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Intracranial pressure elevation following ischemic stroke in rats: Cerebral edema is not the only cause, and short-duration mild hypothermia is a highly effective preventive therapy

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Running Title: **Edema not the only cause of ICP rise post-stroke**

Abstract

In both the human and animal literature it has largely been assumed that edema is the primary cause of intracranial pressure (ICP) elevation post-stroke and that more edema equates to higher ICP. We recently demonstrated a dramatic ICP elevation 24 hours after small ischemic strokes in rats, with minimal edema. This ICP elevation was completely prevented by short-duration *moderate* hypothermia soon after stroke. Here, our aims were to determine the importance of edema in ICP elevation post-stroke and whether *mild* hypothermia could prevent the ICP rise. Experimental stroke was performed in rats . ICP was monitored and short-duration mild (35 °C) or moderate (32.5 °C) hypothermia, or normothermia (37 °C) was induced post-stroke onset. Edema was measured in 3 studies, using wet-dry weight calculations, T_2-weighted magnetic resonance imaging or histology. ICP increased 24 hours post-stroke onset in all normothermic animals. Short-duration mild or moderate hypothermia prevented this rise. No correlation was seen between ΔICP and edema or infarct volumes. Calculated rates of edema growth were orders of magnitude less than normal cerebrospinal fluid production rates. These data challenge current concepts and suggest that factors other than cerebral edema are the primary cause of the ICP elevation 24 hours post-stroke onset.

Keywords: Cerebral edema, hypothermia, intracranial pressure, ischemic stroke, rats

Introduction

Dramatic elevations in intracranial pressure (ICP) may be associated with death following large ischemic stroke (1, 2). This elevation in ICP has been attributed to an increasing volume of cerebral edema, however there is a distinct lack of empirical evidence to support a causative relationship. The idea of edema as the primary cause of ICP elevation following stroke appears to have become a self-fulfilling prophecy, as ICP rise seems only to have been investigated in subjects with large volumes of edema (2-4). Little is known about the levels of ICP in patients with smaller strokes and volumes of edema, due to the invasiveness of available methods for ICP monitoring in patients. Our group has recently shown that ICP is dramatically elevated 24 hours after relatively minor stroke in rats (5), raising doubts about the association between ICP and edema.

Hypothermia is the most promising neuroprotective modality currently available. It has shown overwhelming efficacy in experimental models (6) and cooling has been shown to reduce ICP in stroke and other neurological conditions such as traumatic brain injury and hepatic encephalopathy (7, 8). However rebound ICP elevation is a frequent complication during rewarming, particularly if this is rapid. Clinically, hypothermia is a difficult and complicated therapeutic strategy to implement. In particular, preventing shivering thermogenesis is difficult in awake patients if cooling to temperatures <35 °C. Prolonged cooling increases the risk of developing severe systemic complications such as pneumonia and immunosuppression (9, 10). Our recent study demonstrated that a short duration of moderate hypothermia (32.5 °C) administered shortly after stroke, did not affect ICP during the cooling interval, but completely prevented any subsequent rise in ICP 24 hours post-

stroke onset (5). If a short duration of mild hypothermia (35 °C) was able to limit ICP elevation following stroke, this would be a more achievable target temperature in the clinical setting, and therefore a more widely applicable potential therapeutic strategy.

Understanding the fundamental mechanisms of ICP elevation following stroke and the preventative effects of short-duration hypothermia is important in developing potential stroke treatments. The aims of this study were 1) to determine the importance of edema in the ICP elevation 24 hours post-stroke onset, and 2) to determine whether mild short-duration hypothermia prevents this ICP rise.

Materials and Methods

Animals

Surgery was performed on male outbred Wistar rats at the University of Newcastle, Australia (Studies I and III; n = 48, 315-497g, Animal Services Unit, University of Newcastle), and male Sprague-Dawley rats at the University of Glasgow, UK (Study II; n = 18, 282-372g, Harlan, Bicester, UK). Experimental animal procedures conducted in Australia were in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013) and were approved by the Animal Care and Ethics Committee of the University of Newcastle. Experiments performed in the United Kingdom were performed under license granted by the Home Office, UK, according to the UK Animals (Scientific Procedures) Act, 1986 and approved by the local University Ethical Review Panel.

Experimental Protocols

Study I

In Study I we investigated the importance of edema in ICP elevation at 24 hours using the 'gold-standard' edema calculation, wet-dry weight. Wistar rats underwent experimental stroke (3 hour intraluminal Middle Cerebral Artery occlusion - MCAo). At 1 hour post-MCAo, rats were randomized by sealed numbered envelope to 2.5 hours hypothermia-treatment (32.5 °C, n = 6) or normothermia (37 °C, n = 6). Blood pressure and ICP were measured at baseline, throughout stroke, and at 24 hours post-MCAo, before brains were removed for wet-dry weight calculation of brain edema.

Study II

In Study II our aim was to investigate the effect of moderate hypothermia on ICP post-MCAo in a strain of rat known to have larger strokes, Sprague-Dawley, and to determine the importance of edema in an *in vivo*, intact skull model of edema calculation using magnetic resonance imaging (MRI). Animals underwent experimental stroke (45 min MCAo), followed by 2.5 hours hypothermia-treatment (32.5 °C, n = 6) or normothermia (37 °C, n = 6) commencing 30 minutes post-MCAo. Blood pressure and ICP were measured at baseline, throughout stroke (blood pressure only), and at 24 hours post-MCAo. Infarct volume, edema volume and blood-brain-barrier (BBB) breakdown were assessed using T_2- and T_1-weighted MRI scans.

Study III

Our aim in Study III was to determine whether early short-duration mild hypothermia prevented ICP elevation at 24 hours, as is seen with moderate hypothermia (32.5 °C) (5), and to determine the importance of edema in ICP elevation post-MCAo using histological methods. Wistar rats underwent experimental stroke (3 hour MCAo) and at 1 hour post-MCAo, were randomized to 2.5 hours hypothermia-treatment (35 °C, n = 6) or normothermia (37 °C, n = 3 concurrent + n = 10 historical controls). Given the high consistency of the results of multiple previous experiments using the same paradigm, ten historical controls were included in this study, following the direction of the local ethical review committee. Data for historical controls used in Study III has previously been published (5). Blood pressure and ICP were measured at baseline, throughout stroke, and at 24 hours post-MCAo, before brains were removed for histology.

