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Evaluation of Molecular Mechanisms Involved in Therapeutic Hypothermia-Mediated Neurovascular Protection

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Abstract

Ischaemic stroke continues to be one of the leading causes of mortality and morbidity in the Western World. The availability of recombinant tissue plasminogen activator as a sole therapeutic agent for this condition alongside its limited application due to a short therapeutic window may in large part account for the elevated death and disability rates ascribed to ischaemic stroke. Hence, it is of critical importance to discover novel therapeutic agents or approaches that can be administered beyond the acute phase of stroke to attain long-term neurovascular benefits. Hypothermia has long been considered as an efficacious therapeutic option in cases of ischaemia caused by cardiac arrest or hypoxic-ischaemic encephalopathy in neonates due to its ability to protect tissue function and positively influence neurological and functional outcome. Nevertheless, many aspects of hypothermia as an alternative or supplementary therapy for ischaemic stroke remain largely unknown. Given the prominent involvement of a series of pathologies, notably inflammation, excitotoxicity, oxidative stress and apoptosis in ischaemic stroke-mediated cerebrovascular damage, this review paper selectively discusses how therapeutic hypothermia may affect these pathways in experimental and where possible, clinical settings of stroke.

Keywords: Hypothermia; Ischaemic Stroke; Oxidative Stress; Blood-Brain Barrier; Apoptosis, Neuroinflammation; Neuroexcitotoxicity

Introduction

Stroke continues to be one of the leading causes of mortality in the United Kingdom where each year approximately 150,000 people suffer a first stroke and around 60,000 people die from it. Although most sufferers are over the age of 65 years, all ages including children are at risk of having a stroke. Stroke also continues to be one of the leading causes of morbidity in the United Kingdom where over 250,000 people live with disabilities caused by stroke. Considering all direct (diagnosis, inpatient and outpatient care), indirect (income loss and social security benefit payments) and informal costs (home nursing of disabled survivors), stroke also bears significant financial implications. Indeed, the National Health System spends approximately £9 billion per annum to cover all these costs [1].

There are two main types of stroke; ischaemic and haemorrhagic. The former constitutes about 85% of all strokes in Caucasian populations and can be further divided into two main subtypes; thrombotic strokes and embolic strokes. Thrombotic stroke represents the most common subtype and occurs when a thrombus formed in an atherosclerotic artery occludes blood flow to a part of the brain. This is usually preceded by a transient ischaemic attack or mini-stroke [2]. Embolic stroke, on the other hand, usually arises when a blood clot breaks loose [embolus] and travels through the bloodstream to a part of the brain that is too small to let it pass thereby blocking the blood supply to the distal end of the artery. In most cases the root cause is atrial fibrillation which causes blood to pool in the atria, resulting in blood clots [3]. Haemorrhagic strokes involve leakage or rupture of a cerebral artery within or on the surface of the brain and therefore depending on the location of the haemorrhage are stratified into two; intracerebral haemorrhage

and subarachnoid haemorrhage. Naturally, the extent of cerebral damage and the associated mortality rates stemming from haemorrhagic strokes are often significantly greater than those with ischaemic strokes [4,5].

Despite being the main cause of human cerebral damage, thrombolysis with the recombinant tissue-plasminogen activator (r-tPA) remains the only available medical therapy for ischaemic stroke. However, due to short therapeutic window, i.e. the first 4.5 h of stroke onset, only 5% of patients receive this therapy. Besides, it is contraindicated in the treatment of haemorrhagic stroke because of an elevated risk of further bleeding. Hence, the initial treatment options for the haemorrhagic stroke focus largely on gradual lowering of the raised blood and intracranial pressures. However, the size, location and intraventricular expansion of the haematoma may necessitate surgical interventions [5].

