Research Article

Melatonin and calpeptin synergistically protect against post-reperfusion injury in a rat middle cerebral artery occlusion stroke model

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ABSTRACT

Cerebral ischemia induces oxidative injury and increases the intracellular calcium ion concentration to activate several calcium-dependent proteases such as calpains. Calpain activation leads to various necrotic and apoptotic processes. Calpeptin is a potent, cell-permeable calpain inhibitor. As a strong antioxidant and free radical scavenger, melatonin shows beneficial effect in rodent models of focal cerebral ischemia when given prior to ischemia or reperfusion. This study was focused on the neuroprotective effects of melatonin and/or calpeptin given after onset of reperfusion. For this purpose, right-sided middle cerebral artery occlusion (MCAO) for 90 minutes followed by 24 or 72 hours of reperfusion was performed in male Sprague Dawley rats, then, melatonin 50 or 150 μg/kg, calpeptin 10, 15 or 50 μg/kg or a combination of melatonin 50 μg/kg plus calpeptin 15 or 50 μg/kg were injected via an intracerebroventricular route at 15 minutes after onset of reperfusion. Melatonin or calpeptin tended to reduce the relative infarct volume and significantly decreased the neurological deficit at 24 hours. The combination achieved a greater protection than each of them alone. Melatonin, calpeptin or the combination all decreased Fluoro-Jade B (FJB)+ degenerative neurons and cleaved/total caspase-3 ratio at 24 hours. These treatments did not significantly impact the density of surviving neurons and ED-1+ macrophage/activated microglia. At the 72-hour-reperfusion, melatonin or the combination decreased the relative infarct volume and neurological deficit. Nevertheless, only the combination reduced FJB+ degenerating neurons at 72 hours. In conclusion, a combination of melatonin and calpeptin exerted synergistic protection against post-reperfusion injury in a rat MCAO stroke model.

Key words: melatonin, calpeptin, ischemic stroke, neuroprotection, ischemia, reperfusion.

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1. INTRODUCTION

Stroke is a leading cause of death and a major source of disability in the world (1). Ischemic stroke accounts for 80-85% of all strokes (2) and is caused by a blockage in a

cerebral artery (3). Chemical or mechanical busting of the clot inside the blocked cerebral artery within a short time after onset is effective, but such acute reperfusion treatment is feasible only in a small portion of stroke patients. Given the increasing global burden of stroke, other effective treatments are warranted (4-7). A variety of animal stroke models have been developed to study the pathophysiological processes underlying brain damage induced by transient focal ischemia followed by reperfusion. The rat middle cerebral artery occlusion (MCAO) is the most widely used animal model in studies on focal cerebral ischemia (8-10).

The pathophysiology of cerebral ischemia/reperfusion includes rapid consumption of intracellular adenosine triphosphate stores, glutamate excitotoxicity, mitochondrial dysfunction, free radical production, activation of the immune system, and stimulation of calcium-dependent enzymes. Ischemia/reperfusion eventually leads to cell death (11, 12). During cerebral ischemia, excessive activation of glutamate receptors increases the intracellular influx of sodium and calcium ions. Sodium-influx-related membrane depolarization leads to opening of voltage-controlled calcium channels (13). Calcium ion dysregulation contributes to cell death occurring during the first minutes of reperfusion via different mechanisms including necrosis, apoptosis and autophagy (14). Increased intracellular calcium levels at pathological conditions activate calcium-dependent proteases such as calpain (13, 15). As calcium-dependent enzymes, calpains belong to a unique class of intracellular cysteine proteases participating in multiple calcium-regulated intracellular signaling pathways. Calpains affect many substrates in regulating necrosis, apoptosis and autophagy and play a crucial role in accelerated neuronal loss in various neurological disorders (15).

Calpain inhibitors protect against necrosis and, to a lesser extent, apoptosis (16). Calpeptin, a cell-permeable calpain inhibitor, has been found to alleviate ischemic renal (17), heart (18) and excitatory amino acid-induced neuronal damages (19). In experimental animal models of cerebral ischemia, calpeptin and other calpain inhibitors have been shown to be effective in reducing infarct volume, edema, calcium-activated proteolysis and neurological deficit (10, 20, 21). In a 120-minute rat MCAO model, a prior intracerebroventricular (ICV) injection of 50 µg calpeptin in 5 µL dimethyl sulfoxide (DMSO) at 30 minutes before MCAO has been found to suppress caspase-3 activation (as detected by immunohistochemistry) and reduce neuronal apoptosis (as quantitated using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay) in the hippocampal CA1 sector (10). Calpains may remain inactive during ischemia and be activated during reperfusion (14).

Melatonin (N-acetyl-5-methoxytryptamine) is primarily synthesized in the pineal gland and secreted into the cerebrospinal fluid (CSF) and blood especially at night. Recent evidence reveals that it is likely produced in the mitochondria of all cells (22, 23) and functions as an antioxidant (24). Owing to its direct antioxidant, anti-inflammatory and anti-apoptotic properties, melatonin is a neuroprotective molecule against various neurological diseases (25-28). Some of the products derived from its interaction with reactive oxygen species (ROS) also possess antioxidative and anti-apoptotic properties (29-31). Post-ischemia reperfusion leads to increased prostaglandin synthesis, elevated levels of second messengers, inflammation and mitochondrial dysfunctions with opening of the mitochondrial permeability transition pore and generation of ROS (32). Melatonin has been shown to reduce infarct volume in experimental stroke models in different mammalian species (33-36) and ameliorate ischemia/reperfusion-induced mitochondrial dysfunctions (11) via antioxidative stress, anti-inflammation, and anti-apoptosis (37). Among most animal studies exploring the effects of melatonin against ischemia-reperfusion injury, melatonin was given prior to ischemia or before reperfusion (38-40), but pretreatment with

melatonin was impractical in clinical setting. Melatonin was given after onset of reperfusion in our study, which is more relevant to clinical practice. Either intraperitoneal (IP) or intravenous (IV) administration of melatonin was commonly adopted in previous studies (9, 41). IV administration of melatonin upon reperfusion suppressed the cellular inflammatory response after ischemia-reperfusion in rat MCAO model by decreasing emigration of circulatory neutrophils and macrophages into the injured brain and by inhibiting focal microglial activation (41). ICV administration of melatonin is less commonly used.

