Neuroprotective Effect of Curcumin in an Experimental Rat Model of Subarachnoid Hemorrhage

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ABSTRACT

Background: Subarachnoid hemorrhage (SAH) causes a high mortality rate and morbidity. It was suggested that oxidant stress plays an important role in neuronal injury after SAH. Therefore, we assessed the effect of curcumin on reducing cerebral vasospasm and neurologic injury in a SAH model in rat.

Methods: A double-hemorrhage model was used to induce SAH in rats. Groups of animals were treated with intraperitoneal injection of 20 mg/kg curcumin (curcumin group, n = 24) or dimethyl sulfoxide (vehicle group, n = 33), normal saline (SAH group, n = 34) or normal saline (sham group, n = 22), 3 h after SAH induction and daily for 6 days. Glutamate was measured before SAH induction and once daily for 7 days. Glutamate transporter-1, wall thickness and the perimeter of the basilar artery, neurologic scores, neuronal degeneration, malondialdehyde, superoxide dismutase, and catalase activities were assessed.

Results: Changes of glutamate levels were lower in the curcumin group versus the SAH and vehicle groups, especially on day 1 (56 folds attenuation vs. vehicle). Correspondingly, glutamate transporter-1 was preserved after SAH in curcumin-treated rats. In the hippocampus and the cortex, malondialdehyde was attenuated (30% and 50%, respectively). Superoxide dismutase (35% and 64%) and catalase (34% and 38%) activities were increased in the curcumin rats compared with the SAH rats. Mortality rate (relative risk: 0.59), wall thickness (30%) and perimeter (31%) of the basilar artery, neuron degeneration scores (39%), and neurologic scores (31%) were improved in curcumin-treated rats.

Conclusions: Curcumin in multiple doses is effective against glutamate neurotoxicity and oxidative stress and improves the mortality rate in rats with SAH.

What We Already Know about This Topic

- Oxidant stress may play an important role in neuronal injury after subarachnoid hemorrhage (SAH), and curcumin may be a promising therapeutic agent for reducing cerebral vasospasm and neurologic injury.

What This Article Tells Us That Is New

- In the double-hemorrhage rat SAH model, curcumin in multiple doses was effective against glutamate neurotoxicity and oxidative stress, and improved the mortality rate in rats with SAH.
SUBARACHNOID hemorrhage (SAH) is a serious and debilitating disease. Several putative mechanisms and possible treatments for SAH have been proposed. It is plausible that the inflammatory response to oxidant stress represents a critical step in the pathogenesis of cerebral vasospasm (CVS) pursuant to SAH. In addition, glutamate plays an important role in the pathogenesis of neuronal injury after ischemic injury. Glutamate transporter-1 (GLT-1) predominates among the functional glutamate transporters, which are essential for maintaining a low extracellular glutamate concentration and for preventing glutamate neurotoxicity. In a previous study, we observed down-regulation of GLT-1, neuronal degeneration, increased basilar artery wall thickness, and neuron variability after SAH. It was suggested that an increase in GLT-1 expression or activity would attenuate glutamate-induced excitotoxicity.

Oxidative stress contributes to damage and misfolding of proteins, including glutamate transporters. Therefore, therapeutic strategies that confer antioxidant effects should attenuate the increase in glutamate level and increase GLT-1 expression after SAH.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (diferuloyl methane), possesses antioxidant and antiinflammatory effects. Several studies have indicated a single, high dose of curcumin has protective effects against cerebral ischemia and CVS after SAH, and that they are mediated by its antioxidant effect. We hypothesized that multiple doses of curcumin may reduce CVS and neurologic injury via an antioxidant effect and attenuate glutamate-induced neurotoxicity after SAH in rats.

Materials and Methods

Construction of the Microdialysis Probe
The microdialysis probe with each end of a 4 cm cuprophan hollow fiber (300–380 g) in each group were implanted with a microdialysis loop (2 × 2 cm) after intraperitoneal injection of sodium pentobarbital anesthetic (30 mg/kg). The microdialysis loop was placed into the intrathecal space through the cisternal membrane until the level of fourth cervical vertebra, and externalized on top of head. After then, the rats were allowed to recover for 5 days. The microdialysis probes were perfused once per day to keep the catheter patent. One end of the microdialysis probe was connected to a microdialysis pump (CMA 102; CMA Microdialysis, Holliston, MA) (inflow) for continuous infusion of artificial CSF, and the other end was connected to a polyethylene-10 tube (outflow) to collect the CSF dialysate as mentioned in our previous study.

