Agmatine-Promoted Angiogenesis, Neurogenesis, and Inhibition of Gliosis-Reduced Traumatic Brain Injury in Rats

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Background: The mechanisms of agmatine-induced neuroprotective effects in traumatic brain injury (TBI) remain unclear. This study was to test whether inhibition of gliosis, angiogenesis, and neurogenesis attenuating TBI could be agmatine stimulated.

Methods: Anesthetized rats were randomly assigned to sham-operated group, TBI rats treated with saline (1 mL/kg, intraperitoneally), or TBI rats treated with agmatine (50 mg/kg, intraperitoneally). Saline or agmatine was injected 5 minutes after TBI and again once daily for the next 3 postoperative days.

Results: Agmatine therapy in rats significantly attenuated TBI-induced motor function deficits (62° vs. 52° maximal angle) and cerebral infarction (88 mm³ vs. 216 mm³), significantly reduced TBI-induced neuronal (9 NeuN-TUNEL double positive cells vs. 60 NeuN-TUNEL double positive cells) and glial (2 GFAP-TUNEL double positive cells vs. 20 GFAP-TUNEL double positive cells) apoptosis (increased TUNEL-positive and caspase-3-positive cells), neuronal loss (82 NeuN-positive cells vs. 60 NeuN-positive cells), gliosis (35 GFAP-positive cells vs. 72 GFAP-positive cells), 60 Iba1-positive cells vs. 90 Iba1-positive cells), and neurotoxicity (30 n-NOS-positive cells vs. 90 n-NOS-positive cells; 35 3-NT-positive cells vs. 90 3-NT-positive cells), and significantly promoted angiogenesis (3 BrdU/endothelial cells vs. 0.5 BrdU/endothelial cells; 50 vascular endothelial growth factor-positive cells vs. 20 vascular endothelial growth factor-positive cells) and neurogenesis (27 BrdU/NeuN positive cells vs. 15 BrdU/NeuN positive cells).

Conclusions: Resultantly, agmatine therapy may attenuate TBI in rats via promoting angiogenesis, neurogenesis, and inhibition of gliosis.

Key Words: Traumatic brain injury, Agmatine, Angiogenesis, Neurogenesis, Gliosis.

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MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 275 g ± 16 g were used in these experiments. Animals were kept under a 12/12-hour light/dark cycle and allowed free access to food and water. All experimental procedures conformed to the instructions of National Science Council of Republic of China and were approved by the Chi Mei Medical Center Animal Care and Use Committee to minimize discomfort in the animals during surgery and in the recovery period. The animals were randomly assigned to the sham group, TBI rats treated with normal saline, or TBI rats treated with agmatine. Agmatine was injected 5 minutes after TBI and again once daily for the next three postoperative days. All the tests were blinded, and the animal codes were revealed only at the end of the behavioral and histologic analyses. Agmatine (Sigma

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were the means of left and right side maximal angles.

TBI Model

The animals were anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally [i.p.]; Sigma Chemical Co.), and in a stereotaxic frame, were with the scalp incised sagittally and subjected to a lateral fluid percussion injury (FPI) to induce TBI. After an incision in the scalp was made, a 4.8-mm circular craniotomy was performed midway between lambda and bregma, 3.0 mm to the right of the central suture. A modified leurock connector (trauma cannula), 2.6-mm inner diameter, was secured into the craniotomy with cyanoacrylic adhesive and dental acrylic. A moderate FPI (2.2 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a fluid percussion device (VCU Biomedical Engineering, Richmond, VA). The animal was removed from the device, the acrylic removed, and the incision sutured. Each injured and sham-injured animal for behavioral recovery was closely evaluated immediately after FPI.

Experimental Procedures

In experiment 1, TBI was performed randomly in rats treated with agmatine, and their effects on motor function and infarction volume were assessed 7 days after FPI. In experiment 2, TBI was performed randomly in rats treated with agmatine, and their effects on neuronal loss, astrogliosis, microgliosis, and neuronal and glial apoptosis were assessed 7 days after FPI. In experiment 3, TBI was performed randomly in rats treated with agmatine, and their effects on both neurogenesis and angiogenesis were assessed 7 days after FPI. In Experiment 4, TBI was randomly performed in rats treated with agmatine, and their effects on TBI-induced increased numbers of n-NOS-positive and 3-NT-positive cells were assessed 7 days after FPI.

Inclined Plane

The inclined plane was used to measure limb strength. The animals were placed, facing right and then left perpendicular to the slope of a 20 × 20-cm rubber ribbed surface of an inclined plane starting at an angle of 55°. The angle was increased or decreased in 5° increments to determine the maximal angle an animal could hold to the plane. Data for each day were the means of left and right side maximal angles.

Cerebral Infarction Assay

The TTC staining followed those described elsewhere. All animals killed at 7 days after TBI were saline-perfused intracardially under deep anesthesia (100 mg/kg sodium pentobarbital, i.p.). The brain tissue was successfully removed, immersed in cold saline for 5 minutes, sliced into 2-mm sections, incubated in 2% TTC dissolved in phosphate-buffered saline (PBS) for 30 minutes at 37°C, and transferred to 5% formaldehyde solution for fixation. The volume of infarction, as revealed by negative TTC stains indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based Image Tools Software). The volume of infarction was calculated as 2 mm (thickness of the slice) × (sum of the infarction area in all brain slices [mm²]).

Bromodeoxyuridine Labeling

To evaluate the proliferation of cells, 5-bromodeoxyuridine (BrdU; Roche Diagnostics, IN; 50 mg/kg) dissolved in PBS were administered once daily intraperitoneally for 3 days after TBI. The rats were killed 7 days later.

Immunohistochemistry

Adjacent 50-μm sections corresponding to coronal coordinates 0.20 mm to 0.70 mm anterior to the bregma were incubated in 2 mol/L HCl for 30 minutes, rinsed in 0.1 mol/L boric acid (pH 8.5) for 3 minutes at room temperature and then incubated with primary antibodies in PBS containing 0.5% normal bovine serum at 4°C overnight. After being washed in PBS, the sections were incubated with secondary antibodies for 1 hour at room temperature. The following antibodies were used in this study: rat monoclonal anti-BrdU antibody (1:10; Oxford Biotechnology, Oxfordshire, United Kingdom), mouse anti-VEGF, mouse anti-GFAP, mouse anti-Iba1, mouse anti-GDNF, or mouse anti-neuronal-specific nuclear protein (NeuN) antibody in 1:200 dilution and then detected with Alexa-Fluor, 568 goat anti-mouse (IgG) antibody. For endothelial cells, biotinylated Lycopersicon esculentum (tomato) lectin (1:200; Vector Laboratories, Burlingame, CA) were used in the same manner instead of other primary antibodies and visualized using fluorescein-labeled streptavidin (1:200; Vector Laboratories). For caspase-3 activity, cortical sections were incubated with the cleaved caspase-3 (ASP/75) antibody (cell signaling technology, Inc., Beverly, MA), which only recognizes the large fragment of activated caspase-3 (17–20 KDa), in 3% BSA/PBS/0.1% Tx-100 at 4°C overnight. The sections were then washed in PBS/0.6% Tx-100 and were incubated with FITC-conjugated anti-rabbit IgG (1% BSA/PBS/0.1% Tx-100 for 60 minutes. They were mounted with anti-fade mounting media. The labeled cells were calculated in five coronal sections from each rat and expressed as the mean number of cells per section. For negative coronal sections, all procedures were performed in the same manner without the primary antibodies.

TUNEL Assay

The TUNEL assay was performed using the same brain sections used in histochemical verification. The color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB). The sections were xylene- and ethanol-treated for paraffin sections used in histochemical verification. The color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB). The sections were xylene- and ethanol-treated for paraffin
Statistical Analysis

The data are presented as mean ± SD. The repeated measures analysis of variance was conducted to test the treatment-by-time interactions and the effect of treatment over time on each score. The Duncan’s multiple-range test was used for post hoc multiple comparisons among means. The p value <0.05 was considered evidence of statistical significance.

RESULTS

Agmatine Improved Motor Dysfunction During TBI

Seven days after TBI, behavioral tests revealed that vehicle-treated TBI rats had significantly lower performance in motor function test than they were for sham-operated controls (Fig. 1). The TBI-induced motor dysfunction could be significantly reduced by agmatine therapy (Fig. 1).

Agmatine Decreased Infarct Volume During TBI

The TTC-stained sections at 7 days after TBI showed a significant increase in the infarcted area of the vehicle-treated TBI as compared with those of sham TBI controls (Fig. 2). The TBI-induced infarction volume was significantly decreased by agmatine treatment (Fig. 2).

Agmatine Decreased Neuronal Loss, Astrogliaisis, and Microgliosis during TBI

As evaluated at 7 days after TBI, the vehicle-treated TBI rats had lower numbers of NeuN-positive cells (Fig. 3, A) but higher number of both GFAP-positive cells (Fig. 3, B) and Iba1-positive cells (Fig. 3, C) in the ischemic cortex compared with those of sham TBI controls (Fig. 3, A–C).

Agmatine Decreased Both Neuronal and Glial Apoptosis During TBI

At 7 days after TBI, it was found that vehicle-treated TBI rats had significantly higher numbers of NeuN plus TUNEL positive cells (Fig. 4, A), GFAP plus TUNEL positive cells (Fig. 4, B), and caspase-3-positive cells (Fig. 5) in the ischemic cortex compared with those of sham TBI controls. These figures also showed that the increased numbers of NeuN plus TUNEL-positive cells, GFAP plus TUNEL-positive cells, and caspase-3-positive cells in the ischemic cortex after TBI were significantly decreased by agmatine therapy.

Agmatine Promoted Neurogenesis During TBI

At 7 days after TBI, the vehicle-treated TBI rats had significantly higher numbers of BrdU/NeuN double positive cells (Fig. 6, A) and GDNF-positive cells (Fig. 6, B) in the ischemic cortex compared with those of sham TBI controls. Again, the increased numbers of both BrdU/NeuN double-positive cells and GDNF-positive cells in the ischemic cortex were significantly decreased by agmatine therapy.

Agmatine Promoted Angiogenesis During TBI

As evaluated at 7 days after TBI, the number of both BrdU-positive endothelial cells (Fig. 7, A) and VEGF-positive cells (Fig. 7, B) in the ischemic cortex of vehicle-treated TBI rats were significantly higher than those of sham TBI rats. Again, the increased numbers of both BrdU-positive endothelial cells and VEGF-positive cells in the ischemic cortex after TBI were further significantly increased by agmatine therapy (Fig. 7, A and B).
Agmatine Decreased Both n-NOS and 3-NT Expression During TBI

As revealed at 7 days after TBI, the numbers of both n-NOS-positive cells (Fig. 8, A) and 3-NT-positive cells (Fig. 8, B) in the ischemic cortex of vehicle-treated TBI rats were significantly higher than those of sham TBI rats. Again, the increased numbers of both n-NOS-positive cells and 3-NT-positive cells in the ischemic cortex after TBI were significantly decreased by agmatine therapy (Fig. 8, A and B).

**DISCUSSION**

Previously, we demonstrated that agmatine therapy after TBI in rats caused attenuation of intracranial hypertension, cerebral hypoperfusion, ischemic brain damage, motor dysfunction, and body weight loss that occurred during TBI. The present results further demonstrated that agmatine ther-
apy caused attenuation of TBI-induced neuronal and glial apoptosis (evidenced by increased numbers of both TUNEL-positive cells and caspase-3-positive cells), neuronal loss (evidenced by decreased numbers of NeuN-positive cells), and gliosis (evidenced by increased numbers of both GFAP-positive cells and Iba1-positive cells) in the ischemic cortex during TBI. In addition, our results showed that both angiogenesis (evidenced by increased numbers of both BrdU-positive endothelial cells and VEGF-positive cells) and neurogenesis (evidenced by increased numbers of both BrdU/NeuN double-positive cells and GDNF-positive cells) were promoted in the ischemic cortex by agmatine therapy. Together, these observations indicate that agmatine may improve the outcomes of TBI via promoting angiogenesis, neurogenesis, and inhibition of gliosis (or scar formation).

We found here that agmatine therapy caused attenuation of neuronal loss that occurred during TBI. In the ischemic cortex, 7 days after TBI, generation of proliferated neurons (evidenced by the increased numbers of NeuN plus BrdU double-positive cells and GDNF-positive cells) was promoted by agmatine treatment. It is apparent that agmatine, in addition to preventing neuronal loss, might promote the penetration of new neurons in both the hippocampus and subventricular zones.10–12 The proliferated cells generated from these areas could migrate to and differentiate in the damaged region of the brain.

GDNF, a distant member of the transforming growth factor-β family, is a protein that is essential for the survival of dopaminergic,13–15 motor,16–18 and peripheral neurons.19,20 It was shown that topical application or intracerebral administration of GDNF decreased the size of ischemia-induced brain infarction.9,21 In addition, GDNF treatment might be beneficial in reversing hind limb dysfunction by reducing spinal cord infarction and apoptosis in a spinal cord compression model.22 The present findings further demonstrated that agmatine might improve motor dysfunction and cerebral infarction and apoptosis that occurred during TBI by stimulating production of GDNF in the ischemia brain.

As shown in the present study, agmatine therapy increased the amounts of both BrdU-positive endothelial cells and VEGF-positive cells in the injured brain, attenuated cerebral infarction and apoptosis, and restored normal motor function in a rat TBI model. A rich vascular environment, along with generation of VEGF, might enhance subsequent angiogenesis and neurogenesis.23,24 A more recent report also showed that systemic delivery of Premarin, a soluble estrogen sulfate, attenuated TBI-induced cerebral infarction and apoptosis by increasing the amounts of both VEGF-

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**Figure 5.** Agmatine decreased the TBI-induced increased numbers of active caspase 7 positive cells. *The numbers of active caspase 3 positive cells in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) increased for TBI-injured animals treated with vehicle (○) compared with TBI sham controls (□). †The numbers of active caspase 3-positive cells in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) decreased for TBI-injured animals treated with agmatine (■) compared with vehicle controls. Each column and bar denoted mean ± SD of eight animals per group.

**Figure 6.** Agmatine increased the TBI-induced neurogenesis. *The numbers of both BrdU/Neu double positive cells (A) and GDNF-positive cells (B) in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) increased for TBI-injured animals treated with vehicle (○) compared with TBI sham controls (□). †The numbers of both BrdU/NeuN double-positive cells (A) and GDNF-positive cells (B) in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) increased for TBI-injured animals treated with agmatine (■) compared with vehicle controls. Each column and bar denoted mean ± SD of eight animals per group.
positive cells and BrdU-positive endothelial cells in the injured brain. Thus, agmatine might improve motor outcome during TBI by enhancing neovessel formation and accelerating endogenous neurogenesis.

Decisive evidence indicated that nitric oxide overproduction from neuronal nitric oxide synthase impaired brain tissue. Poor neurologic outcome was also associated with increased levels of nitrotyrosine in the cerebrospinal fluid in human TBI. 3-NT was shown to be involved in the induction of both motor neuron apoptosis in vitro and mitochondrial oxidative damage and dysfunction in a mouse model of focal TBI. Agmatine was believed to be synthesized predominantly by the astroglia cells, then released and taken up into neurons by active transport. Agmatine also acted as an irreversible inactivator of n-NOS. In the current studies, TBI-induced neuronal and glial apoptosis, and overexpression of both n-NOS and 3-NT in the ischemia brain could be

Figure 7. Agmatine increased the TBI-induced angiogenesis. *The numbers of both BrdU positive endothelial cells (A) and VEGF-positive cells (B) in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) increased for TBI-injured animals treated with vehicle (C) compared with TBI sham controls (D). †The numbers of both BrdU-positive endothelial cells (A) and VEGF-positive cells (B) in the ischemic cortex 7 days after TBI were significantly increased for TBI animals treated with agmatine (E) compared with vehicle controls. Each column and bar denoted mean ± SD of eight animals per group.

Figure 8. Agmatine decreased the TBI-induced increased numbers of both n-NOS-positive cells (A) and 3-NT-positive cells (B) in ischemic cortex. *The numbers of both n-NOS-positive cells and 3-NT-positive cells in the ischemic cortex 7 days after TBI was significantly (p < 0.05; n = 8) increased for TBI-injured animals treated with vehicle (C) compared with TBI sham controls (D). †The numbers of both n-NOS-positive cells and 3-NT-positive cells in the ischemic cortex 7 days after TBI were significantly (p < 0.05; n = 8) decreased for TBI-injured animals treated with agmatine (E) compared with vehicle controls. Each column and bar denoted mean ± SD of eight animals per group. Top panels of (A) and (B) depicted representative n-NOS-positive and 3-NT-positive stainings 3 days after TBI, respectively, for a TBI sham control, a vehicle-treated TBI rat, and an agmatine-treated TBI rat.
significantly reduced by agmatine treatment. Together, these results suggested that agmatine might cause attenuation of neuronal and glial apoptosis by reducing overexpression of both n-NOS and 3-NT in the ischemic brain during TBI.

In the present study, 7 days after TBI, gliosis (evidenced by increased numbers of both GFAP-positive cells and Iba1-positive cells) and neuronal and glial apoptosis (evidenced by increased numbers of NeuN plus TUNEL-positive cells, GFAP plus TUNEL-positive cells, and Iba1 plus TUNEL-positive cells) in the ischemia cortex, could be significantly reduced by agmatine therapy. The reduction of both gliosis and neuronal and glial apoptosis in agmatine-treated TBI animals was paralleled by the reduced infarct volume and near normal motor function. These results indicated the agmatine might protect against the delayed infarct expansion caused by activated astrocytes during TBI. Astrocytes are believed to be responsible for most glutamate uptake in synaptic and monosynaptic areas and consequent are the major regulators of glutamate homeostasis. Microglia may secrete cytokines, which can impair glutamate uptake. These observations indicate that agmatine may protect against TBI-induced apoptosis via reducing glutamate-mediated glial injury.3, 4

In summary, TBI-induced neuronal loss and apoptosis, gliosis, glial apoptosis, and neurotoxicity (or increased expression of both neuronal NOS and 3-NT) in the ischemia brain were all significantly reduced by agmatine. In addition, agmatine caused attenuation of TBI-induced cerebral infarct and motor dysfunction. In injured brain, both angiogenesis and neurogenesis were also agmatine promoted. Our data indicated that agmatine improved outcomes of TBI in rats through mechanisms of promoting angiogenesis, neurogenesis, and inhibition of gliosis (or scar formation) in the injured brain.

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