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Combination of Therapeutic Hypothermia and Other Neuroprotective Strategies after An Ischemic Cerebral Insult[#]

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Abstract: Abrupt deprivation of substrates to neuronal tissue triggers a number of pathological events (the "ischemic cascade") that lead to cell death. As this is a process of delayed neuronal cell death and not an instantaneous event, several pharmacological and non-pharmacological strategies have been developed to attenuate or block this cascade. The most promising neuroprotectant so far is therapeutic hypothermia and its beneficial effects have inspired researchers to further improve its protective benefit by combining it with other neuroprotective agents. This review provides an overview of all neuroprotective strategies that have been combined with therapeutic hypothermia in rodent models of focal cerebral ischemia. A distinction is made between drugs interrupting only one event of the ischemic cascade from those mitigating different pathways and having multimodal effects. Also the combination of therapeutic hypothermia with hemicraniectomy, gene therapy and protein therapy is briefly discussed. Furthermore, those combinations that have been studied in a clinical setting are also reviewed.

Keywords: Acute ischemic stroke, clinical, combination therapy, experimental, human, hypothermia, neuroprotection, rodent.

INTRODUCTION

Following an ischemic stroke (IS), intravenously (i.v.) injected recombinant tissue plasminogen activator (rt-PA) reduces the size of ischemic damage and salvages neuronal cells by dissolving the clot obstructing a cerebral artery [1]. Unfortunately, this drug needs to be administered within a therapeutic window of 4.5 h after symptom onset [2, 3], otherwise hemorrhagic complications increase [4]. Another therapeutic strategy is "neuroprotection", which is defined as any strategy, or combination of strategies, that antagonizes, interrupts or slows the propagation of the pathological events that occur following an IS [5]. Abrupt deprivation of oxygen and glucose to neuronal tissue triggers a series of pathological events leading to cell death. This series of destructive events is referred to as an ischemic cascade and can be summarized as cellular bio-energetic failure due to focal cerebral hypoperfusion, followed by excitotoxicity, oxidative stress, blood-brain-barrier (BBB) dysfunction, ischemia-induced microvascular injury, hemostatic activation, post-ischemic inflammation and finally death of neurons, glial and endothelial cells [6-10]. In the last two decades, neuroprotective agents, designed to block this cascade, have been investigated in animal models of cerebral ischemia. The most promising neuroprotectant so far is therapeutic hypothermia (TH), defined as an intentionally induced, controlled reduction of the body temperature below 36 °C [11, 12]. The success of this therapy can be explained by its multifaceted neuroprotective effect [13]. It retards energy depletion by lowering the metabolic and the enzymatic rate [14], restores the neurotransmitter balance [15], reduces the intracellular calcium influx, reduces intracellular acidosis [16], suppresses reactive oxygen species (ROS) formation [17], suppresses infiltration of inflammatory cells [18], prevents BBB disruption [19], suppresses specific cell death pathways or up-regulates cell survival mechanisms [11, 20-22]. However, its beneficial effect is still not completely known [23] and researchers tend to further improve its salvageable beneficial effect by combining it with other neuroprotective strategies. This review will summarize which neuroprotective strategies have already been combined with TH in rodent models of focal cerebral ischemia. A distinction is made between those drugs interrupting only one event of the ischemic cascade and those mitigating different pathways and thereby having multimodal effects. Also the combination of TH with hemicraniectomy, gene therapy and protein therapy is briefly discussed. Furthermore, those combinations tested in a clinical setting are also reviewed and discussed.

Although there are some articles reviewing combination therapies with TH [24, 25], this review is the first to provide a complete overview of all combination strategies with TH in rodent models of IS as well as in a clinical setting. Moreover, the mechanisms involved are detailed. A summary can be found in Table **1**.

THERAPEUTIC HYPOTHERMIA IN ISCHEMIC STROKE

Based on the target temperature, TH can be classified into mild (32 °C-34 °C), moderate (28 °C-32 °C), deep

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[#]Combination therapy with hypothermia

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(11 °C-28 °C), profound (6 °C-10 °C) or ultra-profound (< 5 °C) hypothermia. However, there has been some inconsistency in defining the range of temperature constituting each level of TH. The ideal therapeutic level is still not known, but most authors currently agreed that a temperature range between 32 °C and 34 °C is neuroprotective [26].

Furthermore, TH can also be classified based on the moment of institution, i.e. prior to the insult (protective), during the insult (preservative) or after the insult (resuscitative). In the case of stroke, TH will be induced after the insult (resuscitative TH).

