Safety and Efficacy Evaluation of Carnosine, an Endogenous Neuroprotective Agent for Ischemic Stroke

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Background and Purpose—An urgent need exists to develop therapies for stroke that have high efficacy, long therapeutic time windows, and acceptable toxicity. We undertook preclinical investigations of a novel therapeutic approach involving supplementation with carnosine, an endogenous pleiotropic dipeptide.

Methods—Efficacy and safety of carnosine treatment was evaluated in rat models of permanent or transient middle cerebral artery occlusion. Mechanistic studies used primary neuronal/astrocytic cultures and ex vivo brain homogenates.

Results—Intravenous treatment with carnosine exhibited robust cerebroprotection in a dose-dependent manner, with long clinically relevant therapeutic time windows of 6 hours and 9 hours in transient and permanent models, respectively. Histological outcomes and functional improvements including motor and sensory deficits were sustained on 14th day poststroke onset. In safety and tolerability assessments, carnosine did not exhibit any evidence of adverse effects or toxicity. Moreover, histological evaluation of organs, complete blood count, coagulation tests, and the serum chemistry did not reveal any abnormalities. In primary neuronal cell cultures and ex vivo brain homogenates, carnosine exhibited robust antiexcitotoxic, antioxidant, and mitochondria protecting activity.

Conclusions—In both permanent and transient ischemic models, carnosine treatment exhibited significant cerebroprotection against histological and functional damage, with wide therapeutic and clinically relevant time windows. Carnosine was well tolerated and exhibited no toxicity. Mechanistic data show that it influences multiple deleterious processes. Taken together, our data suggest that this endogenous pleiotropic dipeptide is a strong candidate for further development as a stroke treatment.

Key Words: carnosine ■ efficacy ■ ischemic stroke ■ neuroprotection ■ safety

Despite extensive efforts to develop new treatments for ischemic stroke, many promising experimental drugs have failed in human clinical trials due to intolerable side effects, low efficacy, and short therapeutic time windows.1-4 Despite its benefits, the use of tissue plasminogen activator (tPA), which is the only approved acute drug therapy for ischemic stroke, is limited by the short therapeutic time window and the risk of hemorrhage.5,6 Therefore, an urgent need exists for safe and effective drugs.

Because tissue damage after stroke involves multiple deleterious mechanisms, it is desirable that novel therapeutic drugs favorably influence multiple molecular pathways that contribute to tissue damage.7-9 Carnosine is an endogenous dipeptide composed of alanine and histidine and is expressed in many tissues of the body including the central nervous system.9 Carnosine exhibits pleiotropic biological activities such as antioxidant, cytosolic buffering, heavy metal chelating, and antie excitotoxic activity.10,11 Because of these beneficial and diverse activities, carnosine has been proposed as an attractive therapeutic candidate for ischemic stroke damage.

Carnosine reduces neurological impairment, decreases mortality, and improves functional outcome after global ischemia in gerbils and rats.12,13 In focal ischemia, we have previously shown that intraperitoneally administrated carnosine reduced brain damage in mouse ischemia models induced by permanent middle cerebral artery occlusion (pMCAO).14 We also showed that carnosine is the most effective among several carnosine analogs in reducing pMCAO-induced infarction volumes.15 Nevertheless, more thorough preclinical evaluation is needed for carnosine to meet the Stroke Therapeutic Academic Industry Roundtable guidelines and before clinical testing can take place.16

In the present study, we determined: (1) short- and long-term neuroprotective efficacy of intravenous carnosine in rats using focal ischemia models (permanent and transient); (2)

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the therapeutic time window; (3) the safety and tolerability of carnosine; (4) the effect of carnosine on the thrombolytic activity of tPA; and (5) the influence of carnosine on several specific deleterious ischemia-induced mechanisms. Taken together, our findings provide strong support for the development of carnosine as a therapeutic agent for stroke.

Materials and Methods
More details are provided in the online-only Data Supplement.

Animals
Adult male Sprague-Dawley rats (250–300 g; Harlan) were used after approval from Institutional Animal Care and Use Committee at Michigan State University.

Blinding and Randomization
Treatment groups were allocated in a randomized fashion. Investigators were blind to the allocation.

Carnosine Treatments
Carnosine (Sigma) was dissolved in saline and administered intravenously.

Permanent or Transient Middle Cerebral Artery Occlusion
Permanent and transient focal cerebral ischemia was induced by placing and advancing a silicone-coated intraluminal filament (Doccol Co) in the carotid artery to occlude the middle cerebral artery. The filament was left in place for the permanent ischemia model. For the transient model, reperfusion was produced by withdrawal of the monofilament 3 hours after occlusion. In experiments measuring the therapeutic time window, the filaments were removed 6 hours or 9 hours after onset of ischemia.

Calculation of Infarct Volume by Triphenyl tetrazolium Chloride Staining
At 24 hours after onset of ischemia, rats were euthanized by isoflurane overdose, decapitated, and the brains were rapidly removed. The infarct volume was determined with correction for edema using triphenyl tetrazolium chloride-stained brain slices.

Assessment of Neurological Function
Neurological deficit was evaluated by an 18-point-based scale, adhesive tape removal testing, and accelerated rotaror testing.

Quantification of Brain Damage With Nissl Staining
The serial coronal sections (40 μm) were cut from the frozen brains and stained with cresyl violet. The infarct volume was estimated as the product of the sum of the lesion areas and the distance between sections.

Animal Handling for Safety Assessment
Rats were allowed to survive for 14 days after carnosine treatments for safety assessment.