Although, over the years many therapeutic agents have demonstrated efficacy under in vitro conditions or in animal models of stroke, the subsequent clinical trials conducted with the same agents failed to replicate such favourable results. In case of ischaemic stroke, one of the main drawbacks regarding translational stroke research is that the vast majority of the abovementioned agents used in clinical trials have focused on a single pathway pertaining to either recanalisation of vessels or excitotoxicity to reduce neuronal death, while many other processes are involved spatially and temporally in the pathophysiology of stroke. Given the short duration of excitotoxicity after ischemia and the short time of therapeutic window for recanalisation with r-tPA, it is crucial to discover new mediators or employ applications that can target several mechanisms simultaneously to exert long-term therapeutic effect on neurovascular integrity and function. In this context, hypothermia has long been regarded as an effective therapeutic regimen. It is defined as a core body temperature below 35ºC and is routinely used to minimise the deleterious effects of hypoxia-mediated brain injury in patients after cardiac arrest and in children with hypoxic-ischaemic encephalopathy. It has also been shown to improve neurological integrity after traumatic brain injury and spinal cord injury [6,7]. Although large-scale studies examining the effects of hypothermia on stroke are currently underway, the available studies are presently confined to a few mainly pre-clinical studies [8,9]. As various pathologies such as apoptosis, excitotoxicity, neuroinflammation and oxidative stress are implicated in the pathogenesis of ischaemic stroke and lowering body temperature impairs many physiological processes at molecular, cellular and system level, this paper reviews the effects of therapeutic hypothermia on these pathways in experimental and clinical settings of stroke.

Cerebral Metabolism and Neuroexcitotoxicity

A sudden decline in oxygen and glucose delivery and consequent decreases in ATP and phosphocreatine production during the acute phase of an ischaemic stroke along with a concomitant increase in cellular acidification, characterised by the excessive availability of lactate and H^+, impair mitochondrial function, promote glial cell death and thus cause an extensive damage to the brain tissue [10,11]. Hence, it is assumed that post-ischaemic approaches that can attenuate glucose and oxygen consumption and maintain physiological levels of ATP and pH would effectively prevent cerebral lactate overload and acidosis. In support of this, a recent study, using nuclear magnetic resonance spectroscopy to observe carbon-13 labelled glucose metabolism, has shown that hypothermia (31^{o}C) successfully preserves neuronal integrity, membrane potential and oxidative balance in rodents by shunting an increased proportion of glucose through the pentose phosphate pathway while concomitantly depressing its metabolism via tricarboxylic acid cycle flux [12]. As each degree centigrade drop in body temperature reduces metabolic rate by 6%-10%, the demand for oxygen and thus susceptibility to cellular damage is further diminished by hypothermia [13,14]. A significant relationship between body temperature and glucose utilisation has been reported in 34 out of 38 regions of the central nervous system examined in hypothermic and hyperthermic rats. Despite heterogeneity in results across different brain regions, some regions revealed 35%-50% reduced glucose levels in hypothermic rats [15]. In contrast to the successful maintenance of cerebral ATP levels in animals subjected to focal cerebral ischaemia, hypothermia fails to preserve ATP levels in case of global ischaemia where the blood flow to the brain is completely halted [16-19]. Even so, an accelerated rate of ATP recovery is consistently noted in translational studies where hypothermia could not prevent the initial ATP depletion [20].

Depletion of high energy metabolites i.e. ATP and phosphocreatine in ischaemic settings adversely affects ATP-dependant ion transporters and causes a loss of Na^+ gradient across the cell membrane which in turn stimulates Ca^{2+} influx into the cell through voltage-mediated Ca2+ channels, causes further depolarisation and triggers the release of excitatory glutamate into the synaptic cleft [21,22]. Impairments in active transport of glutamate back into presynaptic terminals and glial cells further increase the extracellular levels of glutamate whereby prolongs postsynaptic N-methyl-D-aspartate (NMDA) glutamate receptor activation, and further stimulates Ca^{2+} influx into neurones. These then promote cellular damage through activation of calpains, endonucleases, phospholipases and excessive release of reactive oxygen species (ROS) [5]. Increased glutamate levels may also damage neurovascular integrity through acidosis and excessive synthesis and release of nitric oxide (NO) by endothelial and neuronal nitric oxide synthases [23,24].

