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The Role of Antioxidant Treatment in Acute Ischemic Stroke: Past, Present and Future

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ABSTRACT

Background: *Oxidative stress in acute ischemic stroke was extensively evaluated in the past years. Attempts to influence it in animal models of stroke showed benefit, but in human clinical trials the results were disappointing.*

Material and Methods: *The oxidative stress was evaluated by sequentially measuring the malondialdehyde levels in a series of 100 consecutive ischemic stroke patients. Stroke subtype was classified according to the TOAST criteria. Patients with undetermined stroke subtype and with other causes of increased oxidative stress were excluded.*

Results: *Oxidative stress was significantly increased only in cardioembolic stroke. This can be explained by the fact that the embolus is not adherent to the vessel wall and often undergoes spontaneous fragmentation and lysis, reestablishing the blood flow. Spontaneous recanalisation of the vessel leads also to haemorrhagic transformation often seen in cardioembolic strokes. On the other hand, reestablishing the blood flow supplies oxygen needed for oxidative stress to develop. These findings can also explain the experimental results found in animal models, in which ischemic stroke was accomplished by temporarily clamping the carotid artery. Further developing the idea, antioxidant therapy could prove beneficial in those stroke subtypes in which blood flow is reestablished, namely embolic stroke, after thrombolysis or mechanical clot retrieval by diminishing the magnitude of reperfusion injuries.*

Conclusion: *It would be worth reevaluating the effect of antioxidant therapy only in cardioembolic stroke and in the context of vessel recanalisation.*

Keywords

Antioxidants, Cardioembolic stroke, Ischemia/reperfusion injuries, Recanalisation.

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.

Brief Historical Background

The story of oxidative stress begins at the middle of the 20th century when Commoner and coworkers [1] discovered the presence of free radicals in biological materials. Soon thereafter, based on the works of Denham Harman [2], free radicals were viewed as a Pandora's Box of evils that accounted for cellular damage, genetic mutations, cancer, and the degenerative processes of biological aging themselves. The discovery of the enzyme superoxide dismutase (SOD) by McCord and Fridovich [3] marked the entry into a second era of the science of free radicals in living organisms. Gradually most researchers became convinced that free radicals are important in biology. Now, at the beginning of the third millennium, a large body of evidence has accumulated showing that living organisms have adapted to the unfriendly coexistence with free radicals and have even developed mechanisms for their advantageous use. Free radicals are known to be involved in the regulation of vascular tone, in the enhancement of signal transduction from membrane receptors, and are used by the immune system to attack and kill pathogens. Neutrophils, for example, contain an enzyme – myeloperoxidase, which uses hydrogen peroxide and chloride to produce hypochlorous acid,

actually the main ingredient in household bleach. Due to the large number of infectious diseases in early childhood the natural selective process favored individuals capable of producing large amounts of oxidants as antimicrobial defenses at the expense of suffering greater collateral damage in diseases occurring beyond the age of reproduction [4].

Oxidative stress and the nervous system

Oxidative damage can occur in any organ or system but the nervous system is especially vulnerable to reactive oxygen species-induced injury due to a series of anatomical, histological, physiological and metabolical particularities, namely [5]:

- The high energy needs of the brain lead to a high rate of oxygen consumption
- The neuronal membranes are rich in polyunsaturated fatty acids (PUFA), which are particularly vulnerable to free radical attack.
- The ratio of membrane surface area to cytoplasmic volume is high.
- The neuromediator glutamate is ubiquitous and is the major effector that causes oxidative stress.
- There is a high calcium traffic across neuronal membranes, and interference of ion transport, both increasing intracellular calcium which often leads to oxidative stress.
- The autooxidation of neurotransmitters (e.g., dopamine) can generate O2 and quinones that reduce glutathione.
- Damage to the cerebral parenchyma releases iron ions capable of catalyzing free radical reactions.
- The antioxidant defense mechanisms are modest, the brain having low levels of catalase, glutathione peroxidase, and vitamin E.

