Chapter 2

Excitotoxicity and Oxidative Stress in Acute Stroke

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Additional information is available at the end of the chapter

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Abstract

Excitotoxicity, defined as cell death resulting from the toxic actions of excitatory amino acids, is actually considered as a major factor contributing to the early stage of ischemic cell death in stroke. In stroke, once vessel occlusion is produced, the disruptions to the blood flow in the affected areas decrease the delivery of oxygen and metabolic substrates to neurons. Consequently, the lack of oxygen interrupts oxidative phosphorylation by the mitochondria and drastically reduces cellular ATP production, which results in a rapid decline in cellular ATP. After several minutes, inhibition of the Na^+/K^+-ATPase function causes a profound loss of ionic gradients and depolarization of regulated neurons, which leads to excessive release of excitatory amino acids—particularly glutamate—to the extracellular compartment. The presence of excessive amounts of glutamate into the synapses and extrasynaptic sites can lead eventually to neuronal death. Excitotoxicity leads to a number of deleterious consequences, including impairment of cellular calcium homeostasis, generation of free radicals and oxidative stress, mitochondrial damage, and activation of several transcription factors and their genes expression. All these mechanisms' acting synergy can cause neuron death by apoptosis. Oxidative stress induced by excitotoxicity is considered to be the main event leading to brain damage after stroke. On the basis of experimental models, there is ample evidence of the role of oxidative stress in ischemic brain damage.

Keywords: stroke, ischemia, excitotoxicity, NMDAR, calcium, oxidative stress, mitochondrial dysfunction, apoptosis

1. Introduction

Cerebral ischemia is defined as insufficient blood flow to the brain to supply an adequate amount of oxygen and nutrients. Cerebral ischemia accounts for about 80% of all strokes; the

other 20% are due to intracranial hemorrhage. Cerebral ischemia occurs when blood flow to the brain or any of its areas is insufficient to supply the oxygen and glucose that the tissue needs to maintain their metabolic activity. Ischemic stroke is the result of total or partial interruption of cerebral arterial blood supply (ischemia) by a thrombus or embolism, which leads to oxygen and glucose deprivation of the tissue that ultimately results in apoptotic and necrotic cell death. Cerebral ischemia may be either permanent (the thrombus occluding the vessel is not removed) or transient (followed by reperfusion). In all cases, a stroke causes dysfunction and death of brain neurons and neurological damage that reflects the location and size of the brain area affected [1, 2]. Brain tissue is very sensitive to ischemia because neurons obtain almost all energy using oxidative metabolism via mitochondrial oxidative phosphorylation where oxygen is the final electron acceptor.

In stroke, once the vessel occlusion is produced, if cerebral arterial blood flow is not restored within a short period, ischemic stroke is the usual result, with subsequent neuron death within the perfusion territory of the vessels affected. Acute ischemic stroke results from acute occlusion of cerebral arteries. Ischemic stroke is characterized by complex sequence of spatial and temporal events evolving over hours and days [1, 2].

Focal ischemia is characterized by an ischemic core surrounded by a "penumbra" region that has partial reduction in blood flow due to the presence of collateral arteries. Lesser reductions in blood flow, which do not lead to apparent functional or metabolic disturbances, are called benign oligemia and do not produce tissue injury [3]. In the ischemic core, a significant reduction of blood flow causes severe deprivation of oxygen and glucose and consequent total bioenergetics failure and neurons are unable to maintain the ionic gradients. As a result, a number of mechanisms that cause altered lipid and protein structural component of cellular membrane are triggered [4]. Neurons are killed rapidly within minutes and the tissue in the ischemic core is irreversibly damaged even if blood flow is reestablished [5].

In the penumbra, neurons become functionally impaired because the ability to fire action potentials is lost but remain metabolically active. Neurons in the penumbra maintain enough energy to sustain their resting membrane potentials, and when collateral blood flow improves, action potential and function are restored. Thus, ischemic penumbra refers to areas of the brain that are damaged during stroke but not killed [6]. In the absence of early reperfusion, the death of neurons in the ischemic penumbra due to ischemic injury progresses, leading to a reduction in penumbra area and expansion of the infarcted core. Tissue injury in the penumbra is the outcome of a complex series of genetic, molecular, and biochemical mechanisms, which contribute either to protecting and repairing the penumbra tissue and recovery of the functional activity or to damaging and then the penumbra area becomes necrotic. The ischemic core is generally considered unsalvageable, whereas the penumbra may be rescued by timely intervention and poses a target for the development of therapeutic treatment.

Results from the recent studies using imaging show that in the early minutes and hours after ischemia onset, the core ischemic contains pockets of injury, which were characterized as "minicores," and surrounded by "minipenumbras" that are heterogeneously distributed. The architecture and reversibility of the penumbra depend on time and location of rCBF reduction in the ischemic brain territory. Depending on the rCBF time, "minicores" coalesce with

"micropenumbras." In this way, if unimpeded, the "minicores" can grow into their respective "minipenumbras" to encompass a larger injury region [7].

2. Pathophysiology of neuron death in the stroke

In the last 20 years, experimental and clinical studies have allowed to identify and characterize the multiple mechanisms that injure the brain tissue in a stroke [1, 2, 5, 8]. Brain damage in ischemic stroke is the result of multiple mechanisms acting synergistically at physiological, biochemical, molecular, and genetic level, which impair neurological functions and may cause neuronal death [1, 8, 9] via mechanisms that promote rupture, lysis, phagocytosis or involution, and shrinkage [10]. Knowledge of the molecular mechanisms that underlie neuron death following a stroke is important if we are to devise effective neuroprotective strategies.

A severe transient or permanent reduction of CBF in a restricted vascular territory causes acute ischemic injury. Physiological values of CBF are between 45 and 60 ml blood/100 g/min. It is well documented that in response to reduced CBF time-dependent neuronal events are triggered [11]. Oxygen supply to the brain below a critical level reduces and eventually blocks the oxidative phosphorylation, drastically decreasing cellular ATP production leading to dysfunction of ATPase pumps and to the collapse of ionic gradients. The neuron activity ceases and if oxygen level is not restored quickly, cells die [12].

The brain is highly vulnerable to ischemia. In part, the vulnerability of brain tissue to ischemia reflects its high metabolic demands. The brain has a relatively high energy consumption and depends almost exclusively on the oxidative phosphorylation for energy production. Although the weight of the human brain is only about 2% of the total body weight, it has high metabolic activity and uses 20% of the oxygen and 25% of the glucose consumed by the entire body [13].

Brain activity involves a high metabolic activity by brain neurons, which require to have large amounts of oxygen and glucose. And since the brain has no oxygen storage capacity, the proper functioning of the brain depends on an abundant and continuous supply of oxygen.

Energy in the brain is mainly formed when glucose is oxidized to CO_2 and water through mitochondrial oxidative phosphorylation. The brain requires large amounts of oxygen to generate sufficient ATP to maintain and restore ionic gradients, although demands due to the activity of inhibitory interneurons [14], astrocytes [15], and other brain cells [16], just like the constant rebuilding of each neuron from its constituent proteins, are also a significant factor in the energy cost of brain function [17, 18].

3. Basic mechanisms of ischemic cell injury

Since the severity of ischemia is heterogeneous, the mechanisms involved in ischemic neuronal damage differ according to the severity of ischemia. Thus, in neurons located in the ischemic

"core" the ischemia leads to the inability of neuron to generate the energy needed to maintain the cell structure with the activation of mechanisms that cause cell death by necrosis, whereas neurons of the ischemic penumbra are able to maintain cell structure and the possibility of recovering the function. The brain injury in acute ischemic stroke is the result of multiple mechanisms acting synergistically [1, 5, 8].

