Combined use of a cytoprotectant and rehabilitation therapy after severe intracerebral hemorrhage in rats

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Abstract

After moderate intracerebral hemorrhage (ICH), both hypothermia (HYPO) and constraint-induced movement therapy (CIMT) improve recovery and reduce the volume of brain injury. We tested the hypothesis that more severe ICH requires both cytoprotection and rehabilitation to significantly improve recovery. Rats were subjected to a unilateral striatal ICH via collagenase infusion. Rats remained normothermic or were subjected to mild HYPO (\textdegree{}2 days) starting 12 h later. Fourteen days after ICH, half of the rats received CIMT (7 days of restraint of the less affected limb plus daily exercises); the remainder were untreated. Walking, limb use and skilled reaching were assessed up to 60 days, at which time animals were euthanized and the volume of tissue lost was determined. The HYPO treatment alone did not improve outcome, whereas CIMT alone provided significant benefit on the limb use asymmetry test. In the staircase test, the greatest benefit was achieved with the combination of HYPO and CIMT treatments. The volume of tissue lost after ICH was similar among groups arguing against cytoprotection as a mechanism of functional recovery. Finally, these findings suggest that, at least under the present circumstances (e.g., severe striatal ICH), CIMT provides superior benefit to HYPO and that combination therapy will sometimes further improve recovery.

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Furthermore, HYPO is a safe and feasible treatment for stroke in humans [41] and provides benefit after out-of-hospital cardiac arrest [43]. Experimentally, however, greater benefit is conferred after mild to moderate insults than after severe global [4,5] or focal ischemia [39]. Thus, additional therapies (e.g., rehabilitation) may be needed in conjunction with HYPO to maximally promote recovery after extensive stroke damage. Constraint-induced movement therapy (CIMT) is a rehabilitation approach that involves restraining the less affected limb (ipsilateral to the lesion) to force the use of the impaired limb during daily activities and rehabilitation exercises. It has been repeatedly shown to improve recovery in ischemic stroke and trauma patients [36,42,45]. In rats, 7 days of CIMT coupled with daily exercises significantly improved motor performance when initiated 1 week after a moderate-sized ICH [12]. This regimen also reduced the volume of tissue lost, perhaps by reducing cell death and atrophy. CIMT is thought to promote functional improvement by overcoming learned nonuse of the affected limb, cortical reorganization [24,27,42] as well as other effects such as enhanced dendritic growth, and alterations in neurotrophins [21,24].

Combinations of cytoprotective agents have been tested and are recommended [47], but the combination of a cytoprotective agent and subsequent rehabilitation therapy has not been assessed after hemorrhagic stroke. Whereas each treatment improves outcome following a moderate striatal ICH [12,30], they may only benefit a larger ICH when combined. Therefore, we created a large ICH in rats and tested the efficacy of HYPO, CIMT, and the combination. We used a 60-day survival time with histopathology and multiple functional endpoints to gauge long-term efficacy, which is of greater clinical concern. Functional outcome was assessed with the staircase, cylinder, and ladder tests, which are all sensitive to striatal injury [12,20,31,30]. We hypothesized that the most benefit would occur with the combination of treatments.

2. Materials and methods

2.1. Animals

Sixty-two male Sprague–Dawley rats were used in this study. Animals were obtained locally and weighed approximately 400 g (~16 weeks old) at the time of ICH. All procedures were approved by the Biosciences Animal Policy and Welfare Committee at the University of Alberta and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Behavior training

2.2.1. Montoya staircase task

Prior to surgery, rats were trained in the staircase test, which measures forelimb reaching ability [37]. Starting 3 days before training, rats were food-deprived to 90% of their free-feeding weight and weighed daily to maintain weight at this level. Rats were trained to retrieve reward food pellets (45 mg each; Bio-Serv, Frenchtown, NJ, USA) twice daily (15 min trials separated by 4 h), 5 days/week over 3 weeks (Fig. 1).