Detection of free radicals

The detection of free radicals is challenging due to their short half-life. Several methods of detecting the presence of free radicals have been employed:

- The direct quantification of reactive oxygen and nitrogen species is a complex task. Approaches include electron spin resonance, fluorescence magnetic resonance, and mass spectrometry techniques, but their use has been limited to cell cultures and other in vitro applications [6,7].
- A different approach is measuring in vivo stable compounds which result from the reaction of free radicals with lipids, proteins, or DNA [8]. The presence of unsaturated double bonds makes polyunsaturated fatty acids (PUFAs) highly susceptible to oxidative damage in the presence of reactive oxygen species (ROS) or free radicals. Malondialdehyde (MDA) is one of the thiobarbituric acid reactive substances which can react with two equivalents of thiobarbituric acid (TBA) to give a pink adduct complex, easily measured by a colorimetric or fluorimetric assay. Despite TBA test for MDA determination being the most frequently used method to evaluate lipid peroxidation, it shows several pitfalls and has been criticized as being too unspecific and prone to artifacts [9,10]. Oxidative modifications of proteins include carbonylation, nitrosilation, breaking of the histidine and tryptophan rings, and hydrolysis of the peptide bond in the presence of proline. Several methods have been developed for the detection of the different kinds of protein modifications. However, the biological and clinical relevance of protein oxidation as a biomarker is still limited.
- The total antioxidant capacity (TAC) is defined as the moles of oxidants neutralized by one liter of body fluids. In plasma, nonenzymatic antioxidants include endogenous (e.g., UA, bilirubin, and thiols) and nutritional (e.g., tocopherols, ascorbic acid, carotenoids, and phenolics) compounds. Obviously, the concentration of these molecules will diminish as they are used up in neutralizing oxidative species [11].
- Recently QUEnch-assiSTed MRI (QUEST MRI) has been developed, which provides a unique measure of in vivo production of endogenous, paramagnetic reactive oxygen species (ROS) [12].

Oxidative Stress in Ischemic Stroke

Oxidative stress in cerebral ischemia

Results from a large number of studies have shown that after cerebral ischemic injury, free radical production is greatly increased and causes redox disequilibrium in the natural endogenous antioxidant system [13-17]. Detoxification mechanisms are inactivated and oxidants are overproduced. In addition, the one widely used and approved therapy for focal occlusion of a cerebral artery, tPA itself has been shown to increase oxidative stress, damage the blood brain barrier (BBB) and promote thereby hemorrhagic complications [18].

The sequence of events begins at the mitochondrial level with a significant reduction in the electron transport chain activity resulting in adenosine-5'-triphosphate (ATP) depletion. Once mitochondrial ATP synthesis is inhibited by global ischemia, ATP is consumed within minutes, causing depolarization of the neuronal plasma membrane, potassium leakage into the extracellular space and sodium entry into cells. In addition, the bioenergetic failure prevents the plasma membrane Ca^{2+}-ATPase from maintaining the normally low concentration of cytosolic calcium. Soon after cessation of blood flow, these metabolic impairments cause a large increase in neuronal activity and enhanced neuromediator release. Glutamate activates several pre- and post-synaptic glutamate receptors resulting in a rise in intracellular calcium leading to excitotoxic injury. Additionally, the elevated extracellular glutamate concentrations can activate neuronal nitric oxide synthase (nNOS). High levels of intracellular calcium, sodium and adenosine diphosphate potentiate the mitochondrial dysfunction leading to the generation of ROS and reactive nitrogen species (RNS) and the activation of proteases, phospholipases, and endonucleases, resulting in cell death [19,20].

Oxidative stress in ischemia-reperfusion injuries

Restoring blood flow to the brain was expected to be a reversal of the ischemia-induced disruption of the cellular milieu. Unfortunately, this is not the case. Experimental evidence shows that reperfusion triggers a set of potentially pathologic events including increased prostaglandin synthesis, elevated production of second messengers,

inflammation, and mitochondrial dysfunction, as indicated by the elevation of ROS and the opening of the mitochondrial permeability transition pore which dissipates the mitochondrial membrane potential and additionally impairs ATP production. Reoxygenation through spontaneous or thrombolytic reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions in the cytosolic compartments, subcellular organelles and mitochondria [21,22]. Two different models of reversible ischemia demonstrated the existence of reflow-induced secondary energy failure and neuronal destruction. In the first study, performed in the gerbil, a 5-minute episode of global cerebral ischemia caused a generalized reduction of ATP and creatine, levels which were restored after reperfusion. The hippocampal CA1 pyramidal neurons survived for several days after the ischemic insult but between days 2 and 4 after reperfusion the levels of ATP dropped significantly and the neurons were destroyed [23]. The second model investigated transient focal cerebral ischemia by occluding the middle cerebral artery in mice for 1 hour, during which time the energy stores in both the ischemic core and the penumbral region were depleted to variable degrees. After reperfusion the concentration of ATP was restored in the cerebral cortex within 1 hour, only to drop after 3 hours and continue for 3 days [24].

