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CURCUMIN ATTENUATES HEMATOMA SIZE AND NEUROLOGICAL INJURY FOLLOWING INTRACEREBRAL HEMORRHAGE IN MICE

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Abstract

Objective—Intracerebral hemorrhage (ICH) is associated with significant patient morbidity and mortality. Acute hematoma enlargement is an important predictor of neurological injury and poor clinical prognosis; however, neurosurgical clot evacuation may not be feasible in all patients and treatment options remain largely supportive. Thus, novel therapeutic approaches to promote hematoma resolution are needed. In the present study, we investigated whether the curry spice, curcumin, limited neurovascular injury following ICH in mice.

Methods—ICH was induced in adult male CD-1 mice by the intracerebral administration of collagenase or autologous blood. Clinically-relevant doses of curcumin (75–300 mg/kg) were administered up to 6 hours after ICH and hematoma volume, inflammatory gene expression, blood-brain barrier permeability, and brain edema were assessed over the first 72 hours. Neurological assessments were performed to correlate neurovascular protection with functional outcomes.

Results—Curcumin increased hematoma resolution at 72 hours post-ICH. This effect was associated with a significant reduction in the expression of the pro-inflammatory mediators, tumor necrosis factor- α , interleukin-6, and interleukin-1 β . Curcumin also reduced disruption of the blood-brain barrier and attenuated the formation of vasogenic edema following ICH. Consistent with the reduction in neuroinflammation and neurovascular injury, curcumin significantly improved neurological outcome scores after ICH.

Conclusion—Curcumin promoted hematoma resolution and limited neurological injury following ICH. These data may indicate clinical utility for curcumin as an adjunct therapy to reduce brain injury and improve patient outcome.

Keywords

Hemorrhagic stroke; vasogenic edema; blood-brain barrier; hematoma

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DISCLOSURES

The authors report no conflicts of interest.

INTRODUCTION

Intracerebral hemorrhage (ICH) induces 50–60% mortality within the first year and is associated with long-term disability in many survivors^{4,33}. Primary ICH may be caused by the rupture of small vessels damaged by chronic hypertension or amyloid angiopathy. This can lead to the accumulation of erythrocytes within the parenchyma and the formation of a space-occupying hematoma. Hematoma volume directly correlates with neurological outcome¹⁰, supporting clot evacuation as a strategy to attenuate brain injury and improve patient prognosis. Unfortunately, a limited number of suitable surgical candidates and/or unfavorable size/location of the mass lesion may restrict the utility of neurosurgical intervention. As such, treatment options remain largely supportive, reinforcing the notion that ICH is the least treatable form of stroke and stressing the need for novel therapeutic approaches.

Acute hematoma enlargement, an independent determinant of both mortality and functional outcome after ICH, is clinically associated with neurovascular damage and increased patient mortality^{8,10,18,23,33}. Early phase predictive markers of acute hematoma growth remain poorly defined; however, an inflammatory reaction correlated with hematoma growth in pre-clinical injury models and with neurological deterioration in ICH patients^{26,31}. Activation of the pro-inflammatory transcription factor, NF κ B, increased the expression of inflammatory mediators associated with cell death, increased blood-brain barrier (BBB) permeability, and induced the development of vasogenic edema after experimental ICH^{20,38,42,48}. Along these lines, interleukin-6 (IL-6) is an independent predictor of hematoma enlargement³¹, and both tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) increase BBB permeability and promote vasogenic edema after brain injury^{5,16,21,29,45}. Thus, a reduction in inflammatory activation may limit neurovascular injury and improve clinical outcomes following ICH.

Recent work by our laboratory demonstrated curcumin, a low molecular weight curry spice derived from the *Curcuma longa*, reduced vascular inflammation and acute injury after subarachnoid hemorrhage (SAH) and traumatic brain injury (TBI) via a reduction in NF κ B activity^{24,42}. Curcumin has been safely consumed by humans for centuries, including use as an anti-inflammatory agent in Ayurveda, an ancient Indian system of medicine^{19,36}. Recent clinical trials also demonstrated that oral administration of curcumin resulted in bioactivity with minimal adverse effects, even when administered at high doses⁶. Given the role for neuroinflammation in the development of secondary neurological injury, we hypothesized that curcumin may restrict neurological demise following ICH.

MATERIALS AND METHODS

ICH model

Animal studies were reviewed and approved by the Committee on Animal Use for Research and Education at the Medical College of Georgia, in compliance with NIH guidelines. Male CD-1 mice (8–10 weeks old; Charles River, Wilmington, MA, USA) were anesthetized with 8 mg/kg xylazine/60 mg/kg ketamine. Throughout all surgical procedures, body temperature was maintained at 37°C using a small-animal temperature controller (David Kopf Instruments, Tujunga, CA, USA). Mice were placed into a stereotactic frame and a 0.5 mm burr hole was formed 2.2 mm lateral to bregma using a high speed dental drill. A 26-gauge Hamilton syringe containing 0.04U of bacterial type IV collagenase in 0.5 μ L saline was lowered 3 mm into the left striatum, as described previously^{3,9}. A blood injection model of ICH was also tested, to confirm the effects observed in the collagenase model. Briefly, 30 μ L of autologous tail blood was injected directly into the striatum via a 22 gauge Hamilton syringe, using the coordinates listed above, as detailed previously³⁵. In both injury models, the syringe was depressed at a rate of 450 nL/min and remained in place for 10 minutes to

prevent reflux. The burr hole was sealed with bone wax and the incision was surgically stapled. Curcumin (>98% purity; Acros Organics, Morris Plains, NJ; see Figure 1) was dissolved in corn oil (vehicle) and administered via intraperitoneal injection following randomization, prior to or up to 6 hours post-ICH. Sham animals underwent the same treatments (corn oil or curcumin) and surgical procedures, but only received an intracerebral saline (vehicle) injection. The surgeon was blinded to drug treatments throughout the experiment. Mice were maintained at 37°C until the recovery of the righting reflex.