Histopathologic Evaluation of Organ Toxicity
Histopathologic evaluation was performed on heart, lung, liver, kidney, brain, and bone marrow from 4 randomly selected animals from the group of saline or carnosine using Hematoxylin and Eosin staining.

Assessment of Complete Blood Count (CBC)/ Coagulation and Serum Chemistry
Four randomly chosen rats from saline- or carnosine-treated group underwent the tests for CBC/coagulation and serum chemistry profiles at 14 days after carnosine administration.

Measurement of Clot Lysis
The effect of carnosine on the fibrinolytic activity of tPA was examined using spectrophotometry.

Primary Neurons/Astrocytes Culture and Determination of Cytotoxicity
Primary cortical neuronal and astrocytic cultures were established as previously described, and used for experiments intravenously on days 7 to 11 and 14, respectively.

Measurement of Reactive Oxygen Species or Mitochondrial Membrane Potential Transition
The intracellular reactive oxygen species levels or the mitochondrial membrane potential transition were evaluated in fluorescence microplate reader using dichlorofluorescein diacetate, acetyl ester (H2DCFDA) or JC-1, respectively.

Brain Mitochondrial Isolation and Mitochondrial Respiratory Activity Measurement
Brain mitochondria was isolated from rats after pMCAO, and the respiratory activity was measured using respiratory control ratio using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, United Kingdom).

Sample Size Estimates
The number of rats to be used per group was determined using a series of power calculations using commercially available software (Janet D. Elashoff, nQuery Advisor Version 2.0, Los Angeles, CA).

Statistics
Statistical analysis was performed using SPSS software (Chicago, IL) as described in online-only Data supplement. In all cases, a probability value of <0.05 was considered significant.

Results
Improvement of Histological and Functional Outcomes in Rat Transient Focal Ischemia
To examine the neuroprotective effect of carnosine, focal ischemic stroke was induced in rats using the intraluminal monofilament technique. No significant differences among the experimental groups were detected in physiological variables of body weight, rectal temperature, and cerebral blood flow before and after ischemia (Supplemental Table 1). Blockade and subsequent restoration of cerebral blood flow was confirmed by laser Doppler. Carnosine did not induce any significant change in cerebral blood flow (Figure 1A).

Carnosine was administered intravenously at 3 hours after ischemia, and the monofilament was removed to allow reperfusion. Along with the functional improvement (Figure 1B), treatment with carnosine (500–2000 mg/kg) significantly decreased brain damage in a dose-dependent manner (Figure 1C). Carnosine treatment significantly decreased infarct volume by 41.9% (P=0.004) and 49.1% (P=0.002) at 1000 mg/kg and 2000 mg/kg dose, respectively.

Therapeutic Time Window
To determine the therapeutic time window, a single dose of carnosine was administered intravenously at increasing time intervals after ischemic stroke. Protective effect
of carnosine was significant up to 6 hours in the transient model (Figure 2A), showing decrease of infarct volume by 41.9% ($P=0.004$), 39.4% ($P=0.024$), and 13.4% ($P=0.882$) for 3 hours, 6 hours, and 9 hours, respectively. The protective effect of carnosine was greater in the permanent ischemic model, where the time window was found to be 9 hours (Figure 2B). Carnosine reduced brain infarct by 57.1% ($P<0.001$), 41.2% ($P=0.006$), 30.7% ($P=0.011$), and 8.5% ($P=0.500$) for 3, 6, 9, and 12 hours post treatment after pMCAO, respectively.

**Influence of Carnosine on Mortality in Permanent and Transient Ischemia**

Stroke is associated with significant mortality. tPA improves disability but not mortality. Our data show that carnosine is not only cerebroprotective but also it improved mortality in both transient and permanent models. Mortality in the tMCAO model was 6.7% versus 0% after 3 hours tMCAO, 13.3% versus 6.7% after 6 hours tMCAO, and 65% versus 35% after 9 hours tMCAO in saline-treated versus carnosine-treated rats, respectively. The decreased mortality in carnosine-treated rats was also observed in the permanent model over all the time-points tested (Supplemental Table 2).

**Extended Benefit of Carnosine in Rat Transient Focal Ischemia**

The neuroprotective effects of carnosine were apparent even 14 days after stroke onset. Rats received saline or carnosine (1000 mg/kg, IV) at 3 hours after ischemia, and reperfusion was initiated immediately after administration of carnosine. On day 14 postischemia, histological brain damage was evaluated by Nissl staining. The brain sections from saline-treated rats exhibited a consistent necrotic lesion both in cortical and subcortical regions of ipsilateral hemisphere (Figure 3A, left). Infarct volumes were significantly decreased by carnosine treatment by 30.5% (Figure 3A, right; $P=0.045$).

**Functional Outcomes**

We also examined whether treatment with carnosine influenced functional outcome. The adhesive tape removal test and the accelerated rotarod tests were used to assess responses/asymmetries and motor coordination/balance, respectively. Rats treated with carnosine showed a significant improvement in the adhesive tape removal test: 38.2% ($P=0.031$) on day 7 and by 44.9% ($P=0.029$) on day 14 after ischemia (Figure 3B). Significant differences between saline- and carnosine-treated rats were also observed on days 1 and 3 after ischemic stroke using the rotarod test (Figure 3C); 54.1% ($P=0.006$) and 71.8% ($P=0.018$), respectively. Differences were not statistically significant for the rotarod test on days 7 and 14. Similarly, improvement in neurological scores was significant at all-time points observed through the14-day survival periods (Figure 3D; $P<0.01$ for all time-points).

