Chapter 6

A Review of Neuroinflammatory Mechanisms in Ischemic Stroke: Background and Therapeutic Approaches

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Abstract

In this review, we will discuss the relevant clinical details of acute ischemic stroke and its currently very limited therapeutic opportunities, sequentially emphasizing its populational and economical burden. Based on our increasing knowledge in molecular and cell biology of immunological mechanisms of ischemic stroke, we will introduce the main processes in the background of arterial vessel occlusion, ensuing tissue damage and following reparation. After that, we will compare the obtained results from animal models with clinical studies and thus the possible causes of foregoing failures. Following this, we will demonstrate the most important drugs tested and/or being tested in human or animal studies from the field of neuroprotection. Finally, we raise possible opportunities that can be considered in development or clinical applications of neuroprotectants.

Keywords: acute ischemic stroke, stroke induced immunodepression, neuro-inflammation, neuroprotection, future perspectives

1. Introduction

In 2013, the Stroke Council of the American Heart Association/American Stroke Association laid an up-to-date definition of ischemic stroke. According to this, it is defined as brain, spinal cord or retinal cell death attributable to ischemia, based on neuropathological, neuroimaging and/or clinical evidence of permanent injury. In a clinical spectrum, it can be accompanied by symptoms or can be asymptomatic. Transient ischemic attack (TIA) is defined as a transient

episode of neurological dysfunction caused by focal brain, spinal cord or retinal ischemia, without acute infarction [1].

Estimates from the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2010) ranked stroke as the second most common cause of death [2] and the third most common cause of disability-adjusted life-years (DALYs) [3] worldwide in 2010. Expressed by numbers, roughly 10% of the 52,769,700 deaths [2] and about 4% of the 2,490,385,000 DALYs [3] worldwide were due to stroke. Further analysis of the GBD study showed that although stroke mortality rates and mortality-to-incidence ratios have decreased in the past two decades, the global burden of stroke in terms of the absolute number of people affected every year, stroke survivors, related deaths, and DALYs lost are great and increasing, with most of the burden in low-income and middle-income countries. If these trends in stroke incidence, mortality, and DALYs continue, by 2030, there will be almost 12 million stroke deaths, 70 million stroke survivors, and more than 200 million DALYs lost globally [4]. Furthermore, stroke changes the lives not only of those who experience a stroke but also of their family and other caregivers [5].

We can classify the stroke subtypes by aetiology. According to this, 80–85% of all stroke events are ischemic, the other 15–20% are of haemorrhagic origin [6]. The theme of our review is about ischemic stroke, so from now on, we will discuss only this subtype—means, that if 'stroke' is written, it refers to ischemic stroke automatically.

The ischemic stroke has its well-known risk factors, some of them are the common vascular risk factors. Among these, we can find so called non-modifiable ones: genetics, age, ethnicity/race, and low birth weight. Fortunately an international case-control study of 6000 individuals found that 10 potentially modifiable risk factors explained 90% of the risk of stroke [7]. These are—with no purpose of detailed description—physical inactivity with or without diet and nutrition failure (containing dyslipidaemia, obesity and body fat distribution, metabolic syndrome, diabetes mellitus) hypertension, cigarette smoking, atrial fibrillation and other cardiac conditions, carotid artery stenosis, sickle cell disease, migraine, alcohol consumption, drug abuse, sleep-disordered breathing [8].

Despite the intensive populational stroke education of these methods of primary prevention, the number of stroke patients increases to date.

After so many years of unsuccessful therapeutic approaches, recombinant tissue plasminogen activator (rtPA) was approved by the U.S. Food and Drug Administration (FDA) in 1996 for the treatment of acute ischemic stroke [9]. Since then, scores of stroke patients have been treated worldwide with this drug, managed by comprehensive stroke centres.

In a selected patient population (see detailed inclusion and exclusion criteria as per applied protocol), intravenous or intra-arterial thrombolysis can be a reliable choice. With this method of recanalisation, the treatment physician must calculate certain complications and a relatively poor outcome in several cases [10].

Most of these severely disabled stroke patients have intra- or extra-cranial large arterial vessel occlusion. In the past decade, a new form of acute revascularisation treatment, the endovascular stroke treatment (EST), appeared. After the failure of the first 'unhappy' trials with

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first-generation devices; in the past few years, smashing successes were achieved with the newer stent retrievers. These results—especially combined with iv thrombolysis—were comparably better than iv thrombolysis alone, and patient safety with risk/benefit ratio is also very promising [11].

Although several patients can benefit from the above mentioned methods of acute stroke treatment, they still have a few significant weak spots, above all, the narrow therapeutic time window.