Hypothermia suppresses neuroexcitotoxicity through synchronous regulation of various elements, notably that of glutamate.

Comparative analyses of glutamate release in the cores of permanent focal cerebral infarcts caused by occlusion of the middle cerebral artery (MCAO) has revealed a substantial decrease in animals subjected to intra-ischaemic hypothermia (33°C). Indeed, while the hypothermic group had a significantly lower glutamate efflux throughout the entire 120 min period studied, rising from an initial value of 5.22±1.3 µmol/ml to a peak of 10.69±3.3 µmol/ml at 50 min after MCAO, the initial glutamate concentration (9.23±2.5 µmol/ml) had peaked 30 min after MCAO (33.95±6.3 µmol/ml) and decreased considerably by 120 min (21.35±6.8 µmol/ml) in the normothermic group [25]. Therapeutic hypothermia also downregulate the astrocytic glutamate transporter, GLT-1 which mediates the reverse transport of glutamate. This is particularly important, as under severe ischaemic conditions, glutamate receptors can functionally reverse to release glutamate and induce further neuronal injury [26]. Similar to glutamate, hypothermia also decreases the concentration of glycine, a glutamate co-agonist (2095±281 vs 3521±631 in normothermic animals) as well as the infarct volume (85±52 vs 226±42 mm3) in rats subjected to MCAO [27]. Furthermore, hypothermia (31°C) is also implicated in diminished entry of Ca^{2+} into neurons in that NMDA and ryanodine receptors appeared to play a role in in vitro settings [28].

Suppression of a glutamate-induced increase in NO synthesis by mild hypothermia after focal cerebral ischaemia may exert some neuroprotection considering its adverse effects on neuronal viability [29,30]. However, increases in NO levels may also be neuroprotective due to its vasodilatory effects which increases cerebral blood flow and decreases infarct size as a consequence [31]. The difference in outcome may be attributed to time in which NO is released. Indeed, the beneficial effects of NO are thought to be limited to the first 30 min after ischaemia onset while NO generated after this point tends to be neurotoxic. Moreover, NO produced by neuronal NOS is largely associated with neurotoxicity whilst that generated by endothelial NOS is largely implicated in neuroprotection [24,32].

Apoptosis

Apoptosis or programmed cell death is an active process that requires intact energy metabolism and protein synthesis to balance the rate of new cell formation in physiological settings [33]. However, apoptosis is also induced in many pathological conditions including cerebral ischaemia. Although the absence of oxygen and high energy metabolites may be the apparent cause of apoptosis after an acute ischaemic attack, other mechanisms like excitotoxicity-evoked neuronal death and necrosis, characterised by disruption of cell membrane, are likely to contribute to this phenomenon [34,35]. Application of therapeutic hypothermia following an ischaemic injury has been shown to diminish the rate of apoptosis in a time-dependent manner in that exposure of MCAO rats to increasing periods of hypothermia (33°C) from the onset of ischaemia appeared to decrease terminal deoxynucleotidyltransferased UTP nick end labelling (TUNEL) staining at 72 h post-ischaemia selectively in groups subjected to hypothermia for 60 or 120 min but not 30 min compared to the respective control groups. Albeit invaluable, this study did not address the question whether hypothermia merely delayed or prevented the onset of apoptosis at 72 h post-ischaemia [36]. Modulation of cytochrome C release, an intermediate in apoptosis, along with the expressions of anti-apoptotic (e.g. Bcl-2) and pro-apoptotic (e.g. Bax) factors may account for the anti-apoptotic effects of hypothermia. In support of this notion, immunohistochemical analysis of the brains obtained from MCAO rats exposed to therapeutic hypothermia has revealed selective decreases in Bax or cytochrome C but not Bcl-2 staining in neurones [37,38]. Post-ischaemic hypothermia also inhibits the activities of cerebral caspase-3 and caspase-7 which are normally induced by intrinsic pathways leading to chromatin condensation and DNA fragmentation [39]. Intriguingly, another study investigating the impact of hypothermia on Bax and Bcl-2 expression and caspase activity in MCAO animals has failed to detect any change in these parameters while reporting a significant decrease in mitochondrial cytochrome C release 5 h after ischaemia onset, implying that the maintenance of mitochondrial integrity, rather than regulation of the intrinsic apoptotic pathway, may be responsible for the ameliorative effects of mild hypothermia [40]. Since regulation of Fas-ligand pathway along with the activities of caspase-8, protein kinase C and c-Jun NH_2- terminal kinase may also explain the beneficial effects of hypothermia, a better scrutiny of their specific involvement is warranted in future studies [41-43].

