Original Contributions

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. (Stroke. 2013;44:00-00.)

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.7,8 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 antiexcitotoxic 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)
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 Triphenyltetrazolium 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 rotorod 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 dichlorofluorescin 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 posts ischemia, 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 the 14-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.21 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 hemisphere22 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.23 Many reasons may account for this,1,24–26 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.28–30 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.7 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.28–30

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.
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.

Sources of Funding

This study was supported by the American Heart Association grant to Arshad Majid, MD and by Basic Science Research Program through the National Research Foundation of Korea (NRF) to Ok-Nam Bae (2012R1A1A3013240).

Disclosures

None.

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*Stroke.* published online December 18, 2012; 
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231 
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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