DRUGS INTERRUPTING ONE EVENT OF THE ISCHEMIC CASCADE

NMDA Antagonists

IS starts with severe focal hypoperfusion which restricts oxygen and substrates delivery to cells leading to energy failure, loss of membrane potential and consequently depolarization of neurons and glial cells [9]. After depolarization, large amounts of excitotoxic amino acids, especially glutamate, are released into the synaptic cleft. This massive glutamate release stimulates the postsynaptic N-methyl-D-aspartate (NMDA) receptors allowing Ca^{2+} influx into neurons. This excess of glutamate release is likely to maintain the activation of these NMDA receptors resulting in further increase of the intracellular calcium concentrations and hence neuronal death [27]. NMDA receptors were therefore considered as potential neuroprotective targets. Various NMDA antagonists have been studied as they limit the subsequent neurodegradative processes through reduction of Ca^{2+} influx through the NMDA-operated Ca^{2+}channels [28]. Also their combination with TH, which reduces the ischemia induced release of glutamate [29], has been evaluated.

The first report of an NMDA antagonist combined with TH dates back to the early 90s where dextromethorphan, a non-competitive NMDA antagonist, was shown to be synergistically protective when combined with post-ischemic hypothermia (30 °C) in a hypotensive rat model of bilateral carotid artery occlusion [30]. Post-ischemic hypothermia has also been combined with the non-competitive NMDA antagonist MK-801 (dizocilpine) in a rat model of permanent focal ischemia. It appeared that hypothermia (33 °C) and MK-801, administered 30 min before MCAO at 1 mg/kg, offer similar cerebroprotective effects when administered separately but do not confer additional cerebroprotection when combined [31].

Other than drugs that antagonize the NMDA receptor, researchers also tested products that enhance the concentration of endogenous NMDA antagonists, such as magnesium. Magnesium is the fourth most abundant cation in the body, essential for cell functions such as preservation of membrane integrity, protein synthesis, energy metabolism, maintenance of ionic gradients and regulation of vascular smooth muscle tone [32]. It has also an anti-excitotoxic property since magnesium ions antagonize calcium entry *via* NMDA receptors [33]. This has been the rationale for the administration of magnesium as a neuroprotective treatment following cerebral ischemia. In a permanent middle cerebral artery occlusion (MCAO) rat model, Campbell *et al*. [34] concluded that magnesium (i.v. loading dose of 360 µmol/kg followed by a 25 h i.v. infusion at 120 µmol/kg/h) is not neuroprotective unless it is combined with TH (35 °C). In a transient MCAO rat model, Song *et al*. [35] infused a 15 °C magnesium sulfate solution (120 mg/kg), which reduced the temperature of the MCA supplied territory to 33-34 °C within 5-10 min after infusion. The authors concluded that the combination of local hypothermia and magnesium is more effective in reducing acute ischemic damage than local hypothermia alone. Meloni *et al*. [36], on the other hand, could not confirm that a combination of TH at 35 °C and magnesium (360 µmol/kg) significantly reduces infarct size in a permanent MCAO rat model.

Another, although indirect, endogenous inhibitor of NMDA receptors is N-acetyl-aspartyl-glutamate (NAAG), an abundant peptide transmitter in the mammalian nervous system, widely co-distributed with glutamate, gamma-aminobutyric acid (GABA) and acetylcholine [37, 38]. NAAG is neuroprotective when administered exogenously at low or moderate doses (2-10 mg/kg intraperitoneally (i.p.)) as it activates group II mGlu receptors that inhibit glutamate releases [39, 40]. In a rat model of transient focal MCAO, it was demonstrated that TH (34 °C) combined with NAAG (10 mg/kg i.p.) either 40 min before or 20 min after the insult does not considerably improve ischemic damage [41].

In conclusion, the advantage of NMDA receptor antagonists resides in their excitotoxic inhibitory pathway. One caveat is that, however, it affects also other pathways necessary for normal neuronal function and survival and generates many adverse side effects necessitating dose reduction beneath the potential neuroprotective dose [42-45]. Furthermore, the protective time window for NMDA receptor antagonists is only 1 to 2 h [46], which limits its clinical use [47].

Oxidative Stress Scavengers

When there is an imbalance between free radical production and degradation, oxidative stress occurs. Under normal conditions, endogenous antioxidants scavenge the ROS produced. However, in ischemic conditions these defense mechanisms fail to protect tissue from oxidative damage because of overproduction of ROS and inactivation of antioxidant enzymes [48]. It was therefore suggested that the administration of therapeutic compounds that have the ability to neutralize ROS, could provide neuroprotection. Several antioxidant agents have therefore been investigated as free radical scavenging effect-providing neuroprotectants after an IS. Some of them have also been combined with TH, which is known to attenuate oxidative DNA damage and DNA damage-triggered pro-death signaling events [49]. In a rat model of transient (2 h) focal ischemia, Nito *et al*. [50] investigated the effect of the antioxidant 3-methyl-1-phenyl-pyrazolin-5-one (edaravone) (3.0 mg/kg), intravenously administered just prior to reperfusion, combined with TH (35 °C). It could be concluded that the combined treatment

Table 1. Summary of all neuroprotective strategies that have been combined with therapeutic hypothermia for the treatment of ischemic stroke.