**Assessment of Safety and Tolerability of Carnosine**

Next we examined the safety and tolerability of carnosine in rats. Based on the Food and Drug Administration guidelines on preclinical acute toxicity studies, daily assessments for systemic signs of toxicity were performed. Body weight, food consumption, activity, and mortality were evaluated for 14 days after single intravenous carnosine treatment (100, 500, 1000, and 2000 mg/kg). No significant differences were found.
between control (saline-treated) and carnosine-treated groups both in body weight change (Figure 4A) and the amount of food consumption (Figure 4B). No rats died in the control group or carnosine-treated groups.

To examine organ-specific toxicity, histopathologic evaluations were performed on bone marrow, cerebellum, cerebrum, brain stem, hippocampus, heart, lung, liver, and kidney in randomly selected animals. Carnosine (2000 mg/kg)
did not induce signs of toxicity in any of the examined organs (Figure 4C).

Effects of Carnosine on Coagulation, CBC, and Serum Chemistry
The effect of carnosine on coagulation, CBC, and serum chemistry were also examined. Fourteen days after treatment with saline or carnosine (2000 mg/kg), rats were euthanized and blood was collected for analysis. No abnormalities were noted in any of the blood variables examined (Supplemental Table 3–5).

Effect of Carnosine on Fibrinolytic Activity of tPA
Next, we determined whether carnosine had any effect on clot lysis by tPA, because coadministration of tPA and carnosine may occur in a future clinical trial. Clots were generated from healthy volunteers. Exogenously added tPA significantly increased clot lysis, and plasminogen activator inhibitor significantly attenuated clot lysis by tPA, demonstrating the validity of the ex vivo assay. Carnosine itself had no apparent clot lysis activity (Figure 5A). Significant clot lysis was obtained with tPA, achieving 50.7±0.8% total clot lysis after 120 minutes. Cotreatment with carnosine (10, 20, and 30 μg/mL) did not affect the thrombolytic activity of tPA (Figure 5B).

Mechanisms Underlying the Protective Effect of Carnosine
To address how carnosine mediates cerebroprotective effects, we used primary cultures of cortical neurons and astrocytes, the major cell types impaired during ischemia. Carnosine reduced neuronal cell death induced by in vitro ischemic insults of oxygen-glucose deprivation by 48.4% or an excitotoxic stimulus of N-methyl d-aspartate by 40.8% (Figure 6A). Protective effects against oxygen-glucose deprivation-induced injury were also observed in primary astrocytes (Figure 6A). In both models, carnosine decreased reactive oxygen species generation, supporting its role as an antioxidant (Figure 6B). Transition of mitochondrial membrane potential was protected by carnosine in cortical neurons as well as in astrocytes (Figure 6C), suggesting that carnosine decreased mitochondrial damage. Moreover, in brain homogenates isolated after focal ischemia (pMCAO), mitochondrial respiratory damage in ipsilateral hemisphere was significantly recovered by carnosine treatment (1000 mg/kg, 6-hour post treatment, Figure 6D).

Discussion
To date, numerous neuroprotective agents have been effective in animal studies, but every agent has failed in clinical trials. Previous therapeutic strategies have targeted single pathways but stroke involves many different deleterious processes that eventually lead to cellular injury and cell death. Another point of concern has been the poor quality of animal studies with inadequate randomization, blinding, and appropriate statistical power. Many studies evaluated only acute histological end points, whereas clinically stroke recovery is determined by functional capacity at delayed time-points. Consequently, guidelines by representatives from academia and industry (Stroke Therapeutic Academic Industry Roundtable) have developed to improve the quality of preclinical studies. Although this study follows Stroke Therapeutic Academic Industry Roundtable recommendations, additional studies of carnosine in females, older animals, and animals with comorbidities are still needed to fully satisfy the recommendations.

The primary goal of this study was to determine preclinical efficacy, therapeutic time window, and safety of carnosine as a cerebroprotective therapy in stroke. We chose carnosine, an endogenous dipeptide, because of its beneficial pleiotropic effects on deleterious mechanisms that contribute to cell death during ischemia. We used intravenous dosing because in a future clinical trial carnosine would be administered intravenously. Our data show that carnosine is highly efficacious in protecting against brain damage when administered intravenously and is safe and well tolerated at doses up to 2000 mg/kg in rats. Importantly, we demonstrate that carnosine is protective against both transient and permanent focal ischemia models. Testing in both models is important because, although permanent occlusion is more common in human patients, recanalization rates of about 30% have been reported. Many previous neuroprotective candidates reported efficacy in only 1 model.

Another remarkable finding of our study is the wide and clinically useful therapeutic time window. A critical issue in
Stroke treatment is that many patients arrive in hospital several hours after their stroke onset. We observed significant protection even when carnosine therapy was initiated 6 hours after the onset of tMCAO or 9 hours after pMCAO (Figure 2). The benefits of tPA have been limited primarily because of its narrow therapeutic time window of 4.5 hours. Many agents that have failed in clinical testing have short preclinical therapeutic time windows but were tested clinically with longer inclusion time windows. This broad window of cerebroprotective efficacy demonstrated in this study makes carnosine an attractive therapeutic candidate.

In most preclinical studies of cerebroprotective agents, efficacy was evaluated by reductions in histologically-determined infarction volumes. However, cerebroprotective efficacy is measured by neurological function in clinical trials. Infarction volumes correlate poorly with functional outcome because small lesions in critical brain areas can result in major functional deficits, whereas large lesions in silent areas can cause little detectable dysfunction. We demonstrated that carnosine showed protective effects both histologically and functionally. We used several functional tests as each functional test represents a specific damage of somatosensory, motor-ambulatory, and fine motor/tactile function. Carnosine improved deficits in all functional tests (Figure 3B–3D), and these protective effects persisted through the 14 day-survival period.