In human practice myocardial reperfusion injuries, likely to be mediated by free-radical-induced oxidation, have been implicated in myocardial stunning, reperfusion arrhythmias, and even the excess first-day mortality which occurs after thrombolysis [25]. In thrombolysed ischemic stroke between 10-26% of patients suffer early neurologic worsening. This may be due to symptomatic intracranial bleeding or edema. However, after excluding the patients with these complications, still up to 12% of patients deteriorate after thrombolysis due to ischemia progression [26]. One of the predictors of END was a larger PWI/DWI mismatch and this indicates a wider penumbra where reperfusion injuries may occur [27].

Another feared complication of thrombolysis is hemorrhagic transformation, connected with an increase in the permeability of the blod-brain barrier (BBB). NADPH oxygenase generates hydrogen peroxide which modifies the endothelial tight junctions by redistributing occluding and tight junction protein 1 (zonula occludens-1). Peroxynitrates degrade collagen and laminin in the basal membrane by activating the matrix metalloproteinase pathways. In addition, free radicals cause lipid peroxidation and protein dysfunction. All these processes lead to BBB breakdown, hemorrhagic transformation and vasogenic edema. Moreover, lipid peroxidation and protein oxidation impair membranal ion transport and result in cytotoxic edema [28].

Antioxidant treatment in cerebral ischemia

The involvement of free radicals in the pathophysiology of cerebral infarction, of reperfusion injuries as well as of hemorrhagic transformation makes them a valid therapeutic target. A significant amount of research has focused on assessing the therapeutic effects of antioxidants. There are three main strategies by which antioxidants work:

(1) Inhibition of free radical production;
(2) Scavenging of free radical production; or
(3) Increasing free radical degradation.

Antioxidant therapy in animal models of cerebral ischemia

In animal models both up-regulation of endogenous antioxidants and delivery of exogenous antioxidants showed promising results.

The three major antioxidant enzymes, SOD, catalase, and glutathione peroxidase, have relatively low concentrations in the brain and their capacity may be overwhelmed during reperfusion, when free radical production is enhanced. In a study of acute reperfusion injury in the gerbil brain, infusion of recombinant human SOD was found to afford significant cerebroprotection if administered during reperfusion [29]. Furthermore, transgenic mice overexpressing a recombinant human SOD gene were found to be highly resistant to reperfusion injury after focal cerebral ischemia [30]. Pretreatment of rats with vitamin E by intramuscular injection 20 minutes before ischemia was found to reduce lipid peroxidation and to improve survival following cerebral post-ischemic reperfusion [31]. A protective effect of vitamin C pretreatment against neural loss was observed in a primate model of focal cerebral ischemia [32]. Iron chelators, like deferoxamine, reduce infarct size and improve neurological status if given 1 hour before and 1 hour after middle cerebral artery occlusion in the rat by inhibiting iron-dependent lipid peroxidation [33]. Tirilazad (10 mg/kg, intraperitoneally) given 10 minutes before unilateral carotid occlusion and immediately after reperfusion in the gerbil protected hippocampal CA1 neurons against ischemic damage [34]. N-Tert-butyl-α-phenylnitrone (PBN), a spin trap agent was shown to inhibit the production of free radicals during ischemia/reperfusion in gerbil brain [35]. Other agents, like allopurinol, a xanthine-oxidase inhibitor, have also been reported to reduce infarct size in rats subjected to middle cerebral artery occlusion [36].