Anesthesia and Monitoring

All animals were anesthetized with isoflurane (5% induction, 1.5-2% maintenance) in 70:30 N_2:O_2 (Studies I and III) or N_2O:O_2 (Study II). Animals spontaneously breathed through a laboratory-manufactured low dead-space nose cone in Studies I and III, and were intubated and artificially ventilated in Study II. A rectal thermocouple provided continual monitoring of core body temperature. Temperature was also recorded every 5 minutes for 24 hours using an intra-peritoneal datalogger (SubCue, Calgary, Canada) in Studies I and III. Blood pressure was continuously monitored under anesthesia via a right femoral arterial cannula. Blood samples from this line were periodically monitored for blood gases in Studies I and II. Heart rate, respiratory rate, and SpO_2 were also continuously monitored throughout surgery. Following stroke surgery, animals were injected subcutaneously with saline (2 x 2.5 mL) to prevent dehydration and returned to their cages with free access to softened laboratory chow and water.

Intracranial Pressure Measurement

ICP measurements were obtained whilst under anesthesia using a fibre-optic microcatheter (SAMBA Sensors, Gothenburg, Sweden), as previously described, with minor changes (11). Briefly, two polyether ether ketone, fluid-filled, hollow screws were inserted bilaterally into the parietal bone at 2 mm lateral and 2 mm posterior from Bregma. The sensor was placed above the dura mater and sealed in place with a caulking material (Silagum, Gunz Dental, Sydney, Australia). Cerebral perfusion pressure (CPP) was calculated as mean arterial pressure – mean ICP.

Experimental Stroke and Hypothermia

Stroke surgery and hypothermia induction were performed as previously described (5, 12, 13). Briefly, a 3 mm silicone-tipped monofilament was passed via the right external carotid artery to occlude the middle cerebral artery. The filament remained in place for 3 hours (Wistar rats, Studies I and III) or 45 minutes (Sprague-Dawley rats, Study II) before being retracted to allow reperfusion. Occlusion and reperfusion of the MCA were confirmed using a laser Doppler flowmetry probe (Studies II and III) that was placed above the MCA watershed area for the entire occlusion.

At 1 hour (Studies I and III) or 30 minutes (Study II) post-MCAo, hypothermia-treated animals were cooled using 70% ethanol evaporation with a sprayer and a fan under anesthesia. Normothermia animals were maintained at 37 °C. Hypothermia/normothermia was maintained for 2.5 hours. ICP and blood pressure monitoring equipment was then removed, and hollow skull screws were sealed with caulking material. Animals were recovered immediately following hypothermia and allowed to self-rewarm by placing ½ the holding cage over a heat mat. At 24 hours post-MCAo, animals were re-anesthetized, the left femoral artery cannulated, physiological equipment reattached and ICP catheter reinserted and sealed for recording of physiological variables for 1 hour.

Neurological Testing

Prior to 24 hour ICP and blood pressure monitoring, animals were tested for stroke induced neurological deficits. The forelimb flexion, torso twist and lateral push tests were used to assess this and a total neurological deficit score given out of 6 (higher score indicating greater deficit) by a blinded assessor (14, 15).

Wet–Dry Weight Calculations- Study I

Water content percentages of the brain tissue were evaluated using the wet-dry weight method. Rats were decapitated immediately under deep anesthesia with isoflurane. Brains were placed in a rat brain matrix, the cerebellum removed and the two hemispheres separated. Each hemisphere was weighed before being placed in an oven at 70 °C for 48 hours. The percentage of tissue water content was calculated using the formula: % water content = [(wet-dry weight) / wet weight] x 100. To allow comparisons with Studies II and III, water content was converted into percentage of contralateral hemisphere: [ipsilateral (wet-dry weight) – contralateral (wet-dry weight) / contralateral (wet weight)] x 100.

Magnetic Resonance Imaging and Analysis- Study II

The ICP probe was removed following the 24 hour ICP and blood pressure monitoring. Animals were then transferred under anesthesia to the MRI scanner. Body temperature was maintained at 37 ± 0.5 °C during the MRI scanning procedure by a closed circuit thermal jacket. All MRI data were acquired using a Bruker Pharmascan 7T/16cm system (Ettlingen, Germany) with a gradient coil (internal diameter = 90 mm, 300 mT/m). A 72 mm birdcage resonator was used to transmit and a 4-channel phased array rat head surface to receive the MRI signal.

Once a pilot sequence had been obtained to ensure correct geometry, a rapid acquisition with relaxation enhancement (RARE) T_2-weighted scan was performed (effective Te 46.6 ms, repetition time TR = 5000 ms, Rare factor 8, averages 2, matrix 256 x 256, FOV 3.0 cm x 3.0 cm, slice thickness 0.75 mm). A baseline T_1-weighted scan was acquired using a RARE sequence with 800 ms repetition time (Echo time Te= 13.5 ms, Rare factor 4, averages 8,

partial FT factor 1.6, matrix 256 x 256, FOV 3.0 cm x 3.0 cm, slice thickness 1.5 mm). Following this scan, 0.1 mL of MultiHance contrast (529 mg/mL, gadobenate dimeglumine, Bracco Diagnostics Inc. NJ, USA) and 0.5 mL of sterile saline were injected through a femoral venous cannula, and the T_1-weighted scan was repeated 5 minutes after contrast agent injection. When injected intravenously, the gadolinium-based contrast accumulates in regions with impairment of the BBB integrity.

Image J software (NIH, Maryland, USA) was used for image analysis. Infarct volume at 24 hours post-MCAo was defined as the hyperintense area on T_2-weighted images. Hyperintensity was traced using Image J software on all slices and areas multiplied by the slice thickness to calculate volume. Contralateral and ipsilateral hemisphere volumes were also determined to assess brain swelling and for edema correction of infarct volume. All volumetric measurements were performed independently by 2 different investigators blinded to treatment allocation, any cases with >10% discrepancy were flagged for review. Infarct volume was corrected for edema using the formula: Corrected infarct volume (mm^3) = infarct volume x (contralateral volume/ipsilateral volume) (16). Edema was calculated by infarct volume minus corrected infarct volume.

Contrast agent enhancement maps were generated from the signal of the pre- and post-contrast T_1-weighted scans according to the formula: (post-contrast – pre-contrast)/(pre-contrast) x 100. Hyperintensity was traced using Image J software on all slices. Areas were then multiplied by the slice thickness to calculate the volume of contrast-induced hyperintensity.

Histological Analysis- Study III

Histological analysis followed the methodology described by McLeod *et al*. (16). Briefly, following the 24 hour ICP monitoring, animals were sacrificed and perfused transcardially with saline followed by 4% paraformaldehyde in 0.2M phosphate buffer. Brains were then fixed in neutral-buffered formalin before being processed, paraffin embedded, 5 µm coronal sections cut and stained with hematoxylin and eosin. Images were scanned using a digital slide scanner (Aperio Technologies Inc., Vista, CA, USA). Image J software was used for image analysis by two investigators blinded to treatment allocation. The edema-corrected infarct and edema volumes for both the concurrent animals and historical controls were calculated as per Study II.