Although ICV administration of drugs is rarely used in the clinical settings (42), direct delivery of a drug or chemical into the lateral ventricle is often used in laboratory animals to explore its therapeutic potentials. Following ICV injection, a drug will rapidly enter the circulation together with CSF flow whilst penetration into the brain tissue may be limited by diffusion property of the drug molecule (43). Nevertheless, ICV injection bypasses the blood-brain barrier and may achieve a higher concentration within the brain when compared to other routes of administration such as IP injection (44). As an example, the effective melatonin dose for ICV is roughly about 1/100 of that for IP-administered melatonin (45). Thus, the doses of melatonin ICV used in the present study were 50 and 150 μg/kg.

As calpeptin reduces necrosis and possesses anti-apoptotic effect and melatonin has both anti-apoptotic and anti-oxidative properties, it is reasonable to hypothesize that combination of these molecules may be more effective in ameliorating brain injury due to ischemia/reperfusion. On the other hand, melatonin may attenuate calpain upregulation (46) via an inhibitory effect on calpains (47). This study will explore the optimal dosage of an ICV administration of melatonin, calpeptin or their combination after onset of reperfusion and to investigate whether the combination exerts synergistic neuroprotective effects in a rat MCAO model at 24 or 72 hours of reperfusion after 90 minutes of focal ischemia.

2. MATERIALS AND METHODS

2.1. Chemicals and agents.

Calpeptin, DMSO, DPX mounting medium, melatonin, paraformaldehyde, protease inhibitor cocktail and phosphatase inhibitor cocktail, 2,3,5-triphenyltetrazolium chloride (TTC) and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Buprenorphine (Reckitt Benckiser Healthcare Ltd, Hull, UK). Enrofloxacin (Bayer Ltd, Kiel, Germany). Sodium pentobarbital (Rhone Merieux, Pinkenba, QLD, Australia). Anti-Bcl-2, anti-Bax and anti-β-actin primary antibodies as well as goat anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Anti-Caspase-3 and anti-cleaved Caspase-3 primary antibodies (Cell Signaling Technology, Beverly, MA, USA). Anti-NeuN monoclonal primary antibody, Fluoro-Jade B and Alexa Fluor®488 goat anti-mouse IgG secondary antibody (EMD Millipore, Temecula, CA, USA). Mouse anti-ED-1 primary antibody (AbD Serotec, Kidlington, United Kingdom). Avidin-biotin complex solution and 3,3'-diaminobenzidine (DAB) (Vector Labs, Burlingame, CA, USA). Bradford protein assay kit and polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). 4',6'-diamidino-2-phenylindole (DAPI) mounting medium (Life Technologies, Carlsbad, California, USA).

2.1. Animals.

All experiments were conducted under the institutional guidelines with the protocol approved by the Committee on Use of Live Animal for Teaching and Research (CULATR; reference number 2678-12), The University of Hong Kong. Normally fed adult male

Sprague Dawley rats weighing between 270 and 290 g were obtained from the Laboratory Animal Unit, The University of Hong Kong. The rats were housed at the room with 22 ± 2 °C, light/dark cycle of 12/12 and free access to water and food (9).

2.2. Right-sided endovascular MCAO.

The rat was subjected to transient focal cerebral ischemia for 90 minutes with reperfusion for 24 or 72 hours using the right-sided endovascular MCAO method (48). Briefly, the rat was anesthetized with an IP injection of sodium pentobarbital (60 mg/kg initially with an additional bolus of 20 mg/kg as required), and a midline cervical incision was made to expose the right carotid arteries. The right external carotid artery (ECA) was dissected free, isolated distally by coagulating its branches (Bipolar Electric Coagulation GN60; Aesculap AG & Co, Hesse, Germany) and transected after placing a distal ligation. A 6-o silk suture was tied loosely around the mobilized ECA stump, and microclips were temporarily placed at both the common carotid artery and internal carotid artery (ICA). A piece of silicone rubber-coated monofilament (4-o medium MCAO suture reusable PK5; Doccol corporation, Sharon, MA, USA) was introduced via the right ECA stump into the right ICA. The silk suture was tightened to prevent bleeding, and the MCAO suture was gently advanced via the right ICA to embed into the right anterior cerebral artery at approximately 12-18 mm from the carotid bifurcation to occlude the right middle cerebral artery at its origin for 90 minutes before withdrawal. After the procedure, the neck incision was closed using 4-o monofilament nylon suture (Ethicon; Johnson & Johnson, Somerville, NJ, USA). Subcutaneous injections of buprenorphine (50 µg/kg every 12 hours) were given to the rat for post-operative pain relief, and an intramuscular injection of enrofloxacin (10 mg/kg) was given for prevention of wound infection. The rat was allowed to recover from the anesthesia. Rectal temperature was maintained 36.5-37.5°C throughout the experiment by using a rectal thermostat probe and a thermostatically regulated heating pad.

2.3. Monitoring of regional cerebral blood flow (CBF).

Regional CBF was monitored using a laser-Doppler flowmeter (MBF3D; Moor Instruments Limited, Axminster, Devon, UK). The rat was placed on a stereotaxic device (SR-6N; Narishige Scientific Instrument Laboratory, Tokyo, Japan), and a low-speed dental drill was applied to make two 2-mm diameter burr holes on the skull (9). The burr hole located at 2 mm posterior and 5 mm lateral to the bregma on the right side was used for monitoring of regional CBF. A laser probe was placed on the dura surface away from visible cerebral vessels and glued to the burr hole. Baseline values remained stable for at least 10 minutes before the induction of focal cerebral ischemia. Successful induction of focal ischemia was revealed by a sudden decline in the regional CBF by 65% or more, and a surge in regional CBF to more than 80% of baseline value upon withdrawal of the MCAO suture would reveal reperfusion. Data on regional CBF at 30 and 60 minutes of ischemia and upon reperfusion were normalized and expressed as percentages of the baseline values (9).

2.4. Melatonin and calpeptin treatment.

The rat was randomly assigned to different treatment groups to receive the vehicle alone, melatonin, calpeptin or their combination via an ICV injection using a 10-µL Hamilton glass syringe (Hamilton Co., Reno, NV, USA) commenced at 15 minutes after the onset of reperfusion. The timelines of experimental protocol are depicted in Figure 1A and 4A. The injection was made through the burr hole at 0.6 mm anterior and 1.5 mm lateral to the

bregma on the right side with tip of the stainless steel needle at 3.5-4 mm beneath the dura surface (49). The volume of ICV injection was 10 µL/kg given at a constant rate of 0.25 µL per minute (10). The vehicle was normal saline containing 25% DMSO. The head incision was closed using 4-o monofilament nylon suture (Ethicon; Johnson & Johnson) at about 10 minutes after completion of the ICV injection.