Antioxidant treatment in human ischemic stroke

In human trials the results have been disappointing. In the largest clinical trial of tirilazad, a lipid peroxidation inhibitor, the substance was administered within 6 hours from the onset of cerebral ischemia. Primary outcome of disability measured by the Glasgow Outcome Scale and Barthel index at 3 months showed no change between groups at an independent interim analysis of 556 patients, and the trial was subsequently terminated [37]. NXY-059, a spin-trapping agent also known as Cerovive, was studied in two large randomised and double-blinded trials known as SAINT I and SAINT II trials. In both trials patients were assigned to receive either a 72 h infusion of NXY-059 or placebo, starting within 6 h of the onset of cerebral ischemia. SAINT I showed a significant improvement in NXY-059 treated patients assessed by the modified Rankin score, but not by the NIHSS scale or Barthel index [38]. However, the subsequent SAINT II trial published entirely negative results in primary and secondary endpoints [39,40]. Ebselen is an inhibitor of glutathione peroxidase-like activity, and also scavenges peroxynitrates. A randomised and blinded trial of 302 ischemic stroke patients who were administered Ebselen at 48 h post ischemia for 2 weeks failed to replicate the protective effects

seen in the pre-clinical models at 3 months, although improvements in the Ebselen treated groups were observed prior to this at 1 month [41]. Citicoline stabilizes cell membranes and reduces free fatty acid release caused by lipid peroxidation during ischemia. Several clinical trials of citicoline for the treatment of stroke have been conducted and reported mixed results. However, in 2012, the International Citicoline Trial on Acute Stroke concluded that the molecule was not efficacious for moderate-to-severe acute ischemic stroke [42]. Edaravone (5-methyl-2-phenyl-4H-pyrazol-3-one) is a free radical scavenger that has been approved for the treatment of stroke in Asia since 2001. Several clinical trials in Japan have shown that Edaravone treatment was beneficial for a subset of stroke patients [43,44]. Animal models also suggested that Edaravone could be a promising agent to expand the time window of rtPA treatment [45].

Possible reasons for failure of antioxidants in human ischemic stroke

A notable difference between the animal studies and human trials is the time window for administering the antioxidants. In animal models, the antioxidants were administered before or soon after the occlusion of the blood vessels and worked. In human trials, the time window extended in some cases up to 48 hours after stroke onset, and they failed. In fact, Kimura and coworker's findings are edifying in this respect [46]. Of the 40 patients enrolled in their study, 23 were assigned to the so-called edaravone group, which was intravenous alteplase infusion with simultaneous intravenous edaravone, and 17 to the non-edaravone group, actually intravenous alteplase infusion FOLLOWED by intravenous edaravone. Early recanalization occurred almost 4 times more frequently in the edaravone group (56.5%) compared to the non-edaravone group (11.8%). Clinically, the rate of patients with 'remarkable' or 'good' recovery was 80% in the edaravone group compared to 45.5% in the non-edaravone group. The authors hypothesized that the administration of edaravone during alteplase infusion inhibited endothelial cell injury in the occluded artery, and maintained the release of the tissue plasminogen activator from endothelial cells, thereby enhancing early recanalization. In addition, the rate of patients with symptomatic intracerebral hemorrhage subsequent to alteplase infusion was negatively correlated with the rate of combined treatment with edaravone in several clinical trials [47].

Many highly cited articles and expert symposia, most importantly the Stroke Therapy Academic Industry Roundtable have tried to further explain the striking lack of success of bench to bedside translation in the stroke field [48]. High up on the list of potential reasons explaining this apparent 'loss in translation' are:

- species differences
- the use of young animals without comorbidity, while human patients have significant comorbidities which can be influenced by the experimental agent, as happened with lubeluzole, which caused heart conduction disorders [49].
- different rates of metabolism of the antioxidant agent , for example women have been subsequently shown to metabolise tirilazad up to 60% more efficiently than men [50], and therefore tirilazad had perhaps not been administered a high enough dose to mediate neuroprotection, reducing the efficacy across the whole trial.
- inappropriate time windows of treatment – as mentioned earlier Cerovive was administered within 6 h from stroke onset, lubeluzole trials used the same time-window, Ebselen was given within the first 48 hours.
- effective drug levels were sometimes not achievable in humans because of toxicity.
- failure to model white matter damage and protect axons.
- incongruent end points (infarct size in animal experiments versus neurologic outcome in clinical trials).
- hetereogeneity of stroke subtypes in human patients.

However, in our opinion, among the reasons for failure of the antioxidant treatment in ischemic stroke is an inappropriate selection of the cases included in the trials. Ischemic stroke is a heterogeneous disease entity. Among the many available classifications, the one most used is the TOAST classification, based on the pathophysiology of the stroke. It classifies ischemic strokes into 5 categories: thrombotic, embolic, and lacunar stroke, stroke of other determined etiology and stroke of undetermined etiology [51]. This allows focusing in secondary prevention on antiplatelets and statins, carotid endarterectomy for thrombotic stroke, on anticoagulants for embolic stroke, and on tight control of risk factors for lacunar stroke [52].