Exclusion Criteria

Since subarachnoid hemorrhage (SAH) is a well-recognized complication using intraluminal thread occlusion models of stroke, and may have a profound effect on both ICP and infarct volume (vasospasm), any evidence of SAH was pre-specified as an exclusion criterion (5). Presence of SAH was assessed by: a) dramatic sudden ICP rise >50 mmHg at MCA occlusion/reperfusion, b) evidence of SAH at post-mortem in animals that died, or c) evidence of subarachnoid blood seen on saline/paraformaldehyde perfused brains post-mortem (blinded assessor).

Statistics

An *a priori* sample size calculation was performed for the mild hypothermia study based on our previously published ICP data (5, 17). Six animals per treatment group (hypothermia or normothermia) were required to detect a 15 mmHg difference in Δ ICP between the

treatment groups (Δ ICP = mean peak ICP – mean baseline ICP) with standard settings of alpha 0.05, power 0.8. Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, Inc. La Jolla, CA, USA).

Intra-rater reliability of infarct volumes was determined in Study II and inter-rater reliability determined in Studies II and III. Intra-observer reliability was assessed by comparing two sets of measurements performed 4 months apart by the same investigator. Inter-rater reliability was assessed by comparing measurements of the same slices performed by two individual investigators. Investigator 2 was blinded to the results of investigator 1 and both investigators were blinded to treatment allocation. Pearson correlation analysis was used to determine reliability.

Student's *t*-tests were performed to compare differences between treatment groups (unpaired *t*-test) or changes from baseline (paired *t*-test). Mann-Whitney *U* tests were performed to compare neurological deficit scores between treatment groups. Neurological deficit scores are presented as median (25th – 75th interquartile range). Pearson correlation analysis was used to determine the relationship between ICP vs. infarct volume and edema volume. Spearman correlation analysis was used to compare ICP and neurological deficit scores. Significant differences were accepted at the $p < 0.05$ level. Data are presented as mean ± standard deviation (SD) unless otherwise stated.

Results

Study I- Effect of moderate hypothermia, wet-dry weight edema

Seven Wistar rats were excluded in total. Reasons for exclusion were: equipment difficulties (2 hypothermia; 1 normothermia; 1 prior to randomization), mortality during surgery (2 normothermia), or SAH (1 hypothermia). There were no significant differences in baseline or 24 hour physiological parameters between groups (Table 1). Mean temperature was 32.8 ± 1.3 °C during hypothermia-treatment and 38.1 ± 1.1 °C during equivalent normothermia period.

ICP increased from 9.7 ± 4.3 mmHg at baseline to 42.1 ± 7.0 mmHg at 24 hours in the normothermic group, $p < 0.0001$. There was no elevation in ICP in the hypothermia-treated group, 8.1 ± 2.1 mmHg at baseline vs. 7.7 ± 3.8 mmHg at 24 hours, $p = 0.24$ (Figure 1-A). ICP did not change significantly from baseline during hypothermia-treatment. CPP decreased significantly from baseline to 24 hours in the normothermic group (90.7 ± 12.1 mmHg vs. 70.7 ± 14.4 mmHg, $p < 0.0001$), and was significantly lower than in the hypothermia-treated group at 24 hours (70.7 ± 14.4 mmHg vs. 120 ± 13.7 mmHg, $p < 0.0001$). No significant change in CPP was seen from baseline to 24 hours in the hypothermia-treated group, $p = 0.10$ (Figure 1-B).

A trend to larger brain-water content was seen in the stroke (ipsilateral) hemisphere of the normothermic group compared to the contralateral hemisphere, 79.5 ± 1.5% vs. 78.4 ± 1.1%, $p = 0.08$. No difference in brain-water content was seen in the hypothermia-treated group, 78.5 ± 1.3% in the ipsilateral hemisphere vs. 78.4 ± 0.8% in the contralateral hemisphere, $p = 0.8$ (Figure 1-C). No difference was seen between normothermia and

hypothermia-treated edema volumes (expressed as percentage of contralateral hemisphere %HLV), 4.1 ± 8.5 vs. 7.4 ± 6.8%, p = 0.47. The growth rate of edema was calculated by dividing the edema volume by 1440 minutes (24 hours). There was no significant difference in edema growth rate between the normothermia and hypothermia-treated groups, 0.005 ± 0.006 µL/min vs. 0.002 ± 0.002 µL/min, p = 0.18. There was no significant difference in neurological deficit scores between the normothermia group, median score of 3 (0.75 - 5) and the hypothermia-treated group, median score 2.5 (1.0 - 3.5), p = 0.86.

Study II- Effect of moderate hypothermia, "in vivo" **edema quantification**

Six Sprague-Dawley rats were excluded in total. Reasons for exclusion were: equipment difficulties (1 normothermia) or SAH (5 normothermia). Heart rate was significantly higher from baseline to 24 hours in the hypothermia-treated group, and was significantly higher than in the normothermic group. There was no significant difference in other physiological parameters (Table 1).

ICP increased from 10.2 ± 3.2 mmHg at baseline to 60.7 ± 21.1 mmHg at 24 hours in the normothermic group, p = 0.0025. There was no elevation of ICP in the hypothermia-treated group, 18.5 ± 8.5 mmHg at baseline vs. 15.5 ± 8.7 mmHg at 24 hours, p = 0.57 (Figure 2-A). CPP decreased significantly from baseline to 24 hours in the normothermic group (79.9 ± 10.9 mmHg vs. 33.1 ± 15.1 mmHg, p = 0.005), and was significantly lower than in the hypothermia-treated group at 24 hours (33.1 ± 15.1 mmHg vs. 79.5 ± 12.9 mmHg, p = 0.0002). No significant change in CPP was seen from baseline to 24 hours in the hypothermia-treated group, p = 0.66 (Figure 2-B).

There was no significant difference in infarct volume between the hypothermia-treated and normothermic group, 71.7 ± 41.7 mm^3 vs. 109.7 ± 65.6 mm^3, p = 0.26 (%HLV, 19.3 ± 11.5% vs. 21.1 ± 12.5%), Figure 2-C, (representative images in Supplementary Figure). Intra-rater and inter-rater reliability of infarct volume measurements was strong, r = 0.97 (p < 0.0001) and r = 0.99 (p < 0.0001), respectively. Similarly, there was no significant difference in edema between the hypothermia-treated group compared to the normothermic group, 9.6 ± 8.1 mm^3 vs. 17.8 ± 18.6 mm^3, p = 0.34 (%HLV, 2.6 ± 2.3% vs. 10.1 ± 7.5%), Figure 2-D. There was no significant difference in edema growth rate between the normothermia and hypothermia-treated groups, 0.012 ± 0.013 µL/min vs. 0.007 ± 0.006 µL/min, p = 0.34. No significant difference in volume of brain with BBB breakdown was detected between the groups, 0.4 ± 0.5 mm^3 in the normothermic group vs. 0.5 ± 0.4 mm^3 in the hypothermia-treated group, p = 0.74 (representative image, Figure 3). A trend towards a higher neurological deficit score was seen in the normothermia group, median score of 4.5 (2.8 - 5.3) when compared to the hypothermia-treated group, median score 2 (0.0 - 3.5), p = 0.09.