Reference	Model	Therapeutic Hypothermia – Target Temperature (°C)	Second Neuroprotective Strategy	Is Combination Therapy More Neuroprotective than Monotherapy?
[30]	Hypotensive rat model of bilateral carotid artery occlusion	30	dextromethorphan (20 mg/kg i.p.)	yes
[31]	Permanent rat MCAO model	33	MK-801 (dizocilpine) (1 mg/kg)	no
[34]	Permanent rat MCAO model	35	magnesium (IV loading dose of 360 μmol/kg followed by a 25 h i.v. infusion at 120 μmol/kg/h)	yes
[35]	Temporal rat MCAO model	33-34	magnesium sulfate solution (15 °C, 120 mg/kg)	yes
[36]	Permanent rat MCAO model	35	magnesium (360 μmol/kg)	no
[41]	Temporal rat MCAO model	34	N-acetyl-aspartyl-glutamate (NAAG) (10 mg/kg i.p.)	no
[50]	Temporal rat MCAO model	35	3-methyl-1-phenyl-pyrazolin-5-one (edaravone, 3 mg/kg)	yes
[53]	Temporal mouse MCAO model	33	rt-PA (10 mg/kg i.v.)	yes
[54]	Rat thromboembolic stroke model	34	rt-PA (10 mg/kg)	yes
[55]	Rat model of embolic stroke	32	rt-PA (20 mg/kg i.v.)	no
[56]	Rat model of thromboembolic occlusion	33	rt-PA (1 mg/100 g)	no
[57]	Clinical study	32-34	rt-PA (0.9 mg/kg)	no
[58]	Clinical study	33	rt-PA (0.9 mg/kg)	no
[65]	Temporal rat MCAO model	35	argatroban (3 mg/kg)	yes
[69]	Temporal rat MCAO model	32-33	atorvastatin pretreatment (1 mg/kg/day, for 10 days before ischemia)	yes
[77]	Temporal rat MCAO model	35	FK506 (tacrolimus) (0.3 mg/kg)	yes
[78]	Temporal rat MCAO model	32-34	ketoprofen (10 mg/kg)	no
[86]	Temporal rat MCAO model	34	citicoline (400 mg/kg i.p.)	yes
[94, 95]	Permanent rat focal embolic stroke model	34	minocycline (45 mg/kg on the first day and 22.5 mg/kg 24 h and 32 h after stroke)	no
[96]	Temporal rat MCAO model	33	minocycline (twice daily 30 mg/kg)	yes
[101]	Temporal rat MCAO model	35	caffeinol (ethanol 0.32 g/kg + caffeine 10 mg/kg)	yes
[106]	Clinical study	33-35	caffeinol (8-9 mg/kg caffeine + 0.4 g/kg ethanol) + rt-PA (0.9 mg/kg i.v.)	combination could not be evaluated as no control group was included
[132]	Temporal rat MCAO model	36	xenon (30%)	yes
[145]	Temporal rat MCAO model	33	magnesium (1 mmol/kg) and tirilazad (3 mg/kg)	yes
[146]	Permanent rat MCAO model	33	magnesium (2x1 mM/kg) + tirilazad (2x3 mg/kg)	yes

Table 1. contd….

Reference	Model	Therapeutic Hypothermia – Target Temperature (°C)	Second Neuroprotective Strategy	Is Combination Therapy More Neuroprotective than Monotherapy?
[147]	Temporal rat MCAO model	33	magnesium (2x1 mmol/l/kg) + tirilazad (2x3 mg/kg)	yes
[157]	Temporal rat MCAO model	33	mannitol (1 g/kg)	no
[158]	Hypertensive rabbit model of temporary focal ischemia	33-34	mannitol (1 g/kg)	no
[162]	Temporal rat MCAO model	33	methohexital (started at a dose of 1 to 1.5 mg/kg/min until an EEG burst-suppression pattern was reached, burst suppression was maintained by an infusion rate of 0.4 to 0.6 mg/kg/min)	no
[166]	Permanent rat MCAO model	33	brain-derived neurotrophic factor (BDNF) (300 µg/kg/h)	yes
[173]	Temporal rat MCAO model	30 local cooling	cold albumin solution (0 °C, 0.5 g/kg)	yes
[175]	Permanent rat MCAO model	32	decompressive hemicraniectomy	yes
[176]	Permanent rat MCAO model	32	decompressive hemicraniectomy	yes
[177]	Clinical study	35	decompressive hemicraniectomy	yes but not significant
[179]	Temporal rat MCAO model	33	gene therapy	yes
[185]	Temporal rat MCAO model	35	protein therapy	yes

significantly reduced edema volume and infarct size. When administered separately, edaravone attenuated only brain edema and TH failed to reduce post ischemic brain damage.