A pilot study of alpha lipoic acid in cardioembolic stroke

The course of oxidative stress differs in the different ischemic stroke subtypes as shown by a study performed by the authors in 2006. Of a series of 150 consecutive ischemic stroke patients 104 cases could be classified according to the TOAST criteria into thrombotic (34.8%), embolic (34.8%), and lacunar stroke (30.3%). Oxidative stress was evaluated by the measurement of serum malondialdehyde levels [53] in samples drawn on admission and on day 3 and 7 from admission after informed consent was given by each patient or by a family member. It is true that the inclusion criteria allowed for inclusion of patients in a time window of 48 hours after stroke onset, which could have biased the results, but only 12% of patients included were admitted later than 24 hours from stroke onset. Another weakness of the study was the measurement of MDA levels in the serum and not CSF, but there is a good parallel between the levels of MDA in plasma and CSF, which both increase in acute ischemic stroke [54].
The results are shown in figure 1.

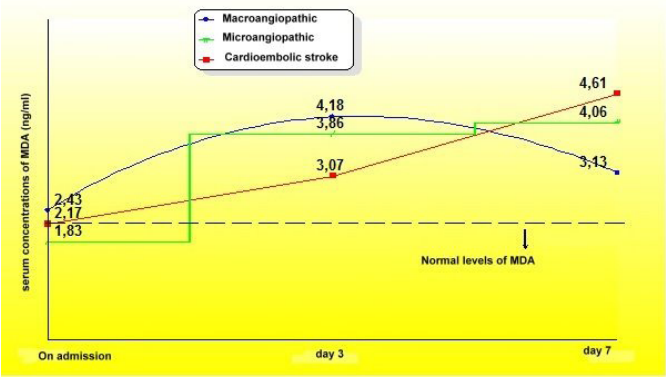

Figure 1: The course of oxidative stress assessed by the sequential measurement of serum malondialdehyde (MDA) levels in thrombotic, lacunar and cardioembolic stroke.

We found a striking difference of the course of oxidative stress in the stroke subtypes. As opposed to non-cardioembolic strokes, in cardioembolisms there was an exponential rise of the serum MDA levels throughout the first week [55].

What differentiates cardioembolic stroke from the rest of ischemic strokes is the high rate of spontaneous recanalization, up to 17% in the first 6 to 8 hours and rising as high as 50% in the first 3 to 4 days [56]. This accounts for some of the clinical characteristics of embolic strokes, like the spectacular shrinking deficit [57], as well as for the histologic and imagistic findings of hemorrhagic transformation. Reestablishing blood flow fuels the damaged mitochondria and other enzymes to produce excess reactive oxygen species and may dramatically increase the oxidative stress. A similar process happens after thrombolysis, the only difference being that the latter is performed in a specified time window. If the subsequent oxidative stress is high enough, it may damage the cerebral parenchyma as well as the blood vessels.

Cerebral ischemia-reperfusion injury is defined as a biochemical cascade that compromises and antagonizes the beneficial effect of recanalization and leads to tissue damage. The pathophysiological mechanisms in the reperfusion phase after cerebral ischemia include the release of excitotoxic neurotransmitters, intracellular calcium accumulation, free radical damage, neuron apoptosis, neuroinflammation, and lipolysis [28]. Among all these complex mechanisms, free radical damage to the brain plays a central role. In addition, cardioembolic stroke resembles most the animal models of ischemic stroke in which the carotid arteries were occluded for variable periods of time, and in these studies antioxidant therapies proved beneficial.

So the following year, from a consecutive series of ischemic stroke patients evaluated and treated in a similar way, 34 patients with cardioembolic stroke received in addition 600 mg of alpha-lipoic acid IV for 10 consecutive days. Obviously, the study had several weaknesses, such as: the comparators were from another series, treated in the previous year; patients were not randomized; the investigators were not blinded to the treatment when judging outcome, lack of assessment after 3 months. Oxidative stress was assessed in the same fashion, in blood samples drawn on days 1, 3, and 7 as well, by measuring the serum MDA levels.

The results showed a reduction of the oxidative stress, as shown in figure 2, and this translated into a better clinical outcome, at least in the acute phase. Although with a higher mean NIHSS score on admission, patients were discharged with a lower disability score after a slightly shorter hospitalization [58]. The data on patient's demographics, stroke severity and length of hospitalization are shown in table 1.