Even in the countries with the best achievements, just like Austria with about 10% of stroke patients, can receive either or other treatment, the others, with wider stroke onset-to-treatment time have no or minimal chance of revascularisation, thus of good clinical outcome.

There is an urgent need to aim this enormous patient population with an effective treatment.

Neuroprotection would be a promising choice for this group, but until now, controversial results came to light in this field.

Hereinafter, we will introduce the main known reactions, immune responses in the brain following acute arterial vessel occlusion and potential therapeutic targets in this process.

2. Immunological background of acute stroke

2.1. First processes that appear immediately after arterial occlusion

The brain is highly dependent on continuous delivery of oxygen and glucose through blood flow, and the permanent interruption of this supply leads to irretrievable brain damage [12]. Cascade of cellular and molecular events caused by sudden lack of blood flow ends in ischemic cell death via necrosis or apoptosis. Among cells traceable in the brain, the neurons are more vulnerable than glia and vascular cells, and suffering from hypoxia-ischemia, these are the first ones to become dysfunctional and die [13]. Neurons can further be divided particularly into sensitive ones by location: the caudate body, putamen, insular ribbon, paracentral lobule, precentral-, middle- and inferior-frontal gyri [14].

In clinical practice, one of the most common types of severe stroke is the occlusion of the middle cerebral artery (MCA). This is also the one that provides a basis for animal models, as will be discussed later. After MCA occlusion, the ischemic damage will be more rapid and severe in the centre of the infracted territory—named the ischemic core— where the blood flow is the lowest. The periphery of the core is a region, the so called penumbra, where neuronal damage develops more slowly, because blood flow from adjacent vascular territories (collateral flow) provides a barely acceptable cerebral perfusion, that is enough to avoid immediate cell death [12]. In the core, a bioenergetic failure develops after a few seconds of arterial occlusion. Without oxygen and glucose, neurons cannot produce ATP needed to fuel the ionic pumps—most of all Na/K ATPase—that maintain the ionic gradient across neuronal membrane. This process results in accumulation of Na and Ca ions and efflux of K contributing to a widespread anoxic depolarisation in the membranes of neurons and glial cells, to

swelling (cytotoxic oedema), degeneration of the cell organelles, loss of membrane integrity and finally the necrotic cell death [13, 15–17]. In contrast with the core, the flow reduction in the penumbra is not sufficient to cause energy failure, and the neurons remain viable for a prolonged period of time [12].

The before-mentioned reduced ATP production also contributes to reduced reuptake of glutamate, the main excitatory neurotransmitter. The resulting over activation of the NMDA type glutamate receptors leads to further cytoplasmatic accumulation of Ca ions. Because of the elevated intracellular Ca level, mitochondrial failure sets in, and Ca-dependent enzymes activate mainly in neurons, rather than astrocytes, such as proteases calpain and caspase and enzymes producing nitric oxide, free radicals (reactive oxygen species: ROS) and arachidonic acid metabolites [13, 18]. Besides these steps, the constant arterial occlusion results in a critical reduction of pO_2 and concomitant elevation of pCO_2 that leads to hypercapnia and falling of tissue pH. This process ends in lactate acidosis and irreversible cell injury mediated by Ca^- permeable acid-sensing ion channels [19–21]. Altogether, these steps lead to necrosis or apoptosis depending on the intensity of the insult and the metabolic state of the neurons.

2.2. Inflammatory mechanisms in the ischemic brain

Cerebral ischemia contributes to both, first the innate and then, the adaptive immunity, the two main branches of human immune system [22]. The innate immunity is germline-coded, rapidly activated and consists of low-affinity receptors to gain wide-range target recognition. In contrast, the adaptive immune system is based on high-affinity receptors (like immunoglobulins and T-cell receptors), that are randomly generated by somatic mutations. The adaptive branch needs several days for activation because of antigen-driven clonal cell expansion, but it retains a memory of this certain antigen exposure [22].

Immediately after vessel occlusion, post-ischemic inflammation begins in the vascular compartment. With appearance of ROS, the procoagulant state increases that means platelet, complement and endothelial cell activation [23, 24]. Besides this effect, ROS—that are produced by NADPH oxidase and iNOS, traceable in almost all inflammatory cells, and the synthesized peroxynitrite and its hydroxyl radical derivates are highly cytotoxic—alters cellular proteins, lipids, RNA, leading to cell dysfunction or death [25]. Blood brain barrier (BBB) breakdown starts—secondary to the pericyte death—as its permeability increases by oxidative stress and inflammatory mediators, impairing the neurovascular unit, that consists of endothelial cells, astrocytes and neurons. Along this process, extravasation of proteins and activation of macrophages initiates [26, 27].

