Title page

Neuroprotective effects of carnosine in a mice stroke model concerning oxidative stress and inflammatory response

Xinran Hu[1], Yusuke Fukui[1], Tian Feng[1], Zhihong Bian[1], Haibo Yu[1], Ryuta Morihara[1]

Xiao Hu[1], Yuting Bian[1], Hongming Sun[1], Mami Takemoto[1], Yumiko Nakano[1],

Taijun Yunoki[1], Koji Abe[1], Toru Yamashita[1]

1) Department of Neurology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-Ku, Okayama 700-8558, Japan

Corresponding author: Dr. Toru Yamashita, Department of Neurology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikatacho, Kitaku, Okayama 700-8558, Japan. Tel: +81-86-235-7365; Fax: +81-86-235-7368; E-mail: uumeko0326@gmail.com

Abbreviations used: AGEs, advanced glycation end products; ANOVA, analysis of variance; DAB, diaminobenzidine; Iba-1, ionized calcium binding adapter protein 1; IL-1β, interleukin-1beta; IL-10, interleukin-10; i.p., Intraperitoneal; I/R, ischemia / reperfusion; IS, ischemic stroke; MCA, middle cerebral artery; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RAGE, receptor for advanced glycation end products; SD, standard deviation; tMCAO, transient middle cerebral artery occlusion; tPA, tissue plasminogen activator; 4-HNE, 4-Hydroxynonenal; 8-OHdG, 8-hydroxydeoxyguanosine；ELISA, Enzyme-linked immuno-sorbent assay；Nrf2, NF-E2-related factor 2；Keap1, Kelch-like ECH-associated protein 1；ARE, Antioxidant Response Element；HSPs: heat shock proteins; HO-1, Haem oxygenase 1；iNOS, inducible nitric oxide synthase；NO，nitric oxide；RNS，reactive nitrogen species

Abstract

Carnosine (β-alanyl-L-histidine) is a natural dipeptide with multiple neuroprotective properties. Previous studies have advertised that carnosine scavenges free radicals and displays anti-inflammatory activity. However, the underlying mechanism and the efficacies of its pleiotropic effect on prevention remained obscure. In this study, we aimed to investigate the anti-oxidative, anti-inflammative, and anti-pyroptotic effects of carnosine in the transient middle cerebral artery occlusion (tMCAO) mouse model. After a daily pre-treatment of saline or carnosine (1000mg / kg / day) for 14 days, mice (n=24) were subjected to tMCAO for 60 min and continuously treated with saline or carnosine for additional 1 and 5 days after reperfusion. The administration of carnosine significantly decreased infarct volume 5 days after the tMCAO (*$p < 0.05$) and effectively suppressed the expression of 4-HNE, 8-OHdG, Nitrotyrosine 5 days, and RAGE 5 days after tMCAO. Moreover, the expression of IL-1β was also significantly suppressed 5 days after tMCAO. Our present findings demonstrated that carnosine effectively relieves oxidative stress caused by ischemic stroke and significantly attenuates neuroinflammatory responses related to IL-1β, suggesting that carnosine can be a promising therapeutic strategy for ischemic stroke.

Keywords: ischemic stroke; carnosine; middle cerebral artery occlusion; oxidative stress; inflammation; pyroptosis

1. Introduction

Stroke is the leading cause of severe long-term disability and reduces motility in more than half stroke survivors. Around 795,000 people continue to experience a new or recurrent stroke (ischemic or hemorrhagic) each year, about 87 % of all strokes are ischemic strokes (Tsao CW et al.,2022). Acute treatments for ischemic stroke are usually thrombectomy and thrombolysis combined tissue plasminogen activator (tPA). However, both therapies have a limitation, short therapeutic time windows of 4.5-6 hrs in most cases. In addition to the thrombectomy and thrombolysis, preventing another stroke is also important since having a stroke increases the risk of getting another one.

Many mechanisms are involved in neural damage during cerebral ischemia, such as excitotoxicity, oxidative/nitrosative stress, ionic imbalance, and apoptotic-like cell death (Lo et al., 2013). Carnosine is a natural, free radical scavenger that can interact with lipid peroxidation molecular products and superoxide anion and hydroxyl radicals (Boldyrev AA, 2012) and exhibits anti-inflammatory activity based on its anti-glycation properties (Nagai K, 1980; Shimada et al., 1999). Previous studies have shown that carnosine has multipotent neuroprotective components with various biological effects (such as antioxidant, free radical scavenging, and anti-glycosylation) in the tMCAO animal model as a candidate therapy for ischemic stroke (Lo et al., 2013; Bae et al., 2013; Rajanikant et al., 2013). However, the underlying mechanism and the efficacies of its pleiotropic effect on prevention remained obscure.

Therefore, in the present study, we investigated the anti-oxidative, anti-inflammatory, and anti-pyroptosis efficacies of carnosine as a protective agent in the

tMCAO mouse model.

