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Original Research Communication

Curcumin Attenuates Vascular Inflammation and Cerebral Vasospasm After Subarachnoid Hemorrhage in Mice

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Abstract

Cerebral vasospasm is a major cause of death and disability after subarachnoid hemorrhage (SAH); however, clinical therapies to limit the development of cerebral vasospasm are lacking. Although the causative factors underlying the development of cerebral vasospasm are poorly understood, oxidative stress contributes to disease progression. In the present study, curcumin (150 or 300 mg/kg) protected against the development of cerebral vasospasm and limited secondary cerebral infarction after SAH in mice. The protective effect of curcumin was associated with a significant attenuation of inflammatory gene expression and lipid peroxidation within the cerebral cortex and the middle cerebral artery. Despite the ability of curcumin to limit the development of cerebral vasospasm and secondary infarction, behavioral outcome was not improved, indicating a dissociation between cerebral vasospasm and neurologic outcome. Together, these data indicate a novel role for curcumin as a possible adjunct therapy after SAH, both to prevent the development of cerebral vasospasm and to reduce oxidative brain injury after secondary infarction. *Antioxid. Redox Signal*. 11, 35–45.

Introduction

NEUROVASCULAR diseases, such as ischemic and hemorrhagic stroke, are a complex group of human pathologies and an important cause of death and disability. Subarachnoid hemorrhage (SAH), a type of hemorrhagic stroke, occurs without regard for age, race, or gender, with a prevalence of 1 in 10,000 people (comprising ~7% of all strokes) (19). Although the overall incidence of stroke has steadily declined, the frequency of SAH remains stable despite medical advancements and behavioral modifications. SAH patients exhibit a 30-day mortality rate of 30–40% and sustain a loss of productive life years comparable to that of ischemic stroke patients, due in part to the young age at onset and poor clinical outcome (19). Along these lines, approximately half of all SAH patients experience permanent disability, including deficits in verbal and nonverbal memory, psychomotor speed, executive function, and visual–spatial function (23), attesting to the devastating nature of this injury.

Neurologic dysfunction after SAH involves a complex, multifactorial series of events (32). One contributing factor, delayed cerebral vasospasm, is characterized by the chronic narrowing of the cerebral arteries beginning at day 3 and peaking 4–14 days after a hemorrhage, culminating in reduced brain perfusion, increased secondary cerebral infarction, and sometimes death (20, 32, 42, 62). Although the development of cerebral vasospasm has been long regarded as the primary determinant of neurologic injury after SAH (6, 32), this notion was recently challenged by clinical studies showing that vasospasm could be attenuated without a beneficial effect on patient outcome (24, 32). These data suggest other factors, including acute vasoconstriction, the delayed effects of global cerebral ischemia, thromboembolism, oxidative stress, disruption of the blood–brain barrier, increased cerebral edema, microcirculatory dysfunction, and cortical spreading depression may also contribute to the observed neurologic dysfunction and poor clinical outcome after SAH (3, 4, 7, 17, 21, 32, 44).

Oxidative stress likely contributes to the development of cerebral vasospasm (29, 34, 41), as mice overexpressing su-

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peroxide dismutase (SOD) exhibit less severe vasospasm after experimental SAH. Furthermore, intracisternal injection of SOD reduced vasospasm in cat and rabbit models of brain hemorrhage, supporting the involvement of superoxide anions in the generation of cerebral vasospasm. However, targeted genetic overexpression of antioxidant genes (*e.g.*, SOD) in the brain is technically challenging (10, 64), and direct intrathecal administration of SOD failed to prevent SAH-induced cerebral vasospasm in primates, likely because of bioavailability issues (32). These findings suggest that a reduction in oxidative stress may limit SAH-induced injury, although improved therapeutic approaches are needed.

Curcumin, a low-molecular-weight curry spice derived from the rhizome *Curcuma longa*, reduces oxidative damage and cognitive deficits in animal models of Alzheimer disease, ischemic stroke, and traumatic brain injury (9, 30, 60). Curcumin has been safely consumed by humans for centuries, including use as an antiinflammatory agent in Ayurveda, an ancient Indian system of medicine (22, 55). Recent clinical trials demonstrated that oral administration of curcumin resulted in bioactivity with minimal adverse effects (8, 60). Given the reported ability of curcumin to limit the production of superoxide (40, 51, 56), an important causative factor in the development of cerebral vasospasm, we hypothesized that curcumin may reduce cerebral vasospasm and neurologic injury in a mouse model of SAH. A second goal of this study was to determine whether curcumin limits vascular inflammation and oxidative stress after SAH in mice, which may also contribute to brain injury.

Materials and Methods

Animals

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) were anesthetized with 8 mg/kg xylazine/60 mg/kg ketamine. Throughout all surgical procedures, body temperature was maintained at 37°C by using a small-animal temperature controller (David Kopf Instruments, Tujunga, CA).

SAH model

SAH was induced as described previously (34, 47). The common carotid artery was exposed *via* a midline incision, and the external carotid artery (ECA) was isolated and ligated. A blunted 5-0 monofilament nylon suture was placed into the ECA and advanced into the internal carotid artery (ICA). The suture was advanced to the anterior cerebral artery (ACA)–middle cerebral artery (MCA) bifurcation, where resistance was encountered. The suture was then advanced 2 mm, perforating the ACA, and then immediately withdrawn, allowing reperfusion. In sham-operated mice, the suture was advanced only until the point of resistance, but arterial perforation was not performed. After suture removal, the skin was surgically stapled, and animals were returned to cages until recovery of the righting reflex. Curcumin (150 or 300 mg/kg; MP Biomedical, Solon, OH) was dissolved in corn oil and given *via* intraperitoneal injection immediately after SAH. The SAH procedure was associated with an overall mortality rate of 13%, comparable to that of others using this model (34).

Assessment of cerebral ischemia

To quantify secondary ischemic injury after the development of vasospasm, animals were intracardially perfused with saline, and 1-mm coronal brain sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC; 2% wt/vol in saline; Sigma, St. Louis, MO), followed by fixation in 2% paraformaldehyde. Digital images were quantified by using NIH Image software and expressed as percentage of the hemisphere infarcted, as described by our group (36).