Injured and dying cells play a key role in post-ischemic inflammation by releasing danger signals, the so-called damage-associated molecular patterns (DAMPs). These molecules activate macrophages via pattern recognition receptors (PRR, i.e. toll like receptors—TLR) and inflammasomes. The first pathway involves such pro-inflammatory factors that get released by nuclear gene expression mediated by transcriptional mediators, activated by TLR.

Studies highlighted importance of these TLRs in mediation of inflammation. In transgenic mice, lacking TLR2 and TLR4, much smaller brain infarcts were observed [28, 29]. In a clinical trial, patients, who exhibited reduced expression of TLR 4, had a better outcome [30].

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The second mechanism means caspase-1 activation that results in pro-inflammatory IL-1, IL-6 and IL-18 activation [31, 32]. Besides macrophage activation and thus pro-inflammatory cytokines release, this process also results in mast cells releasing vasoactive mediators, proteases and tumour necrosis factor (TNF).

As part of the innate immune system, complement system is also involved in cerebral ischemic tissue changes. Mannose-binding lectin (MBL) and MBL-associated serine proteases initiate lectin pathway of complement activation. Animal experiment with transgenic mice lacking MBL showed reduced infarct size. Clinical observation showed that patients with low MBL genotype expressed lower levels of C3-C4 complement and C-reactive protein (CRP), showing better functional outcome [33–35].

Monocytes also take part in the regulation of inflammation and tissue repair. They can be found early in the infarcted area shortly after vessel occlusion [36, 37]. In acute stroke, these cells increase in number in peripheral blood and show such phenotypic changes as reduced expressions of antigen presenting molecules, and low production of pro-inflammatory TNF, and unchanged production of anti-inflammatory IL-10 [38, 39]. These cells will differentiate into two sub-types. M1 macrophages promote strong T-helper1 (Th1), while M2 macrophages support Th2 response, playing a part in the resolution of inflammation [36]. Macrophages also play a great role in clearance of debris and damaged cells at later stages as a regenerative process [36].

Immune cell extravasation is initiated by interaction of adhesion molecules and selectins that contributes to a rolling mechanism of leukocytes on endothelium followed by adhesion and to subsequent transmigration to the brain parenchyma (leukocyte infiltration).

In response to ischemia, glial cells develop an inflammatory phenotype and release such mediators that attract neutrophils, monocytes and lymphocytes.

The increasing number of these cells and the produced pro-inflammatory mediators by them, rapidly results in a progrediation of inflammation and thus ischemic tissue damage. The before-mentioned DAMPs induced release of interleukins by macrophages and microglia, leading to further leukocyte infiltration and activation of antigen presentation between dendritic cells (DC) with MHC II receptors and T-cells [40, 41].

These infiltrating T-cells are the main source of interferon gamma that is responsible for delayed neurotoxic effect of the brain tissue [42]. Blockage of lymphocyte invasion of the brain by FTY720 improved stroke outcome in animal models, but was not verified by other study [43]. Gamma-delta T-cell sub-population and IL-17, IL-23 have a crucial role of damage progression. These cells are activated by infiltrating macrophages and DAMPs, produce pro-inflammatory IFN gamma and IL-17. Depletion of these cells or pharmacological blockade of IL-17 and IL-23 pathways suppressed brain damage in a mouse model [44]. Depletion of CD4 and CD8 T cells and ablation of perforin that mediates cytotoxicity of CD8 T cells also reduced infarct size and improved stroke outcome in experimental stroke model [42].

Cell damage and disruption lead to exposition of such brain epitopes that were previously hidden from the immune system. The antigen presenting cells (APCs) recognise these epitopes and take part in lymphocyte priming and auto-reactive activation. These auto-reactive lymphocytes worsen the local inflammation resulting in poor outcome. In clinical experiments,

neuronal and myelin epitopes were found in cervical lymph nodes and palatine tonsils of acute stroke patients. Relative neuronal predominance of these epitopes was associated with poorer clinical outcome [45].

2.3. Cytoprotective effects and reparation of damaged tissue

Besides so many damaging effects of the immune system, there are cytoprotective ones as well.

In animal models, T-cells that are specific to myelin antigens can reduce secondary neurodegeneration, enhance neurogenesis and promote recovery after stroke [46]. Local immune suppression can be achieved with tolerized lymphocytes to CNS antigens. In rodent model, immunisation with intra-nasal or oral administration of MBP or MOG weeks before induced stroke showed better outcome [47]. According to these studies, patients with a history of stroke may benefit of CNS antigen immunisation, and we could prevent a recurrent stroke, but recent studies raised concerns on this. We can induce a deleterious auto-immune process against the brain by such drugs [48, 49].

T reg cells are the main protectors of the brain after ischemia. They can exert anti-inflammatory effects by either direct cell-cell interaction or secretion of tumour growth factor beta (TGF-β) and IL-10 as well [50]. Depletion of these cells in mice increased infarct size [42] and enhanced activation of invading pro-inflammatory T-cells [51].