After the onset of a stroke, ischemic stroke begins with the occlusion of a brain artery, with the consequent restriction in the delivery of basic nutrients to a cerebral region. The affected area of the brain receives insufficient oxygen and glucose. Severe deficit of oxygen and glucose due to disruption of blood flow leads to dysfunction in mitochondrial oxidative phosphorylation resulting in insufficient production of ATP and irreversible failure of energy metabolism [19] and an increase in the formation of superoxide radical [4]. Insufficient ATP leads to the inability of the neuron to maintain the ionic homeostasis; the increase in the formation of superoxide radical attached to a decrease in antioxidant activity can lead to an oxidative stress. The rise of the free radicals leads the mitochondria to increase the production of free radicals and an oxidative stress in the cell, resulting in the oxidation of proteins and lipids components of the structure of the cell membrane and DNA fragmentation. The result is necrotic cell death [20, 21]. It could be said that under these conditions of severe ischemia, neurons die by the direct action of the lack of oxygen and glucose, independently of the influence of neighboring cells. The situation is more complex when it comes to the penumbra zone.

Within the ischemic penumbra, multiple mechanisms have been identified that irreversibly damage brain tissue. After ischemic onset, while there are potential reserves of alternative substrates to glucose, such as glycogen, lactate, and fatty acids, for both glycolysis and respiration, there is no alternative capable of assuming the lack of oxygen and maintain mitochondrial oxidative phosphorylation, the main source of ATP in neurons. Consequently, in situations of severe oxygen deficit, as in the ischemic penumbra, ATP production by mitochondria is insufficient to maintain the activity of ATPase responsible for maintaining ionic gradients. Reduced ATP stimulates the glycolytic metabolism of residual glucose and glycogen, causing an accumulation of protons and lactate, which leads to rapid intracellular acidification and increases the depletion of ATP [4]. After several minutes, inhibition of the Na^+/K^+-ATPase function causes a profound loss of ionic gradients and the depolarization of neurons and astrocytes [1]. This leads to an uncontrolled depolarization of neurons affected in the penumbra, activation of voltage-gated calcium channels and to excessive release of neurotransmitters excitatory amino acids—particularly glutamate—in the presynaptic terminal (**Figure 1**). Simultaneously, neurotransmitter reuptake from the extracellular space is reduced [22, 23]. The presence of excessive amounts of excitatory amino acids into the synapses and extrasynaptic sites can eventually lead to neuronal death, in a process known as excito-toxicity [24], and it is defined as cell death resulting from the toxic actions of excitatory amino acids. Because glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) where it plays important roles in neuronal growth and axon guidance, in brain development and maturation, and in synaptic plasticity, and constitutes the basis of synaptic transmission in about 10^{14} synapses in the human brain, excitotoxicity usually refers to the injury and death of neurons arising from prolonged intense exposure to glutamate [25–

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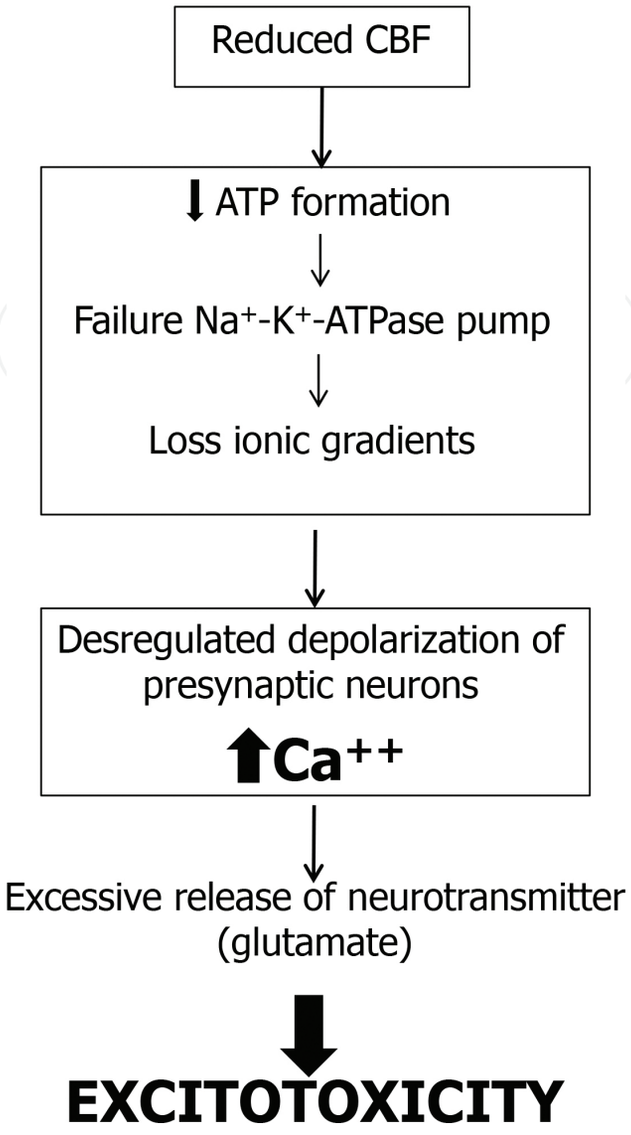

Figure 1. Excessive glutamate release by neurons in stroke. In neuron, the lack of oxygen and glucose due at reduced cerebral blood flow interrupts oxidative phosphorylation by mitochondria and reduces the cellular ATP production. Low ATP levels cause inhibition of the ATPase pump leading to profound loss of cellular membrane ionic gradient, cellular membrane depolarization, and the excessive glutamate release.

27], although other cells such as astrocytes may also suffer damage as a result of excessive levels of glutamate [28].

4. Excitotoxicity and acute ischemic stroke

Excitotoxicity is actually considered as a major factor contributing to the early stage of ischemic cell death in stroke [27, 29–31]. Excitotoxic death requires the excessive influx of the extracellular Ca^{2+} via receptor-operated channels or voltage-sensitive Ca^{2+} channels [32, 33]. The excessive intracellular Ca^{2+} initiates a series of molecular events that culminate in neuronal death [25, 34].

The rise in the extracellular glutamate concentration initiates a positive feedback loop, with further activation of glutamate receptors in neighboring neurons, and as a result, more Na^+ inflow to neurons via monovalent ion channels that decrease ionic gradients and consume ATP, both of which promote further release of glutamate [30]. A marked and prolonged rise in the extracellular glutamate concentration kills central neurons [2, 10]. Excessive glutamate in the synapses leads to glutamate receptors, at a pathophysiological level, triggering a cascade of events that can result in neuronal dysfunction and death.

Excitotoxicity leads to a number of deleterious consequences, including impairment of cellular calcium homeostasis, generation of free radicals and oxidative stress, activation of the mitochondrial permeability transition, secondary excitotoxicity, and activation of several transcription factors and their genes expression.

4.1. Overactivation of glutamate receptors and calcium overload by excitotoxicity

The excitatory effects of glutamate are mediated through two kinds of glutamate receptors: ionotropic receptors, the ligand and the ion channel are part of the same molecular receptor complex, and metabotropic receptors linked to G-protein [35]. They are located in the pre- and postsynaptic neuron membranes of the central nervous system. Glutamate ionotropic receptors are ligand-gated cation channels permeable to Ca^{2+}. Although practically all glutamate receptors are involved in excitotoxic processes, the N-methyl-D-aspartate receptor (NMDAR) is the key initiator of excitotoxic damage [36]. The glutamate overload leads to prolonged stimulation of AMPA and NMDA ionotropic receptor subtypes, which enhance the excessive influx of calcium, sodium, and water into neurons.