Animal Model
The current investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, Maryland) and was approved by the Animal Care and Use Committee of the National Defense Medical Center (Taipei, Taiwan, Republic of China).

Animals were randomly allocated to the following groups: sham (n = 22), SAH (n = 34), vehicle (n = 33), or curcumin (curcumin 20 mg/kg; n = 24). Dimethyl sulfoxide, 10%, was used as a vehicle for curcumin. Ten male Wistar rats (300–380 g) in each group were implanted with a microdialysis loop 12 h after the first blood injection was administered 48 h after the first injection. A second blood injection was administered 48 h after the first injection. The first SAH induction was defined as day 1.

Materials and Methods

Construction of the Microdialysis Probe
The microdialysis probe with each end of a 4 cm cuprophan hollow fiber (300–380 g) in each group were implanted with a microdialysis loop (2 × 2 cm) after intraperitoneal injection of sodium pentobarbital anesthetic (30 mg/kg). SAH was induced using a prechiasmatic approach in which a needle with a rounded tip and a side hole (Withcare spinal set, 0.41 × 90 mm; Becton Dickinson, Madrid, Spain) was stereotactically inserted into the prechiasmatic cistern. We tilted the needle 30° anteriorly and put 7.5 mm anterior to the bregma in the midline. With the aperture directing to right, the needle was lowered until the tip came to the skull base 2–3 mm anterior to the chiasma, about 10 mm to the brain surface. Autologous arterial blood (200 μl) was injected by a pump to maintain intracranial pressure at the mean arterial blood pressure level. In the beginning, the burr hole was left open without filling the wax, which resulted in CSF leakage in some animals; consequently, the intracranial pressure increased only minimally. A second blood injection was administered 48 h after the first injection. The first SAH induction was defined as day 1. Sham rats received normal saline instead of blood. Arterial blood pressure was monitored via a 4-French intra-arterial sheath placed in the right femoral artery.

CSF Sample Collection and Measurement of Glutamate Concentrations
On day 6 after microdialysis loop implantation, a mini-osmotic pump with a pump rate of 5 μl/h was filled with...
artificial CSF and attached to the dialysis loop for 2 h to achieve equilibration, after which dialysate was collected before the first SAH induction (baseline) and daily for 7 days thereafter. The collected dialysate samples were frozen at −80°C until analysis.

Analysis of extracellular concentrations of glutamate in the microdialysates was performed using high-pressure liquid chromatography (Agilent 1100; Agilent Technologies, Palo Alto, CA), with fluorescence detection (Gilson model 121, set at 428 nm; Gilson, Middleton, WI) after precolumn derivatization with o-phthalaldehyde reagent and iodoacetamide/methanol scavenger. Derivatization was performed by adding 4 ml of o-phthalaldehyde/t-butylthiol reagent to 40 ml of sample and shaking the mixture for 2 min to enable it to react. Four milliliters of reagent B (185 mg of iodoacetamide/ml of methanol) was then added and the mixture was again shaken for 2 min. The sample was then injected into a C18 reverse-phase column and eluted at a flow rate of 0.45 ml/min. A linear gradient from 100% eluent A [0.1 M sodium acetate buffer, pH 6.8/acetonitrile (80:20)] to 100% eluent B [acetonitrile/double-distilled water (80:20)] was used to separate glutamate. All solvents were vacuum-filtered through a 0.22-μm membrane (Millipore, Billerica, MA) and degassed by sonication before use. Solutions containing 0, 10−8, 10−7, 10−6, and 10−5 M of glutamate standard were run before and after running each sample group. The dialysate samples from the survival rats were analyzed. The technicians who performed the analysis were blinded to treatment group.

Drug Preparation and Treatment Schedule
Curcumin (purity ≥94%) was purchased from Sigma-Aldrich (St. Louis, MO). Three hours after the first SAH induction and daily for 6 days, 0.25–0.3 ml curcumin/dimethyl sulfoxide solution was administered intraperitoneally to the curcumin group. Equal volumes of 10% dimethyl sulfoxide solution were given to the vehicle group, whereas equal volumes of normal saline were administered intraperitoneally to the sham and SAH groups.

Mortality Rate and Neurologic Assessment
The rats’ mortality rate was recorded during and after the SAH procedure. Neurologic scoring (n = 6 in each group) was done in a blinded fashion at 6 h and days 1, 3, 5, and 7 after first SAH induction. This assessment evaluated motor function based on spontaneous activity (0 to 3), symmetry of limb movements (0 to 3), and climbing the walls of a wire cage (1 to 3). Sensory score was examined by responses to touch on vibrissae or on the sides of the trunk. Each scored from 1 to 3. The neurologic score of each rat at the end of the evaluation was the summation of all six individual scores. The minimum neurologic score was 3 and the maximum was 18.