Regulatory B-cells are also reported as beneficial ones after experimental models. Lack of these cells resulted in increased inflammatory cell infiltration and conversely, transfer of these cells reduced infarct size and pro-inflammatory cytokine production of T-cells [52].

The immunological processes following brain ischemia is self limiting, and numerous factors play a role in the immunosuppressive activity. The first mechanism in the reparative phase is performed by microglia and macrophages. These phagocytes remove the dead cells and accompanying immunoregulatory cytokines can facilitate tissue repair. Concomitant growth factors (like TGF-β and insulin like growth factor) released by neurons and astrocytes help in cell sprouting, neurogenesis, angiogenesis and matrix reorganisation as well. Angiogenesis also required the concomitant action of vascular endothelial growth factor (VEGF) and neutrophil matrix metallo-proteases (MMPs) [53–55].

2.4. Stroke-induced immunodepression and related post stroke infections

Acute stroke can result a stroke-induced immune depression syndrome (SIID) [56, 57].

The central nervous system has multiple pathways to modulate the systemic immunity. Among these are hypothalamic-pituitary-adrenerg axis, the vagus nerve, and the sympathetic nervous system. Complex functional reactions between these systems together suppress the peripheral release of inflammatory cytokines from T-cells, monocytes and macrophages and promote the release of anti-inflammatory IL-10. The released noradrenalin from nerve terminals and adrenal medulla induces an anti-inflammatory type of lymphocytes, monocytes and macrophages. These mechanisms together reduce the inflammation in the infarcted brain area, but parallel to this, suppress the systemic immune responses, giving a chance for post-stroke infections.

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An unknown proportion of patients are affected by this condition, because there is no commonly accepted definition for this, and representative investigations in large stroke patient population are missing. It can only be estimated, as about 30% post-stroke infections were reported [56].

It was confirmed in a meta-analysis of 87 studies involving more than 130,000 patients, published in 2011, that the overall infection rate was 30%, most commonly pneumonia and urinary tract infections [58]. According to a multicenter retrospective cohort study including consecutive patients with ischemic stroke admitted to Regional Stroke Centers participating in the Registry of Canadian Stroke Network in July 2003-March 2007 pneumonia increased 30 days and 1 year mortality as well [59]. Statistical analysis showed that half of the pneumonia cases occurred in the first two days after stroke onset, but almost all cases occurred in the first week [60]. The most susceptible patients for aquiring pneumonia as a concomitant disease, are the ones who have dysphagia, even with aspiration, are of older age, and the male sex, stroke severity, chronic obstructive pulmonary disease, coronary artery disease and pre-stroke dependency (greater than 2 points in modified ranking scale mRS) are also independent risk factors for pneumonia [61, 62].

According to urinary tract infections, a study showed 16% prevalence in stroke patients mostly in the first two weeks after stroke onset [63].

A few trials detected correlation between the locations of infarction in the brain, resulting in a higher risk for post-stroke infection. Affection of the anterior MCA cortex, and the insula caused more infections; however, other study found that the extent of the infarcted brain tissue is a better prognostic factor for this [64–66].

Many clinical studies were conducted to evaluate the benefit of prophylactic use of antibiotics in stroke patients, but according to the latest AHA/ASA guideline for secondary stroke prevention [10], routine use of prophylactic antibiotics has not been shown to be beneficial (class III, level B recommendation).However in selected patient population, it can be considered.

3. Challenges in neuroprotection

Neuroprotection can be defined as a therapy that enhances the brain's resilience to ischemia to improve the clinical outcome of affected patients. It aims not only the neurons but also is equally applicable to other brain constituents like cells of vessels and glia. A neuroprotective drug is designed to target one or more components of ischemic cascade. The disappointing results of the neuroprotective trials to date raise the question that successes in pre-clinical animal models can even translate in clinical practice [67–69].

4. Reasons why neuroprotective agents have failed in human stroke trials

It is almost impossible to create a true, representative model for human acute ischemic stroke (AIS). In laboratory, scientists work with animals. They are mostly rats, or other rodents, in that several physiological processes differ from ones in human. In addition, these animals are basically healthy and not suffering from such civilisational concomitant diseases, like

hypertonia, diabetes and dyslipidemia. They are not exposed to smoke, and not influenced by other unhealthy conditions, just to emphasize some factors.

As a basic of stroke, patients usually develop atherosclerotic disease over decades, causing decaying cerebral autoregulation, poorer collateral system, etc. while in laboratory, animals are tested as a sudden occlusion of the MCA on the ground, besides healthy vessel system. Many patients take medications, prescribed for cardiovascular (CV) risk factors, that affect the ischemic process and that can interact with the investigational neuroprotective drugs [70–73].

