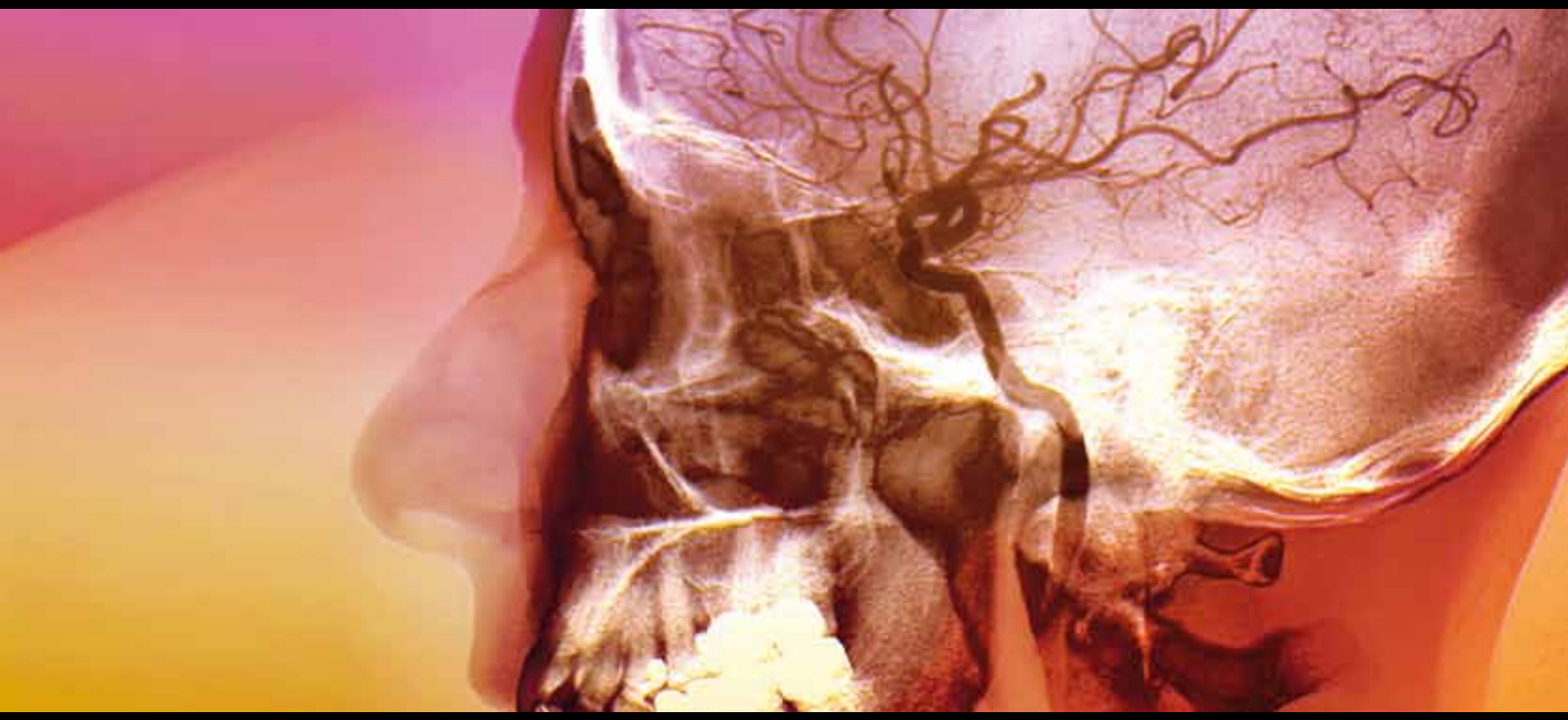
Stroke Research and Treatment

Therapeutic Hypothermia in Stroke

Guest Editors: Midori A. Yenari, Fred Colbourne, Thomas M. Hemmen, Hyung Soo Han, and Derk Krieger



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Editorial

Therapeutic Hypothermia in Stroke

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For nearly 10 years, therapeutic mild hypothermia has become increasingly recognized to positively influence neurological outcome in humans following acute brain injuries, namely, ischemic brain injury due to cardiac arrest and hypoxic-ischemic encephalopathy in neonates. In the laboratory, hypothermia is perhaps the most robust and consistent neuroprotectant studied to date. It has been shown to both suppress and enhance many factors leading to ultimate tissue preservation and better outcome. Now that it has been shown to be effective in humans, this special issue surveys the various directions this area of research is taking, from using it as a model of neuroprotection in the laboratory to refining the technique and exploring ways of applying hypothermia to other neurological conditions, especially stroke.

Y. Shintani et al. provide an overview of how state-of-the-art molecular gene and protein profiling technologies can be applied to models of hypothermic neuroprotection to reveal potentially new signaling pathways and to identify potential therapeutic targets. This strategy was then demonstrated by H. S. Han et al. who discovered that hypothermia upregulates extracellular signal regulated kinase (ERK-1/2) and that ERK-1/2 upregulation might be responsible for the anti-inflammatory properties of cooling.

Therapeutic hypothermia has become increasingly embraced by the medical community as a means of improving neurological outcome in certain conditions. However, this intervention still has its limitations. Since these trials were published, centers have struggled to determine optimal cooling techniques, such as surface cooling versus endovascular methods. A. F. Caulfield et al. report their experience with both approaches at an academic stroke center. Another critical question is whether therapeutic cooling can be offered in community settings or whether patients need to be referred to tertiary centers. The study by M. P. Shah and colleagues show that it is possible to implement a cooling protocol in a community hospital, provided that there is a team approach in place, involving relevant medical services.

Encouraging clinical studies in cardiac arrest and hypoxic-ischemic encephalopathy patients has fueled further interest in pursuing therapeutic cooling in stroke victims. However, stroke poses additional challenges since most stroke victims are elderly with many comorbidities. Stroke patients are also generally awake, making cooling more difficult due to the problem of shivering. Nevertheless, preclinical studies continue to refine optimal parameters for such an intervention in stroke models, including defining the limits of hypothermic protection, whether cooling might be combined with other therapies and understanding where preclinical models might fall short of the clinical condition. These issues are explored in reviews by H. G. Zhao and Steinberg and Zgavc et al. Still, there is an urgent need for larger, prospective studies to determine if hypothermia could be used in stroke patients. Bench scientists, translational researchers, and clinicians alike must work together towards this goal.

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Review Article

Molecular Mechanisms Underlying Hypothermia-Induced Neuroprotection

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Stroke is a dynamic event in the brain involving heterogeneous cells. There is now compelling clinical evidence that prolonged, moderate cerebral hypothermia initiated within a few hours after severe ischemia can reduce subsequent neuronal death and improve behavioral recovery. The neuroprotective role of hypothermia is also well established in experimental animals. However, the mechanism of hypothermic neuroprotection remains unclear, although, presumably involves the ability of hypothermia to suppress a broad range of injurious factors. In this paper, we addressed this issue by utilizing comprehensive gene and protein expression analyses of ischemic rat brains. To predict precise target molecules, we took advantage of the therapeutic time window and duration of hypothermia necessary to exert neuroprotective effects. We proposed that hypothermia contributes to protect neuroinflammation, and identified candidate molecules such as MIP-3α and Hsp70 that warrant further investigation as targets for therapeutic drugs acting as "hypothermia-like neuroprotectants."

1. Introduction

Stroke is the second leading cause of death in the world [1], and is a primary cause of long-term disability in adults [2, 3]. One potential approach for treating acute stroke involves neuroprotective agents. Although more than 100 clinical trials of potential agents have been conducted, none of these agents proved to be clinically efficacious except for a free radical scavenger edaravone [4–6]. However, recent progress in understanding the disease have unveiled the cellular and molecular events underlying ischemic cell death, such as loss of metabolic stores, excessive intracellular calcium accumulation, oxidative stress, and neuroinflammatory response. For instance, expression of stress, apoptosis, and inflammation-related genes is known to be upregulated upon reperfusion and reoxygenation [7, 8].

Therapeutic hypothermia is a promising neuroprotective intervention shown to improve outcome from brain ischemia in humans. The neuroprotective role of hypothermia has been well established in experimental animals [9–11] and in patients with cardiac arrest [12, 13]. Although the key mechanism has not been clarified, hypothermic neuroprotection may provide insight into stroke pathology and suggest novel therapeutic drug targets. To date, reactive oxygen species production [14], NF-κB activation [15], neutrophil infiltration [16] and cytochrome c release [17] have been observed to be inhibited in hypothermia-treated ischemic brains. However, it is difficult to discern an intrinsic hallmark of hypothermia from sequential outcomes by retrospective approaches. To overcome this drawback, we performed comprehensive gene and protein expression analyses using ischemic brains with or without hypothermia collected at appropriate time points. DNA microarray and proteomic approaches are revolutionary technologies for performance of high-throughput analyses of global gene and protein expression. However, additional devises are needed to elucidate the essential molecules and to identify appropriate targets for therapeutic intervention from massive amount of expression profiles. In this series of study, we focused on the therapeutic time window and duration

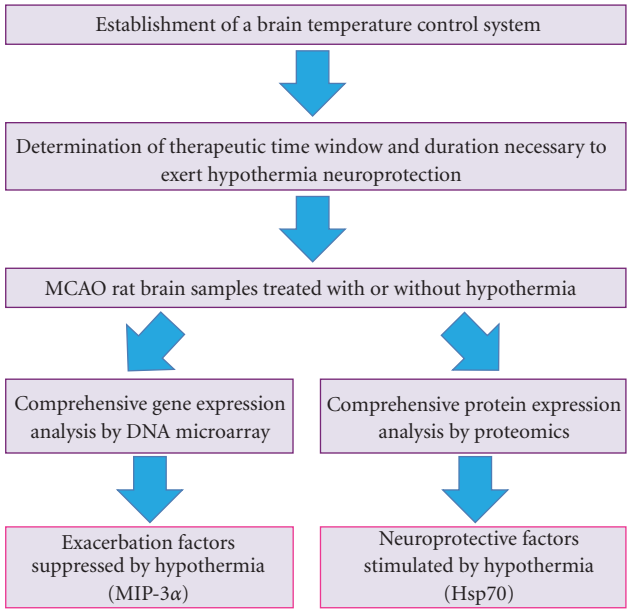

Figure 1: An experimental strategy to identify potential drug targets associated with hypothermia.

of hypothermia required for exerting neuroprotective effects (Figure 1).

2. Therapeutic Time Window and Duration of Hypothermia

It is not known precisely when the series of events leading to final cell death and dissolution becomes irreversible. However, there is overwhelming evidence that the early recovery phase, before the start of secondary deterioration, represents the effective window of opportunity for initiation of postinsult cooling [18]. A continuous comparison of stroke damage with or without hypothermia would provide massive amount of information that would be difficult to interpret precisely; therefore, it is necessary to compare appropriate samples according to the therapeutic time window and duration of hypothermia required to exert a neuroprotective effect. Precise control of brain temperature is critical for the evaluation of the efficacy of mild postischemic hypothermia and for the acquisition of reproducible gene and protein expression patterns. To predict the effective phase of hypothermia, we established a rat experimental stroke model: a 2-hr transient intraluminal occlusion of the middle cerebral artery (MCAO), which resulted in severe brain infarct with almost complete striatal and extensive cortical damages, was combined with a multichannel computer-controlled brain temperature control system that enabled us to maintain a constant brain temperature for more than 2 days [19]. Rats were implanted with a telemetry brain temperature probe with its temperature sensor inserted approximately 4 mm from the skull surface (lower layer of the cerebral cortex). The rats were individually housed in acrylic cages with wooden chips. To induce hypothermia, each cage was placed in a room maintained at 4°C, and the cortical temperature was continuously and individually monitored from the freely moving rats. Cooling fans attached to the cage lid were turned on when the brain temperature was higher than 35°C and were stopped when the brain temperature was below 34°C. To maintain normothermia, rats were kept under room temperature. Because the brain temperature increased during MCAO, the term "normothermia" does not refer to a certain controlled temperature. For instance, in our rat model, normothemic rat brain temperature increased over 38°C for more than 18 hr [19]. Similar brain temperature control systems that used water sprays for cooling instead of cold rooms have been reported [20, 21]. The surgical placement of the temperature probes into the brain on the day prior to MCAO should be taken into consideration during further experimentation because the stab wounds might have had a preconditioning effect. The therapeutic time window is considered to depend upon the severity of the insult, depth of damage, and duration of cooling [22–24].

We identified the therapeutic time window of mild hypothermia at 35°C in rats subjected to hypothermia after a 2-hr MCAO [19]. The brain infarct volumes measured 2 days after MCAO were 288.6 ± 21.5 mm^3 in normothermic rats. In contrast, postischemic hypothermia significantly reduced the volume when introduced at 0 hr (120.0 ± 21.7 mm^3, $P < .005$), 2 hr (147.0 ± 26.3 mm^3, $P < .005$), and 4 hr (209.9 ± 33.5 mm^3, $P < .025$) after normothermic reperfusion. No significant reduction was observed when postischemic hypothermia was introduced 6 hr after reperfusion (244.3 ± 35.5 mm^3). The therapeutic time window of hypothermia was therefore concluded as 4 hr after reperfusion in our model. However, even 4 hr of hypothermia when introduced immediately after reperfusion did not significantly reduce brain infarct volumes (182.3 ± 29.4 mm^3) as compared to the volumes in normothermic rats (219.3 ± 29.9 mm^3). The infarct volume after 24 hr of hypothermia (63.1 ± 28.2 mm^3) was significantly smaller than that after normothermia (219.2 ± 39.7 mm^3, $P < .01$), suggesting that hypothermia should be maintained for longer than 4 hr in our model to obtain neuroprotective effects. This finding is consistent with reports that mention the importance of prolonged hypothermia for persistent neuroprotection [25, 26]. Hypothermia may exert neuroprotective effects in ischemic brains by regulating particular injurious factors within specific time intervals.

3. DNA Microarray Analysis

DNA microarray analysis is a useful tool for determining gene expression [27–29]. A number of endogenous proteins have been reported to be induced during ischemia, including several involved in inflammation and neuronal death such as the MAP kinase protein and heat shock proteins (HSP) that play roles in both tissue damage and reorganization [30–35]. Lu et al. [36] reported upregulation of immediate early genes, transcription factors, and HSPs as early as 30 min after MCAO in 30 min or 2 hr transient rat MCAO models, and upregulation of genes related to inflammation, apoptosis, the cytoskeleton, and metabolism that peaked within 4–24 hr after MCAO. Figure 2 shows expression patterns of several

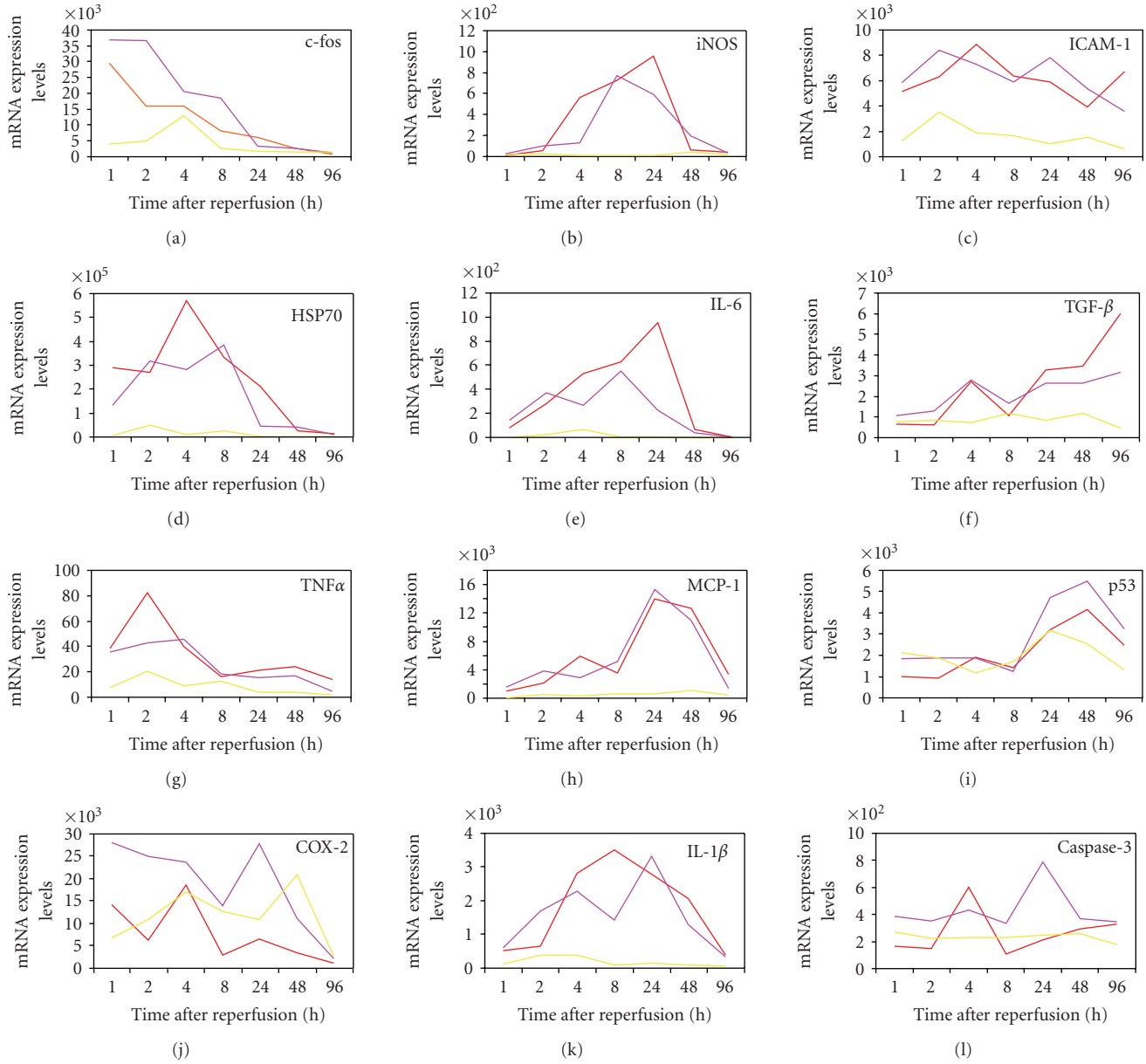

Figure 2: Stress-related genes expression in rat brains after reperfusion under normothermia. The expression levels of each mRNA in ischemic core (red), penumbra (purple), and normal (yellow) regions were quantified by real-time PCR, and their mRNA expression levels were shown as a gene copy number per 1 ng of polyA$^+$ RNA. The procedure used has been previously described [17].

stress-related genes after reperfusion in ischemic brains of our rat model under normothermia. The expression level of *c-Fos* mRNA reached the peak during MCAO and sustained at least for 1 h after reperfusion. *Hsp70* mRNA expression was also high in the beginning of reperfusion. Interestingly, tumor necrosis factor-α (*TNF-*α), interleukine-1β (*IL-1*β), inducible nitric oxide synthase (*iNOS*), and *IL-6* mRNAs were transiently and sequentially induced after reperfusion. In contrast, monocyte chemotactic protein-1 (*MCP-1*), transforming growth factor-β (*TGF-*β), and *p53* mRNAs gradually increased along with progression of the disease. Gene expression analysis provides a large amount of information regarding expression changes of thousands of genes, and time-dependent changes should therefore be analyzed to grasp the entire picture. Hence, the information of therapeutic time window and time duration of hypothermia was exploited. The therapeutic time window of hypothermia in our model was 4 hr, so we hypothesized that gene expression changes occurring prior to this time point are important for hypothermic neuroprotection. Of the 8000 transcripts represented on the Affymetrix Rat U34A GeneChip, 45 ± 8% showed a present call (a specific signal with statistical significance) in both hypothermia and normothermia brains during the 4 hr reperfusion [19]. Approximately 400 of the genes were upregulated more than 2-fold, but no genes were reproducibly suppressed.

Of the upregulated genes, 22 were upregulated more than 20-fold, including *Hsp70* mRNA. Interestingly, most of the genes whose expression was significantly suppressed by hypothermia were inflammatory-related molecules including IL-1β and osteopontin (OPN), suggesting that the suppression of neuroinflammation primarily contributes to neuroprotection. OPN has a proinflammatory role in some diseases [37–40], although the neuroprotective role of OPN is also reported [41]. Further studies are needed to elucidate the roles of OPN in ischemic pathophysiology.

Meanwhile, we also speculated that the interruption of hypothermia is implicated in ischemic damage because the duration necessary to exert hypothermic neuroprotection was longer than 4 hr. To identify genes involved in the recovery phase before the secondary deterioration, we compared gene expression in ischemic brains kept in hypothermia for 8 hr during reperfusion with those kept in hypothermia for the first 4 hr followed by 4 hr normothermia. We identified 12 genes functionally related to neuroinflammation, including *c-Fos*, early growth response 1 and 4 (*Egr-1*, *and Egr-4*), MAPK phosphatase-1 (*MKP-1*), macrophage inflammatory protein-3α (*MIP-3*α), *MCP-1*, and *IL-1*β [19]. Egr-1 is a master switch activated by ischemia to trigger the expression of pivotal regulators of inflammation, such as IL-1β, MCP-1, and macrophage inflammatory protein-2 (MIP-2), in addition to coagulation and vascular hyperpermeability [43]. Deng et al. [44] demonstrated that postischemic mild hypothermia decreases inflammatory responses by suppressing intercellular adhesion molecule-1 (ICAM-1), an essential molecule for peripheral leucocyte recruitment into brain parenchyma. Stroke-mediated release of proinflammatory cytokines and adhesion molecules is a relevant component of the complex pathophysiological response [45]. Those inflammatory mediators enable leukocytes to adhere to the vascular endothelial cells and to infiltrate inflammatory cells from the circulation into the ischemic brain within hours after ischemic damage. Studies addressing efficacy of mild hypothermia also indicate the importance of microglia activation [16, 44], nuclear-factor kappaB (NF-κB) activation [46], or nitric oxide production [47].