Because several neuroprotective candidates were withdrawn from clinical testing owing to their adverse effects, preclinical assessments of the safety and tolerability of a putative neuroprotective agent represent a critical translational step in moving a therapeutic from animals to humans. We observed the effect of carnosine on CBC, serum biochemistry, and coagulation. In addition, histopathologic evaluations of several organs were performed to evaluate organ-specific adverse effects; however, in all analyses, no adverse effects were seen (Supplemental Table 2–4 and Figure 4). Moreover, we tested the effect of carnosine on the ability of tPA to thrombolyse clot because carnosine may be coadministered with tPA in a future trial, and found that carnosine did not influence the thrombolytic function of tPA (Figure 5). Future studies are planned that will test carnosine with tPA in a focal ischemia model. These studies will also determine the influence of carnosine on edema formation and hemorrhagic transformation of infarction.

A significant strength of carnosine is its beneficial effects against brain damage resulting from ischemic stroke and is likely to be mediated through multiple mechanisms. The interruption of cerebral perfusion during stroke initiates a cascade of multiple detrimental events leading to cell death. These detrimental events include secondary inflammation, enhanced matrix metalloproteinase activity, excitotoxicity, apoptosis, free radical injury, and microglial activation. Previous neuroprotection strategies have focused on targeting single pathways. Recent studies have suggested that an ideal cerebroprotectant should favorably influence multiple pathways. Carnosine is reported to exhibit antioxidant, pH buffering, heavy metal chelating, antiexcitotoxic, and vasodilating effects in many cell types including neurons as well as under various disease states. Carnosine may enhance neurogenesis, which may also contribute to recovery after stroke. Here we demonstrated that carnosine reduced neuronal and astrocytic cell death against ischemia-like insults.

![Figure 6. Underlying mechanisms for carnosine neuroprotection.](image)

A to C, Primary cortical neurons or astrocytes were isolated from neonatal mice and were exposed to ischemic stimulus of N-methyl d-aspartate or oxygen-glucose deprivation (OGD). Cell death (A), generation of reactive oxygen species (B), and transition of mitochondrial membrane potential (C) were decreased by carnosine. *P<0.05 and **P<0.01 vs ischemic stimulus. PI, Propidium iodide; JC-1, 5,5',6,6'-tetrachloro-1',1',3,3'-tetraethylbenzimidazol-carbocyanine iodide. D, Effect of carnosine on respiratory control ratio was examined in ex vivo rat brain homogenates after permanent middle cerebral artery occlusion (pMCAO). *P<0.05 and **P<0.01. P/M indicates Pyruvate and malate; A, ADP; O, Oligomycin; A; C, CCCP; and S, Succinate. A: n=4, B: n=3, C: n=3, D: n=4. All values are means±SEM and analyzed by ANOVA tests or by Student t test. Representative tracings of oxygen consumption are shown.
such as oxygen-glucose deprivation and N-methyl d-aspartate (Figure 6A). Consistent with previous reports, carnosine showed antioxidant and mitochondrial-protecting activities in neuronal and astroglial cells. Of note, we documented that carnosine treatment at 6 hours after pMCAO rescued mitochondrial respiratory function, which is critical for cell survival during ischemic stroke.20,22 Future studies will evaluate functional improvement at longer intervals after ischemia onset. Moreover, studies are planned in aged animals and animals that have comorbidities like hypertension and diabetes mellitus. Although we and others have shown that carnosine can penetrate the blood-brain barrier of rodents,15,40,44 detailed data on brain pharmacokinetics have not been generated.

In summary, using both histological and functional outcome metrics, we documented that intravenous carnosine confers enduring brain protection against focal ischemic stroke. Carnosine was safe and well tolerated at efficacious doses and has a wide, clinically useful therapeutic time window. Our findings support the therapeutic potential of carnosine for ischemic stroke and will be used to plan further preclinical and clinical testing to fully satisfy Stroke Therapeutic Academic Industry Roundtable before clinical testing.

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Disclosures None.

References


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SUPPLEMENTAL MATERIAL

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Materials and Methods

**Animals** All experiments were conducted using adult male Sprague-Dawley rats weighing 250 to 300 g (Harlan) and performed in accordance with the NIH Policy and Animal Welfare Act under the approval by Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

**Blinding and Randomization** Treatment groups were allocated in a randomized fashion using a Researcher Randomizer Program (http://www.randomizer.org/). Investigators were blind to the allocation of treatment during surgeries and outcome evaluations.

**Carnosine treatments** Carnosine was obtained from Sigma and dissolved in saline. For safety/toxicity assessment, saline or carnosine (100, 500, 1000, or 2000 mg/kg BW) were administered over 5 min into an indwelling intravenous catheter in the left femoral vein. The lateral tail vein was used for the administration for the focal ischemia models.