Anticoagulants

In acute IS, the endogenous fibrinolysis is usually outweighed by ongoing activation of the coagulation cascade and platelet activation [51]. Components of the coagulation cascade have therefore been attractive targets for neuro-protective agents. rt-PA is currently the only approved treatment for acute IS [52]. Since rt-PA needs to be administered within the first 4.5 h after stroke onset [2, 3], and TH is most effective when initiated as soon as possible after the insult, the question raised whether rt-PA could be combined with TH. In a study by Liu *et al*. [53], mice subjected to MCAO and receiving rt-PA in hypothermic circumstances (33 °C) had smaller infarcts than those receiving rt-PA in normothermic circumstances. In a rat model of thromboembolic stroke, the combination of TH (34 °C) with rt-PA treatment, both induced 1.5 h after stroke onset, was superior to thrombolysis alone and resulted in a reduction of infarct volume as well as a mitigation in the breakdown of the BBB [54]. On the other hand, Meden *et al*. [55] concluded that, although rt-PA and a 3-h TH (32 °C) reduced the infarct volume remarkably, the combination did not show any further protection in a rat model of embolic stroke. Similarly, Kollmar *et al*. [56] could not show an additive effect when rt-PA, administered 1 or 3 h after embolization, was combined with TH (33 °C), initiated 1 h after embolization and maintained for 4 h, in a rat model of thromboembolic occlusion of the middle cerebral artery. The safety and efficacy of TH combined with intravenous rt-PA were also investigated in acute IS patients. Based on the National Institutes of Health Stroke Scale (NIHSS) score and the Barthel Index (BI), it was concluded that the combination of TH (32-34 °C), induced locally on the surface of the lesion side of the head, and rt-PA (0.9 mg/kg, 10% of the dose as a bolus and the remainder given as a constant infusion over 60 min) provided no benefit compared to rt-PA alone [57]. Also Hemmen *et al*. [57, 58] could not demonstrate statistically significant differences between hypothermic (33 °C) and normothermic patients receiving an rt-PA treatment (0.9 mg/kg) [58]. Nevertheless, both studies indicated that it is safe to combine TH with rt-PA.

Next to rt-PA, argatroban also has been combined with TH. Argatroban directly inhibits free and clot-bound thrombin and, hence, thrombin-induced activities [59]. It has predictable anticoagulant effects [60], causes less bleeding compared to heparin for the same anticoagulant effect [61] and is well-tolerated [62]. It has been shown that argatroban is beneficial in models of acute IS [63, 64]. In an MCAO rat model, Kamiya *et al*. [65] investigated and confirmed that argatroban (3.0 mg/kg), continuously injected for 24 h after onset of ischemia, combined with TH (35 °C), significantly

decreased the cortical infarct volume compared with vehicle treated groups and animals treated with only argatroban.

Statins

Improvement in the microcirculation can be achieved by statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, originally introduced as inhibitors of cholesterol biosynthesis, but also exerting neuroprotective effects by up-regulating endothelial nitric oxide (NO) synthase (eNOS) [66]. As nitric oxide relaxes vascular smooth muscle, it increases cerebral blood flow and thus improves microcirculation [67, 68]. Lee *et al*. [69] demonstrated that atorvastatin pretreatment (1 mg/kg, daily for 10 days before ischemia) enhances the efficacy of neuroprotection conferred by TH (32-33 °C) in acute IS. The simultaneous administration of atorvastatin and TH showed a greater reduction of infarct size than each treatment modality alone. Moreover, atorvastatin pretreatment also extended the therapeutic time window of TH from 3 h to 6 h after stroke. As statins are safe and effective drugs, it is an attractive strategy to combine them with TH.

Anti-Inflammatory Agents

In ischemic brain parenchyma, inflammatory reactions are triggered which may amplify further tissue damage by triggering other deleterious mechanisms [70, 71]. Ischemic injury induced inflammation can be characterized by the rapid synthesis of pro-inflammatory cytokines and chemokines and the coordinated infiltration of neutrophils, T-lymphocytes and macrophages [72]. Because inflammation reactions occur rapidly and persist for a few days after ischemic brain injury [73], these responses are potential targets for human therapy.

FK506 or tacrolimus is a drug that suppresses the release of inflammatory cytokines and decreases NO synthase resulting in a reduced production of NO. It has been demonstrated that FK506 provides neuroprotective effects against transient and permanent focal ischemia [74], global ischemia [75] and chronic hypoperfusion [76]. In a rat model of transient MCAO, Nito *et al*. [77] combined TH (35 °C) with FK506 (0.3 mg/kg) and found that TH further reduced the infarct and edema damage. TH also expanded the therapeutic time window for FK506 administration. A low dose of FK506 (0.3 mg/kg) was shown to be neuroprotective when administered at 1 h, but not at 2 h. However, the combination of FK506 and TH significantly improved infarct size and edema volume, even when administered 2 h after MCAO. Although the precise mechanisms of this neuroprotective effect remain unclear, this combination may be useful in future treatment for acute stroke.

Another drug tested was ketoprofen, a non-steroid agent with a very potent analgesic and anti-inflammatory action. At a dose of 10 mg/kg body weight, combined with TH (32-34 °C), it was not more effective than the isolated action of these two neuroprotective therapies [78].

Drugs Interrupting Multiple Ischemic Cascades

Next to those agents interrupting only one pathway of the ischemic cascade, there are several agents that have the ability, like TH, to influence numerous pathways simultaneously. Some of these agents have also been combined with TH.