25% DMSO was used as the vehicle because of a low solubility of calpeptin in DMSO at lower concentrations. The effects of melatonin at 50 or 150 µg/kg, calpeptin at 10, 15 or 50 µg/kg, and melatonin at 50 µg/kg plus calpeptin at 15 or 50 µg/kg were first compared with the vehicle at 24 hours post-reperfusion regarding the infarct volume and neurological deficit. The effects of melatonin alone at 50 µg/kg, calpeptin alone at 15 µg/kg or melatonin at 50 µg/kg plus calpeptin at 15 µg/kg were compared with the vehicle at 24 hours post-reperfusion regarding degenerating neurons, surviving neurons, macrophage/microglia infiltration, apoptotic markers and caspase-3 cleavage. In addition, these four groups were compared at 72 hours post-reperfusion regarding infarct volume, neurological deficit, body weight, degenerating neurons, surviving neurons and macrophage/microglia infiltration.

2.5. Neurobehavioral assessment.

Rats were assessed for their neurobehavioral performance using the neurological deficit scoring system (NDSS) (50-52). Points were given to indicate impaired performance in different neurobehavioral tests, including spontaneous-activity test, floor-walking test, tail-raising test, limb-placing tasks and beam-balance test. Rats which suffer from more severe deficit will get higher NDSS points, and the maximum is 21. All assessments were performed by an observer who was blinded to the group identity.

2.6. Measurement of infarct volume.

At 24 or 72 hours after reperfusion, the rat was deeply anesthetized with an IP injection of sodium pentobarbital at 100 mg/kg before decapitation. The brain (between 4 mm anterior and 8 mm posterior to the bregma) was carefully removed and cut into 2-mm thick coronal slices before reacting with 1.5% TTC for 20 minutes at room temperature to reveal the cerebral infarction. After TTC reaction, the brain slices were fixed in pre-cooled 4% paraformaldehyde at pH 7.4. Digital photographs were taken from the coronal slices for evaluation of infarct and hemisphere volumes using the Image J software (1.36b; NIH, Rockville Pike, MD, USA). The infarct volume was expressed as a percentage of the total ipsilateral hemispheric volume.

2.7. Western blot.

Activated caspase-3 is at the convergence point for different signaling pathways in apoptosis, and Bax/Bcl-2 ratio is also a marker of apoptosis (53). At 24 hours after reperfusion, the rat was deeply anesthetized with an IP injection of sodium pentobarbital at 100 mg/kg before decapitation. The brain was quickly removed, and the right cerebral hemisphere between 2 mm anterior and 2 mm posterior to the bregma was dissected on ice and separated into the striatum and cortex with the cortex used for subsequent western blot analysis. Briefly, the radioimmune precipitation assay lysis buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail were added to exact the proteins. After sonicating for 15 seconds to allow the brain specimens to fully mix with the buffer, the lysate was kept on ice for 30 minutes before centrifugation at 12,000 g for 20 minutes; the supernatants were collected carefully without disturbing the pellets. The concentration

of protein was measured using a Bradford protein assay kit. Depending on the molecular weight of the target proteins, 30-50 μg of the protein was mixed with loading buffer and separated using 12-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Next, the proteins were transferred to polyvinylidene difluoride membranes at 4°C. After immersion in 5% non-fat milk in Tris-HCl-based buffered saline with 0.1% Tween 20 (TBST) for blocking at pH 7.4 and room temperature for 30 minutes, the membranes were incubated with the primary antibody overnight at 4°C. On the next day, the membranes were washed three times with TBST for 15 minutes each time before incubating with a horseradish peroxidase-conjugated goat secondary antibody (anti-mouse or anti-rabbit, 1: 5000 dilution) at room temperature for 2 hours. After washing three times with TBST for 15 minutes each time, the protein bands were visualized using advanced chemiluminescence (GE Healthcare Life Sciences, Hong Kong) and recorded by GelDoc-2000 Imagine System (Bio-Rad, Hercules, CA, USA). The protein expression levels were analyzed using the Image J software (1.36b; NIH). The band density was adjusted according to the beta-actin band and expressed as a percentage of the vehicle group for analysis. The primary antibodies used included Bcl-2 (1: 3000 dilution), Bax (1: 2500 dilution), caspase-3 (1:300 dilution), cleaved caspase-3 (1:300 dilution) and β-actin (1: 500 dilution).

2.8. Immunofluorescence.

At 24 or 72 hours after reperfusion, the rat was deeply anesthetized with an IP injection of sodium pentobarbital at 100 mg/kg before transcardial perfusion with pre-cooled normal saline first and then ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.5 as a fixative. The brain was quickly removed, fixed overnight in 4% paraformaldehyde at 4 °C, and cryoprotected in 30% sucrose in phosphate buffered saline (PBS) for approximately 3 days. 30-μm thick coronal cryosections were obtained from each of three bregma levels (−2.4 to −2.04 mm, −0.6 to −0.24 mm, and +1.08 to +1.44 mm) to study neuronal survival and degeneration. Three brain sections per rat (with one brain section per bregma level) were affixed on Superfrost Plus slides (Menzel-Glaser, Braunschweig, LS, Germany) and air-dried overnight. Before incubating, the slides were boiled in citrate buffer at 90°C for 8 minutes to increase the cell permeability.

NeuN staining was used to label surviving neurons. Briefly, the brain sections were stained with immunofluorescent mouse NeuN monoclonal antibody (1:500 dilution) diluted with non-specific blocking solution (10% goat serum) overnight at 4°C. Secondary antibody (Alexa Fluor®488 goat anti-mouse IgG, 1:500 dilution) was applied on the next day for 2 hours at room temperature after washing with PBS three times for 10 minutes each, and then coverslipped with (DAPI mounting medium. Photomicrographs were taken over the peri-infarct cortex under 400x high power lens using a fluorescence microscope (Nikon, Minato City, Tokyo, Japan). FITC Green fluorochrome (green) for NeuN on the sections was excited by a laser beam at 488 nm, and positively stained cells were identified manually with the number counted using Image J software (1.36b; NIH) from 6 fields per brain section. The average number per high power field was used as the cell density for analysis. Results were expressed as percentage of the mean of the vehicle group.