**Permanent or transient middle cerebral artery occlusion (MCAO)** After induction of general anesthesia by isoflurane inhalation, rats were maintained under anesthesia through the surgical period. Rectal temperature was maintained at 37°C and the cerebral blood flow (CBF) was measured with laser Doppler (Perimed, North Royalton, OH). Permanent or transient focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO). Briefly, the left common carotid artery (CCA) and the external carotid artery (ECA) was exposed and ligated by a suture. The occipital artery of the ECA was coagulated. The internal carotid artery (ICA) was exposed and the pterygopalatine artery was ligated. Ischemia was produced by advancing a silicone-coated 4-0 monofilament nylon suture (Doccol Co.). The monofilament was inserted into the CCA and advanced into the ICA to the origin of the MCA (18.0 mm from the bifurcation). The filament was left in place for the permanent ischemia model. For the transient model, reperfusion was produced by
withdrawal of the monofilament 3 hrs after occlusion. In experiments measuring the therapeutic time window, the filaments were removed 6 hr- or 9 hr after onset of ischemia. Rats were excluded from the study when the CBF was not decreased below 30% of baseline after occlusion, or CBF was not restored above 80% of basal CBF after reperfusion. Sham animals underwent the same anesthesia and surgical procedure besides the intraluminal monofilament insertion.

**Calculation of infarct volume by TTC staining** Rats were euthanized by isoflurane overdose, decapitated, and the brains were rapidly removed. Brains were cut into 2 mm sections, stained with 2% triphenyltetrazolium chloride (TTC), and fixed in 4% paraformaldehyde. Each section was scanned to a digital image, and analyzed using the NIH ImageJ software. The infarct volume for each section was determined and edema correction was performed by the measurement of the infarcted and control hemisphere.

**Assessment of neurological function** Neurological functional deficit was evaluated by functional tests at 1, 3, 7 and 14 d after tMCAO. The 18-point-based scale includes the following six tests (maximum of 3 points per test): spontaneous activity, symmetry of movements, symmetry of forelimbs, climbing, reaction to touch on either side of trunk, and response to vibrissae touch. Final scoring was obtained by summing the scores recorded in each individual test. Maximum score was 18.

For the adhesive tape removal test, somatosensory deficit was measured both before and after surgery. All rats were trained for the tests for 5 consecutive days before focal ischemia. Small pieces of adhesive were used as bilateral tactile stimuli on the wrist of each forelimb. The time to remove each stimulus from forelimbs was recorded on 3 trials per day. (Maximum time allowed to remove the tape was 180 sec). The mean values from the left and right forelimb times were used for statistical analysis. Motor–ambulatory function was assessed with a rotorod test, a well-established procedure for testing the balance and
coordination aspects of motor performances in rats and mice. Rats were placed on the rotorod and then tested with acceleration speeds. Latencies to fall off or the 395 sec cutoff were recorded for analysis with 3 trials per day. Rats were trained 3 consecutive days before ischemia.

**Quantification of brain damage with Nissl staining** Fourteen days after ischemia, rats were perfused with 4% paraformaldehyde and decapitated. The brains were stored in situ in 4% paraformaldehyde for 24 hr, cryoprotected in 30% sucrose/PBS and frozen. To assess infarct localization and volume, serial coronal sections (40 μm) were cut from the frozen brains at 24 equidistant planes (500 μm apart, covering the entire forebrain). These sections were stained with cresyl violet, converted to digital file by scanning and analyzed using Image J. The infarct volume was estimated as the product of the sum of the lesion areas and the distance between sections. Analyses were conducted by an investigator who was blind to the experimental treatment of the animals.

**Animal handling for safety assessment** A PE-50 catheter was inserted into the femoral vein of anesthetized rats. The femoral vein catheter was tunneled subcutaneously to the posterior cervical region. The catheter was held in place by a shoulder harness during the drug administration. Saline or carnosine (100, 500, 1000, or 2000 mg/kg BW) was administered over 5 minutes into an indwelling intravenous catheter. The harness and catheter were removed after 24 hr after treatment and the incision was closed. Rats were allowed to survive for 14 days for the daily assessment of food consumption, body weight, activity and mortality. After 14 days, rats were euthanized, and the organs or blood was rapidly removed for toxicity assessment.

**Histopathological evaluation of organ toxicity** Histopathological evaluation was performed on heart, lung, liver, kidney, brain (cerebrum, cerebellum, hippocampus, brain stem), and bone marrow from four randomly selected animals from the group of saline, mid-
and high-dose of carnosine (500 and 2000 mg/kg BW). Organs were rapidly harvested and fixed in 10% formalin. After staining with Hematoxylin and Eosin (HE), organ sections were visibly examined by Diagnostic Center for Population and Animal Health at Michigan State University.

**Assessment of complete blood count/ coagulation and serum chemistry** Four randomly chosen rats from saline- or carnosine (2000 mg/kg)-treated group underwent the tests for complete blood count/coagulation and serum chemistry profiles at 14 days after carnosine administration. The analysis was completed in Clinical Pathology, Diagnostic Center for Population and Animal Health at MSU. Four weight and age matched rats without any surgical procedure or drug treatment were used to assess normal physiologic values.

**Measurement of clot lysis** The fibrinolytic activity of tPA and/or carnosine was examined according to our previous report using spectrophotometry. Recombinant tPA was obtained in single-chain form from Calbiochem. Briefly, the dye Coomassie Brilliant Blue R-250 was bound to proteins in diluted plasma from human donors, and clots were formed by adding thrombin. The clots were washed, fresh diluted plasma was added, and the amount of lysis in the presence of various agents was measured by increasing absorbance at 540 nm due to the release of dye-bound proteins from the clot. Measurements were taken every 20 minutes for 3 hours. Experiments were performed in blood from 5 healthy volunteers.