Citicoline

Citicoline is an endogenous compound, originally identified as the key intermediary in the biosynthesis of phosphatidyl-choline [79]. Since it was recognized as a neuroprotectant, it has been used in a plethora of experimental and clinical trials. It has some neuroprotective ability to improve phosphatidyl-choline synthesis in the injured brain [80]. It stabilizes membrane function [81], reduces free radical formation [82, 83], inhibits free fatty acid release [84] and it may also interact with the apoptotic cascade by inhibiting pro-caspase expression and caspase activation [85]. Sahin *et al*. [86] investigated the effect of citicoline (400 mg/kg i.p.) combined with TH (34 °C) in a rat model of transient focal cerebral ischemia. The authors concluded that the combination therapy is more effective in mitigating cerebral damage than either therapy used alone. The combination resulted in a greatly reduced expression of the tested apoptotic proteins; it also significantly affected the expression of the anti-apoptotic protein Bcl-2 and reduced apoptotic cell death, thus preventing neuronal damage.

Minocycline

Minocycline is a second-generation tetracycline which easily crosses the BBB [87]. Besides its antimicrobial action, it has also neuroprotective properties through inhibition of microglial activation [88, 89], attenuation of apoptosis [90], suppression of free-radical production [91] and inhibition of matrix metalloproteinases (MMPs) [92, 93]. Two research groups investigated the combination of minocycline and TH. In a permanent focal embolic stroke model, it was demonstrated that TH (34°C) combined with minocycline, administered i.p. at 1 h and 4 h after embolization on the first day (45 mg/kg body weight), and again after 24 h and 32 h (22.5 mg/kg), reduced infarct volume [94, 95]. However, there was no additive effect compared to the group receiving minocycline alone. Moreover, the group that received only TH, showed no reduction in infarct size compared to the control group [94, 95]. It was suggested this might be due to the insufficient duration of hypothermia, i.e. only 2 h. Therefore, Nagel *et al*. [96] investigated, in a transient MCAO model, the effect of a prolonged hypothermic phase (4 h, 33 °C) combined with minocycline, administered twice daily (30 mg/kg). They concluded that the combined therapy was only slightly superior to either treatment alone. Nevertheless, since minocycline has only minimal side effects and is well tolerated in humans, it could be a useful drug to treat stroke patients.

Caffeinol

Caffeine (1,3,7-trimethyxantine) and ethanol are among the most widely and frequently used psychoactive drugs in most societies. Both are rapidly absorbed from the gastrointestinal tract achieving high concentrations in the brain *via* the blood stream. Caffeine is a competitive antagonist at the adenosine receptor. Its chronic administration up-regulates the brain adenosine receptor [97]. This receptor up-regulation, in combination with increased brain adenosine

concentrations produced in response to ATP breakdown during ischemia, could increase stimulation of inhibitory adenosine transduction pathways resulting in neuro-protection [98]. Ethanol, on the other hand, acts on the brain *via* $GABA_A$ receptor stimulation and inhibition of NMDA receptors [99]. It was shown to reduce neuronal death in a gerbil model of global ischemia [100].

Aronowski *et al*. [101] demonstrated that caffeinol, a mixture of ethanol (0.32 g/kg) and caffeine (10 mg/kg), combined with intra-ischemic TH (35 °C), initiated 1 h after a 2.5 h transient MCAO, enhanced the neuroprotective effect of caffeinol or TH alone by >50%. They also demonstrated that it is very important to apply ethanol and caffeine at the same time in order to achieve optimal neuroprotection. Animals that were treated with ethanol and 2 h later with caffeine showed no benefit [101]. However, despite the positive effects of caffeinol, a disadvantage is the development of tolerance to its neuroprotective effect. Adenosine receptors are known to desensitize rapidly in response to repetitive activation [102]. Analogously, repetitive treatment with ethanol up-regulates *N*-methyl-D-aspartate (NMDA) receptor activity, down-regulates $GABA_A$ receptor function [103] and potentiates susceptibility to glutamate excitotoxicity [104]. Daily administration of caffeinol, for 2 to 3 weeks, before stroke builds up a tolerance to its neuroprotective properties [105]. In a study by Martin-Schild *et al*. [106], it was demonstrated that it is feasible to combine a 2 h caffeinol infusion (caffeine 8-9 mg/kg + ethanol 0.4 g/kg), started 4 h after symptom onset, and TH (33-35 °C, started 5 h after symptom onset) in patients with acute stroke treated with rt-PA. No adverse events were attributed to caffeinol and there was no apparent increase in brain hemorrhage by linking caffeinol and cooling to rt-PA. However, as no control group was included in the study, the efficacy of the combination therapy could not be evaluated.