	Group 1	Group 2	Significance level in the Student's t test
Age (years, mean ± standard deviation)	69.6 ± 11.1	72.6 ± 13	P > 0.05
NIHSS score on admittance (mean± standard deviation)	9.8 ± 6.6	12.2 ± 6.5	P > 0.05
NIHSS score at discharge (mean ± standard deviation)	4.56 ±4.13	4.07 ± 4.05	P > 0.05
Improvement in the NIHSS score (mean ± standard deviation)	5.27± 4.25	8.92 ± 4.71	P < 0.05
Length of hospitalization (days, mean ± standard deviation	15.1 ± 1.7	11.9 ± 1.3	P < 0.05
Serum MDA levels on admittance (ng/ml, mean ± standard deviation	2.19 ± 1.78	1.77 ± 0.81	P > 0.05
Serum MDA levels on day 3 (ng/ml, mean ± standard deviation	2.94 ± 1.80	2.09 ± 0.89	P > 0.05
Serum MDA levels on day 7 (ng/ml, mean ± standard deviation	4.61 ± 1.91	2.75 ± 0.89	P = 0.003

Table 1: Data on demographics, stroke severity, outcome, and oxidative stress in the 2 groups of patients with cardioembolic stroke. Group 1 included 36 patients treated conventionally; group 2 consisted of 34 patients who received in addition to the conventional treatment 600 mg alpha lipoic acid daily intravenously for 10 consecutive days.

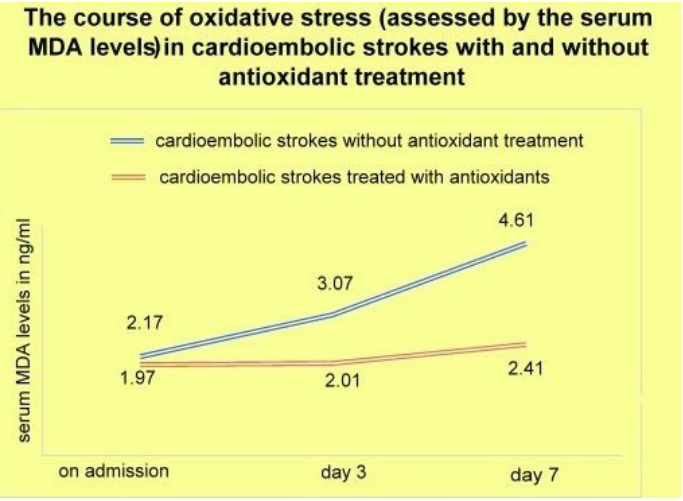

Figure 2: Oxidative stress assessed by the sequential measurement of serum malondialdehyde (MDA) levels in cardioembolic strokes treated with and without alpha-lipoic acid.

Alpha lipoic acid was chosen because both α-lipoate and especially dihydrolipoate have been shown to be potent antioxidants, to regenerate through redox cycling other antioxidants like vitamin C and vitamin E, and to raise intracellular glutathione levels [59]. Current research reveals protective effects of these compounds in cerebral ischemia-reperfusion, excitotoxic amino acid brain injury, mitochondrial dysfunction, diabetes and diabetic neuropathy, inborn errors of metabolism, and other causes of acute or chronic damage to brain or neural tissue. It was well tolerated, without side effects by all 34 patients receiving it.

Conclusion and Future Perspectives

In conclusion, it is our true belief that antioxidants have not yet reached the end of their story in acute ischemic stroke. Most of the antioxidants used in recent trials – uric acid, edaravone, lipoic acid - were well tolerated and caused no serious adverse effects. Obviously, in our effort to mitigate reperfusion injuries, antioxidants should be administered as soon as possible. Starting antioxidant treatment in potentially eligible patients for thrombolysis while awaiting for the necessary evaluations as well as to ischemic strokes of presumably cardioembolic origin may prove beneficial and cost-effective. In the future it is more likely that a multimodal approach will be used, with broad spectrum (pluripotential) neuroprotective therapies, such as therapeutic hypothermia and high dose albumin therapy, or simultaneous or serial (field and emergency room) administration of multiple neuroprotectants with different mechanisms of action. Due to the rising incidence and prevalence of this disease [60] no effort should be spared to reduce stroke-caused disability.

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