4. Proteomic Analysis

Proteomic analysis is another powerful tool for examining the physiopathologies of diseases. Hence, proteomic studies using brain tissues, blood, or cerebrospinal fluids of stroke patients [48] or experimental models [42, 49] have been performed to elucidate the pathogenesis of stroke and to identify novel diagnostic biomarkers. Proteomic profiling generally consists of 2 technologies: two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for the comparison of protein expression and LCQ-advantage micro-LC/MS/MS system for protein identification. In our experiments, the criteria for altered protein expression between hypothermia and normothermia were set as a ratio of greater than 1.5-fold or less than 1/1.5-fold. Out of approximately 1200 spots on 2D-PAGE, 44 were identified with a statistically significant difference in mean values. Thirteen of the 44 spots were upregulated by hypothermia more than 1.5-fold (1.50–1.85) compared with normothermia. Among them, a spot identified as Hsp70 was studied further because it was upregulated by MCAO and was further induced by hypothermia, although hypothermia itself did not induce Hsp70 in nonischemic brains [50]. This observation was confirmed by Hsp70-specific enzyme-linked immunosorbent assay (ELISA), western blotting, and immunohistochemistry of ischemic brain tissues. Hsp70 is a chaperone protein that can fold or refold proteins, coordinate protein trafficking, inhibit protein aggregation or degradation, and exhibit antiapoptotic and anti-inflammatory activities under physiological conditions [51–53]. Several reports have suggested that Hsp70 inhibits NF-κB transcription by stabilizing NF-κB and preventing its phosphorylation [54]. Zheng et al. [55] recently demonstrated that the interaction between Hsp70 and the NF-κB:IκB complex significantly inhibits NF-κB activation and leads to a decrease in NF-κB-regulated genes in MCAO-treated Hsp70 transgenic mice. The expression of Hsp70 and Hsp27 can be induced in glial cells and neurons by a wide range of noxious stimuli including ischemia [56, 57], epileptic seizure [58], and hyperthermia [59]. Neuroprotective effects of Hsp70 against ischemic injury have been reported for drugs that induce Hsp70 expression [60] and in Hsp70 transgenic mice [61]. However, the induction of Hsp70 by hypothermia has not been reported to date. Hsp27 was also induced by ischemia, but was slightly suppressed by hypothermia (Figure 3). GAP43, a constitutive protein in the brain, and other Hsps, including Hsp40, Hsp90, Grp78, Grp94, PDI, and ORP150, were detectable under normal conditions and were not affected by either hypothermia or ischemia (Figure 3 and unpublished data). Hence, Hsp70 accumulation by hypothermia was unique. Recently Hagiwara et al. [62] reported that hypothermia at 34°C increased the Hsp70 expression level in lipopolysaccharide (LPS)-stimulated RAW264.7 cells, although IL-1β, IL-6 and TNF-α expression levels were reduced under the same conditions. In contrast to the protein level of Hsp70, *Hsp70* mRNA expression in our model was suppressed by hypothermia. Rubtsova et al. [63] reported that the 5′-untranslated region of human *Hsp70* mRNA represents an internal ribosome entry site (IRES) with relative activity similar to that of the classical picornaviral IRESs, implying posttranscriptional regulation of Hsp70. While it is unknown whether there is a similar IRES element in rat *Hsp70* mRNA and if the IRES is activated under hypothermia, this finding merits further investigation because it hints at the identity of neuroprotectants that increase Hsp70 by a novel mechanism. The neuroprotective roles of Hsp70 in hypothermia are also worthy of further investigation.

5. Novel Targets for Therapeutic Drugs Acting as "Hypothermia-Like Neuroprotectants"

Neuroinflammation is involved in the pathogenesis of many CNS diseases. In stroke, excess inflammatory activation results in brain injury and ultimately causes severe neuronal apoptosis [64]. Anti-inflammatory therapies using immunosuppressants [65] or biogenetics such as an anti-ICAM-1-neutralizing antibody [66] have been applied in

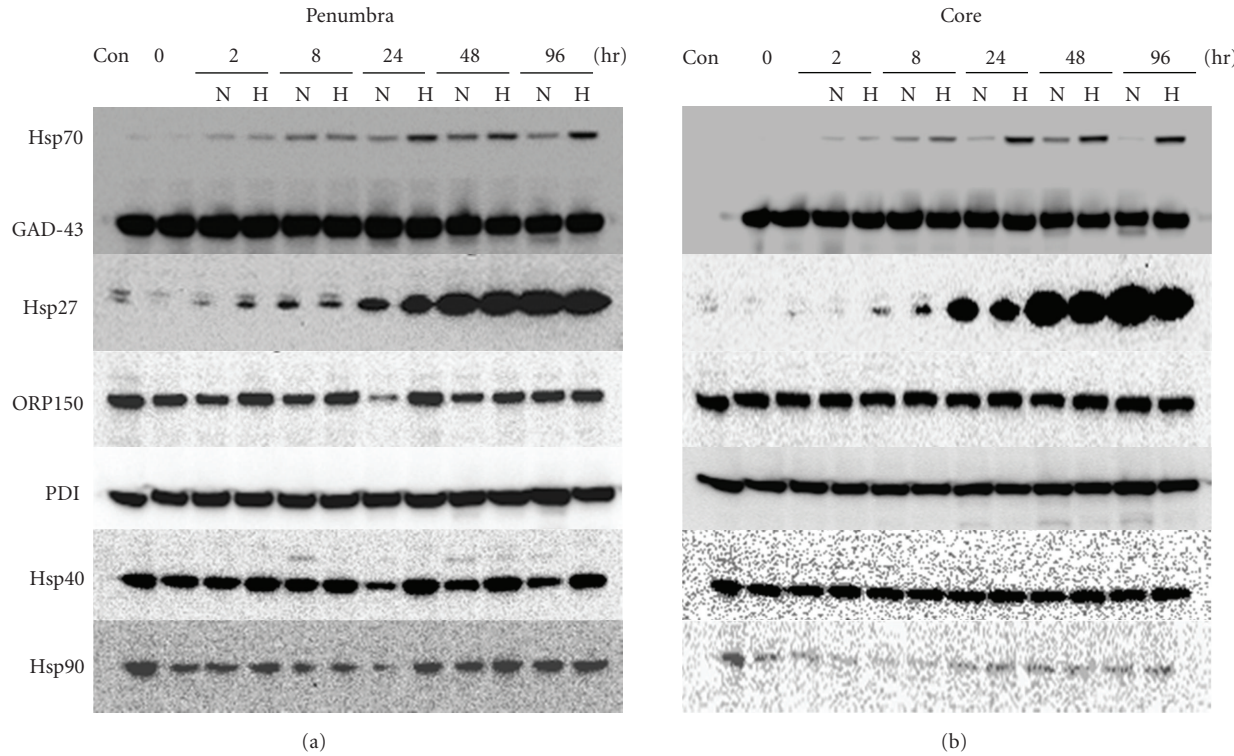

Figure 3: Effect of occlusion of the middle cerebral artery (MCAO) and hypothermia on chaperones expression in rat brains. Western blot analysis for Hsp70, Hsp27, Hsp40, Hsp90, ORP150, PDI, and GAD-43 was performed using protein samples from the penumbra and core regions of rat ischemic brains at the indicated time points after reperfusion. GAD43 protein served as an internal control. N: normothermia, H: hypothermia. "Con" denotes protein samples from normal rat brains. The procedure used has been previously described [42].

both preclinical and clinical contexts. MCP-1 is also considered a promising drug target due to its possible role in exacerbating ischemic injury, controlling blood-brain barrier permeability, and driving leukocyte infiltration into the brain parenchyma in stroke [67, 68]. Our DNA microarray analysis revealed that both cerebral *MIP-3α* and *CC-chemokine receptor 6 (CCR6)* genes were significantly induced in the core and penumbra regions of MCAO rat brains, and hypothermia suppressed expression of both genes [69]. The MIP-3α chemokine is expressed in macrophages, dendritic cells, and lymphocytes. Depending on the conditions, MIP-3α acts both constitutively and inducibly and serves as a chemoattractant, especially in epithelial immunological systems such as those of the skin and mucosa [70, 71]. In CNS, MIP-3α expression has been reported in autoimmune encephalomyelitis [72] and stroke [36, 73], but its full role has not yet been determined. CCR6, the only receptor for MIP-3α, is expressed in multiple leukocyte subsets, and is implicated in diverse inflammatory responses in animal models, such as allergic airway disorders, inflammatory bowel disease, and autoimmune encephalitis [74, 75]. Strikingly, intracerebral administration of an an anti-MIP-3α-neutralizing antibody significantly reduced infarct volumes in MCAO rats ($187.5 \pm 37.0\,mm^3$) compared with those of vehicle ($304.0 \pm 32.8\,mm^3$) and of control mouse IgG ($291.3 \pm 33.5\,mm^3$), suggesting that MIP-3α-CCR6 signaling is dominant in neuroinflammatory cascades involved in brain ischemia. Interestingly, MIP-3α administration into the striatum dose-dependently induced *CCR6*, but not *CCR1* or *CCR2*, gene expression. Intrastriatal injection of IL-1β and TNF-α upregulated *MIP-3α* and *CCR6* mRNA expression levels in a sequential fashion. Robust induction of IL-1β and TNF-α was observed at an acute phase of MCAO prior to MIP-3α expression, suggesting that these cytokines may be directly involved in MIP-3α production.

MIP-3α mRNA expression was markedly induced by IL-1β and TNF-α in rat astrocytes but not in microglia or neurons. Astrocytes are reported to emerge around the damaged area after ischemic injury [76] and to produce MIP-3α in a mouse experimental autoimmune encephalomyelitis model [77]. In contrast, rat primary microglia constitutively expresses the *CCR6* gene in normal culture conditions while astrocytes or neurons do not. Strikingly, the expression of both iNOS and IL-1β were induced in MIP-3α-treated microglia. Microglia are activated and accumulate around the injured area following ischemia [78]. Because hypothermia reportedly inhibits NF-κB directly by decreasing IκB kinase activity in MCAO rat brains [15], one of the suppressive effects of hypothermia on MIP-3α induction may be a direct inhibition of NF-κB activity in astrocytes. Another possibility is that the upregulation of Hsp70 expression by hypothermia inhibits MIP-3α expression by the interaction

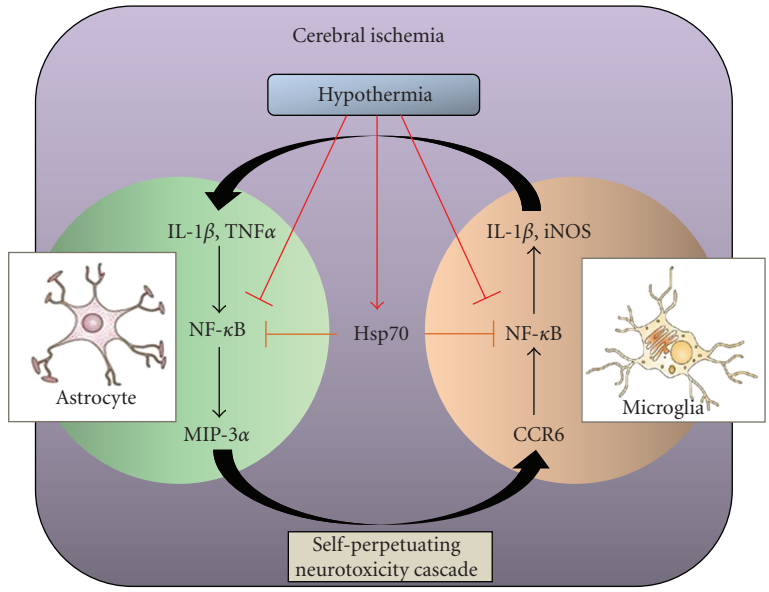

Figure 4: A putative neuroinflammatory cascade exacerbated by MIP-3α-CCR6 signaling and protected by Hsp70 in ischemic rat brains. In ischemic brains, the activation of neuroinflammatory cascades including MIP-3α-CCR6 signaling is speculated to progress as follows: astrocytes activated by proinflammatory cytokines like IL-1β and TNFα stimulate MIP-3α release. MIP-3α activates microglia through CCR6 and the cells produce other stress-related proteins like iNOS and IL-1β. Production of IL-1β further accelerates astrocyte activation. The self-perpetuating neurotoxicity cascade may damage surrounding neurons and brain tissues. On the other hand, hypothermia suppresses the cytokines and chemokines production in part by direct inhibition of NF-kB transcription via decreased IkB kinase activity. Our experiment has also elucidated the potential effect of hypothermia, which stimulated Hsp70 production in MCAO brains. Accumulated Hsp70 may inhibit NF-kB transcription by directly interacting with the NF-kB:IkB complex in astrocytes and microglia.

of Hsp70 with the 5′-untranslated regulatory region of MIP-3α mRNA that contains NF-κB binding sites, as described by Zheng et al. [55]. The activation of astrocytes and microglia may accelerate brain injury-induced neuroinflammation via MIP-3α-CCR6 signaling, whereas hypothermia would suppress this signaling via Hsp70-dependent and independent pathways (Figure 4).

The physiological roles of MIP-3α-CCR6 signaling in the CNS have yet to be fully determined, because various roles of chemokines have recently been proposed in brain, including as neurotransmitters and neuromodulators [79, 80]. The interactions between MIP-3α-CCR6 signaling and other pathways involved in the ischemic pathology, such as excitotoxicity, acidotoxicity, oxidative stress, and apoptosis, also should be addressed. For instance, oxidative stress is considered one of the primary risk factors that exacerbate the damage by cerebral ischemia, and several components of ROS are generated after ischemia/reperfusion injury [81]. Kil et al. [82] have reported that ROS production by leukocytes and microglia in the ischemia brain is strongly suppressed by hypothermia. Apoptosis is another important cause because of the significant contribution to the cell death subsequent to ischemia/reperfusion injury [83]. Jong et al. [84] have reported the reduction in levels of matrix metalloproteinases (MMPs) and increased expression of tissue inhibitor of metalloproteinase (TIMP)-2 in response to mild hypothermia therapy in experimental stroke. Precise evaluation of ROS, MMPs, and TIMPs in MCAO rat brains treated with an anti-MIP-3α-neutralizing antibody may provide insights into these interactions.

6. Conclusions

This paper described the utilization of current technologies such as DNA microarray and proteomics to reveal the specific mechanisms and key molecules involved in the neuroprotective effects of hypothermia against ischemic injury. The therapeutic time window and duration of hypothermia necessary to exert neuroprotective effects were determined using a brain temperature control system. Comprehensive gene expression analysis showed that suppression of neuroinflammatory cascades including MIP-3α-CCR6 signaling may primarily contribute to the neuroprotective effects of hypothermia. In contrast, proteomics identified Hsp70 as a potential effector. Although the physiological roles and potential cross-talk between these molecules remain to be elucidated, these approaches may be promising for the discovery of antistroke pharmaceutical drug targets.

Acknowledgments

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Research Article

Mild Hypothermia Attenuates Intercellular Adhesion Molecule-1 Induction via Activation of Extracellular Signal-Regulated Kinase-1/2 in a Focal Cerebral Ischemia Model

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Intercellular adhesion molecule-1 (ICAM-1) in cerebral vascular endothelium induced by ischemic insult triggers leukocyte infiltration and inflammatory reaction. We investigated the mechanism of hypothermic suppression of ICAM-1 in a model of focal cerebral ischemia. Rats underwent 2 hours of middle cerebral artery occlusion and were kept at 37°C or 33°C during occlusion and rewarmed to normal temperature immediately after reperfusion. Under hypothermic condition, robust activation of extracellular signal-regulated kinase-1/2 (ERK1/2) was observed in vascular endothelium of ischemic brain. Hypothermic suppression of ICAM-1 was reversed by ERK1/2 inhibition. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) in ischemic vessel was attenuated by hypothermia. STAT3 inhibitor suppressed ICAM-1 production induced by stroke. ERK1/2 inhibition enhanced phosphorylation and DNA binding activity of STAT3 in hypothermic condition. In this study, we demonstrated that hypothermic suppression of ICAM-1 induction is mediated by enhanced ERK1/2 activation and subsequent attenuation of STAT3 action.

1. Introduction

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily and the principal ligand for leukocyte function-associated antigen-1 (LFA-1), a member of the integrin superfamily. ICAM-1/LFA-1 adhesion system assists leukocyte movement into the tissue. LFA-1-positive leukocytes are induced to adhere to ICAM-1-positive endothelial surface [1, 2], and then to pass through the basement membrane into the tissue [3, 4]. Many animal and human studies indicate that ICAM-1 is implicated in the pathogenesis of ischemic cardiovascular and cerebrovascular disorders [5–8]. Especially during reperfusion period of stroke, infiltrated leukocytes contribute to the secondary injury by producing toxic substances that damage the brain cells and disrupt the blood-brain barrier [9, 10]. Since ICAM-1 is an important factor of leukocyte infiltration and reperfusion injury in stroke, intervention of ICAM-1 induction has been a promising therapeutic strategy against stroke.

The remarkable benefit of mild hypothermia in brain ischemia has long been recognized and remains one of the most powerful neuroprotective strategies in cerebral ischemia both experimentally and clinically [11]. Many studies indicate that inflammatory response contributes significantly to the secondary injury after ischemia [12, 13],

and protection by mild hypothermia is associated with anti-inflammatory processes [14–16]. Even though there is considerable interest in the potential therapeutic role of induced hypothermia, the molecular basis of hypothermic protection remains mostly unknown.

Previously, we and others have demonstrated that hypothermia attenuated ICAM-1 induction [17–21] and neutrophil infiltration after stroke attack [15, 16, 22]. In most studies, hypothermia was applied during ischemic or few hours after ischemia. Therefore, there is a time gap between hypothermia application and ICAM-1 induction when the temperature was already returned to normal body temperature. Since hypothermia is known to interfere with some ischemia related signaling pathways and gene expression [23, 24], we hypothesize that hypothermia applied during ischemia interferes the upstream pathway of ICAM-1 expression and investigated the molecular mechanism in the vascular endothelium of the ischemic brain.

2. Materials and Methods

2.1. Animal Model. Experiments were carried out according to the guidelines for the animal care and use of laboratory animal protocols approved by our university administrative panel on laboratory animal care. Rats were housed with food and water available ad libitum under diurnal lighting conditions and temperature-controlled environment until the day of experiment.

2.2. Focal Cerebral Ischemia by Transient Middle Cerebral Artery Occlusion (MCAO). Male Sprague-Dawley rats weighing 290 to 320 g were anesthetized with enflurane and maintained during surgical procedures. Physiological parameters were monitored and maintained in the normal range as shown previously [14]. Ischemia was induced using an occluding intraluminal suture. An uncoated 30 mm long segment of 3–0 nylon monofilament suture with the tip rounded by flame was inserted into the stump of the common carotid artery and advanced into the internal carotid artery approximately 19-20 mm from the bifurcation in order to occlude the ostium of middle cerebral artery. After 2 hours of ischemic period, the suture was removed and the animal was allowed to recover. Sham-operated animals were treated in the same manner as the ischemic animals, but no ischemia was applied. During surgery, rectal temperature was maintained between 37-38°C. Mild hypothermia (33°C of rectal temperature) was achieved as previously described using paradigms associated with neuroprotection [14]. Cooling began upon ischemia onset, maintained for 2 hours and terminated immediately after reperfusion. To inhibit ERK1/2 activation, U0126 (0.5 mg/kg, Cell Signaling Technology) was administered via tail vein 30 minutes before MCAO. Cucurbitacin I or JSI-124 (0.1 mg/kg; selective JAK/STAT inhibitor, Calbiochem) was injected intraperitoneally 1 hour before MCAO.

2.3. Brain Endothelial Cell Culture and Oxygen Glucose Deprivation (OGD) Study. bEnd.3 cells, mouse brain endothelial cell line was purchased from American Type Culture Collection (Rockville, MD). The cells were cultured with DMEM containing 10% FBS at 37°C in a humidified 5% CO_2 incubator. JSI-124 (10 μM) or U0126 (10 μM) was treated 30 min before OGD. OGD was performed by transferring cells to an anaerobic chamber (Forma) with an atmosphere of 5% CO_2, 5% H_2, and 90% N_2. The culture medium was replaced three times with deoxygenated PBS and serum free media for OGD. Cultures were placed in a humidified 37°C or 33°C incubator within the anaerobic chamber for 4 hr. Oxygen tension was monitored with an oxygen electrode meter and was kept under 0.02%. OGD was ended by adding 5.5 mM glucose to the culture medium, and the cultures were returned to the normoxic (37°C) or hypothermic (33°C) incubator (reperfusion). The cultured cells and media were harvested for further study.

2.4. Tissue Preparation and Infarct Area Measurement. Rats were euthanized by carbon dioxide overdose and perfused with cold normal saline immediately. The brain was quickly removed and sectioned into 2 mm thick slices starting at the frontal pole using a brain matrix slicer. Some slices were immersed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma) and incubated at 37°C for 20 minutes. To assess lesion area, TTC-stained slices were photographed and analyzed by Image-J analysis software (public domain software developed at NIH, available at http://rsb.info.nih.gov/ij/). Lesion area was determined as the percentage of the total ipsilateral hemispheric area. Other slices were processed for further studies.

2.5. Immunohistochemistry. From paraffin embedded brain slices, 6 μm thick sections were cut. After deparaffinization, sections were treated with 0.03% H_2O_2 and blocked in 1% bovine serum albumin and 5% normal serum. Following incubation with primary antibodies against ICAM-1 (1 : 100, Serotec), phosphorylated ERK1/2 (1 : 200, Santa Cruz), total ERK1/2 (1 : 200, Santa Cruz), STAT3 phosphorylated at Ser727 (1 : 200, Cell Signaling), and total STAT3 (1 : 200, Santa Cruz), respectively, biotin-labeled anti-IgG secondary antibody (1 : 200, Vector Labs) was treated. Antibodies were detected using the Vector ABC kit (Elite Vectastain ABC kit, Vector Labs) and colorized with 0.05% diaminobenzidine (DAB, Vector Labs). Negative controls were run in parallel using adjacent sections incubated with IgG instead of the primary antibody. For fluorescence staining, we used FITC or Cy3-labeled anti-IgG secondary antibody (1 : 200, Jackson) instead of biotin-labeled antibody.