FJB staining was used to label degenerating neurons. Briefly, the brain sections were immersed in 80% ethanol containing 1% sodium hydroxide for 5 minutes, and this was followed by immersion in 70% ethanol and then deionized water for 2 minutes each. Next, the brain sections were placed in 0.06% potassium permanganate solution for 15 minutes of oxidization before rinsing three times in double distilled water for 2 minutes each. The brain sections were then reacted with 0.004% solution of Fluoro-Jade B in 0.1% acetic acid for 20 minutes. Finally, the slides were rinsed with double distilled water, dried at 55°C for 20

minutes, cleared with xylene, and coverslipped with DPX mounting medium. Photomicrographs were taken over the peri-infarct cortex under 400x high power lens using a fluorescence microscope (Nikon). Positively stained cells were identified manually with the number counted using Image J software (1.36b; NIH) from 6 fields per brain section. The average number per high power field was used as the cell density for analysis. Results were expressed as percentage of the mean of the vehicle group.

2.9. Immunohistochemistry.

The ED-1 antigen is expressed on most macrophage populations including activated microglia as well as on monocytes and is considered as a pan-macrophage marker in the rat. At 24 or 72 hours of reperfusion, the brain was obtained with the 30-µm thick coronal cryosections prepared from the three bregma levels using the protocol as for immunofluorescence study. Three brain sections per rat were immunohistochemically reacted with mouse anti-ED-1 antibody (1:200 dilution) diluted with non-specific blocking solution (10% goat serum) overnight at 4°C. Biotinylated goat anti-mouse secondary antibody (1:500) was applied the next day for 2 hours at room temperature after washing with PBS three times for 10 minutes each. To amplify the signals, avidin-biotin complex solution (1:200 dilution) was used. After 30 minutes of incubation, slides were rinsed in PBS, and the signal was visualized by DAB for detection of the peroxidase signal. After washing with PBS for 3 more times, brain sections were dehydrated with 95% ethanol for 5 min twice followed by 100% ethanol for 5 min twice and xylene for 10 minutes. Slides were then coverslipped with DPX mounting medium. Photomicrographs were taken over the peri-infarct cortex under 200x high power lens using a light microscope (Axio Vision Control; Carl Zeiss, Munich, Germany). Positively stained cells were identified manually with the number counted using Image J software (1.36b; NIH) from 6 fields per brain section. The average number per brain section was used as the cell density for analysis. Results were expressed as percentage of the mean of the vehicle group.

2.10. Study design and statistical analysis.

The study was not pre-registered. No sample size calculation was performed. Sample size for animal experiments were approximated based on past studies (8, 50, 54). Inclusion criteria included rats weighing between 270 and 290 g and successful MCAO revealed by a sudden decline in the regional CBF by 65% or more and a surge in regional CBF to more than 80% of baseline value upon reperfusion. The rats were excluded if weighing outside the range of 270-290 g, MCAO unsuccessful or presence of intracerebral hemorrhage in the brain specimen. All the data were normally distributed. No outlier exclusion was performed. Data were expressed as mean ± standard error of the mean (SEM). Data on regional CBF over different time points were analyzed using two-way repeated measures analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) or Games-Howell post-hoc tests. In the experiments on melatonin groups or calpeptin groups, data on infarct volume and NDSS were compared with vehicle group using independent samples t tests to indicate if any of the treatment may be beneficial. In the experiments on melatonin plus calpeptin groups, data on infarct volume and NDSS were analyzed using one-way ANOVA followed by Tukey HSD post-hoc tests. In both the 24 and 72-hour post-reperfusion experiments involving vehicle only, melatonin alone at 50 µg/kg, calpeptin alone at 15 µg/kg and melatonin at 50 µg/kg plus calpeptin at 15 µg/kg, data on FJB, NeuN, ED-1, Bax/Bcl-2 ratio and cleaved/total caspase 3 ratio were analyzed using one-way ANOVA followed by Dunnett post-hoc tests; the same analyses were applied to infarct

volume at 72 hours. In the 72 hours post-reperfusion experiments, body weight and NDSS data over different time points were analyzed using two-way repeated measures ANOVA followed by Tukey HSD or Games-Howell post-hoc tests. SPSS (version 21; IBM Corporation, Armonk, NY, USA) was used in all statistical analyses, and a two tailed *P*-value of 0.05 or less was taken to infer statistical significance.

3. RESULTS

3.1. Effects of melatonin or calpeptin on post-reperfusion neuronal injury.

The experimental design of 90-minutes focal ischemia followed by 24 hours of reperfusion was summarized in Figure 1A. The significant overall changes in regional CBF ($P<0.001$; Figure 1B) were similar in all groups with a significant reduction at 0, 30 and 60 minutes of focal ischemia ($P<0.001$ when compared to baseline) and with a significant surge upon reperfusion (with no significant difference from baseline). The brain infarct at 24 hours was revealed by TTC staining (Figure 1C). When compared to the relative infarct volume of 41.04 ± 3.52% in the vehicle group (Figure 1D), a single ICV injection of melatonin at 50 and 150 µg/kg dose-dependently reduced it to 32.10 ± 3.25% (not significant) and 27.19 ± 4.06% ($P<0.05$), respectively. A single ICV injection of calpeptin at 10, 15 and 50 µg/kg tended to dose-dependently reduce the relative infarct volume to 38.16 ± 6.21% (not significant), 32.93 ± 6.85% (not significant) and 26.75 ± 6.67% (not significant), respectively. The NDSS points (Figure 1E) were 13.36 ± 1.50 in the vehicle group, 9.05 ± 0.99 ($P<0.05$) and 9.41 ± 0.84 ($P<0.05$) following treatment with melatonin at 50 and 150 µg/kg respectively, and 11.75 ± 2.98 (not significant), 8.0 ± 0.93 ($P<0.05$) and 8.0 ± 0.99 ($P<0.05$) following treatment with calpeptin at 10, 15 and 50 µg/kg, respectively. Thus, significant reduction in NDSS points was seen in all treatment groups except the group treated with calpeptin at the lowest dose.

3.2. Effects of melatonin plus calpeptin on post-reperfusion neuronal injury.

The same experimental design (Figure 1A) was adopted for the combinational treatment. The significant overall changes in regional CBF ($P<0.001$; Figure 2A) were similar in all groups with a significant reduction at 0, 30 and 60 minutes of focal ischemia ($P<0.001$ when compared to baseline) and with a significant surge upon reperfusion (with no significant difference from baseline). The brain infarct at 24 hours was revealed by TTC staining (Figure 2B), and the inter-group difference was significant ($P<0.01$). When compared to the relative infarct volume of 38.96 ± 1.84% in the vehicle group (Figure 2C), a single ICV injection of melatonin at 50 µg/kg plus calpeptin at 15 and melatonin at 50 µg/kg plus calpeptin at 50 µg/kg significantly reduced it to 20.95 ± 4.88% ($P<0.01$) and 25.60 ± 2.03% ($P<0.05$), respectively; there was no significant difference between the two treatment groups. The NDSS points (Figure 2D) also showed a significant inter-group difference ($P<0.05$) with a reduction from 13.25 ± 0.96 in the vehicle group to 7.17 ± 1.55 ($P<0.05$) and 8.17 ± 1.75 ($P<0.05$) following treatment with melatonin plus calpeptin at 15 or 50 µg/kg, respectively; there was no significant difference between the two treatment groups.