**Primary neurons/astrocytes culture** Primary cortical neuronal and astrocytic cultures were established using enzymatic dissociation with papain as previously described with slight modification (Yim et al., 2010; Danilov et al., 2009; Pastor et al., 2009). Cell culture media and reagents (Neurobasal A, B27, DMEM, glutamine, and penicillin/streptomycin) were obtained from Invitrogen (Carlsbad, CA). For cortical neurons, cerebral cortices were isolated from C57BL/6 newborn mice at postnatal day 0 and dissociated in dissection media (81.8 mM Na₂SO₄, 30 mM K₂SO₄, 5.8 mM MgCl₂, 0.252
mM CaCl₂, 1.5 mM HEPES, 20 mM glucose, and 0.001% phenol red, pH 7.6) supplemented with 4 mM L-cysteine, 10 U/ml papain (Worthington), and 1000 U/ml DNase (Roche) for 30 min at 37°C. After dissociation, cells were washed with Neurobasal A and then triturated with pipette. 1×10⁶ cells were plated onto poly-D-lysine-precoated 12 well plates. Three days after plating, 50% of the medium was changed, and subsequently replaced every 3 days with Neurobasal A supplemented with 2% B27. Neuronal cultures were maintained in CO₂ incubator (5% CO₂/ 95% air balance) at 37°C, and used between days in vitro (DIV) 7 and 11. These cultures contained >90% neurons as revealed by NeuN/ beta tubulin-immunohistochemistry. Primary cultures of astrocytes were prepared using the same dissociation protocol described for neuronal cultures. Cerebral cortices were isolated from 1 day-old C57BL/6J mice and dissociated and digested using papain. Cells were plated at a density of 1.5×10⁷ cells/flask in 75 cm² flasks coated with poly-D-lysine using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Seven days after plating, the flasks were shaken for 4 h at 37 °C to dislodge contaminated cells such as oligodendroglial and microglial cells that were loosely attached to the astrocyte monolayer. On DIV 14, astrocytes were detached by trypsinization and plated on poly-D-lysine precoated wells. Astrocytes were used between DIV 18-21, when they reach maximal sensitivity to OGD-induced cytotoxicity (Danilov et al., 2009). These cultures contained >90% astrocytes as revealed by immunohistochemistry against glial fibrillary acidic protein (GFAP).

**Determination of cytotoxicity in neurons/astrocytes**  Protective effect of carnosine against neuronal cytotoxicity was measured after NMDA- or OGD stimulation. Primary neuronal cells were treated with NMDA-containing media and incubated at 37°C for 20 min on DIV 9. Exposure to NMDA was terminated by replacement with the original media collected before NMDA treatment. NMDA-induced cytotoxicity was measured at 24 hour
after NMDA exposure using propidium iodide (PI)-staining or LDH assay. Briefly, cultured neurons were stained with 5 μg/mL PI at 37°C for 30 min, and examined by fluorescent microscopy (Nikon) or fluorescent microplate reader (Ascent, Thermo Lab systems, Franklin, MA). To detect the loss of membrane integrity, a typical marker of cytotoxicity, the extent of lactate dehydrogenase (LDH) release was measured in conditioned media using the Cytotox 96R Non-Radioactive Cytotoxicity Assay (Promega Corporation). Cell viability in sister cells treated with 100 μM NMDA was used to induce near complete neuronal death and used the total cell death (100%).

Oxygen-glucose deprivation (OGD) on primary cortical neurons or primary astrocytes was performed to simulate in vivo hypoxic condition. On DIV 9, the medium in neuronal culture was replaced by glucose-free Earle’s balanced salt solution (EBSS), and the cells were placed in an anaerobic chamber (Billups-Rothenberg Inc, Del Mar, CA) saturated with 5% CO2 and 95% N2 for 2 hr. Control cells were incubated in EBSS with glucose in a normoxic incubator for the same period. OGD was terminated by switching back to normal culture conditions. OGD on astrocytes was performed with the same procedure, except the cells were used on DIV 18-21 and OGD was applied for 6 hr. Cytotoxicity was determined at 24 hour after OGD using PI-staining or LDH assay as described above.

**Reactive oxygen species (ROS) measurement** The intracellular neuronal ROS levels were evaluated using Dichlorofluorescein diacetate, acetyl ester (H2DCFDA) in fluorescence microplate reader. Neurons or astrocytes were pretreated with carnosine for 30 min and exposed to lethal stimuli of NMDA or OGD, respectively. Cells were incubated with DCF (1 μM, Molecular Probes) for 30 min at 37°C in dark. After DCF staining, cells were washed with PBS twice and counterstained with DAPI. ROS level was determined by DCF fluorescence normalized by DAPI.

**Detection of mitochondrial membrane potential transition** Mitochondrial
membrane potential transition was examined in a fluorescence microplate reader using JC-1. Neurons or astrocytes were pretreated with carnosine for 30 min and exposed to lethal stimuli of NMDA or OGD, respectively. Cells were incubated with mitochondria specific fluorescent dye JC-1 (2 μM, Molecular Probes) for 30 min at 37°C in dark. Red fluorescence from JC-1 aggregates in normal mitochondria is detected, while green fluorescence from monomer can appear in damaged mitochondria. After JC-1 staining, cells were washed with PBS twice. Mitochondrial membrane potential transition was determined by JC-1 green fluorescence.