2.6. Western Blot Analysis. Brain samples were homogenized in Laemmli's lysis buffer plus protease inhibitors. Aliquots containing 30 μg of protein were subjected to 10% SDS-PAGE. Protein bands were transferred to polyvinylidene difluoride membrane (Millipore), probed by incubating with the primary antibodies, and followed by a horseradish peroxidase conjugated secondary antibody (1 : 2000, Santa Cruz). We used the following primary antibodies raised against ICAM-1 (1 : 500, Serotec), phosphorylated ERK1/2

(1 : 1000, Santa Cruz), total ERK1/2 (1 : 1000, Santa Cruz), phosphorylated STAT3 (1 : 1000, Cell Signaling), and total STAT3 (1 : 1000, Santa Cruz). To determine the specificity of the primary antibodies, we used antibodies preabsorbed with blocking peptides instead of primary antibodies. Blots were visualized using the ECL system (Amersham) according to the manufacturer's directions, and exposed to X-ray film. Equal protein loading was confirmed by measuring β-actin (1 : 5000, anti β-actin, Sigma). Densitometric measurements were made from the film using a GS-700 imaging densitometer (Bio-Rad), then quantified using Multi-Analyst (Bio-Rad). For quantification of relative protein expression, the optical density of the protein band of interest was normalized to the optical density of β-actin on the same gel.

2.7. Microwell Colorimetric STAT3 DNA Binding Assay. The binding ability of STAT3 to its DNA consensus sequences was measured using a commercially available kit (TransAMTM STAT3, Active Motif). In this assay, tissue lysates were isolated from the brain tissue and tested for their ability to bind to a double-stranded oligonucleotide probe containing the consensus binding sequence for STAT3. Samples were homogenized in 3 mL ice-cold lysis buffer (20 mmol/L HEPES, pH 7.5; 350 mmol/L NaCl; 20% glycerol; 1% Igepal-CA630; 1 mmol/L MgCl2; 0.5 mmol/L EDTA; 0.1 mmol/L EGTA) per gram tissue. Lysates were centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was used to measure the protein content by a Bradford-based assay (Bio-Rad). STAT3 activity was determined by the sample's ability to bind to consensus sequences (5′-GGGACTTTCC-3′) in a 96-well plate. A primary antibody that recognizes an epitope on STAT3 and is accessible only when STAT3 is activated and bound to its target DNA was added to the wells, followed by a secondary horseradish peroxidase-conjugated antibody. Developing solution (tetramethylbenzidine) was added and the colorimetric reaction was stopped by adding stop solution (0.5 mol/L H_2SO_4). After stopping the reaction, absorbance was measured on a spectrophotometer within 5 minutes at 450 nm with a reference wavelength of 625 nm. HeLa whole-cell extract was used as positive control. The STAT3 wild-type and mutated consensus oligonucleotides were used in order to monitor the specificity of the assay.

2.8. Statistical Analysis. Data are given as means ± SEM. Comparisons between groups were performed using standard statistical methods using SigmaStat (SPSS). The data were analyzed by one-way ANOVA, Kruskal-Wallis one-way ANOVA on ranks, or unpaired t-test. Statistical significance was determined at the $P < .05$ level.

3. Results

3.1. Hypothermia Potentiated ERK1/2 Phosphorylation in the Vascular Endothelium of the Ischemic Brain. We observed the presence of phosphorylated or activated ERK1/2 in the ischemic brain using immunohistochemistry (Figure 1(a)) and demonstrated its localization in the endothelium using fluorescence double-labeling with antibody against CD31, an endothelial cell marker from the brain tissue at 2 hours after MCAO (Figure 1(b)). To find out the evidence that ERK1/2 pathway in the endothelium is related with ICAM-1 induction, we first investigated the effect of hypothermia on the activation of ERK1/2. From the immunohistochemistry study, the number and intensity of phosphorylated ERK1/2 immunoreactivity were higher in hypothermia group than normothermia (Figure 1(a)). To obtain quantitative data, we performed Western blot analysis and observed that ischemia-induced phosphorylated ERK1/2 level was significantly higher in hypothermia group than normothermia while total ERK1/2 was not affected by the temperature difference (Figure 2(a)).

3.2. ERK1/2 Inhibitor Reversed Hypothermic Suppression of ICAM-1. To demonstrate the role of ERK1/2 in the hypothermic attenuation of ICAM-1 induction, we administered U0126, a MEK inhibitor suppressing phosphorylation of ERK1/2, to the animals and measured the ICAM-1 level in the ischemic brain. The optimal dose of U0126 was evaluated in the pilot study, and activation of ERK1/2 in brain was almost completely suppressed when U0126 (0.5 mg/kg) was administered 30 minutes before MCAO onset (Figure 2(b)). In U0126 untreated animals, ICAM-1 induction was reduced by hypothermia. But after U0126 treatment, ICAM-1 induction was not suppressed by hypothermia (Figure 2(b)).

3.3. STAT3 Phosphorylation Was Induced by Ischemia and Attenuated by Hypothermia. Even though hyperactivation of ERK1/2 seems to suppress ICAM-1 induction, the association between ERK1/2 phosphorylation and ICAM-1 expression is not clearly reported. So we first investigate the signal pathways implicated in the regulation of ICAM-1 expression in our model. During ischemia and until few hours after reperfusion initiation, phosphorylated STAT3 was observed in the ischemic brain, especially in the vessels (Figure 3(a)). At 24 hours after ischemia, STAT3 was not observed in the vessels. Western blot analysis demonstrated that ischemia increased STAT3 phosphorylation at 2 and 6 hours and it was declined to the basal level at 24 hours. Hypothermic attenuation of STAT3 phosphorylation was observed both in immunohistochemically stained tissues and Western blotted gel images (Figures 3(a) and 3(b)).

3.4. STAT3 Inhibitor Attenuated ICAM-1 Induction. The presence of phosphorylated STAT3, which acts as a transcription factor, in the vessels and its suppression by hypothermia imply that STAT3 is important in ICAM-1 expression. To get more evidence of STAT3's role in ICAM-1 induction, we treated animals with JSI-124, an inhibitor of STAT3, at 1 hour before MCAO. The protein level of ICAM-1 was compared between the STAT3 inhibitor-treated and vehicle-treated groups. ICAM-1 induction at 24 hours after ischemia was significantly inhibited by JSI-124 treatment (Figure 3(c)).

3.5. ERK1/2 Inhibition Enhanced STAT3 Phosphorylation and DNA Binding Activity. Based on the data so far, it seems that

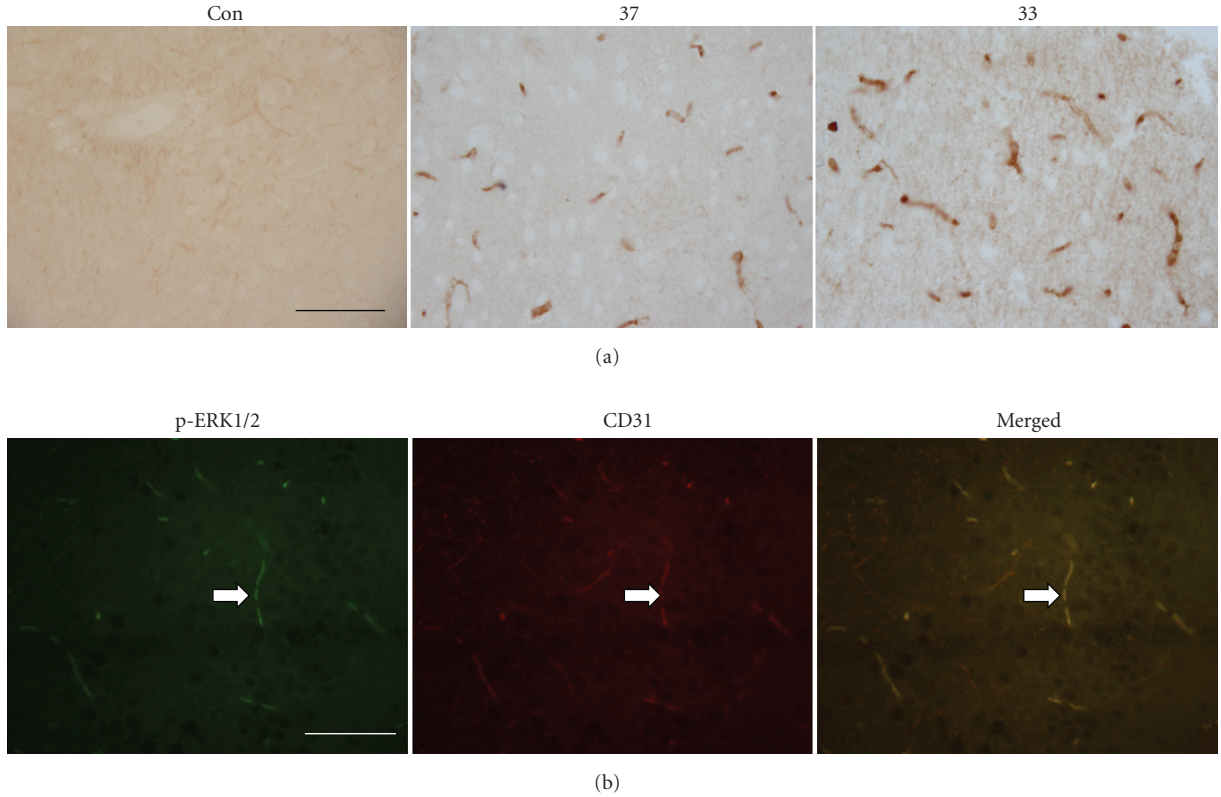

Figure 1: Photomicrographs of the cerebral cortex in the ischemic brain with immunohistochemical staining for phosphorylated ERK1/2. (a) Phosphorylated ERK1/2 is detected in the ischemic brain under normothermic (37) or hypothermic (33) condition but not in the nonischemic control brain (sham) at 2 hours after MCAO initiation. The number and intensity of ERK1/2 immunoreactive vessels were higher in hypothermic group. (b) Fluorescence double labeling illustrates the colocalization of CD31 (red), an endothelial marker, and phosphorylated ERK1/2 (green) in the vessels of the hypothermia group at 2 hours after MCAO initiation. Scale bar: 100 μm.

enhanced ERK1/2 activation and reduced STAT3 activation are the key role players of hypothermic suppression of ICAM-1 induction. To investigate whether these two factors are working dependently or not, we treated U0126 and observed phosphorylation and DNA binding activity of STAT3. Under hypothermic condition, STAT3 activation at 2 hours after MCAO was reduced. But when U0126 was treated to the hypothermic group, phosphorylated STAT3 was increased to the level of normothermia condition (Figure 4(a)). Since activity of STAT3 as a transcription factor can be indirectly estimated using the binding ability of STAT3 to its consensus sequence in the promoter area, we measured DNA binding activity of STAT3 as well. Tissue lysates from ischemic brain tissue of the hypothermia group were taken from the animals treated with or without ERK1/2 inhibition. U0126 treatment significantly enhanced the binding activity of STAT3 (Figure 4(b)).

4. Discussion

This study is designed to investigate the mechanism of hypothermic suppression of ICAM-1 induction following brain ischemia. Our data are summarized as follows. ICAM-1 induction is suppressed by hypothermia after stroke. Stroke activates ERK1/2 mildly under normothermic condition and hypothermia potentiates ERK1/2 activation robustly. Stroke activates STAT3 under normothermic group and hypothermia attenuates this. ERK1/2 inhibition increases STAT3 activation and attenuates hypothermic effect. STAT3 inhibitor attenuates ICAM-1 induction. Based on these results, we suggest that hypothermia enhances ERK1/2 activation, inhibits STAT3 activation, and then leads to suppression of ICAM-1 induction.

Our first finding is potentiation of ERK1/2 activation by hypothermia. Even though it is well accepted that most of the metabolic and enzymatic pathways are down-regulated by hypothermia, increased activity of ERK1/2 by hypothermia was reported by many studies [25–29]. Since phosphorylated ERK1/2 from the brain tissue is a sum from mixed cell types, it cannot clearly indicate pure endothelial component. Our *in vitro* work (Supplementary Figure 2 available online at doi:10.4061/2011/846716) which demonstrated hypothermic enhancement of ERK1/2 activation in cultured mouse brain endothelial cells after oxygen-glucose deprivation should be a strong support for the hypothermic effect in the brain vascular endothelium. Roberts and colleagues also reported hypothermic activation

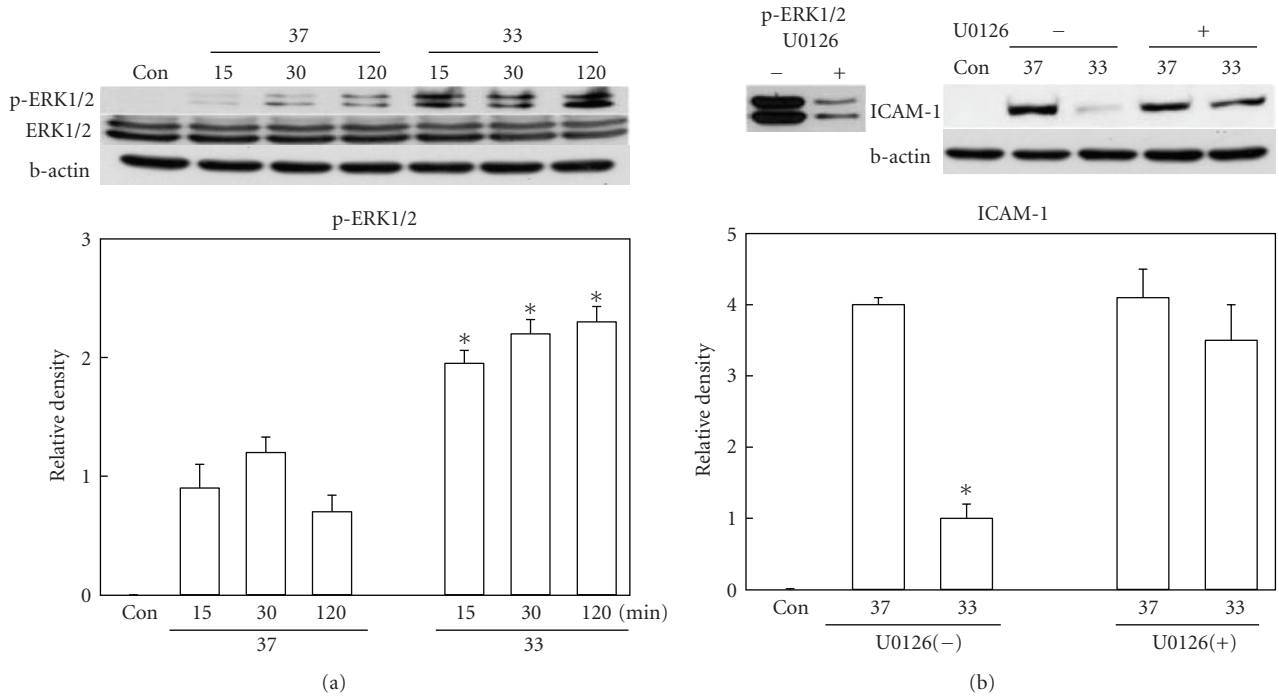

Figure 2: Western blot analysis of phosphorylated ERK1/2 and ICAM-1. (a) Phosphorylation of ERK1/2 was significantly higher in hypothermic (33) brains than normothermic (37) ones at 15, 30, and 120 minutes after ischemic insult ($n = 6$ per group). Total ERK1/2 level was not changed. (b) Ischemia-induced increase of ICAM-1 at 24 hours after MCAO was suppressed by hypothermia. But this suppression was not observed when ERK1/2 inhibition was done (U0126, $n = 4$) at the dose which almost completely inhibited ERK1/2 phosphorylation. $^*P < .05$ versus normothermia.

of ERK1/2 in endothelial cells [30]. When hypothermia was applied without ischemic stimuli, it was not strong enough to activate ERK1/2. There was no difference between normothermia and hypothermia in nonischemic brain. Cultured brain endothelial cells showed the same result (Supplementary Figure 1). Even though we do not have a clear idea, it seems that hypothermia potentiates ERK1/2 activation when ERK1/2 activation is triggered first by ischemia. Hypothermia may have a direct influence on ERK1/2 itself or can affect one of the upstream factors of ERK1/2 pathway. Even though we did not investigate the upstream factors, we can draw valuable clues from 3 reports [25, 28, 29]. Chan and colleagues showed that hypothermic stress leads to activation of Ras in rat fibroblasts, and Raf-Mek-ERK cascade is rapidly activated when hypothermic cells are returned to physiologic temperature [28]. Sakurai and colleagues reported that mild hypothermia protects cells from TNF-alpha-induced apoptosis, at least partly, via induction of cold-inducible RNA-binding protein (CIRP), and that CIRP protects cells by activating the ERK pathway [25]. At our hands, CIRP gene expression was detected a few hours later than ERK1/2 activation in response to hypothermia (unpublished data). This implies that CIRP cannot be in the upstream of ERK1/2 pathway in our model. Atkins and colleagues demonstrated ERK activation in a traumatic brain model using similar hypothermia model as ours [29]. So we speculate that hypothermia acts on Raf-Mek-ERK cascade. Since the role of p38 or c-Jun N-terminal kinases (JNK) in the inflammation is well known, we also investigated p38 and JNK activation in the preliminary experiment. Active form of JNK was not detected in the vessel at the earlier time period after stroke, and phosphorylated p38 was observed in the vessels almost at the time window of ERK1/2 but showed no difference between normothermic and hypothermic conditions (unpublished data).

To elucidate the transcription factor which might be down-regulated by hypothermia and ERK1/2 signal, we searched references on ICAM-1 expression regulatory system. Studies on ICAM-1 promoter demonstrated the presence of STAT1/3 binding motif sequence, interferon response element (IRE) [31, 32], activator protein 1 (AP-1), nuclear factor kappa B (NFkappaB), Ets, CCAAT/enhancer binding protein (c/EBP), and SP1 [33, 34]. Among these transcription factors, we need to find the candidate which is the key factor of ICAM-1 induction in our model. In the preliminary experiment, we investigated a couple of transcription factors using immunohistochemistry and Western blot analysis. It is well known that NFkappaB system is the major pathway of inflammation [35, 36] and our previous study [37] also demonstrated that hypothermic suppression of NFkappaB system led to suppression of inflammation in stroke. Even though the role of NFkappaB in ICAM-1 induction was reported in many studies [38–40], Wen and colleagues [41] showed that nuclear translocation of NFkappaB observed in the ischemic area was mostly in the neurons and astrocytes.

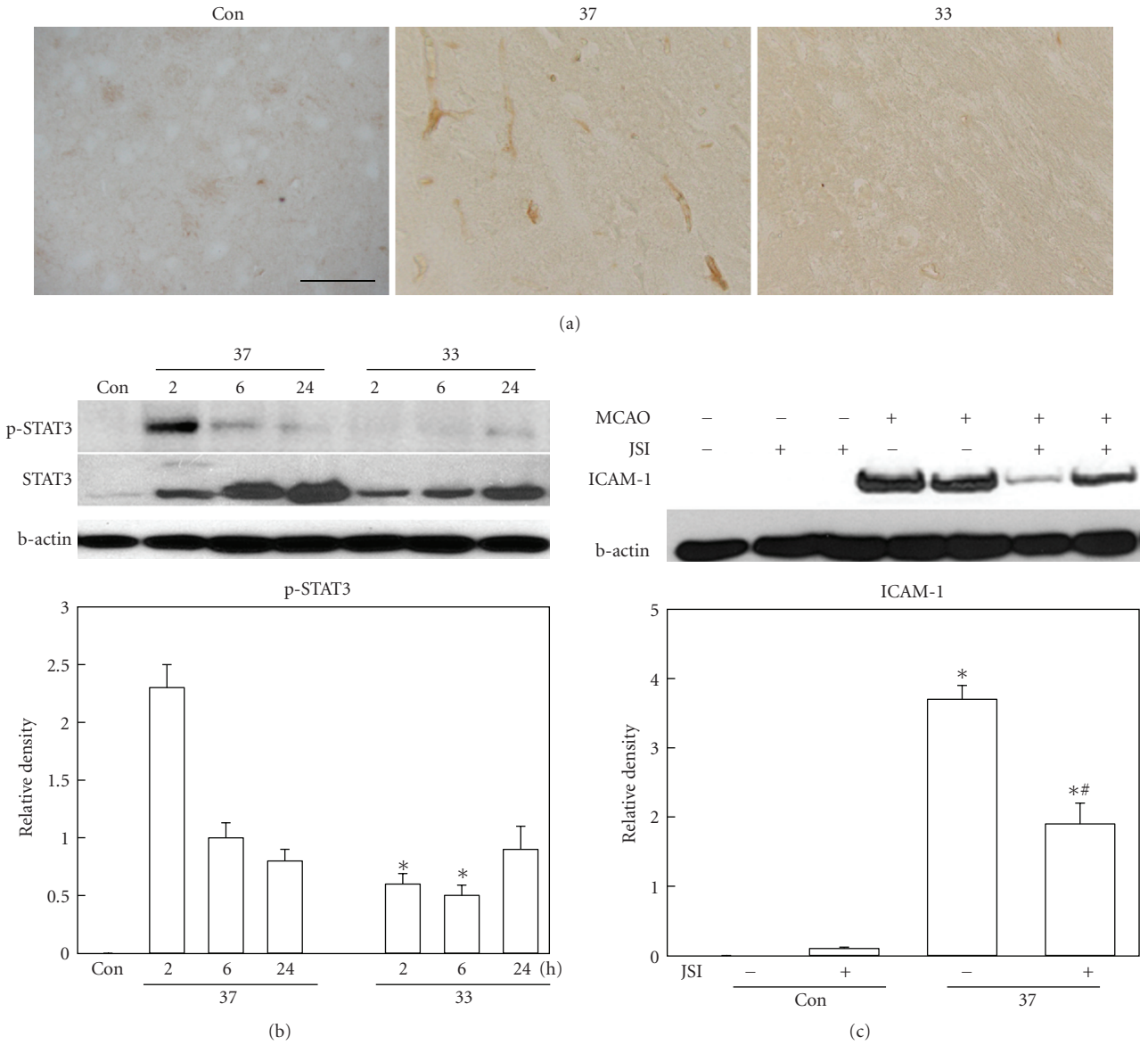

FIGURE 3: (a) STAT3 phosphorylation in the cerebral cortex of the ischemic brain. Photomicrograph illustrates the presence of phosphorylated STAT3 in the vessels of the ischemic brain at 2 hours after ischemic insult. Positive immunoreactivity is not observed in nonischemic brain (sham), and hypothermia (33) reduced the number of phosphorylated STAT3 positive cell compared with normothermic group (37). Scale bar: 100 μm. (b) Western blot analysis shows that ischemia-induced STAT3 phosphorylation at 2 and 6 hours after MCAO onset in normothermic brain (37, $n = 6$) is reduced in hypothermia group (33, $n = 6$). $^{*}P < .05$ versus normothermia. (c) Western blot analysis demonstrates that ICAM-1 induction at 24 hours after MCAO is reduced by STAT3 inhibitor treatment (JSI, $n = 4$). $^{*}P < .05$ versus sham; $^{\#}P < .05$ versus ischemia without JSI-124.