Xenon

Xenon, a noble gas with anesthetic properties, provides neuroprotection in experimental models of neonatal asphyxia [107, 108], focal IS [109-111], cardiopulmonary bypass [112] and cardiac arrest [113]. It is known to interact with the adenosine triphosphate potassium channel [114, 115] and the glutamatergic NMDA receptor [116-118]. In addition, xenon may also be neuroprotective by reducing overall neurotransmitter release [119], inhibition of the α-amino-3-hydroxy-5-methyl-4-isoxazolole propionate and kainate receptors, which are 2 subtypes of glutamate receptor channels [120], reduction in cytosolic pro-apoptotic Bax protein expression and enhanced Bcl-xL expression [121-123], activation of 2-pore domain K^+ channels [124], inhibition of calcium/calmodulin-dependent protein kinase II [119], and increased phosphorylation of transcription factor cAMP-response element binding protein, which may, in turn, up-regulate cAMP-response element binding protein-dependent pro-survival genes [125, 126]. Since TH and xenon have been shown to have both anti-excitotoxic [127, 128] and anti-apoptotic properties [129-131], the combination has been studied in a rat model of temporary focal ischemia [132]. The results showed that xenon (30%) and TH (36 °C) exhibit synergistic neuroprotective properties when administered together. Xenon may thus be a promising agent with effective neuroprotective properties and has no adverse effects when administered at sub-anesthetic concentrations [109, 110, 112]. However, there are two drawbacks related to the use of xenon. First, it is described that xenon inhibits tPA thrombolysis and should only be administered after tPA-induced reperfusion [133]. Second, xenon delivery capability systems are currently very expensive, making their wide use less probable.

Magnesium and Tirilazad

Magnesium is a naturally occurring calcium antagonist that promotes vasodilation of cerebral arteries [134]. Magnesium ions (Mg^{2+}) participate in a voltage-sensitive blockade of ion channels, resulting in a non-competitive antagonism of NMDA receptors [33, 134]; they compete with extracellular calcium ions to reduce calcium entry into cells [135] and inhibit the release of intracellular calcium ions [136] and excitatory amino acids [137]. The 21-aminosteroid tirilazad mesylate, on the other hand, is a free radical scavenger and lipid peroxidation inhibitor [138]. Both drugs have been shown to have beneficial effects in numerous animal studies of focal and global cerebral ischemia [139, 140], traumatic brain injury [141], subarachnoid hemorrhage [142] and spinal cord ischemia [143]. Also the combination of these pharmacological agents has been shown to be synergistically neuroprotective in a rat model of transient focal cerebral ischemia [144]. Schmid-Elsaesser *et al*. [145] investigated the effect of TH (33 °C) combined with tirilazad (3 mg/kg) and magnesium ($MgCl_2$, 1 mmol/kg), administered both before and after ischemia, in rats subjected to MCAO for 90 min and concluded that the combination significantly reduced infarct size. Even in a model of permanent focal cerebral ischemia of 6 h, the combined therapy provided neuroprotection [146]. In a follow-up paper, Zausinger *et al*. [147] defined the therapeutic window of this combination and concluded that, in a temporal MCAO rat model (90 min), this therapeutic strategy is even efficient when applied up to 3 h after the ischemic insult.

Mannitol

Mannitol has neuroprotective properties in transient [148] and permanent MCAO models [149, 150] as well as in models of hemorrhagic stroke [151]. It is a hypertonic agent that reduces blood viscosity up to 2 h after i.v. administration [152] and thus improves cerebral blood flow in ischemic situations [153, 154]. It also reduces cerebral edema because of its osmotic effect [155] and acts as a free-radical scavenger [156]. The combination of mannitol with TH was evaluated by Karibe *et al*. [157] who concluded that, in a rat model of transient focal ischemia, mannitol (25%, 1 g/kg) added no significant protection to TH (33 °C). Also in a hypertensive rabbit model of temporary focal ischemia, mannitol (1 g/kg of body weight) combined with TH (33-34 °C) did not result in significantly smaller infarct volumes compared with the individual therapies [158]. The use of mannitol in combination with TH is therefore not indicated. Furthermore, as the effects of mannitol change with time after administration, it is an unsuitable agent to use. It was found that within 30 min of administration, intracranial pressure (ICP) drops

followed by an increase in ICP secondary to a rebound effect [159, 160].

Methohexital

The protective properties of barbiturates include stabilization of cell membranes, improvement of blood flow into the ischemic brain tissue as well as counteraction of oxidative and excitotoxic processes, resulting in an inhibition of intracellular calcium overload [161]. Westermaier *et al*. [162] studied whether methohexital, a barbiturate, provides an additional protective effect under hypothermic conditions. Sodium methohexital infusion started 30 min before ischemia at a dose of 1 to 1.5 mg/kg/min until an EEG burst-suppression pattern. Next, it was kept at an infusion rate of 0.4 to 0.6 mg/kg/min. TH (33 °C) was also induced 30 min before the ischemic insult. Although the total infarct volumes were significantly smaller in TH vehicle-treated animals and in TH methohexital-treated animals, compared with normothermic vehicle-treated controls and normothermic methohexital-treated animals, the barbiturate therapy did not provide a significant additional protection under hypothermic conditions [162].