2. Materials and Methods

2.1 Animals

In the present study, all experimental procedures were under the protocol approved by the Animal Committee of the Graduate School of Medicine and Density, Okayama University (OKU-2020480). Male C57BL/6J mice (7-8 weeks old, body weight: 22-27 g) were purchased from Japan SLC Inc. and maintained in the 12/12 hrs light-dark cycle room under the constant humidity and temperature of around 23 ℃ for one week. The mice were fed with standard pellets (MF; Oriental Yeast, Tokyo, Japan).

2.2 Experimental groups and administrate

For behavioral and immunohistochemical analysis, mice were randomly split into six groups as indicated in Figure 1: sham group 1 day after sham surgery (physiological saline, n=3), vehicle group 1 day after tMCAO (physiological saline, n=4), carnosine group 1 day after tMCAO (1000 mg/kg/day, Sigma-Aldrich Co. LLC, St. Louis, USA, n=4), sham group 5 days after sham surgery (physiological saline, n=6), vehicle group 5 days after tMCAO (physiological saline, n=8), and carnosine group 5 days after tMCAO (1000 mg/kg/day, n=8). Physiological saline or carnosine was administered by intraperitoneal (i.p.) injection from 8 to 9 weeks old. After 14 days of pre-treatment, mice in the vehicle and the carnosine groups were subjected to tMCAO surgery, and in the sham group were subjected to sham operation.

For quantitative analysis of antioxidative activities and oxidative stress, Extra mice (n=20) were purchased from Japan SLC Inc for quantitive analysis. All mice were randomly and equally separated into vehicle and carnosine groups. After 14 days of pre-treatment, mice in the vehicle and the carnosine groups were subjected to tMCAO surgery and sacrificed after 5 days. 8 of 20 mice were excluded due to the surgery problem and death.

2.3 Focal cerebral ischemia

As previously reported, focal cerebral ischemia was assessed in mice (Abe et al.,1992; Yamashita et al., 2017). In brief, mice were anesthetized with a nitrous oxide/oxygen/halothane (69%:30%:1%) by an inhalation mask. A 6-0 nylon filament thread coated with silicon and marked at 0.9 cm from the tip was through the right common carotid artery to occlude the right middle cerebral artery (MCA). After 60 min occlusion of tMCAO, the nylon thread was slowly pulled out to recover cerebral blood flow in the MCA. For the sham group, the sham operation involved surgical procedures without occlusion of the MCA. For successful MCA occlusion, a laser-doppler flowmeter (model ALF21; Advance, Tokyo, Japan) was used before and during the surgery. Measure the cerebral blood flow of the right frontoparietal cortical region. During the entire surgical procedure, a heating pad (BWT-100; Bio Research Center, Aichi, Japan) was used to check and maintain the body temperature at 37 ± 0.3 °C.

2.4 Behavioral analysis

Bodyweight measurement and behavioral tests were operated at 0 and 1 day after reperfusion in 1 day sacrificed groups and at 0, 1, and 5 days after reperfusion in 5 days sacrificed groups by an independent researcher blinded to the experimental groups. Corner test was administered to measure impairment of sensorimotor function (Zhang et al., 2002). In brief, mice were placed in the middle of cardboards at an angle of 30 degrees and waited for the mouse to walk deep and then turn its head. The mouse turns left or right, and due to an ischemic mouse preferentially turning toward the nonimpaired side, the number of rights sides was recorded. This test was repeated ten times on each mouse (Zhang et al., 2002). Based on our previous report, the mice were also detected by a rotarod test (MK-610A; Muromachi Kikai Co., Tokyo, Japan) (Yamashita et al., 2017). Mice were trained on the rod for three days before surgery to get used to the machine at first. Mice were put on a rod that was not rotated. The speed of the rod was slowly accelerated from 0 rpm to 45 rpm over 5 min. The mice were allowed a maximum of three trials to remain on the rotarod for 5 min for each test. The maximum time of three trials was recorded.

2.5 Tissue preparation and infarct size analysis

All mice were deeply anesthetized by i.p. injection of pentobarbital (40 mg/kg) and transcardially perfused with 20 ml of ice-cold phosphate-buffered saline (PBS) followed by 20 ml of ice-cold 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer. The brains were removed and post-fixed in 4% PFA over 24 hrs at 4 ºC. Floating coronal sections (50 μm thickness) were sliced with a vibrating blade microtome

(LEICA VT1000S; Leica, Germany). Six sections at a 0.6 mm interval each between 1.0 mm anterior and 2.0 mm posterior to the bregma were pasted onto a microscope glass slide and dried for two days to measure the infarct volume and stained with Cresyl Violet as Nissl staining. The infarct volume was calculated by counting pixels using image processing software (Adobe Photoshop 2021, mac version 23.1.1, Adobe Inc., U.S.A.) (Shi et al., 2018).