Study III- Effect of mild hypothermia, H&E edema

Ten animals were excluded in total. Reasons for exclusion were: equipment difficulties (1 hypothermia, 1 prior to randomization), mortality during surgery (1 hypothermia, 2 prior to randomization), or SAH (2 hypothermia, 1 normothermia, 2 prior to randomization). Core temperature was significantly higher at 24 hours compared to baseline in the hypothermia-treated group but was not different to normothermic group at 24 hours. There was no significant difference in other physiological parameters (Table 1). Mean temperature was 34.6 ± 0.8 °C during hypothermia-treatment and 37.4 ± 1.1 °C during equivalent normothermia period.

ICP increased from 7.0 ± 2.7 mmHg at baseline to 33.2 ± 8.6 mmHg at 24 hours in the normothermic group, $p < 0.0001$ (ICP at 24 hours was 38.4 ± 3.0 mmHg in the concurrent controls and 31.6 ± 9.3 mmHg in the historical controls). There was no significant ICP rise in the animals that received 35 °C hypothermia-treatment, 8 ± 3.2 mmHg at baseline vs. 13 ± 7.1 mmHg at 24 hours, $p = 0.12$ (Figure 4-A). ICP did not change significantly from baseline during hypothermia-treatment. CPP decreased significantly from baseline to 24 hours in the normothermic group (90.3 ± 13.1 mmHg vs. 64.3 ± 13.8 mmHg, $p < 0.0001$), and was significantly lower than in the hypothermia-treated group at 24 hours (64.3 ± 13.8 mmHg vs. 95.2 ± 19.4 mmHg, $p = 0.02$). No significant change in CPP was seen from baseline to 24 hours in the hypothermia-treated group, $p = 0.75$ (Figure 4-B).

Infarct volume was significantly smaller in the hypothermia-treated group compared to normothermia group, 11.2 ± 11.5 mm^3 vs. 55.6 ± 45.9 mm^3, $p = 0.03$ (%HLV, 5.6 ± 6.0% vs. 14.8 ± 15.2%), Figure 4-C. Inter-rater reliability of infarct volume measurements was strong, $r = 0.99$ ($p < 0.0001$). Edema volumes were small in both groups, with a non-significant trend to lower values in hypothermia-treated rats, 3.1 ± 0.65 mm^3 vs. 27.9 ± 6.5 mm^3 in controls, $p = 0.15$ (%HLV, 0.3 ± 0.6% vs. 1.9 ± 2.8%), Figure 4-D. There was no significant difference in edema growth rate between the normothermia and hypothermia-treated groups, 0.005 ± 0.006 µL/min vs. 0.0004 ± 0.0008 µL/min, $p = 0.14$. There was no significant difference in neurological deficit scores between the normothermia group, median score of 3 (1.5 - 4.3) and the hypothermia-treated group, median score 2 (0.75 - 3.25), $p = 0.29$.

Correlations

No significant correlations were found between Δ ICP over 24 hours and infarct volume (%HLV), cerebral edema volume (%HLV) or neurological deficit scores in the normothermia and hypothermia-treated groups of each study (Table 2 and Figure 5).

Discussion

In these studies we have made several findings that challenge the conventional thinking about ICP following stroke. We confirmed our previous findings that ICP is consistently and dramatically elevated 24 hours following even small strokes in Wistar rats and we showed even greater ICP elevation in the larger strokes induced in Sprague- Dawley rats. We also confirmed that a short duration of moderate hypothermia soon after the stroke prevents this later dramatic ICP elevation. We extended upon the latter finding by showing that mild hypothermia to 35 °C also prevented the ICP elevation. Finally, we have shown no correlation between ICP and volume of edema, indicating that there must be mechanisms other than edema contributing both to ICP elevation following stroke, and to its prevention by hypothermia.

The data indicate that the 24 hour ICP elevation seen in these studies was not primarily caused by cerebral edema. Our previously published data demonstrated that animals with small strokes and little edema had a dramatic elevation in ICP (5). Using three separate methods of edema calculation, this study has confirmed those findings. The edema volumes that were observed in the normothermic groups of Studies I and III were small in comparison to the dramatic ICP elevations. Furthermore, hypothermia-treated Sprague-Dawley rats had larger volumes of edema, but no ICP rise. This evidence suggests that while

edema may be a contributory factor to ICP rise following stroke, dramatic ICP elevation is possible in the absence of significant edema. Additionally, large volumes of edema were not accompanied by any ICP rise (in hypothermia-treated animals). Therefore edema appears neither necessary, nor sufficient to cause ICP rise (Figure 5-B). This was further confirmed by the lack of any correlation between ICP and edema in any of the individual studies. Furthermore, the rate of edema growth over 24 hours in each study was at least 2 orders of magnitude less than the natural rate of cerebrospinal fluid (CSF) production in rats, 2-3 µL/min (18). Even if we assumed that all of the edema growth occurred in the final 6 hours, the growth rate would still be < 0.05 µL/min. Previous investigators have shown that to artificially increase ICP to levels seen in this study (> 30 mmHg), a constant infusion rate of 4.8 µL/min into the spinal subarachnoid space was required (19). Our own data using lateral ventricular infusions suggests even higher flow rates may be required (unpublished). If ICP elevation is not primarily caused by cerebral edema, what then is the mechanism involved? The Monro-Kellie doctrine states that since the cranial cavity is fixed, any increase in volume of an intracranial component (tissue, CSF or blood) must be compensated by a decrease in the other two (20, 21). ICP will elevate if this compensation fails. Blood volume and CSF are not parameters that have previously been considered important in ICP elevation following stroke. Our data suggest that they are definitely worthy of further investigation.

Do patients with small strokes have a similar transient ICP elevation at 24 hours as we have seen in the rats? Despite the wealth of rat experimental stroke studies, including hypothermia (6), this early ICP rise in rats has gone largely unrecognized until our recent work. The idea that cerebral edema is the cause of ICP elevation appears to have become a self-fulfilling prophecy – with ICP elevation only being looked for in big strokes. Other

papers have reported an early ICP peak in animals with smaller strokes, but the authors chose to focus on the secondary peak at day 3 in those with significant edema (22, 23). This secondary peak is likely to correspond to the raised ICP seen at 48-72 hours following large human stroke. Could an early ICP rise have gone unnoticed in small stroke patients? We believe it is quite possible, since invasive monitoring is not performed in such patients, hence ICP elevation would not be apparent (as is the case in rats with experimental stroke). Papilloedema (optic disc swelling) occurs in response to ICP elevation, but takes days to develop. Headache may be reported, but is a common enough phenomenon after stroke that a self-limited headache of 4-6 hours would not be remarked upon. If a similar rise in ICP does occur following small human strokes, the implications would be very important to the outcome of those patients. Blood flow to the ischemic penumbra is known to be CPP-dependent since normal cerebral blood flow autoregulation is lost in the ischemic penumbra (24), so ICP elevation would be predicted to reduce penumbral perfusion. The principle of increasing CPP to enhance collateral flow and penumbral perfusion has been exploited in several clinical trials (25-31), however to date these have all focused on mean arterial pressure (MAP) rather than intracranial pressure (CPP = MAP- ICP).