Our preliminary data also showed no NFkappaB translocation in the vessels during the early period ahead of ICAM-1 induction. Therefore, we tried other candidates. During ischemia and few hours after reperfusion initiation, c-Fos and phosphorylated STAT3 were observed in the ischemic brain. Phosphorylated STAT3 localized exclusively in the vessels while c-Fos were found in vessels and other cell types as well. When hypothermia was applied, there was no temperature difference in c-Fos (data not shown) but STAT3 phosphorylation was reduced by hypothermia. This suggests that STAT3 is a temperature sensitive transcription factor of ICAM-1. Yang and colleagues showed the evidence of STAT3 as a transcription factor of ICAM-1 in renal ischemia/reperfusion model [42]. By blocking Janus kinase (JAK)/STAT signal with selective JAK2 inhibitor tyrphostin AG490, expression of ICAM-1 was significantly inhibited, renal function was improved, and histological lesions and apoptosis were reduced [42]. In our stroke model, we also observed that STAT3 inhibitor JSI-124 effectively reduced ICAM-1 induction. These data might support that STAT3 plays as a key transcription factor of ICAM-1 expression in our system.

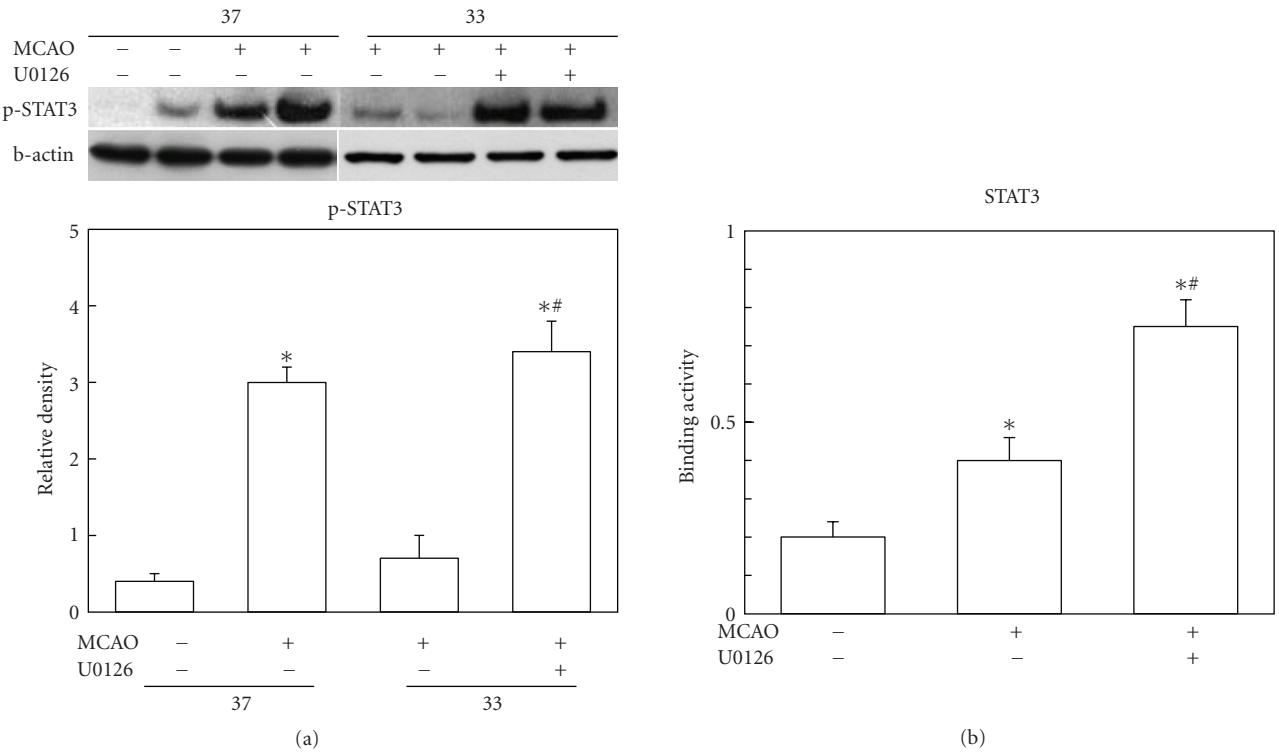

Figure 4: Effect of ERK1/2 inhibition on the STAT3 phosphorylation and DNA binding activity. (a) Western blot analysis demonstrates that STAT3 activation at 2 hours after ischemic insult is increased in normothermic group (37, $n = 6$). This increase was reduced by hypothermia (33, $n = 6$). In hypothermic group, U0126 treatment (33, $n = 4$) enhanced STAT3 phosphorylation to the level of normothermia group (37, $n = 6$). $^{*}P < .05$ versus sham; $^{\#}P < .05$ versus hypothermic ischemia without U0126. (b) DNA binding activity assay of STAT3 using nuclear fraction from ischemic brain under hypothermia condition shows increased binding activity of STAT3 at 2 hours after MCAO ($n = 4$). STAT3 binding was further augmented by U0126 treatment ($n = 4$). $^{*}P < .05$ versus sham; $^{\#}P < .05$ versus ischemia without U0126.

To evaluate whether STAT3 is regulated by ERK1/2 in addition to hypothermia, we tested the effect of ERK1/2 inhibition. When ERK1/2 activity was reduced, STAT3 phosphorylation and DNA binding activity were enhanced. Even though ERK1/2's role as the upstream regulator of STAT3 can be expected by ERK1/2 inhibition, it is not clear how ERK1/2 regulates STAT3 pathway. We just speculate that ERK1/2 might inhibit STAT3 directly or indirectly via one of the factors located in the upstream of STAT3 pathway. Even though JSI-124 effectively blocked the ICAM-1 induction, infarction size was not prevented or reduced in contrast to our expectation. We assume that there is a reason to explain this disappointing result. Many investigators have suggested STAT3 signal pathway as a neuroprotection mechanism. Shyu and colleagues [43] showed that secretoneurin's effect on reduced cerebral infarction improved motor performance, and increased brain metabolic activity in MCAO model was mediated via the JAK2/STAT3 pathway. Yamashita and colleagues [44] found that endogenous IL-6 plays a critical role in the neuroprotection, and its role may be mediated by STAT3 activation. In general it seems that STAT1 activation is related to cell death, whereas STAT3 activation is often associated with cell survival [45]. We also observed STAT3 immunoreactivity in neuron at 6 and 24 hours after MCAO while in the vessels at 2 and 6 hours. In spite of the immunohistochemistry data, we tried to confirm STAT3 phosphorylation in the endothelial cells. By repeating *in vivo* experimental conditions in the cultured brain endothelial cell system, we could demonstrate the hypothermic effect on STAT3 phosphorylation and effect of U0126 on STAT3 phosphorylation (Supplementary Figure 3). Based on these facts, STAT3 might have two opposite roles, promotion of ICAM-1 induction in the vessel and protection of cell in the neurons. This can explain why STAT3 inhibitor is not protective in our model. Even though STAT3 inhibitor used in this study was not protective against stroke, the better outcome can be expected when the endothelium-specific STAT3 inhibitor is developed.

5. Conclusions

We demonstrated that mild hypothermia has a robust suppressive effect on induction of ICAM-1 through regulation of ERK1/2 and STAT3. Even though mild hypothermia has been shown to have clinical efficacy in some settings, routine cooling of stroke victims may not always be practical or feasible. For those cases, ERK1/2 and STAT3 can be the good target of pharmaceutical treatment.

Abbreviations

ICAM-1: Intercellular adhesion molecule-1
ERK1/2: Extracellular signal-regulated kinase-1/2
STAT3: Signal transducer and activator of transcription 3
MCAO: Middle Cerebral Artery Occlusion
TTC: 2,3,5-triphenyl tetrazolium chloride
DAB: Diaminobenzidine
JAK: Janus kinase
CIRP: Cold-inducible RNA-binding protein
JNK: c-Jun N-terminal kinases
IRE: Interferon response element
AP-1: Activator protein 1
NFkappaB: Nuclear factor kappa B
c/EBP: CCAAT/enhancer-binding protein.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Review Article

Experimental and Clinical Use of Therapeutic Hypothermia for Ischemic Stroke: Opportunities and Limitations

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Stroke remains a disease with a serious impact on quality of life but few effective treatments exist. There is an urgent need to develop and/or improve neuroprotective strategies to combat this. Many drugs proven to be neuroprotective in experimental models fail to improve patient outcome in a clinical setting. An emerging treatment, therapeutic hypothermia (TH), is a promising neuroprotective therapy in stroke management. Several studies with TH in experimental models and small clinical trials have shown beneficial effects. Despite this, implementation into the clinical setting is still lacking due to methodological considerations as well as hypothermia-related complications. This paper discusses the possible opportunities and limitations of the use of TH in animal models and the translation into the clinic.

1. Introduction

Stroke is the third leading cause of death in industrialized countries, after cardiovascular diseases and cancer, and is the main cause of severe and long-term disability. Moreover, as our society ages, the incidence of stroke will increase and will become a considerable socioeconomic burden on society due to the excessive costs of long hospitalisations, nursing care, and rehabilitation [1–5].

So far, two major strategies in the treatment of thrombotic stroke exist: thrombolysis and neuroprotective therapies. Thrombolysis (or recanalization) with recombinant tissue plasminogen activator (rt-PA) is the only approved and effective therapy after thrombotic stroke thus far. Unfortunately, treatment with rt-PA has important limitations such as a short treatment window (3-4.5 h), reperfusion-associated injury, and hemorrhagic complications. Consequently, less than 10% of all patients with stroke can be treated with thrombolytic agents [1–4, 6–8]. Although other agents including platelet inhibitors (aspirin), anticoagulants (ancrod, heparins), and prourokinase have been used in the management of acute stroke, there is only poor or no evidence that these approaches improve outcome [4, 9, 10]. Alternatively, neuroprotection after an acute ischemic stroke antagonizes, interrupts, reduces, or slows down injurious biochemical and molecular events [11]. Neuroprotective strategies mostly focus on reducing damage in the penumbra and thus improving the outcome after stroke [12]. Some neuroprotective agents have shown promising results in animal studies; however, very few clinical trials show the same neuroprotective effect in patients with an ischemic stroke [13–15].

Therapeutic hypothermia (TH) is one of the best studied neuroprotective therapies. Small decreases in brain temperature are well tolerated and have been shown to confer a significant degree of neuroprotection in several animal models of cerebral ischemia as well as a reduction of the extent of ischemic brain damage and improvement of neurological function [16–21]. Clinical trials and experimental studies in acute stroke indicate that there is an association between body temperature, initial stroke severity, infarct volume, and clinical outcome [22–24]. Increased brain temperature is

associated with deleterious effects on injured brain tissue [24]. Fever, even a mild one, during the first few days of acute stroke is associated with a worse clinical outcome [22, 24, 25]. This is a concern as twenty-five percent of stroke patients have a body temperature of 37.5°C or more within the first 6 hours after the onset of the symptoms [6]. In contrast, clinical observations have revealed that patients whose body temperature was low at admission to hospital have a better neurological outcome than patients with normal or high body temperature [25]. In addition, controlling body temperature below 36.5°C has proven to correlate with good clinical outcome [26]. Therefore, the conclusion can be drawn that increases in brain temperature escalate neuronal damage whereas lowering the brain temperature reduces brain damage [5]. Thus, although a fever is considered to be a normal physiological defensive response to infection, inflammation, and several other conditions, avoiding hyperthermia and controlling the body temperature are necessary to prevent a worse outcome after cerebral ischemia [19, 27, 28].

Understanding the ischemic cascade, including excitotoxicity, free radical production, and inflammatory and apoptotic pathways, is mandatory in order to develop new therapeutic strategies for stroke. Several extensive reviews on the complex ischemic cascade after stroke have been published [2, 3, 25, 29–32]. Briefly, when a brain vessel is occluded, a series of cellular and molecular events occur, starting with a decrease in cerebral blood flow which can interfere with ion homeostasis. Indeed, within minutes to a few hours, the occlusion leads to increased intracellular calcium levels and glutamate release, causing excitotoxicity and a spreading depression throughout the ischemic region. Cells start to depolarize, causing even more calcium influx and glutamate release. Additionally, water will passively follow the ion influx, resulting in cytotoxic edema [1, 31, 33]. After a few hours to a few days, the high intracellular calcium levels lead to an overactivation of several proteolytic enzyme systems that degrade cytoskeletal proteins. Activation of Ca^{2+}-dependent enzymes, including phospholipase A_2, cyclooxygenase, and neuronal nitric oxide synthase results in additional cellular damage and the generation of reactive oxygen species (ROS), which subsequently damage membranes, mitochondria, and DNA. As a result of all these stimulatory influences, an apoptotic and neuroinflammatory response develops [3, 29–31, 34]. Repair and regeneration over the following months will determine the ultimate damage [35]. In all cases, the location of the original occlusion, and whether the occlusion was permanent or transient, is critical in determining the size and severity of the insult [25, 36].

TH protects the brain in various ways. Among these, it retards energy depletion by lowering the metabolic and the enzymatic rate, restores the neurotransmitter balance, reduces the intracellular calcium influx, reduces intracellular acidosis, suppresses the generation of ROS, suppresses infiltration of inflammatory cells, prevents blood-brain barrier disruption, suppresses specific cell death pathways, or upregulates cell survival mechanisms [17, 23, 34, 37, 38]. A review of the mechanisms of action of hypothermia was not in the scope of this manuscript. Therefore, the reader is referred to a number of reviews on this subject [17, 23].

2. Experimental Studies

Several experimental stroke models have been developed over the last decade to mimic human stroke. Of these, occlusion of the middle cerebral artery (MCA) is the closest to the patient's situation. Several strategies have been used to induce focal cerebral ischemia near the MCA including the embolic model, the intraluminal suture MCA occlusion model, the photothrombosis model, and the endothelin-1 model. These models are well described by Durukan and Tatlisumak [3]. The cerebral insult in stroke models may vary from permanent to transient occlusions and transient models are diverse in the occlusion time, varying from 30 minutes to 3 hours [23]. Due to the range of model variables, the severity of functional and structural outcome changes depend on the exact experimental protocol, and thus it is important to emphasise which experimental model is used [39].

In stroke models, different types of animals are used, including rats, mice, rabbits, and gerbils. The rat is most commonly used in experimental studies for several reasons including the resemblance between human and rat cerebrovascular anatomy and physiology, the moderate size of the animal which allows easy monitoring of physiological parameters as well as cytological analysis of the brain, and the relative homogeneity within strains [3]. Mice are preferred when genetic modifications to investigate specific differences in the pathophysiology and ischemic cascade of stroke are required. Transgenic mice offer the advantage of a short gestation time (18.5–21 days) and perhaps most importantly a well-developed set of technologies for introducing genetic modifications [40].

While experimental stroke models are an important tool to identify new treatment strategies, there are a few important pitfalls. There is not "one" ideal ischemic stroke model since human stroke itself is a diverse condition [3]. Each model only mimics some aspects of the pathophysiology and the progression of the human stroke. Furthermore, the species and the strain that are used, even within the same laboratory, may determine the outcome of a treatment strategy. Portelli et al. [41] found that several pharmacological, behavioural, and neurochemical factors were significantly different from one breeding location to another and could even vary in time between rats coming from the same breeding location. So, it is likely that genetic differences have an effect on infarct outcomes in the various stroke models. Ren et al. [42] demonstrated significant differences in temporal window for hypothermic protection among rats from different strains in which MCA occlusion was induced. Furthermore, even if the same model is used, technical manipulations can vary between investigators of different laboratories. The location and the intensity of the lesion as well as its progression can vary accordingly [42].

Currently, experiments are generally performed in young healthy rodents. This is in contrast with the clinical situation, where most patients are elderly and have concomitant pathologies which can affect the outcome of an ischemic incident. Hyperglycemia, for example, plays a role in the severity of stroke and it has been associated with exacerbated

cerebral damage and increased morbidity and mortality in acute ischemic stroke [43, 44]. It is important to take these risk factors and comorbidities into account when testing on animals. Experiments performed in aged rodents suffering from hypertension, hypercholesterolemia, and/or diabetes are therefore more relevant to clinical practice [45, 46]. These conditions were formulated in the original Stroke Therapy Academic Industry Roundtable (STAIR) criteria [47].

Technical complications also compromise the accuracy of animal stroke models. While brain temperature is the critical issue in regard to neuroprotection in stroke, most experimental studies rely on core temperature measurements, which are usually estimated by sampling rectal temperature because it is easy and inexpensive [45]. A few researchers have used telemetry probes to measure temperature, most often in the core but also in the brain [48, 49]. Although some authors claimed that rectal temperature can nicely predict brain temperature, there is conflicting data about this correlation [18, 49]. One study in rodents showed that the core temperature is about 0.7°C higher than brain temperature [49] whereas another study in rodents found that brain temperature was 0.2 to 0.7°C higher than rectal temperature [18]. Since the temperature is not consistent throughout the body or brain, there is no consensus in which brain region measurements should be performed. Not only the brain region is important, but there is also a difference in temperature between the ischemic and the contralateral hemisphere. Even the way TH is induced could even influence the correlation between the core and brain temperature. Measuring the temperature in freely moving animals or animals under anaesthesia may be an important consideration. As minimal changes in brain temperature can have protective effects, standard methods of measuring temperature should be elaborated to allow comparison between different studies.

Knowing the optimal depth of hypothermia is important for clinical studies because of the increasing side effects associated with increased temperature reduction. Depending on the depth of cooling, TH has been classified in different levels: minimal (35°C), mild (32–34°C), moderate (28–32°C), deep (20–28°C), profound (5–20°C), and ultraprofound (<5°C) [17]. From the literature, it is not clear which is the most appropriate target temperature, but mild and moderate hypothermia have been shown to be protective in animal models and cause fewer side effects than deep hypothermia [18, 20, 50]. An animal study comparing mild and moderate TH showed that 33°C was better tolerated than 30°C in a rat model of transient focal cerebral ischemia [18]. Another study showed that treatment with hypothermia of 33-34°C in the reperfusion period of focal cerebral ischemia is superior to all other applied temperatures [51]. A third study showed that postischemic hypothermia of 32°C produced a larger reduction in infarct volume than 27°C [52]. Therefore, it seems that mild TH is superior to moderate TH.

An important observation is that in many animal studies, TH is initiated before or at the onset of the insult. However, this is in contrast with the clinical situation, where most patients reach the hospital several hours after symptom onset. Consequently, neuroprotective agents targeting early events in the ischemic cascade are unrealistic, especially because at that time a significant amount of cerebral tissue is already impaired [1]. Animal research suggests that intraischemic hypothermia is more effective than postischemic hypothermia [23]. However, to better mimic clinical settings, initiation of the TH should be delayed in experimental settings and the optimal time window for inducing hypothermia should be determined. Recently, we showed [53] that in the endothelin-1 rat model, a 2-hour mild hypothermia treatment delayed by 1 hour after the insult is still neuroprotective and improves neurological outcome whereas a delay of 2 hours was ineffective. In other MCA occlusion models, similar results have been reported. For instance, Maier et al. [54] showed that 2 hours of hypothermia reduced the infarct volume, even when delayed up to 90 minutes after a 2-hour MCA occlusion. A study by Ohta et al. [55] showed that the hypothermic treatment could be delayed up to 4 hours after 2 hours MCA occlusion if cooling was prolonged for 48 hours. So it seems that hypothermia is neuroprotective when applied early after the insult and remains beneficial, if the duration of the cooling is prolonged.

Most patients, in clinical trials, receive the hypothermic treatment for several days (1 to 3 days) [56]. However, this is in contrast with most experimental studies, where animals mostly receive short periods of cooling (few hours). A rat study showed that a mild hypothermic treatment (using core temperature telemetry measurements) of 48 hours provided superior protection compared to a hypothermic treatment of 12 hours [48]. Another study showed that 22 hours of mild hypothermia (using rectal temperature measurements) was superior to a 3-hour treatment [57]. These data suggest that short periods of cooling may lead to transient neuroprotection. Therefore, prolonging hypothermia treatment might provide permanent neuroprotective effects. As most patients reach the hospital several hours after the cerebral insult, it is possible that longer treatment durations may be necessary when the initiation of cooling is delayed.

The rewarming phase after hypothermic treatment is also important as rapid rewarming may lead to rebound phenomena and enhanced deleterious ischemic effects. Berger et al. [58] compared fast rewarming (within 20 minutes) with slow rewarming (within 2 hours) and showed that slow rewarming significantly reduced the infarct volume compared to fast rewarming. Therefore, as the rewarming phase plays a crucial role in influencing the outcome after stroke, it is important that the rewarming phase should be studied in experimental studies as well or at least be elaborated upon in the experimental protocol to allow comparison.