Last but not the least, a few more practical factors to consider.

- Clinical trials have real life difficulties like time window (pre-clinical studies usually using such short time window that are unfeasible in real life).
- The optimal duration of neuroprotectant administration is unknown (only the start of administration seems to be clear as early as possible).
- Outcome measure is different in pre-clinical trials, where particularly infarct size is judged to evaluate efficacy of a drug, while clinical trials use clinical and functional scales, such as National Institutes of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS), Barthel index, etc.
- Pre-clinical trials evaluate mainly early outcomes, while clinical ones rely on late assessments.
- In laboratory, scientists use just a very few stroke models, most widespread are the transient and permanent middle cerebral artery occlusion models (tMCAO and pMCAO), while in human studies, patients develop a broad pathophysiological heterogenity of stroke in duration, extent, location and severity [74].

In spite of all these difficulties, there is still a massive trend of drug development in this field. In the course of past unsuccessful trials, we have become more and more aware of each molecular or cellular step of the ischemic cascade, and the knowledge gained from these experiments helped us to understand the immunological processes beyond tissue damage after stroke.

5. Therapeutic approaches in the field of neuroprotection

In pre-clinical phase of studies, over a thousand of potential neuroprotective agents have been investigated to date and many of them seemed to be promising. They aimed one or more steps of the above mentioned ischemic cascade to minimize the tissue injury. Different therapeutic approaches, tried on animals and/or in clinical trials will be discussed below sorted by mechanism of action [73, 74].

As discussed before, immediately after ischemia onset, a few processes lead to excessive activation of excitatory amino acid receptors, accumulation of intra-cellular calcium and release of toxic products that all results in cell damage.

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5.1. NMDA antagonists

The most widely studied neuroprotective agents are the N-methyl-D-aspartate (NMDA) receptor antagonists. The first neuroprotective drug applied in human trial was also a non-competitive NMDA antagonist. It was *dextrorphan*, a metabolite of the cough suppressant dextrometorpohan. Further investigation was stopped because of occurred hallucinations and hypotension as adverse events [75].

A competitive NMDA antagonist *CGS 19755 or selfotel* reduced infarct size by 50% with 40 mg/kg dose in animal studies [76], but in a phase III trial, with a total of 567 enrolled patients, a higher mortality rate was observed than in the placebo group, so it was terminated [77, 78].

Two non-competitive NMDA antagonists were tested in placebo controlled trials (*MK801 or dizocilpine and aptiganel HCL-Cerestat*) but they were terminated because of poor risk-benefit ratio and hallucination [79, 80]. These frightening adverse reactions mimic those seen with phencyclidine which binds at a similar site.

This recognition led to development of indirect NMDA antagonists that connects with the glycine site of the NMDA receptor and thus has fewer side effects [81]. Prominent representative of this glycine antagonist group was *GV150526*, which was well tolerated and reported as safe in a trial with 1367 patients but showed no positive effect in 3-month outcome measures, so no further investigations were planned [82].

In a recent pre-clinical study, investigating NMDA modulation with post-synaptic density-95 (PSD-95) protein inhibitor *NA-1* that uncouple PSD-95 from neurotoxic signalling pathways in neurons, showed promising result. This trial was conducted not with rodents, but primate class cynomolgus macaques, in which animals have closer genetic, anatomic and behavioural relationship with humans. They found reduced infarct volumes and significantly better neurological function in neuro-behavioural tests in the NA-1 treated group [83]. NA-1 was also tested in patients, who underwent endovascular aneurysm coiling, to prevent small embolic strokes, that can occur during such procedure (evaluating neuroprotection in aneurysm coiling therapy [ENACT]). It was a multicentre, double-blind, placebo-controlled, randomized trial, with 185 participants. They received either a single dose of NA-1 infusion or saline at the termination of the coiling procedure and were evaluated with MRI and neuro-psychological tests in a 30-day follow-up period. The subjects, who received NA-1, developed fewer emollitions, and by patients with ruptured aneurysms, NA-1 reduced the number of stroke events, volume of tissue damage and improved neurological outcome in 30 days [84].

5.2. Free-radical scavengers and antioxidants

In a murine model of stroke, animals treated with *carnosine*, a naturally occurring dipeptide with several neuroprotective properties, developed significantly decreased infarct size when administered both before and after induction of ischemia. This neuroprotective effect was related to decreased level of reactive oxygen species, preserved glutathione levels and attenuated matrix metallo-protease (MMP) levels and activity [85, 86].