NMDAR consist of four subunits: two GluN1 (NR1) and two regulatory subunits that can be GluN2A (NR2A) through GluN2D (NR2D) and GluN3A (NR3A) or GluN3B (NR3B) [37, 38]. Subunit NR1 contains the site where the glutamate is united to the receptor, whereas subunit NR2 contains the site where the glycine is united [39]. The subunit combination and alternative splicing determine the functional properties of the receptors. The pharmacological and biochemical properties mediated by NMDA receptors are largely determined by the type of NR2 subunits incorporated into the heteromeric NR1/NR2 complex [40, 41]. Specific NR2 subtypes appear to play a pivotal role in strokes [42].

The blocking NMDARs containing NR2A enhanced neuron death and prevented the induction of ischemic tolerance, whereas inhibiting NMDARs that contained NR2B attenuated ischemic cell death and enhanced preconditioning-induced neuroprotection in an occlusion model of transient global ischemia in rats [43]. It has been suggested that excitotoxicity is triggered by the selective activation of NMDARs containing the NR2B subunit [43, 44].

Because NR2A and NR2B are the predominant NR2 subunits in the adult forebrain, NMDA receptors that contain NR2A and NR2B may play different roles. NMDARs that contain NR2A subunit would be involved in supporting neuronal survival, whereas NMDAR containing NR2B subunit would be involved in neuron death, and hence have opposing impacts on excitotoxic brain damage after acute brain insults, such as a stroke or brain trauma [42, 43].

NMDARs are found at synaptic or extrasynaptic sites [45, 46]. NMDARs are trafficked to the synapse throughout development and in adulthood, but a significant proportion remain extrasynaptic. The locations of NMDARs in different parts on cellular membrane of neurons that perform different functions suggest that it could be a determining factor in excitotoxicity after a stroke [45, 47]. Thus, participation of NMDARs in synaptic activity plays an important role in neuron survival, whereas activation of NMDARs in extrasynaptic activity appears to be associated with neuronal death [42]. In this regard, it has been observed that stimulation of synaptic NMDAR induces the expression of prosurvival proteins, such as brain-derived neurotrophic factor (BDNF), whereas activation of extrasynaptic NMDAR leads to expression of proapoptotic proteins and suppression of survival pathways [46, 48]. However, it has also been suggested that the apparent differences in excitotoxicity mediated by NMDARs could be due to differences in molecular composition between synaptic/extrasynaptic NMDARs as opposed to the location of the receptors *per se*. In adult brain, NMDARs located in synapses predominantly contain the NR2A subtype while extrasynaptic NMDARs predominantly contain NR2B [49, 50]. Although there is little evidence that differences in subunit composition explain the different effects of glutamate in the functioning of the synaptic or extrasynaptic location of the NMDAR, the activation of NMDARs containing NR2B subunits tends to promote neuron death, irrespective of the location, whereas activation of NMDARs containing NR2A subunits promotes survival [42]. However, NR2A-NMDARs are capable of mediating excitotoxicity [51] and NR2B-NMDARs are capable of mediating both prosurvival and prodeath signaling, depending on the stimulation paradigm [49]. In older neurons expressing comparable levels of NR2A- and NR2B-containing NMDARs, amelioration of Ca^{2+} overload required the inhibition of extrasynaptic receptors containing both NR2 subunits [52].

NMDARs interact with multiple intracellular synaptic and cytoskeletal proteins, mainly through the cytoplasmatic C-termini of the NR1 and NR2 subunits [53, 54]. NMDARs exit in multiprotein complexes that determine the efficiency with which their activation leads to specific signaling events into neuron. It has further been proposed that lethal Ca^{2+} signaling trough NMDARs is determined by interacting between the molecular complex and the NMDARs [55]. At the synapse, NMDAR receptors are found localized within electron-dense structures known as the postsynaptic densities (PSDs) where they form large and dynamic multiprotein signaling complexes [56, 57]. The PSD is a multiprotein complex that includes a group of proteins called membrane-associated guanylate kinases (MAGUKs) [53, 54]. The MAGUK proteins contain several PDZ (postsynaptic density-95/large/area occludens-1 disks) domains. These domains consist of 80–90 amino acids, which involved in the formation of large protein complexes in cells [57]. One PDZ domain, the PSD-95 (postsynaptic density-95), is involved in the formation of NMDAR complex intracellular signaling proteins and enzymes [53, 54]. The scaffolding of protein PSD-95 causes the translocation of nNOS from cytosol to membrane where it is linked to NMDAR through the complex NMDAR-PSD95-nNOS [58]. In ischemic brain, excessive Ca^{2+} influx through NMDAR activates nNOS, which leads to the production of excessive levels of nitric oxide (NO) [56]. This excessive NO together with the hydrogen peroxide produced from Superoxide dismutase (SOD) enzyme cause the production of the highly reactive free radicals peroxynitrites, which promote cellular damage and ultimately neuron death [59, 60]. Thus, during ischemia, Ca^{2+} influx through NMDARs

promotes cell death more efficiently than through other Ca^{2+} channels [61], suggesting that NMDAR ion channel is involved in excitotoxicity. The efficiency by which calcium ions activate excitotoxic signals through molecules such as nNOS can be reduced by disruption of the NMDAR-PSD-95 or nNOS-PSD-95 complexes. Suppression of PSD-95 selectively blocks NO production induced by glutamate in NMDARs without affecting NOS expression in cortical neurons [56]. In experimental animals, the use of small peptides that disrupted the interaction of NMDARs with PSD-95 improves the resistance of neurons to focal cerebral ischemia [62]. The importance of the interaction of NMDAR/PSD-95 complex in cerebral ischemia is reinforced by results showing that the inhibition of binding of PSD-95 with NMDAR prevents ischemic brain damage, while the physiological function of the NMDAR remains intact [63]. In neuron cultures, the block of protein-protein interactions of NMDAR/PSD-95 using small peptides that bind to the PDZ domains of PSD-95 modifying their molecular structure protected neurons from excitotoxicity. When these small peptides are used in rats subjected to transient focal cerebral ischemia, a dramatically reduced cerebral infarction and effectively improved their neurological function in rats was observed. The treatment was effective when applied either before or 1 h after the onset of excitotoxicity both *in vitro* as *in vivo* cerebral ischemia [63]. The vulnerability of neurons to excitotoxicity and ischemia was reduced when the NMDAR/PSD-95 interactions were disturbed using small peptides that comprise the NR2B subunit. Proteomic and biochemical analysis of all the human PDZs examined shows that only neurons lacking PSD-95 or nNOS exhibited reduced excitotoxic vulnerability. Only PSD-95 and nNOS participated significantly in excitotoxicity signaling. Thus, it has been shown that despite the ubiquity of proteins that contain the PDZ domain, PSD-95 and nNOS play a more important role in mediating NMDAR-dependent excitotoxicity than any other PDZ proteins [51, 64].