3.3. Effects of melatonin, calpeptin or their combination on degenerating neurons, surviving neurons, macrophage/activated microglia and apoptosis in the cortex at 24 hours post-reperfusion.

The same experimental design (Figure 1A) was adopted in the study exploring the effects of melatonin at 50 μg/kg, calpeptin at 15 μg/kg or their combination on degenerating neurons, surviving neurons, macrophage/activated microglia and apoptosis in the cortex. Degenerating neurons were labelled by FJB (Figure 3A), and the inter-group difference was significant (P<0.01). When compared to the relative density of FJB+ cells of 99.99 ± 8.09% in peri-infarct cortex of the vehicle group (Figure 3B), a single ICV injection of melatonin, calpeptin or melatonin plus calpeptin significantly reduced it to 57.47 ± 14.97% (P<0.05), 64.18 ± 7.24% (P<0.05) and 42.01 ± 2.02% (P<0.01), respectively.

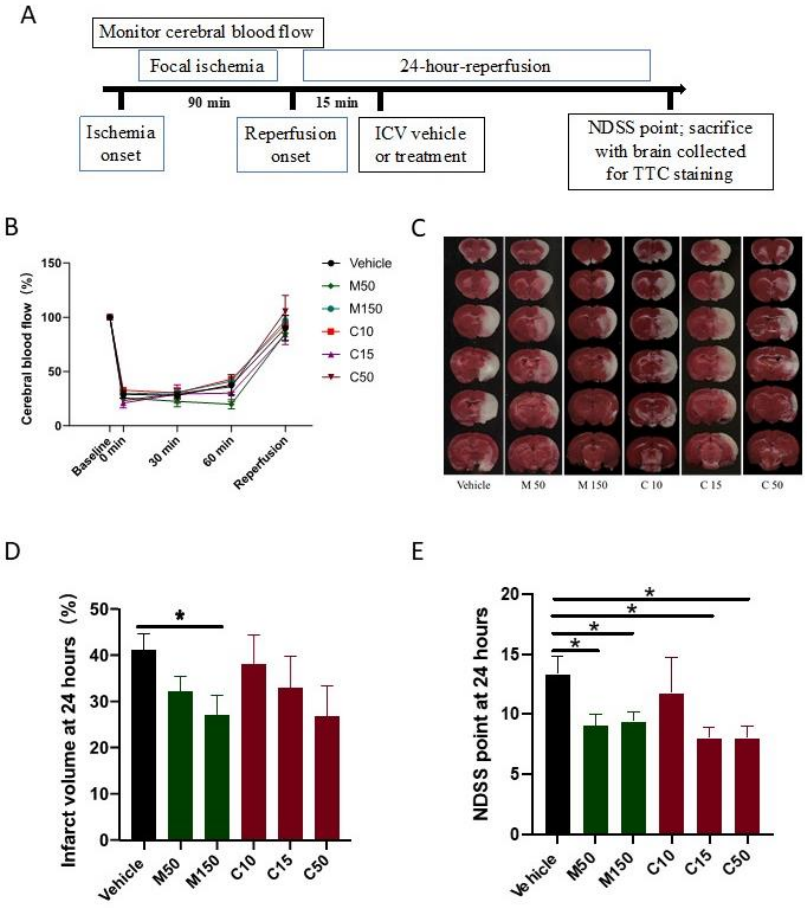

Fig. 1. Effects of ICV administration of melatonin or calpeptin on post-reperfusion neuronal injury.

*A single ICV dose of melatonin or calpeptin at different concentrations was given at 15 minutes after onset of reperfusion following 90 minutes of MCAO. (A) Experimental design with timeline. (B) Normalized regional CBF (%) at different time points of transient MCAO. (C) Representative images of different groups showing the infarct as a pale area on the TTC-reacted 2-mm thick coronal brain slices between bregma levels +4 and −8 mm. (D) Infarct volume as percentage of right hemispheric volume. (E) NDSS point. Results are expressed as mean ± SEM (Vehicle n=7, M50 n=11, M150 n=10, C10 n=4, C15 n=6, and C50 n=7). Data on regional CBF over different time points were analyzed using two-way repeated measures ANOVA followed by Tukey HSD or Games-Howell post-hoc tests. Data on infarct volume and NDSS point were compared with vehicle only group using independent samples t tests. *P<0.05 compared with the vehicle group.*

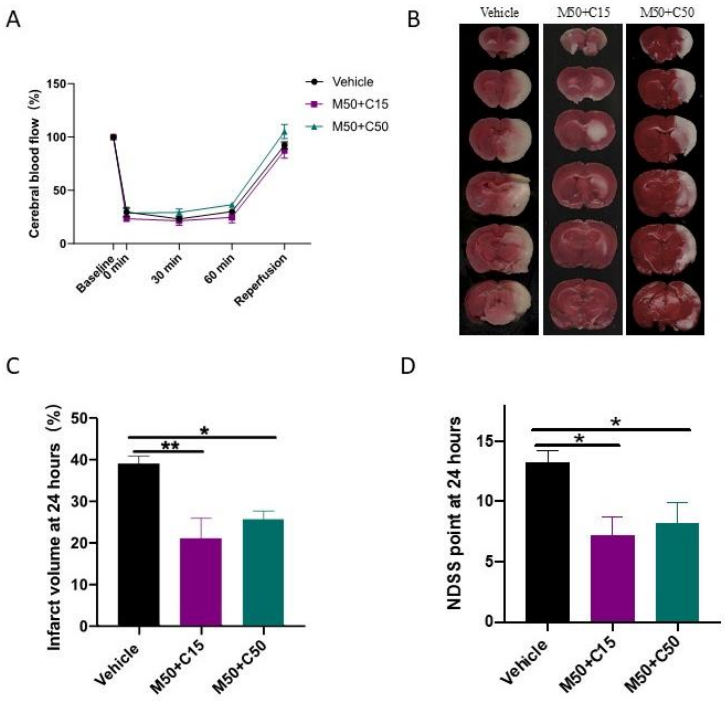

Fig. 2. Effects of ICV administration of melatonin plus calpeptin on post-reperfusion neuronal injury.