Anthocyanins (isolated from tart cherry) are strong antioxidants and thus have anti-inflammatory effects. In a mouse model, it reduced infarct volume significantly by 27% pre-treated and by 25% in delayed treatment and was associated with better functional outcome in both pre- and post-treated group. These effects are considered to decrease level of superoxide in brain and blockage of apoptosis-inducing factor released from mitochondria [87].

Ebselen, a seleno-organic compound with antioxidant effect mediated by a glutathione peroxidase like action, was evaluated in a multicentre, double blind, placebo-controlled trial with 302 patients [88]. A positive effect was found at 1 and 3 months follow-up in functional scores. However, the final results are still not available [89].

Edaravone, another free radical scavenger was investigated in more clinical studies. Administered in 72 hours of stroke onset, better functional scores were observed compared with placebo, especially in small vessel disease [90, 91]. However, there were no significant differences in outcome after 1 year against placebo group [91].

NXY-059, also a free radical trapping agent, was found to be effective in animal models [92].

Two large trials were conducted to evaluate this drug. In SAINT I., administered in 6 hours after stroke onset, it showed controversial results in functional scales (better outcome by mRS, but not by NIHSS). Following SAINT II, NXY-059 was found safe but ineffective compared to placebo. This result was confirmed by another study by Diener et al. [93–95].

Citicoline is an intermediate in the biosynthesis of phosphatidylcholine (PtdCho) showed reduced infarct volume in animal model of stroke [96]. It enhances the synthesis of PtdCho and sphingomyelin, attenuates lipid peroxidation and restores Na/K ATP-ase activity. In ICTUS study, with 2298 patients from 2006 to 2011, it was not effective in the treatment of stroke [97]. In a clinical trial, that compared edaravone and citicoline in AIS, edaravone was more effective and showed a better neurological outcome at 3-month follow-up [98].

Cerebrolysin, a porcine brain-derived preparation of low-molecular-weight neuropeptide and free amino acids, showed improved functionality in animal models, but not in clinical trials (CASTA) [99].

Tirilazad is a non-glucocorticoid, 21-aminosteroid that inhibits lipid peroxidation. It was effective in animal models of focal ischemia, by reducing infarct volume by 29.2% and improved neuro-behavioral scores by 48.1%. However, it did not show any significant effects in clinical trials of AIS [100, 101].

5.3. Excitotoxicity and magnesium

Magnesium has a multi-pathway neuro-protective effect, as it antagonizes calcium channels, it is also a non-competitive NMDA antagonist, inhibits excitatory neurotransmitter release and relaxes vascular smooth muscles. It was observed that magnesium also antagonize the vaso-constrictive effect of endothelin 1, thus ameliorates cerebral blood flow [102, 103]. Eighty percent of acute stroke patients show a significantly decreased serum ionized magnesium level [104]. As a phase III, double blind, placebo-controlled, randomized trial, with 1700 patients, the FAST-MAG was the first of the pre-hospital administered neuroprotectant trials.

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It was designed to overcome the drawback of the delayed administration of neuro-protective agents in prior clinical trials, which has demonstrated that initiation of magnesium by paramedics in the field within 2 hours of symptom onset is feasible and safe; however, it did not improve disability outcomes at 90 days [105]. Based on the somewhat positive results, a large Phase III clinical trial is ongoing [106], to investigate if magnesium is effective when administrated by emergency medical service personnel between 15 min and 2 hours after stroke onset.

5.4. GABA agonists

Clomethiazole, as a gamma-aminobutyric acid (GABA) agonist, can decrease excitatory neurotransmission by increasing activity of inhibitory pathways. It is widely used as anti-convulsant or sedative. In pre-clinical trials, clomethiazole appeared to be effective both in focal and global cerebral ischemia, at plasma concentrations known to be well-tolerated by patients [107]. In the CLASS I, randomized, double blind, multicentre, placebo-controlled trial with 1198 severe stroke patients, this drug did not improve disability, or reduced infarct volume, administered as a 24 hours intravenous infusion within 12 hours of stroke onset. As predicted, primary side effect was sedation [108].

5.5. AMPA antagonists

The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor is a non-NMDA-type ionotropic transmembrane receptor for glutamate that mediates fast synaptic transmission in the central nervous system (CNS).

ZK 200775 reduced infarct size in animal model of tMCAO [109]. In clinical trial, it transiently worsened the neurological condition of the stroke patients by neuronal dysfunction, glial cell toxicity and sedation [110].

YM872 pre-clinically showed reduced infarct volume in rats and was neuro-protective by restoring cortical cells and decreasing oedema in traumatic brain injury [111]. Two clinical trials were conducted, the ARTIST MRI and ARTIST+, in the second one, with combined administration of alteplase. The drug was given within 6 hours of stroke onset in a 24-hour infusion. Both of them stopped after interim analysis, but results are still not available since 2003 [112].