The consideration of the activation of NMDARs, regardless of whether their participation in excitotoxic mechanisms is based on the type of subunit [42, 50] or by their different location [45, 46], as the main responsible mechanism of the disruption of calcium homeostasis in ischemic neurons is perhaps oversimplistic since others mechanisms may be involved [65]. TRP channels inhibitors reduce calcium entry into neurons exposed to excitotoxicity. Members of the TRP family [66], TRPM7 and TRPM2 are membrane channels that are activated during ischemia and contribute to the rise in intracellular calcium [65, 67]. Also, cerebral ischemia increases the calcium permeability about 18-folds in the AMPA glutamate receptors, contributing to the increase in the cytosol Ca^{2+} levels [68]. Other pathways involved in calcium influx into neurons that contribute to the accumulation of calcium in the cytosol of ischemic neurons include Ca^{2+} entry through gated voltage-channels, Ca^{2+}-permeable acid-sensing ion channels [69], activation of metabotropic glutamate receptors via the release of Ca^{2+} from endoplasmic reticulum, and via a cleavage of Na^{+}/ Ca^{2+} exchangers [70]. These data suggest that the main factor involved in the neuron death in stroke is the disruption of Ca^{2+} homeostasis leading to the accumulation of free cytosolic Ca^{2+} and not the route of entry.

In short, the main effect of excessive extracellular glutamate accumulation on the membrane of the neuron is caused by excessive accumulation of calcium in the cytosol and increased NOS activity leading to an increase in intracellular levels of NO.

4.2. The role of the cytoplasmic calcium overload in excitotoxicity

Calcium plays a critical role in the excitotoxic cascade. Thus, excitotoxic cascade does not occur when the Ca^{2+} is removed from extracellular medium [71] or preventing Ca^{2+} release from mitochondria by uncouplers [72]. It is now widely accepted that disturbance of cellular Ca^{2+} homeostasis is key in the death of neurons following a stroke [61, 64, 73]. It is well established that there is a close relationship between the neuronal damage initiated by the excessive release of glutamate during stroke with an excessive calcium influx into the injured neurons [2, 8, 74]. After ischemia, cytoplasmic Ca^{2+} levels in the ischemic neurons rise to 50–100 μM. Such excessive Ca^{2+} levels can trigger many downstream neurotoxic cascades, including the activation and overstimulation of proteases, lipases, phosphatases, and endonucleases [71, 73, 74]. The results include the activation of several signaling pathways, mainly causing an overproduction of free radicals, mitochondrial damage, cell membrane disruption, and DNA fragmentation, which synergistically cause neuron death [1, 2, 64, 75] (**Figure 2**).

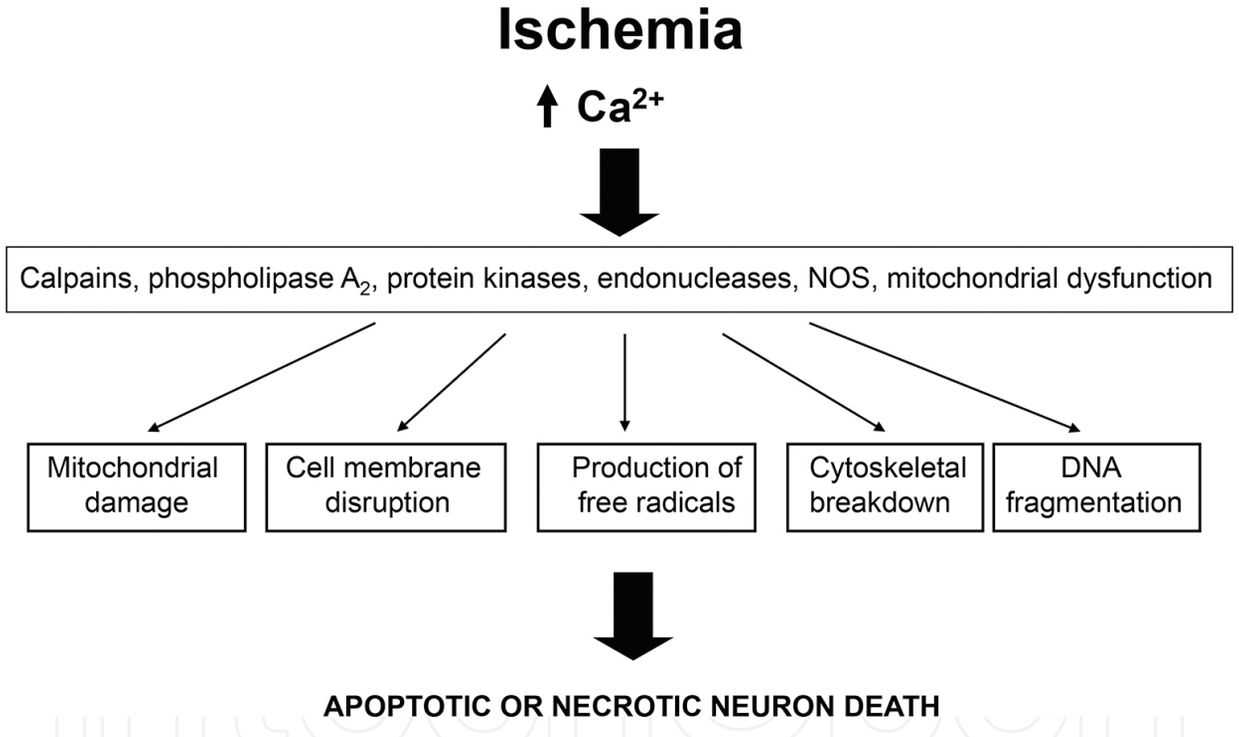

Figure 2. Effects of excessive accumulation of free cytosolic calcium in neurons. Calcium overload induced by excitotoxicity leads to the activation and overstimulation of proteases, lipases, phosphatases, and endonucleases that mainly results in mitochondrial damage, cell membrane disruption, and excessive production of free radicals, which act synergistically causing apoptotic or necrotic neuron death.

4.3. Oxidative stress in acute ischemia damage

Major excitotoxic events promoted by cytoplasmic Ca^{2+} overload due to massively activated glutamate receptors include oxidative/nitrosative stress, calpain activation, and mitochondrial damage, although each of these may individually cause cell death, in ischemia they act synergistically (**Figure 3**).