*A single ICV dose of melatonin at 50 µg/kg plus calpeptin at 15 or 50 µg/kg was given at 15 minutes after reperfusion following 90 minutes of MCAO. (A) Normalized regional CBF (%) at different time points of transient MCAO. (B) Representative images of different groups showing the infarct as a pale area on the TTC-reacted 2-mm thick coronal brain slices between bregma levels +4 and −8 mm. (C) Infarct volume as percentage of right hemispheric volume. (D) NDSS point. Results are expressed as mean ± SEM (n=6). Data on regional CBF over different time points were analyzed using two-way repeated measures ANOVA followed by Tukey HSD or Games-Howell post-hoc tests. Data on infarct volume and NDSS point were analyzed using one-way ANOVA followed by Tukey HSD post-hoc tests. *P<0.05 and **P<0.01 vs the vehicle group.*

Surviving neurons in peri-infarct cortex were labelled by NeuN (Figure 3C), and the inter-group difference was not significant. When compared to the vehicle group (Figure 3D), melatonin led to a non-significant decrease in NeuN+ cell density within peri-infarct cortex. Indeed, none of the treatment had significant effect on the relative density of NeuN+ cells. Macrophage/activated microglia in peri-infarct cortex was labelled by ED-1 (Figure 3E), and the inter-group difference was not significant. When compared to the vehicle group (Figure 3F), melatonin led to a non-significant decrease in ED-1+ cell density within peri-infarct cortex. None of the treatment had significant effect on the relative density of ED-1+ cells. Apoptosis was indicated by Bax/Bcl-2 ratio and cleaved/total caspase-3 ratio (Figure 3G). Melatonin led to a non-significant increase in Bax/Bcl-2 ratio within peri-infarct cortex. Whilst there was no significant inter-group difference to indicate an effect of any of the treatment on Bax/Bcl-2 ratio (Figure 3H), cleaved/total caspase-3 ratio within peri-infarct cortex showed a significant inter-group difference ($P<0.05$). Melatonin alone ($P<0.05$) and the combination of both ($P<0.05$) significantly suppressed cleaved/total caspase-3 ratio (Figure 3I).

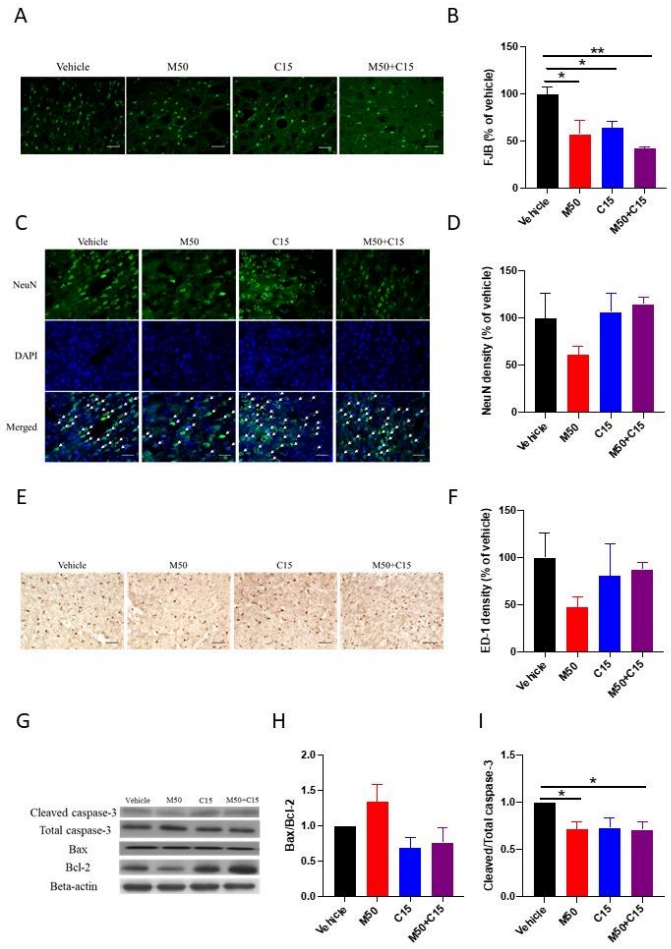

Fig. 3. Effects of ICV administration of melatonin, calpeptin or their combination on FJB+ cell density, cleaved/total caspase-3 ratio, NeuN+ cell density and ED-1+ macrophage/activated microglia at 24 hours of reperfusion.

*A single ICV dose of melatonin at 50 µg/kg, calpeptin at 15 µg/kg or melatonin at 50 µg/kg plus calpeptin at 15 µg/kg was given at 15 minutes after onset of reperfusion following 90 minutes of MCAO. (A) Representative fluorescence photomicrographs of FJB-stained brain sections at 400x magnification from different groups showing degenerating neurons in the peri-infarct cortex. (B) Relative FJB density (number of FJB+ cells per high power field) of different groups as percentage of the vehicle group. (C) Representative fluorescence photomicrographs of NeuN-stained brain sections at 400x magnification from different groups showing surviving neurons in the peri-infarct cortex. (D) Relative NeuN density (number of NeuN+ cells per high power field) of different groups as percentage of the vehicle group. (E) Representative light photomicrographs of ED-1-stained brain sections at 200x magnification from different groups showing macrophage/activated microglia in the peri-infarct cortex. (F) Relative ED-1 density (number of ED-1+ cells per high power field) of different groups as percentage of the vehicle group. (G) Representative images of expression of protein markers of apoptosis using western blot. (H) Relative band density of Bax/Bcl-2 ratio from different groups. (I) Relative band density of cleaved/total caspase 3 from different groups. Results are expressed as mean ± SEM (Vehicle n=4, M50 n=5, C15 n=4, and M50+C15 n=4 for (B), (D) and (F); n=6 for (H) and (I)). Data were analyzed using one-way ANOVA followed by Dunnett post-hoc tests. *P<0.05 and **P<0.01 compared with the vehicle group. Scale bar is 40 µm in (A) and (C) and 80 µm in (E).*

3.4. Effects of melatonin, calpeptin or their combination on regional CBF, infarct volume, neurological deficit, degenerating neurons, surviving neurons, and macrophage/activated microglia in the cortex at 72 hours post-reperfusion.