Another key finding was that treatment with mild hypothermia also prevents subsequent ICP elevation. This has important implications in terms of clinical relevance, since cooling below 35 °C in conscious patients becomes progressively more difficult due to the need to prevent shivering thermogenesis. One fascinating aspect of these findings with great potential clinical importance was that neither mild nor moderate hypothermia influenced ICP at the time of cooling. Our previous work also showed no effect during rewarming (5). Hypothermia is well known to lower ICP, and is used to treat otherwise uncontrollable ICP

elevation in a number of conditions. A major problem in this setting is rebound ICP elevation during attempted rewarming. This has also been problematic in clinical trials of hypothermia in stroke (3, 10). Our recent data suggest that if cooling/rewarming is completed before the ICP elevation begins, it acts in a "switch-like" manner to prevent subsequent ICP increase even many hours after restoration of normothermia.

Hypothermia is the best studied neuroprotectant in experimental stroke. However, the significant logistical difficulties and risk of iatrogenic complications from long-duration, deep hypothermia are hindering its potential as a post-stroke treatment. There has been a paradox in the attempted clinical translation to date. The vast majority of animal studies have cooled for <6 hours (6), yet all the clinical trials to date have cooled for >12 hours, and the majority for >24 hours (3, 9, 10, 32, 33). Do we know that short and long duration cooling are equivalent, or that long duration is superior? Several studies have suggested that long durations of hypothermia may be more effective than short. However, the interpretation of these studies is difficult because of the wide variation in what is considered short or long duration. Some have compared 12 hours versus 24-48 hours of hypothermia (34), all of which are considerably longer than the majority of experimental hypothermia studies. Whereas others have compared the effect of durations from 1-4 hours (35). Previous investigations have also indicated that short-duration hypothermia is effective when administered early post-stroke onset and when present during reperfusion (6), as was seen in the current study. The current thinking in clinical hypothermia therapy is that "longer and cooler are better" but given the data presented here and elsewhere, the benefits of hypothermia may be able to be induced with shorter and less severe reductions

in temperature. If a short duration, mild hypothermia therapy is all that is needed, it may become much more clinically achievable and deserves further pre-clinical investigation.

This study had some unavoidable limitations. First, the use of the wet-dry weight technique for edema calculation in Study I prevented infarct volume from being calculated in these animals. Confirmation of ischemic insult, however, was determined from 24 hour neurological deficit scores. Second, the studies were powered to detect ICP reduction, not infarct reduction, since there have already been >100 studies and a meta-analysis of hypothermia-induced neuroprotection in experimental stroke (6). The point estimates of infarct volumes were all consistent with that from the meta-analysis (6).

In conclusion, mild and moderate hypothermia prevented the dramatic ICP elevation that was observed at 24 hours after minor stroke, and this elevation in ICP was not primarily caused by cerebral edema. This challenges the orthodoxy of nearly a century regarding the control of ICP elevation and begs the question about whether similar (as yet unknown) mechanisms also occur in other neurological conditions associated with ICP elevation such as traumatic brain injury, idiopathic intracranial hypertension, parenchymal intracerebral hemorrhage or subarachnoid hemorrhage? There is a strong impetus to investigate the mechanism responsible for the observed ICP elevation, and it's relevance in other conditions since it has clear implications for therapy, particularly given the observed benefits of mild or moderate short-duration hypothermia.

Conflicts of Interest/Disclosures: None

Supplementary information is available at the Journal of Cerebral Blood Flow & Metabolism website- www.nature.com/jcbfm

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Table 1. Physiological Parameters

		Study I		Study II		Study III	
		0 h	**24 h**	**0 h**	**24 h**	**0 h**	**24 h**
Temp. *(°C)*	Normo.	37.1 ± 0.2	37.3 ± 0.4	37.4 ± 0.6	36.8 ± 0.2	37.6 ± 1.2	38.0 ± 0.5
	Hypo.	37.4 ± 0.0	37.4 ± 0.3	37.0 ± 0.2	36.9 ± 0.2	36.6 ± 0.7	38.1 ± 0.6†
RR *(BPM)*	Normo.	62.2 ± 10.2	60.6 ± 9.1	~	~	65.6 ± 17.9	61.0 ± 12.6
	Hypo.	66.0 ± 5.7	56.6 ± 11	~	~	58.0 ± 11.4	60.7 ± 21.2
HR *(BPM)*	Normo.	380 ± 32	399 ± 39	373 ± 22	397 ± 19	376 ± 43	401 ± 26
	Hypo.	400 ± 46	406 ± 27	386 ± 15	418 ± 7.0*†	393 ± 25	382 ± 32
MAP *(mmHg)*	Normo.	100 ± 9.4	112 ±10.1	90.1 ± 8.1	93.8 ± 9.2	97.2 ± 13.6	96.0 ± 11.0
	Hypo.	107 ± 10.3	128 ± 12.2	93.0 ± 7.6	94.9 ± 6.4	98.4 ± 10.3	109 ± 17
SpO_2 *(%)*	Normo.	99.0 ± 0.7	99.4 ± 0.9	99.2 ± 0.0	98.3 ± 2.0	98.7 ± 1.5	98.6 ± 1.4
	Hypo.	97.8 ± 1.6	99.3 ± 0.5	98.2 ± 1.2	99.0 ± 0.5	96.0 ± 3.9	98.7 ± 0.8
paO_2 *(mmHg)*	Normo.	138 ± 18	174 ± 63	165 ± 3	152 ± 48	~	~
	Hypo.	111 ± 21	155 ± 27	128 ± 28	152 ± 15	~	~
$paCO_2$ *(mmHg)*	Normo.	46.2 ± 5.6	48.0 ± 4.6	33.9 ± 2.8	25.8 ± 5.6	~	~
	Hypo.	48.4 ± 5.0	52.8 ± 9.5	46.1 ± 12.3	34.3 ±12.8	~	~
pH	Normo.	7.41 ± 0.04	7.44 ± 0.04	7.48 ± 0.03	7.48 ± 0.02	~	~
	Hypo.	7.39 ± 0.04	7.43 ± 0.04	7.42 ± 0.10	7.44 ± 0.11	~	~

Study I- n = 6/group; Study II- n = 6/group; Study III- n = 6 hypothermia, n = 3 concurrent + n = 10 historical controls; Temp. = temperature; RR = respiratory rate; HR = heart rate; BPM = breaths (RR) or beats (HR) per minute; MAP = mean arterial pressure; SpO_2 = oxygen saturation; paO_2 = arterial partial pressure of oxygen; $paCO_2$ = arterial partial pressure of carbon dioxide. 0h =immediately prior to MCAo *$p < 0.05$ versus normothermia; †$p < 0.05$ versus 0 h.