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a well-characterized neurotrophic factor which plays an important role in proliferation, differentiation, maintenance, plasticity, survival and function of neurons in the central and peripheral nervous system [163]. Considering its actions, several studies reported a neuroprotective effect in various ischemia models. In a rat model of temporary focal cerebral ischemia, administration of BDNF through an intraventricular route [164], as well as intravenously [165], has been shown to reduce infarct size and improve neurological outcome. Potential mechanisms of the neuroprotective role of BDNF in focal cerebral ischemia include counter-regulation of Bax and Bcl-2 proteins within the ischemic penumbra [165]. However, the exact mechanisms are still under debate. Berger *et al*. [166] investigated, in a rat model of IS, whether TH (33 °C) and intravenous administration of BDNF at 300 µg/kg/h for 2 h, applied 30 min after stroke onset, acted synergistically. They concluded that, when applied separately, TH and BDNF reduced infarct size by 20% and 19%, respectively. However, the combined strategy reduced the infarct size by approximately 40% as compared to non-treated control animals. Both therapies seem to reduce glutamate and the extracellular accumulation of endogenous BDNF. Important to notice is that the BDNF dose used in this study was rather high, i.e. 300 µg/kg/h, compared to studies where BDNF was administered intraventricularly [164] or when other growth factors were used to treat focal cerebral ischemia [167]. The authors mention to have chosen this dose since lower daily doses of 60-80 µg/d were not neuroprotective when given intravenously after transient forebrain ischemia. This may be explained by the fact that, after peripheral delivery, exogenous BDNF may encounter two major obstacles in arriving at the central nervous system, i.e. degradation during circulation in blood and the presence of the BBB which limits transport of BDNF to the brain [168]. Surprisingly, in that study, it seems that TH does not influence the level of endogenous BDNF. Also in a rat model of global ischemia [169] and a pig model of hypoxic ischemic (HI) brain injury [170], it has been shown that endogenous BDNF levels remain unaltered by TH.

Albumin

Albumin is an endogenous plasma protein with neuroprotective properties. It is known to protect both parenchymal and vascular elements of the brain, diminish brain edema, maintain microvascular integrity, inhibit endothelial cell apoptosis and exert antioxidant effects [171]. However, both moderate and high-dose systemic human albumin therapies have been shown to cause severe dose-related adverse effects in clinical stroke studies [172]. Therefore, Chen *et al*. [173] investigated the neuroprotective effect of low-dose albumin (0.5 g/kg), infused locally at the ischemic site, in a rat model of 2 h MCAO. By infusing albumin as a cold (0 °C) solution, it was furthermore possible to induce local brain hypothermia. Temperature was significantly reduced within 3 min in the cerebral cortex and the striatum (from ± 37 °C to ± 30 °C) and the reduced temperature was sustained up to 45 min. The local low-dose cold albumin infusion into the ischemic area, which offered a combination of regional brain hypothermia and albumin administration, resulted in a significant reduction in infarct volume and a significant improvement in neurological and motor function. This method has the potential to be used in a clinical setting as it has the advantage that cooled low doses of albumin can be infused, which reduces the risk of systemic adverse dose-related effects. Furthermore, it produces local TH much faster than systemically induced whole body TH. However, the local infusion of albumin in human brain is very invasive and risky as well as the long-term neuroprotective effect of this strategy in animal models remains to be investigated.

DECOMPRESSIVE CRANIECTOMY

Regardless of the underlying pathology, all types of stroke are accompanied with brain edema. Because the brain is encased by the walls of the bony skull, this swelling leads to an increased ICP and results in an enlarged area of brain damage. Craniectomy, a technique in which a portion of the skull is removed and the dura is opened, is one of the most effective ways to relieve the increased ICP secondary to cerebral edema following IS [174]. The effect of combined decompressive craniectomy and TH was first investigated by Doerfler *et al*. [175]. In a rat model of permanent MCAO, it was demonstrated that craniectomy combined with TH (32 °C, maintained for 5 h), yields a significant additional benefit as the combination resulted in additional reduction in infarct size and improvement in neurological outcome compared with controls. Using the same model, Jieyong *et al*. [176] demonstrated that the combined therapy up-regulates Bcl-2, an anti-apoptotic protein, and down-regulates Bax, a pro-apoptotic protein, resulting in a reduction in cell apoptosis and infarct size.

This combination strategy was also evaluated in patients with malignant supratentorial cerebral ischemia [177]. It seemed that patients who received a combination of TH (35 °C) and hemicraniectomy performed better compared to patients who were treated with decompressive hemicraniectomy

alone [177]. However, the difference was not statistically significant, due to the small sample size, and therefore, no definitive conclusions could be drawn.

GENE THERAPY

A novel therapeutic strategy to treat IS is gene therapy or the transfer of genetic material into host cells with the intention of expressing the protein of interest. It is known that cerebral ischemia alters the expression of many genes. Those genes possessing neuroprotective properties may thus be ideal candidates for gene therapy.