Histology: Tissue Preparation
On day 7 after the first SAH induction, the rat was decapitated and the brain was harvested right after anesthesia. The brains were postfixed in 4% paraformaldehyde for 3 days. The brain tissue with the basilar artery and the pons was sectioned to a thickness of 6 μm at 200-μm intervals using a cryostat (Leica CM3050 S; Leica Microsystems, Wetzlar, Germany). Every eighth section of the brain was collected on a slide and stained with hematoxylin and eosin. Six rats from each group were used for histologic analysis.

Measurements of Wall Thickness and Perimeter of Basilar Artery
The mean thickness and the perimeter of the basilar artery wall were measured using Imaging-Pro-Plus software (Media Cybernetics, Bethesda, MD) and an Olympus microscope (Olympus, Optical Tokyo, Japan). Light microscopic sections of basilar artery were projected as digitized video images. From each animal, three slices of the basilar artery were taken from the proximal, distal, and middle portions of the vessel, and measurements were made at four points (at the hours 3, 6, 9, and 12, as if on the face of a clock), from which the mean wall thickness and perimeter were calculated. A blinded technician took the measurements.

Quantitative Analysis of Neuronal Cell Bodies in the Hippocampus
The tissues from the rats used for measuring wall thickness and perimeter were studied using a bright-field microscope (Eclipse E600W microscope; Nikon, Tokyo, Japan) and photographed by a digital camera (Nikon DP70; Nikon). Degeneration of the neuron (shrinkage of the neuron, hyperchromasia, and nuclear pyknosis) was evaluated by a light microscopy. The dentate gyrus, cornu ammonis 1 and cornu ammonis 3 were scored semiquantitatively for the presence and extent of neuronal degeneration. The scoring was 1 point for normal appearance; 2 points for a few degenerated neurons among normal neurons; 3 points for a lot of degenerated neurons with scattered normal neurons; and 4 points for complete degeneration with no residual normal neurons. Each of the three regions of the hippocampus was scored. “Degeneration score” was designated as the sum of these three scores, and the means were analyzed for statistical significance by a blinded observer.

Western Blotting
The harvested brain tissues were subjected to Western blots as described in our previous study. Six rats from each group were sacrificed on day 7 after SAH. The hippocampus and cerebral cortex were dissected out and homogenized in a 10 mM Tris buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM Na3VO4, 5 mM dithiothreitol, and a mixture of protease inhibitors followed by centrifugation for 30 s at 11,000 × g. The resulting pellets were dissolved in gel-loading buffer and centrifuged for 30 min at 20,000 × g. For gel
Electrophoresis, each lane was loaded with approximately 40 μg of protein from the supernatant, and the amount of protein was determined using the Coomassie blue protein assay. Each sample was denatured by heating at 90°C for 10 min in an equal volume of sodium dodecyl sulfate sample buffer, separated on a 10% sodium dodecyl sulfate gel, and transferred to a polyvinylidene difluoride membrane (Millipore). After separation by electrophoresis using 10% polyacrylamide gels, the resolved proteins were electro-transferred onto a nitrocellulose filter (Bio-Rad Laboratories, Hercules, CA). The filters were placed in a blocking buffer for 1 h at room temperature and were then incubated overnight (16 h) with anti-GLT-1 (1:600; Chemicon, Temecula, CA) antibodies. After three 10 min washes with phosphate-buffered saline-containing 0.1% Tween 20, the filters were then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:1,500; Amersham, Piscataway, NJ). After two washes with phosphate-buffered saline-containing 0.1% Tween 20, the blots were incubated for 1 min with the enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL) and exposed immediately to Hyperfilm-enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL). The filters were then reprobed with 50% dismutation of the superoxide radicals.23 SOD activity was determined with a SOD assay kit (Cayman, Ann Arbor, MI). The assay kit was added to the mixture, which was centrifuged at 1,000 g for 5 min. The maximum absorbance was 532 nM. Serial dilutions of 1,1,3,3-tetraethoxypropane were used as standards.22 Malondialdehyde concentration was expressed as nM/g tissue.