Table 2. Correlations between intracranial pressure change (24 hour – baseline) and infarct volume (%HLV), edema volume (%HLV) and neurological deficit score (Neuro. Score).

		Infarct Volume		Edema Volume		Neuro. Score	
		r^2	*p*-Value	r^2	*p*-Value	r	*p*-Value
Study I	*Normo.*	~	~	0.21	0.37	0.23	0.65
	Hypo.	~	~	0.05	0.67	-0.18	0.67
Study II	*Normo.*	0.03	0.72	0.07	0.6	-0.29	0.56
	Hypo.	0.36	0.21	0.33	0.23	0.26	0.67
Study III	*Normo.*	< 0.01	0.83	< 0.01	0.87	0.39	0.19
	Hypo.	0.21	0.36	0.24	0.33	-0.47	0.3

Study I- n = 6/group; Study II- n = 6/group; Study III- n = 6 hypothermia, n = 3 concurrent + n = 10 historical controls. Pearson correlation analysis was used to determine the relationship between ICP vs. infarct volume and edema volume. Spearman correlation analysis was used to compare ICP and neurological deficit scores.

Titles and Legends to Figures

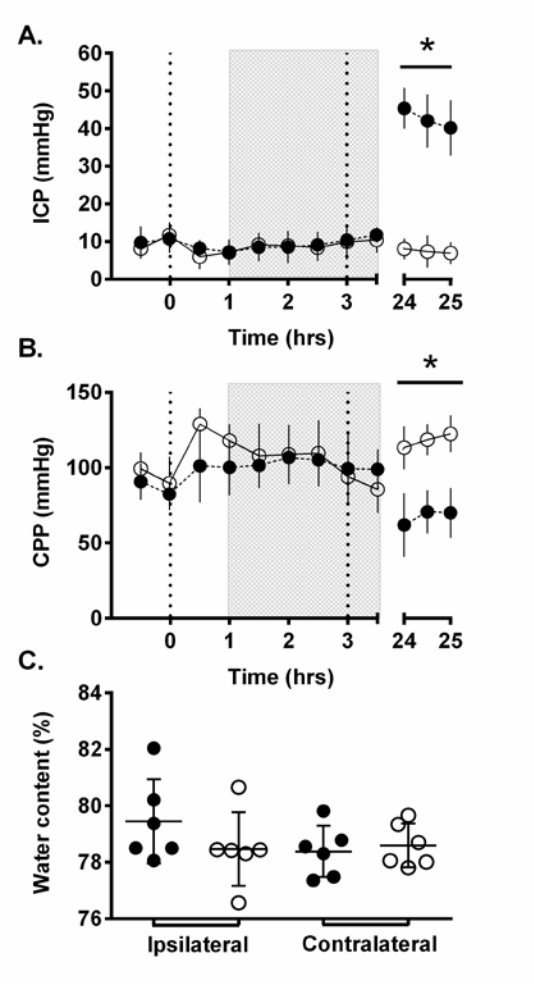


Figure 1. Study I - moderate hypothermia (32.5 °C), Wistar rats **A.** Intracranial pressure (ICP) 0 - 3.5 hours and 24 - 25 hours post-MCAo in hypothermia-treated (open circles) and normothermia (closed circles) animals, the shaded region represents the cooling interval; MCAo is between the dotted vertical lines **B.** Cerebral perfusion pressure (CPP). CPP was calculated as arterial pressure minus ICP **C.** Brain-water content measured with wet-dry weight calculations for ipsilateral and contralateral hemispheres in hypothermia-treated (open circles) and normothermia (closed circles) animals. **A.** and **B.** data plotted as mean ± SD. **C.** Individual animals, mean ± SD. *p < 0.0001, for t-tests between respective hypothermia (open circles; n = 6) and normothermia (closed circles; n = 6) groups.