Hematoma assessment

Mice were intracardially perfused with saline and 2-mm coronal sections were immediately prepared. Anterior and posterior views of each brain section were digitized and a region of interest (ROI) was traced around the perimeter of the hematoma. Hematoma area was quantified by calculating the number of pixels within the ROI using Adobe Photoshop software. Hemoglobin content, a sensitive measure of hematoma volume, was quantified in the ipsilateral or contralateral cerebrum using a QuantiChrom Hemoglobin Assay kit (Bioassay Systems, Hayward, CA, USA)^{7,32}.

Blood-brain barrier permeability

BBB permeability was quantified following administration of Evans blue (20 mg/mL in PBS, i.v.) 2h prior to sacrifice. Blood (100 µL) was obtained by cardiac puncture, centrifuged, and then the plasma was diluted in N, N-Dimethylformamide (1:1000). Following perfusion with saline, brains were weighed, solubilized in N,N-dimethylformamide, and then incubated at 78°C for 18h. Absorbance was then determined in brain and blood samples at 620 nm using a Synergy HT plate reader (Bio-Tek, Winooski, VT). The concentration of Evans blue (µg/µl) in each sample, a measure of BBB permeability, was calculated using a standard curve, and permeability was equal to [(Evans blue concentration of brain/weight of brain) / (Evans blue concentration of plasma/circulation time)], as reported previously⁴⁷

Assessment of cerebral edema

Brain water content, an established measure of cerebral edema, was quantified in a 2 mm coronal tissue sections of the ipsilateral or corresponding contralateral striatum, as detailed by our laboratory and others^{11,37,42}. Tissue was immediately weighed (wet weight), then dehydrated at 65°C. Samples were reweighed 48h later to obtain a dry weight. The percentage of water content in each sample was calculated as follows: % Brain water content = [((wet weight – dry weight)/wet weight)*100].

RNA isolation and qRT-PCR

Total RNA was isolated (SV RNA Isolation kit, Promega, Madison, WI, USA) and qRT-PCR performed on a Cepheid SmartCycler II (Cepheid, Sunnyvale, CA, USA) using a Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA), as described by our laboratory^{25,42}. Primers were as follows: IL-1β: (FP 5'-GCCATCCTCTGTGACTCAT-3'; RP 5'-AGGCCACAGGTATTTTGTGCG-3'), IL-6: (FP 5'-AGTTGCCTTCTTGGACTGA-3'; RP 5'-TCCACGATTCCAGAGAAC-3'), TNF-α: (FP 5'-CGTCAGCCGATTTGCTATCT-3'; RP 5'-CGGACTCCGCAAAGTCTAAG-3'), and RPS3: (FP 5'-AATGAACCGAAGCACACCATA-3'; RP 5'-ATCAGAGAGTTGACCGCAGTT-3'). Product specificity was confirmed by melting curve analysis and visualization of a single, appropriately sized band on a 2% agarose gel. Gene expression levels were quantified using a cDNA standard curve^{12-14,25,34,42} and data were normalized to RPS3, a housekeeping gene that was unaffected by the experimental manipulations (data not shown). Data were expressed as fold change vs. sham.

Neurological outcomes

Neurological injury was determined using a modified 24-point scale, as detailed previously^{3,9}. This scale was comprised of six behavioral tests, each of which were graded from 0 (performs with no impairment) to 4 (severe impairment). Prior to experimentation, mice were pre-tested and any mouse unable to climb without impairment was excluded from further study. A composite score was calculated as the sum of the grades on all six tests.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Student Newman-Keuls or Two-way ANOVA followed by Bonferroni *post-hoc* test were used for multiple group comparisons and a t-test was used for two group comparisons, as indicated in the figure legends. Data are expressed as mean \pm SEM. A *p* value of <0.05 was considered to be significant.

RESULTS

Curcumin attenuates hematoma size and brain hemoglobin content after ICH

Administration of curcumin (150 mg/kg) significantly reduced gross hematoma size within the ipsilateral cortex following ICH. Notably, no beneficial effect was observed prior to 72h post-injury, indicating curcumin did not reduce collagenase activity or limit acute hematoma expansion (Figure 2a). Quantification of the hematoma at 72h post-injury indicated a significant reduction in the curcumin-treated group ($10.6 \pm 1.2 \text{ mm}^2$), as compared to placebo-treated mice ($16.1 \pm 1.6 \text{ mm}^2$, $p < 0.01$, $n=8/\text{group}$) (Figure 2b). Conversely, significant differences in hematoma size were not observed at either the 24h or 48h timepoints. These changes mirrored the temporal pattern of hemoglobin content within the brain, a validated measure of hematoma volume. Specifically, acute administration of curcumin reduced hemoglobin content by 28%, as compared to placebo-treated mice ($p < 0.01$ vs. ICH) using the collagenase ICH model (Figure 2c, **$n=8/\text{group}$**). Similarly, curcumin reduced hematoma volume by 42%, as compared to placebo-treated mice, using an autologous blood clot model of ICH (Figure 3).