Measurement of Malondialdehyde, Superoxide Dismutase, and Catalase

Malondialdehyde was assayed on brain samples using a thiobarbituric assay. In brief, brain samples were homogenized in ice-cold phosphate buffer. The homogenate was mixed with thiobarbituric acid and butylated hydroxytoluene and heated in a water bath at 90°C for 45 min. After cooling, n-butanol was added to the mixture, which was centrifuged at 1,000 × g for 5 min. The maximum absorbance was 532 nm. Serial dilutions of 1,1,3,3-tetraethoxypropane were used as standards.22 Malondialdehyde concentration was expressed as nM/g tissue.

Superoxide dismutase (SOD) activity was determined using a SOD assay kit (Cayman, Ann Arbor, MI). The assay kit involved detection of superoxide radicals generated by xanthine oxidase and hypoxanthine using a tetrazolium salt. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals.23 SOD concentration was expressed as U/mg protein.

Catalase activity was determined with catalase assay kit (Cayman). The method was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H₂O₂. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromagen.25 Catalase activity was expressed as mg · nm⁻¹ · min⁻¹ protein.

Statistical Analysis

The data are presented as mean and corresponding 95% CI. Mortality rate was analyzed by Fisher exact test. The mean values of glutamate concentration and neurologic scores over time were compared using a two-way ANOVA with group as a between-subjects factor and time as a repeated-measures factor. If the comparison was significant, post hoc analysis using Bonferroni correction for multiple comparisons was performed. The basal artery wall thickness and perimeter, neuronal degeneration scores, GLT-1, malondialdehyde, SOD, and catalase were measured with one-way ANOVA analysis among four groups; if analysis of variance results were significant, post hoc analysis with Bonferroni correction for multiple comparisons between groups was performed. A P value <.05 with two-tailed testing was considered to be significant. Analysis of variance was performed using GraphPad Instat (GraphPad Software, San Diego, CA).

Results

Changes in Glutamate Concentrations

Significantly lower glutamate levels were observed between day 1 and day 7 in the curcumin group compared with the SAH and vehicle groups (both P < 0.001). However, glutamate levels in the curcumin group were higher than in the sham group on days 1, 2, and 3 after SAH (fig. 1; P < 0.01). The mean percentage changes (95% CI) in glutamate levels from baseline for the sham (n = 10), SAH (n = 6), vehicle (n = 6), and curcumin (n = 8) groups were −14.5 (−45.2–16.2) %, 135.9 (57.3–214.5) %, 130.2 (19.0–241.5) %, and 2.3 (22.7–27.4) % on day 1, −17.1 (−40.8–6.7) %, 65.8 (−17.7–149.4) %, 63.7 (10.0–117.4) %, and 3.6 (12.5–19.6) % on day 2, −15.5 (−44.7–13.7) %, 43.3 (25.2–61.5) %, 47.6 (3.4–91.7) %, and −9.4 (−36.6–17.8) % on day 3, −5.7 (−28.6–17.2) %, 27.5 (4.6–50.4) %, 32.1 (17.2–47.0) %, and 1.5 (−23.6–26.7) % on day 4, 1.4 (−21.3–24.1) %, 68.1 (41.1–95.1) %, 60.9 (22.6–99.2) %, and 2.3 (−29.2–33.8) % on day 5, −17.0 (−25.4–−8.6) %, 13.8 (7.4–20.3) %, 12.4 (7.7–17.2) %, and −6.5 (−31.6–18.7) % on day 6, and −10.0 (−30.9–10.9) %, 18.0 (1.7–34.3) %, 17.7 (3.0–32.4) %, and 2.5 (−0.1–5.2) % on day 7, respectively. Curcumin attenuated the percent increase of glutamate levels by 56-fold than the vehicle group on day 1.

Mortality Rate

No animal in the sham group (n = 22) died after the first SAH induction. The mortality rate was significantly lower in the curcumin group (8.3%) than in the SAH (35.3%) and vehicle (33.3%) groups: Both P = 0.03; relative risks were 0.58 and 0.59; 95% CI: 0.405 to 0.841 and 0.406 to 0.860, respectively (table 1).

Neurologic Scores

None of the rats showed neurologic injury after intrathecal catheterization. The SAH and vehicle groups had the clini-
cally significant lowest neurologic scores compared with the sham and curcumin rats after SAH induction on day 1 (136.0–17.4) versus 12.3 (7.5–14.5) versus 15.5 (9.2–15.4) versus 15.8 (14.0–17.5), respectively; \( P \leq 0.05 \), day 3 (14.5–17.3) versus 10.3 (6.7–11.7) versus 15.9 (7.1–13.5) versus 14.4 (12.1–16.6), respectively; \( P \leq 0.05 \), day 5 (13.5–16.9) versus 9.6 (7.3–12.3) versus 15.2 (6.7–12.5) versus 15.6 (14.7–16.5), respectively; \( P \leq 0.05 \), and day 7 (13.6 (16.0–17.4) versus 13.4 (10.9–16.3) versus 16.7 (10.6–16.2) versus 15.8 (14.8–16.7), respectively; \( P \leq 0.05 \). Neurologic scores in the curcumin-treated rats increased 31% compared with the vehicle rats. However, the curcumin group had a clinically lower neurologic score compared with the sham group only on day 3, after the first SAH induction (fig. 2).

**Histologic Examination**

Representative cross-sectional images of the basilar artery on day 7 after the first SAH induction are shown (fig. 3A). The mean (95% CI) wall thickness of the basilar artery was thinner in the sham group [27.9 (24.7–31.1) \( \mu \text{m} \)] than in the SAH [43.4 (39.5–47.3) \( \mu \text{m} \), \( P \leq 0.028 \)] and the vehicle group [43.6 (36.1–51.2) \( \mu \text{m} \), \( P \leq 0.004 \)]. Wall thickness of the basilar artery in the curcumin group [30.3 (19.7–40.9) \( \mu \text{m} \)] was decreased compared with that of the SAH and vehicle groups (\( P \leq 0.012 \) and \( P \leq 0.028 \), respectively). However, basilar artery wall thickness in the sham and curcumin groups was similar (fig. 3B). The mean perimeter of the basilar artery was significantly less in the SAH group [262

### Table 1. Mortality Rate of Rats during Experimental Period

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>Less than 6 h</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Sham (n = 22)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Subarachnoid Hemorrhage (n = 34)</td>
<td>5 (14.7%)</td>
<td>4 (11.7%)</td>
<td>2 (6.9%)</td>
<td>1 (2.9%)</td>
<td>12 (35.3%)*</td>
</tr>
<tr>
<td>Vehicle (n = 33)</td>
<td>5 (15.2%)</td>
<td>3 (9.1%)</td>
<td>2 (6.1%)</td>
<td>1 (3.0%)</td>
<td>11 (33.3%)*</td>
</tr>
<tr>
<td>Curcumin (n = 24)</td>
<td>1 (4.2%)</td>
<td>1 (4.2%)</td>
<td>0</td>
<td>0</td>
<td>2 (8.3%)</td>
</tr>
</tbody>
</table>

Fisher exact test used. The curcumin group is subarachnoid hemorrhage rats treated with curcumin; the vehicle group is subarachnoid hemorrhage rats treated with dimethyl sulfoxide.

\( * P = 0.03 \) versus rats in the curcumin group and \( P = 0.002 \) versus rats in the sham group.
(199.8 – 300.0) μm] and the vehicle group [241 (194.9 – 290.6) μm; P < 0.001 and P = 0.008, respectively] and curcumin group [292 (239.1 – 345.1) μm; P = 0.019 and P = 0.041, respectively] (fig. 3C). The cerebral vasospasm was attenuated by 30% by wall thickening and by 31% of perimeter narrowing of the basilar artery compared with the vehicle group.

The morphology of neurons in the dentate gyrus, cornu ammonis 1 and cornu ammonis 3 are shown in figure 4. Histopathologic analysis revealed that the morphology of neurons in the dentate gyrus, cornu ammonis 1 and cornu ammonis 3 was normal in the sham group (figs. 4A, E, and I, respectively). The curcumin rats exhibited mild to moderate degenerative changes in the dentate gyrus, cornu ammonis 1 and cornu ammonis 3 (figs. 4D, H, and L, respectively). The SAH (figs. 4B, F, and J, respectively) and vehicle (figs. 4C, G, and K, respectively) rats exhibited severe degenerative changes in the dentate gyrus, cornu ammonis 1 and cornu ammonis 3 (shrunken cytoplasm, extensive dark pyknotic nuclei, dark cells, and vacuolar spaces). The mean (95% CI) degeneration scores for the sham, SAH, vehicle, and curcumin groups were 3.3 (2.8 – 3.8), 8.0 (7.3 – 8.7), 7.7 (6.7 – 8.5), and 4.7 (4.1 – 5.3), respectively. There was less neuronal degeneration in the sham and the curcumin groups compared with the SAH and vehicle rats (P < 0.001). There was a clinically significant difference in neuronal degeneration between the sham and curcumin groups (P < 0.05). The neuronal degeneration score attenuated 39% compared with the vehicle group.

Western Blot Analysis of GLT-1
The mean (95% CI) GLT-1 expression in the hippocampus (fig. 5A) and cortex (fig. 5B) of the SAH group [70.0 (0.53 – 0.87)% and 87.0 (0.74 – 1.0)%] and the vehicle group [69.0 (0.59 – 0.81)% and 81.0 (0.69 – 0.93)%] were decreased compared with the sham (P = 0.002, P = 0.003, P = 0.017 and P = 0.012, respectively) and curcumin (P = 0.001, P = 0.002, P = 0.019 and P = 0.036, respectively) rats. There was no clinically significant difference between the sham and curcumin rats [99.0 (0.84 – 1.14)% and 92.0 (0.72 – 1.12)%] in GLT-1 expression.

Malondialdehyde, SOD and Catalase
The mean (95% CI) contents of malondialdehyde (nM/g tissue) in the hippocampus and cortex were increased in SAH...
The rats in the sham group showed no neuronal degeneration in the dentate gyrus, cornu ammonis 1 or cornu ammonis 3 (A, E, and I, respectively). In the rats in the subarachnoid hemorrhage group (B, F, and J) and vehicle group (C, G, and K), there was moderate to severe neuronal degeneration in the dentate gyrus, cornu ammonis 1, and cornu ammonis 3. In the curcumin group, there was normal to mild neuronal degeneration in the dentate gyrus, cornu ammonis 1, and cornu ammonis 3 (D, H, and L, respectively). Normal neuron cell bodies with distinct nuclei and nucleoli are evident. Degenerated neurons have pyknotic nuclei, dark cells, and vacuolar spaces (indicated with arrows). Magnified at 400×; n = 6 in each group; scale bar = 50 μm. The curcumin group is subarachnoid hemorrhage rats treated with curcumin; vehicle group is subarachnoid hemorrhage rats treated with dimethyl sulfoxide. CA1 = cornu ammonis 1; CA3 = cornu ammonis 3; Cur = curcumin; DA = dentate gyrus; SAH = subarachnoid hemorrhage.

**Fig. 4.** Effect of curcumin on hippocampus neuronal degeneration after subarachnoid hemorrhage. The samples were obtained from rats on day 7. The rats in the sham group showed no neuronal degeneration in the dentate gyrus, cornu ammonis 1 or cornu ammonis 3 (A, E, and I, respectively). In the rats in the subarachnoid hemorrhage group (B, F, and J) and vehicle group (C, G, and K), there was moderate to severe neuronal degeneration in the dentate gyrus, cornu ammonis 1, and cornu ammonis 3. In the curcumin group, there was normal to mild neuronal degeneration in the dentate gyrus, cornu ammonis 1, and cornu ammonis 3 (D, H, and L, respectively). Normal neuron cell bodies with distinct nuclei and nucleoli are evident. Degenerated neurons have pyknotic nuclei, dark cells, and vacuolar spaces (indicated with arrows). Magnified at 400×; n = 6 in each group; scale bar = 50 μm. The curcumin group is subarachnoid hemorrhage rats treated with curcumin; vehicle group is subarachnoid hemorrhage rats treated with dimethyl sulfoxide. CA1 = cornu ammonis 1; CA3 = cornu ammonis 3; Cur = curcumin; DA = dentate gyrus; SAH = subarachnoid hemorrhage.

The mean (95% CI) SOD levels (U/mg protein) in the hippocampus and cortex were decreased in SAH [18.8 (17.0–20.5) and 23.2 (20.2–26.2)] and vehicle [19.0 (15.5–22.4)] rats compared with the sham rats [31.1 (18.3–43.9) and 35.9 (21.4–50.4)] on day 7 after SAH induction ($P = 0.001$, $P = 0.002$, $P = 0.028$, and $P = 0.025$, respectively). Treatment with curcumin decreased malondialdehyde content compared with the SAH and vehicle rats in the hippocampus [496.3 (437.0–555.5)] and cortex [259.7 (229.5–290.0)] ($P = 0.01$, $P = 0.034$, $P = 0.048$, and $P = 0.031$, respectively). The malondialdehyde levels in the hippocampus and the cortex were attenuated by 30% and 50% compared with the vehicle group (figs. 6A and B).

The mean (95% CI) catalase activities (mg H2O2/min mg protein) in the hippocampus and the cortex were decreased in SAH [25.7 (23.2–28.2)] compared with the sham rats [32.2 (30.8–33.5)]. Treatment with curcumin preserved catalase levels in the hippocampus [36.5 (34.1–39.0)] and cortex [45.9 (43.5–48.3)] compared with the SAH and vehicle rats ($P = 0.032$, $P = 0.023$, $P < 0.001$, and $P = 0.027$, respectively). The SOD activity in the hippocampus and the cortex were increased by 34% and 39% compared with the vehicle group (figs. 6E and F).

**Discussion**

We showed that postinjury intraperitoneal administration of curcumin significantly attenuated the glutamate surge and preserved GLT-1 expression. It also attenuated the increase in malondialdehyde and preserved SOD and catalase activities. The basilar artery wall thickening was attenuated, but the basilar artery wall perimeter was preserved. Curcumin also reduced mortality rate and improved neurologic score after SAH induction.

**The Effect of Curcumin on Glutamate Production and GLT-1 Expression**

Curcumin attenuated glutamate levels from day 1 to day 7 after SAH. It is well established that a high extracellular glutamate concentration causes neuronal damages. In increased release or decreased uptake of glutamate have neuro-
toxic effects.\textsuperscript{25} In this study, curcumin attenuated glutamate levels after SAH, which may explain the neuroprotective effect. Germano \textit{et al.}\textsuperscript{26} showed that N-methyl-D-aspartate excitotoxicity is involved in the central dysfunction after SAH, and a N-methyl-D-aspartate receptor antagonist reduced behavioral deficits. In our previous study, an increase in glutamate level, down-regulation of GLT-1 expression, and neuronal degeneration were also observed after SAH.\textsuperscript{7} In addition, previous studies\textsuperscript{27,28} reported that curcumin has a concentration- and time-dependent neuroprotective effect against glutamate toxicity in rat cerebral cortical neurons \textit{in vitro}. These reports are consistent with our \textit{in vivo} study, as we showed that curcumin attenuated glutamate levels after SAH and improved survival and neurologic functions.

The preserved GLT-1 expression observed in this study may explain the attenuated glutamate levels after SAH. Recently, it was suggested that increased GLT-1 expression or activity attenuates glutamate excitotoxicity in primary human fetal astrocytes.\textsuperscript{8} Reduced glutamate uptake contributed to the elevation of extracellular glutamate because of a reduction in GLT-1 expression by astrocytes.\textsuperscript{29,30} Ouyang \textit{et al.}\textsuperscript{31} found that up-regulation of GLT-1 expression protected CA1 neurons from forebrain ischemia. Therefore, we suggest that the neuroprotective effect of curcumin may be exerted \textit{via} preserved GLT-1 expression and removal of excess glutamate after SAH.

\textbf{Fig. 5.} Western blot analysis of glutamate transporter-1 in hippocampus (A) and cortex (B). Western blot analysis showed a decrease in glutamate transporter-1 in rats in the subarachnoid hemorrhage and vehicle groups, but not in the curcumin group. \( ^* P < 0.05 \) for subarachnoid hemorrhage and vehicle groups versus sham group. \( ^# P < 0.05 \) for curcumin group versus subarachnoid hemorrhage and vehicle groups. All data were analyzed using one-way ANOVA; \( n = 6 \).

The curcumin group is subarachnoid hemorrhage rats treated with curcumin; the vehicle group is subarachnoid hemorrhage rats treated with dimethyl sulfoxide. Cur = curcumin; GLT-1 = glutamate transporter-1; SAH = subarachnoid hemorrhage.

\textbf{The Effect of Curcumin on Neurologic Outcome and CVS}

Recent studies on rats and gerbils have demonstrated the neuroprotective effect of curcumin against cerebral ischemic injury. A single dose of curcumin (200 mg/kg, intraperito-

\textbf{Fig. 6.} Effect of curcumin on oxidative stress after subarachnoid hemorrhage. (A and B) The malondialdehyde levels in hippocampus and cortex were lower in rats in the curcumin group than in rats in the subarachnoid hemorrhage and vehicle groups, but not than the rats in the sham group. (C and D) The superoxide dismutase activities in hippocampus and cortex were higher in the curcumin group than in subarachnoid hemorrhage and vehicle groups, but not than the sham group. (E and F) The catalase activities in hippocampus and cortex were higher in the curcumin group than in subarachnoid hemorrhage and vehicle groups, but not than the Sham group. Data were expressed as mean (95\% CI) and measured on day 7 after subarachnoid hemorrhage. \( ^* P < 0.05 \) for subarachnoid hemorrhage and vehicle groups versus sham group. \( ^# P < 0.05 \) for curcumin group versus subarachnoid hemorrhage and vehicle groups. \( P \) values were analyzed using one-way ANOVA; \( n = 6 \) in each group. The curcumin group is subarachnoid hemorrhage rats treated with curcumin; the vehicle group is subarachnoid hemorrhage rats treated with dimethyl sulfoxide. Cur = curcumin; MDA = malondialdehyde; SAH = subarachnoid hemorrhage; SOD = superoxide dismutase.
neally) 30 min after ischemia significantly reduced the ischemia/reperfusion-induced oxidative stress. Moreover, in a global cerebral ischemia model induced in Mongolian gerbils by transient occlusion of the common carotid arteries, a single dose of curcumin (30 mg/kg, intraperitoneally) significantly attenuated ischemia-induced neuronal death. In addition, single intraperitoneal injection curcumin doses (100 or 300 mg/kg) 30 min after middle cerebral artery occlusion in rats resulted in a clinically significant reduction of infarct volume and edema in a dose-dependent manner. Most of these studies explored the effect of curcumin on ischemic stroke. Only one study, by Wakade et al., demonstrated that a single intraperitoneal injection of curcumin (150 or 300 mg/kg) attenuated CVS after experimental SAH via an antioxidant effect. In this study, we used multiple doses of curcumin, which resulted in significantly improved survival rate and neurologic scores. This might be associated with attenuation of glutamate level and increase in malondialdehyde level, as well as preserved SOD and catalase activities after SAH. In addition, we demonstrated that the basilar artery wall thickening and the perimeter were significantly preserved after SAH induction in curcumin-treated rats, which may also have contributed to the neurologic outcome and survival rate. Our results are consistent with those of previous studies showing that a pharmacological approach involving an antioxidant effect is a potential strategy for treatment of CVS after SAH.

The Dosage of Curcumin

A wide range of curcumin doses has been used in experimental brain ischemia studies (30–300 mg/kg, intraperitoneally). Huang et al. reported that a single dose of curcumin (20 or 40 mg/kg, intraperitoneally) was effective in inhibiting the increase in glutamate level in the hypothalamus during lipopolysaccharide-induced systemic inflammation in rabbits. In addition, Chen et al. reported that a single intraperitoneal injection of curcumin (20 mg/kg) attenuated airway hyperreactivity induced by ischemia-reperfusion of the pancreas in rats. Moreover, inflammation may be a common pathogenic pathway in CVS after SAH and may persist for days. Based on these studies, we prescribe curcumin (20 mg/kg, intraperitoneally) within 3 h of SAH and then daily for 6 days thereafter.

We suggest that curcumin has a potential clinical application as a treatment for SAH-mediated brain injury, as prophylactic SAH treatment is not feasible because of its unpredictability. In our study, we gave curcumin within 3 h of SAH to mimic the clinical situation of patients in the emergency department. These results imply that curcumin may be useful as a neuroprotective drug for treatment of patients suffering from SAH and ischemic cerebral stroke. A pharmacokinetic study conducted with mice showed a plasma curcumin concentration of 2.25 μg/ml after 15 min after intraperitoneal administration of 100 mg/kg curcumin and a brain curcumin concentration of 0.41 μg/g after 1 h. Disruption of the blood-brain barrier after SAH has been reported. In this condition, the permeability of the brain to curcumin may be higher than normal. Further investigation is needed to associate the duration of the neuroprotective effect of curcumin after brain injury with the dosage administered. However, extrapolation of the results of neurologic treatments from rats to humans is seldom successful.

This study has limitations. We did not examine astrocyte or measure their GLT-1 activity directly; therefore, we cannot verify the relationships between astrocytes, extracellular glutamate levels, GLT-1 expression, and neuronal damage after SAH. Multiple varieties of GLT-1 function after brain ischemia have been reported, and changes in GLT-1 function may influence extracellular glutamate concentration and the associated neuronal damage. The second limitation was that we did not examine the antioxidant effect of curcumin on glutamate transporters, but it is known that glutamate transporters activity is inhibited by oxidative damage caused by reactive oxygen species and lipid peroxidation products. Therefore, neuronal injury is mostly associated with oxidative stress and glutamate-induced neurotoxicity, with cross talk between the two phenomena. Further studies are warranted to explore the relationships among the variables used in this study.

In conclusion, we have shown that treatment with multiple intraperitoneal doses of curcumin after SAH reduced the mortality rate, and improved functional and histologic outcomes after SAH in rats. Possible mechanisms responsible for these effects include attenuation of glutamate level via preservation of GLT-1 protein expression, attenuation of the increase in malondialdehyde level, and preservation of SOD and catalase activities. Curcumin could be a potential treatment for SAH in humans.

References

Curcumin in Experimental Subarachnoid Hemorrhage