5.6. Ion channel blockers or modulators and chelators

More than 50% of the total synthesized ATP in the brain is for maintaining the energy-dependent ion pumps, the ones that are responsible for maintaining the ionic balance between intra- and extra-cellular compartments. In two minutes after stroke, total ATP level in the brain significantly decreases, triggering an ionic imbalance of intra-cellularly decreasing K and increasing Na ions, that results membrane depolarization [113, 114]. After this anoxic depolarization, neuronal death starts along with the opening of the voltage gated Ca channels, thus causing intra-cellular Ca overload, resulting in cell death pathways discussed earlier.

Animal studies revealed that in the penumbra, a rapid de- and re-polarisation happens, causing further neuronal damage [115].

Na channel blockers (phenytoin, carbamazepine, lamotrigine, sipatrigine (619C89) and riluzole) are believed to reduce the elevated Na influx and thus the following damaging pathways in AIS and are investigated in clinical trials. The ones with sipatrigine and fosphenytoin were terminated due to toxicity and lack of efficacy [116–120].

A drug, named lubeluzole, has effects that are not completely clear. It blocks Na channels and reduces the release of nitric oxide. Trials could not confirm its positive effects in AIS patients [121, 122].

Ca channel blockers were also strongly investigated, unfortunately without significant positive effect. A clinical trial with nimodipin (VENUS) was terminated after enrolling 439 acute stroke patients within 6 hours of symptom onset because of lack of efficacy [123]. A large meta-analysis of 22 T- and L-type Ca antagonist trials in AIS found no benefit of this family of drug. A possible explanation for this can be that such Ca antagonists caused hypotension and thus impaired auto-regulation of cerebral circulation by acute stroke patients, and this effect is known to be harmful for penumbral neurons to survive [124]. For future trials, N-type Ca channel blocker can be a promising choice with less hypotensive effect.

K channel modulators showed hopeful results in animal stroke models. However, BMS-204352 (MaxiPost), a fluorooxindole K channel opener, did not reveal significant benefit in an extensive phase III trial (POST), that enrolled 1978 AIS patients in 200 centres worldwide [125].

Zinc is neurotoxic in cerebral ischemia in an environment of impaired ionic homeosthasis. *Zinc chelator DP-b99* presented positive effect in pre-clinical and phase II trials, but not in a phase III, double blind, placebo-controlled, multicentre, randomized study (MACSI), where severe stroke patients were included within 9 hours of stroke onset, and received four dose of drug infusion in four consecutive days. Unfortunately it did not show any positive effect.

5.7. Albumin

Animal studies highlighted that albumin's neuroprotective effects are related to its antioxidant properties, preservation of microvascular integrity, decreasing endothelial cell apoptosis, hemodilution, and mobilization of free fatty acids [126]. In the ALIAS study, 82 acute stroke patients received either iv. albumin and rtPA or iv. albumin alone. Albumin-related adverse events like pulmonary oedema were mild or moderate in severity. Patients who received a higher dose of albumin presented significantly better clinical outcome than subjects treated with lower dose of the drug [127]. These findings led to ALIAS 2 trial, where albumin therapy was compared with placebo. It was terminated on the basis of interim analysis [128].

5.8. Inflammatory cascade inhibition

In ischemic stroke, tissue damage develops by processes such as endothelial activation, pro-inflammatory and pro-thrombotic interactions between endothelium and different circulating blood elements, resulting in thrombogenesis [129]. Cell adhesion is directed by different adhesion molecules. Animal stroke studies proved that administered anti-adhesion antibody can decrease infarct size [130].

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Enlimomab, a mono-clonal anti-ICAM antibody, was investigated in a phase III trial (EAST) in patients with AIS. Six hundred patients within 6 hours of stroke onset received either boluses of enlimomab or placebo for five days. Unfortunately, it was associated with greater mortality, compared with the placebo group. Patients in enlimomab group often had fevers and developed an immune response to the murine antibody [131]. Humanized antibody could be a rational choice for research to avoid this adverse reaction.

Hu23F2G or LeukArrest is a humanized IgG1 antibody against CD18, thus blocking leukocyte infiltration in AIS. In a phase III trial, with patients within 12 hours of stroke onset, that allowed concomitant use of rtPA, LeukArrest had unfavourable results.

Tacroliums (FK506), a drug for prevention of organ rejection after transplantation, was confirmed to have neuro-protective effect in animal model of pMCAO administered within 4 hours of stroke onset [132].

Statins have such pleiotropic effects, besides decreasing cholesterol levels, like modulating the immune system, increasing cerebral perfusion by up-regulating angiogenesis and by activation of survival signals [133]. Lovastatin was investigated in a phase IB trial (Neu-START) and showed the drug to be safe in different doses up to 3 days after AIS [134].