Hematoma volumes were significantly attenuated at 72h post-ICH by administration of curcumin at 15 minutes prior to injury; however, this pre-treatment regimen may not be clinically feasible. Thus, the therapeutic window for acute curcumin administration to limit hematoma size was established. As was observed using a pre-treatment paradigm, administration of curcumin up to 3h post-ICH significantly reduced hemoglobin content within the ipsilateral cortex at 72h. A 0.5h post-treatment with 150 mg/kg curcumin was associated with a 38.0% reduction in hematoma volume ($p < 0.001$ vs. ICH, $n=8$) whereas a 3h post-treatment induced a 20.5% reduction in hematoma size ($p < 0.05$ vs. ICH, $n=8$) (Figure 4). This protective effect was lost when post-treatment was delayed by 6h or more after ICH (data not shown), suggesting the molecular mechanisms responsible for hematoma resolution after curcumin treatment are restricted to the acute time period following injury.

Curcumin attenuates inflammatory gene expression after ICH

We next investigated whether curcumin limits inflammatory gene expression following ICH. Pre-treatment with curcumin (150 mg/kg) significantly reduced the expression of IL-6, IL-1 β , and TNF- α , inflammatory mediators strongly implicated in the pathophysiology of ICH, adjacent to the hematoma by 24h post-ICH. Expression of IL-6, a cytokine that this clinically correlated with hematoma expansion, was increased by 18.2 ± 6.0 fold ($p < 0.01$ vs. sham) within the peri-hematoma region. This increase was significantly reduced by pre-treatment with curcumin (2.4 ± 1.3 fold vs. sham; $p < 0.01$ vs. ICH; $n=8$) (Figure 5a). Similarly, curcumin reduced the expression of IL-1 β (0.4 ± 0.2 fold vs. sham; $p < 0.001$ vs. ICH, 29.5 ± 8.2 fold vs. sham; $n=8$) (Figure 5b) and TNF- α (1.5 ± 1.0 fold vs. sham; $p < 0.01$

vs. ICH, 5.9 ± 1.5 fold vs. sham; $n=8$) (Figure 5c). Notably, inflammatory gene expression within the contralateral hemisphere following ICH did not significantly differ from sham-operated mice (data not shown).

Curcumin reduces BBB permeability and cerebral edema after hemorrhagic injury

Production of pro-inflammatory mediators, such as those elevated following ICH, is associated with disruption of the BBB and the development of vasogenic edema. Consistent with this assertion, curcumin significantly reduced the extravasation of Evans blue dye, a sensitive estimate of BBB permeability, at 6h ($p<0.001$ vs. ICH, $n=10$ /group), 12h ($p<0.01$ vs. ICH, $n=10$ /group), and 24h ($p<0.001$ vs. ICH, $n=10$ /group) post-ICH (Figure 6a–c). Similarly, brain water content was significantly decreased in curcumin-treated mice ($81.3 \pm 0.9\%$); $p<0.05$ vs. ICH, $n=10$ /group), as compared to placebo-treated mice ($83.7 \pm 0.3\%$) at 24h (Figure 7a), with a maximal effect noted at 72h post-ICH (Figure 7b). Significant differences in edema were not observed in the contralateral hemispheres in any of the treatment groups across all time points (data not shown).

Neurological outcome is improved in curcumin treated animals

Consistent with the effect on hematoma volume and cerebral edema, curcumin improved neurological outcomes following ICH, as determined using a 24-point scale. Specifically, curcumin significantly improved neurobehavioral scores at 48h, with a maximal improvement noted at 72h ($n=10$ /group) (Figure 8). Significant differences in baseline neurological score were not observed between any of the experimental groups.

DISCUSSION

Despite maximal surgical intervention and supportive care, ICH is associated with significant morbidity and mortality, in part, due to a lack of viable treatment options^{4,33}. Acute or subacute hematoma expansion can occur in the days following the initial rupture. This persistent or recurrent bleeding exacerbates the space-occupying mass lesion, which subsequently increases neurovascular injury and promotes clinical deterioration. That surgical clot evacuation is associated with a more favorable outcome suggests hematoma size may dictate clinical prognosis³⁰; however, many ICH patients are poor surgical candidates and/or present with a hemorrhage which is not amenable to neurosurgical intervention. Thus, new strategies to promote clot resolution are needed.

The present study demonstrates a novel beneficial effect of clinically-achievable doses of the curry spice, curcumin, in promoting hematoma resolution and neurological improvement in separate pre-clinical models of ICH. Hematoma formation was unaffected by curcumin treatment, implying neither the hemorrhagic activity of collagenase nor altered blood coagulation parameters were adversely influenced following drug administration. In contrast, curcumin reduced hematoma volume and neurological deficits by post-ICH days 3 and 7, respectively. Interestingly, administration of curcumin before injury or within the first hour following ICH was required for the maximal protective effect. While this narrow therapeutic window may diminish the ultimate translation of curcumin into the clinic, these data indicate the activation of cellular signaling pathways within the first hour after vascular rupture may contribute to subsequent neurovascular injury.

A dose of 150 mg/kg curcumin was optimal for promoting clot resolution and reducing neurological injury following ICH. This finding is consistent with reports from our laboratory and others showing a maximal beneficial effect of similar doses of curcumin in pre-clinical models of cerebral ischemia^{40,48}, TBI²⁴, and SAH⁴². Notably, pharmacokinetic studies in mice showed peak plasma concentrations ($\sim 1.6 \mu\text{M}$) within 15 minutes and brain

accumulation within 1 hour after intraperitoneal administration of 100 mg/kg curcumin²⁵. These levels reflect the serum concentrations observed in patients following daily oral ingestion of 8 grams and more importantly, functionally inhibited pro-inflammatory signaling in peripheral blood mononuclear cells from pancreatic cancer patients^{6,15}. Thus, the doses used to promote hematoma resolution in this study may simulate clinically-achievable steady-state serum concentrations. Unfortunately, curcumin exhibits relatively poor oral bioavailability and a short serum half-life (<45 minutes), which could also contribute to the limited therapeutic window observed in this study. Thus, the use of liposomes or nanoparticles may improve drug delivery, overcome bioavailability issues, and could extend the therapeutic window¹. Alternatively, the development of curcumin analogues exhibiting an improved pharmacological profile (e.g. improved solubility, half life, brain distribution), may providing a longer therapeutic window and/or increased activity with respect to reducing the volume of the hematoma.

Inflammatory activation correlates with hematoma expansion, neurological deterioration, and a poor functional recovery^{20,26,31,48}. Along these lines, plasma concentrations of IL-6 and TNF- α were significantly higher in patients exhibiting acute hematoma enlargement following a cerebral hemorrhage³¹. Similarly, expression of IL-1 β and TNF- α were acutely increased within the perihematoma tissue using multiple species and models of experimental ICH^{2,27,28,41,43,46}. Although the functional significance of the inflammatory responses remains largely unresolved, both IL-1 β and TNF- α are associated with increased BBB permeability and the formation of vasogenic edema^{5,16,21,29,45}. Further supporting this assertion, early elevations in plasma concentrations of TNF- α and IL-6 clinically correlated with the development of brain edema, mass effects, and patient outcome following ICH^{5,17,22,44}.

The pro-inflammatory transcription factor, NF κ B, is a positive regulator of IL-1 β , IL-6, and TNF- α expression. Interestingly, NF κ B activation was observed within minutes and was sustained for several days following ICH in rats⁴⁸. Curcumin is a potent inhibitor of NF κ B³⁹ and our recent work suggests curcumin reduces neuroinflammatory expression, cerebral edema, and neurological injury after SAH and TBI⁴². Herein, we show for the first time, that curcumin reduces the expression of classical pro-inflammatory cytokines within the perihematoma tissue following collagenase- (Figure 4) or autologous blood-induced ICH (data not shown). As pro-inflammatory mediators, such as those studied in this report, often induce the expression of other inflammatory mediators, the ability of curcumin to limit acute expression could limit the progressive increase in neurovascular injury observed following ICH. These findings may also explain how the acute administration of curcumin produces a dramatic reduction in neurovascular injury observed days later.

CONCLUSION

Curcumin or structural analogues of curcumin may provide a novel, safe therapeutic approach to limit hematoma volume and neurovascular injury following ICH.

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MDK and DJM performed all brain injury modeling and endpoint assessments. MFW and SEM performed the hematoma volume measurements. All authors contributed to data analysis and final manuscript editing. KMD and CHA conceptualized the study and wrote the manuscript.

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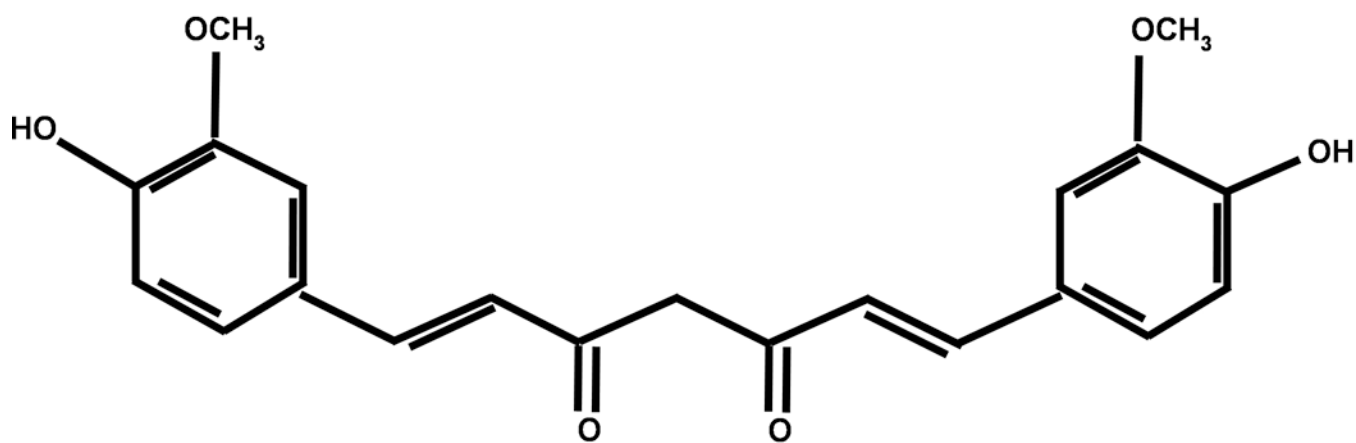


Figure 1. Chemical structure of curcumin

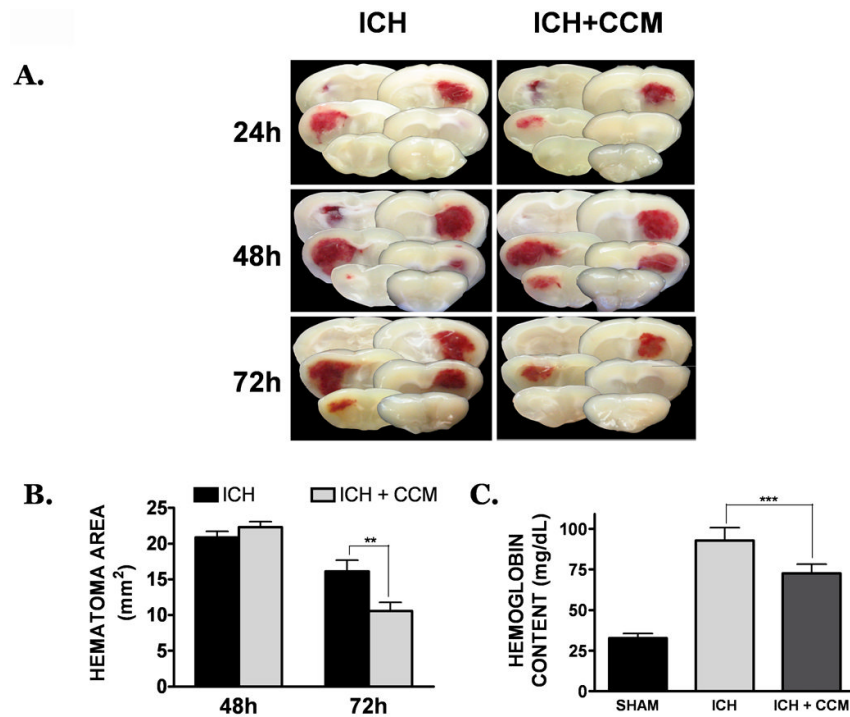


Figure 2. Curcumin promotes hematoma resolution after collagenase-induced ICH

(A) Mice were treated with placebo (left panels) or curcumin (150 mg/kg; right panels) at the time of ICH. At 24h, 48h, or 72h post-ICH, coronal brain sections were prepared and digitally captured. Each panel depicts serial brain sections from a single, representative mouse per group. (B) Determination of hematoma area (mm²) at 48h or 72h post-ICH. Hematoma size was estimated by calculating the area inside a region of interest, as detailed in the Materials and Methods section (C) Hematoma volume was quantified by determining the hemoglobin content of each hemisphere at 72h post-ICH. Data are expressed as mean \pm SEM and were analyzed by t-test or One-Way ANOVA (n=8 mice/group) followed by Student Newman-Keul's post-hoc test (**p<0.01, ***p<0.001).

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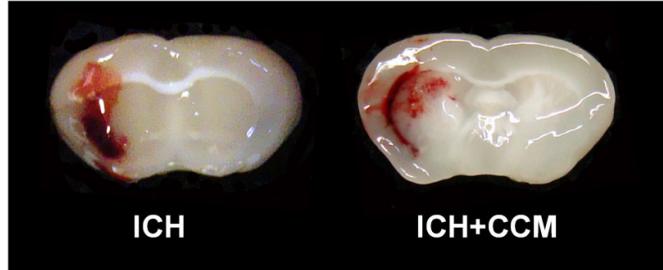
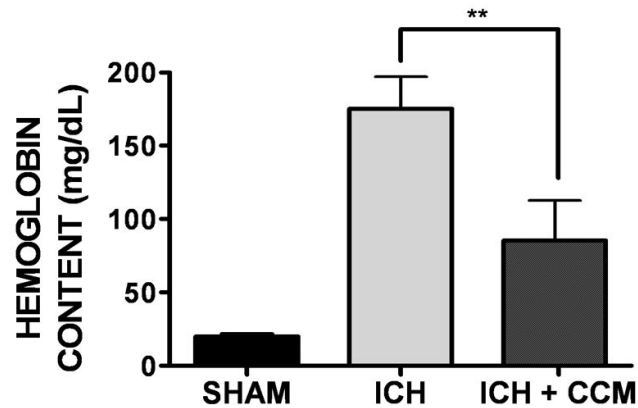
A.**B.**

Figure 3. Curcumin promotes hematoma resolution after autologous blood clot-induced ICH (A) Mice were treated with placebo (left panel) or curcumin (150 mg/kg; right panel) at the time of ICH. At 72h post-ICH, coronal brain sections were prepared and digitally captured. Each panel depicts a brain sections from a single, representative mouse per group (n=8 mice/group). (B) Hematoma volume was quantified by determining the hemoglobin content of each hemisphere at 72h post-ICH. Data are expressed as mean \pm SEM (n=8 mice/group) and were analyzed by One-Way ANOVA followed by Student Newman-Keul's post-hoc test (**p<0.01).