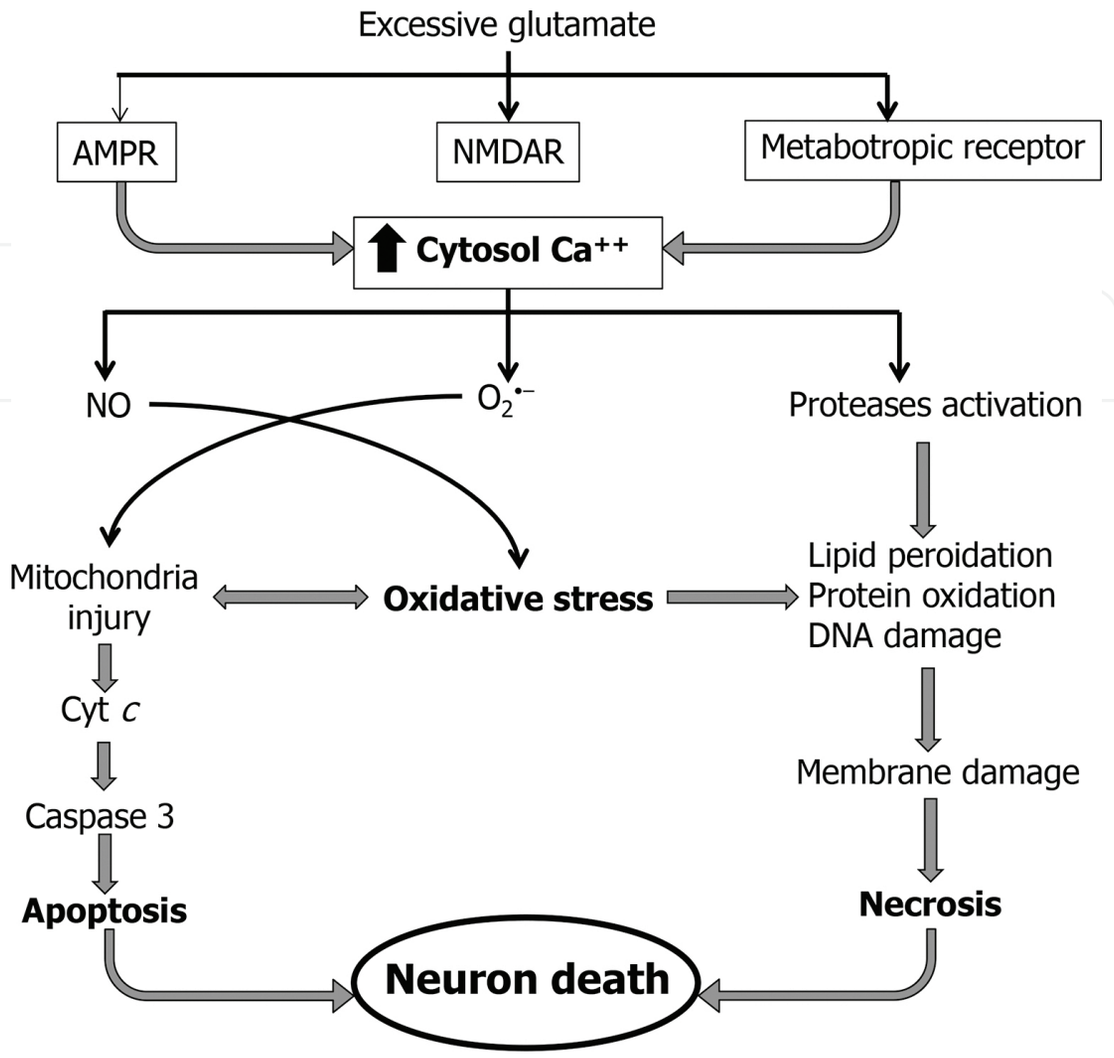

Figure 3. Major cellular mechanisms induced by overstimulation of glutamate receptors in neurons. As a result of excessive activation of glutamate receptors, there is an excessive Ca^{2+} accumulation in the cytosol of neurons leading to oxidative stress and mitochondrial dysfunction. Both situations trigger processes that ultimately cause the death of the neuron.

Oxidative stress is generally defined as an imbalance that favors the production of free radicals over their inactivation by antioxidant defense systems [76, 77]. Oxidative stress describes a condition in which cellular antioxidant defense are insufficient to keep the levels of free radicals below a toxic threshold. This may be either due to excessive production of "free radicals," loss of antioxidant defenses, or both. A "free radical" is any chemical species (atom, molecule) capable of independent existence having one or more unpaired electrons. Free radicals are highly reactive and can directly oxidize and damage macromolecules such as proteins [78], lipids [79], and DNA [80]. Through such interactions, free radicals may irreversibly destroy or alter the function of the target molecule and to worsen the cellular structural architecture and ultimately to cell death. Indirectly, free radicals may also initiate reactions, which may finally lead to neuron death. These reactions include mainly mitochondrial dysfunction [81], cascade apoptotic activation [82], and signal transduction pathways activation [83].

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Many lines of evidence demonstrate that free radicals play a pivotal role in excitotoxic death in the brain after stroke [1, 81, 84–86]. The most common free radicals induced by excitotoxicity are molecular derived from oxygen and nitric oxide, called reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively. Several ROS, including the superoxide radical ($O_2^{\bullet}-$), hydroxyl radical ($OH^{\bullet}$), and certain nonradicals that are either oxidizing agents or easily converted into radicals, such as hydrogen peroxide (H_2O_2) and RNS nitrogen-derived molecules, such as nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$), are generated after stroke. Although NO itself is not a radical, it has not been reported that high levels of NO are toxic; however, in the presence of H_2O_2 and $O_2^{\bullet}-$, NO reacts spontaneously leading to the formation of $OH^{\bullet}$ and peroxynitrite acid, which are very cytotoxic.

Although initially free radicals have been identified as major contributors to damage in biological organisms, under physiology conditions free radicals are continuously generated during oxidative metabolism and homeostatic levels are maintained because they play an important role in physiological control of the cell function [87–89]. Intracellular sources of ROS include the mitochondrial electron transport chain (ETC), NADPH oxidases, xanthine oxidase, and arachidonic acid.

Healthy mitochondria use oxygen to generate ATP by oxidative phosphorylation at the mitochondrial respiratory chain. While passing through the mitochondrial electron transport chain, some electrons escape from the mitochondrial ETC, especially from complexes I and III, and react with O_2 to form superoxide anion radicals ($O_2^{\bullet}-$) in the mitochondrial matrix [90–93]. $O_2^{\bullet}-$ is rapidly converted to H_2O_2 either spontaneously, particularly at low pH, or by the superoxide dismutase [90]. In normally respiring (uninhibited) mitochondria, the formation of superoxide and hydrogen peroxide is barely detectable [94–96].

Other important source of ROS is through NADPH oxidase (NOX), an enzyme that uses NADPH to reduce O_2, thus generating large amounts of $O_2^{\bullet}-$. NOX was originally discovered in neutrophils, and subsequently identified in many other cell types including neurons [97]. NOX is a multisubunit complex composed of membrane-associated subunits, cytosolic subunits, and one small rho GTP-binding proteins. In its active form, the NOX transports electrons from NADPH complex in cytosol to the extracellular space. In general, the electron acceptor is oxygen and the product of the electron transfer reaction is $O_2^{\bullet}-$. Thus, overactivation of NOX is a main source of $O_2^{\bullet}-$ in stroke [97]. NOX behaves as an important pro-oxidant enzyme. Protein expression and NOX activity rise after ischemia increasing oxidative stress in the brain tissue in the mice [98]. The postischemic $O_2^{\bullet}-$ generation in neurons was reduced when NOX is inhibited. This reduction of the $O_2^{\bullet}-$ levels due to NOX inhibition was associated with a decrease in the amount of lipid peroxidation or DNA fragmentation [98].

RNS are molecules derived from the nitric oxide ($NO^{\bullet}$). It is now well established that NO is a physiological messenger in the central nervous system and is synthesized by the NO synthase (NOS)-catalyzed reaction [99]. At least three isoforms of NOS have been characterized in brain cells. Neurons produce $NO^{\bullet}$ mainly by Ca^{2+}-dependent activation of neuronal NOS (nNOS or NOS1), which is constitutively expressed in these cells [100]. $NO^{\bullet}$ has a relatively long half-life (approx. 1 s) and whose reactions with biological molecules are slow due to its very rapid diffusion into the blood and consequent removing by reacting with oxyhemoglobin to form

nitrate. Along with its important physiological functions, excessive production of $NO^{\bullet}$ is an important pro-oxidant radical because of its ability to combine with $O_2^{\bullet-}$ and H_2O_2 to form $OH^{\bullet}$ and peroxynitrite ($ONOO^-$). The latter is readily protonated at cellular pH to peroxynitrous acid (ONOOH), which is very cytotoxic [99, 101].