2.6 Quantitively analysis of Antioxidative activities and oxidative stress

Blood was collected from mice via cardiac puncture after deeply anesthetized by i.p. injection of pentobarbital. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for 30 minutes. Then remove the clot by centrifuging at 3000rpm for 10 minutes in a refrigerated centrifuge (S500FR; KUBOTA, Tokyo, Japan) and the resulting supernatant be collected. A protein sample was collected after being perfused with PBS. Tissue samples from the infarct cerebral hemisphere were cut and immediately put into the tissue protein extraction reagent (T-PER; 78510; Thermo Fisher Scientific, Massachusetts, U.S.A.) containing 1% phosphatase inhibitor (07574-61; Nacalai Tesque, Kyoto, Japan), and 1% protease inhibitor (25955-24; Nacalai Tesque). Then homogenized with BioMasher (9790A; TaKaRa, Tokyo, Japan). The homogenates were centrifuged at 12,500 rpm for 5 min at 4 °C and the supernatant was collected (CR21F; HITACHI, Tokyo, Japan).

To evaluate the antioxidant capacity and ROS in serum, OXY-adsorbent and d-ROMs tests were assayed with a spectrophotometer based on a previous report (Taira

et al., 2020). The OXY-adsorbent kit (OX191207; Wismerll, Tokyo, Japan) and the d-ROMs kit (DI-003b; Wismerll) were used in this experiment.

For quantitive analysis of the representative oxidation marker in the stroke mouse brain, tissues from mice were analyzed for HNE adducts using the OxiSelect HNE Adduct Competitive ELISA kit (STA-838; Cell Biolabs, California, U.S.A) according to the manufacturer's specifications.

2.7 Immunohistochemistry

For immunohistochemical staining, brain sections were immersed in 0.6% periodic acid to block intrinsic peroxidase and treated with a mixture of 5% bovine serum in 50 mM PBS and 0.1% Triton X-100 for 3 hrs to block any non-specific antibody expressions, then incubated at 4 ºC overnight with the primary antibody. We used the following primary antibodies: mouse anti-4-HNE antibody (1:50, MHN-020P; JaICA, Shizuoka, Japan), mouse anti-8-OHdG antibody (1:20, MOG-020P; JaICA), rabbit anti-Nitrotyrosine (1:500, 06-284; Millipore, Massachusetts, U.S.A.), rabbit anti-RAGE antibody (1:200, ab3611; Abcam, Cambridge, UK), rabbit anti-Iba-1 antibody (1:1000, 019-19741; Wako, Osaka, Japan), rabbit anti-IL-1β antibody (1:100, AF-401-NA; R&D Systems, MN, U.S.A.), rat anti-IL-10 antibody (1:100, ab33471; Abcam), rabbit anti-cleaved-Gasdemind-D antibody (1:200, 50928; Cell Signaling Technology, Massachusetts, U.S.A.), and anti-cleaved-caspase-1 antibody (1:100, AB_2554702; Thermo Fisher Scientific, Massachusetts, U.S.A.). The sections were washed in PBS

and biotinylated secondary antibodies corresponding to the respective hosts (1:500, Vector Laboratories; California, U.S.A.) for 2 hrs at room temperature. Then treated with avidin-biotin-peroxidase complex solution (1:100, PK-6104; Vector Laboratories) for 30 min and incubated with diaminobenzidine (DAB) standard dissolved in PBS. The treated sections were pasted on a glass slide, dried for 2 days, and visualized with a light microscope (BX-51; Olympus, Tokyo, Japan).

2.8 Semiquantitative analysis

For semiquantitative analysis, three sections per mouse were selected, and five random positions were taken in each peri-infarct area by a digital microscope (Olympus BX-51, Tokyo, Japan). Those fifteen pictures per mouse were analyzed in total. In 4-HNE, 8-OHdG, nitrotyrosine, RAGE, IL-1β, IL-10, and cleaved-caspase-1 staining, the positive cells number of each position were counted by image processing software (Image J, version 2.1.0; NIH, U.S.A.), then the average positive cells number per 0.14 mm^2 were calculated. In Iba-1 and cleaved-Gasdemind-D staining, the average pixel intensity of the signal was analyzed. Measured pixel intensity processing was accomplished by image processing software (Image J).

2.9 Statistical analysis

Statistical software (Prism 9 for macOS version 9.1.1; Graphpad Software, LLC) was used in the data analysis in this experiment. All results are expressed as the mean

± SD. One-way analysis of ANOVA followed by Tukey's multiple comparisons test was performed to verify the differences in the immunohistochemistry expression. In all statistical analyses, $p < 0.05$ were considered statistically significant.

3. Results

3.1 Cerebral ischemic infarction

To evaluate the cerebral ischemic infarction, we conducted Nissl staining (Fig. 2A). Statistical analysis of infarct volume showed that the carnosine group 1 day after tMCAO (39.1 ± 9.9 mm^3) displayed a decreasing trend than the vehicle group (27.8 ± 11.1 mm^3). However, the infarct volume at 5 days (32.2 ± 8.4 mm^3) was significantly decreased in the carnosine group compared with the vehicle group (24.6 ± 4.2 mm^3, $*p < 0.05$) (Fig. 2B).

3.2 Clinical Scores

Compared to the vehicle group, carnosine treatment did not change the body weight at 1 day after tMCAO (vehicle: 20.6 ± 1.2 g, carnosine: 20.4 ± 1.9 g), but significantly attenuated it at 5 days after tMCAO (vehicle: 18.6 ± 3.5 g, carnosine: 21.7 ± 2.9 g, $*p < 0.05$) (Fig. 3A). In the result of survival rates, there was no significant difference between the vehicle group and the carnosine group at 1 day after tMCAO (vehicle: 79.3 %, carnosine: 86.9 %) or at 5 days after tMCAO (vehicle: 65.5 %,

carnosine: 78.2 %) (Fig. 3B). Although carnosine treatment did not significantly improve the score of the rotarod test, it showed a tendency to improve it compared with the vehicle at 1 day (vehicle: 156.5 ± 100.7 secs, carnosine: 166.3 ± 95.2 secs) and especially 5 days (vehicle: 149.9 ± 118.1 secs, carnosine: 221.9 ± 124.5 secs) (Fig. 3C). Besides, corner test scores were not improved by carnosine treatment than the vehicle group at 1 day after tMCAO (vehicle: 7.8 ± 1.9 times, carnosine: 6.0 ± 2.9 times) and 5 days after tMCAO (vehicle: 8.3 ± 2.1 times, carnosine: 7.3 ± 2.4 times) (Fig. 3D).

3.3 **Quantitively analysis in serum and tissue protein**

As compared with the vehicle group, carnosine did not reduce oxidative stress (d-ROMs) 5 day after tMCAO (Vehicle: 97.9 ± 14.5 CARR U, carnosine: 102.2 ± 22.7 CARR U), (Fig. 4A). On the other hand, OXY-Adsorbent showed a reduction tendency in carnosine at 5 days after tMCAO (Vehicle: 273.1 ± 36.2 μmol HClO/mL, carnosine: 291.0 ± 100.8 μmol HClO/mL), but again not significant (Fig. 4B).

The concentration of lipids peroxidation product HNE showed a significant decrease in the carnosine group (Vehicle: 6.2 ± 7.1 μg/ml, carnosine: 2.3 ± 0.9 μg/ml) compared with the vehicle group (Fig. 4C).

3.4 Oxidative stress marker expression

At 1 and 5 days after tMCAO, the oxidative stress markers 8-OHdG, 4-HNE, Nitrotyrosine, and RAGE were strongly expressed in the peri-infarct area, which was significantly increased in the vehicle and the carnosine group compared with the sham group (Fig. 5A-H).

In the number of the 8-OHdG positive cells, which was mainly observed in the cytoplasm of neural cells, there was no significant difference between the vehicle and the carnosine group at 1 day after tMCAO (vehicle: 32.8 ± 5.9 cells / 0.14 mm², carnosine: 29.1 ± 5.9 cells / 0.14 mm²), but carnosine treatment significantly decreased it at 5 days after tMCAO (vehicle: 29.9 ± 2.5 / 0.14 mm², carnosine: 25.6 ± 3.4 / 0.14 mm², $^{**}p < 0.01$) (Fig. 5A, B). In the number of lipid peroxidation marker 4-HNE positive cells which expressed at cytoplasm and cytomembrane of neural cells, there was no significant difference between the vehicle and the carnosine group at 1 day after tMCAO (vehicle: 31.1 ± 4.2 / 0.14 mm², carnosine: 26.3 ± 5.3 / 0.14 mm²). However, the vehicle group was significantly decreased at 5 days after tMCAO (vehicle: 29.5 ± 2.9 / 0.14 mm², carnosine: 25.9 ± 3.5 / 0.14 mm², $^{*}p < 0.05$) (Fig. 5C, D). Nitric Oxide superoxide interactions marker, Nitrotyrosine expressed in neural cells' nuclei and foamy cytoplasm. There was no significant difference in the number of positive cells at 1 day after tMCAO between the vehicle and the carnosine group (vehicle: 24.2 ± 2.0 / 0.14 mm², carnosine: 20.5 ± 4.6 / 0.14 mm²), whereas carnosine group were significantly attenuated at 5 days after tMCAO (vehicle: 14.0 ± 3.2 / 0.14 mm², carnosine: 10.8 ± 2.3 / 0.14 mm², $^{*}p < 0.05$) (Fig. 5E, F). Semiquantitative analyses showed the number of RAGE positive cells, which was strongly observed in the

cytoplasm and cytomembrane of neural cells, were significantly decreased in the carnosine group both at 1 day (vehicle: 57.8 ± 8.1 / 0.14 mm², carnosine: 44.4 ± 4.4 / 0.14 mm², $^{\delta}p < 0.05$) and 5 days after tMCAO (vehicle: 62.8 ± 16.0 / 0.14 mm², carnosine: 43.9 ± 8.8 / 0.14 mm², $^{*}p < 0.05$) (Fig. 5G, H).

3.5 Neuroinflammation marker expression

The microglia marker Iba-1 and the inflammation cytokine IL-10 and IL-1β were expressed in the peri-infarct area at 1 and 5 days after tMCAO, which was significantly promoted in the vehicle and the carnosine group compared with the sham group (Fig. 6A-F).

There was no significant decrease in the pixel intensity of Iba-1, which was observed in activated microglia, between the vehicle and the carnosine group at 1 day (vehicle: 6.0 ± 2.4 pixels fold to sham 1d, carnosine: 3.5 ± 0.9) or 5 days after tMCAO (vehicle: 15.8 ± 1.3, carnosine: 14.4 ± 1.9) (Fig. 6A, B). The anti-inflammation cytokine IL-10 is mainly expressed in the cytoplasm of neural cells. There was no significant difference between the vehicle and the carnosine group at 1 day (vehicle: 27.8 ± 8.4 / 0.14 mm², carnosine: 29.2 ± 6.8 / 0.14 mm²) and at 5 days after tMCAO (vehicle: 27.0 ± 7.4 / 0.14 mm², carnosine: 26.3 ± 5.3 / 0.14 mm²) (Fig. 6C, D). There was no significant difference in the number of pro-inflammation cytokine IL-1β positive cells, which was observed in the cytoplasm of neural cells, between the vehicle and the

carnosine group at 1 day after tMCAO (vehicle: 31.9 ± 1.9 / 0.14 mm², carnosine: 31.6 ± 3.2 / 0.14 mm²). On the other hand, a significant difference was observed at 5 days after tMCAO (vehicle: 26.8 ± 3.2 / 0.14 mm², carnosine: 19.0 ± 4.4 / 0.14 mm², ***$p < 0.001$) (Fig. 6E, F).

3.6 Pyroptosis in mice brain

At 1 and 5 days after tMCAO, typical pyroptosis marker cleaved-Gasdemind-D and cleaved-caspase-1 were observed in the peri-infarct area, which was increased in the vehicle group and carnosine group compared with the sham group (Fig. 7A-D).

The pyroptosis marker cleaved-Gasdemind-D and cleaved-caspase-1 are expressed in the cytoplasm and cytomembrane of the cells. Semiquantitative analyses of cleaved-Gasdemind-D showed no significant difference between the vehicle and the carnosine group at 1 day (vehicle: 22.2 ± 8.7 / 0.14 mm², carnosine: 21.1 ± 2.6 / 0.14 mm²) or 5 days after tMCAO (vehicle: 21.5 ± 2.4 / 0.14 mm², carnosine: 21.1 ± 8.4 / 0.14 mm²) (Fig. 7A, B). Correspondingly the pixel intensity of cleaved-caspase-1 also showed no significant difference at 1 day (vehicle: 2.2 ± 0.8 pixels fold to sham 1d, carnosine: 2.1 ± 0.7) or 5 days after tMCAO (vehicle: 1.3 ± 0.5, carnosine: 0.9 ± 0.7) (Fig.7C, D).

4 Discussion

The present study investigated whether carnosine effectively ameliorated behavioral function and neurological damage in a transient ischemia mouse model and explored its potential mechanisms. The experimental results demonstrated that carnosine significantly alleviated the infarct volume (Fig. 2), attenuated the expression of oxidative stress-related markers (Fig. 4c, Fig. 5), and the inflammatory factor IL-1β (Fig. 6). Moreover, we demonstrated that it tended to decrease the expression of pyroptosis markers (Fig. 7) and approve the antioxidative capacity (Fig. 4B) with improved behavioral function (Fig. 3)**.**

Stroke involves a cascade activation of multiple deleterious pathways (Saleem et al., 2008; Moskowitz et al., 2010; Majid A, 2014). In particular, oxidative stress increases brain injury after ischemia/reperfusion by inducing the peroxidation of nucleic acids, lipids, and proteins (Abe et al., 1995). Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage (Birnboim HC, 1986). To maintain homeostasis and restore balance, it is considered feasible to use low doses of stressors to induce an adaptive response in cells and organisms which is called Hormesis (Lopez-Otin C, Kroemer G., 2021; Calabrese V et al., 2018). Hormesis is a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition. Hormetic dose responses excite the endogenous cellular defense pathway mediated Kelch-like ECH-associated protein 1 (Keap1)/NF-E2-related factor 2 (Nrf2)/ Antioxidant Response Element (ARE) pathway. Modulating endogenous cellular defense mechanisms represents an innovative approach to

therapeutic intervention in diseases causing chronic tissue damage, such as neurodegeneration (Calabrese V et al., 2010). Carnosine is a natural dipeptide that can induce the Keap1/Nrf2/ARE pathway, which collectively controls the expression of more than 100 genes that are concerned with cellular protection to rescue lipid peroxidation, mitochondria metabolism, and proteotoxicity (Zhao J et al., 2007; Calabrese V et al., 2010). Carnosine reacts particularly with low molecular weight aldehydes such as AGEs and the aldehydes manufactured by unsaturated fatty acids have an excellent scavenging effect on peroxide products, including malondialdehyde, acrolein, and 4-HNE in vivo and in vitro via the histidine imidazole moiety (Hipkiss et al., 1995, 1998; Andrea et al., 2005). In the present study, we initially reported that carnosine effectively reduced the expression of lipid peroxidation product (4-HNE) and nucleic acid peroxidation product (8-OHdG) 1 and 5 days after tMCAO (Figure 5A, B), indicating that carnosine has a potential role in oxidative stress reduction, possible by inducing an adaptive response in cells and organisms.

The term vitagenes refers to a group of genes that are strictly involved in preserving cellular homeostasis during stress conditions and the vitagene family includes heat shock proteins (HSPs), including haem oxygenase-1 (HSP32, HO-1) and HSP70. The products of the genes actively operate in detecting and controlling diverse forms of stress and cell injuries. Recent evidence demonstrates that carnosine prevents the upregulation of inducible nitric oxide synthase (iNOS) and the induction of both HO-1. The upregulation of HO-1 protein course the following increase in biliverdin (Calabrese, V et al., 2005; Mancuso, C et al., 2004,2006; Calabrese, V et al., 2007).

Compelling evidence has shown that biliverdin can serve as an endogenous scavenger of both nitric oxide (NO) and reactive nitrogen species (RNS), thus protecting against subsequent in vitro peroxynitrite damage in which mitochondria damage (Drake, J et al., 2003; Mancuso, C et al., 2006). Our study showed that carnosine effectively reduced the expression of NO-superoxide interactions product (Nitrotyrosine) 5 days after tMCAO, and glycosylated receptors (RAGE) 1 and 5 days after tMCAO (Figure 5C, D), indicating that carnosine has a potential role in preserving cellular homeostasis possible by active the vitagenes network.

Microglial polarization results in the classical activation (M1) pathway and the alternate activation (M2) pathway (Hu et al., 2015). The M1 microglia is a pro-inflammatory cell character releasing inflammatory cytokines such as IL-1β (Higashi et al., 2017). M2 phenotype expresses an anti-inflammatory state in which cells express anti-inflammatory mediators like IL-10 (Jin and Yamashita, 2016; Collmann et al., 2019). In this experiment, carnosine significantly attenuated the expression of IL-1β at 5 days after tMCAO (Fig. 6E-F) but did not significantly affect the expression of active microglia markers (Iba-1) or the inflammatory suppressor (IL-10) (Fig. 6A-D). The decrease of IL-1β at 5 days after tMCAO may not be the result of the direct action of carnosine on M1 microglia but was closely related to oxidative stress.

Pyroptosis is a type of programmed cell death distinct from apoptosis. Previous studies have shown that pyroptosis is an essential pathway for neuronal death in an acute ischemic stroke. Caspase-1 inhibition prevents neuronal death (Li et al., 2020).

The Gasdermin-D N-terminal is sufficient to drive pyroptosis, which can be a potential therapeutic target to reduce brain I/R injury (Shi et al., 2015; Zhang et al., 2019). However, carnosine did not significantly affect the expression of cleaved-caspase-1 or cleaved-Gasdermin-D in this experiment (Fig. 7). Because the expression of the two markers peaked within 24 hrs after surgery (Zhang et al., 2019; Li et al., 2020), carnosine may have inhibited pyroptosis at an earlier time point without being detected in this study.

In conclusion, we demonstrated that carnosine effectively relieved oxidative stress caused by ischemic stroke and significantly attenuated neuro-inflammatory responses in specific pathways. Our results also suggest that carnosine is a potent Hormetic dose response inducer and activates endogenous cellular defense programs through multiple pathways. It can be a promising therapeutic strategy for ischemic stroke if combined with supplements that substantially affect inflammation or improve cerebral microcirculation.

Acknowledgments

Funding

This study was partly supported by a Grant-in-Aid for Scientific Research (C) 20K09370, 20K12044, Challenging Research 21K19572, Young Research 20K19666, 21K15190, and by Grants-in-Aid from the Research Committees (Toba K, and Tsuji S) from the Japan Agency for Medical Research and Development.

Authorship contributions

TY, KA designed the experiments. HXR, TF, and HX conducted the experiments. HXR, BZH, YHB, and SHM collected the data and analysis. HXR, FY drafted the paper manuscript. TY, RM, MT, YN, and TY afford administrative, technical, and material support.

Conflicts of interest

The authors disclose no conflicts of interest.

Reference

1. Tsao CW, Aday AW, Almarzooq ZI, Alonso A, Beaton AZ, Bittencourt MS, et al. Heart Disease and Stroke Statistics-2022 Update: A Report From the American Heart Association. Circulation. 2022;145(8):e153-e639.

2. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nature Reviews Neuroscience. 2003;4(5):399-414.

3. Boldyrev AA. Carnosine: new concept for the function of an old molecule.

Biochemistry (Mosc). 2012;77(4):313-26.

4. Nagai K. [The inhibition of inflammation by the promotion of spontaneous healing with L-carnosine (author's transl)]. Langenbecks Arch Chir. 1980;351(1):39-49.

5. Shimada T, Watanabe N, Ohtsuka Y, Endoh M, Kojima K, Hiraishi H, et al. Polaprezinc down-regulates proinflammatory cytokine-induced nuclear factor-kappaB activiation and interleukin-8 expression in gastric epithelial cells. J Pharmacol Exp Ther. 1999;291(1):345-52.

6. Bae ON, Serfozo K, Baek SH, Lee KY, Dorrance A, Rumbeiha W, et al. Safety and efficacy evaluation of carnosine, an endogenous neuroprotective agent for ischemic stroke. Stroke. 2013;44(1):205-12.

7. Rajanikant GK, Sugunan S. Commentary: linking productive autophagy to neuroprotection: potential implications for anti-ischemic therapy. CNS Neurol Disord Drug Targets. 2013;12(3):298-9.

8. Abe K, Kawagoe J, Araki T, Aoki M, Kogure K. Differential expression of heat shock protein 70 gene between the cortex and caudate after transient focal cerebral ischaemia in rats. Neurol Res. 1992;14(5):381-5.

9. Yamashita T, Liu W, Matsumura Y, Miyagi R, Zhai Y, Kusaki M, et al. Novel Therapeutic Transplantation of Induced Neural Stem Cells for Stroke. Cell Transplant. 2017;26(3):461-7.

10. Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniego P, Li Q, et al. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. J Neurosci Methods. 2002;117(2):207-14.

11. Shi X, Ohta Y, Shang J, Morihara R, Nakano Y, Fukui Y, et al. Neuroprotective effects of SMTP-44D in mice stroke model in relation to neurovascular unit and trophic coupling. J Neurosci Res. 2018;96(12):1887-99.

12. Taira Y, Yamashita T, Bian Y, Shang J, Matsumoto N, Sasaki R, et al. Antioxidative effects of a novel dietary supplement Neumentix in a mouse stroke model. J Stroke Cerebrovasc Dis. 2020;29(8):104818.

13. Saleem S, Zhuang H, Biswal S, Christen Y, Dore S. Ginkgo biloba extract neuroprotective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury. Stroke. 2008;39(12):3389-96.

14. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron. 2010;67(2):181-98.

15. Majid A. Neuroprotection in stroke: past, present, and future. ISRN Neurol. 2014;2014:515716.

16. Abe K, Aoki M, Kawagoe J, Yoshida T, Hattori A, Kogure K, et al. Ischemic delayed neuronal death. A mitochondrial hypothesis. Stroke. 1995;26(8):1478-89.

17. Birnboim HC. DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumor promoters are repaired slowly compared to breaks induced by ionizing radiation. Carcinogenesis. 1986;7(9):1511-7.

18. Calabrese EJ, Giordano JJ, Kozumbo WJ, Leak RK, Bhatia TN. Hormesis mediates dose-sensitive shifts in macrophage activation patterns. Pharmacol Res. 2018; 137:236-49.

19. Lopez-Otin C, Kroemer G. Hallmarks of health. Cell. 2021;184(7):1929-39.

20. Calabrese V, Cornelius C, Dinkova-Kostova AT, Calabrese EJ, Mattson MP. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. Antioxid Redox Signal. 2010;13(11):1763-811.

21. Zhao J, Moore AN, Redell JB, Dash PK. Enhancing expression of Nrf2-driven genes protects the blood brain barrier after brain injury. J Neurosci. 2007;27(38):10240-8.

22. Hipkiss AR, Michaelis J, Syrris P. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. FEBS Lett. 1995;371(1):81-5.

23. Hipkiss AR. Carnosine, a protective, anti-ageing peptide? Int J Biochem Cell Biol. 1998;30(8):863-8.

24. Guiotto A, Calderan A, Ruzza P, Borin G. Carnosine and carnosine-related antioxidants: a review. Curr Med Chem. 2005;12(20):2293-315.

25. Calabrese V, Colombrita C, Guagliano E, Sapienza M, Ravagna A, Cardile V, et al. Protective effect of carnosine during nitrosative stress in astroglial cell cultures. Neurochem Res. 2005;30(6-7):797-807.

26. Mancuso C. Heme oxygenase and its products in the nervous system. Antioxid Redox Signal. 2004;6(5):878-87.

27. Mancuso C, Pani G, Calabrese V. Bilirubin: an endogenous scavenger of nitric oxide and reactive nitrogen species. Redox Rep. 2006;11(5):207-13.

28. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat

Rev Neurosci. 2007;8(10):766-75.

29. Drake J, Sultana R, Aksenova M, Calabrese V, Butterfield DA. Elevation of mitochondrial glutathione by gamma-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. J Neurosci Res. 2003;74(6):917-27.

30. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization-new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56-64.

31. Higashi Y, Aratake T, Shimizu S, Shimizu T, Nakamura K, Tsuda M, et al. Influence of extracellular zinc on M1 microglial activation. Sci Rep. 2017;7:43778.

32. Jin X, Yamashita T. Microglia in central nervous system repair after injury. J Biochem. 2016;159(5):491-6.

33. Collmann FM, Pijnenburg R, Hamzei-Taj S, Minassian A, Folz-Donahue K, Kukat C, et al. Individual in vivo Profiles of Microglia Polarization After Stroke, Represented by the Genes iNOS and Ym1. Front Immunol. 2019;10:1236.

34. Li J, Hao JH, Yao D, Li R, Li XF, Yu ZY, et al. Caspase-1 inhibition prevents neuronal death by targeting the canonical inflammasome pathway of pyroptosis in a murine model of cerebral ischemia. CNS Neurosci Ther. 2020;26(9):925-39.

35. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526(7575):660-5.

36. Zhang D, Qian J, Zhang P, Li H, Shen H, Li X, et al. Gasdermin D serves as a key

executioner of pyroptosis in experimental cerebral ischemia and reperfusion model both in vivo and in vitro. J Neurosci Res. 2019;97(6):645-60.

Figure legends

Fig. 1 Schematic diagram of the experimental groups and procedure. Mice were divided into six groups. The black arrows present i.p. injection of saline, and the white arrows present i.p. injection of carnosine.

Fig. 2 Nissl staining and quantitative analysis of infarct volume at 1 and 5 days after tMCAO in the vehicle and carnosine groups. Note that the infarct volume at 5 days was significantly decreased in the carnosine group compared with the vehicle group. (Scale bar: 50 μm, *$p < 0.05$ versus vehicle group).

Fig. 3 Clinical scores of (A) Body weight, (B) Survival rate, (C) Rotarod test, and (D) Corner test on surgery day, 1 and 5 days after tMCAO in the vehicle and carnosine groups (*$p < 0.05$ versus vehicle group).

Fig. 4 Quantitively analysis of Antioxidative activities and oxidative stress. Serum levels of reactive oxygen metabolites (A) and antioxidant capacity (B) and HNE Adduct Competitive ELISA result (C) at 5 days after tMCAO (*$p < 0.05$ versus vehicle group).

Fig. 5 Expression of oxidative stress markers in tMCAO mice brains. Immunohistochemical staining of (A) 8-OhdG, (C) 4-HNE (E) nitrotyrosine, and (G) RAGE in the peri-ischemic area at 1 and 5 days after tMCAO of sham, vehicle, and carnosine groups. Quantitative analysis of (B) 8-OhdG, (D) 4-HNE (F) nitrotyrosine, and (H) RAGE positive cells after tMCAO (Scale bar : 50 μm; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ versus sham group; *$p < 0.05$, **$p < 0.01$versus vehicle group at 5 days; $^{\delta}p < 0.05$ versus vehicle at 1 day).

Fig. 6 Expression of inflammation markers in tMCAO mice brains. Immunohistochemical staining of (A) Iba-1, (C) IL-10, and (E) IL-1β in the peri-ischemic area at 1 and 5 days after tMCAO of sham, vehicle, and carnosine groups. Quantitative analysis of (B) Iba-1, (D) IL-10, and (F) IL-1β positive cells after tMCAO (Scale bar: 50 μm; ##$p < 0.01$, ####$p < 0.0001$ versus sham group; ***$p < 0.001$ versus vehicle at 5 days).

Fig. 7 Expression of pyroptosis markers in tMCAO mice brains. Immunohistochemical staining of (A) cleaved-GSDMD, and (C) cleaved-caspase-1 in the peri-ischemic area at 1 and 5 days after tMCAO of sham, vehicle, and carnosine groups. Quantitative analysis of (B) cleaved-GSDMD, and (D) cleaved-caspase-1 positive cells after tMCAO (Scale bar: 50 μm; #$p < 0.05$ versus sham group).

.