**Brain mitochondrial isolation** Brains were rapidly removed at 24 hours after MCAO. Whole ipsilateral ischemic and contralateral nonischemic hemispheres were dissected out and placed in homogenizer containing isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH adjusted to 7.2 with KOH). The tissue was homogenized, and 30% Percoll in isolation buffer was added. The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll solution, followed by a 24% Percoll solution, and finally the sample in a 15% Percoll solution. The density gradients were spun in a Sorvall RC-5C plus superspeed refrigerated centrifuge (Asheville, NC) in a fixed angle SE-12 rotor at 30,400g for 10 minutes. Following centrifugation, band 3 (nonsynaptic mitochondria) were separately removed from the density gradient. The final mitochondrial pellet was resuspended in isolation buffer without EGTA and protein concentration was determined using the BCA protein assay (Thermo Scientific, Rockford, IL).

**Measurement of mitochondrial respiratory activity** The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK). Approximately 100 μg protein/ml of isolated nonsynaptic or synaptic mitochondria were suspended in a sealed, constantly stirred, and thermostatically...
controlled chamber at 37 °C in KCl respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM MgCl₂, 2.5 mM KH₂PO₄, pH 7.2). The rate of oxygen consumption was calculated based on the slope of the response of isolated mitochondria to the successive administration of oxidative substrates (5 mM pyruvate and 2.5 mM malate): 150 μM ADP added twice in 1 minute intervals; 1 μM oligomycin; 1 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazine); and finally 1 mM succinate. The respiratory control ratio (RCR) was determined by dividing the rate of oxygen consumption/min for state III (in the presence of ADP, second addition) by state IV (in the absence of ADP and presence of oligomycin). The states of mitochondrial respiration were also calculated in KCl respiration buffer as nmol of oxygen consumed/mg of protein.

**Sample Size Estimates** We conducted a series of power calculations using commercially available software (Janet D. Elashoff, nQuery Advisor Version 2.0, Los Angeles, CA) to determine the number of rats to be used per group. Previous work in our lab as well as other published data using this model of ischemia has shown that the average infarct volume for untreated rats is about 150 mm³ with a standard deviation of 40 mm³. A 30% reduction in infarct size by carnosine would give an average infarct volume of 100 mm³. Assuming a common standard deviation of 40 mm³, the effect size for carnosine treatment would therefore be (150-100)/40 = 1.25. Using a 2 group t-test comparison it can be shown that to identify this effect size with a power of 80% and standard alpha of 0.05, the number of rats required per group would be 12.

**Statistics** We calculated the means and standard errors of means (SEM) for all treatment groups. The data were subjected to student t-test or one-way ANOVA followed by Duncan’s test to determine the significant differences between treatment groups. Statistical analysis was performed using SPSS software (Chicago, IL). In all cases, a p value of <0.05 was considered significant.
Supplemental Table S1. Physiological variables in rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Control</th>
<th>Carnosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>285.0 ± 9.75</td>
<td>286.3 ± 3.47</td>
<td>279.5 ± 3.12</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.2 ± 0.16</td>
<td>37.3 ± 0.09</td>
<td>37.3 ± 0.08</td>
</tr>
<tr>
<td>CBF before MCAO</td>
<td>316.8 ± 5.72</td>
<td>309.3 ± 4.72</td>
<td>314.4 ± 3.52</td>
</tr>
<tr>
<td>CBF after MCAO</td>
<td>306.6 ± 3.59</td>
<td>52.9 ± 3.08</td>
<td>55.0 ± 3.06</td>
</tr>
<tr>
<td>CBF before reperfusion</td>
<td>-</td>
<td>56.8 ± 2.86</td>
<td>61.8 ± 3.17</td>
</tr>
<tr>
<td>CBF after reperfusion</td>
<td>-</td>
<td>182.8 ± 4.40</td>
<td>182.9 ± 5.95</td>
</tr>
<tr>
<td>CBF at 15 min after reperfusion</td>
<td>-</td>
<td>307.9 ± 3.99</td>
<td>317.1 ± 4.95</td>
</tr>
<tr>
<td>Body weight at 14 d after MCAO/reperfusion (g)</td>
<td>333.0 ± 7.52</td>
<td>299.7 ± 8.32</td>
<td>299.7 ± 8.32</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are means ± SEM. MCAO, middle cerebral artery occlusion; CBF, cerebral blood flow.

MCA was not occluded in Sham rats. Other surgical procedures were same besides MCAO.
## Supplemental Table S2. Mortality