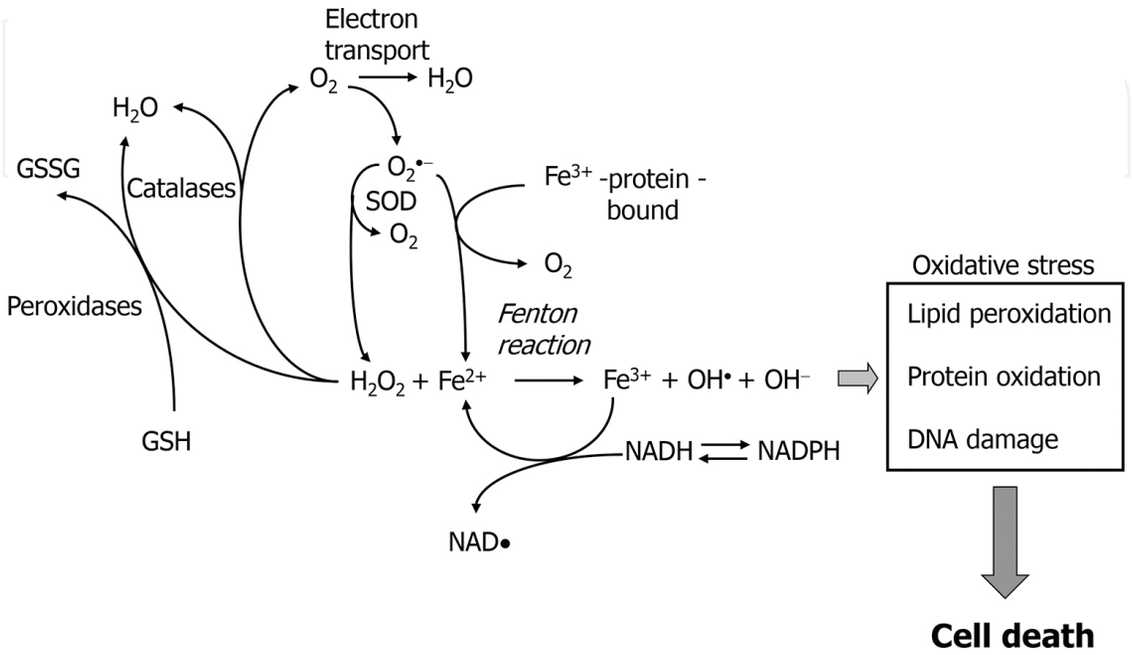

Figure 4. Endogenous antioxidant enzyme systems. Cellular reactions that cause oxidative damage to lipids, proteins, and DNA via Fenton reaction and cell protection by the endogenous antioxidant enzymes principles (SOD, catalases, and peroxidases). Homeostatic level of $O_2^{\bullet-}$ is regulated by SOD isoforms to O_2 and H_2O_2. The latter is a potential source of $OH^{\bullet}$ via Fenton reaction. Catalases and peroxidases regulate the levels of H_2O_2.

To maintain the homeostatic balance and cope with the continuous production of free radicals, cells are equipped with a sophisticated system of enzymes and nonenzymatic antioxidants [76]. Enzymatic components mainly comprise superoxide dismutase (SOD) [102] that converts $O_2^{\bullet-}$ to H_2O_2, the catalase [67] that converts H_2O_2 to H_2O and O_2, and the glutathione peroxidases (GPX) that converts H_2O_2 to H_2O in a reaction that oxidizes glutathione (GSH) to its disulphide form (GSSG). In turn, GSH is regenerated from GSSG by glutathione reductase [103, 104] (**Figure 4**). Also, small molecular nonenzymatic antioxidants, including ascorbic acid, pyruvate, α-tocopherol, and glutathione (GSH), are important in the detoxification of free radicals, provision of antioxidant defense, and prevention of tissue damage [76, 77]. The cell can also combat oxidative stress by regulating ROS generation by eliminating ROS with the help of neutralizing enzymes and scavenger molecules [105], and by repairing those lipids, proteins, or DNA that have been affected by oxidative stress [106].

Oxidative stress induced by excitotoxicity is considered the main event leading to brain damage after cerebral ischemia [73, 81, 82, 107]. Several lines of research indicate that oxidative stress is a primary mediator of neurologic injury following cerebral ischemia [84, 85, 107]. After cerebral ischemia and particularly reperfusion, robust oxidants are generated including superoxide and hydroxyl radicals, which overwhelm endogenous scavenging mechanisms

[108, 109] and are directly involved in the damage of cellular macromolecules, such as lipids, proteins, and nucleic acids, eventually leading to cell death [1, 2, 110] (**Figure 5**). Reperfusion provides oxygen to sustain neuronal viability and also as a substrate for numerous enzymatic oxidation reactions that produce reactive oxidants. During reperfusion to the vessel, oxygen replenished is crucial for neuron survival in the ischemic tissue. However, oxygen can also be used by the mitochondria and by pro-oxidant enzymes to produce more free radicals. The presence of a situation of oxidative stress in the perfused ischemic brain tissue results in several detrimental processes, including overproduction of oxygen radicals, and consumption and failure to adequately replenish the antioxidant systems [108–110].

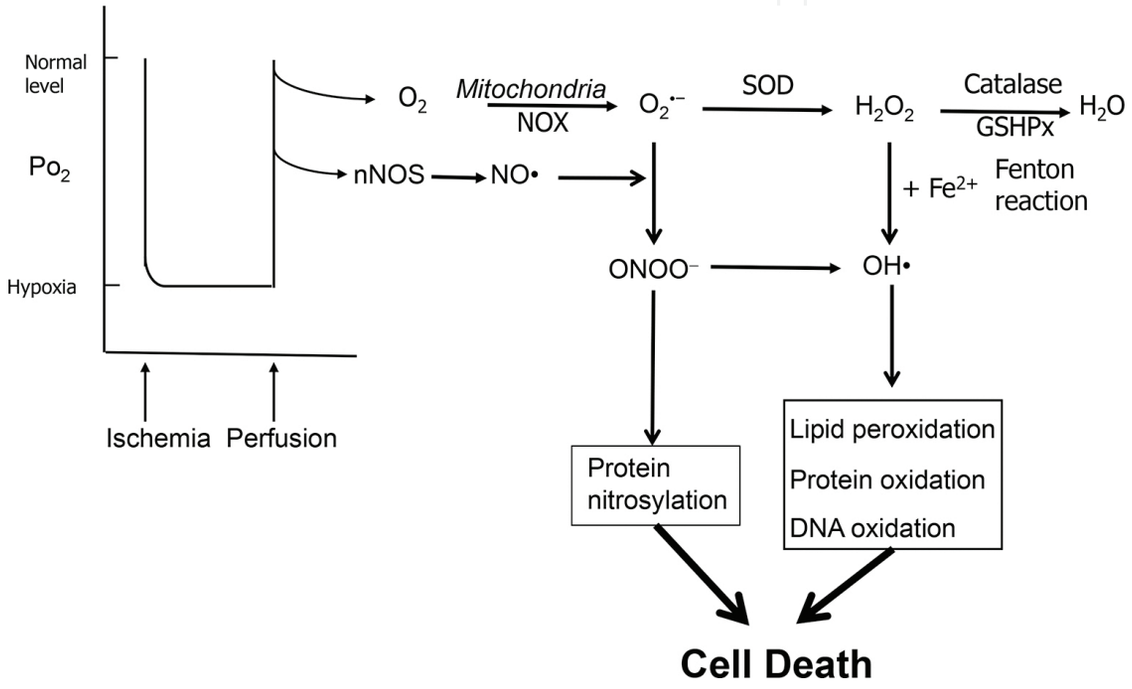

Figure 5. Major sources of ROS and RNS and antioxidant systems during cerebral ischemia and reperfusion. Generated reactive oxygen species by mitochondria and reactive nitrogen species by nitric oxide synthase. SOD converts superoxide radical to H_2O_2 which is converted to H_2O by catalase or GSHPx. Formation of peroxynitrite ($ONOO^-$) and subsequent hydroxyl radical production can directly damage lipids, proteins, and DNA and lead to cell death.