The experimental design of 90-minutes focal ischemia followed by 72 hours of reperfusion was summarized in Figure 4A. The significant overall changes in regional CBF ($P<0.001$; Figure 4B) were similar in all groups with a significant reduction at 0, 30 and 60 minutes of focal ischemia ($P<0.001$ when compared to baseline) and with a significant surge upon reperfusion (with no significant difference from baseline). The brain infarct at 72 hours was revealed by TTC staining (Figure 4C), and the inter-group difference was significant ($P<0.05$). When compared to the relative infarct volume of 44.08 ± 4.27% in the vehicle group (Figure 4D), a single ICV injection of melatonin at 50 µg/kg significantly reduced it to 29.74 ± 4.27% ($P<0.05$). A single ICV injection of calpeptin at 15 µg/kg tended to reduce the relative infarct volume to 32.73 ± 3.65% (not significant). A single ICV injection of both melatonin at 50 µg/kg and calpeptin at 15 µg/kg significantly reduced the infarction volume to 30.03 ± 3.39% ($P<0.05$). Body weight decreased after MCAO, and the variation over time points was significant ($P<0.001$); the changes were similar in all groups (Figure 4E).

The variation of NDSS points over time was significant ($P<0.005$; Figure 4F) to indicate a time-dependent decline in NDSS points. The interaction between time factor and group factor was significant ($P=0.05$). There was an overall significant difference in NDSS points between the vehicle group and the melatonin group ($P<0.05$) and between the vehicle group and the combined treatment group ($P<0.05$). The significant difference in NDSS points was seen at 24 ($P<0.01$) and 72 hours ($P<0.05$). The NDSS points at 24 hours post-reperfusion were 8.00 ± 0.96 in the vehicle group, 4.82 ± 1.04 ($P<0.05$ when compared to the vehicle group) in the melatonin group, 5.32 ± 0.82 (no significant difference from the vehicle group) in calpeptin group and 4.15 ± 0.51 ($P<0.01$ when compared to the vehicle group) in the melatonin plus calpeptin group, respectively. The NDSS points at 48 hours post-reperfusion were 6.42 ± 0.70 in the vehicle group, 4.5 ± 0.88 in the melatonin group, 4.64 ± 0.64 in calpeptin group and 4.12 ± 0.57 in the melatonin plus calpeptin group, respectively; there was no significant difference between the vehicle group and any of the treatment groups. The NDSS points at 72 hours post-reperfusion were 6.31 ± 0.75 in the vehicle group, 3.36 ± 0.67 in the melatonin group, 5.05 ± 0.80 in calpeptin group and 4.19 ± 0.55 in the melatonin plus calpeptin group, respectively. Only the difference between the vehicle group and melatonin group was significant ($P<0.05$).

The inter-group difference in FJB+ cell density within peri-infarct cortex was significant at 72 hours ($P<0.01$). When compared to the relative density of FJB+ cells of 100.00 ± 11.31% in peri-infarct cortex of the vehicle group (Figure 4G and 4H), the reduction achieved by a single ICV injection of melatonin was not significant (67.14 ± 6.72) and the reduction achieved by a single ICV injection of calpeptin at 15 µg/kg was also not significant (77.00 ± 15.68%). Only melatonin plus calpeptin achieved a significant reduction to 36.49 ± 7.01% ($P<0.01$). The inter-group difference in NeuN+ cell density within peri-infarct cortex was not significant. When compared to the vehicle group, none of the treatment had any significant effect on the relative density of NeuN+ cells within peri-infarct cortex (Figure 4I and 4J). The inter-group difference in ED-1+ cell density within peri-infarct cortex was not significant. When compared to the vehicle group, calpeptin treatment and melatonin plus calpeptin treatment led to non-significant reductions in ED-1+ cell density within peri-infarct cortex. Indeed, none of the treatment had any significant effect on the relative density of ED-1+ cells (Figure 4K and 4L).

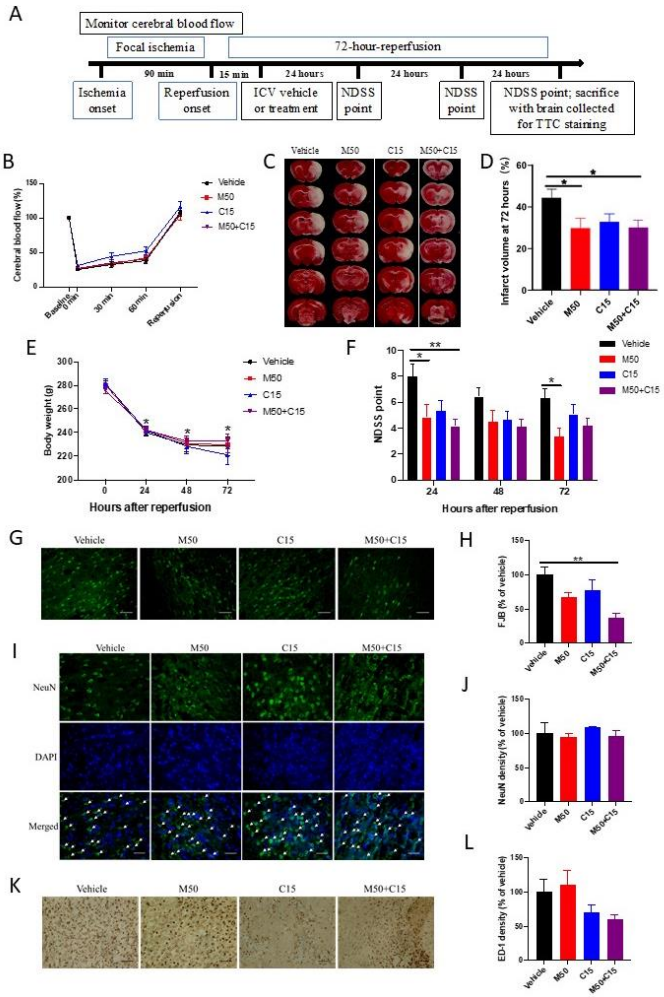

Fig. 4. Effects of ICV administration of melatonin, calpeptin or their combination on post-reperfusion neuronal injury at 72 hours.