Most animals are sacrificed within a few days after ischemic onset preventing the study of long-term events [45, 46, 59]. However, because complex processes of the ischemic cascade can last up to some months after the ischemic insult, it is desirable to monitor the animals for a longer period of time than is currently used.

Currently, most experiments are carried out on male animals only, but researchers should consider the possibility

that the sex and the hormone changes might influence stroke outcome. Suzuki et al. [60] showed that estrogens protect the brain against stroke injury which points to an urgent need for studies comparing males and females, and the hormonal changes with it. Besides sex hormones, poor outcome was also associated with high levels of stress hormones. Studies in rodents showed that stress delays or diminishes recovery of cognitive functions after cerebral ischemia [61].

Another important issue to be addressed is the anaesthetic used in experimental studies. Studies using TH are carried out on anesthetized animals to avoid shivering. However, the choice of the anaesthetic can influence the clinical outcome [62] and therefore, the use of a sham group is mandatory. Furthermore, as the method of cooling depends on the choice of the anaesthetic (such as pharmaceuticals and inhalation anaesthetics), it is important to stress these experimental conditions as well. An important contrast between experimental research and the clinical situation is the use of sedation and ventilation. In the clinical setting, the use of anaesthetics has decreased the last few years because of the potential side effects [22]. While in animal models, most studies are still carried out on anesthetized animals when cooling techniques are used. Animals are mostly cooled via surface cooling techniques, such as with fans, water or alcohol spray, use of a cold room, or administration of some chemicals. Selectively cooling the brain, for example with an extracranial cooling coil, is becoming more popular in experimental rat models [63]. In the clinical situation, however, surface cooling combined with endovascular cooling is commonly used [26, 27, 37, 38, 64, 65].

A selection of outcome parameters should be used when evaluating the neuroprotective effect of TH in animal studies. While histological endpoints are important for demonstrating reductions in infarct volume, these assessments may not necessarily be associated with improved functional and neurological outcome [45]. Several laboratories have consistently shown that TH reduces both the extent of neurological damage and improves neurological function [37]. However, simple measurements of recovery are not sufficient to detect improvements in neurological function, as they do not reflect the complexity of functional impairment and may not accurately predict neuroprotection [45]. Functional outcome should be tested with several sensorimotor behavioural and cognitive tests, such as limb placing, beam walking, grid walking, rotarod, sticky label test, staircase test, and Morris water maze [3, 66]. Subsequently, combined functional and histological endpoints are necessary for examining the protective effect of hypothermia or drugs in stroke models [67].

Physiological parameters are strictly controlled during experimental studies, which is in contrast with the clinical setting. It is possible that neuroprotective agents convey a beneficial effect in animal models because some physiological parameters (such as the body temperature, blood pressure, etc.) are controlled in animal experiments. Clinical evidence suggests that admission of acute stroke patients to a stroke unit with intensive monitoring can lead to improved outcome [68]. Optimal medical care is important to reduce the risk of complications and to stabilize a number of acute physiological parameters that may worsen ischemia, such as high or low blood pressure, fluid and electrolyte management, hypoxemia, body temperature, infections, and blood glucose [12]. A strict control of the physiological parameters in stroke patients should improve the outcome.

In summary, a suitable experimental animal model should meet the following criteria: it should be reliable, minimally invasive, and yield reproducible lesions with minimum variability. Age and comorbidities should be taken into account before conducting an experimental study. The physiological variables should also be monitored and maintained within normal ranges [3]. Not only the acute effects of TH, but also the long-term effects should be studied. The model has to be relevant to human stroke and consider all the above-mentioned impediments when these experiments are translated into clinical settings. Although further research is needed to investigate the optimal conditions for TH, the current knowledge suggests that TH should be initiated as soon as possible, and if the treatment is delayed longer, cooling should be considered. Several studies also suggest that mild hypothermia is neuroprotective and produces fewer complications than deep cooling. As the rewarming phase strongly influences the outcome after stroke, it is crucial that all studies use slow rewarming phases.

3. Clinical Studies

There is an urgent need to improve the translation of preclinical research into the clinic. To help the translation of animal studies to human clinical trials, the STAIR criteria were set up to improve the understanding of experimental data and extrapolation to a clinical setting. The STAIR provides recommendations for clinical development of acute stroke therapies, including elimination randomization bias, calculation of sample size, specification of the inclusion and exclusion criteria, blind assessment of the outcome, use of multiple species, and reporting animals excluded from the analysis [1, 47].

As the ischemic cascade is so complex, it is possible that different types of stroke (ischemic or hemorrhagic) benefit from different therapeutic protocols [1]. When running a clinical trial, patients with the same stroke severity should be enrolled, in order to produce comparable results. Of these, patients who have had an earlier stroke or other comorbidity, should be placed in a subgroup during the analysis as it might influence the outcome [11]. Before including patients in a clinical trial, inclusion and exclusion criteria should be well defined and optimized. The protocol has to include cutoff points for the National Institute of Health Stroke Scale (NIHSS) and the Barthel index and should have clear outcome measurements. As hypothermia is less effective in ischemia without reperfusion, any neuroprotective strategy should, if possible, be combined with recanalization therapy.

A number of small clinical trials of hypothermia have already been performed in stroke patients (Table 1), such as the "Copenhagen Stroke Study" [70], the "Cooling for Acute Ischemic Brain Damage" (COOL AID) study [73], and the "Intravascular Cooling in the Treatment of Stroke-Longer

Table 1: Clinical studies with therapeutic hypothermia in acute stroke.

First author, year [ref.]	Number of patients	Sedated or awake (+ antishivering drugs)	Method of cooling	Time to treatment (h)[1]	tPA[2]	Target temperature (°C) + place of measurement	Duration of cooling	Rewarming rate	Outcome
Schwab et al., 1998 [69]	25	Sedated with fentanyl and propofol	Cooling blanket, cold infusion, cold washing	4–24 Mean 14	−	33 Bladder	48–72 h	Passive rewarming in 24 h	SSS[4] and BI[5] (4 weeks and 3 months)
Kammersgaard et al. 2001, [70]	17	Awake (pethidine)	Cooling blanket, cold air	<12 Mean 3	−	35.5 Tympanic and rectal	6 h	In 4 h to 36.5°C	SSS[4] (6 months)
Schwab et al., 2001 [71]	50	Sedated with midazolam or propofol and morphine or fentanyl	Cooling blanket, alcohol, ice bags	4–75 Mean 22	−	33 Bladder	24–72 h	Passive rewarming in 11–24 h	NIHSS[6] (4 weeks) mRS[7] (3 months) BI[5] (3 months)
Georgiadis et al., 2001 [72]	6	Sedated with midazolam andfentanyl	Endovascular cooling	12–58 Mean 28	−	33 Bladder	48–72 h	1°C/8 h Max 0.2°C/h	NA[3]
Krieger et al., 2001 [56]	10	Sedated with propofol	Cooling blanket, ice water	Mean 6	+	32 Bladder	12–72 h	0.21°C/h	mRS[7] (3 months)
De Georgia et al., 2004 [73]	18	Awake (meperidine and buspirone)	Endovascular cooling, warming blanket	<12 Mean 9	−	33 Esophageal	24 h	0.2°C/h	NIHSS[6] and mRS[7] (30 +7 days)
Guluma et al., 2006 [22]	10	Awake (meperidine and buspirone)	Endovascular cooling, warming blanket	<6	+	33 Blood	24 h	0.3°C/h 12 h	NA[3]
Kollmar et al., 2009 [74]	10	Awake (pethidine and buspirone)	Ice-cold saline, warming blanket	<3 Mean 2	+	35.5 Tympanic	NA[3]	NA[3]	NIHSS[6] (1 day and at discharge (± 4.5 days))
Hemmen et al., 2010 [75]	58	Awake (meperidine and buspirone)	Endovascular cooling, warming blanket	0–3 3–6	+	33 Blood	24 h	0.3°C/h 12 h	NIHSS[6] (1, 30 and 90 days) mRS[7] (90 days)

[1]Time from the insult to initiation of treatment; [2]tPA: tissue plasminogen activator (+: administered; −: not administered); [3]NA: not available; [4]SSS: Scandinavian Stroke Scale; [5]BI: Barthel Index; [6]NIHSS: National Institutes of Health Stroke Scale; [7]mRS: modified Rankin scale.

tPA Window" (ICTuS-L) study [75]. The "Copenhagen Stroke Study" showed that modest hypothermia (35.5°C) by surface cooling can be achieved in conscious patients following an acute stroke [70]. The COOL AID study showed that endovascular cooling (33°C) is feasible in conscious patients with moderate to severe ischemic stroke [73]. The ICTuS-L study showed that endovascular cooling (33°C) can be combined with standard thrombolytic therapy [75]. However, further research with larger patient groups is necessary to evaluate the efficacy of hypothermia after ischemic stroke as recent studies were not able to show reduced mortality after an insult [22, 56, 69–75].

Early initiation of hypothermia after stroke gives the best outcomes, but if hypothermia is delayed, longer cooling times may also be protective [38, 76] and thus, as most stroke patients reach the hospital several hours after the insult, many trials use long therapeutic windows compared to animal studies (see Table 1). Although a long cooling time seems attractive, it may increase the risk of complications [17, 37, 76]. Prolonged hypothermia not only increases the risk for side effects, it also has an influence on every organ system [59].

Although several small clinical trials show that TH could be safe for stroke patients, there are still a number of complications that need to be overcome [22, 56, 69]. The most common complications related to hypothermia are pneumonia, hypovolemia, arrhythmias, hyperglycemia, bradycardia, thrombocytopenia, hypertension, hypotension, increased intracranial pressure, electrolyte abnormalities like hypokalemia, and metabolic acidosis [16, 27]. There are also complications related to fast rewarming, such as intracranial hypertension [27].

As mentioned above, besides the control of the body temperature, it is also important to control other physiological parameters such as blood glucose, blood pressure, fluid and electrolyte management, hypoxemia, cardiac arrhythmias, and infections [12]. Clinical evidence suggests that admission of acute stroke patients to a stroke unit with intensive monitoring can improve the outcome [68]. Most studies agree that it is difficult to keep all of the metabolic and physiologic variables that might influence the outcome constant across different centers [27]. However, this should be their goal in order to reduce the risk of a bad outcome and the risk of complications in stroke patients. Blood markers, such as NSE and S100β, should also be included, as they can give an indication of the prognosis and the outcome after stroke.

TH seems a very promising treatment for stroke if investigators can reduce the complications related to the treatment, but there are also other limitations. Endovascular cooling compared to surface cooling may be better to control the body temperature more precisely but it is more invasive [22]. Patients treated with TH have to be admitted to an intensive care unit, this may generate practical and logistical problems [46].

Finally, the lack of translation of experimental studies into human clinical trials reflects the lack of resources as well as shortage of sponsoring from companies and governments [46, 67]. There is an urgent need for larger clinical trials with a sufficient number of patients, which might reveal small but significant differences in outcome and low-incidence complications [22]. Despite all limitations, researchers still believe that TH could become the standard therapy in stroke management, as it already showed promising results in cardiac arrest patients [22, 50, 77].

4. Conclusion

As only a small percentage of stroke patients can be treated with thrombolytic agents, there is an urgent need to develop and to improve neuroprotection strategies. TH seems to be the most promising neuroprotective therapy in experimental models with encouraging results in the clinical setting. The current knowledge from both experimental and clinical research suggests that TH should be initiated as soon as possible after stroke onset in order to achieve the best outcome. Prolonged cooling should be considered if the initiation of the hypothermia treatment is delayed. As the rewarming phase strongly influences the outcome after stroke, it is crucial that all studies use slow rewarming phases. Currently, it can be considered that mild hypothermia is neuroprotective and produces less complications than deep cooling. At the same time, controlling physiological parameters and the temperature during the hypothermic treatment are essential for successful clinical or experimental trials.

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Clinical Study

Therapeutic Hypothermia after Cardiac Arrest: Experience at an Academically Affiliated Community-Based Veterans Affairs Medical Center

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At laboratory and clinical levels, therapeutic hypothermia has been shown to improve neurologic outcomes and mortality following cardiac arrest. We reviewed each cardiac arrest in our community-based Veterans Affairs Medical Center over a three-year period. The majority of cases were in-hospital arrests associated with initial pulseless electrical activity or asystole. Of a total of 100 patients suffering 118 cardiac arrests, 29 arrests involved comatose survivors, with eight patients completing therapeutic cooling. Cerebral performance category scores at discharge and six months were significantly better in the cooled cohort versus the noncooled cohort, and, in every case except for one, cooling was offered for appropriate reasons. Mean time to initiation of cooling protocol was 3.7 hours and mean time to goal temperature of 33°C was 8.8 hours, and few complications clearly related to cooling were noted in our case series. While in-patient hospital mortality of cardiac arrest was high at 65% mortality during hospital admission, therapeutic hypothermia was safe and feasible at our center. Our cooling times and incidence of favorable outcomes are comparable to previously published reports. This study demonstrates the feasibility of implementing, a cooling protocol a community setting, and the role of neurologists in ensuring effective hospital-wide implementation.

1. Introduction

Comatose survivors of cardiac arrest notoriously have poor outcomes including significant neurological deficits and persistent vegetative state. Although there is variation in incidence and outcomes, epidemiologic studies suggest that between one and five of every 1,000 patients admitted to a hospital in Western countries suffer an in-hospital cardiac arrest [1], with the National Registry of Cardiopulmonary Resuscitation citing an incidence of 0.175 events per bed in a sample of American hospitals [2]. There is a wide range of reported survival to hospital discharge, from 0% to 42%, with larger studies approaching an average of 20% [1].

There is considerable evidence that mild therapeutic hypothermia after cardiac arrest is protective in preventing cerebral injury. Animal and human studies have demonstrated that hypothermia reduces brain metabolism and thus oxygen and ATP consumption [3, 4], reduces the release of excitotoxic glutamate via regulation of transmembrane electrolyte transport [5], protects against oxidative stress and lipid peroxidation [6, 7], and alters gene expression (see also reviews by Holzer [8], Liu and Yenari [9], and Yenari et al. [10]) to promote brain cell survival after cerebral ischemia. These physiologic effects were shown to have clinical significance with the publication in 2002 of two major clinical trials of therapeutic hypothermia in comatose survivors of cardiac arrest. These studies, by Bernard and colleagues [11] in Australia and by the Hypothermia after Cardiac Arrest Study Group [12] in Europe, showed that cooling these patients improved neurological outcomes and reduced rate of death.

Since the publication of these trials, numerous medical centers around the world have replicated the efficacy of therapeutic hypothermia in this patient population and also

established its overall feasibility and safety [13–15] (see also review by Sagalyn et al. [16] and meta-analysis by Arrich et al. [17]). Followup from the European multicenter trial also included data from patients who had in-patient cardiac arrests and patients who initially had pulseless electrical activity (PEA) or nonventricular fibrillation or ventricular tachycardia rhythms. While return of spontaneous circulation (ROSC) was faster for in-hospital compared to out-of-hospital patients, there was no statistically significant difference in neurologic outcome or mortality in comparing hypothermia or normothermia within the in-hospital cohort. Within the PEA cohort, mortality was lower for patients who received therapeutic hypothermia [18].

In spite of this growing body of literature, therapeutic hypothermia has not been universally accepted and pursued at all medical centers [19], with cited barriers including lack of institutional protocols, resources, and limited prior experience [20]. Some authors have called upon neurologists to become more active in management of these patients beyond offering prognosis and in helping create hospital-wide policies given that therapeutic hypothermia remains one of the only proven treatments for improving neurologic outcome [21], and a recent report from Prior and colleagues demonstrated the success of planning and implementing a therapeutic hypothermia protocol in a community-based hospital setting [20]. As such, in this retrospective analysis, we report our experience with implementation of therapeutic hypothermia for comatose cardiac arrest survivors and its outcomes in an academically affiliated community-based Veterans Affairs medical center in which the majority of events were in-hospital.

2. Methods

The San Francisco Veterans Affairs Medical Center is a 378-bed hospital that serves more than 310,000 veterans in Northern California. It is a teaching hospital affiliated with the University of California San Francisco medical center. The hospital is staffed 24 hours a day by an inpatient neurology consultation service. In November 2007, a protocol for mild therapeutic hypothermia after cardiac arrest was written and reviewed by neurology attending physicians at the medical center and was discussed with the chiefs of the ICU services in December 2007. In February 2008, all housestaff and ICU nursing staff began in-service training regarding appropriateness and implementation of treatment including training on the use of cooling blankets and devices, monitoring during protocol implementation, and awareness of potential complications. Treating services were asked to consult neurology service in cases where therapeutic hypothermia was being considered.

This was a retrospective chart review study. In November 2007, a log was kept of every Code Blue on the medical center campus including the Emergency Department. After approval of study design from local IRB, each case was retrospectively reviewed from November 2007 to August 2010. Initial demographic information including age, gender, medical comorbidities, and events leading to the Code Blue alarm was obtained for each case. In those cases of documented cardiac arrest and loss of perfusing rhythm, information regarding the code was obtained including setting and location of the arrest; initial cardiac rhythm; medical cause of arrest if known at time of event or later; treatment during arrest including pharmacologic, electrical, and surgical intervention; duration of code; time at which spontaneous circulation returned; whether the patient survived the code; presence of coma after arrest. In those cases in which the patient was found to be in a coma, the chart was reviewed to determine if neurology service was consulted for possible cooling and any reason why they were not consulted was noted. Appropriate cases were reviewed for initiation of therapeutic hypothermia, and reasons for not starting protocol were also recorded. If started, the time of initiation in relation to return of spontaneous circulation was recorded, as was the cooling method, time at which goal temperature was achieved, total duration of cooling, rewarming time, complications related to cooling (defined as occurring during or up to one week after completion of rewarming), and any technical or unexpected difficulties were noted, including reasons for early termination of protocol. The lowest temperature recorded during treatment was also noted as measure of possible hypothermia overshooting. Cerebral Performance Categories (CPC) Scale scores of outcome (from 1 to 5, with 1 being conscious and alert with good cerebral performance, 2 being conscious with moderate cerebral disability, 3 being conscious with severe disability and dependent on others for activities of daily living, 4 being comatose or persistent vegetative state, and 5 being brain death or death from other causes) [22] were determined for all possible cases at hospital discharge, and, where appropriate, at six-month and two-year followup.

Inclusion criteria for initiation of cooling protocol included age greater than or equal to 18, woman with negative pregnancy test, and coma after cardiac arrest with no eye opening or response to noxious stimulation after resuscitation. Return of spontaneous circulation following loss of perfusing rhythm and subsequent stable cardiac rhythm was also required, with blood pressure greater than 90 mmHg systolic. Any nonperfusing initial rhythm during cardiac arrest including ventricular fibrillation, ventricular tachycardia, PEA, or asystole was deemed appropriate for treatment upon return of spontaneous circulation. Exclusion criteria included noncomatose state or other reasons for coma beyond cardiac arrest, pregnancy, known advanced directive or goals of care that would preclude therapeutic hypothermia and prolonged life support measures, and known severe coagulopathy including administration of full dose tPA during arrest or active bleeding. Cooling was stopped if any of the exclusion criteria developed after initiation.

Under the protocol, surface cooling—with ice packs placed under the axilla and near the neck, torso, and limbs—was initiated once patient was hemodynamically stable after acute treatment for cardiac arrest and if there were no other clear contraindications to initiation of hypothermia. This was done to prevent delay in initiation of cooling. The neurology consult service would then evaluate the

patient and history and recommend whether cooling should continue or be discontinued. If cooling was to be continued, a cooling blanket or suit was deployed and nursing staff, primary physicians, and neurology service would work together to achieve goal temperature of 33°C as quickly as possible, ideally within 8 hours of return of spontaneous rhythm. Temperature was then maintained at 32°C to 33°C, and temperature sensing was done with a Foley catheter bladder probe when possible, *otherwise*, *rectal temperatures were recorded*. All patients were mechanically ventilated, and midazolam and fentanyl were used for sedation. Vecuronium was sometimes used as a paralytic and to help control shivering, and EEG monitoring was placed if there was concern for seizures under discretion of neurology consult service. All other ICU management and treatment plans were continued as deemed appropriate by the critical care team. Twenty-four hours after initiation of therapeutic hypothermia, passive rewarming was initiated using normal blankets and the removal of cooling measures, with goal of reaching 37°C at a rate of 0.3–0.5°C increase per hour. Paralytics and then sedation would then be discontinued as appropriate, and any subsequent hyperthermia was treated with cooling blanket and antipyretics.

Fisher's exact test and analysis of variance (ANOVA) were used to look for significant differences across the cardiac arrest groups including baseline demographics, mechanism and duration of cardiac arrest, medical comorbidities, and outcomes at discharge and six months. Within the cohort of patients who completed cooling protocol, correlation analysis between CPC score and time to initiation of protocol and time to goal temperature after ROSC was done by calculating Pearson's correlation coefficient (r) and the variance (r^2). Total duration of cooling and time over which rewarming was completed were also noted. All the statistical analysis was performed using a computer software program, R version 2.12.1 (The R Foundation for Statistical Computing, 2010).

3. Results

From November 2007 to August 2010, there were 130 "Code Blue" alerts at the San Francisco Veterans Affairs Medical Center (see Figure 1). Of these, 12 of the alarms were done in error or involved cases in which there was no loss of a perfusing cardiac rhythm. Of the remaining 118 alerts involving 100 patients, 38 cardiac arrests occurred in which the patient did not survive. In these cases, the mean age of the patients was 72 years and 97% of the patients were men. Thirty-four arrests were in-hospital (89%) and the initial cardiac rhythm was PEA or asystole in 95% of arrests, with 5% ventricular tachycardia or fibrillation arrests. The mechanism of arrest involved a primary pulmonary arrest in 8 arrests (21%), primary cardiac in 12 (32%), gastrointestinal bleeding or catastrophe in 5 (13%), intraoperative event in 2 (5%), sepsis or infection in 4 (11%), and unknown or other etiology in 7 (18%). Comorbidities within this cohort included coronary artery disease (CAD) or congestive heart failure (CHF) in 17 patients (45%), cardiovascular risk factors defined as hypertension, diabetes, dyslipidemia, or tobacco use disorder in 21 (55%), cancer diagnosis in 9 (24%), pulmonary disease in 8 (21%), gastrointestinal bleeding or end-stage liver disease in 7 (18%), end-stage renal disease in 5 (13%), human immunodeficiency virus (HIV) or hepatitis infection in 2 (5%), and history or ongoing substance or alcohol abuse in 5 (13%).