During the ischemic phase, some Ca^{2+}-dependent enzymes, such as phospholipase A_2 (PLA_2) and cyclooxygenase (COX), produce oxygen-free radicals. The activation of PLA_2 by Ca^{2+} results in the generation of arachidonic acid from the phospholipids, which are metabolized by cyclooxygenase to thromboxane and leukotrienes. Both eicosanoids activate NOX and thus contribute to increased free radical formation [111]. COX induces the prostaglandin PGG_2 production from arachidonic acid, which in turn is rapidly peroxidized to PGH_2 with a simultaneous release of $O_2^{\bullet -}$. In the ischemic tissue, activation of PLA_2 and cyclooxygenase generates free radical species, which contribute to lipid peroxidation and membrane damage [111]. However, mitochondrial dysfunction and NOX as sources of RNS are considered the main source of free radical production causing oxidative stress during ischemia/perfusion [107, 112].

4.4. The role of calpains in excitotoxicity

It is now well established that the induced Ca^{2+} overload in neurons after ischemia causes a massive activation of calpains, proteins belonging to the family of calcium-dependent, cysteine proteases, which contribute to excitotoxic cell death [47]. All calpains can act in two modes: under physiological conditions they undergo controlled activation (involving only a few molecules of calpain), whereas during sustained calcium overload under pathological conditions they undergo hyperactivation (involving all the available calpain molecules) [47]. Calpains in the CNS play an important role in the synaptic function and memory formation [47]. In models of stroke in animals, the use of Ca^{2+}-dependent calpain protease inhibitors showed neuroprotective effect [113, 114]. The main mechanism by which calpains contribute to excitotoxic damage is by their ability to cleavage the Na^{+}/Ca^{2+} exchangers (NCX), which is critical to regulate the concentration of calcium into neurons [115]. Thus, calpains contribute to the accumulation of calcium during ischemia [70, 116].

4.5. Mitochondrial dysfunction

The excitotoxicity can contribute to neuron death by altering the functions of mitochondria. Mitochondrial disturbance is the result of both oxidative-nitrosative stress and a direct effect of excessive Ca^{2+} intracellular levels [75, 117, 118]. In ischemia, the excess of cytosolic-free Ca^{2+} caused by overstimulation of glutamate receptors may overload the mitochondrial proton circuit, which leads to the failure in oxidation and an increase in ROS into mitochondria [81, 91]. In mitochondria, surpassed antioxidant protection and oxidative stress ROS can lead to

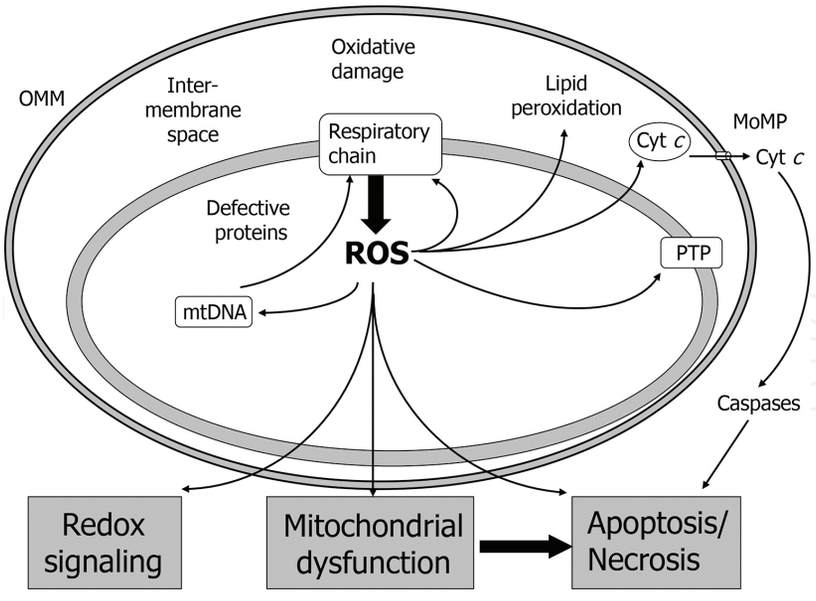

Figure 6. Excessive ROS production by mitochondria leads to direct oxidative damage of mitochondrial proteins, membranes, and DNA, causing mitochondrial dysfunction and finally death neuron by apoptosis or necrosis. Excessive ROS within mitochondria can also induce changes in the mitochondrial permeability transition pore mPTP, which renders the inner membrane permeable to small molecules including cytochrome *c* (cyt *c*). Activation of proapoptotic proteins Bcl-2 produces a pore in the outer membrane of mitochondria that allows the release of cytochrome *c* to the cytosol where it triggers the apoptotic cascade of caspases (modified from Ref. [91]).

oxidative damage to mitochondrial proteins, membranes, and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions. Mitochondrial oxidative damage can also enhance the release of proteins located in the mitochondrial inner membrane, including cytochrome *c*, to the intermembrane space. Activation of proapoptotic proteins Bcl-2 enhance the formation of permeability pore in the outer membrane of mitochondria that allows the release of cytochrome *c* to the cytosol where it activates the apoptotic machinery of the cell [91] (**Figure 6**).

During stroke, electron microscope analyses show that Ca^{2+} accumulates in mitochondria very soon after global ischemia and this state persists for several hours [119]. Two events seem to play an important role in the death of neurons in the brain stroke due to the mitochondrial dysfunction induced by the cytosol-free Ca^{2+} accumulation: The oxidative stress due to antioxidant/pro-oxidant imbalance in favor of the second [107, 112], and the activation of the intrinsic apoptotic pathway of caspases [120, 121].

5. Apoptosis

In stroke, neuron death is the result of apoptosis or necrosis [82, 122]. That neuronal death occurs by one or another mechanism depending mainly on the time elapse since the onset of stroke, the severity of blood flow reduction, and the level of metabolic activity that produce the energy in the neuron.

Neuronal cell death by necrosis occurs mainly when the decrease or absence of blood flow implies a severe deficit or absence of oxygen and glucose (OGD) in ischemic brain area and leads to the formation of the ischemic core [20, 21, 123]. Death of neurons by apoptosis occurs mainly in ischemic areas when the existence of a certain level of blood flow provides insufficient oxygen and glucose to maintain the functional activity in the neuron but sufficient to maintain the survival of neurons and leads to the formation of ischemic penumbra. If the blood flow is not restored in this area, excitotoxicity may induce neuronal death by apoptosis [120].