5.9. Other agents

Tetracycline class of antibiotics reduces leukocyte infiltration and improves stroke outcome. In addition, minocycline proved to inhibit caspase, inducible NOS and P38 MARK and can generate hypothermia. It can penetrate to CNS, has low cost and had positive effects in animal studies, so it is a good neuroprotectant candidate. A dose finding IB study of minocycline in patients within 6 hours of stroke resulted in significantly better outcome compared with placebo group [135]. It was also safe and tolerable to administer with combination of rtPA. It can decrease matrix metallo-protease 9 (MMP 9) levels, which occurs with rtPA associated cerebral haemorrhage. Based on these result, a phase III study Neu-MAST is ongoing.

Antiplatelet antibodies inhibit platelet aggregation, thus prevent additional ischemic injury. Abciximab (ReoPro) was investigated in a phase III AIS trial (AbESTT II), but the lack of efficacy and the increased rate of symptomatic and fatal intra-cranial haemorrhage led to termination of the study [136].

Citicoline is used in membrane biosynthesis. It can stabilize membranes and decrease free radical formation. A phase II, then a few phase III trials were conducted, with somewhat promising trend, but finally no such significant positive effect was found that would led the drug to clinical practice [97, 137–139].

Fiblast, a fibroblast growth factor, can regulate neuronal healing after ischemic injury. After promising first data from clinical trials, a large study that evaluated its efficacy in stroke patients within 6 hours of stroke onset, was terminated because of poor risk-to-benefit ratio [140].

Autologous mesenchymal stem cell therapy has also promising data, additional trials are in progress [141].

GSK249320 is an anti-MAG (myelin-associated glycoprotein) antibody. MAG inhibits axon outgrowth after neuronal injury. Dose finding studies were completed till now [142].

Hypothermia showed significant pre-clinical efficacy in animal models. In stroke patients, it can be executed either by surface cooling or in an endovascular way. COAST II, CHILI, Euro-Hyp trials investigate the process in stroke patients [143, 144].

Uric Acid was evaluated in a phase III study (URICO-ICTUS). It was administered together with rtPA within 4.5 hours of stroke onset. It was finished in Oct. 2013, but no study results are available yet [145].

Studies with *Cromolyn, Dapsone, Cyclosporin A and Pioglitazone* are still in progress [146–149].

6. Conclusion and future perspective

Currently, the predominant atmosphere in the field of neuroprotectant research is frustrating. In the past decades, over a thousand neuro-protective agents were proved to be safe and effective in animal trials but failed to show proper effect in human trials. Since 1996, when FDA approved rtPA, there has not been any other drug that could be suitable in the treatment of AIS.

The main differences between animal and human studies were discussed above that can be a possible explanation for ineffectiveness. Therapeutic benefit from neuroprotection can only be appraisable, before ischemic damage is complete (we can only save tissue, if there is something left to save). Most studies aim the penumbra, the potentially salvageable region after ischemic stroke. An ideal neuroprotectant would be effective in safely halting or slowing stroke progression and improve clinical outcome. Unfortunately, the extent of the penumbra is highly individual and depends not only on stroke onset time, but also on a few other factors, such as site of arterial occlusion, collateral circulation, blood pressure, pre-stroke condition of the affected brain region, etc.

Another practical difficulty in neuroprotectant studies is that in developed countries, the rtPA is a standard of care and can be administered in a 3–4.5 hours time window. Even endovascular therapy of AIS is spreading in stroke centres recently, with a wider therapeutic timeframe (even 6 hours or more from stroke onset in selected patients). This is exactly the same time range, when neuroprotectants could also be effective according to animal studies, and clinical experience as well. It leads to a conflict in application of a test drug because only a heterogeneous patient population remains to enrol to a study, who somehow could not participate in either or other re-canalisation therapy (too mild or too sever stroke, haemorrhagic stroke, medical or surgical history, blood tests, etc.).

The first pre-hospital trial, the FAST-MAG evaded this difficulty, when the neuroprotectant was administered before hospital arrival, reaching a very favourable onset to treatment time. However, these populations with stroke-like symptoms were really heterogeneous, with no preceding brain imaging, and thus the lack of proper patient selection. But, this study raised the concept of safely administering a neuroprotective drug complementary with an already

approved re-canalisation therapy, because these drugs not necessarily display adverse effects in patients treated otherwise.

In connection with mechanism of action, there is also a concept that an administered neuroprotectant should aim not only a single target of the ischemic cascade, but more, or administering a 'stroke cocktail' to a patient with a selection of drugs is also an interesting idea.

Anyway, researchers with grim determination are still on the issue of developing neuroprotectant drugs, and hopefully, we will have positive results in the near future.

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