Baseline demographics and characteristics of cardiac arrest are summarized for the 80 cardiac arrests involving 70 unique surviving patients in Table 1. The overall mean age of these cases was 70.7 years, and 95% were men. Ninety-three percent of these arrests occurred in-hospital, and the initial perfusing rhythm was PEA or asystole in 94% of cases and ventricular tachycardia or fibrillation in 6%. Although comparisons were limited by small sample size, return of spontaneous circulation occurred faster in patients who were not in a coma after arrest ($F = 29.6$, $P = 3.9 \times 10^{-9}$). The 17 patients who were in a coma after arrest and did not undergo therapeutic cooling also had a higher rate of unfavorable CPC at discharge compared to the other groups ($P = 7.6 \times 10^{-5}$) and between the comatose survivor groups at six months after arrest ($P = 0.05$).

Amongst the 51 cardiac arrests in which the survivor was not comatose afterwards, 14 were treated with attempted electrical cardioversion (27%) and one patient received full-dose tPA (2%). In one case, a chest tube was placed due to hemothorax. Neurology was consulted in one case for consideration of therapeutic hypothermia but patient was noted to be moving extremities and was not comatose; the service was consulted for six other patients later in their hospital course typically for prognosis or management of other complications. Of note, one patient was initially noted to not be in a coma by primary team but was noted to be comatose by neurology service 72 hours after arrest and has since remained in persistent vegetative state.

Of the 18 cardiac arrests in which the survivor was comatose but not cooled, one patient was treated with attempted electrical cardioversion (6%) and three with full-dose tPA (17%). One patient was taken to the operating room urgently for cardiac surgery and was placed on extra-corporeal membrane oxygenation. Neurology service was consulted for five cases regarding initiation of therapeutic hypothermia and two times for other reasons including prognosis. The reasons why patients were not cooled are listed in Figure 1. Of note, neurology was not called initially by primary team for one patient due to prolonged duration of code (30 minutes before ROSC); that patient was later declared brain dead by neurology service. In addition, one patient was felt to have a focal examination concerning for focal cerebral process after cardiac arrest rather than global hypoxia and so neurology service advised against cooling; that patient remained neurologically devastated and was eventually transitioned to hospice care and died one month later.

Amongst the 11 cases where cooling was initiated, 3 were treated with attempted electrical cardioversion (28%), and 2 were treated with bedside cardiac massage. Two were treated with bedside cardiac massage. Table 2 displays data specific to this group of patients, including description of discharge and

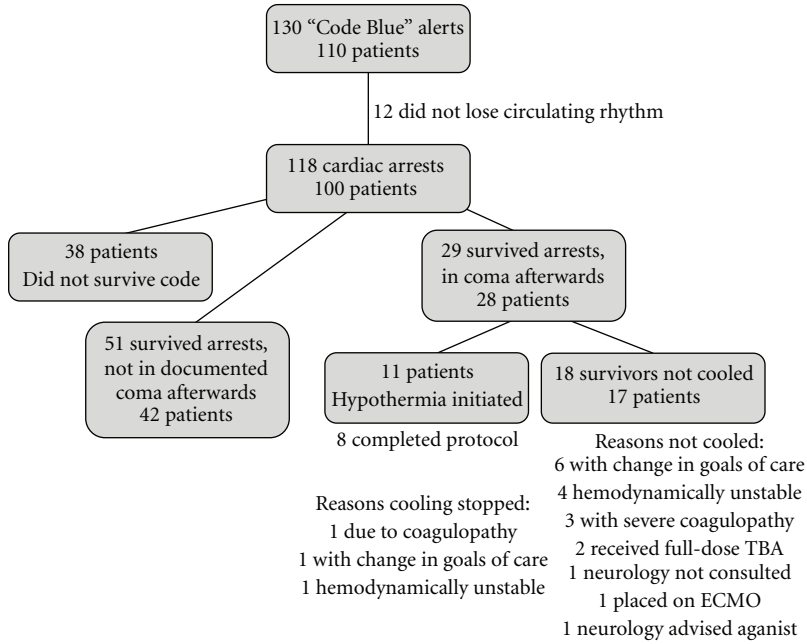

Figure 1: "Code Blue" Alerts at San Francisco VA Medical Center from November 2007 to August 2010. The diagram breaks down the numbers and types of "Code Blue" arrests including brief categorization of reasons of why patients who were comatose after cardiac arrest were not cooled or why cooling was stopped after initiation.

six-month outcomes and details regarding cessation of cooling therapy in three cases. For the 8 patients that completed the cooling protocol, mean time to initiation of cooling protocol was 3.7 hours (range 2 to 6 hours) and mean time to goal temperature of 33°C after ROSC was 8.8 hours (range 3 to 17 hours). Cooling was continued for an average of 23.6 hours (range 22 to 24 hours). Rewarming occurred over an average of 13.9 hours (range 4 to 24 hours). Five of these 8 patients had favorable CPC score of 1 or 2 after 72-hour evaluation (63%) and three expired before discharge (38%). Three patients continued to have CPC score of 1 or 2 at six months after cardiac arrest (38%). Complications noted during therapeutic cooling or during the subsequent week after cardiac arrest included cardiac arrhythmia requiring treatment with medication in 3 patients (38%), pneumonia in 3 patients (38%), another infection in 1 patient (13%), and deep venous thrombosis or subsegmental pulmonary embolus in 2 patients (25%). There were no instances of acute kidney injury, skin breakdown or rash, or bleeding or hemorrhage during this same time period.

Neither the time to initiation of cooling protocol ($r = 0.48$, $r^2 = 0.23$) nor the time to goal temperature of 33°C ($r = 0.05$, $r^2 = 0.003$) was associated with CPC scores at discharge.

4. Discussion

We report our experience of therapeutic cooling in comatose survivors at our academically affiliated community-based Veterans Affairs medical center. We show that it is possible to implement such a cooling protocol in this setting where the majority of arrests occurred in hospital. Of the patients who completed the therapeutic hypothermia protocol, 63% (5 of 8) had a favorable neurologic outcome at the time of hospital discharge. Although this is a small sample size, this rate is similar to those reported in the original trials (49% and 55%) [11, 12] and in subsequent series [20]. This percentage of favorable outcomes is also consistent with larger registries that have noted that 60 to 85% of patients who survive in-hospital arrests tend to have a favorable neurologic outcome [2, 23].

In terms of the initiation of the cooling protocol from time of ROSC, our average of 3.7 hours is comparable to another series from a community hospital [20] and the average time to goal temperature of 8.8 hours was also comparable to previous reports, which ranged from 5.0 to 9.2 hours [15, 18, 20]. Given that most of the arrests were in-hospital, it would be hoped that time to initiation would be even shorter. However, there was no association with time from ROSC to initiation of protocol or time to goal temperature and CPC score at discharge, although the small sample size likely contributed to the lack of statistical significance.

In comparing the survivors of cardiac arrest, there was a significantly faster ROSC in the cases where the survivor was not in a coma afterwards. Although this data was not available in all cases, faster return of circulating rhythm was also associated with higher probability for favorable CPC given that more patients who were not in a coma were neurologically intact at discharge and at six months compared to the patients who were comatose. Although these noncomatose survivors were similar in age and comorbidities, and had high rates of initial PEA or asystole during arrest compared to the comatose survivors,

Table 1: Characteristics of cardiac arrests in which patient survived.

	Not in coma after arrest (n = 51 arrests, 42 patients)	In coma after arrest, not cooled (n = 18 arrests, 17 patients)	In coma after arrest, cooling started (n = 11 arrests, 11 patients)	Statistical test for significance
Sex				
Male	40 (95)	17 (100)	10 (91)	$P = 0.53$[e]
Age, in yrs	70.4 ± 11.1	72.4 ± 12.4	69.5 ± 13.0	$F = 0.24, P = 0.79$[f]
In-hospital Arrest	47 (92)	16 (94)	11 (100)	
Initial cardiac rhythm				
PEA/asystole	47 (92)	18 (100)	10 (91)	$P = 0.51$[e]
VTach/VFib	4 (8)	0 (0)	1 (9)	
ROSC in minutes	5.2 ± 4.6 range (0.1–20)[a]	33.5 ± 21.2 range (5–60)[b]	21.9 ± 11.9 range (5–35)[c]	$F = 29.6, P < 0.001$[f]
Mechanism of arrest				
1° Cardiac	24 (47)	4 (22)	2 (18)	$P = 0.07$[e]
1° Pulmonary	13 (25)	8 (44)	3 (27)	$P = 0.33$[e]
1° Gastrointestinal	1 (2)	2 (11)	1 (9)	$P = 0.18$[e]
Sepsis/infection	5 (10)	2 (11)	0 (0)	$P = 0.72$[e]
Intraoperative	2 (4)	2 (11)	0 (0)	$P = 0.30$[e]
Unknown/other	6 (12)	0 (0)	5 (45)	$P = 0.005$
Comorbidities				
CAD or CHF	24 (57)	9 (53)	7 (64)	$P = 0.83$[e]
Vascular RFs	26 (62)	13 (76)	6 (55)	$P = 0.47$[e]
Cancer	8 (19)	5 (29)	3 (27)	$P = 0.66$[e]
Pulmonary disease	8 (19)	3 (18)	1 (9)	$P = 0.91$[e]
GI/liver	5 (12)	2 (12)	1 (9)	$P = 1.0$[e]
ESRD	6 (14)	3 (18)	0 (0)	$P = 0.41$[e]
HIV, hepatitis	3 (7)	1 (6)	0 (0)	$P = 1.0$[e]
Substance abuse[d]	3 (7)	1 (6)	2 (18)	$P = 0.48$[e]
Expired before discharge	13 (31)	16 (94)	6 (55)	$P < 0.001$[e]
CPC at discharge				
Favorable (1-2)	27 (64)	1 (6)	5 (45)	$P < 0.001$[e]
Unfavorable (3–5)	15 (36)	16 (94)	6 (55)	
CPC at 6 Months				
Favorable (1-2)	19 (45)	0 (0)	3 (27)	$P = 0.001$[e]
Unfavorable (3–5)	23 (55)	17 (100)	8 (73)	

Values as whole number and (percentage), except in case of continuous variable, given as mean ± standard deviation.
ROSC: return of spontaneous circulation; 1°: primary; VTach, ventricular tachycardia; VFib: ventricular fibrillation; vascular RFs: cardiovascular risk factors.
Values as whole number and (percentage), except in case of continuous variable, given as mean ± standard deviation.
[a]Data available for 34 of 51 arrests; [b]available for 10 of 18 arrests; [c]available for 8 of 11 arrests; [d]substance abuse includes alcohol abuse; [e]Fisher's exact test; [f]analysis of variance (ANOVA).

there was a trend towards a higher proportion of primary cardiac etiologies ($P = 0.07$). Similarly, only one patient out of 18 arrests in the noncooled comatose survivor group was treated with electrical cardioversion, compared to more than 25% of the arrests in the noncomatose and cooled comatose survivor groups, suggesting increased evolution of rhythm to ventricular fibrillation or tachycardia in these latter arrests. Thus, there were some differences in the baseline characteristics in these patient groups which likely affected the duration of arrest. This also meant that, as expected, patients with longer periods of impaired cerebral perfusion were more likely to become comatose.

We should also point out that, within this sample, 38 patients did not survive the arrest itself and overall 65 patients (65%) died before hospital discharge. This rate is higher than reported in previous registries of in-hospital cardiac arrest [1, 2] and may be related to the large majority of patients having an initial rhythm at time of arrest of PEA or asystole (94% overall), which is associated with poorer outcome and has been noted to be the predominant initial

Table 2: Comatose survivors of cardiac arrest that were treated with therapeutic hypothermia.

Patient no.	Date of arrest	Age	Mechanism of arrest	Duration pulseless	Time to initiation	Time to 33°C	Time cooled	Lowest temp.	Rewarming time	Comments/CPC at discharge
1	11/22/2007	55	Suicide attempt, hanging	35 mins	3.5 hrs	11 hrs	24 hrs	N/A	16 hrs	At 72 hrs, intact brainstem reflexes but persistent coma, care withdrawn (CPC 5)
2	1/26/2008	83	Unknown	20 mins	—	—	—	—	—	Cooling stopped when goals of care changed after family arrived; died next day (CPC 5)
3	5/2/2008	59	Variceal bleeding, aspiration	20 mins	—	—	—	—	—	Cooling stopped due to coagulopathy and severe GI bleeding; died next day (CPC 5)
4	5/27/2008	85	Aspiration	30 mins	6 hrs	9 hrs	24 hrs	32.1°C	12 hrs	Neurology called late due to concern for coagulopathy; persistent coma at 72 hrs; care withdrawn a week later (CPC 5)
5	7/27/2008	83	Cardiac Arrhythmia	35 mins	2 hrs	6 hrs	23 hrs	31.3°C	24 hrs	Discharged to rehab unit with short-term memory deficits, required assistance with walking (CPC 2); later diagnosed with gastric cancer, deceased 8 months later
6	8/20/2008	61	Pericardial tamponade	N/A	4 hrs	9 hrs	24 hrs	32.4°C	9 hrs	Initially treated in OR for tamponade; at 72 hrs in persistent coma with myoclonic status epilepticus; ethics consult called and care withdrawn (CPC 5)
7	2/21/2009	57	Hyperkalemia, postextubation	N/A	4 hrs	6 hrs	22 hrs	33.0°C	10 hrs	At 72 hr exam, neurologically normal examination; discharged to rehab. unit, still alive and neurologically intact (CPC 1)
8	2/24/2009	85	Possible vagal hyperactivity	5 mins	2.5 hrs	3 hrs	24 hrs	32.3°C	14 hrs	At 72 hrs, awake and alert, neurologically intact aside from mild confusion (CPC 2); had another arrest 3 weeks later and died
9	5/29/2009	59	Pulmonary embolus, sepsis	5 mins	0.5 hrs	4 hrs	5 hrs	N/A	—	Cooling stopped due to persistent hypotension and multiorgan failure; deceased two days later (CPC 5)
10	2/20/2010	78	COPD, aspiration	N/A	5 hrs	9 hrs	24 hrs	30.5°C	22 hrs	At 96 hrs, alert, briskly following commands; remained ventilator dependent, transferred to rehab. unit (CPC 2); died 6 months later
11	8/10/2010	59	Unknown	25 mins	2.5 hrs	17 hrs	24 hrs	32.0°C	4 hrs	Myoclonus while cooled; rewarming occurred quickly and febrile afterwards; despite normal neuroexamination at 72 hrs; discharged to home, still alive (CPC 1)

rhythm in other in-hospital arrest series as well [24, 25]. Among patients who received cooling, 2 died within the following six months (18%). These observations are also in line with a recent study that demonstrated decreased chances of good outcome from cooling in patients with such nonshockable rhythms [26]. It is possible that patients in the latter category may have more severe disease, but this study did not suggest that such patients were harmed by cooling.

Of the 18 cases in which the survivor was comatose and therapeutic hypothermia was not initiated, these patients were not felt to be candidates for therapeutic hypothermia due to change in goals of care status, hemodynamic instability, or severe coagulopathy and hemorrhage. There were 2

cases where patients might have benefitted from cooling and neurology was not consulted initially. These cases occurred before a formal cooling protocol was adopted by our hospital and relevant staff were educated in its implementation. Since staff training occurred, no further cases were identified. Moving forward, one way to prevent this in the future would be to have every case in which the patient was comatose after cardiac arrest discussed with the on-call neurologist, even in cases where there is a clear contraindication. Other measures to improve our current protocol might include providing a dedicated neurology code pager in code blue protocols and staff training and reorientation at regular intervals.

At our institution, hypothermia was instituted using surface cooling methods and cooling blankets or suits. While our time to target temperature was comparable to previously published studies, it might be possible to further shorten the cooling time by induction with chilled intravenous saline solution. Further, there has also been some concern that surface cooling has been associated with overshooting of goal temperatures which could lead to higher complication rates. Studies have shown that endovascular catheter-assisted hypothermia allows for better control of temperature and faster cooling rates [27]. As such, the incorporation of endovascular cooing catheters might help achieve cooling at a faster rate and prevent overshooting of mild hypothermia goals. However, placement of these catheters requires a trained physician and this can often delay the initiation of cooling if the physician is not readily available [28]. Many community hospitals, including ours, do not always have such trained individuals on staff. Thus, our experience demonstrates that it is still possible to implement a cooling protocol where advanced technologies are not available. It should be noted that, in our series, there were two cases of overshooting beyond 32°C but both of these patients had a favorable CPC score at discharge.

Complications that were noted during the cooling phase and in the week after induction of hypothermia included pneumonia, cardiac arrhythmia requiring administration of medication, deep venous thrombosis, and, in one case, subsegmental pulmonary embolus. It is unclear whether these complications were necessarily related to cooling, as these are also common complications that occur in noncooled cardiac arrest patients. However, a recent study of therapeutic cooling in stroke patients did report a somewhat higher incidence of pneumonia that did not adversely affect eventual patient outcome [29]. Our rates were similar to those described by Prior and colleagues in their series at a community-based hospital [20]. There were no complications associated with rewarming, and although one patient was warmed faster than expected, this was likely related to underlying fever leading to rapid increase in temperature once therapeutic hypothermia was completed.

Limitations of this study are largely related to the retrospective nature of this analysis. Some data points were not recorded in each case, and determination of timing often involved estimates based on ICU nursing charting and by extrapolating from time of arrest indicated by treating physicians. The objective of this study was not to show evidence of the efficacy of therapeutic hypothermia which has been well established but the implementation of a cooling protocol in this particular hospital setting. The lack of a true control group makes it difficult to interpret the overall significance of therapeutic hypothermia beyond comparison of results with previous trials. Although there were patients who were comatose and not cooled, the baseline characteristics of these arrests and the fact that they were *actively* not cooled precludes direct comparison with the patients who were cooled.

5. Conclusion

This work describes our experience over the last three years with therapeutic hypothermia for comatose survivors of cardiac arrest. In contrast to previous reports, the vast majority of our arrests occurred in-hospital, the initial rhythm was PEA or asystole, and many of these patients died prior to hospital discharge. With the exception of 2 cases, therapeutic hypothermia was appropriately started for patients who were comatose and had no contraindication to cooling and the rate of favorable CPC scores at hospital discharge were comparable in our small series to previous reports and the original landmark trials. Clearly, the mortality of in-hospital cardiac arrest is high but we were able to offer a treatment where prior clinical trials showed clear benefit in terms of neurologic recovery. We describe our experience with this treatment which was readily implemented and completed. Reviewing these cases will also help in improving our protocols in the future and demonstrate the role of neurologists in initiating and managing therapeutic hypothermia.

Disclosures

The authors have no relationships or other items to disclose.

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Research Article

A Comparison of Cooling Techniques to Treat Cardiac Arrest Patients with Hypothermia

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Introduction. We sought to compare the performance of endovascular cooling to conventional surface cooling after cardiac arrest. *Methods*. Patients in coma following cardiopulmonary resuscitation were cooled with an endovascular cooling catheter or with ice bags and cold-water-circulating cooling blankets to a target temperature of 32.0–34.0°C for 24 hours. Performance of cooling techniques was compared by (1) number of hourly recordings in target temperature range, (2) time elapsed from the written order to initiate cooling and target temperature, and (3) adverse events during the first week. *Results*. Median time in target temperature range was 19 hours (interquartile range (IQR), 16–20) in the endovascular group *versus*. 10 hours (IQR, 7–15) in the surface group ($P = .001$). Median time to target temperature was 4 (IQR, 2.8–6.2) and 4.5 (IQR, 3–6.5) hours, respectively ($P = .67$). Adverse events were similar. *Conclusion*. Endovascular cooling maintains target temperatures better than conventional surface cooling.

1. Introduction

Each year an estimated 165,000 people in the United States have an out-of-hospital cardiac arrest, and many more undergo in-hospital resuscitation [1–4]. Those that survive may have devastating neurological impairments from global ischemic brain injury. Mild-to-moderate therapeutic hypothermia for 12 to 24 hours has been shown to improve neurological outcome in two randomized clinical trials of comatose survivors following out-of-hospital ventricular fibrillation arrest [5, 6]. Based on the results of these trials, the 2005 American Heart Association guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care recommended to consider the use of therapeutic hypothermia for unconscious adult patients with return of spontaneous circulation after witnessed out-of-hospital ventricular fibrillation arrest (Class IIA) and nonventricular fibrillation and in-hospital cardiac arrest (Class IIB) [7]. The 2010 guidelines continue to recommend therapeutic hypothermia in these patients, now as Class I and Class IIB recommendations, respectively [8].