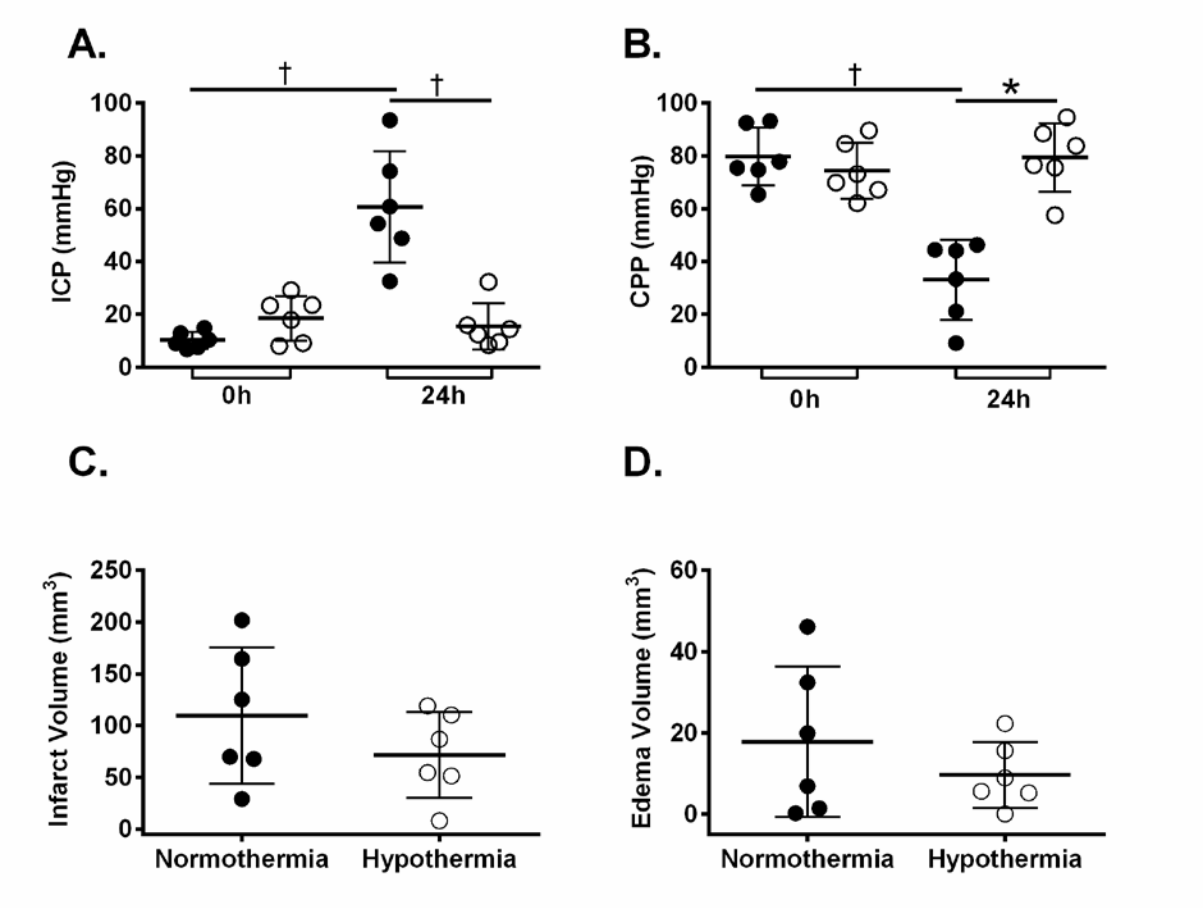


Figure 2. Study II - moderate hypothermia (32.5 °C), Sprague-Dawley rats **A.** Intracranial pressure (ICP) at baseline (pre-stroke, 0 hours) and 24 hours post-MCAo in hypothermia-treated (open circles) and normothermia (closed circles) animals **B.** Cerebral perfusion pressure (CPP). CPP was calculated as arterial pressure minus ICP **C.** Infarct volume measured with T_2-weighted magnetic resonance imaging (MRI) **D.** Cerebral edema volume measured with T_2-weighted MRI. Individual animal data plotted, and mean ± SD. *p < 0.0001, †p < 0.05 for t-tests between respective hypothermia (open circles; n = 6) and normothermia (closed circles; n =6) groups.

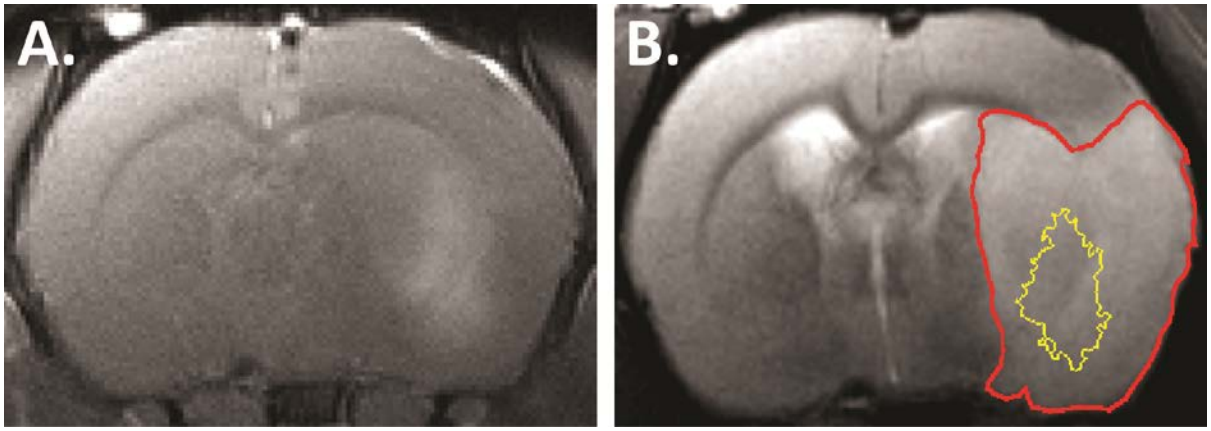


Figure 3. Study II - representative magnetic resonance imaging (MRI) scans at 24 hours post-MCAo. **A.** Gadolinium contrast was infused intravenously and a T_1-weighted MRI scan was obtained to determine the area of blood-brain-barrier (BBB) breakdown. The area of BBB breakdown is depicted by the area of pallor. **B.** This area was traced and overlaid onto the same slice on the T_2-weighted MRI scan (yellow line). The area of hyperintensity of the T_2-weighted scan is representative of infarct (red line).