2.2.2. Limb use asymmetry (cylinder) test

Seven days before ICH, rats were placed in a Plexiglas cylinder (45 cm high and 33 cm in diameter) and allowed to spontaneously explore the walls for 5 min. Forelimb placements were videotaped from below the cylinder and analyzed according to Tillerson et al. [44]:

\[
\text{Contralateral forelimb use/}(\text{Ipsilateral forelimb + contralateral forelimb use}) \times 100
\]

2.2.3. Horizontal ladder walking test

Seven days before surgery, rats were videotaped while walking across the middle 0.5 m segment of a 1 m long horizontal ladder with variably spaced rungs (1 to 3 cm apart). The number of errors (limb slips through the rungs) made with each forelimb was determined over three trials [35].

2.2.4. Tray task

To determine paw preference, rats were trained in the tray task for 30 min per day over 5 days before surgery. Briefly, rats were placed in a Plexiglas box (length: 27 cm, width: 19 cm; height: 25 cm) and allowed to reach through 2 mm wide vertical bars separated by 1 cm to obtain pellets (chicken feed, 20–40 mg; Masterfeeds, Edmonton, Alberta) placed in a tray that measured 4 cm wide and 5 mm deep [48].

2.3. Surgical procedures

All surgical procedures were performed aseptically under isoflurane anesthesia (4% induction; 1.5–2% main-

![Fig. 1. Time line of major procedures (days are relative to the day of ICH) in the HYPO + CIMT group. Behavioral training and testing were the same in the other three groups. Rats were euthanized 60 days following ICH.](image-url)
tenance in 70% N₂O and 30% O₂). A local anesthetic (Marcaine; Sanofi Canada, Markham, Ontario, Canada) and antibiotic ointment were applied to the wound at the end of surgery. Body weight was recorded daily for 7 days after ICH surgery and weekly thereafter.

2.3.1. Core temperature probe implantation

Four days prior to ICH, core temperature telemetry probes (model TA10TA-F40; Transoma Medical, St. Paul, MN) were implanted into the peritoneal cavity, and the wound was sutured closed [30]. Temperature and activity were sampled every 30 s, and data from the complete 24 h period prior to surgery served as a baseline.

2.3.2. Intracerebral hemorrhage

Rats were anesthetized and placed in a stereotaxic frame. A midline incision was made, and a hole was drilled 3.5 mm lateral to Bregma, contralateral to the preferred paw as determined in the tray task (i.e., the limb making >50% of the reaches). To create a severe ICH, we injected 1.0 μL of sterile saline containing 0.20 U of bacterial collagenase into the striatum [40]. Briefly, a 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV, U.S.A) was lowered 7 mm below the surface of the skull, and 0.5 μL of sterile saline containing 0.10 U of bacterial collagenase was injected over 5 min. This was followed by a second equal injection 5.5 mm below the skull. The needle remained in place for 5 min after each injection. A metal screw (model MX-080-2; Small Parts, Miami Lakes, FL) sealed the hole, and the wound was closed with staples. Core temperature was maintained at 37 °C throughout the surgery (~45 min) using an electric heating pad. The abdomen and back were shaved in all groups to facilitate cooling and to prevent knowledge of group identity subsequently.

2.4. Temperature control after surgery

For 12 h after ICH, all animals were maintained near normothermia (~37 °C). Half of the rats were then cooled according to MacLellan et al. [30]. Briefly, starting 12 h after surgery, rats were cooled (1 °C per 30 min) to 33 °C for 24 h and then 35 °C for an additional 24 h (HYPO). The others were maintained at normothermia (NORMO). Temperature was precisely (~0.3 °C) controlled with a servo-controlled system using fans and fine water misters to cool and infrared lamps to warm [9]. Core temperature was monitored in all animals for 7 days. Telemetry probes were removed 21 days after ICH under brief (~10 min) isoflurane anesthesia.

