

Review

Effects of Therapeutic Hypothermia Combined with Other Neuroprotective Strategies on Ischemic Stroke: Review of Evidence

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[Received May 7, 2017; Revised June 27, 2017; Accepted June 28, 2017]

ABSTRACT: Ischemic stroke is a major cause of death and disability globally, and its incidence is increasing. The only treatment approved by the US Food and Drug Administration for acute ischemic stroke is thrombolytic treatment with recombinant tissue plasminogen activator. As an alternative, therapeutic hypothermia has shown excellent potential in preclinical and small clinical studies, but it has largely failed in large clinical studies. This has led clinicians to explore the combination of therapeutic hypothermia with other neuroprotective strategies. This review examines preclinical and clinical progress towards developing highly effective combination therapy involving hypothermia for stroke patients.

Key words: ischemic stroke, neuroprotection, therapeutic hypothermia, combination therapy

Hypothermia is a remarkable neuroprotectant and can mitigate brain injury induced by stroke, trauma, cardiac arrest or hypoxic-ischemic encephalopathy in newborns [1-3]. Yet how hypothermia protects the brain and what are the optimal conditions for protection are unclear. Studies suggest that initiating hypothermia as soon as possible after ischemia is better [4, 5], and that longer periods of hypothermia are superior to shorter periods [6]. Hypothermia appears to give better results when reperfusion occurs, though it is unclear whether hypothermia provides benefit in the case of permanent ischemia [5, 7]. The depth and duration of hypothermia strongly influence the risk of complications such as

infection [8] and arrhythmias [9]. These adverse events and the lack of optimized procedures have limited the application of hypothermia in the clinic.

To overcome these limitations, preclinical and clinical studies over the last few decades have been exploring the combination of hypothermia with a second neuroprotective strategy (Table 1). Such combination therapy is intended to prolong the therapeutic window of both treatments while reducing or eliminating adverse effects, thereby enhancing the magnitude and duration of neuroprotection. The present review discusses various combination approaches and summarizes their preclinical and clinical application in ischemic stroke.

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Table 1. summary of the second neuroprotective strategies combined with hypothermia in ischemic stroke.

Main Function of combined strategies	Combined strategies	Specified treatment
Reduce energy consumption	Anesthetics	Methohexital Thiopentone sodium Xenon Dexmedetomidine
Suppress calcium overload	Psychotropic agents NMDA receptor antagonists	Chlorpromazine and promethazine Dextromethorphan MK-801 Delfotel Magnesium with or without tirilazad
Increase blood supply	Ryanodine receptor inhibitor Reperfusion	Dantrolene t-PA Intra-arterial recanalization
	Vessel dilator Induce arteriogenesis	Statin Granulocyte colony stimulating factor (G-CSF)
Anti-inflammation	Antibiotics	Tacrolimus Minocycline
Anti-oxidative stress	Oxidative stress scavengers	Edaravone Mannitol
Repair damaged cells Increase oxygen supply	Biosynthesis of cell component Oxygen	Citicoline Normobaric oxygen Hyperbaric oxygen
Reduce intracranial pressure Anti-apoptosis	- Anti-apoptosis protein Gene of anti-apoptosis protein	Decompressive craniectomy FNK protein Bcl-2 gene
Multiple protection	-	Caffeinol (caffeine and ethanol) Insulin-like growth factor-1 (IGF-1) Brain-derived neurotrophic factor (BDNF) Albumin

1. Combination therapy involving antagonists of the *n*-methyl-D-aspartate (nmda) receptor

Therapies aiming at reducing excitotoxicity (preclinical evidence)

In this approach, hypothermia is combined with the administration of NMDA receptor antagonists in order to reduce excitotoxicity. To date, this approach has been tested in preclinical studies. The approach is based on the fact that the abrupt deprivation of oxygen and energy depolarizes neurons and glial cells, which release large amounts of excitatory amino acids into the synaptic cleft. These amino acids bind to several receptors, one of which is the NMDA receptor, which in response triggers calcium influx that leads to programmed cell death. Not surprisingly, efforts to develop neuroprotective therapies have been focusing on NMDA receptors for decades, and various antagonists have been studied on their own or combined with hypothermia.

The first studies were conducted using the non-competitive antagonists dextromethorphan and MK-801. In one study, animals were exposed to MK-801, hypothermia (33°C) or both and then subjected to permanent middle cerebral artery occlusion [10]. Either MK801 or hypothermia on their own reduces infarct volume, while the combination of both did not show an additive protective effect. In another study, animals were subjected to transient middle cerebral artery occlusion, followed by hypothermia (30°C, 3 h) and later by MK-801 therapy given 3-7 days after ischemia [11]. The combination of post-ischemia hypothermia and delayed MK-801 therapy attenuated neurobehavioral deficits at various follow-up points, and neuroprotection in the CA1 hippocampus was observed histologically up to 2 months after ischemia [12].

It is difficult to understand the mechanism of combination therapies involving hypothermia and non-competitive antagonists such as MK-801, since this compound by itself induces hypothermia [13, 14]. As a result, it is difficult to optimize combination therapy

parameters. In addition, non-competitive blockade of NMDA receptors may cause severe adverse effects [16, 17] because low-dose NMDA is necessary for normal neuronal function and survival [15].

Therefore, research has also focused on competitive NMDA receptor antagonists such as selfotel (CGS-19755). Despite being a potent antagonist, this compound did not provide significant neuroprotection in gerbils when administered alone or with hypothermia 30 min after 5-min forebrain ischemic insult [18]. In contrast, the compound helped protect various parts of the brain when administered with hypothermia after a series of three transient ischemic insults lasting 3 min each and delivered 1 h apart [19].

An alternative to exogenous antagonists is to use endogenous antagonists such as magnesium ion (Mg^{2+}). Mg^{2+} exerts anti-excitotoxic effects by antagonizing calcium entry via the NMDA receptor [20]. On its own, Mg^{2+} does not confer neuroprotection in transient or permanent stroke, but when combined with hypothermia, it has shown promising benefits in both injury contexts [21, 22]. In fact, the combination of magnesium and hypothermia proved superior to hypothermia alone in an animal model of transient middle cerebral artery occlusion [23]. On the other hand, a study of permanent middle cerebral artery occlusion failed to find a benefit of the combination of Mg^{2+} and hypothermia after ischemic stroke [24].

These precedents suggest that combination therapy shows potential but that further work is needed to optimize when it is delivered relative to the stroke and at what doses. Obtaining optimal neuroprotection will likely require a deeper understanding of how the two components of the combination therapy act separately and together.

2. Combination therapy involving reperfusion

Therapies aiming at increasing brain perfusion (preclinical evidence)

In this approach, hypothermia is combined with recombinant tissue plasminogen activator therapy, which is the only treatment for acute ischemic stroke approved by the US Food and Drug Administration. The rationale behind combining hypothermia and plasminogen activator therapy is that reperfusion helps to rapidly restore oxidative metabolism in surviving cells, resolve cytotoxic edema and reduce levels of excitatory amino acids. On the other hand, reperfusion can trigger NO release and superoxide formation as well as increase permeability of the blood brain barrier (BBB), which can lead to further excitotoxicity and hemorrhagic

transformation. Hypothermia induced shortly after reperfusion can reduce NO efflux in the brain and BBB leakiness, minimizing reperfusion injury [25, 26].

One study reported that combining hypothermia and plasminogen activator reduced infarct size as well as the hemorrhagic transformation ratio that plasminogen activator therapy usually increases [27]. Hypothermia also alleviated the adverse effects of plasminogen activator therapy, such as larger infarct volume and brain edema, and it mitigated reperfusion injury caused by matrix metalloproteinase-1 (TIMP-1) and soluble intercellular adhesion molecule-1 (sICAM-1) [28].

Nevertheless, the benefits of this combination therapy are not without controversy. Studies of embolic stroke reported no therapeutic enhancement when the two treatments were combined [29, 30]. Other studies showed that hypothermia yielded neuroprotection in transient ischemia, but it gave mixed results in permanent ischemia, it gave mixed results in permanent ischemia [5, 7]. Those studies suggested that hypothermia may give better results when performed with vascular recanalization.

These results suggest a somewhat inconsistent picture of therapeutic benefits of combination therapy, highlighting the need for more detailed mechanistic insights.

Therapies aiming at increasing brain perfusion (clinical evidence)

On the basis of the strong benefits reported for thrombolysis or hypothermia on their own, clinical trials have explored the safety and efficacy of combining the two for treating acute ischemic stroke patients. The ICTuS-L trial recruited acute ischemic stroke patients within 6h of symptom onset and administered intravenous thrombolysis with or without 24-h endovascular cooling (33°C) [31]. Based on assessment using the modified Rankin Scale (mRS), combination therapy was not superior to thrombolysis alone. In another clinical trial, thrombolysis was administered to ischemic patients within 6 h of symptom onset with or without 1-h local hypothermia therapy (32-34°C) on the surface of the lesion side. Once again, combination therapy was not superior to thrombolysis alone, based on assessments using the National Institutes of Health Stroke Scale (NIHSS) score or the Barthel Index (BI) [32].

Therapeutic benefit of combination therapy may depend on the relative timing of the procedures: performing hypothermia after thrombolysis has been shown to reduce edema and hemorrhagic transformation [33] as well as improve clinical outcomes [34]. Further work is needed to clarify the influence of timing. At the very least, the available evidence clearly shows that the

combination of hypothermia and thrombolysis is safe and feasible in acute ischemic patients. These trials have also demonstrated the safety of intra-arterial hypothermia, which can rapidly reduce brain temperature significantly while avoiding adverse effects associated with systemic cooling.

Endovascular mechanical thrombectomy (intra-arterial recanalization) is an alternative to plasminogen activator therapy that has significantly improved the success rate of stroke treatment in recent years [35, 36]. One clinical study showed good results when acute ischemic stroke patients were subjected, within 8 h after stroke onset, to intra-arterial hypothermia followed by intra-arterial recanalization. Clots were mechanically removed using a stent retriever inserted through an angiographic catheter [37]. The combination therapy was performed without technical errors in all 26 patients, without obvious complications related to the hypothermia.

These clinical studies show the feasibility and safety of combining hypothermia with plasminogen activator therapy or intra-arterial recanalization in acute stroke patients. They also highlight the feasibility of intra-arterial hypothermia as a potentially safer alternative to systemic cooling. Further work is needed to determine whether such combination therapies are superior to thrombolysis or recanalization on their own.

3. Combination therapy involving anti-inflammatory factors

Therapies aiming at anti-inflammation (preclinical evidence)

This approach has been tested so far only in preclinical studies. It is well known that neuroinflammation is a key element in the ischemic cascade after cerebral ischemia, and that it leads to the damage and death of neurons in the subacute phase. This death and especially the release of necrotic cell debris triggers inflammation, strongly activating phagocytic cells [38]. Induction of mild-to-moderate hypothermia in the ischemic brain inhibits local inflammation and is believed to contribute to neuroprotection [6, 39]. In addition, FK506 (Tacrolimus), an agent that suppresses the release of inflammatory cytokines and decreases NO synthase expression, was found to protect animals against ischemic injury [40, 41], though its therapeutic window was shorter than 2 h, limiting its clinical application. Combining FK506 with hypothermia (35°C) prolonged the therapeutic window and led to smaller infarct volume and edema than either treatment on its own [42].

The anti-inflammatory effects of minocycline, a second-generation, semi-synthetic tetracycline, are thought to contribute to its ability to protect neurons from ischemic injury in preclinical models [43]. Various studies suggest that it may act by inhibiting the activity of inducible nitric oxide synthase (iNOS) [44] and matrix metalloproteinase (MMPs) [45], inhibiting the activation of caspase-1 and -3 [46], and enhancing the effects of Bcl-2 [47]. One study involving transient middle cerebral artery occlusion found a small, albeit non-significant, increase in therapeutic benefit when minocycline was combined with hypothermia (33°C, 4 h) relative to either treatment on its own [25]. Further work is required to explore this combination therapy since a study involving permanent middle cerebral artery occlusion found no such additive effect [48]. Delaying the use of minocycline can still provide neuroprotection, but combining delayed minocycline with delayed hypothermia (34-35°C) did not enhance protective effects [49]. Clearly the research into combination therapy involving hypothermia and anti-inflammatory treatments is in very early stages.

4. Combination therapy involving statins

Therapies aiming at increasing brain perfusion (preclinical evidence)

This approach aims to increase brain perfusion in order to protect neurons from ischemic injury, and so far, it has been evaluated only in preclinical studies. Statins up-regulate endothelial nitric oxide (NO) synthase (eNOS) and are recommended for treating patients with atherosclerotic ischemia [50]. NO is a major vasodilator produced by cerebrovascular endothelium to maintain sufficient cerebral blood flow and normal brain function [51]. Combining atorvastatin with hypothermia (32-33°C, 2 h) led to smaller infarct volume than either therapy separately and it expanded the therapeutic window of hypothermia from 3 to 6 h after ischemia [52]. This promising approach should be investigated in greater detail in preclinical studies and ultimately in clinical trials.

5. Combination therapy involving oxidative stress scavengers

Therapies aiming at scavenging free radical (preclinical evidence)

This approach, which has been tested so far only in preclinical studies, aims to scavenge excess free radicals produced when cerebral ischemia and reperfusion injury perturb the balance between free radical production and degradation [53]. Since 2002, for example, the free radical

scavenger edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) has been approved for the treatment of stroke in Asia [54]. This scavenger is believed to interact with peroxy and hydroxyl radicals, creating a radical intermediate that forms stable oxidation products [55, 56]. The combination of edaravone and hypothermia (35°C) was found to significantly reduce edema and infarct volume in rats subjected to transient focal cerebral ischemia [57]; in contrast, edaravone on its own reduced edema, while hypothermia on its own showed no neuroprotective effect.

Mannitol is a free-radical scavenger also used clinically as a dehydrating agent because it can reduce intracranial pressure by creating an osmotic pressure gradient [58]. Combining mannitol and hypothermia (32-34°C) that was begun 1 h before permanent middle cerebral artery occlusion and continued for 2 h afterwards led to infarct volume similar to that with hypothermia alone [59]. Similarly, combining mannitol with hypothermia (33°C) lasting 1 h through the ischemic process did not enhance the therapeutic effects of hypothermia in a rat model of transient ischemia [60].

6. Combination therapy involving citicoline

Therapies aiming at biosynthesis of cell component (preclinical evidence)

This approach, which has been evaluated so far only in preclinical studies, takes advantage of the ability of citicoline to mimic cytidine-5'-diphosphocholine, which is an intermediate in the generation of phosphatidylcholine and is essential for the biosynthesis of membrane phospholipids [61]. Citicoline restores the activity of mitochondrial ATPase and membrane Na⁺/K⁺ ATPase, and it inhibits the activation of phospholipase A₂, the formation of free radicals and the release of free fatty acids [62, 63]. Citicoline can influence the ischemia cascade at different levels to produce neuroprotective effects [64]. Combining citicoline with hypothermia (34°C) was more effective than either therapy alone at suppressing apoptosis [65].

7. Combination therapy involving psychotropic agents

Therapies based on multiple mechanisms (preclinical evidence)

The combination of low-dose caffeine and ethanol, termed caffeinol, can effectively reduce brain damage in rodent models of focal cerebral ischemia [66], perhaps by stimulating adenosine-mediated transduction pathways that inhibit gamma-aminobutyric acid (GABA) and NMDA receptors [67]. The neuroprotective effect of

caffeinol can be enhanced by combining it with hypothermia (35°C) in animal models of transient occlusion of the middle cerebral artery [68]. The therapeutic window needs to be determined carefully, since administering caffeinol daily for two weeks before ischemic stroke onset eliminated its neuroprotective effects.

Therapies based on multiple mechanisms (clinical evidence)

In addition to preclinical studies, the combination of caffeinol and hypothermia has been explored in patients. In one study in which caffeinol, tissue plasminogen activator and hypothermia (33-35°C) induced from 5 to 24 h after stroke onset were given to patients with acute ischemic stroke, caffeinol did not enhance the effects of plasminogen activator [69].

Therapies aim at reducing energy consumption (preclinical evidence)

Phenothiazines are among the oldest synthetic anti-psychotic drugs. They block dopaminergic receptors to induce neuroleptic effects, but they also reduce energy consumption by inhibiting calmodulin, protein kinase C, and P-glycoprotein transport, suppressing cell proliferation [70, 71]. Combining chlorpromazine and promethazine (both 1 mg/kg) with 5-min hypothermia (35°C) at the initiation of reperfusion following 2-h occlusion of the middle cerebral artery led to smaller infarct volume and long-term neurological deficit [72]. In contrast, neither treatment on its own showed neuroprotection.

8. Combination therapy involving anesthetic-related agents

Therapies aiming at reducing energy consumption (preclinical evidence)

Numerous preclinical studies have explored the combination of hypothermia with barbiturates and volatile anesthetics, which on their own have been shown to protect against cerebral damage in animal models of focal ischemia [73, 74]. These drugs may exert protective effects by reducing cellular energy requirements, improving blood flow into ischemic brain tissue, and counteracting oxidative stress and excitotoxicity [75, 76].

Methohexital administered 30 min before stroke reduced infarct volume and neurological score in a preclinical study, and these benefits were not enhanced by adding hypothermia [77]. In a study involving cortical

neuron cultures exposed to prolonged hypoxia (24-48 h) [78], the combination of thiopentone sodium with mild hypothermia (32°C) or deep hypothermia (22°C) provided greater neuroprotection than either therapy on its own. Xenon, a potent anesthetic, has shown neuroprotective effects in adult rats subjected to transient brain ischemia [79]. The mechanisms underlying this neuroprotection may involve interaction with NMDA receptors, down-regulation of cytosolic pro-apoptotic Bax protein, up-regulation of Bcl-xL expression and increased phosphorylation of transcription factor cAMP-response element binding protein [80, 81]. Xenon and hypothermia have shown additive therapeutic effects in hypoxia-ischemia models [82, 83], as well as in a model of transient middle cerebral artery occlusion [84]. Administering the $\alpha 2$ agonist dexmedetomidine before ischemia significantly reduced subsequent levels of plasma catecholamines and decreased neurological comorbidities [85], potentially by reducing oxidative stress as well as inhibiting inflammation [86] and apoptosis [87]. Combining dexmedetomidine (given 30 min before ischemia) with hypothermia (35°C) from 1 h before ischemia until 1 h after reperfusion onset showed no additional benefit relative to either therapy on its own [88].

The anesthetic dantrolene, an inhibitor of ryanodine receptors, shows good preclinical promise for enhancing the therapeutic benefits of hypothermia after stroke. It blocks the release of Ca^{2+} stores from endoplasmic reticulum, protecting neurons from oxygen-glucose deprivation toxicity [89] and apoptosis [90]. Dantrolene can also accelerate body temperature cooling [91] and reduce incidence of shivering and shivering thermogenesis [92], both common side effects of hypothermia. For these reasons, dantrolene has been approved for treating malignant hyperthermia after anesthesia. One study found that combining dantrolene with hypothermia (33°C) helped protect cerebral cortex neurons from oxygen-glucose deprivation, increasing neuronal survival and mitochondrial membrane potential as well as reducing DNA fragmentation and apoptosis [93].

9. Combination therapy involving normo- and hyperbaric oxygen

Therapies aiming at increasing oxygen supply (preclinical evidence)

One of the initiators of ischemic pathophysiology is shortage of oxygen to the brain. Normobaric hyperoxia (NBO), induced by breathing air containing 21-100% oxygen at a pressure of 1 absolute atmosphere, effectively

reduced infarct volume and neurological deficits in rodents following acute ischemic stroke [94, 95]. NBO can also reduce BBB permeability and extend the therapeutic window of reperfusion therapy [96, 97]. In animals subjected to 1-h occlusion of the middle cerebral artery, the combination of tissue plasminogen activator-induced reperfusion, NBO (60%) and 3-h hypothermia (33°C) starting 1 h after infarction reduced infarct volume, neurological deficit and production of reactive oxygen species [98]. These effects were associated with an increase in pyruvate dehydrogenase activity and protein expression. Combining NBO with hypothermia reduced reperfusion injury by modulating NADPH oxidase activity and attenuating hyperglycolysis [99, 100].

Hyperbaric oxygen (HBO) may provide superior neuroprotection than NBO during transient and permanent cerebral ischemia [101, 102]. At the same time, HBO has been shown to induce oxidative stress in animal studies, likely reflecting prolonged exposure to highly concentrated oxygen [103, 104]. Performing HBO (2 absolute atmospheres, 100% oxygen, 60 min) during rewarming from moderate hypothermia (31°C, 60 min) preserved CA1 pyramidal neurons better than hypothermia alone [105].

10. Combination therapy involving granulocyte colony-stimulating factor

Therapies aiming at inducing arteriogenesis and brain perfusion (preclinical evidence)

Hematopoietic cytokines, by binding receptors on the membranes of neurons and glial cells in the central nervous system, stimulate intracellular signaling pathways that can help protect against injury and/or support neurogenesis [106, 107]. The cytokine granulocyte colony-stimulating factor (G-CSF) stimulates growth and differentiation of hematopoietic cells and is clinically used to treat chemotherapy-induced neutropenia [108]. It induces arteriogenesis in the hypo-perfused brain of rats and mice, restores cerebral blood flow, and mitigates stroke severity [109-112]. G-CSF may exert neuroprotective effects by mobilizing stem cells, preventing apoptosis, promoting neurogenesis, and reducing inflammation [113, 114]. Combining G-CSF with mild, 30-min hypothermia (33.5-35°C) initiated at reperfusion significantly reduced mortality, edema and neurological deficit after transient middle cerebral artery occlusion [115].

11. Combination therapy involving growth factors

Therapies based on multiple protection (preclinical evidence)

Insulin-like growth factors (IGFs) are peptide hormones with significant structural homology to insulin. IGF-1 is a potent cardiomyocyte growth and survival factor [116], which can regulate cell proliferation and inhibit cell apoptosis and necrosis after ischemic stroke [117-119]. The combination of IGF-1 and hypothermia was tested in a study in which rats were subjected to global ischemia lasting 8 min. Afterwards, animals were treated with hypothermia (32°C, 4 h) and/or IGF-1 (0.6 U/kg), then spatial memory was assessed at 21 days and CA1 neurons were counted on day 7 or 28 [120]. The combination of both treatments starting at the onset of reperfusion preserved CA1 structure and memory at 28 days better than either treatment on its own. On the contrary, another study indicated that after cerebral ischemia lasting 30 min, white matter damage was similar between animals treated with the combination of IGF-1 (3 µg, ventricularly) and hypothermia (30-33°C) from 5.5 h after ischemia until 72 h after ischemia, and animals treated with hypothermia alone, based on immunohistochemistry and numbers of caspase 3-positive cells [121].

12. Combination therapy involving brain-derived neurotrophic factor (bdnf)

Therapies based on multiple protection (preclinical evidence)

Brain-derived neurotrophic factor (BDNF) plays an important role in proliferation, differentiation, maintenance, plasticity, survival and neurite extension in the central and peripheral nervous systems [122]. BDNF administered intraventricularly or intravenously can protect the brain from transient or permanent ischemic insult [123, 124]. It may exert these effects by countering the activity of Bax and Bcl-2 proteins within the ischemic penumbra [125], inhibiting glutamate and NO neurotoxicity [126] and reversing NMDA-induced inactivation of protein kinase C [127]. In a model of the hyperacute phase of permanent middle cerebral artery occlusion, combining BDNF [300 mg/(kg/h), 2 h] with hypothermia (33°C) at 30 min after occlusion onset reduced post-ischemic glutamate concentration and infarct volume [128].

13. Combination therapy involving magnesium and tirilazad

Therapies aiming at reducing excitotoxicity (preclinical evidence)

Mg²⁺ is a non-competitive endogenous NMDA antagonist [10], and tirilazad (U-74006F) is a non-glucocorticoid, 21-aminosteroid that inhibits lipid peroxidation. Tirilazad is thought to inhibit iron-dependent lipid peroxidation within membranes through free-radical scavenging of lipid peroxyl and hydroxyl groups. This reduces the formation of hydroxyl radicals and maintains the levels of endogenous antioxidants [129]. Administering tirilazad before ischemia reduced stroke lesion size, neuronal necrosis, brain injury, and cerebral edema in rodents exposed to permanent or transient ischemia, while administering it after ischemia provided no protection [130, 131]. The combination of Mg²⁺ and tirilazad given pre-ischemia resulted in better neurologic function and smaller infarct volume [132]. Combining these two agents with 3-h hypothermia (33°C) starting 20 min before transient ischemia further decreased subsequent infarct volume and improved electroencephalography amplitude and neurological function [133]. The same researchers also showed that the triple combination of Mg²⁺, tirilazad and hypothermia gave better results than the combination of nimodipine, mannitol, dexamethasone, and methohexital [134]. At least in a model of transient infarction, the triple combination of Mg²⁺, tirilazad and hypothermia showed potent neuroprotection when administered before ischemia onset or up to 3 h afterwards [135].

14. Combination therapy involving albumin

Therapies based on multiple protection (preclinical evidence)

This approach, which has been evaluated only in preclinical studies, is based on the ability of albumin to inhibit endothelial apoptosis and to act as an antioxidant [136]. It also causes hemodilution, helps maintain microvascular permeability and regulates the enzyme pyruvate dehydrogenase. Administering low-dose cold albumin (0.5 g/kg, 0°C) via the middle cerebral artery to induce 45-min local hypothermia (30°C) in the ischemic lesion led to smaller infarct volume and better neurological outcomes than normothermia albumin [137].

15. Combination therapy involving decompressive craniectomy

Therapies aiming at decreasing intracranial pressure (preclinical evidence)

Decompressive craniectomy, in which part of the skull is removed and the dura is opened, can reduce intracranial

pressure; edema is a lethal complication of malignant supratentorial stroke, and it leads to high mortality [138]. This surgical procedure is superior to conservative treatment at reducing mortality [139]. While hypothermia on its own can also reduce intracranial pressure, the pressure can rebound during or after rewarming and cause lethal herniation [140]. Combining 5-h hypothermia (32°C) and decompressive craniectomy in rat models of permanent middle cerebral artery occlusion significantly reduced infarct volume and improved neurological outcome [141]. Applying this combination therapy even up to 6 h after stroke gave better results than either therapy on its own, based on infarct volume, neurological score and BBB integrity [142].

Therapies aiming at decreasing intracranial pressure (clinical evidence)

The combination of hypothermia and decompressive craniectomy has also been evaluated in clinical trials. The combination of surgery and hypothermia (35°C) induced immediately afterward led to better clinical outcomes than surgery alone in patients with malignant supratentorial stroke [143].

16. Combination therapy involving protein

Therapies aiming at anti-apoptosis (preclinical evidence)

This approach, which has been evaluated only in preclinical studies, takes advantage of the fact that Bax promotes apoptosis, whereas Bcl-2 and Bcl-xL inhibit apoptosis by blocking the translocation of cytochrome C. Substituting three amino acids in Bcl-xL enhances the anti-apoptotic activity, and the resulting variant, called FNK, can be fused to the protein transduction domain of HIV/Tat protein and efficiently introduced into cells [144]. In cell culture studies, FNK provided greater protection than Bcl-xL against oxidative stress, calcium ionophores, growth factor withdrawal, heat, or treatment with anti-Fas agents, cell cycle inhibitors or a protein kinase inhibitor [145]. FNK has been shown to ameliorate ischemic damage *in vivo* and *in vitro* [146]. Combining FNK with mild, 2-h hypothermia (35°C) starting from ischemia onset until reperfusion enhanced neuroprotection in rats following transient middle cerebral artery occlusion, potentially by inhibiting pro-apoptotic pathways mediated by caspase-12, and not by promoting anti-apoptotic pathways mediated by Bcl-2 [147].

17. Gene-based combination therapy

Therapies aiming at anti-apoptosis (preclinical evidence)

The rationale of gene therapy is to insert genes encoding neuroprotective proteins into neurons to promote their survival against cerebral insults [148]. Such therapy can lead to protective effects only after hours or even days. Administering simplex virus (HSV) vector expressing Bcl-2 led to neuroprotection via Bcl-2 overexpression when it was given 1.5 h after stroke but not 5 h after stroke [149]. To prolong the therapeutic window of gene therapy, researchers have combined it with hypothermia. Indeed, in one study, combining Bcl-2 gene treatment with hypothermia (33°C) induced after ischemia prolonged the therapeutic window of Bcl-2 overexpression from 1.5 h to 5 h, and it blocked the release of cytochrome C up to 48 h after ischemia [150].

Conclusions

The combination of diverse neuroprotective strategies with hypothermia has been extensively investigated for the alleviation of ischemic injury arising from cardiac arrest, hypoxic-ischemic encephalopathy or spinal cord impairment. The literature applying such combination therapy to focal stroke is more limited. In addition, studies of such combination therapy often report inconsistent results about the efficacy for treating stroke (Table 2). Several differences among studies may help explain these discrepancies, such as how stroke was modeled, when treatment was initiated relative to stroke, how long treatment lasted, and what was the depth of cooling. As far as neuroprotective drugs are concerned alone, heterogeneity of human stroke and lack of methodological agreement between preclinical and clinical studies may lead to failure of translating experimental success to clinical. Another potential problem is that drug metabolism may differ at cooler temperatures from at normal body temperature. The finding by several studies that combination therapy failed to improve on the results of monotherapy may reflect in some cases a “ceiling effect”: the monotherapy achieved the optimal efficacy, such that additional benefit from the other therapy was undetectable.

Table 2. Summary of outcomes of neuroprotective treatments combined with therapeutic hypothermia in ischemic stroke.

Reference	Permanent(P)/ Transient (T) Ischemia	Temperature degree(°C)	Combined Treatment	Aim of study	Yes /No
Animal					
[10]	P	33	Mk-801	Enhanced effect	No
[11]	T	30	Mk-801	Effective	Yes
[12]	T	30	Mk-801	Enhanced effect	Yes
[18]	T	34	Selfotel	Enhanced effect	No
[19]	Repetitive	34	Selfotel	Enhanced effect	Yes
[21]	T	35.4	Magnesium	Enhanced effect	Yes
[22]	P	35	Magnesium	Enhanced effect	Yes
[23]	T	33-34	Magnesium	Enhanced effect	Yes
[24]	P	35	Magnesium	Enhanced effect	No
[27]	T	33	t-PA	Reduce the side effect of t-PA	Yes
[28]	T	34	t-PA	Reduce the side effect of t-PA	Yes
[29]	T	32	Delayed t-PA	Enhanced effect	No
[30]	T	33	t-PA	Enhanced effect	No
[42]	T	35	Tacrolimus	Enhancec /expand time window of tacrolimus	Yes
[52]	T	32-33	Atorvastatin	Enhanced effect / expand time window of hypothermia	Yes
[57]	T	35	Edaravone	Enhanced effect	Yes
[65]	T	34	Citicoline	Enhanced effect	Yes
[25]	T	33	Minocycline	Enhanced effect	Yes
[48]	P	34	Minocycline	Enhanced effect	No
[49]	P	34-35	Minocycline	Enhanced effect	No
[69]	T	35	Caffeinol	Enhanced effect	Yes
[72]	T	35	Chlorpromazine and Promethazine	Enhanced effect	Yes
[77]	T	33	Methohexital	Enhanced effect	No
[84]	T	36	Xenon	Enhanced effect	Yes
[88]	Incomplete	35	Dexmedetomidine	Enhanced effect	No
[98]	T	33	t-PA and Normobaric Oxygen (NBO)	Enhanced effect	Yes
[99]	T	33	t-PA And normobaric oxygen (NBO)	Enhanced effect	Yes
[105]	-	31	Hyperbaric oxygenation (HBO2)	Enhanced effect	Yes
[100]	T	33	t-PA and normobaric oxygen (NBO)	reduce the side effect of t-PA	Yes
[115]	T	33.5-35	Granulocyte-Macrophage Colony-Stimulating Factor (G-CSF)	Enhanced effect	Yes
[120]	T	32	Insulin-Like Growth Factors -1(IGF-1)	Enhanced effect	Yes
[121]	T	30-33	Insulin-Like Growth Factors -1(IGF-1)	Enhanced effect	No
[128]	P	33	Brain-Derived Neurotrophic Factor (BDNF)	Enhanced effect	Yes
[133]	T	33	Magnesium and Tirilazad	Enhanced effect	Yes
[134]	T	33	Magnesium and Tirilazad	Effective	Yes
[135]	T	33	Magnesium and Tirilazad	Enhanced effect	Yes
[59]	P	32-34	Mannitol	Enhanced effect	No
[60]	T	33	Mannitol	Enhanced effect	No
[137]	T	30-31	Albumin	Enhanced effect	Yes
[141]	P	32	Decompressive Craniectomy	Enhanced effect	Yes
[142]	P	29-31	Decompressive Craniectomy	Enhanced effect	Yes
[147]	T	35	PTD-FNK	Enhanced effect	Yes
[150]	T	33	Gene of Bcl-2	Expand time window/Enhanced effect	Yes
Neuronal culture					
[78]	-	22/32	Thiopentone Sodium (TPS)	Enhanced effect	Yes
[93]	-	33	Dantrolene	Enhanced effect	Yes
Clinical					
[31]	T	33	t-PA	Enhanced effect	No
[32]	T	32-34	t-PA	Enhanced effect	No
[33]	T	<35.5	t-PA	Feasible/ improve outcome	Yes
[34]	T	34.5	t-PA	Reduce the side effect of tPA	Yes
[37]	T	decrease 2 in brain	Intra-Arterial Recanalization	Feasible and safe	Yes
[69]	T	33-35	Caffeinol and T-PA	Feasible	Yes
[143]	P	35	Decompressive Craniectomy	Enhanced effect	Yes

An important consideration when assessing evidence about efficacy of combination therapy is whether the study was conducted in preclinical models or in patients. Hypothermia has shown clear neuroprotective effects in animal studies, but not sure in clinical trials. Hypothermia is induced differently in preclinical animal models and in patients and hypothermia complications such as shivering and infections are generally ignored in the laboratory but can pose substantial problems in the clinic. At the very least, the studies reviewed here make clear that hypothermia induction is feasible in stroke patients, though the optimal conditions still need to be explored.

This review also illustrates the broad range of neuroprotective treatments that have shown promise in preclinical studies, but have not yet been evaluated in the clinic or have failed to show success in patients. So far, only tissue plasminogen activator, intra-arterial recanalization, caffeineol and decompressive hemicraniectomy have been combined with hypothermia in stroke patients. Much more work remains to be done, which should take into account the heterogeneity of human stroke.

Acknowledgements

This study was funded by the China National Funds for Distinguished Youth Scientist (81325007), Distinguished Professor of Cheung Kong Scholars Program (T2014251), National Natural Science Foundation (81571114) and Wenzhou Municipal Sci-Tec Bureau Programs (Y20120154 and Y20140686) and Projects of International Cooperation and Exchanges NSFC (81620108011).

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