Neuroinflammation

Despite being a physiological response to acute ischaemic injury and a prerequisite for the repair and recovery of the damaged tissue, inflammation during the subacute and chronic stages of ischaemic stroke are likely to be deleterious [44]. Cerebral ischaemia triggers an innate immune response which augments production of many pro- and anti-inflammatory mediators including cytokines, chemokines and matrix metalloproteinases (MMPs) and activates microglia and astrocytes. These collectively elevate neuronal vulnerability to apoptosis, disrupt blood-brain barrier (BBB) and stimulate gliosis [45,46]. The enhanced availability of pro-inflammatory cytokines, namely interleukin-1 (IL-1), IL-6, IL-18 and TNF-α in turn induce the production of adhesion molecules and thus mediate the adherence and extravasation of leukocytes to the ischaemic tissue in association with chemokines [47,48]. This along with excessive release of ROS in reperfused and chronically hypoperfused neural tissue further exacerbate the extent of ischaemic damage [5,49].

In animal models of ischaemic stroke, treatments with ther-

apeutic hypothermia has been found to negate the impact of inflammation on neural tissue by decreasing the level of leukocyte infiltration into the ischaemic brain and concurrently suppressing the expression of pro-inflammatory cytokine TNF-α its receptor (TNF receptor 1) and downstream effector, NF-κB [50-53]. Regulation of cytokines (IL-10 and TGF-β), monocyte chemoattractant protein-1, basement membrane-degrading enzymes (MMP-9) and glial cells are also involved in the anti-inflammatory effects of hypothermia [50,54-56]. However, given the immunosuppressive role of hypothermia, the duration and depth of this therapeutic approach should be well-adjusted to minimise the risk of infection.

Activation of high-mobility group box (HMGB) family of proteins, especially that of HMGB1 in neurons and immune cells, during ischaemic injury can also stimulate neuroinflammation [57]. By acting as a pro-inflammatory cytokine HMGB1 activates transcription factor, NF-κB to induce expression of many pro-inflammatory mediators involved in stroke pathogenesis e.g. inducible NOS (iNOS), IL-1β, ICAM-1, COX-2, IL-6 and TNF-α, triggers microglial activation and evokes excitotoxicity-mediated apoptosis [52,58,59]. Pre- or post-ischaemic treatments with moderate hypothermia has been shown to reduce cerebral and plasma levels of HMGB1 in animal models of transient MCAO following 120 min of reperfusion compared to the respective control groups [60]. In accordance with these findings, HMGB1 blood levels in neonates with ischaemic encephalopathy who received brain hypothermic therapy were found to be markedly lower than those who had not (7.9±5.1 vs 61.6±32.4 ng/ml) 1 day after birth [61].

NF-κB is normally sequestered outside the nucleus by a group of inhibitory proteins known as IκB. Once freed from NF-κB-IκB complex after activation by signals usually originating from outside of the cell, proceeded by phosphorylation of IκB and ubiquitination of IκB inhibitor molecules, NF-κB enters the nucleus to induce specific gene expression [52]. Mild hypothermia (33^{o}C) has been shown to inhibit NF-κB translocation in experimental stroke by diminishing the levels IκB phosphorylation and activity compared to animals kept under normothermic conditions [62]. Despite exerting detrimental effects on neuronal survival through upregulation of various pro-inflammatory protein expressions, NF-κB is also associated with cell survival and growth. Given the varied degrees of NF-κB activation during different phases of ischaemic injury and the ability of hypothermia to influence cell genesis and survival in the rat brain following global ischaemia, these findings suggest a necessity for exploring the effect of hypothermia on NF-κB activity in translational and clinical stroke in a comprehensive manner [8,63].

Oxidative Stress

Oxidative stress refers to a condition in which the cells are subjected to excessive levels of molecular oxygen or its chemical derivatives i.e. ROS. It is commonly observed in many physiopathological conditions such as ageing, cancer and ischaemic stroke [64,65]. The brain is particularly vulnerable to oxidative stress due to its low level antioxidant capacity, high rate of oxygen consumption and possession of easily peroxidisable polyunsaturated fatty acids as well as iron and ascorbate which may act as pro-oxidants in pathological states [66]. Superoxide anion (O_2^-) constitutes the foundation molecule of all ROS and is largely generated by a variety of pro-oxidant enzymes such as xanthine oxidases, cyclooxygenases and more importantly NADPH oxidase or Nox [64]. Once generated O_2^- is readily converted to other ROS by enzymatic or non-enzymatic means. Indeed, due to the enhanced availability of H+ atoms after an ischaemic episode through anaerobic generation of lactate or reperfusion of penumbra and other areas with low and intermittent blood supply, O_2^- is either dismutated to H_2O_2 by superoxide dismutases or is simply protonated to form reactive hydroperoxyl radical [66]. In this context, activation of neuronal NOS, stimulation of N-methyl-D-aspartate receptors by glutamate and dysfunction of mitochondria cannot be ruled out [64,67].

Intriguingly, ROS conduct multiple beneficial roles in physiological settings where they may act as secondary messengers and be involved in mitogenesis and cell adhesion [68]. They can activate T lymphocytes and play an important role in neutralising the invading pathogens via respiratory burst [69]. Moreover, mitochondrial ROS may also be used as a marker for sensing oxygen tension by carotid bodies [70]. In contrast, specifically after an ischaemic stroke, ROS induce lipid peroxidation, protein denaturation, DNA modification and cell signalling which collectively promote tissue damage and cell death. They also affect vascular contractility, endothelial barrier permeability, platelet aggregability and the formation of focal lesions [64,71]. Interestingly, as the molecular targets of oxidative stress in vascular cells are similar to that of intracellular Ca^{2+}, the actions of neuroexcitotoxicity and oxidative stress appear to be synergistic [72].

Regulation of oxidative stress plays a critical role in the beneficial effects of hypothermia in that therapeutic hypothermia substantially reduced H_2O_2 levels in fibrosarcoma cells and retained their cellular morphology [73] while exposure of rats, subjected to hypoxic insult through breathing 10% oxygen, to deep hypothermia (22^{o}C) markedly suppressed the generation of NO metabolites (23±2.7 vs 17.8±1.9 μmol/L) which increased from 10.8±0.4 to 23±2.7 μmol/l in a control group [74]. Despite limitations pertaining to transferability of the latter study to clinical scenarios due to depth of hypothermia and exclusive assessment of acute hypoxia, a key but not the sole pathology in ischaemic stroke, on NO metabolites or oxidative stress, this is an important study to exhibit the restraining effect of hypothermia on oxidative stress. A notion corroborated by a study demonstrating that hypothermia (33^{o}C)

effectively controls the levels of a biomarker for oxidative stress, total hydroperoxide, in infants with ischaemic encephalopathy in the first few days after birth compared to their counterparts maintained in normothermic conditions [75].