2.5. Rehabilitation

Half of the NORMO and HYPO rats did not receive additional therapy. The other rats received 7 days of constraint-induced movement therapy (CIMT) which included restraining the forelimb ipsilateral to the lesion using a sleeveless jacket (model RJO2, Lomir Biomedical, Inc) attached to a metal wrist bracelet [12] and daily exercises. The jacket was worn continuously, while the bracelet restraint was used between 8 AM and 4 PM. Daily exercises included 30 min of the tray reaching task and 30 min of running wheel. The CIMT therapy started 14 days following ICH. In summary, there were four groups in this study: NORMO (N = 15), HYPO (N = 15), CIMT (N = 16), and HYPO + CIMT (N = 16).

2.6. Behavioral testing

At 6 and 8 weeks after ICH, spontaneous forelimb use was assessed in the limb use asymmetry test, and limb error rate was measured in the horizontal ladder walking test. Skilled reaching was also measured in the staircase test on days 49–53 (two 15 min trials/day, separated by 4 h). To assess overall performance, rats were ranked (best to worst) on their performance at week 8 for each test (e.g., contralateral forelimb use in cylinder test). These ranks were averaged to produce a composite behavioral score for each rat.

2.7. Histopathology

Rats were euthanized 60 days after ICH with an overdose of sodium pentobarbital (80 mg/kg, i.p.). Animals were then perfused with saline followed by 10% formalin. Fifty-micrometer coronal sections were taken every 600 μm with a cryostat and stained with cresyl violet. An experimenter, blinded to group identity, manually determined the volume of tissue lost (e.g., cavity) plus atrophy (e.g., ventricular enlargement) by using Scion Image J 4.0 (Scion Corporation, Frederick MD, USA). Damage was expressed as [12,30]:

Volume of tissue lost = remaining volume of normal hemisphere − remaining volume of injured hemisphere

Volume of a hemisphere = (average area of complete coronal section of the hemisphere − area of the ventricle − area of damage) × interval between sections × number of sections.

2.8. Data analysis

All behavioral (training, testing) and histological procedures were done by experimenters blind to group identity. Using SPSS (v 12.0) software, data were analyzed using 1-, 2-, or 3-factor (HYPO vs. no HYPO; CIMT vs. no CIMT, test TRIAL) ANOVAs followed by LSD post-hoc tests when necessary. All data are expressed as mean and standard error of the mean.

3. Results

In total, 3 animals were excluded from this study. One HYPO + CIMT animal was excluded due to technical error...
during cooling; one CIMT animal was euthanized 7 days post-ICH because of excessive weight loss; one HYPO + CIMT animal was excluded due to surgical error.

3.1. Body weight

Body weight was regulated as desired during training and testing. All rats lost weight (~10%) by the first day after ICH which reached a 20% loss in the HYPO and HYPO + CIMT groups. All groups were near their pre-surgical weight (within 5%) by day 14 (data not shown).

3.2. Temperature control

Baseline body temperature collected the day before ICH surgery was similar among groups (overall mean: 37.0 ± 0.2 °C; main effects: P ≥ 0.439; data not shown). Similarly, there was no significant difference in the mean temperature for the first 12 h after collagenase infusion (overall mean: 37.1 ± 0.2 °C; main effects: P ≥ 0.181; Fig. 2). Temperature was subsequently regulated as desired (see Materials and methods).

3.3. Staircase test

All groups performed similarly during training (data not shown), and all groups showed substantial impairments after ICH (Fig. 3). For instance, the NORMO group was significantly impaired (vs. baseline) with the contralateral (12.88 ± 2.47% of baseline; P < 0.001) and ipsilateral forelimbs (31.91 ± 7.37%; P < 0.001). A 3-way ANOVA (HYPO, CIMT, TRIAL) on the contralateral forelimb data revealed a significant HYPO × CIMT interaction (P = 0.034), and thus the data were then analyzed by a 2-way ANOVA (GROUP, TRIAL) with post-hoc (LSD) comparisons. With this analysis, only the HYPO + CIMT group retrieved significantly more pellets (P ≤ 0.029 vs. each of the other groups). The TRIAL repeated measures factor was significant (P < 0.001), but the GROUP × TRIAL interaction was not (P = 0.088). Analysis (3 factor ANOVA) of reaching success with the ipsilateral forelimb (Fig. 3B) revealed no main effect of HYPO (P = 0.743) or CIMT (P = 0.906), and the 2- and 3-way interactions were not significant (P ≥ 0.105). However, the TRIAL repeated measures factor was significant (P < 0.001), indicating improved performance over test trials.

3.4. Limb use asymmetry test

Analysis of contralateral forelimb use during exploration of the cylinder walls at week 6 revealed that the HYPO (P = 0.709) and CIMT main effects (P = 0.420) and the interaction (P = 0.281) were not significant (overall mean, 20.44 ± 2.04%). The HYPO main effect (P = 0.594) and the interaction were not significant at week 8 (P = 0.946). However, CIMT significantly increased the frequency of contralateral forelimb use (P = 0.018; Fig. 4).

3.5. Horizontal ladder walking test

There was no effect of HYPO (P ≥ 0.565), CIMT (P ≥ 0.228), or the interaction (P ≥ 0.553) for the contralateral forelimb error rate on week 6 (overall mean, 12.89 ± 1.29) or week 8 (overall mean, 12.57 ± 1.14%; Fig. 5). The error rate for the ipsilateral forelimb was small (<3.00%), and the HYPO and CIMT main effects and the interaction were not significant at both test sessions (P ≥ 0.418; data not shown).
3.6. Composite behavioral score

Analysis of the mean rank across all behavioral tests revealed no significant effect of HYPO ($P = 0.237$), whereas CIMT significantly improved performance overall ($P = 0.002$). There was no further improvement with the combination of HYPO and CIMT ($P = 0.178$, Fig. 6).

3.7. Histopathology

Untreated NORMO rats sustained damage primarily to the striatum, but also to the globus pallidus, internal capsule, thalamus, and corpus callosum (Fig. 7B). The volume of tissue lost and atrophy at 60 days in the NORMO group was not significantly affected by any treatment (main effects, $P \geq 0.199$; Fig. 7A).

4. Discussion

Our study examined the interaction of two putative therapies (HYPO, CIMT) currently undergoing clinical investigation for ischemic stroke and with potential to be used for ICH. Our results indicate that CIMT provides small but significant functional benefits after severe subcortical ICH in rats. Use of HYPO alone was neither beneficial nor harmful. These findings contrast with our earlier studies that show substantially improved recovery with HYPO [30] and CIMT [12] following a moderate-sized striatal ICH. We anticipated less benefit following a very large ICH, which was approximately twice that previously used. Indeed, in relation to our earlier work, our findings suggest that lesion size dramatically affects efficacy of both cytoprotectant and rehabilitation interventions. We created a large ICH in order to clearly test the hypothesis that dual treatment would be needed, which received some support (contralateral forelimb reaching success in staircase test). Neither the single nor the combination treatment affected lesion size in contrast to earlier studies in our laboratory that showed reduced injury with both HYPO [30] and CIMT [12] treatments following a moderate ICH. Overall, these findings indicate that: rehabilitation may work for a wider range of insult severities than cytoprotection; dual treatments may further augment recovery, but not necessarily on all measures; and functional recovery may occur despite no apparent effect on total injury.

With respect to HYPO treatment, there are several possible explanations for the present failure (vs. [30]). First, perhaps little tissue remained to be rescued after such a
severe insult. Indeed, several cytoprotectants, including HYPO, improve outcome following mild to moderate ischemic insults but fail to provide benefit after a larger or more severe lesion [2, 5, 39, 49]. Second, HYPO may effectively treat components of hemorrhagic injury (e.g., edema [22, 46], MacLellan et al., unpublished data) that may not always translate into improved functional outcome, even if the mortality rate is attenuated. Third, HYPO might have provided transient benefit that no longer existed by week 6. Thus, earlier testing may have shown greater effects. Nonetheless, the significant interaction with CIMT in the staircase test argues that some benefit was long-lasting. Perhaps HYPO rescued some peri-hematoma neurons that CIMT then acted upon (e.g., neuronal remodeling). Unfortunately, such a reduction in cell death would not likely be detected by our lesion volume measurement and would not be easily quantified at the extended survival time used. Interestingly, the staircase test is the most sensitive to gradations in hemorrhagic injury [31], and it might have been difficult to detect smaller effects with other tests (e.g., cylinder). Indeed, this is consistent with our previous HYPO [30] and CIMT [12] studies where treatments did not consistently improve outcome on all tests.

The CIMT findings are largely, but not entirely, in accordance with our recent study [12]. Specifically, the lesion volume was not reduced in the present study despite significant functional improvement. Early rehabilitation treatments (within 1 week) with either CIMT [12] or forced treadmill running [26] may be needed to attenuate cell death, which largely occurs in this period [15]. We chose a 2-week delay to allow sufficient time for rats to recover lost body weight after ICH and HYPO treatment. Although a CIMT-induced reduction in selective neuronal injury cannot be excluded, it appears that a reduction in total lesion size is not needed for functional improvement after ICH as argued previously [12]. Further study is therefore needed to elucidate mechanisms of rehabilitation-facilitated recovery in ICH models, which will likely include overcoming learned nonuse, promoting cortical and subcortical reorganization, synaptogenesis, and angiogenesis. Unfortunately, most rehabilitation studies examine cortical lesions caused by ischemia or trauma, and there are little data on recovery after subcortical or hemorrhagic injury.

There are several ways to improve HYPO and CIMT treatments. First, the HYPO regimen used was not necessarily optimal for this insult. In other words, further improvements to the therapy should make it useful for more severe insults, which is an area we are currently exploring. Second, greater benefit may be achieved with CIMT treatments that include additional rehabilitation exercises or by using a therapy that is initiated sooner or longer. Notably, many clinical CIMT studies use much longer daily periods of rehabilitation exercises [36, 42, 45]. Unfortunately, this is time consuming and difficult to conduct in rats especially if food deprivation and reward are involved (e.g., tray task) as the rats become satiated and cease doing the task. An alternative approach is to use forced exercise (e.g., running wheel) or environmental enrichment, but these have not worked in this model of severe ICH [18], and they may not work well when given soon after HYPO treatment. Earlier CIMT may improve efficacy, but the danger is that it may also aggravate injury and/or impeded recovery as found with ischemic [1], electrolytic [25], and devascularization lesions [13]. At least for the latter, it appears that CIMT-induced localized brain hyperthermia accounts for exacerbated injury [13]. Given that mild hyperthermia does not appear to worsen outcome after moderate ICH [29], it is possible that early CIMT would improve outcome after ICH. Nevertheless, an additional concern with adding early rehabilitation to a cytoprotectant is that the rehabilitation therapy may kill some rescued neurons, which may be especially vulnerable to cellular stressors. Indeed, Corbett and colleagues found that environmental enrichment exacerbated cell death in a forebrain ischemia model where ischemic preconditioning was used [16]. Likewise, attenuation of CA1 sector cell death afforded by postischemic HYPO treatment was partly mitigated by delayed transient ischemic attacks that otherwise caused no cell death [10]. Finally, an additional concern with CIMT following severe ICH was that it might have impeded recovery of the ipsilateral limb, which is also affected [29]. The ipsilateral data in staircase test show no such effect, however.

In summary, this is the first study to assess the combination of a cytoprotective agent and delayed rehabilitation after severe ICH in rats. Whereas HYPO alone failed to benefit a severe ICH, CIMT significantly improved recovery, which was further enhanced on one test with co-treatment. Thus, our findings support the growing evidence that CIMT or similar therapies will facilitate motor recovery in animal stroke models [24] and stroke patients [36, 38, 45] including those treated with cell saving therapies. Further work must be done to examine potential interactions among treatments, especially those given soon after stroke, as it cannot be assumed that rehabilitation treatments will always provide benefit or that therapies will provide additive or synergistic effects. Lastly, it is clear that functional impairments after severe injury to the basal ganglia will not be easily resolved and will likely require more effective combination therapy than that presently used.

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