Lawrence *et al*. [178] showed that overexpression of the anti-apoptotic protein Bcl-2 prevents apoptosis and improves striatal neuron survival when delivered 1.5 h after stroke. When delivered 5 h after stroke, no protection was observed. However, post-ischemic TH (33 °C) prolonged the therapeutic window for Bcl-2 gene therapy from 1.5 to 5h, and Bcl-2 plus TH blocked cytochrome *c* release 48 h after the ischemia onset. These data demonstrate a synergistic effect of TH and Bcl-2 overexpression, suggesting a potential clinical application of TH combined with gene therapy [179].

PROTEIN THERAPY

Gene therapy may be a potential treatment strategy to improve stroke related brain damage. However, it takes considerable time to deliver a gene of interest into the brain and to express a sufficient amount of therapeutic proteins. As an alternative approach, delivering therapeutic proteins directly to ischemic brain tissue has been proposed [180]. This is possible through protein transduction domains (PTDs), which are small peptides that are able to ferry much larger molecules into cells independent of classical endocytosis [181]. PTDs have made it possible to design strategies for delivering pharmacologically potent macro-molecules and even active enzymes to brain tissue by crossing the BBB and cell membranes [182]. One of these macromolecules is FNK, an artificial protein derived from the anti-apoptotic protein Bcl-xL by substituting three amino acids [183]. It is the first mutant with a gain-of-function phenotype among the mammalian anti-apoptotic factors and shows anti-necrotic as well as anti-apoptotic activity [183]. By fusing it with the human immunodeficiency virus type 1/trans-activator of transcription (HIV-TAT) protein transduction domain, it can be transduced into neuronal cells rapidly [184]. The protein transduction domain fused FNK (PTD-FNK) protein has been shown to be significantly protective against rat brain focal ischemia, even when administered 3 h after ischemia [184]. Recently, Sakurazawa *et al*. [185] evaluated whether PTD-FNK combined with TH (35 °C) is more effective than monotherapy in a rat model of MCAO. It could be concluded that a hypothermic PTD-FNK treatment significantly reduces infarct volume compared to a normothermic PTD-FNK treatment [185]. The combined therapy is thus an effective and safe strategy for neuronal protection against cerebral ischemia.

COMBINATION THERAPY TESTED IN NEONATAL RATS

Several of the combination treatments mentioned above were also tested in a neonatal rat model of HI brain injury. HI injury is a devastating complication in childbirth [186] that occurs at a frequency of 1-4 per 1,000 live births [187]. It causes long-term neurological and behavioral impairment in the developing brain. To date, hypothermia is the only intervention that improves outcome. As with stroke, several research groups evaluated whether a second intervention could augment the protection afforded by TH.

Ma *et al*. [131] demonstrated that concurrent administration of xenon (20%) and TH (35 °C) synergistically reduced long-term damage in a rat model of neonatal asphyxia. In a follow-up study, the authors also concluded that even asynchronous administration of xenon and hypothermia at a 1 h interval could produce a significant reduction in infarct volume [123]. The combination was also evaluated by Hobbs *et al*. [188] who concluded that xenon (50%) and TH (32°C) additively confer greater protection than either treatment alone. These findings are in accordance with the study of Sheng *et al*. [132], who demonstrated that xenon (30%) and TH (36 °C) exhibit synergistic neuroprotective properties when administered together in a temporal rat MCAO model.

Another combination that was tested in neonatal rats was TH plus MK-801. Ikonomidou *et al*. [189] concluded that lowering the body temperature by 2.5 °C together with MK-801 pretreatment provides total protection against HI brain damage in infant rat pups. Also Alkan *et al*. [190] demonstrated that, in a rat HI model, the combined treatment significantly reduced mortality rate and offered better protection in terms of neuron survival. However, these findings are in contrast with those of Frazzini *et al*. [31] who evaluated the neuroprotective effect of this combination in a rat MCAO model but could not demonstrate an additive degree of cerebroprotection. This finding demonstrates that the results depend on the model used.

CONCLUSION

A better understanding of the underlying patho-physiology of IS has led to the development of several pharmacological and non-pharmacological neuroprotective agents. Despite the promising results in experimental studies, not all success at the bench has translated to success at the bedside [191]. Therefore, the development of additional acute stroke therapies represents a large unmet need with many remaining challenges but also opportunities to incorporate novel approaches [192]. Because of the poor response to the neuroprotective monotherapy, several research groups have evaluated the neuroprotective potential of combination therapies. As TH is currently the most promising neuroprotective strategy, it is an ideal candidate to combine with other neuroprotective compounds. Of all agents summarized in this review, dextromethorphan, edaravone, argatroban, atorvastatin, tacrolimus, citicoline, BDNF, albumin and magnesium, whether or not combined with tirilazad, were found to be synergistically protective when combined with TH in rodent models of acute IS. However, their neuroprotective potential still needs to be confirmed in clinical trials. To date, only rt-PA, caffeinol and decompressive hemicraniectomy have been combined with TH in IS patients. Unfortunately, no definitive conclusions concerning the efficacy of these treatment strategies can be

drawn yet. Further researches, both experimental and clinical, are needed.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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