Apoptosis is one of the fundamental mechanisms of cell death that occur during ischemic brain injury [2, 120, 124]. There are two general pathways for activation of apoptosis: the "extrinsic apoptosis," initiated by the ligation of cell surface death receptors such as tumor necrosis factor receptors and FAS receptor, and "intrinsic/mitochondrial apoptosis" pathways [125]. The mitochondrial dysfunction plays a central role in the apoptotic pathway in stroke [120, 126, 127]. Studies of tissue from patients and of animal models have shown that mitochondria-mediated apoptosis is the mode by which many neurons die after an acute stroke [121, 127]. Oxidative stress and cytotoxic accumulation of intracellular Ca^{2+} initiate a series of cytoplasmic and cellular events, including the triggering of the intrinsic apoptotic pathway [2, 34, 115, 121]. Increased ROS/RNS and intracellular-free Ca^{2+} levels mediate induction/activation of proapoptotic proteins leading to changes in the mitochondrial membrane permeability (MMP) [128]. The family Bcl-2 proteins determine the integrity of mitochondria in the face of apoptotic insult [127]. The complex interplay between Bcl-2 proteins regulates the integrity of the mitochondrial outer membrane. Thus, the mitochondrial integrity may be protected by

antiapoptotic members of the Bcl-2 family (Bcl-2 and Bcl-xl) together with antiapoptotic kinases Akt and ERK, which inhibit the proapoptotic members of the Bcl-2 family (Bid, Bim, Bax, Bak, and Bad). In cerebral ischemia, proapoptotic stimuli activate the intrinsic apoptotic pathway breaking the antiapoptotic/proapoptotic balance leading to mitochondrial network damage in the neuron [127, 129].

Two members of Bcl-2 family, Bcl-2-associated X protein (BAX) or Bcl-2-associated killer (BAK) seem crucial to cell death. Without them, cells are resistant to majority of apoptotic stimuli [130]. BAX is a cytosolic protein that actively translocates to the mitochondrial outer membrane during apoptosis to participate in membrane damage while that BAK is constitutively expressed at the mitochondrial outer membrane [131]. More recent studies propose that BAX is actively trafficked to the cytosol, a process termed "retrotranslocation" [132, 133]. A differential mitochondrial retrotranslocation has also been proposed by BAK [134].

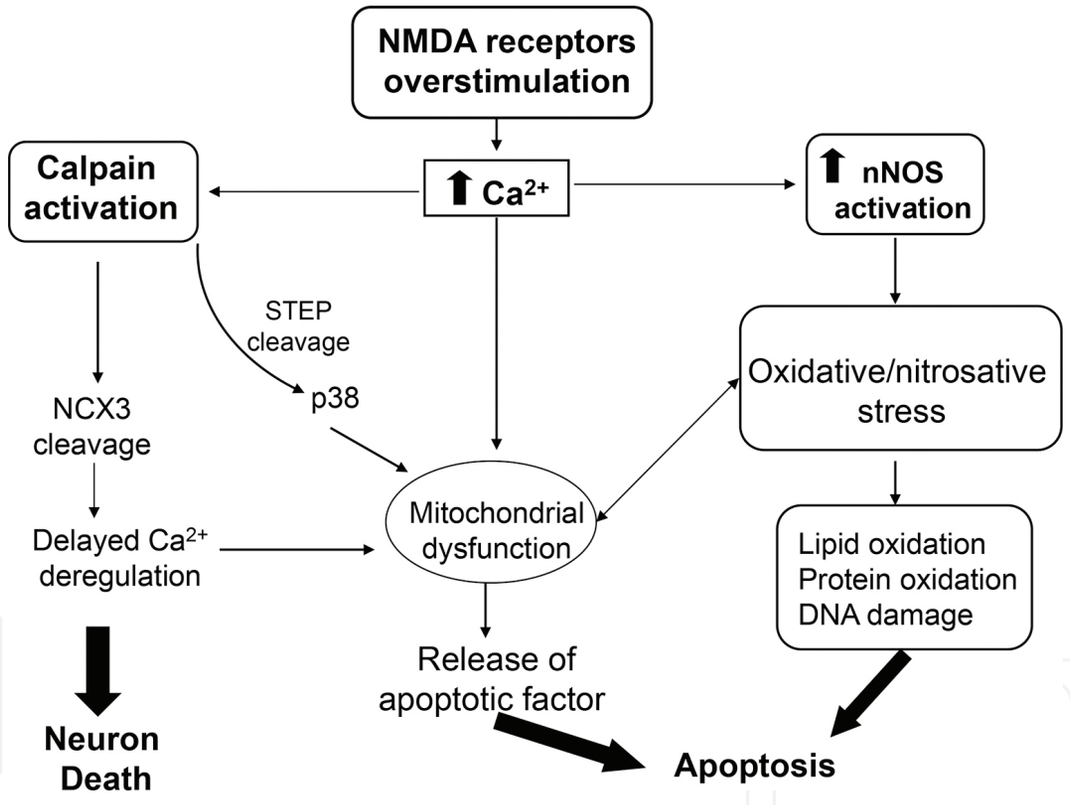

Figure 7. Schematic overview of excitotoxic events during ischemic stroke in the neurons. Overstimulation of NMDAR by glutamate causes excessive increase in calcium concentration in the cytosol. Disruption of intracellular calcium homeostasis leads to calpain and nNOS activation, and mitochondrial dysfunction. Activation of these mechanisms causes a state of oxidative stress. The presence of excess ROS and RNS directly damages essential macromolecules of the cell membrane and otherwise contribute to produce mitochondrial dysfunction. This, in turn, leads to increase oxidative stress and trigger the chain apoptotic events causing neuronal death and contribute to ischemic brain damage.

One of the decisive steps of the apoptotic cascade involves the mitochondrial permeability transition pores (mPTPs) [135, 136]. Under oxidative stress conditions, transient opening of mPTPs in the mitochondrial inner membrane produces the fall of the mitochondrial trans-

membrane potential and elicits the release of cytochrome *c* as well as other proapoptotic molecules that together initiate the apoptotic cascade. Once released into the cytosol from the mitochondrial intermembrane space, cytochrome *c* binds with apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9 to form an "apoptosome," which actives caspase-9 and subsequently caspase-3. There is a large body of evidence suggesting that cerebral ischemia can cause activation of aspartate-specific proteases, the caspases, which can cleave a larger number of cellular substrates [120, 121]. Caspases are cysteine proteases constitutively presents in cells as zymogens and require proteolytic cleavage into the catalytic active heterodimer. Inhibiting the activation of caspases suppresses the ability of cells to undergo apoptosis or causes a switch from apoptosis to necrosis [137]. Upregulation and activation of caspase-3 have been found to precede neuron death in focal and global cerebral ischemia [120]. Activated caspase-3 cleavage the endonuclease inhibitor ICAD freeing CAD (caspase-activated DNAase), which can cleave the nuclear DNA causing DNA damage and the neuron death [138].

In summary, numerous studies report the important involvement of excitotoxicity and oxidative stress in the complex processes that cause neuronal death in acute ischemic stroke [139]. The hypoxia and the low glucose levels caused by the blood flow reduction in the penumbra zone lead to oxidative stress and excessive release of glutamate. Oxidative stress can cause death of neurons by oxidation of structural macromolecules of the membranes, such as lipids and proteins, and DNA. Mediated by NMDA receptor and by the homeostasis calcium deregulation, excitotoxicity contributes not only to injury of neuron death, but also to transduction of apoptotic signals. Mitochondrial dysfunctions occur as a consequence of cerebral ischemia and promote ischemia-induced neuronal cell death, especially by apoptotic intrinsic pathway (**Figure 7**).

Acknowledgements

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