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

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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 effects 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](https://example.com/figure1.png)

![Figure 2](https://example.com/figure2.png)

**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, \( P < 0.001 \).
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
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
through the attenuation of ROS activity.

was adjusted to 30° maintained for 3 h. Group 3, early hypothermia: hypothermia was induced at ischemic onset and

has yet to be determined.

whether mild-to-moderate hypothermia can be successfully

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 clin-
cial trials. For instance, we demonstrated that hypothermia

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

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.
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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