Assessment of cerebral vasospasm

A craniotomy was made over the irrigation area of the MCA to visualize the superficial pial vessels, by using a stereotaxic apparatus. Polyethylene tubing with heat-flared ends was affixed over the cranial window with Vetbond adhesive (3M, Minneapolis, MN) to serve as a positioning guide for the laser Doppler probe. The guides were fixed to the skull with dental acrylic cement, and the scalp incision was closed around the probe guides with 4-0 silk suture. Mice recovered for 1 week to allow stabilization of CBF after cranial window surgery, and then a baseline CBF reading was made over a 30-min period by using a laser-Doppler flowmeter (model 5000; PeriMed, Stockholm, Sweden). At days 2 to 6 after SAH, CBF was measured as a percentage of baseline (58). The magnitude of cerebral vasospasm in the MCA was documented by a blinded investigator with the gelatin-casting method, as detailed previously (33, 34). In brief, 72 h after SAH, animals were intracardially perfused with phosphate-buffered saline, followed by a 15-min perfusion with 10% formalin. Gelatin–India ink solution was then perfused at 60–80 mm Hg for 10 min. Mice were then refrigerated for 24 h to induce gelatin solidification. Brains were harvested and stored in 4% neutral-buffered formaldehyde. Imaging was performed with a dissecting microscope (x6 magnification) connected to an image analyzer. Analysis was performed on the narrowest portion of the proximal MCA (1 mm or less from the ACA–MCA bifurcation), which undergoes the most prominent vasospasm in this model (33). To correct for differences due to perfusions, the diameter of the basilar artery was measured in all mice as a negative control.

Assessment of neurologic injury

To determine the effect of curcumin treatment on SAH-induced neurologic injury, the rotarod test of motor function and the open-field activity test were conducted, exactly as described previously (11). For the measurement of open-field activity, mice were placed in a 14×14-inch black box that was divided into a 2×2-inch square grid (49 squares in total). The open-field activity was measured by the number of crosses within a 3-min trial. For the rotatod test, mice were placed on a rod rotating at 20 rpm. Performance was measured by the amount of time that mice remained on the rotarod. No differences were noted in the rotarod test between any of the groups (data not shown).

Superoxide quantification

Hydroethidine (HEt; AnaSpec Inc., San Jose, CA), which readily enters the central nervous system, was used to quantify superoxide anion production in vivo, as previously demonstrated by our group, with minor modifications (5). Superoxide selectively converts HEt to ethidium (Et), which stably binds DNA and emits red fluorescence. HEt (200 μl; 1 mg/ml in 1% DMSO with saline) was administered intravenously 15 min before endovascular perforation. After injury, mice were perfused with phosphate-buffered saline, and then the fluorescence from the cerebral cortex or middle cerebral artery homogenates was quantified (excitation $\lambda = 520$ nm; emission $\lambda = 610$ nm) (Synergy HT; Bio-Tek, Winooski, VT). Arbitrary fluorescence units were normalized to total protein content, as measured by using a bicinchoninic acid (BCA) Protein Assay (Pierce Biotechnology, Inc., Rockford, IL), and data are expressed as a fold change *versus* sham-operated control mice.

Peroxynitrite formation

Nitrotyrosine formation, a marker of peroxidative injury, was quantitated in cerebral cortex or middle cerebral artery homogenates by using a specific enzyme immunoassay system (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's protocol. Data were quantified by using a nitrotyrosine standard curve, normalized to the protein content of the sample, and expressed as a fold change versus sham-operated animals.

Measurement of lipid peroxidation

Lipid peroxidation was assessed by measuring the colorimetric formation of malondialdehyde at 24 h after SAH (12). In brief, an aliquot of tissue homogenate (100 μl) was added to a reaction mixture containing 100 μl of 8.1% sodium dodecyl sulfate, 750 μl of 20% acetic acid (pH 3.5), 750 μl of 0.8% thiobarbituric acid, and 300 μl of distilled water. Samples were boiled for 1 h and then centrifuged at 4,000 *g* for 10 min. The absorbance of the supernatant was measured with spectrophotometry at 532 nm and normalized to total protein content. Thiobarbituric acid–reactive species (TBARS) formation, a measure of oxidative stress and lipid peroxidation, was expressed as nanomoles per milligram protein.

NF-κB–binding assays

Nuclear extracts were prepared by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Nuclear extract, 7.5 μg, was used to determine NF-κB activation (TransAM; Active Motif). This method rapidly detects activated NF-κB complex binding (p50 or p65 subunits), possessing a detection limit of <0.5 μg nuclear extract or <0.4 ng recombinant p50 or p65 protein/well, and is up to 5 times more sensitive than a traditional electromobility shift assay. Nuclear extracts prepared from TPA-stimulated Jurkat cells (2.5 μg/well) were used as a positive control. To demonstrate binding specificity, a 20-fold excess of an NF-κB wild-type consensus oligonucleotide (20 pmol/well) was used as a competitor to block specific NF-κB binding to the well. Conversely, a mutated consensus NF-κB oligonucleotide had no effect on NF-κB binding, further demonstrating the specificity of the reaction. This entire procedure was detailed by our laboratory (15, 16).

RNA isolation and qRT-PCR

Total RNA was isolated by using a commercially available kit (SV RNA Isolation; Promega, Madison, WI). qRT-PCR was performed on a SmartCycler II (Cepheid, Sunnyvale, CA) by using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). Primers used were as follows: iNOS: (FP 5′-CCTTGTTCAGCTACGCCTTC-3′; RP 5′-AAGGCCAAACACAGCATACC-3′), ICAM: (FP 5′-TTCACACTGAATGCCAGCTC-3′; RP 5′-GTCTGCTGAGACCCCTCTTG-3′), VCAM-1: (FP 5′-ATTTTCTGGGGCAGGAAGTT-3′; RP 5′-ACGTCAGAACAACCGAATCC-3′), IL-1β: (FP 5′-GCCCATCCTCTGTGACTCAT-3′; RP 5′-AGGCCACAGGTATTTTGTCG-3′), COX-2: (FP 5′-AGAAGGAAATGGCTGCAGAA-3′; RP 5′-GCTCGGCTTCCAGTATTGAG-3′), TNF-α: (FP 5′-CGTCAGCCGATTTGCTATCT-3′; RP 5′-CGGACTCCGCAAAGTCTAAG-3′), IL-6: (FP 5′-AGTTGCCTTCTTGGGACTGA-3′; RP 5′-TCCACGATTTCCCAGAGAAC-3′), RPS3 (FP 5′-AATGAACCGAAGCACACCATA-3′; RP 5′-ATCAGAGAGTTGACCGCAGTT-3′). Samples were quantified by using serial cDNA dilutions, as described by our laboratory (14–16), and data were normalized to the housekeeping gene, RPS3, which was unaffected by experimental manipulations. Data were expressed as fold change *versus* sham. Primer specificity was confirmed by melting curve analysis and electrophoresis of PCR products on a 2% agarose gel to confirm the presence of a single band of the predicted size.

Physiological measurements

To assess changes in blood parameters after treatments, 200 μl of blood was drawn from anesthetized mice *via* direct cardiac puncture and immediately quantified by using a CG8+ blood-gas cartridge and iStat1 handheld blood-gas analyzer (Abbott, Inc., East Windsor, NJ). The effect of curcumin treatment on blood pressure was assessed by using a noninvasive cuff (Advanced Blood Pressure Monitor; Harvard Apparatus, Holliston, MA). Data were expressed as mean ± SEM ($n = 4$/group).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Student–Newman–Keul's or Dunnett's *post hoc* tests 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 ± SEM. A p value < 0.05 was considered to be significant.

Results

Pathogenesis of SAH in a murine endovascular perforation model

A significant blood clot was observed in the subarachnoid space within 1 h of SAH (Fig. 1A). In contrast, no clot was observed in the sham-operated animals, which underwent the same surgical procedures without vascular perforation. The clot persisted for several hours after SAH and was largely cleared by 24 h after SAH. After SAH, cerebral blood flow (CBF) was acutely reduced to 59.1 ± 3.7% ($p < 0.01$ *vs.*

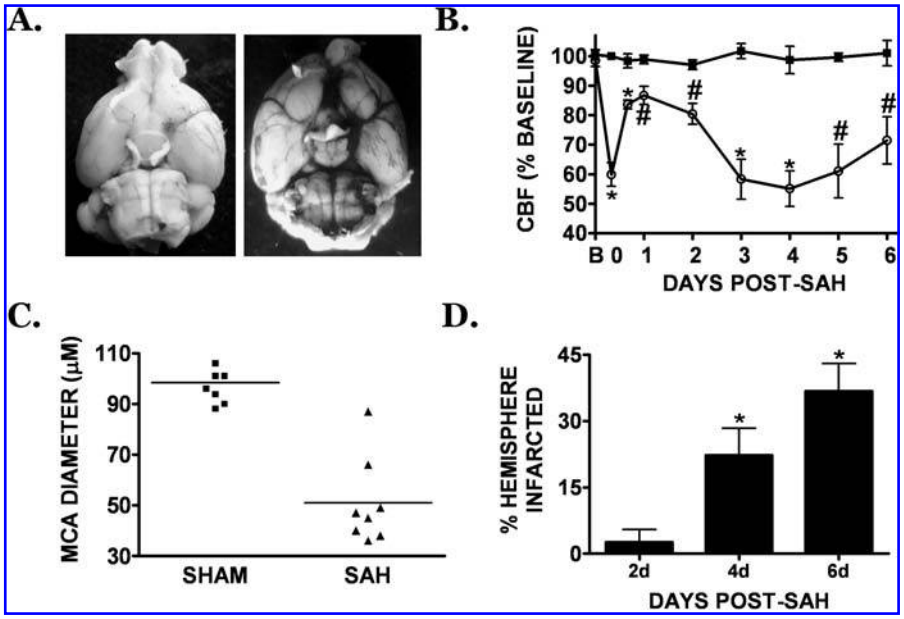

FIG. 1. (A) Ventral image of the mouse brain 1 h after SAH. Sham-operated animals (*left*) showed no injury, whereas SAH animals (*right*) exhibited a significant blood clot. (**B**) Cerebral blood flow (CBF) changes after SAH ($n = 6$ mice; $n = 4$ sham-operated mice), as measured by laser Doppler. B, baseline; time 0 was measured within 1 h of SAH. Delayed vasoconstriction was observed between days 3 and 6 after SAH, suggesting the development of cerebral vasospasm. Data are expressed as %CBF as compared with baseline controls, which were taken before SAH or sham, and time-point comparisons were made by *t* test (#$p < 0.05$; *$p < 0.01$ *vs*. sham). (**C**) Lumen diameter of the MCA at 72 h after SAH, as assessed by gelatin casting. Horizontal lines represent group means, and each data point represents the diameter of the MCA from an individual animal. (**D**) Temporal pattern of cerebral infarction after SAH ($n = 5$ animals/time point). Data are shown as the percentage of hemisphere infarcted, as estimated by TTC staining. *$p < 0.01$ *vs*. sham-operated mice.

sham) of baseline, demonstrating a transient vasoconstriction within hours of SAH. CBF returned to near baseline [86.4 ± 2.4% of baseline ($p < 0.05$ *vs*. sham)] between days 1 and 2 before undergoing delayed cerebral vasospasm beginning on day 3 after SAH [57.1 ± 7.2% of baseline ($p < 0.01$). CBF remained at 50.1–55.6% of baseline throughout the duration of the study, although CBF appeared to be returning toward baseline by day 6 (Fig. 1B). Serial CBF determination may not be a reliable measure of cerebral vasospasm; thus, the diameter of the middle cerebral artery (MCA) also was assessed by using gelatin-casting methods. Sham-operated mice exhibited a MCA lumen diameter (mean ± SD; $n = 8$ mice/group) of 98.4 ± 7.9 μm at 72 h after SAH. In contrast, the diameter of the MCA was reduced to 51.0 ± 17.3 μm after SAH, supporting the reduction in CBF and development of cerebral vasospasm (Fig. 1C). Secondary cerebral infarction, as assessed by TTC staining, was not observed at 48 h of SAH (2.6 ± 2.9% hemisphere infarcted); however, a significant infarct was observed by day 4 after SAH (22.3 ± 6.1% hemisphere infarcted; $p < 0.05$ vs. day 0), with a maximal injury by day 6 (36.7 ± 6.3% hemisphere infarcted) (Fig. 1D).