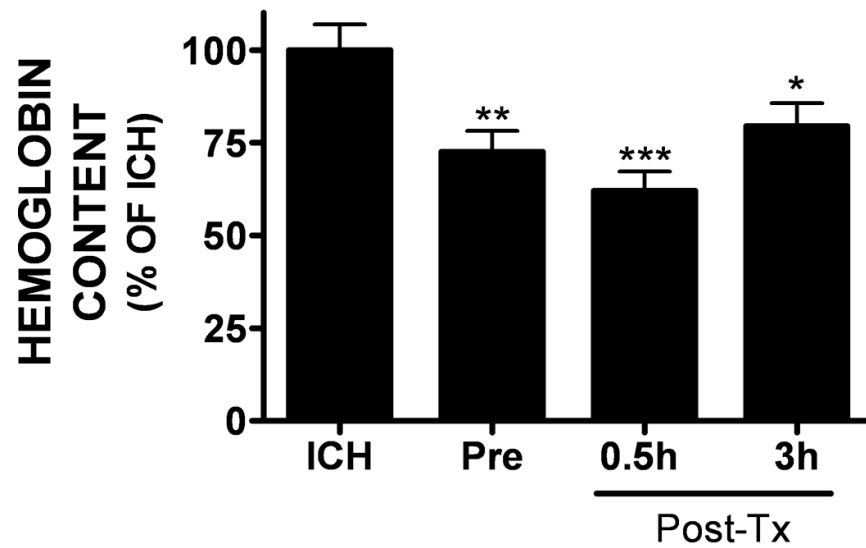


Figure 4. Establishment of a therapeutic window for curcumin to reduce hematoma volume (A) Mice were treated with placebo or curcumin (150 mg/kg) at 15 minutes prior to injury or 0.5h, 1h, or 3h following collagenase-induced ICH. Hematoma volume was quantified 72h later using a hemoglobin content assay. Data are expressed as mean \pm SEM (8 mice/group) and graphed as “% change from placebo-treated ICH mice”. Groups were analyzed using a One-Way ANOVA followed by Student Newman Keul’s post-hoc test (* p <0.05, ** p <0.01, *** p <0.001 vs. ICH). Data are expressed as mean \pm SEM (n=8 mice/group) and analyzed using One-Way ANOVA followed by Student Newman-Keul’s post-hoc test (* p <0.05, ** p <0.01).

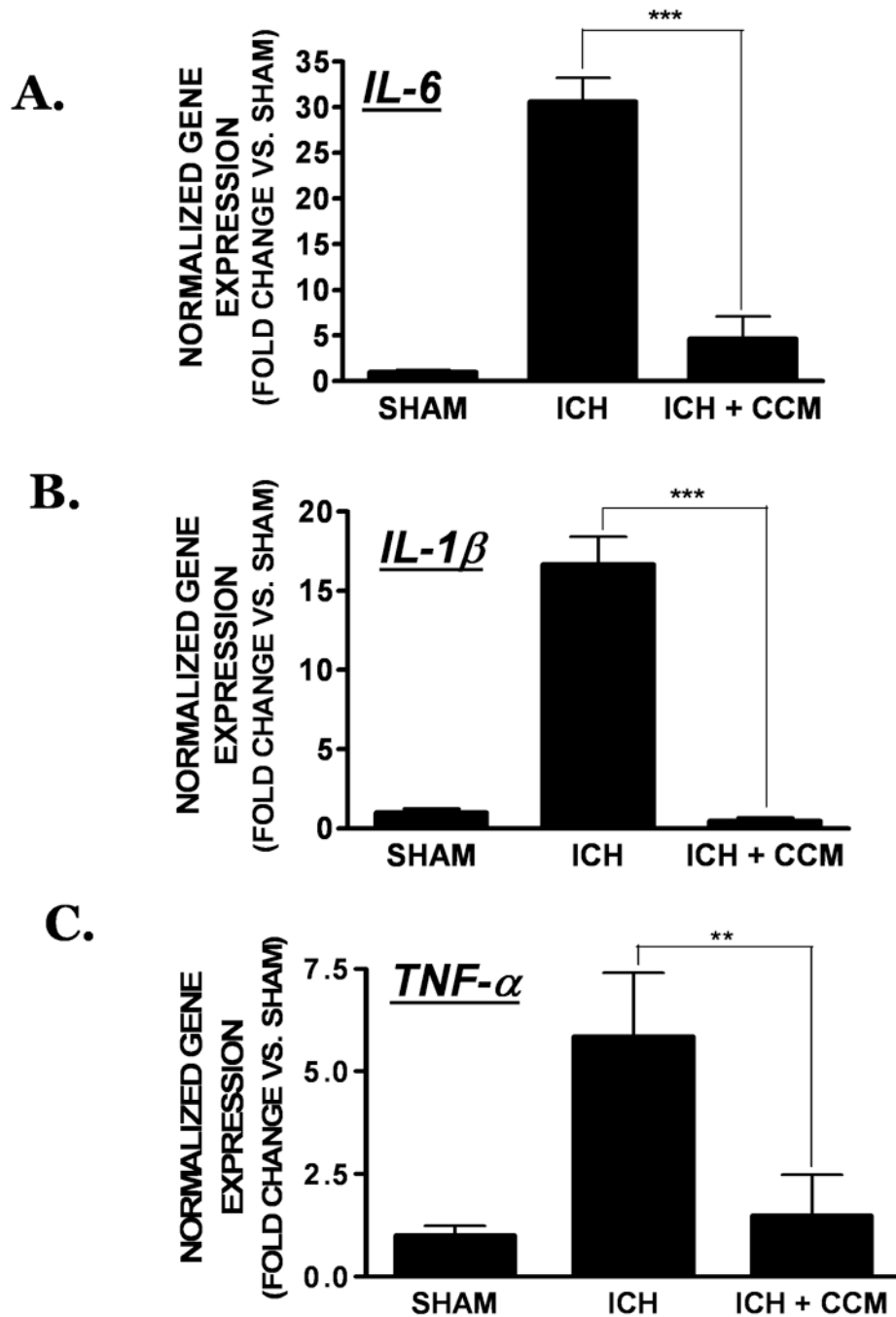


Figure 5. Effect of curcumin on inflammatory gene expression

Mice were treated with curcumin (150 mg/kg) at the time of collagenase-induced ICH following by the quantification of inflammatory gene expression. (A) IL-6, (B) IL-1 β , and (C) TNF- α expression was assessed in the peri-hematoma region at 24h post-ICH using qRT-PCR. Data were normalized to RPS3 and expressed as fold change vs. sham-operated mice (mean \pm SEM; n=8 mice/group) Data were analyzed using One-Way ANOVA followed by Student Newman-Keul's post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs. sham).