<table>
<thead>
<tr>
<th>Figure number</th>
<th>Injection time</th>
<th>model</th>
<th>Mortality (%)</th>
<th>Mortality (%)</th>
<th>Mortality (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>vehicle</td>
<td>Carnosine (mg/kg)</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>1B,C</td>
<td>3 hr after</td>
<td>tMCAO</td>
<td>6.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2A</td>
<td>6 hr after</td>
<td>tMCAO</td>
<td>13.3</td>
<td>-</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>2A</td>
<td>9 hr after</td>
<td>tMCAO</td>
<td>65.0</td>
<td>-</td>
<td>35.0</td>
<td>-</td>
</tr>
<tr>
<td>2B</td>
<td>3 hr after</td>
<td>pMCAO</td>
<td>6.7</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>2B</td>
<td>6 hr after</td>
<td>pMCAO</td>
<td>13.3</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>2B</td>
<td>9 hr after</td>
<td>pMCAO</td>
<td>6.7</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>2B</td>
<td>12 hr after</td>
<td>pMCAO</td>
<td>13.3</td>
<td>-</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3 hr after</td>
<td>tMCAO</td>
<td>15.0</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>FVC*</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* FVC: Femoral Vein Cannulation
**Supplemental Table S3. Complete blood count in rats at 14 days after treatment**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control</th>
<th>Carnosine (2000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tot Protein (g/dL)</td>
<td>5.9 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>RBC (x 10^6/μL)</td>
<td>7.2 ± 0.3</td>
<td>6.7 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>Hgb (g/dL)</td>
<td>13.9 ± 0.3</td>
<td>13.4 ± 0.3</td>
<td>13.7 ± 0.1</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>39.8 ± 1.3</td>
<td>38.4 ± 0.8</td>
<td>39.8 ± 0.5</td>
</tr>
<tr>
<td>HCT Spun (%)</td>
<td>40.3 ± 0.6</td>
<td>39.8 ± 0.5</td>
<td>41.5 ± 0.6</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>55.2 ± 0.3</td>
<td>56.9 ± 0.9</td>
<td>57.4 ± 0.7</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.3 ± 0.3</td>
<td>19.8 ± 0.3</td>
<td>19.8 ± 0.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>34.9 ± 0.4</td>
<td>34.8 ± 0.2</td>
<td>34.5 ± 0.3</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>35.3 ± 0.1</td>
<td>34.5 ± 0.1</td>
<td>34.2 ± 0.3</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11.7 ± 0.0</td>
<td>12.1 ± 0.2</td>
<td>12.3 ± 0.1</td>
</tr>
<tr>
<td>HDW (g/dL)</td>
<td>2.8 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Platelet (x 10^3/μL)</td>
<td>858.3 ± 39.6</td>
<td>812.3 ± 30.3</td>
<td>747.0 ± 42.5</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>7.4 ± 0.2</td>
<td>7.5 ± 0.1</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>WBC (x 10^3/μL)</td>
<td>7.1 ± 0.4</td>
<td>7.7 ± 0.6</td>
<td>8.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. RBC, red blood cell; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; CHCM, cell hemoglobin concentration; RDW, red blood cell distribution width; HDW, hemoglobin distribution width; MPV, Mean platelet volume; WBC, white blood cell; MCAO, middle cerebral artery occlusion; CBF, cerebral blood flow.
### Supplemental Table S4. Coagulation tests in rat serums at 14 days after treatment

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control</th>
<th>Carnosine (2000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (sec)</td>
<td>16.1 ± 0.2</td>
<td>16.9 ± 0.3</td>
<td>17.0 ± 0.1</td>
</tr>
<tr>
<td>aPTT (sec)</td>
<td>13.3 ± 0.5</td>
<td>14.1 ± 0.4</td>
<td>14.3 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. PT, prothrombin time; aPTT, activated partial thromboplastin time.
Supplemental Table S5. Serum chemistry in rats at 14 days after treatment

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control</th>
<th>Carnosine (2000 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mmol/L)</td>
<td>140.5 ± 1.0</td>
<td>142.5 ± 1.6</td>
<td>141.8 ± 0.3</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5.3 ± 0.1</td>
<td>5.6 ± 0.0</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>102.3 ± 1.2</td>
<td>100.8 ± 0.6</td>
<td>101.3 ± 1.1</td>
</tr>
<tr>
<td>TCO₂ (mmol/L)</td>
<td>27.5 ± 0.6</td>
<td>27.0 ± 0.7</td>
<td>27.0 ± 0.4</td>
</tr>
<tr>
<td>Na/K</td>
<td>26.4 ± 0.7</td>
<td>25.7 ± 0.3</td>
<td>25.6 ± 0.7</td>
</tr>
<tr>
<td>Anion Gap (mmol/L)</td>
<td>16.1 ± 1.1</td>
<td>20.3 ± 1.8</td>
<td>19.1 ± 1.3</td>
</tr>
<tr>
<td>Osmolarity (mOs/L)</td>
<td>299.0 ± 2.0</td>
<td>303.0 ± 3.3</td>
<td>302.4 ± 0.9</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.6 ± 0.1</td>
<td>9.7 ± 0.1</td>
<td>9.6 ± 0.0</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>7.7 ± 0.2</td>
<td>8.7 ± 0.2</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td>Magnesium (mg/dL)</td>
<td>2.0 ± 0.0</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Iron (μg/dL)</td>
<td>218.5 ± 12.7</td>
<td>167.0 ± 30.4</td>
<td>174.0 ± 9.9</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>179.3 ± 12.5</td>
<td>201.8 ± 26.1</td>
<td>224.8 ± 18.1</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>SDH (U/L)</td>
<td>6.8 ± 0.9</td>
<td>11.3 ± 1.8</td>
<td>8.4 ± 1.3</td>
</tr>
<tr>
<td>ALT(U/L)</td>
<td>44.5 ± 2.3</td>
<td>43.0 ± 4.1</td>
<td>47.5 ± 3.1</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>75.8 ± 5.9</td>
<td>76.8 ± 6.5</td>
<td>77.8 ± 4.0</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>5.4 ± 0.0</td>
<td>5.3 ± 0.1</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.8 ± 0.0</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.0</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.6 ± 0.0</td>
<td>2.7 ± 0.0</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>21.0 ± 0.9</td>
<td>21.5 ± 1.3</td>
<td>22.0 ± 0.4</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>Amylase (U/L)</td>
<td>577.8 ± 23.3</td>
<td>560.8 ± 44.6</td>
<td>576.5 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM 1</td>
<td>Mean ± SEM 2</td>
<td>Mean ± SEM 3</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>315.3 ± 34.0</td>
<td>267.0 ± 33.2</td>
<td>286.3 ± 29.1</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>189.8 ± 4.5</td>
<td>185.5 ± 9.6</td>
<td>198.3 ± 13.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>84.0 ± 4.0</td>
<td>89.0 ± 4.4</td>
<td>84.5 ± 7.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. ALP, alkaline phosphatase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase