Resuscitation from experimental traumatic brain injury by agmatine therapy

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Summary Both nitric oxide and glutamate contribute to ischaemic brain injury. Agmatine inhibits all isoforms of nitric oxide synthase and blocks N-methyl-p-aspartate receptors. In this study, we gave agmatine intraperitoneally and assessed its effect on fluid percussion brain injury in rats. Anaesthetised rats, immediately after the onset of fluid percussion traumatic brain injury (TBI), were divided into two major groups and given the vehicle solution (1 mL/kg) or agmatine (50 mg/kg) intraperitoneally. Mean arterial pressure, intracranial pressure, cerebral perfusion pressure, and levels of glutamate, nitric oxide, lactate/pyruvate ratio, and glycerol in hippocampus were monitored continuously within 120 min after TBI. The weight loss was determined by the difference between the first and third day of body weight after TBI. The maximal grip angle in an inclined plane was measured to determine motor performance whereas the percent of maximal possible effect was used to measure blockade of proprioception. The triphenyltetrazolium chloride staining procedures were used for cerebral infarction assay. Compared to those of the sham-operated controls, the animals with TBI had higher values of extracellular levels of glutamate, nitric oxide, lactate-to-pyruvate ratio, and glycerol in hippocampus and intracranial pressure, but lower values of cerebral perfusion pressure. Agmatine administered immediately after TBI significantly attenuated the TBI-induced increased hippocampal levels of glutamate, nitric oxide, lactate-to-pyruvate ratio, and glycerol, intracranial hypertension, and cerebral hypoperfusion. In addition, the TBI-induced cerebral infarction, motor and proprioception deficits, and body weight loss evaluated 3 days after TBI were significantly attenuated by

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Introduction

Traumatic brain injury (TBI) is a major burden for the families and the community in terms of cost, suffering, and disability in all developed countries. TBI is the second largest killer in the world after conventional stroke. In spite of the seriousness of this problem, limited neuroprotective drugs are available for those suffering TBI. Basic and clinical research should continue to provide important and significant steps toward the development of new and effective therapeutic drugs for head-injured patients.

Evidence has accumulated to suggest that both nitric oxide (NO) and glutamate contribute to hypoxia-ischaemia brain injury. Inhibitors of nitric oxide synthase (NOS) and antagonists of N-methyl-D-aspartate (NMDA) glutamate receptors are neuroprotective in hypoxia-ischaemia brain injury. Agmatine is thought to be synthesised in the mammalian brain. Agmatine is able to inhibit all isoforms of NOS as well as the NMDA subclass of glutamate receptors. Treatments with agmatine are neuroprotective in both rat ischaemic brain injury and rat spinal cord injury. The effects of agmatine in TBI have not previously been tested, nor has correlation been studied between the neuroprotective properties of agmatine and production of brain NO and glutamate in TBI.

In the present study, we have used the fluid percussion brain injury model to evaluate the neuroprotective potency of agmatine. The purpose of the study was to evaluate the effects of agmatine on brain injury and production of NO and glutamate by using intracerebral microdialysis technique. In addition, this study compared the temporal profiles of cardiovascular dysfunction, behavioural deficits, and cerebral infarction during TBI in the rat with or without agmatine therapy.

Materials and methods

Adult male Sprague–Dawley rats weighing 280±15 g were used in these experiments. Animals were kept under a 12/12-h light/dark cycle and allowed free access to food and water. All experimental procedures conformed to the NIH guide lines and were approved by the Chi Mei Medical Center Animal Care and Use Committee to minimise discomfort in the animals during surgery and in the recovery period.

Animals were randomly assigned to sham-operated group (n = 10), TBI rats treated with normal saline (1 mL/kg, i.p.) (n = 10), or TBI rats treated with agmatine (50 mg/kg, i.p.) (n = 10). All tests were run blinded, and the animal codes were revealed only at the end of the behavioural and histological analyses. We injected 50 mg/kg of agmatine (Sigma Chemical Co., St. Louis, MO, USA) mixed in 0.9% saline solution immediately after TBI for the agmatine-treated group of rats. Animals used for histological or behavioural studies were provided food and water ad libitum throughout the study.

Animals were anaesthetised with sodium pentobarbital (25 mg/kg, i.p.; Sigma Chemical Co.) and a mixture containing ketamine (44 mg/kg, i.m.; Nankuang Pharmaceutical, Taiwan), atropine (0.02633 mg/kg, i.m.; Sintong Chemical Ind. Co., Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer, Germany). Both the femoral artery and vein on the right side were cannulated with PE50 polyethylene tubing for monitoring blood pressure and blood gas analysis. After cannulation, the wound was sutured, and animals were turned into the prone position. They were placed in a stereotaxic frame, and the scalp was incised sagittally. Animals were 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 leu- lock 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, USA). The animal was removed from the device, the acrylic removed, and the incision sutured. Each injured and sham injured animal for fluid percussion model was evaluated closely immediately after TBI for behavioural recovery.

The right femoral artery of rats was cannulated with polyethylene tubing (PE50) under sodium pentobarbital anaesthesia for blood pressure monitoring. For measurement of intracranial pressure (ICP), the animals were positioned in a stereotaxic apparatus (Kopf 1406; Grass Instrument, Quincy, MA) to insert probes for a Statham P23AC transducer via 20-gauge stainless-steelneedled probe (diameter = 0.8 mm) to insert probes for a Statham P23AC transducer via 20-gauge stainless-steel needled probe (diameter = 0.8 mm).
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...ter 0.90 mm; length 38 mm), which was introduced into the left cerebral ventricle according to the stereotaxic coordinates of Paxinos and Watson\(^{12}\): A, interaural, 7.7 mm; L, 2.0 mm from the midline; and H, 3.5 mm from the top of the skull. All recordings were made on a four-channel Gould multichannel analyser. The temperature (Tco) was monitored continuously by a thermocouple, and mean arterial blood pressure (MAP) and heart rate (HR) were monitored continuously with a pressure transducer.

Each animal was anaesthetized with sodium pentobarbital intraperitoneally. The animal’s head was mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the nose bar positioned 3.3 mm below the horizontal line. After a midline incision, the skull was exposed and a burr hole was made in the skull for the insertion of a dialysis probe (4 mm in length, CMA/2; Carnegie Medicine, Stockholm, Sweden). The microdialysis probe was stereotaxically and obliquely (anterior 4.3 mm) implanted into the right hippocampus according to the atlas and coordinates of Paxinos and Watson\(^{12}\): P, 8 mm; R, 3 mm; H, 5 mm. According to the methods described previously,\(^{13}\) the microdialysis was perfused at 2.0 \(\mu\)L/min and the dialysates were sampled in microvials. The dialysates were collected every 20 min in a CMA/140 fraction collector (Carnegie Medicine). Aliquots of dialysates (5 \(\mu\)L) were injected onto a CMA 600 microdialysis analyser (Carnegie Medicine) for measurement of lactate, glycerol, pyruvate, and glutamate. The \(\text{NO}_2^-\) concentrations in the dialysates were measured with the Eicom ENO-20 NO\(_2^-\) analysis system (Eicom, Kyoto).\(^{14}\) Only experiments in which the hippocampal localisation of the microdialysis probes was confirmed histologically were included in the results. In this series of experiments, no animal was excluded due to incorrect microdialysis probe position.

Experiment 1, fluid percussion injury was performed randomly in rats treated with an i.p. dose of agmatine (50 mg/kg), and their effects on MAP, HR, ICP, and CPP were assessed. A transient hypertensive response, apnoea, and seizure was observed immediately following fluid percussion injury and used as the criteria for separating the animals into sham-operated group and fluid percussion injured group. No hypotension, apnea, or seizure was noted in sham-operated rats. In this series of experiments, 24 rats were divided equally into 3 groups: sham-operated controls (\(n=8\)), agmatine-treated FPI rats (\(n=8\)), and vehicle-treated FPI rats (\(n=8\)).

Experiment 2, FPI, was performed randomly rats treated with an i.p. dose of agmatine (50 mg/kg), and their effects on Tco, MAP, and extracellular levels of \(\text{NO}_2^-\), glutamate, glycerol, and lactateto-pyruvate ratio in hippocampus were assessed. Again, in this series of experiments, a transient hypertension, apnea, and seizure response immediately after FPI was used as the criteria for the success of FPI. In this series of experiments, 24 rats were equally divided into 3 groups: sham-operated controls (\(n=8\)), agmatine-treated FPI rats (\(n=8\)), and vehicle-treated FPI rats (\(n=8\)).

Experiment 3, FPI was randomly performed in rats treated with an i.p. dose of agmatine (50 mg/kg), and their effects on weight loss, motor behaviour, proprioception, and cerebral infarction were assessed. In this series of experiments, in order to avoid femoral artery occlusion, a transient apnea and response was used as the criteria for the success of FPI. On this series of experiments, 24 rats were separated equally into 3 groups: sham-operated controls (\(n=8\)), agmatine-treated FPI rats (\(n=8\)), and vehicle-treated FPI rats (\(n=8\)).

The inclined plane was used to measure limb strength. Animals were placed facing right and then left, perpendicular to the slope of a 20 by 20 cm rubber ribbed surface of an inclined plane starting at an angle of 55\(^\circ\).\(^{15}\) The angle was increased or decreased in 5\(^\circ\) increments to determine the maximal angle an animal could hold to the plane. The data for each day was the mean of left and right side maximal angles.

Proprioception evaluation was based on the resting, posture and postural reactions (‘‘tactile placing’’ and ‘‘hopping’’).\(^{16}\) The functional deficit was graded as 3 (normal or 0% maximal possible effect [MPE]), 2 (slightly impaired) 1 (severely impaired), and 0 (Completely or 100% MPE).\(^{17}\) This test was performed by lifting the front half of the animal off the ground and lifting one hind limb at a time off the ground so that the animal was standing on just one limb. Then, the animal was moved laterally, which normally evoked a prompt hopping response with the weight-bearing limb in the direction of movement to prevent the animal from falling. A predominant motor block would cause a prompt but weaker than normal response. Conversely, a predominantly proprioceptive block causes a delayed hopping followed by greater lateral hops to prevent the animal from falling. In the case of full blockade, there would be no hopping manoeuvers.\(^{18}\)

The triphenyltetrazolium chloride (TTC) staining procedures followed those described elsewhere.\(^{19}\) All animals were sacrificed at third day after fluid percussion injury. Under deep anaesthesia (Sodium pentobarbital, 100 mg/kg, i.p.) animals were perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0-mm sections with a tissue...
slicer. The brain slices were incubated in 2% TTC dissolved in PBS for 30 min at 37°C, and then 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 computerised 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)].

Physiological data and microdialysis data were analysed with a repeated-measures analysis of variance for differences between time points and groups, followed by Fisher’s post hoc test. Repeated ANOVAS and post hoc Fisher test were also used to determine effects of treatment over the two test sessions using volume of infarction, incidence of infarction, weight loss, and maximal angle as a single parameter. Ordinal measurement such as % MPE were evaluated using the non-parametric Kruskal–Wallis ANOVA with individual non-parametric Mann–Whitney U-tests. A P-value <0.05 was considered statistically significant.

Results

Both Figures 1 and 2 depict the effects of FPI on several cerebrovascular variables as well as extracellular levels of glycerol, glutamate, lactate/pyruvate ratio, and NO₂⁻ in hippocampus in rats treated with vehicle solution, and in rats treated with agmatine. In vehicle-treated FPI group, the HR, ICP, and hippocampal levels of glycerol, glutamate, lactate/pyruvate ratio, and NO₂⁻ were all significantly higher at 10–120 min after the start of FPI than they were for sham-operated controls. In contrast, the values for CPP were significantly lower than those of sham-operated controls. Resuscitation with agmatine immediately after FPI significantly attenuated the FPI-induced intracranial hypertension, cerebral hypoperfusion and overproduction of cellular ischemia and injury markers in hippocampus. The basal levels of cerebrovascular parameters and ischaemia and injury markers measured in sham-operated rats treated with agmatine (50 mg/kg, i.p.) were indistinguishable from those of sham-operated rats received no treatment (data not shown).

Figure 3 shows that FPI rats treated with vehicle solution immediately after injury have higher amounts of weight loss compared to those of sham-operated controls. The weight loss denotes the difference in body weight between the first and third day after FPI. However, agmatine therapy (50 mg/kg, i.p.) immediately after FPI significantly reversed the FPI-induced weight loss.

Figure 1  Effects of fluid percussion injury (FPI) on mean arterial pressure (MAP), heart rate (HR), intracranial pressure (ICP), and cerebral perfusion pressure (CPP). (○) Values in eight rats treated with vehicle solution (1 mL/kg, i.p.) immediately after FPI. Another eight rats treated with sham operation served as a control (●). (▼) Values in eight rats treated with agmatine (50 mg/kg, i.p.) immediately after FPI as indicated by dash lines. Points represent mean ± S.E.M. (*P < 0.05 in comparison with sham-operated control values; †P < .05 in comparison with vehicle-treated group).
As compared with those of the sham-operated control rats, the maximal angle animals treated with vehicle solution could cling to an inclined plane significantly decreased 72 h after FPI (as shown in Figure 4). However, the FPI-induced reduction in maximum grip angle measured 72 h after FPI was reversed significantly by agmatine therapy (50 mg/kg, i.p.) ($P < 0.05$).

The percent of MPE of proprioception blockade 72 h after FPI increased significantly in vehicle solution-treated FPI animals compared to those of sham-operated controls (Figure 5). Again, the % of MPE of proprioception blockade obtained 72 h after FPI was reversed significantly by agmatine administration ($P < 0.05$).

Triphenyltetrazolium chloride staining revealed that the marked increase in cerebral infarction in FPI rats treated with vehicle solution (Figure 6C). Compared to those of sham-operated controls, FPI induced a significant increase in cerebral infarction.

**Figure 2.** Effects of fluid percussion injury (FPI) on core temperature ($T_c$), and extracellular levels of glutamate, glycerol, and lactate/pyruvate ratio in hippocampus. (○) Values in eight FBI rats treated with vehicle solution (1 mL/kg, i.p.) immediately after FBI, as indicated by dash lines. Another eight rats treated with sham operation served as a control (●). (▼) Values in eight rats treated with agmatine (50 mg/kg, i.p.) immediately after FBI. Points represent mean±S.E.M. (*$P < .05$ in comparison with sham-operated control values; †$P < .05$ in comparison with vehicle-treated group).

**Figure 3.** FPI-induced body weight loss. □ Vehicle-treated FPI animals showed higher amounts of weigh those compared to those of sham-operated controls ($P < 0.05$, $n = 8$). *However, animals given an i.p. dose of agmatine (50 mg/kg) immediately after FPI had a significant reduction in weight loss ($P < 0.05$; $n = 8$).
Figure 4 Maximal angle animals could cling to an inclined plane. *Maximum grip angle 4 days following FPI injury was significantly ($P < 0.05$; $n=8$) decreased for FPI injured animals treated with vehicle (mL/kg, i.p.) (□) compared to FPI sham controls. Maximum grip angle 4 days following FPI injury was significantly ($P < 0.05$; $n=8$) increased for FPI injured animals treated with an i.p. dose of agmatine (■) (50 mg/kg) compared to vehicle controls.

Figure 5 Percent of maximal possible effect (%MPE) of proprioception blockade by RBI in rats. ($n=8$ in each group of different treatments). *The percent of MPE 4 days following TBI injury was significantly ($P < 0.05$; $n=8$) increased for LEP injured animals treated with an i.p. dose of saline (□) (1 mL/kg) compared to LFP sham controls (△). The percent of MPE 4 days following TBI injury was significantly decreased of LEP injured animals treated with an i.p. dose of agmatine (50 mg/kg) compared to vehicle controls ($P < 0.05$; $n=8$).

Discussion

Excessive concentrations of glutamate and the lactate/pyruvate ratio are the well-known marker of cellular ischaemia, that is, an inadequate supply of oxygen and glucose. Glycerol is a cellular marker of how severely cell membranes are damaged by the ongoing pathology. In the current study, the microdialysis probe was implanted into the hippocampus of the...
ipsilateral brain that is the most susceptible to cerebral ischaemia and injury after FPI. The microdialysates obtained from the hippocampal region was assayed for measurement of cellular ischaemia (e.g. glutamate and lactate/pyruvate ratio) and injury (e.g. glycerol) markers during FPI. It was found that FPI brain injury caused increased levels of glutamate, lactate/pyruvate ratio, and glycerol in the hippocampus as well as infarction in several brain areas. Although the post-traumatic rise in extracellular ischaemia and injury markers is presented during short period of 120 min in our study, intracerebral microdialysis studies performed on human head-injured patients at the bedside have shown that glutamate concentrations may be elevated for an extended period of time (days) following clinical head injury. Indeed, as shown in the present results, when evaluated 3 days after FPI, the FPI rats treated with vehicle solution also displayed motor and proprioception deficits, body weight loss, and cerebral infarction. Furthermore, we showed successfully that the FPI-induced intracranial hypertension, cerebral hypotension, cerebral infarction, motor and proprioception deficits, and body weight loss all were suppressed by agmatine therapy adopted immediately after FPI. The current findings are consistent with those of several other brain ischaemia models. For example, treatment with agmatine is neuroprotective in gerbil ischaemic brain injury, adult rat ischaemia brain injury, and spinal cord injury.

In fact, depending on its source, nitric oxide may be toxic or protective to the brain under ischaemic conditions. Decisive evidence tends to indicate that excessive accumulation of nitric oxide from endothelial nitric oxide synthase protects brain tissue by maintaining regional cerebral blood flow; however, nitric oxide overproduction from either neuronal or inducible nitric oxide synthase leads to neurotoxicity. It was realised that agmatine is synthesised in the mammalian brain. From distribution studies of arginine decarboxylase in primary cell cultures, agmatine is thought to be synthesised predominantly by the astroglia cells, then released and taken up into neurons by active transport. Agmatine also inhibits all isoforms of nitric oxide synthase with highest reported activity as an irreversible inactivator of neuronal nitric oxide synthase. In the current studies, the excessive accumulation of nitric oxide in the brain following FPI was significantly reduced by agmatine treatment. Putting these observations together, it seems that agmatine may reduce cerebral infarction and dysfunction by reducing neuronal nitric oxide synthase-dependent nitric oxide formation in brain. Of course, this needs further verification in future studies.

In the rat, within 3 h following FPI, an acute decrease in NMDA but not AMPA/KA receptor binding in the hippocampus, dentate gyms, and neocortex was observed. Activation of the metabotropic glutamate receptor subtype mGlur1 appeared to be associated with cell death following in vitro neuronal traumatic injury. Pretreatment with antisense oligodeoxynucleotides directed against the NMDA-R1 receptor subunit or phencyclidine (a noncompetitive NMDA receptor antagonist) enhances survival and neurological motor recovery following lateral FPI in rats. Administration of higher doses (1 mg/kg) of MK-801 (a noncompetitive NMDA receptor antagonist) at 1 h following lateral FPI improved neurological motor deficits and reduced regional cerebral edema after weight-drop injury in rats. Whatever the mechanism of action may be, evidence from animal models suggest that glutamate-induced toxicity may be responsible for some of the post-traumatic sequelae and that these effects can be blocked by various antagonists. Our results indicate that agmatine may cause attenuation of traumatic brain injury resulting from intracranial hypertension, cerebral hypoperfusion, and cerebral ischaemia and injury by blocking the NMDA subclass of glutamate receptors.

The animal model currently used in the present study is compromised by the use of sodium pentobarbital. Sodium pentobarbital suppresses the central nervous system functions. This may exacerbate the extent of traumatic brain injury.

In summary, compared to the sham-operated controls, the animals with FPI had higher values of extracellular levels of glutamate, nitric oxide, lactate-to-pyruvate ratio, and glycerol in brain and intracranial pressure, but lower values of cerebral perfusion pressure. Agmatine given intravenously and immediately after FPI significantly attenuated the FPI-induced increased levels of glutamate, nitric oxide, and other ischaemia and injury markers, intracranial hypertension, and cerebral hypoperfusion. In addition, the FPI-induced cerebral infarction, motor and proprioception deficits, and body weight loss evaluated 3 days after FPI were significantly attenuated by agmatine therapy. Since agmatine possesses modest affinities for various receptors, including as an inhibitor of the NMDA subclass of glutamate receptors as well as an irreversible inactivator of neuronal nitric oxide synthase. The present data indicate that agmatine may attenuate traumatic brain injury by reducing the excessive accumu-
lation of both glutamate and nitric oxide in brain.

Conflict of interest
None.

References


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