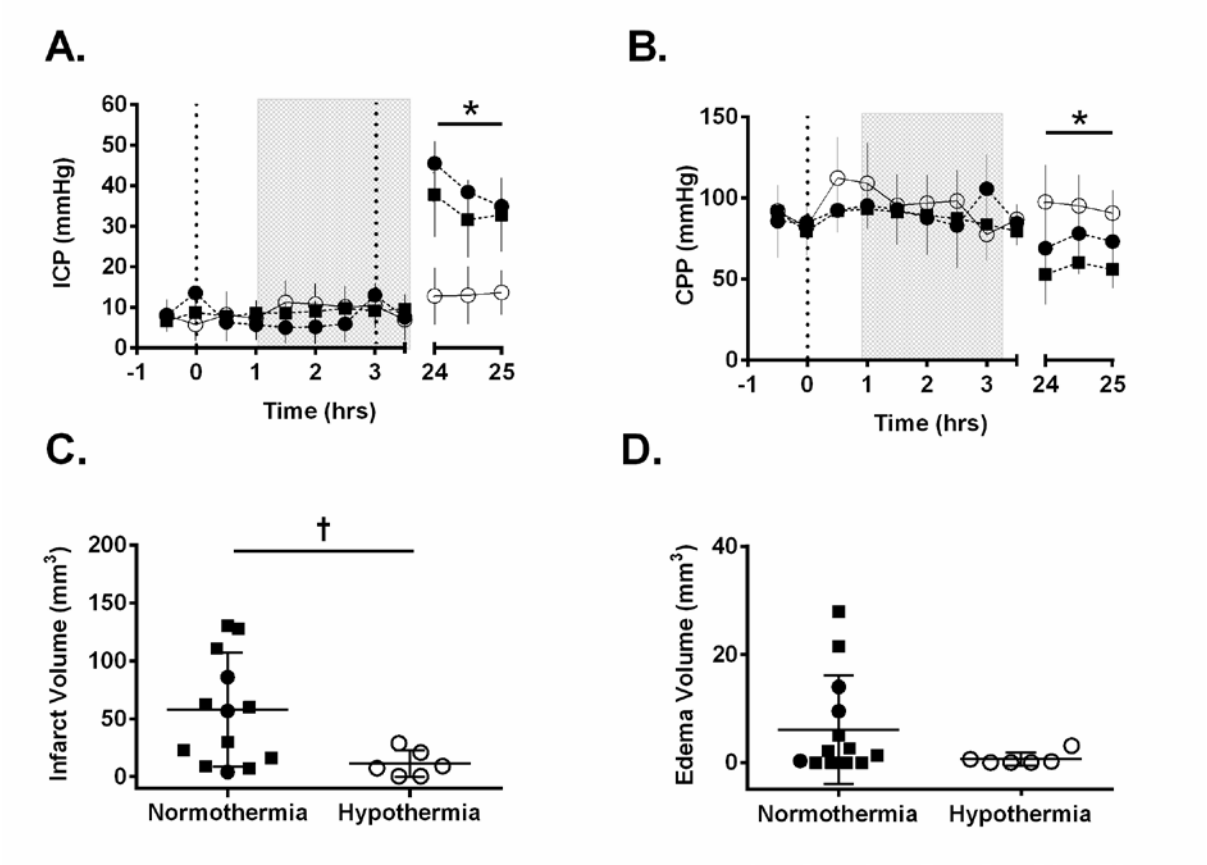


Figure 4. Study III - mild hypothermia (35 °C), Wistar rats **A.** Intracranial pressure (ICP) 0 - 3.5 hours and 24 - 25 hours post-MCAo in hypothermia-treated (open circles) and normothermia (concurrent controls, closed circles; historical controls, closed squares) animals; the shaded region represents the cooling interval; MCAo is between the dotted vertical lines **B.** Cerebral perfusion pressure (CPP). CPP was calculated as arterial pressure minus ICP **C.** Infarct volume measured with H&E histology **D.** Cerebral edema volume measured with H&E histology. **A.** and **B.** mean ± SD. **C.** and **D.** Individual animals, mean ± SD. *$p < 0.0001$, †$p < 0.05$ for t-tests between respective hypothermia (open circles; n = 6) and normothermia (closed circles/squares; n = 3 concurrent + n = 10 historical controls) groups.

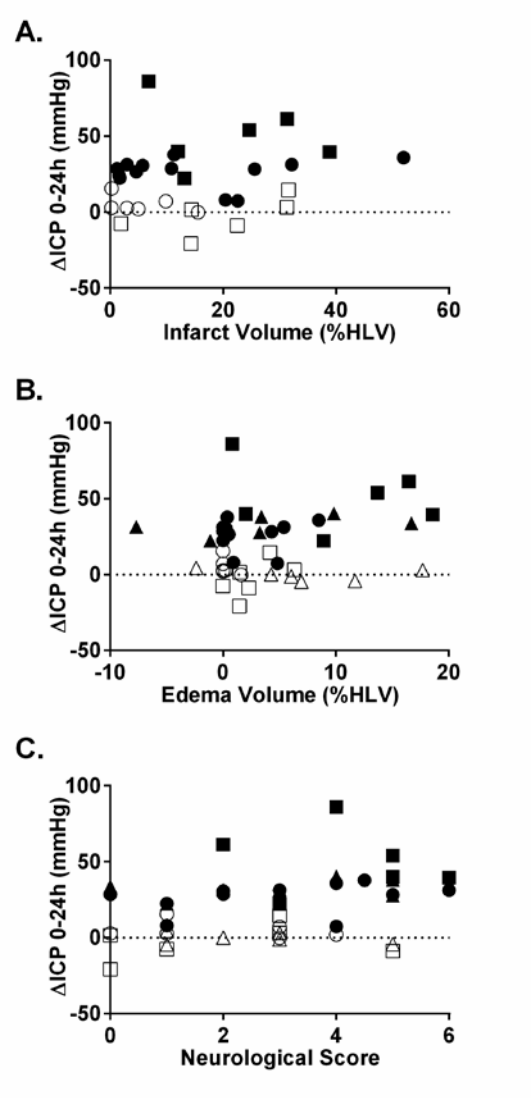
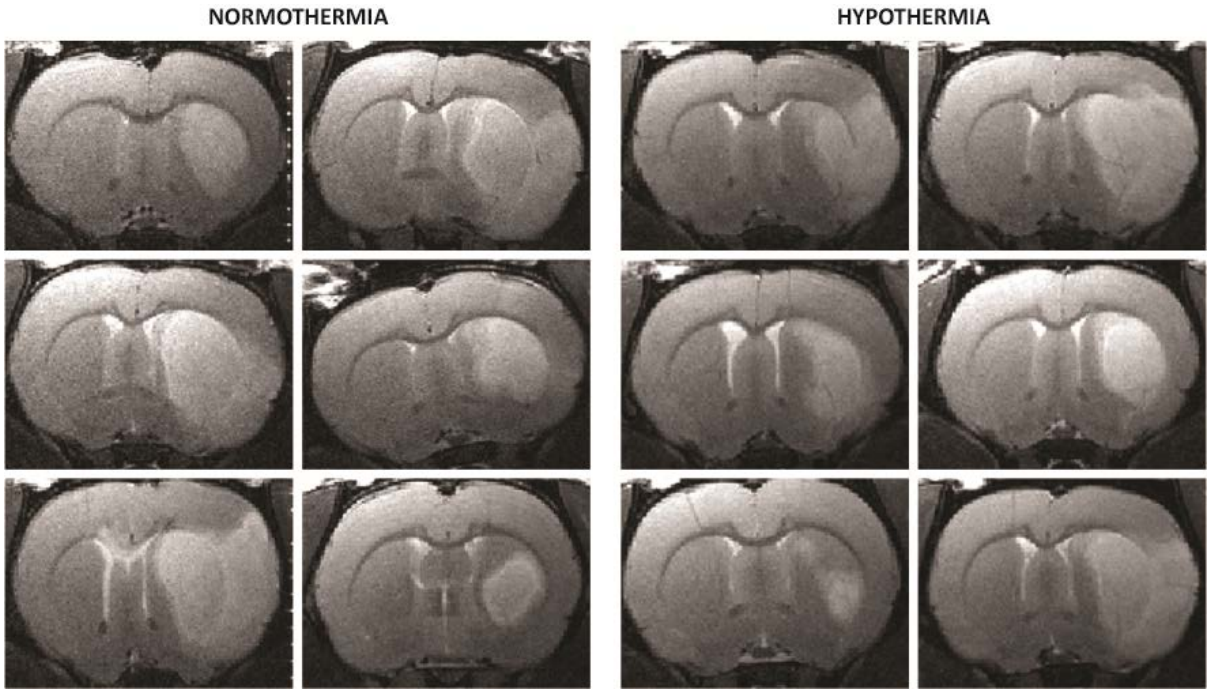


Figure 5. Studies I-III: Illustrative comparison of change in intracranial pressure (ΔICP) versus **A.** infarct volume (%HLV), **B.** cerebral edema volume (%HLV), and **C.** neurological deficit score. Study I is represented by triangles; Study II is represented by squares; Study III is represented by circles. Normothermia closed shapes, hypothermia open shapes. To account for minor differences in baseline ICP between Wistar and Sprague-Dawley rats, data are presented as (delta) ICP. No significant correlation of ΔICP with infarct volume (%HLV), edema volume (%HLV) or neurological deficit scores was seen in each study. Study I- n = 6/group; Study II- n = 6/group; Study III- n = 6 hypothermia, n = 3 concurrent + n = 10 historical controls



Supplementary Figure. A representative T2-weighted magnetic resonance imaging (MRI) scan for each animal in the normothermia and hypothermia groups in Study II. The area of infarct is depicted by the area of hyperintensity.