Vascular Permeability and the Blood-Brain Barrier

The BBB separates the cerebral vasculature from the surrounding tissue through physical and metabolic modalities and is tasked with the maintenance of cerebral homeostasis [76]. Ischaemic stroke and ensuing release of pro-inflammatory cytokines, proteinases and ROS adversely affect the integrity and function of the BBB [77]. Recent findings indicate that NADPH oxidase, the main pro-oxidant enzyme, acts upstream to TNF-α and mediates its barrier-disruptive effects and that silencing of TNF-α via its specific antibody effectively preserves cerebral integrity and function and may therefore be an important therapeutic target after an ischaemic stroke [78,79]. Similarly, NADPH oxidase also mediate the actions of MMPs which degrade many structural components of the BBB including fibronectin, laminin, collagen type IV and tight junction proteins and thus compromise the barrier integrity [80]. Since cerebral oedema is more prevalent in stroke patients with diabetes mellitus, hyperglycaemia is likely to play a crucial role in the initiation and/or exacerbation of the BBB damage [81]. Activations of protein kinase C-β/βI subunits, NADPH oxidase and MMP-2 are also reported to be involved in this phenomenon under in vitro settings [82-84]. A recent study looking at the comparative effects of hyperthermia and normothermia on non-ischaemic and ischaemic hemispheres of MCAO rat brains has also shown significant increases in MMP-2 and MMP-9 expressions as well as laminin and collagen type IV degradations in vivo in ischaemic hemispheres and particularly in those subjected to 4 hours of hyperthermia, indicating the importance of core body temperature in the regulation of MMP expression [85]. Indeed, mild hypothermia (33^{o}C) administered for 6 hours has been shown to reduce the expression of MMP-9 alongside the markers of cellular and extracellular damage such as Tau-1, B-amyloid precursor protein and TIMP-1 and consequently improve neurological function 2 weeks post-stroke [86]. Moreover, humans treated with core body cooling to 33^{o}C for 48-72 h within 12 h of stroke onset had significantly lower MMP-9 levels than in patients treated with r-tPA. Conversely, intact laminin levels were lower in patients treated with r-tPA than in patients treated with hypothermia, which strengthens the theory of less MMP activity at lower body temperatures [87].

Inhibition of pericyte migration from the vascular wall, an important factor in the maintenance of BBB integrity, may be one of the reasons behind the barrier-protective effect of moderate hypothermia which appeared to preserve the BBB integrity for up to 5 days after treatment. This study implies that the beneficial effects of hypothermia on the BBB extends well beyond the duration of hypothermic treatment [88]. In addition to regulating MMP and laminin levels, delayed local hypothermia (31°C), administered for 44 h following 4 h of reperfusion in a rodent model of transient MCAO, has significantly reduced infarct and brain oedema volumes and exerted vasculoprotective effects as evidenced by inhibition of inflammatory glial activation and conservation of neurovascular unit via preservation of tight junction proteins and aquaporin 4, a water channel found on astrocytes [89].

Conclusion

Therapeutic hypothermia is a promising treatment regime in the field of ischaemic stroke. There is emerging evidence for what may be the optimal conditions in administrating this treatment regarding the time of onset, the duration of the cooling period, the rewarming rate and the cooling depth. Along with this, combination therapy with pharmacological or mechanical thrombolysis has also shown considerable promise. Nonetheless, further evidence is still needed in order to come up with a standard protocol that maintains the balance between maximal neuroprotection and minimal complications.

Understanding the molecular mechanisms after ischaemic stroke and how therapeutic hypothermia is able to provide neuroprotection against this is crucial in order to ascertain the full potential of this treatment and how it can be combined with other therapies. Consequently a large proportion of this paper has discussed this in detail. Laboratory evidence of hypothermia mitigating the effects of oxidative stress, excitotoxicity, apoptosis, inflammation and on the BBB is fairly extensive. This is exciting as hypothermic therapy differs from other more targeted neuroprotective therapies in that its beneficial actions extend across multiple areas and phases of the ischaemic cascade. Further pre-clinical and clinical trials are needed to reveal more of the molecular basis for mitigation, as well as find out the true value of this treatment in the ischaemic stroke setting.

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