In spite of the data and guidelines hospitals in the United States have been slow to adopt therapeutic hypothermia in the routine management of comatose postcardiac arrest patients [9]. This may be in part explained by physician unfamiliarity with therapeutic hypothermia and in part by the labor intensiveness and inaccuracy of surface cooling using ice bags and cooling blankets [10]. Most studies to date have used conventional surface-based cooling techniques (ice bags and cool air or water blankets), which are generally

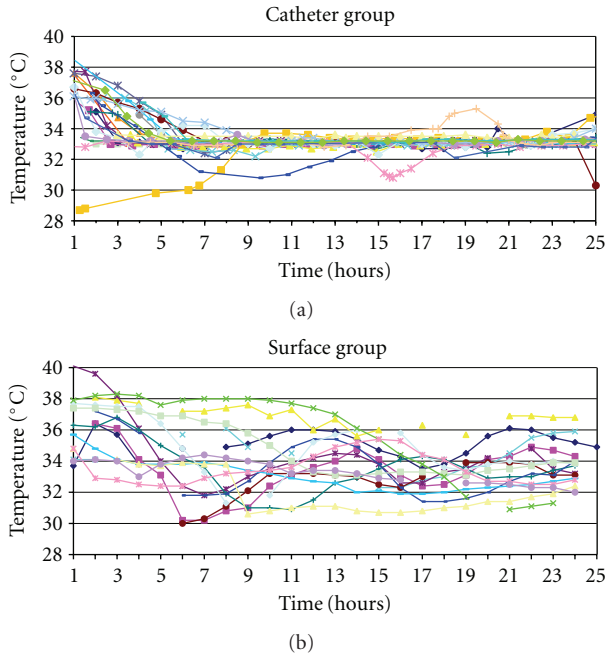

Figure 1: Hourly bladder temperature recordings of each patient from the time point that the cooling protocol was initiated to the end of active cooling (24 hours). (a) Endovascular-cooled group ($n = 26$). (b) Surface-cooled group ($n = 15$).

slow and imprecise in achieving and maintaining target temperature. Animal models suggest that a delay in cooling abates the neurological benefits of mild hypothermia and that deep hypothermia has no added benefit over mild-to-moderate hypothermia [11, 12]. Endovascular cooling techniques have shown promise in providing more rapid and precise temperature regulation than conventional surface cooling techniques and have been shown to be feasible in postcardiac arrest patients, but data on their utility is scarce [13–16]. The purpose of this study is to compare the performance of endovascular versus conventional surface-based cooling in comatose postcardiac arrest patients.

2. Materials and Methods

This was a longitudinal comparative study of consecutive comatose postcardiac arrest survivors, who were treated with our institution's hypothermia protocol (see the appendix). All patients were sedated and paralyzed to ensure comfort and prevent shivering. Target core temperature measured by bladder temperature was 32.0–34.0°C for 24 hours followed by spontaneous or passive rewarming over 12 hours. The start of the 24-hour cooling period was designated as the time of the written order to initiate the therapeutic hypothermia protocol and included the organizational time required for setting up the surface or endovascular cooling equipment and for the catheter insertion. All patients had bladder temperatures recorded hourly from the initiation of the cooling protocol. This study was approved by our institutional review board.

The study spanned the time period between February 2004 and September 2006 (~2.5 years). Between February 2004 and February 2005, hypothermia was induced using cold-water-circulating cooling blankets (Mul-T-Blanket with Gaymar Medi Therm III, Gaymar Industries, Orchard Park, NY) and ice bags. In February 2005 our institution converted to endovascular cooling using the Celsius Control System (Innercool Therapies, San Diego, CA) catheter. This catheter system has a feedback loop controlling target temperature by using a temperature sensing esophageal probe. In patients who underwent endovascular cooling, surface cooling was initiated until the catheter could be inserted. Patients did not receive ice-cooled intravenous fluids. The catheter was removed at the end of the 24-hour cooling period, and spontaneous or passive rewarming occurred over 12 hours.

The performance of endovascular versus surface-based cooling was compared by assessing the following variables: (1) number of hourly recordings in target temperature range (32–34°C) during the 24-hour cooling period, (2) time elapsed from the written orders to initiate the cooling protocol and the target temperature achieved (time required to insert the catheter was included in this time period), and (3) frequency of predefined adverse events possibly related to hypothermia or the use of an endovascular catheter or surface cooling technique during the first 7 days after cardiac arrest. Data was collected using the patients' medical records. Details of the diagnostic criteria for adverse events may be found at the bottom of Table 1.

2.1. Statistical Analysis. Continuous variables were analyzed with the Wilcoxon Rank-Sum test. Categorical variables were analyzed with the Fisher's exact test. Group differences were considered significant at $P < .05$.

3. Results

Forty-one cardiac arrest patients underwent hypothermia between February 2004 and September 2006 at our hospital: 15 with surface and 26 with endovascular cooling. In five patients in whom hypothermia was considered using endovascular cooling, surface cooling was used instead, because of a failed attempt at catheter placement ($N = 1$), a contraindication to catheter placement ($N = 2$) or the general ICU team preferred surface cooling ($N = 2$). There were no differences in baseline characteristics between the two groups except for a higher proportion of patients with ventricular fibrillation arrest in the endovascular group and a strong trend towards a higher proportion of renal failure on admission in the surface cooling group (Table 1). Figure 1 shows the temperature curves of each patient in both groups. The median duration within target temperature range was 19 of the 24 hours (interquartile range (IQR), 16–20) for the endovascular group versus 10 hours (IQR, 7–15) for the surface cooling group ($P = .0001$). Median time from initiation of the cooling protocol to target temperature was 4 hours (240 minutes) (IQR, 2.8–6.2 hours) in the endovascular group versus 4.5 hours (270 minutes) (IQR, 3–6.5 hours) in the surface group ($P =$ NS). All patients in the endovascular group reached target temperature, but

Table 1: Baseline characteristics and adverse events during the first week in the endovascular-($N = 26$) and surface-cooled groups ($N = 15$).

Characteristic/adverse event	Endovascular number (%)	Surface number (%)	P value[a]
Baseline characteristic			
Mean age (years)	63 ± 17	58 ± 15	.28
Sex (males)	18 (69%)	12 (80%)	.22
Mean weight (kg)	82 ± 21	95 ± 37	.15
Duration of the arrest (min)	31	24	.13
Ventricular fibrillation arrests	12 (46%)	1 (7%)	.008
Median time from arrest to initiation of cooling protocol (min)	277	481	.16
Median Bladder T at initiation of cooling (°C)	36.4	36.4	.76
Renal failure on admission[b]	10 (38%)	10 (67%)	.06
Adverse Events			
Hypotension[c]	10 (38%)	4 (27%)	.21
Bradycardia[d]	18 (69%)	8 (53%)	.16
Other arrhythmias	6 (23%)	4 (27%)	.28
New infection in 1st week	16 (62%)	8 (53%)	.23
Pneumonia	14 (54%)	7 (47%)	.23
Sepsis	1 (4%)	0 (0%)	.63
Pancreatitis	0 (0%)	0 (0%)	—
Renal failure 1st week[e]	6 (23%)	4 (27%)	.25
Hemodialysis	1 (4%)	1 (7%)[f]	.48
Coagulopathy[g]	6 (23%)	4 (27%)	.77
Groin hematoma	0 (0%)	N/A	—
Skin injury	0 (0%)	0 (0%)	—
Transfusion pRBC	6 (23%)	4 (27%)	.28
Transfusion of platelets	1 (4%)	0 (0%)	.63
Seizures[h]	2 (8%)	2 (13%)	.33
Deep venous thrombosis[i]	3 (12%)	1 (7%)	.38
Pulmonary embolism	1 (4%)	0 (0%)	.63

[a] P values calculated from Fisher's exact test and Wilcoxon rank-sum test; [b] creatinine ≥1.5 mg/dL; [c] mean arterial pressure <70 mm Hg and requiring pressors; [d] heart rate <60/min; [e] rise in creatinine ≥0.5 mg/dL; [f] already on hemodialysis as outpatient; [g] INR >1.5 or PTT > 40 sec within 48 hours of cooling; [h] not including clinical evidence of status myoclonus.

one patient in the surface group never did. Three patients (12%) in the endovascular group had temperatures below 32°C for more than one hour, compared to 7 (47%) in the surface group ($P = .022$). However, one of the endovascular patients was initially treated with surface cooling and started off below target temperature. Median time from actual physical insertion of the catheter to target temperature was 114 minutes (IQR, 30–199). The frequency of predefined adverse events related to cooling or cooling technology in the first week was similar in both groups (Table 1). There was a trend towards more frequent hypotension and arrhythmias in the endovascular group. No catheter-related procedural complications and no skin injuries were observed. Mortality was 62% (16/26) for the endovascular group and 73% (11/15) for the surface group. Twenty-six percent of the patients in the endovascular group were discharged home, 4% to a rehabilitation facility, and 8% to a nursing home. Of the surface group, 7% went home, 7% went to a rehabilitation facility, and 13% went to a nursing home.

4. Discussion

The results of this study demonstrate that temperature control using the Celsius Control System Innercool catheter is more accurate in keeping patients in the target temperature range than surface cooling with ice bags and cooling blankets. Two similar studies also found that endovascular cooling is superior to surface cooling in maintaining a target temperature [15, 16]. Further, another study found that unintentional overcooling occurred in the majority of patients (63%) treated with ice bags and conventional cooling blankets [17]. In our study this occurred in 12% of the patients in the endovascular group and 47% of the surface group. Accurate temperature control is important

as body temperatures above target temperature may offer less neuroprotection and body temperatures below target may produce more pronounced side effects. The observed trend of higher frequencies of hypotension and bradycardia in our endovascular group probably may reflect an overall longer duration of lower body temperatures in this group during the cooling maintenance phase compared to the surface group but it may also be related to the position of the catheter in relative proximity to the heart.

We felt that a pragmatic comparison between the two cooling methods in our study was the most appropriate and decided to compare them from the time point that it was decided to initiate the cooling protocol to reaching target temperature, hence including the organizational time required to implement each one of the two cooling techniques. After all, if one technique cools patients faster than another, but requires more time to initiate because of process issues, its apparent benefit may be negated. We observed a trend to reach target temperatures faster in the endovascular-cooled group despite the additional time required to move the patient to an intensive care unit bed or the cardiac catheterization laboratory prior to insertion of the catheter. Furthermore, sometimes a neurointensivist traveled from home to place the catheter. Adjunct cooling methods, such as ice-cooled intravenous fluid infusions, might have decreased the time to target temperature in both groups but were not used during the study period [18].

There was a trend towards better neurologic outcomes in the endovascular group. This finding may be based on imbalances in baseline characteristics and should not be interpreted as a suggestion of better clinical outcomes with endovascular cooling. Further, with increasing familiarity with the use of hypothermia at our institution, the time from arrest to initiation of cooling protocol decreased over time and tended to be less in the endovascular group. Thus, data of this study cannot be used to compare the clinical benefit of endovascular with surface cooling.

While this study did not specifically measure nursing satisfaction with the two cooling techniques, part of the reason we changed to endovascular cooling from surface cooling was that the nursing staff found the surface method very labor intensive. The application and removal of the ice bags and cooling blankets to maintain the target temperature was difficult and time consuming for the nursing staff. Despite the nurses efforts some patients would remain above or below the target range. The endovascular method, once in place, frees the nurse to focus on other duties, because temperature is automatically maintained in target range. Both techniques require an additional piece of equipment to be in the patient's room and make transporting a patient more cumbersome. Special attention must be paid by the nurse when turning or transporting the endovascularly cooled patient as the patient's leg must be kept straight on the insertion side. For the physician the endovascular technique is more labor intensive, and for those unfamiliar with the device it will require procedural training for placement.

Limitations of this study include its nonrandomized design and imbalances in the baseline characteristics between the two groups; however, we intended to compare performance of cooling technologies and not patient outcome. It is possible that newer, more sophisticated surface cooling technology, such as adhesive surface devices with patient temperature feedback to computer-controlled temperature management systems, performs even better than endovascular cooling techniques. Similarly, there may also be differences in performance among various commercially available cooling catheters. Other studies have reported effective temperature control and safety with the Icy catheter and CoolGard system [13, 14]. Finally we did not do a cost comparison between the two groups.

5. Conclusions

Endovascular cooling to mild-to-moderate hypothermia is feasible in comatose postcardiac arrest patients. When compared with conventional surface cooling it is more accurate in maintaining target temperature, but it is not faster in terms of reaching target temperature, because of time lost to logistic issued associated with catheter insertion and setting up the cooling console. Further studies are needed to assess if differences in cooling accuracy translate into better clinical outcomes.

Disclosure

The authors have reported no conflicts of interest. The use of the Celsius Control System Innercool catheter for cooling postcardiac arrest patients is offlabel usage of the device.

Appendix

A. Stanford Therapeutic Hypothermia after Cardiac Arrest Protocol

This is adapted from the University of California San Francisco hypothermia after cardiac arrest cooling protocol with permission.

A.1. Inclusion Criteria

(1) Age 18 years or older.

(2) Women must be over 50 or have a negative pregnancy test.

(3) Cardiac arrest with return of normal sinus rhythm.

(4) Persistent coma as evidenced by no eye opening to pain after resuscitation (no waiting period required).

(5) Blood pressure can be maintained at least at 90 mm Hg systolic either spontaneously or with fluid and pressor (no aortic balloon pump unless approved by cardiology).

(6) Modified Rankin scale 0–2 prior to the arrest.

A.2. Exclusion Criteria

(1) Any other overt reason to be comatose (e.g., sedating drugs, drug overdose, status epilepticus).

(2) Pregnancy.

(3) A known terminal illness preceding the arrest.

(4) Known pre-existing coagulopathy or bleeding.

(5) Pre-existing do not intubate code status and patient not intubated as part of the resuscitation efforts.

A.3. Protocol (Goal Temperature 33.0°C to Be Achieved as Soon as Possible)

(1) Hypothermia should be initiated as quickly as possible. For out-of-hospital arrests the ED attending in conjunction with the neurocritical care/stroke team will make the decision to implement the protocol. For in-hospital arrests, the neurocritical care/stroke team in conjunction with the CCU or ICU fellow in charge of the patient will make the decision.

(2) Page Neurology resident in house for immediate neurological assessment prior to pharmacologic paralysis. Do not delay initiation of cooling pending assessment.

(3) Immediately place ice bags under the armpits, next to the neck, on the torso and limbs.

(4) Temperature sensing Foley catheter should be placed, otherwise rectal or tympanic temperatures should be used (in that order) until Foley placement.

(5) Two cooling blankets should be used, one under and one over the patient. Both should be set to maximum cooling.

(6) The room thermostat should be turned off.

(7) Administer midazolam and fentanyl for sedation or other sedatives as ordered by the primary team.

(8) Once sedation is started and effective, give a vecuronium bolus, then continuous drip for paralysis or other paralyzing agent as ordered by the primary team. Titrate the drip to keep 1–2/4 twitches on train of four.

(9) Patients should be on sliding scale insulin to maintain glucose between 151–200 mg/dL, daily aspirin, pressors to maintain blood pressure, and any antiarrhythmics as necessary. Place nasogastric tube for meds.

(10) Patients may receive other cardiac interventions including systemic thrombolysis, anticoagulation, and urgent cardiac catheterization interventions as needed. Hypothermia should proceed concurrent with these interventions.

(11) Once the patient reaches 34°C, remove the ice packs and top cooling blanket if necessary. The goal is for the patient's temperature to remain between 32–34°C.

(12) Begin passive rewarming 24 hours after the beginning of cooling (not 24 hours after target temperature is reached). The goal is to reach 36.5–37°C (If temperature increases over 37.5°C restart cooling blankets).

(a) Turn room thermostat up to normal.
(b) Discontinue cooling blankets.
(c) May use regular blankets.
(d) Do not use warm air blankets unless temperature not at 36°C after 12 hours of passive rewarming.

(13) Paralysis, then sedation, may be discontinued after rewarming to 36.5°C.

A.4. Supplement—Use of Endovascular Cooling Catheter.

(1) The neurocritical care/stroke team should be paged as part of the standard protocol for considering hypothermia following cardiac arrest. The neurocritical care/stroke team will evaluate the patient for endovascular cooling.

(2) External cooling should be initiated immediately as per the standard protocol, pending evaluation for endovascular cooling. Patient must be able to tolerate large-bore catheter (as large as 14 F) into the inferior vena cava via a femoral vein.

(3) If possible inform family members of the procedure and its risks and benefits and make a note of this in the chart.

(4) Equipment needed for endovascular cooling:

(a) innercool console,
(b) console-related equipment,
(c) cooling catheter equipment.

(5) The neurocritical care/stroke team will place the endovascular catheter. Cooling will then be initiated using the innercool console to a target temperature of 33°C for 24 hours. All external cooling elements may be discontinued. Rewarming will take place passively over 12 hours to a target temperature of 36.5–37°C.

(6) An abdominal X-ray should be obtained following placement of the catheter, but initiation of the endovascular cooling should not be delayed pending the result of the study.

(7) Sedation, paralytics and mechanical ventilation should be employed as part of the standard hypothermia after cardiac arrest protocol.

(8) Patients may receive other cardiac interventions, including systemic thrombolysis, anticoagulation and urgent cardiac intervention as needed.

(9) The neurocritical care team will remove the catheter at the end of the 24-hour cooling period.

(10) Start heparin subcutaneously 5000 unit q 12 hours, 12 hours after the catheter is removed.

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Review Article

Limited Therapeutic Time Windows of Mild-to-Moderate Hypothermia in a Focal Ischemia Model in Rat

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Although many studies have shown the great potential of induced hypothermia in stroke treatment, we recognize that there are limitations to the protective effects of hypothermia even in the laboratory. Here, we review our experiments on the protective effects of mild-to-moderate hypothermia in rats. Focal ischemia was induced by bilateral common carotid artery (CCA) occlusion for 1 to 2 hours combined with permanent or transient middle cerebral artery (MCA) occlusion. We compared the effects of mild (33°C) and moderate (30°C) hypothermia, evaluated therapeutic time windows, and studied the underlying mechanisms. On review, our findings revealed that the protective effects of induced mild hypothermia (33°C) were limited, and the therapeutic time window of even moderate hypothermia (30°C) was very short in our specific models, although this limitation might be due to the relatively brief periods of hypothermia used. In addition, we found that hypothermia reduced brain injury by preserving Akt activity, PTEN phosphorylation and εPKC activity, while inhibiting ROS production, and δPKC activity.

1. Introduction

One of the gold standards of neuroprotectants against stroke in animal experiments [1, 2] induced mild (33 to 36°C) to moderate (28 to 32°C) hypothermia has been the focus of several clinical trials for the treatment of cerebral ischemia. In the past decade, prospective randomized controlled studies have demonstrated that induced hypothermia improves neurological function in patients suffering cardiac arrest from ventricular fibrillation [3] and reduces risk of death or disability in neonates following hypoxic-ischemic encephalopathy [4, 5]. However, the clinical translation of hypothermia for acute stroke treatment is still in its early stages. Many barriers remain, including onset time, duration, and depth of hypothermia [6].

In the process of extrapolating animal studies to human patients, significant gaps exist even between the design of laboratory experiments and clinical trials. For instance, many previous animal models used complete reperfusion [7–9], while most stroke patients suffer from permanent cerebral artery occlusion [10, 11]. Even with t-PA treatment, slightly less than one third of patients achieve complete reperfusion, one-third achieve partial reperfusion, and in the rest reperfusion is absent [11, 12]. Therefore, the ability to select animal stroke models that properly mimic clinical stroke is a critical step in evaluating the protective effects of induced hypothermia.

Our laboratories have studied the protective effects of mild-to-moderate hypothermia for nearly two decades [6, 13–17]. Our recent hypothermia studies use a focal ischemic model with partial reperfusion in rats [16, 18, 19]; a model which is less frequently used in other laboratories. In this model, stroke is induced by bilateral common carotid artery (CCA) occlusion combined with permanent distal middle cerebral artery (MCA) occlusion [16, 20–22]. The bilateral CCAs are reopened 1 to 2 hours later while the distal MCA remains occluded [16, 19, 23, 24]. This technique therefore allows partial reperfusion [25, 26]. As discussed above, this model mimics many stroke patients who receive partial reperfusion, with or without t-PA treatment. However, to compare the protective effects of hypothermia in focal ischemia with partial reperfusion and complete reperfusion,

we also used a model with transient three-vessel (bilateral CCAs and distal MCA) occlusion [18].

Several excellent articles have reviewed the protective effects of hypothermia as function of onset time, duration, and depth of hypothermia, as well as its underlying protective mechanisms [27–30]. Particularly, van der Worp et al. have comprehensively reviewed past hypothermic studies [29], which either used temporary or permanent occlusion models. However, the protective affects of hypothermia in stroke models using partial reperfusion as described above have received significantly less attention. Therefore, this paper focuses mainly on our studies of the past several years on therapeutic time windows and the unique model of partial reperfusion.

2. Intraischemic Moderate Hypothermia Offers Strong and Long-Term Protection in a Focal Ischemic Model with Partial Reperfusion

In our first implementation of an ischemic model [16], we cauterized the distal MCA above the rhinal fissure and transiently occluded the bilateral CCAs for 1 hour. This model generates a well-delineated ischemic area limited to the cortex [20, 22]. Moderate hypothermia (30°C) monitored at the core body temperature was induced 10 minutes before ischemia onset and maintained for 1 hour after ischemia onset [16]. Although we did not directly monitor brain temperature, we previously observed a high correlation between rectal temperature and brain temperature in hypothermic rats [21]. We should add that because brain temperature in normothermic rats drops spontaneously during occlusion, core temperature may not accurately reflect brain temperature [2, 31]. Even so, we did not experimentally adjust any potential changes in brain temperature in order to minimize the introduction of possible artificial factors, which would likely exacerbate ischemic injury once the brain was heated. Our results showed that hypothermia reduced infarct size more than 80% compared with normothermia at 2 days after stroke (Figure 1(a)) [16]. Because some neuroprotectants offer transient protection, we also measured brain injury 2 months later and found similar protective effects at 60 days and 2 days (Figure 1(b)), suggesting that hypothermia decreases ischemic damage over the long term rather than merely delaying its emergence. This protective effect is further strengthened by the effects of hypothermia on behavioral deficits after stroke, which showed that hypothermia improved neurological functioning for up to 2 months [16].