Curcumin reduces brain injury after SAH

A single administration of curcumin (150 or 300 mg/kg), at the time of injury, significantly limited secondary cerebral infarction (9.9 ± 2.8% and 3.3 ± 1.0% hemisphere infarcted, respectively, $p < 0.01$ *vs*. placebo; 34.2 ± 5.2% hemisphere infarcted) at 6 days after SAH (Fig. 2A). In a corresponding manner, curcumin attenuated the acute vasoconstriction occurring within the first hours after SAH (63.0 ± 5.1% of baseline for placebo/SAH animals vs. 80.9 ± 2.6% for curcumin/SAH animals). Curcumin also reduced the development of delayed cerebral vasospasm beginning at 3 days after SAH, maintaining CBF at 84 ± 5.6% *versus* 56.3 ± 4.2% for SAH) (Fig. 2B). The ability of curcumin to limit the development of cerebral vasospasm was further documented by using gelatin casting. Whereas curcumin treatment alone did not influence MCA diameter in sham-treated animals (97 ± 6.3 μm in curcumin-treated sham vs. 99.4 ± 7.9 μm in placebo-treated sham), curcumin significantly reduced SAH-induced constriction of the MCA at 3 days after injury (Fig. 2C). The reduction in MCA diameter in placebo-treated mice after SAH (49.8 ± 11.8 μm; $p < 0.01$ *vs*. sham) was completely blocked by administration of curcumin at the time of injury (90.6 ± 13.9 μm; $p < 0.01$ *vs*. SAH animals, $p > 0.05$ *vs*. sham), but vasospasm was not significantly attenuated by a 1, 3, or 24 h after treatment with curcumin (data not shown). In contrast, the lumen diameter of the basilar artery was not significantly different between the placebo-treated SAH group (139.3 ± 6.9 μm) and the curcumin-treated SAH group (141.7 ± 7.3 μm), documenting the specificity of the vasospasm observed in the middle cerebral artery (data not shown). In contrast to the beneficial effect of curcumin on cerebral vasospasm and secondary infarction, cotreatment at the time of injury or posttreatment at 1, 3, or 24 h after injury failed significantly to reduce SAH-induced neurologic injury, as assessed by open-field activity measurement (Fig. 3). Neither the SAH procedure nor the administration of curcumin significantly influenced any of the physiological parameters investigated (Table 1).

Curcumin limits vascular inflammation and NF-κB activation after SAH

Inflammatory gene expression, including cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), was acutely increased in the cerebral cortex within 1.5–3 h of SAH (Fig. 4A), as compared with sham-operated mice. Administration of curcumin (150 mg/kg), at the time of SAH, significantly

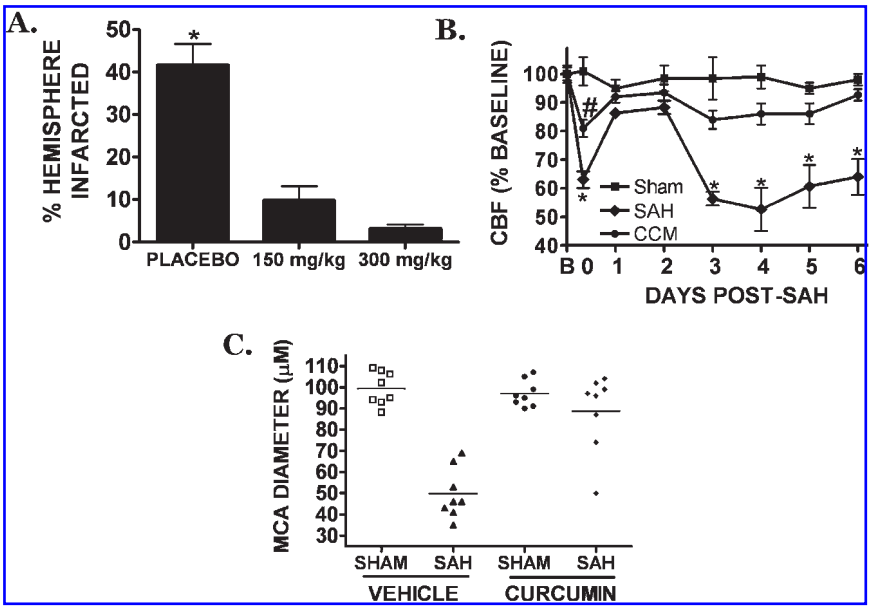

FIG. 2. (A) Mice ($n = 10$/group) were injected with corn oil (placebo) or curcumin (CCM; 150, 300 mg/kg) at the time of SAH. Cerebral infarction was quantified after 6 days and expressed as percentage of the hemisphere infarcted. *$p <$ 0.01 *vs*. sham-operated mice. (**B**) Effect of CCM (300 mg/kg) administration at the time of injury on CBF changes after SAH ($n = 5$–7 animals/group). B, baseline; time 0 was measured within 1 h of SAH. Comparisons were made within individual time points by using a one-way ANOVA (#$p < 0.05$; *$p < 0.01$ *vs*. sham). (**C**) Lumen diameter of the MCA 3 days after sham/SAH ($n = 8$ mice/group) after placebo or curcumin (150 mg/kg) treatment. Bars indicate means, and each data point represents the lumen diameter of an individual animal.

attenuated inflammatory gene expression in the cerebral cortex as compared with placebo-treated SAH mice ($p < 0.05$) (Fig. 4B, supporting an antiinflammatory role. A reduction in the cortical expression of the vascular inflammatory markers, ICAM-1 and VCAM-1, indicated a possible effect of curcumin on the vasculature. Similar to that observed in the cerebral cortex, curcumin attenuated inflammatory gene expression in the MCA within 3 h of SAH ($p < 0.05$ *vs*. placebo-treated SAH mice) (Fig. 4C), further supporting the notion that curcumin could limit cerebral vasospasm via a reduction in vascular inflammation. The NF-κB transcription factor regulates inflammatory gene expression; thus, the activation of NF-κB was studied after SAH. The binding of both the p50 and p65 subunits of NF-κB were significantly elevated in the cerebral cortex and in the MCA at 3 h after injury (Fig. 5A and B). As was observed with the protection against cerebral vasospasm and inflammatory gene expression, NF-κB activation was completely blocked by administration of curcumin (150 mg/kg) at the time of SAH.

SAH-induced oxidative stress is attenuated by curcumin

Superoxide anions are implicated in the development of cerebral vasospasm, suggesting that oxidative stress may be a viable therapeutic target after SAH. A significant increase in the production of superoxide, as quantified by the conversion of HEt to Et, was detected in the cerebral cortex and MCA within 3 h of SAH ($p < 0.05$ *vs*. sham). Superoxide production was maximal in the MCA at 3 h after SAH, whereas brain production did not plateau until 12 h after injury. In both the cerebral cortex and MCA, curcumin (150 mg/kg) administration at the time of SAH completely blocked superoxide production ($p < 0.05$ *vs*. SAH; $p > 0.05$ *vs*. sham) (Fig. 6A and B), although the relative magnitude and temporal pattern of superoxide production differed between the cerebral cortex and the MCA. Nitric oxide and superoxide combine to form peroxynitrite, a potent and damaging radical; thus, we next assessed the effect of curcumin on peroxidative injury. As was observed with inflammatory gene expression and superoxide production, SAH significantly increased nitrotyrosine formation, a marker of peroxynitrite-mediated injury, in the cerebral cortex (Fig. 7A) and in the MCA (Fig. 7A) within 1–24 h after SAH ($p < 0.05$ *vs*. sham). This effect was more pronounced in the cerebral cortex, where acute peroxidative injury was observed within 1 h of SAH. In contrast, a significant increase in nitrotyrosine formation was not observed until nearly 6 h after SAH in the MCA, with a maximal increase by 24 h after injury ($p < 0.01$ *vs*. sham). Nitrotyrosine formation was significantly attenuated by curcumin at all times, both within the cerebral cortex and the MCA, after SAH ($p < 0.05$ *vs*. SAH). SAH also increased lipid peroxidation, a measure of oxidative stress. Within the cerebral cortex, administration of curcumin (150 mg/kg) completely reversed SAH-induced TBARS formation at 4 and 24 h after SAH (1.56 ± 0.16 nmol and 1.88 ± 0.19 nmol TBARS/mg protein in SAH *vs*. 1.06 ± 0.21 nmol and 1.19 ± 0.26 nmol TBARS/mg protein for SAH + curcumin at 4 and 24 h, respectively) ($p < 0.05$ *vs*. SAH mice; $p > 0.05$ *vs*. sham-operated mice (0.99 ± 0.09 nmol TBARS/mg protein and 1.12 ± 0.08 nmol TBARS/mg protein, at 4 and 24 h, respectively) (Fig. 7C). In the MCA, TBARS formation was not different between curcumin treatment

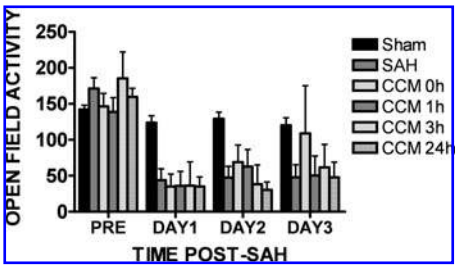

FIG. 3. Effect of curcumin on open-field behavioral activity after SAH. Curcumin (150 mg/kg, i.p.) was administered at the time of injury (CCM-0h), 1 h after injury (CCM-1h), 3 h after injury (CCM-3h), or 24 h after injury (CCM-24h). Open-field activity was assessed at day 1, 2, or 3 after SAH. Data are expressed as mean ± SEM from 5–7 mice/group.

TABLE 1. PHYSIOLOGIC PARAMETERS AFTER SAH AND/OR CURCUMIN TREATMENT

	Sham	*CCM*	*SAH*	*SAH ± CCM*
pH	7.13 ± 0.03	7.14 ± 0.03	7.18 ± 0.03	7.10 ± 0.02
pCO_2	53.5 ± 10.3	57.8 ± 6.3	72.3 ± 5.9	74.9 ± 3.2
pO_2	37.3 ± 6.3	47.3 ± 10.0	52.0 ± 4.3	50.3 ± 5.4
HCO_3	19.5 ± 4.1	19.8 ± 1.2	26.9 ± 1.8	23.2 ± 1.1
TCO_2	20.7 ± 4.3	21.7 ± 1.2	29 ± 2.1	25.5 ± 1.2
SO_2	52.7 ± 11.3	64 ± 13.3	74.3 ± 4.1	68.3 ± 7.2
Na^+	133 ± 5.1	145 ± 3.2	144.7 ± 3.7	145.5 ± 1.6
K^+	2.83 ± 0.6	3.83 ± 0.4	3.13 ± 0.3	2.85 ± 0.1
Glucose	281.0 ± 42.4	229.3 ± 16.4	320.7 ± 64.6	361.0 ± 37.0

and either sham or SAH at 4 h after injury (0.60 ± 0.03 nmol TBARS/mg protein for sham; 0.79 ± 0.10 nmol TBARS/mg protein after SAH; and 0.61 ± 0.11 nmol TBARS/mg protein for SAH + curcumin) (Fig. 7D). In contrast, curcumin decreased lipid peroxidation in the MCA at 24 h after injury (0.61 ± 0.06 nmol TBARS/mg protein for sham; 0.96 ± 0.21 nmol TBARS/mg protein after SAH; and 0.64 ± 0.13 nmol TBARS/mg protein for SAH + curcumin) ($p < 0.05$ *vs.* SAH; $p > 0.05$ *vs.* sham-operated mice).

Discussion

The present study demonstrated that the natural polyphenolic compound, curcumin, reversed SAH-induced cerebral

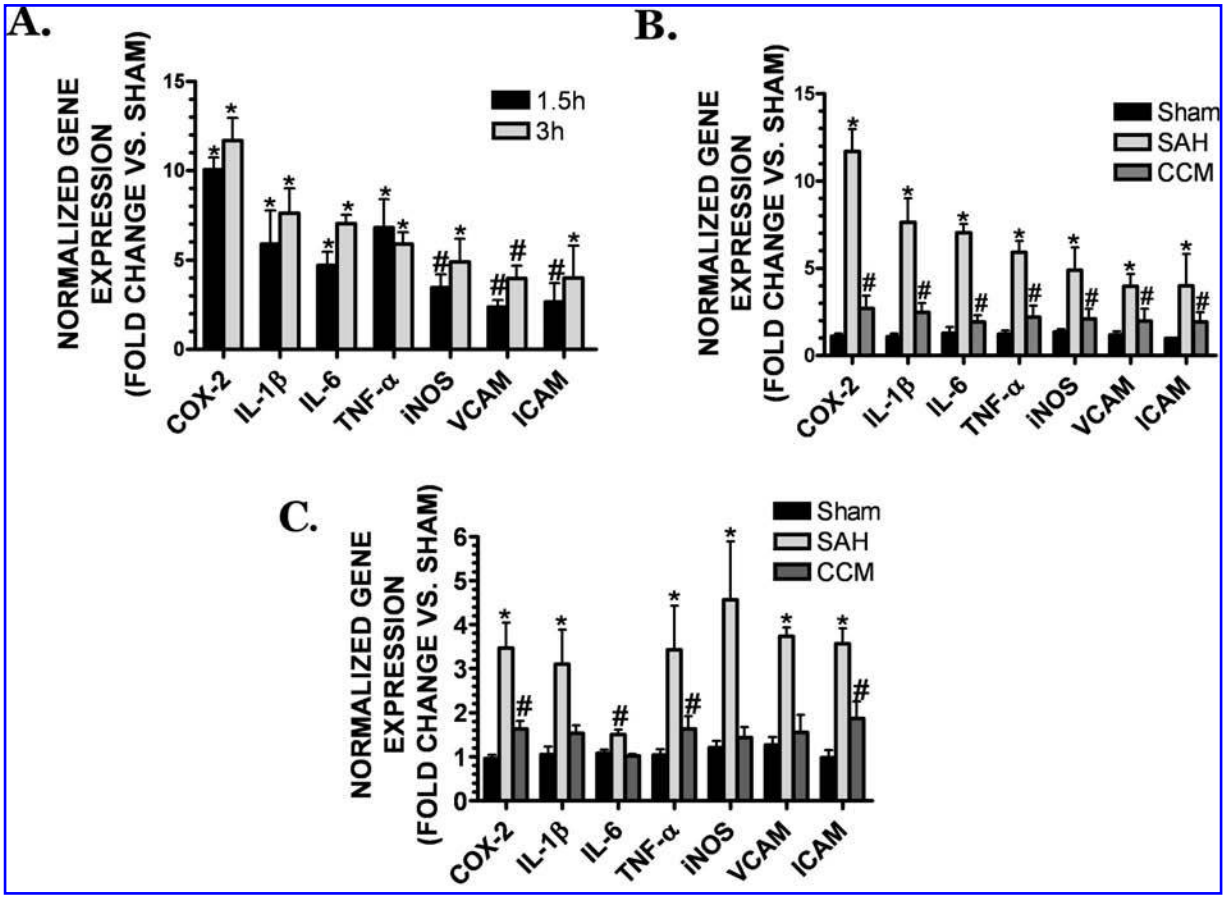

FIG. 4. (A) Inflammatory gene expression in the cerebral cortex at 1.5 or 3 h after SAH. Data are expressed as a fold change *versus* sham-operated mice ($n = 8$ mice/group). Individual gene comparisons were made by t test (#$p < 0.05$; *$p < 0.01$ *vs.* sham). (**B**) Reduction in inflammatory gene expression in the cerebral cortex at 3 h after SAH after curcumin treatment (CCM; 150 mg/kg). (**C**) CCM reduces inflammatory gene expression in MCA at 3 h after SAH. For (**B**) and (**C**), data are expressed as a fold change *vs.* sham-operated mice (#$p < 0.05$; *$p < 0.01$ *vs.* sham).

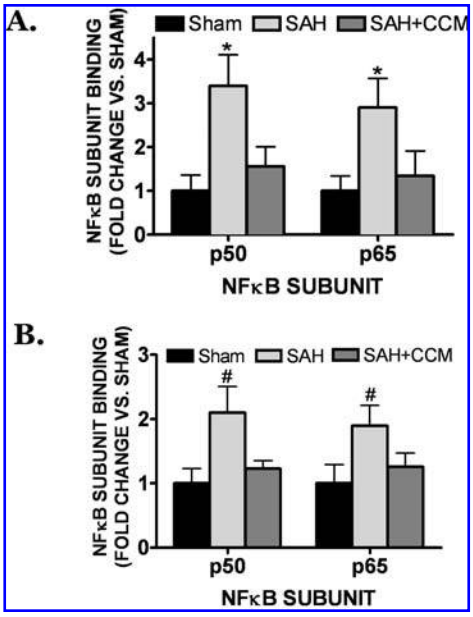

FIG. 5. Effect of curcumin treatment (CCM; 150 mg/kg) on NF-κB (p50 and p65 subunits) binding in (A) cerebral cortex or (B) MCA at 3 h after SAH. After injury, nuclear extracts were prepared and analyzed by using an enzyme-linked immunoassay to detect differences in NF-κB subunit binding. Data are expressed as a fold change *vs*. sham-operated mice (#$p < 0.05$; *$p < 0.01$ *vs*. sham).

vasospasm and attenuated secondary cerebral infarction. This effect was associated with reduced activation of both p50 and p65 subunits of NF-κB after SAH, resulting in decreased neuroinflammation and oxidative stress. Cerebral vasospasm afflicts >70% of SAH patients and contributes to symptomatic brain ischemia and a poor prognosis in more than one third of these patients (54); therefore, curcumin may represent a clinically safe (8, 60) and novel therapeutic for the prevention of cerebral vasospasm after SAH.

The present studies were performed in a murine endovascular perforation model of SAH, which exhibited acute vasoconstriction (in the first hour after SAH) and a significant and sustained decrease in CBF within 72 h of SAH. These changes in CBF support the pattern of SAH in other species, including humans, and are consistent with the temporal pattern of delayed cerebral vasospasm observed by others with this model (33, 34, 58). We extended these previous reports by documenting an increase in secondary cerebral infarction after vasospasm. That ischemic injury was not detected before the reduction in CBF suggests that transient occlusion of the ICA during endovascular perforation did not likely induce the subsequent brain injury. Consistent with the reports of others using this model of injury, the basilar artery, which is not supplied by the ICA, remained unaffected by SAH, providing further confidence in the specificity of cerebral vasospasm in the MCA (34). As a whole, this model of SAH reproducibly recapitulates the essential aspects of aneurysmal SAH in humans, including the development of secondary cerebral infarction and neurologic injury (49, 62).

Oxidative stress clinically correlates with neurologic injury after aneurysmal SAH (21, 46). Although completely unstudied after SAH, curcumin limits oxidative brain injury in preclinical models Alzheimer disease, ischemic stroke, and traumatic brain injury (9, 30, 60). Vascular NADPH oxidase, an enzyme that generates large quantities of superoxide, is implicated as a causative mediator in the development of cerebral vasospasm after SAH (29, 65). Interestingly, curcumin inhibits NADPH oxidase more potently than does apocynin (13), an effective inhibitor of endothelial superoxide production (39). Curcumin also limits hemoglobin oxidation, another important source of superoxide after SAH (43, 61), suggesting that curcumin may limit oxidative brain injury *via* multiple mechanisms. The synthesis of novel curcumin analogues with differential biologic activity will be useful to demonstrate further whether a reduction in oxidative stress underlies curcumin-mediated protection after SAH.

Oxidative brain injury is frequently secondary to neuroinflammation. Inflammatory mediators are elevated in the cerebrospinal fluid (CSF) of SAH patients (25, 52) and directly correlate with the development of cerebral vasospasm (18, 48, 66). In addition to serving as an antioxidant, curcumin exhibits antiinflammatory properties (37). Clinically achievable concentrations of curcumin limited acute inflammation (*e.g.*, iNOS) within the cerebral cortex and the MCA after SAH, in parallel to the reduction in superoxide production. Functionally, inhibition of iNOS attenuates cerebral vasospasm in rats (53, 63), suggesting a possible damaging role for nitric oxide (NO) after SAH. It appears paradoxic that NO, a potent vasodilator, contributes to SAH-induced

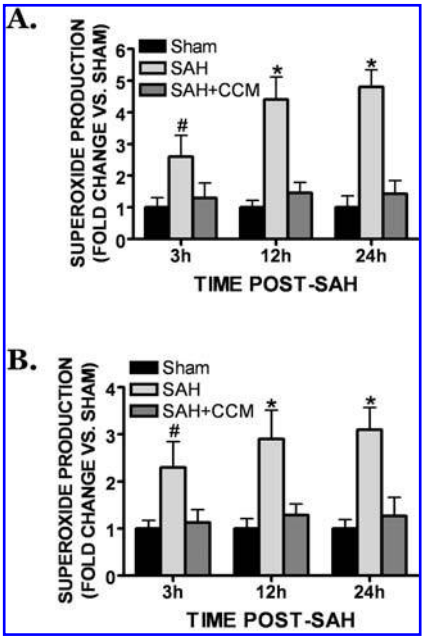

FIG. 6. SAH-increased superoxide production is attenuated by curcumin administration (CCM; 150 mg/kg) in (A) cerebral cortex and (B) MCA at 3, 12, or 24 h after SAH. Data were expressed as a fold change *vs*. sham-operated mice, and graphs depict means ± SEM from 6–8 mice/group (#$p < 0.05$; *$p < 0.01$ *vs*. sham).

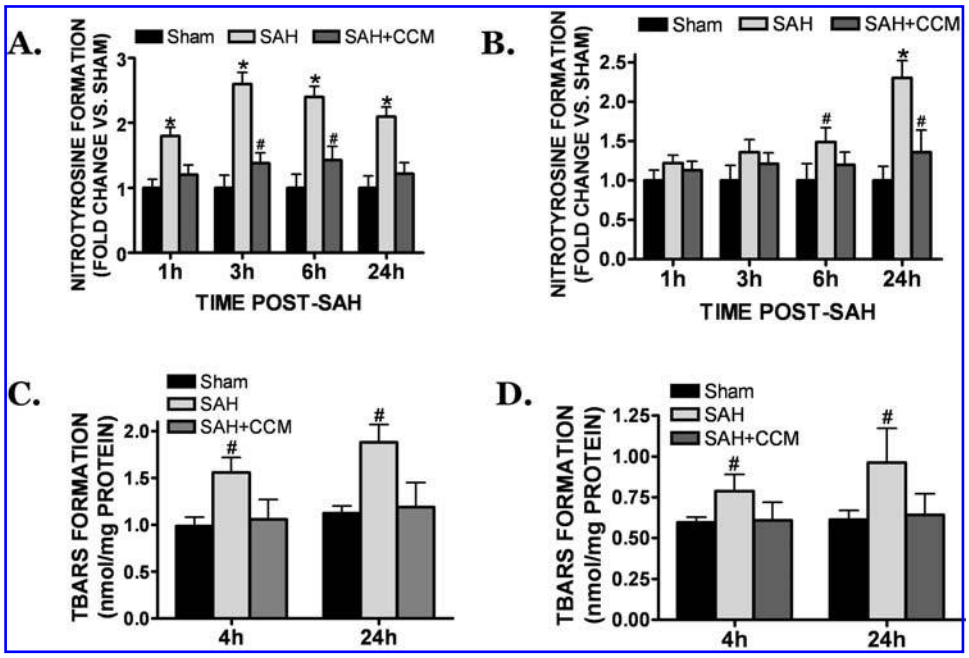

FIG. 7. Time course of nitrotyrosine formation or lipid peroxidation in (A, C) cerebral cortex or (B, D) MCA. Mice were treated with placebo or curcumin (CCM; 150 mg/kg) at the time of SAH, and then nitrotyrosine formation, a marker of peroxidative injury, or lipid peroxidation was estimated at indicated time points after SAH. Data represent the mean ± SEM from 6–8 mice/group (#$p < 0.05$; *$p < 0.01$ *vs.* sham).

vasoconstriction; however, NO may combine with superoxide to form peroxynitrite, a vasoconstrictor implicated in vascular injury (35). This possibility is supported by the detection of peroxidative injury markers in the CSF of humans after SAH (31). In the present study, curcumin attenuated SAH-induced peroxidative injury in the cerebral cortex and in the MCA, raising the possibility that curcumin may prevent vasospasm by at least two important mechanisms. First, by limiting superoxide generation and iNOS expression, the precursors for peroxynitrite formation would be removed. Peroxidative injury enhances phosphatidylcholine and phosphatidylethanolamine metabolism in vascular smooth muscle, increasing the accumulation of intracellular diacylglycerol (DAG), an endogenous activator of protein kinase C (PKC) (2). DAG-mediated activation of PKC and increased phosphatidylcholine and phosphatidylethanolamine metabolism directly correlate with delayed cerebral vasospasm after SAH (41, 59). Thus, curcumin may reduce peroxynitrite-mediated activation of this signaling pathway, functionally preventing endothelial dysfunction and cerebral vasospasm. Second, superoxide may function as an "NO sink," converting NO from a protective and vasodilatory species into a damaging, vasoconstrictive species (*e.g.*, peroxynitrite) (35). A reduction in superoxide would limit peroxynitrite generation, which would, in turn, attenuate vasoconstriction and endothelial injury after SAH. That curcumin may protect the vasculature after SAH is supported by a previous observation demonstrating that curcumin prevents neurologic injury after focal cerebral ischemia, at least in part, by attenuating peroxynitrite-induced injury in cerebral endothelial cells (27). As a whole, our data suggest that curcumin reduces cerebral vasospasm *via* an antiinflammatory and antioxidant mechanism.

The proinflammatory transcription factor, NF-κB, regulates iNOS, IL-1β, and TNF-α (28), and is a molecular target of curcumin (57). The notion that NF-κB may represent a therapeutic target after SAH is supported by a reduction in vascular inflammation and cerebral vasospasm in rabbits after intracisternal administration of pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor (66). Consistent with this possibility, clinically achievable doses of curcumin reduced the activation of both p50 and p65 subunits of NF-κB after SAH. Although our data strongly support a reduction in vascular inflammation *via* inhibition of NF-κB, we cannot exclude the possibility that infiltrating immune cells may also contribute to the observed inflammation, as ICAM-1 and VCAM-1, which recruit leukocytes, are induced within hours of SAH. Nonetheless, treatment of cultured cerebral microvessel cells with hemolysate, which mimics a hemorrhagic injury, induced inflammatory gene expression *via* an NF-κB–dependent mechanism (M.L. and K.M.D., unpublished observations), suggesting that curcumin likely reduces vascular inflammation after SAH.

Clinical diagnosis of SAH may be initiated well beyond the ictus; therefore, an ideal therapeutic drug would limit neurologic injury with a delayed therapeutic window. Unlike treatment at the time of SAH, a 1-, 3-, or 6-h posttreatment with curcumin did not reverse cerebral vasospasm, suggesting that acute (<1 h after SAH) prooxidant and/or proinflammatory effects mediate the subsequent response to injury. Consistent with this possibility, co-treatment with curcumin, which has a short half-life in serum (see next section), attenuated the acute reduction in CBF within the first 10–30 min after SAH. The functional significance of this finding remains unclear; however, acute vasoconstriction directly contributed to subsequent ischemic injury after ex-

perimental SAH (4). These data may, therefore, provide a mechanism whereby curcumin could limit secondary infarction, which develops up to 72 h after the initial injury. Similarly, this provides a mechanistic explanation for the inability of curcumin posttreatment, which preceded acute vasoconstriction, to limit secondary brain injury.

Interestingly, curcumin attenuated cerebral vasospasm and secondary ischemic injury, yet neurologic outcome was not significantly improved, when it was administered at the time of injury. Although unexpected, this finding is consistent with a report suggesting a dissociation between the development of cerebral vasospasm and neurologic outcome (38). Additionally, these findings are in agreement with a recent clinical trial reporting a reduction in vasospasm without an improvement in patient outcome (20, 24). Unfortunately, the therapeutic window for curcumin to prevent cerebral vasospasm was limited to the time of injury; however, a 48-h posttreatment limited secondary infarction independent of cerebral vasospasm (data not shown), suggesting a possible direct neuroprotective mechanism. Consistent with this possibility, clinically achievable serum concentrations of curcumin (1–5 μM) protected purified cortical neurons from oxidative injury paradigms, but not against camptothecin-induced neuronal apoptosis, which directly damages DNA (C.W. and K.M.D., unpublished observations). Together, these studies add to an emerging literature that challenges the dogmatic view of SAH, suggesting that delayed cerebral vasospasm promotes secondary cerebral infarction and, in turn, neurologic dysfunction and poor outcome.

Curcumin is poorly absorbed in the gastrointestinal tract and is reported to undergo significant biotransformation (1, 8, 26, 50). Phase I clinical trials determined that oral administration of up to 8 g/day of curcumin safely exhibits bioactivity, with peak serum levels ($\sim$1.8 μM) reached within 1–2 h, followed by a gradual decline in blood levels within 12 h of dosing (8, 60). Similarly, curcumin blood levels were undetectable between 2 and 12 h after oral dosing of 1 g/kg in rats (8). Intraperitoneal administration of curcumin (100 mg/kg) to mice, a dose approximating those used in this study, produced brain concentrations of $\sim$1.1 μM and plasma levels of 1.6 μM, with most curcumin excreted in the feces (45). Future studies to reconcile the issue of seemingly suboptimal doses of curcumin exerting biologic activity in preclinical models of brain injury are therefore needed (9, 30, 60). For example, physiologic metabolites of curcumin, which are not well characterized *in vivo*, could mediate at least some of the biologic activity of curcumin. Nonetheless, it is noteworthy that the effective dose and route of administration used in the present study produce clinically achievable blood levels, supporting the potential clinical validity of these findings.

As a whole, the present study suggests a potentially novel use for curcumin in the management of SAH, in part by limiting vascular inflammation and secondary brain injury. Treatment of SAH is frequently initiated well beyond the ictus and the resultant acute vasoconstriction; thus, it is notable that curcumin was also directly neuroprotective, independent of cerebral vasospasm. Given the scarcity of therapeutics for the management of SAH and the documented clinical safety of curcumin, these data may provide a framework for the future exploration of curcumin and curcumin-based analogues as an adjunct therapy to limit cerebral vasospasm and secondary infarction after SAH.

Abbreviations

CBF, Cerebral blood flow; CCA, common carotid artery; CCM, curcumin; COX-2, cyclooxygenase-2; ECA, external carotid artery; Et, ethidium; HEt, hydroethidine; ICA, internal carotid artery; ICAM, intracellular adhesion molecule-1; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MCA, middle cerebral artery; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor κB; NO, nitric oxide; qRT-PCR, quantitative real time-polymerase chain reaction; PDTC, pyrrolidine dithiocarbamate; RPS_3, ribosomal protein S3; SAH, subarachnoid hemorrhage; SOD, superoxide dismutase; TBARS, thiobarbituric acid–reactive species; tMCAo, transient middle cerebral artery occlusion; TNF-α, tumor necrosis factor-α; TTC, 2,3,5-triphenyltetrazolium chloride; VCAM-1, vascular cell-adhesion molecule-1.

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