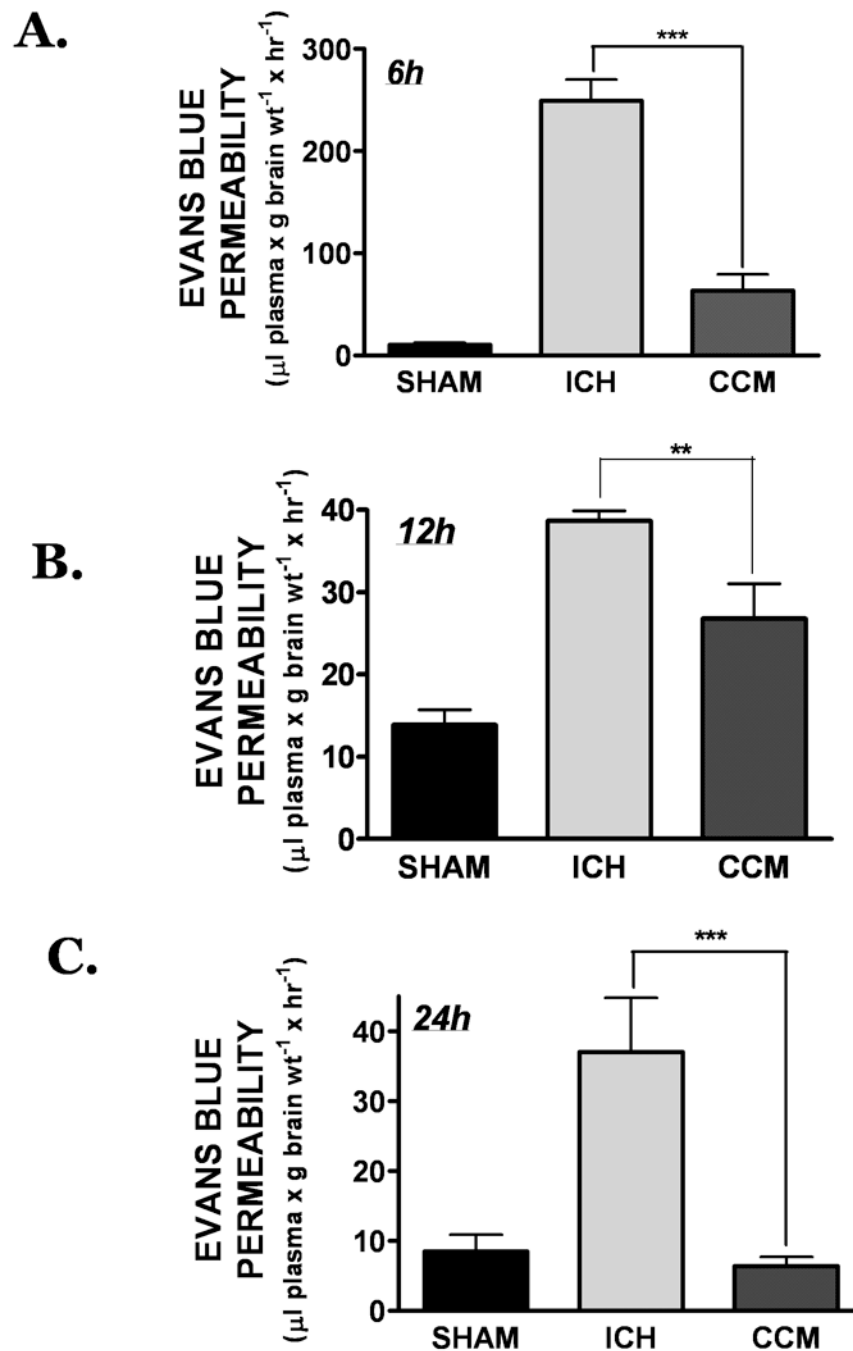


Figure 6. Curcumin reduces BBB permeability after ICH

Mice were administered curcumin (150 mg/kg) at the time of collagenase-induced ICH. Evans blue extravasation, a sensitive measure of BBB disruption, was assessed at (A) 6h or (B) 12h or (C) 24h post-ICH. Comparisons between treatments groups were done using a one-way ANOVA followed by Student Newman Keul's post-hoc test (** $p < 0.01$, *** $p < 0.001$). Data are expressed as mean \pm SEM from 10 mice/group.

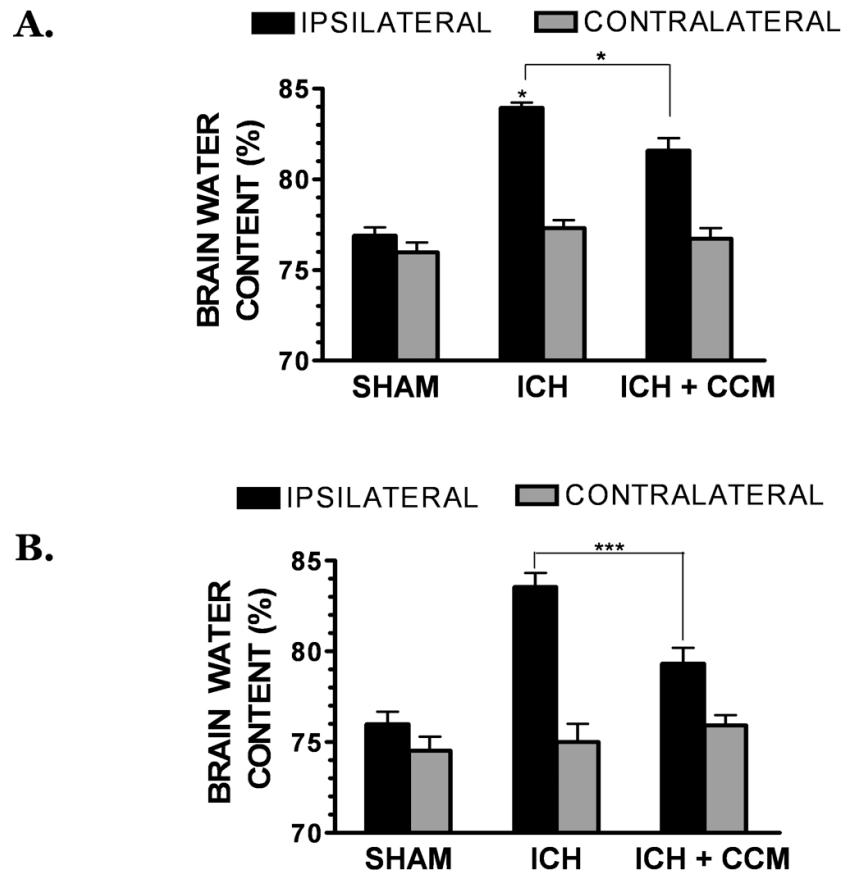


Figure 7. Curcumin attenuates brain edema following ICH

Mice were administered curcumin (150 mg/kg) at the time of collagenase-induced ICH. Brain water content, a measure of cerebral edema, was assessed in the ipsilateral and contralateral hemispheres at (A) 24h or (B) 72h following ICH. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Student Newman Keul's post-hoc test (* $p < 0.05$, *** $p < 0.001$). No significant differences were observed between groups in the contralateral hemispheres. Data are expressed as mean \pm SEM from 10 mice/group.

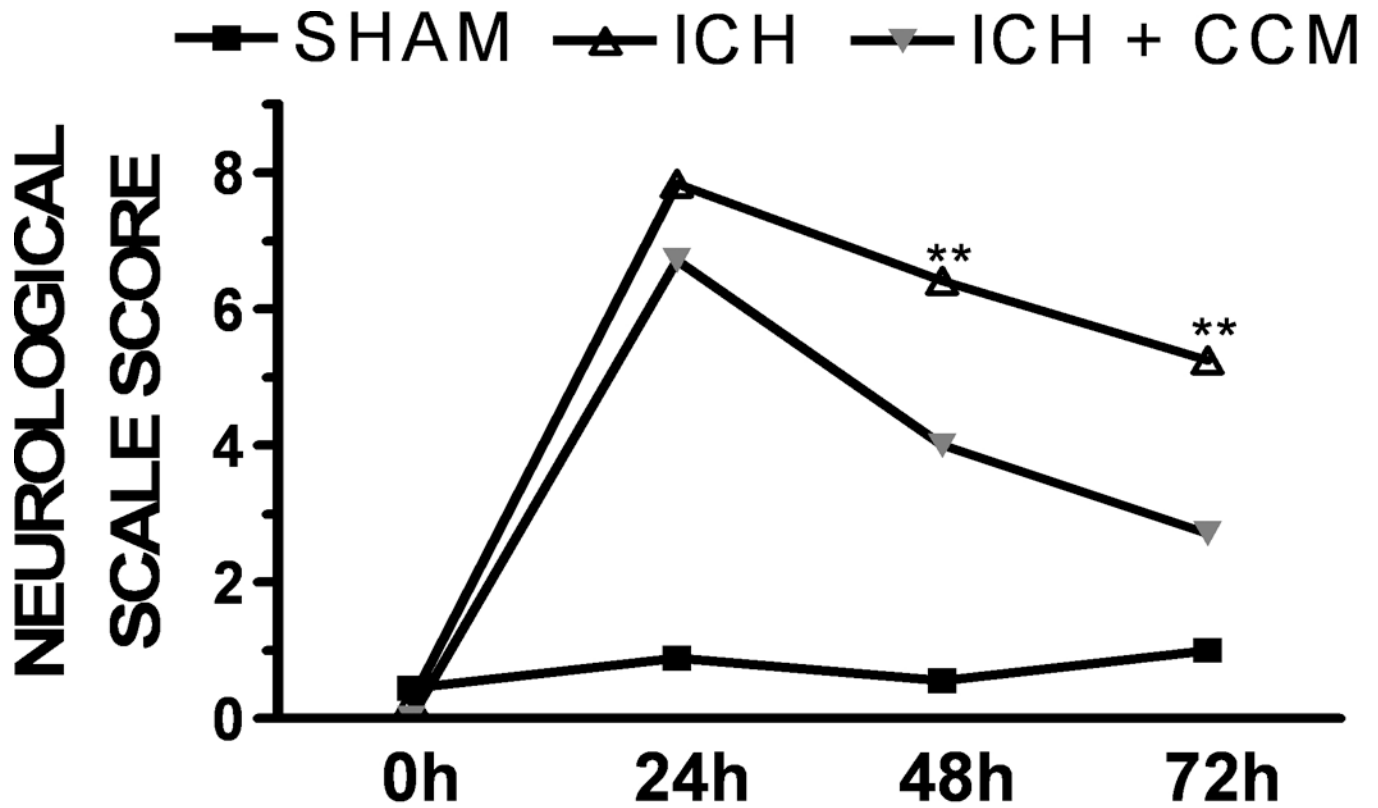


Figure 8. Curcumin improves neurological outcomes following ICH

Mice were treated with placebo or curcumin (150 mg/kg) at the time of collagenase-induced ICH. Neurological outcomes were then assessed at 24h, 48h, or 72h following sham injury or ICH (n=10 mice/group). Data are the mean \pm SEM and were analyzed using a repeated measures analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (**p<0.01 vs. sham).