also severe motor retardation, leading ultimately to catatonia, which may occur with the highest doses. Milder adverse effects have been noticed with cerestat, a non-competitive NMDA antagonist.

b. GABA agonists - While glutamate is a primary EAA in the brain, GABA is the most important inhibitory amino-acid. The potential action of GABA may reverse the toxic effects of glutamate through hyper-polarization of the neuronal membrane. A GABA agonist chlomethiazole has been found to reduce infarction size in a variety of stroke models. This approach is at phase III of research.

c. AMPA antagonists - This is another possibility of action in neuroprotection, as AMPA also plays an important role in the rising of Ca²⁺ inside the cells. The most studied drug in this group is the NBQX, but unfortunately this drug is very nephrotoxic and clinical studies not optimistic.

d. To reduce intracellular Ca²⁺ mobilization is other possibility to oppose excitotoxicity. Some drugs have been studied: GM-1, that inhibits the translocation of CPK; dantrilene sodium; some drugs that inhibit Ca²⁺ dependent enzymes (AT 877, under study).

e. Inhibitors of the pathways of NO modulation - Selective inhibition of nNOS inhibiting the specific production of toxic NO can offer a protection to neuronal tissues during the stroke. The NO made from nNOS plays an important role in the production of free radicals and its selective inhibition brings good results. 7-nitroindazole is still under clinical studies; it inhibits the nNOS and has no action on eNOS. Genetic deletion of nNOS also conferred dramatic resistance to focal ischemic...
injury. Lubeluzole inhibits the production of the NO pathways and also helps preventing the increase of extracellular glutamate in the penumbra and in the normalization of excitability of neurons in this area. Lubeluzole has caused few side effects and phase II and III.

f. Free radicals scavengers - Well-known free radical scavengers like tocopherol, selenium, b-carotene, have little action on neuronal tissue during ischemic processes. Tirilazad is a new scavenger under clinical study; the initial results had shown to be quite interesting.

g. Sodium channel antagonists - Na⁺ plays also a role in the ischemic process; the inhibition of pre-synaptic sodium channels, leads to a neuronal membrane stabilization which in turn, leads to the inhibition of pre-synaptic glutamate release and may be useful in stroke. Some drugs, like a number of anti-epileptics, have this property: lamotrigine, phenytoin, fos-phenytoin, riluzole, lifarizine (under clinical tests).

h. Glutamate release inhibitors - Another strategy which can be used in neuroprotection. Several studies are under way with different drugs with inconclusive results. Omega-canotoxins; synthetic toxin SNX-111, has an important side-effect; it blocks adrenaline release leading to severe hypotension; nalmepe (opioid receptor antagonist); dexamethasone has an important side-effect in this issue, because it can increase the glutamate release or decrease glutamate re-uptake.

i. Growth factors - Some growth factors might have a neuroprotection function. Angiogenic factors might be neuroprotective and are determinant for neuronal survival. PDGF (platelet derived growth factor) is highly expressed in the white matter suggesting that PDGF may exert its function in white matter, participating either in the regeneration of damaged axons or in glial scar formation. Thus PDFG is likely to be angiogenic and neuroprotective in stroke.

j. Acidosis - Experimental evidence indicates that glutamate receptors can be inactivated by extreme acidosis, in vitro, at least. In experimental studies, the combination of extracellular acidosis and glutamate-receptor antagonists provides greater neuronal protection than the glutamate receptor antagonist alone.

l. Hypothermia can reduce the glutamate release and can be used as a neuroprotective action. Hypothermia has an opposite effect.

m. Potassium channel activators - Recent evidence suggests that activators of potassium channel on neurons, can have neuroprotective effects on neuronal ischemia and potential therapeutic implications. They maintain vascular responses of cerebral arterioles after infarction or hemorrhage. Probably these drugs may counteract ischemia depolarization by stimulating the efflux of potassium ions from cellular compartments, and consequently protect cerebral circulation. Several drugs are under experimental tests.

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