We then used this model to study the underlying protective mechanisms related to the PI3K/Akt cell signaling pathway [16]. The PI3K/Akt kinase pathway is known to promote neuron survival postischemia (reviewed by [32]) Figure 2. Akt activity is regulated by phosphorylation at Ser-473 and Thr-308 via upstream molecules, such as PDK1 and PTEN. While activated PDK1 phosphorylates Akt, activated PTEN dephosphorylates Akt. Activated Akt then blocks caspase/cytochrome c-mediated apoptosis by phosphorylating Akt substrates, such as FKHR and GSK3β. In our study,

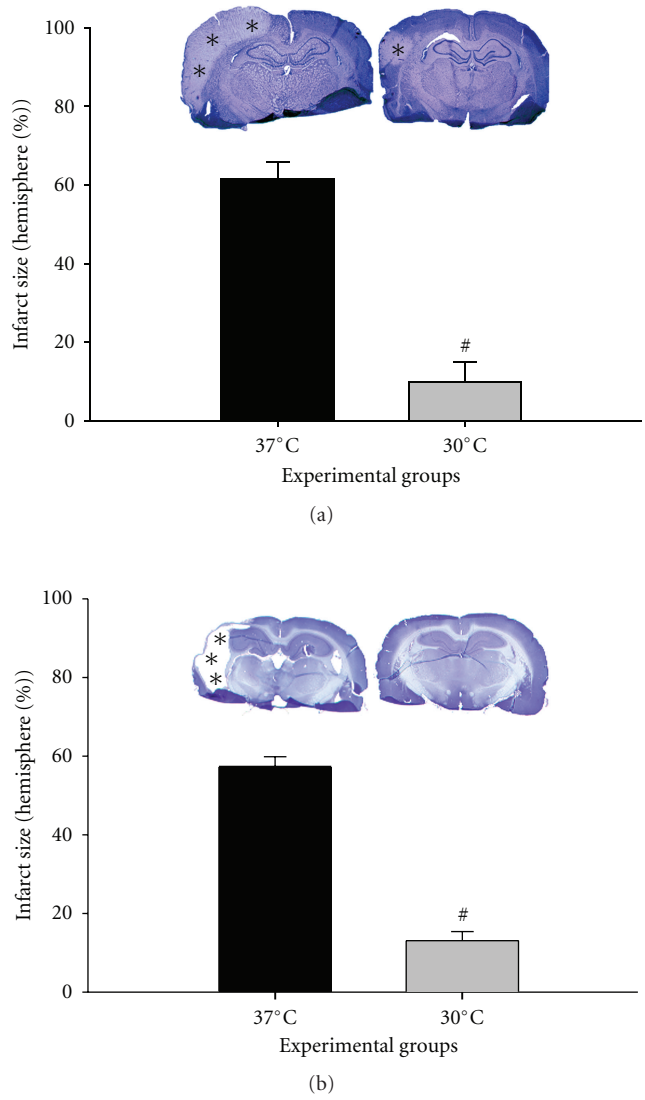

Figure 1: (Revised from [16]). Intraischemic moderate hypothermia (30°C) reduces infarct size in a focal ischemia with partial reperfusion. Focal ischemia was induced by 1 h of bilateral CCA occlusion and permanent dMCAo. Body core temperature was lowered to 30°C 10 min before stroke onset by spraying 70% alcohol on the rat body. (a) The upper panel shows representative infarcts stained with cresyl violet from rats euthanized 2 d after stroke. The pale area with asterisks represents the infarct region. Normothermic ischemia damaged the cortex ipsilateral to the occluded MCA, whereas hypothermia spared all or most of the injured cortex. Only a small lesion was observed in the presented section from a hypothermic rat. The bar graphs represent statistical analysis of infarct size 2 d after stroke. Two-way ANOVA (two factors, temperature and brain section level) was used to compare the effect of temperature on the infarct size at each level (data not shown) and on the mean of all 4 levels. Hypothermia ($n = 7$) reduced the mean infarct size by 80% compared with normothermia ($n = 7$; $P = 0.001$). (b) The upper panel shows representative sections stained with cresyl violet from animals surviving 2 months after stroke. Most of the cortex in the infracted hemisphere was lost in normothermic but not hypothermic rats. The lower panel of bar graphs shows infarct size 60 d after stroke. Hypothermia ($n = 9$) reduced infarct size 60 d after stroke compared with normothermia ($n = 8$; $P = 0.001$). # versus 37°C, $P < 0.001$.

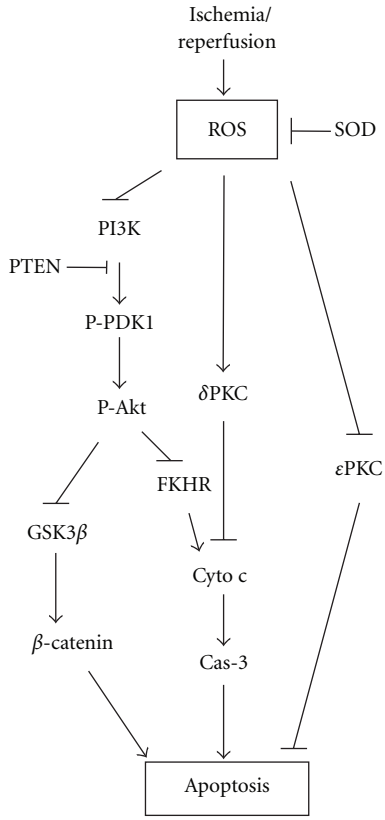

Figure 2: Diagram showing the major cascades that occur after stroke reviewed in this paper. AD: anoxic depolarization; AIF: apoptosis-inducing factor; BBB: blood brain barrier; CBF: cerebral blood flow; cyto c: cytochrome c; Fas L: Fas ligand; FKHR: Forkhead homologue in rhabdomyosarcoma; Glu: glutamate; GSK 3 β: glycogen synthase kinase 3β; MMP: matrix metalloprotease; NOS: nitric oxide synthesis; NO: nitric oxide; $ONOO^-$: peroxynitrite; PI3K: phosphoinositide 3-kinase; PIP2: phosphatidyliositol-4,5-bisphosphate; PIP3: phosphatidyliositol-3,4,5-bisphosphate; PKC: protein kinase C; P-Akt: phosphorylated Akt; PTEN: phosphatase and tensin homologue deleted on chromosome 10; P-PDK1: phosphorylated phosphoinositide-dependent protein kinase-1; ROS: reactive oxygen species; RTK: receptor tyrosine kinase; VDCC: voltage-dependent calcium channel.

stroke resulted in transient increases in phosphorylated Akt (P-Akt) levels, but led to a reduction in phosphorylation levels of PTEN, PDK1, GSK3β, and FKHR [16]. However, in vitro Akt kinase assays showed that true Akt activity was decreased after stroke. Although hypothermia blocked the increase in P-Akt after stroke, it maintained true Akt activity. A functional role for this hypothermia-maintained activity is supported by the finding that the PI3K/Akt inhibitor, LY294004, enlarged infarct size in hypothermic animals. In addition, hypothermia attenuates a decrease in P-PTEN after stroke onset. Taken together, our results suggest that the PI3/Akt pathways play a critical role in the neuroprotection observed in intraischemic moderate hypothermia [16].

We also studied the potential roles of two critical components in the protein kinase C (PKC) pathway: δPKC [24] and εPKC [23]. δPKC is a kinase strongly implicated in executing ischemic damage while εPKC is neuroprotective [33]. We found that intraischemic hypothermia (30°C) blocks translocation of δPKC to the mitochondria and nucleus and attenuates δPKC cleavage [24], but it promotes εPKC activity, as evidenced by increased εPKC phosphorylation levels [23]. Therefore, our results suggest that both δPKC and εPKC may participate in the protective effects of intraischemic moderate hypothermia.

3. Intraischemic Mild Hypothermia (33°C) Fails to Offer Protection in a More Severe Ischemic Model with Partial Reperfusion

In our second study we compared the protective effects of mild (33°C) and moderate hypothermia (30°C) [19] either transiently induced during or after CCA occlusion or maintained during and after CCA occlusion. For stroke models, we extended the bilateral CCA occlusion period from 1 to 2 hours, while the distal MCA remained occluded (Figure 3) [19]. The hypothermic duration at both temperatures was either 2 hours during or after CCA occlusion or 4 hours during and after CCA occlusion. We found that 2 hours of mild hypothermia (33°C) induced either during or after CCA occlusion did not confer protection [19]. This was unexpected because our previous study showed that 2 hours of intraischemic hypothermia (33°C) reduced infarct size in a 2-hour MCA suture occlusion model in rats [14]. In addition, as van der Worp et al. [29] reviewed, previous studies have reported a substantial reduction in infarction even at 35°C, when hypothermia commenced before or at the start of MCA occlusion, with protective effects that were not clearly time dependent.

In our study, however, 4 hours of mild hypothermia applied during and after CCA release slightly, but significantly, reduced infarct size by 22%. When we further reduced hypothermia from 33°C to 30°C, 2 hours of moderate hypothermia during CCA occlusion increased protection, significantly reducing infarct size by 46% (Figure 3). Nevertheless, 2 additional hours of moderate hypothermia (4 hours total) did not offer additional protection, suggesting a limited effect of prolonged moderate hypothermia applied during and after CCA release [19].

Using confocal microscopy and Western blotting, we found that when intraischemic hypothermia reduced infarct size, the subcellular translocation of cytochrome c and apoptosis-inducing factor (AIF) was blocked in the ischemic penumbra. However, when hypothermia (either intraischemic or delayed mild hypothermia) did not reduce infarct size, no effect was observed on these proapoptotic factors [19]. This suggests that inhibition of cytochrome c and AIF release corresponded to the protective effect of hypothermia.

4. Limited Therapeutic Time Windows of Moderate Hypothermia (30°C) in a Focal Ischemia with Complete Reperfusion

After comparing the protective effects of both mild and moderate hypothermia in severe ischemic models with

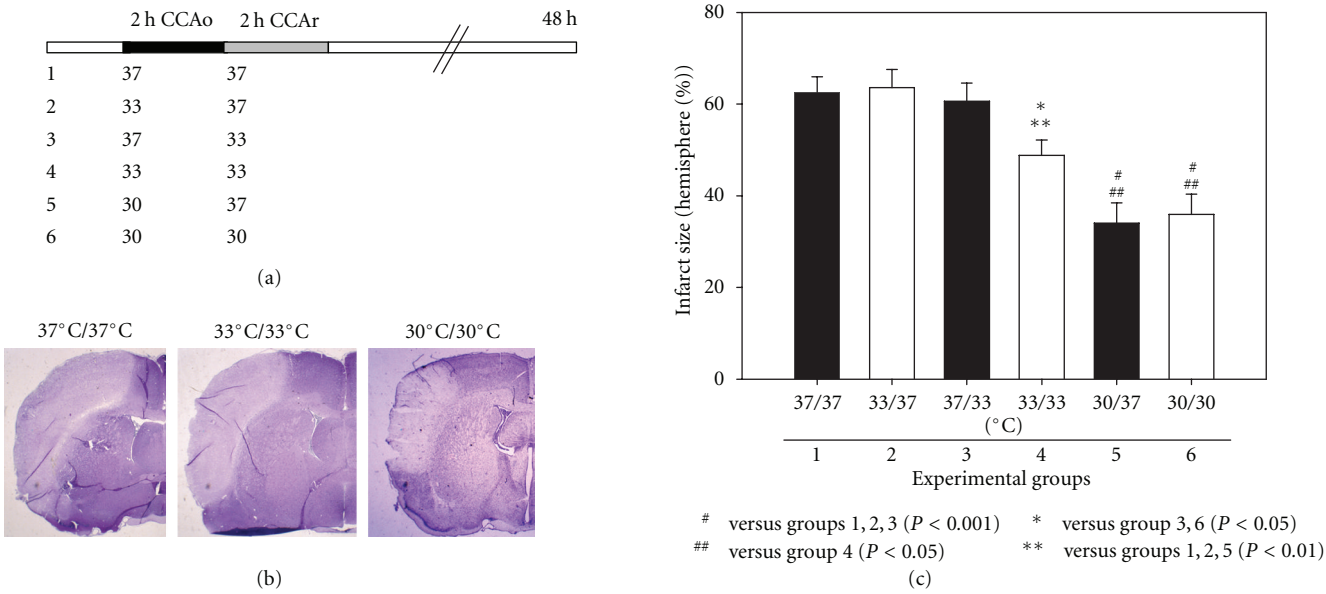

Figure 3: (Revised from [19]) (a) Protocols for surgery and temperature management. Six groups of rats were studied. The distal MCA was occluded permanently. The *black portion* of the bar represents bilateral CCA occlusion (CCAo) for 2 h, and the *gray portion* indicates 2 h of temperature management after CCA release (CCAr), including 30°C, 33°C, and 37°C. Rats were allowed to survive for 48 h after stroke. (b) Photographs of representative infarct sections after cerebral ischemia from groups 1, 4, and 6. Permanent distal MCA occlusion plus 2 h of bilateral CCA occlusion caused an infarct in the ipsilateral cortex of the occluded MCA (*left,* group 1). A coronal section from Level 2 is presented. Four hours of mild hypothermia (*center,* group 4) mildly decreased infarct size. When the temperature was reduced to 30°C robust protection was observed (*right,* group 6). (c) Bar graph showing that hypothermia reduces infarct size after stroke only under certain conditions. A mean infarct size for each group was calculated as the sum of all 4 levels for each animal divided by the number of animals in each group. The infarct size did not differ among groups 1 through 3. However, the infarct in group 4 was reduced about 22% relative to group 1. When the temperature was decreased to 30°C (group 5) robust protection was observed; an additional 2 h of hypothermia in group 6 did not further reduce infarct size.

permanent distal MCA occlusion, we were not optimistic that mild hypothermia (33°C) could achieve protection. Thus, we focused on the therapeutic time window for moderate hypothermia (30°C) in a transient focal ischemic model with 1 hour of CCA and distal MCA occlusion, which allows complete reperfusion (Figure 4) [18]. Our aim was to determine the potential therapeutic time window for a brief moderate hypothermia in a less severe ischemic model. We found that 3 hours of moderate hypothermia started immediately after stroke onset spared almost all infarction (Figure 4(b)), and 3-hours of early moderate hypothermia induced 45 minutes after CCA occlusion markedly reduced infarction by more than 80%, whereas delayed hypothermia initiated 15 minutes after reperfusion did not prevent ischemic damage (Figure 4(b)) [18]. Together, these results suggest a very short therapeutic time window for a brief, moderate hypothermia.

Our study on therapeutic time windows is limited by the short 3-hour duration of hypothermia. It is highly likely that the delayed onset of hypothermia would have been protective if prolonged hypothermia had been used. For instance, Colbourne et al. found that prolonged hypothermia (24 hours of 33°C plus 24 hours of 35°C) started 2.5 hours after the onset of ischemia robustly reduced infarct volume and attenuated behavior deficits in a focal ischemia model with a 90-minute MCA occlusion in rats [34]. Clark et al. reported that hypothermia (33°C) lasting 12, 24, or 48 hours was required to reduce infarct size and improve functional outcomes when hypothermia was instituted 1 hour after permanent distal MCA and CCA occlusion, and prolonged hypothermia (24 or 48 hours) was better than shorter hypothermia (12 hours) [35]. Furthermore, delayed hypothermia beginning 1 hour after ischemia appears to require prolonged periods (12 to 24 hours) to generate protection even for global ischemia lasting just 5 minutes [36]. Therefore, the limited therapeutic effects of post-ischemic hypothermia in our studies may be specific to the experimental settings in our laboratory.

Consistent with its protective effects, early hypothermia, but not delayed hypothermia, blocked TUNEL positive staining, a marker for apoptosis or cell death [18]. In addition, we found that early hypothermia attenuated the generation of superoxide compared with normothermia. However, both early and delayed hypothermia attenuated reductions in Mn-SOD protein levels and δPKC cleavage in the ischemic penumbra, suggesting that both Mn-SOD and δPKC cleavage may not be responsible for the differential protective effects of early and delayed hypothermia [18]. In addition, both early and delayed hypothermia preserved Akt phosphorylation. Nevertheless, only early hypothermia, but not delayed hypothermia, maintained PTEN phosphorylation (P-PTEN) [18], suggesting that P-PTEN may play

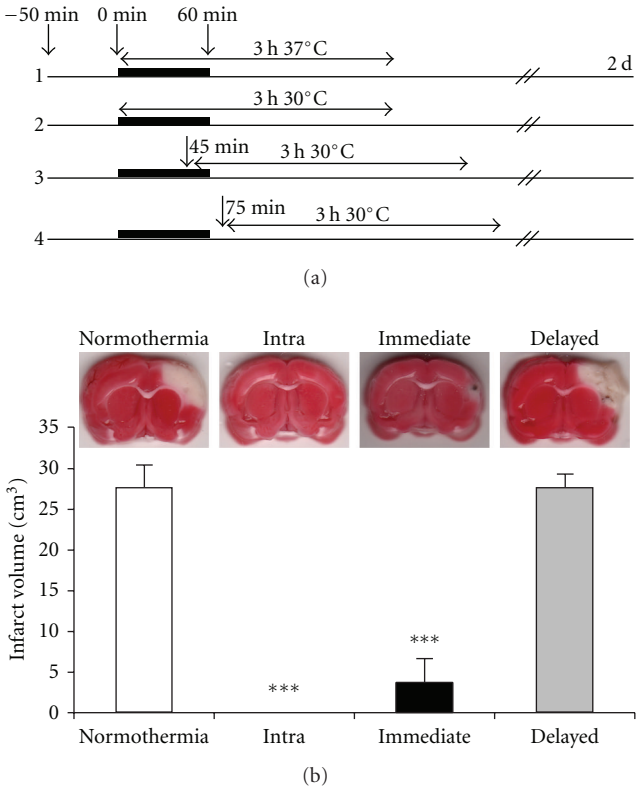

Figure 4: (revised from [18]) Limited therapeutic time windows for post-ischemic moderate hypothermia in a focal ischemia with complete reperfusion. (a) A diagram for experimental procedures comparing the protection of hypothermia. Rats were divided into 4 groups. Group 1, normothermia: body temperature was maintained at 37°C throughout the experiment. Group 2, intraischemic hypothermia: hypothermia was induced at ischemic onset and maintained for 3 h. Group 3, early hypothermia: body temperature was adjusted to 30°C 15 min before reperfusion and maintained for 3 h. Group 4, delayed hypothermia: body temperature was adjusted to 30°C 15 min after reperfusion and maintained for 3 h. (b) The upper panel shows representative infarcts stained by TTC. White areas are the infarct regions. The lower panel shows quantitation of infarct volumes. Values are mean ± S.E.M. ($n = 8$ per each group). $^{***}P < 0.0001$, versus normothermia.

a critical role in the protective effects of early hypothermia through the attenuation of ROS activity.

5. Discussion

As we have discussed, hypothermic studies performed in the laboratory have led to clinical investigations for cerebral ischemia. Significant enthusiasm for this approach still exists in the scientific community. A number of preliminary clinical trials (mostly phase I) to confirm the feasibility and safety of induced mild hypothermia for stroke patients have been completed, and several phase II clinical trials are currently in progress (http://clinicaltrials.gov/). However, whether mild-to-moderate hypothermia can be successfully translated clinically or, if successful, how long this will take has yet to be determined.

The purpose of our basic research using animal models is to provide the rationale for clinical translation, although we cannot directly extrapolate settings from the laboratory to clinical trials. As discussed, our laboratory experiment is limited due to the short 3-hour duration of hypothermia, which contrasts to human clinical trials where hypothermia may last a few days. In addition, our study used infarct size as the criteria for evaluating the protective effects of hypothermia and not neurological function, as is often the case in clinical studies. Despite these limitations, our results serve as a warning of the persistent challenges we must confront as we seek to translate hypothermia to the clinic.

First of all, the most strikingly disappointing results from our studies are the limited protective effects of hypothermia, including mild hypothermia, and the short therapeutic time window of moderate hypothermia. If these observations are true, successful clinical translation of induced hypothermia may prove to be more difficult than anticipated to achieve.

For example, we demonstrated that even intraischemic mild hypothermia (33°C) induced before ischemic onset failed to reduce infarct size in a focal ischemia model with permanent distal MCA occlusion and partial reperfusion upon bilateral CCA release. This model may be more severe than the model of MCA suture occlusion with reperfusion used by most laboratories, but we have no reason to believe it is more severe than strokes in humans. As previously discussed, many stroke patients suffer from permanent cerebral artery occlusion without reperfusion. To achieve protection, even our experimental ischemic models required reducing intraischemic hypothermia to 30°C or prolonging intraischemic mild hypothermia beyond CCA release. However, applying intraischemic hypothermia before stroke onset in clinical trials is nearly impossible, and inducing hypothermia in stroke patients beyond 33°C to 30°C is very difficult. Clinical trials often use mild rather than moderate hypothermia, and it takes significantly longer to reach the target temperature compared to experimental stroke in animal models.

Nevertheless, as we reviewed previously [6], other groups have shown that intraischemic mild hypothermia elicits protection even in permanent MCA occlusion models, in contrast to our recent studies. Our negative findings may simply reflect our specific setting and use of a unique model.

Second, the therapeutic time window for moderate hypothermia is extremely narrow after stroke onset, even in the 1-hour transient focal ischemic model. To achieve protection, 3 hours of moderate hypothermia must be induced as early as 45 minutes after stroke onset; a 30-minute delay rendered the moderate hypothermia ineffective. Again, it is highly unlikely that most stroke patients can receive hypothermic treatment within 1 hour of stroke onset. In most clinical studies, mild-to-moderate hypothermia was initiated as late as 5 to 6 hours after stroke, and one to several hours were required to reach target temperatures [37, 38]. In addition, patients may not have reperfusion, or if there is reperfusion, it may occur at a very late stage.

Our studies on the underlying protective mechanisms may also offer some alternative clues or applications for clinical trials. For instance, we demonstrated that hypothermia

reduces infarct size by preserving Akt activity and PTEN phosphorylation and by inhibiting ROS activity. If possible, pharmacological agents may be developed that improve Akt activity while inhibiting PTEN activity, or attenuating ROS production, and such pharmacological agents may be used in combination with induced hypothermia.

In summary, despite confounding issues, laboratory studies have provided strong rationale for clinical application of hypothermia for acute stroke treatment. In clinical settings, a number of crucial variables need to be considered, including the onset time of hypothermia, its depth, and whether the strokes studied include reperfusion. Early reperfusion and rapid hypothermia initiation should be used to achieve maximal protection.

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