A single ICV dose of melatonin at 50 µg/kg, calpeptin at 15 µg/kg or melatonin at 50 µg/kg plus calpeptin at 15 µg/kg was given at 15 minutes after onset of reperfusion following 90 minutes of MCAO. (A) Experimental design with timeline. (B) Normalized regional CBF (%) at different time points of transient MCAO. (C) Representative images of different groups showing the infarct as a pale area on the TTC-reacted 2-mm thick coronal brain slices between bregma levels +4 and −8 mm. (D) Infarct volume as percentage of right hemispheric volume. (E) Body weight at 0, 24, 48 and 72 hours of reperfusion. (F) NDSS point at 24, 48 and 72 hours of reperfusion. (G) Representative fluorescence photomicrographs of FJB-stained brain sections at 400x magnification from different groups showing degenerating neurons in the peri-infarct cortex. (H) Relative FJB density (number of FJB+ cells per high power field) of different groups as percentage of the vehicle group. (I) Representative fluorescence photomicrographs of NeuN-stained brain sections at 400x magnification from different groups showing surviving neurons in the peri-infarct cortex. (J) Relative NeuN density (number of NeuN+ cells per high power field) of different groups as percentage of the vehicle group. (K) Representative light photomicrographs of ED-1stained brain sections at 200x magnification from different groups showing infiltrating macrophage/microglia in the peri-infarct cortex. (L) Relative ED-1 density (number of ED-1+ cells per high power field) of different groups as percentage of the vehicle group. Results are expressed as mean ± SEM (Vehicle n=11, M50 n=8, C15 n=9, and M50+C15 n=13 for (B), (C), (D), (E) and (F); n=4 for (H), (I) and (J)). Data on regional CBF, body weight and

*NDSS point over different time points were analyzed using two-way repeated measures ANOVA followed by Tukey HSD or Games-Howell post-hoc tests. Data on infarct volume, FJB density, NeuN density and ED-1 density were analyzed using one-way ANOVA followed by Dunnett post-hoc tests. *P<0.05 and **P<0.01 compared with the vehicle group. Scale bar is 40 µm in (G) and (I) and 80 µm in (K).*

4. DISCUSSIONS

The overall objective of the present study is to explore the potential benefits of an ICV injection of melatonin, calpeptin or their combination in protection against post-reperfusion neuronal injury in rats and the possible underlying mechanisms. First, melatonin or calpeptin given at 15 minutes after onset of reperfusion appears to achieve a dose dependent reduction of infarct volume when compared to the vehicle group after 90 minutes of focal ischemia and 24 hours of reperfusion. The reduction is significant for treatment with melatonin at 150 µg/kg. The benefit in amelioration of neurological deficit is more evident, and the difference is significant for all treatment groups except for the treatment with calpeptin at 10 µg/kg. Second, either melatonin at 50 µg/kg plus calpeptin at 15 µg/kg or melatonin at 50 µg/kg plus calpeptin at 50 µg/kg given at 15 minutes after onset of reperfusion significantly reduced the infarct volume and neurological deficit at 24 hours. No significant difference was observed between the two combinations.

Third, melatonin at 50 µg/kg, calpeptin at 15 µg/kg, and melatonin at 50 µg/kg plus calpeptin at 15 µg/kg were compared with the vehicle group regarding degenerating neurons, surviving neurons, macrophage/activated microglia and activation of apoptosis in peri-infarct cortex. FJB was used to identify neuronal degeneration following cerebral ischemia due to its higher affinity than Fluoro-Jade (55). As a sensitive marker for acute neuronal injury, FJB may not be suitable for a delayed assessment of neuronal degeneration; the number of FJB+ cells would increase to a maximum between 24 and 72 hours and then decrease to the basal level by 1 week after stroke (56). The beneficial effect of treatment with melatonin, calpeptin or their combination can be partly attributed to a reduction in neuronal degeneration within peri-infarct cortex. NeuN is a neuronal marker (57) and can be used to reveal surviving neurons. The insignificant reduction in density of NeuN+ cells after treatment with melatonin and a lack of inter-group difference in density of surviving neurons within peri-infarct cortex are surprising findings which warrant further investigations.

ED-1 is a marker for macrophage and activated microglia (58). The insignificant reduction in density of ED-1+ cells within peri-infarct cortex after treatment with melatonin alone and a lack of inter-group difference in density of macrophage/activated microglia suggest that the benefit of ICV administration of melatonin and/or calpeptin after onset of reperfusion is not mediating via activated microglia/infiltrating macrophages. Microglia provide the first and main form of active immune defense in the central nervous system. Similar to peripheral macrophages, activated microglia can stimulate activated CD4+ T cells to differentiate into Th1 and Th2 cells and then generate pro-inflammatory and anti-inflammatory cytokines, respectively (59). Indeed, the activated microglia can release anti-inflammatory cytokines and remove apoptotic or necrotic neurons by phagocytosis (60). On the other hand, activated microglia can release pro-inflammatory cytokines and chemokines to aggravate neuroinflammation (61). Following ischemic stroke, monocytes from peripheral blood will provide a second source of macrophages (62). A plausible explanation of the present results of ED-1+ cells within peri-infarct cortex is that the density of activated microglia/infiltrating macrophages is not as relevant as their exact roles in neuroinflammation, viz. protective/anti-inflammatory vs. damaging/pro-inflammatory

phenotypes. In addition, some activated microglia/infiltrating macrophages may not be detected by ED-1. For example, microglia in the ischemic infarct are positive for both Iba1 and ED-1 whilst Iba1+ and ED-1– microglia are found in peri-infarct cortex (63, 64). Further studies are needed.

Necrosis is a form of traumatic cell death resulting from acute cellular injury. In contrast, apoptosis is a highly regulated and controlled process. Bax binds with Bcl-2 to activate the intrinsic pathway of apoptosis, making Bax/Bcl-2 ratio an upstream marker of apoptosis (53). Activation of both intrinsic and extrinsic pathways of apoptosis leads to activation of caspase 3, making cleaved/total caspase 3 ratio, a reliable marker of apoptosis similar to TUNEL assay (53, 65). Treatment with melatonin at 50 µg/kg or melatonin plus calpeptin at 15 µg/kg significantly suppresses cleaved/total caspase-3 ratio within the peri-infarct cortex. It is intriguing to find that the suppression in cleaved/total caspase-3 ratio achieved by calpeptin at 15 µg/kg fails to reach statistical significance. None of the treatments hassignificant effect on Bax/Bcl-2 ratio within the peri-infarct cortex. The present findings support the anti-apoptotic effects of melatonin and calpeptin and suggest an important role of extrinsic pathway activation in our 90-minute rat MCAO model. More studies are warranted.

Finally, the effects of an ICV administration of melatonin at 50 µg/kg, calpeptin at 15 µg/kg, and melatonin at 50 µg/kg plus calpeptin 15 µg/kg given at 15 minutes of reperfusion after 90 minutes of focal ischemia were compared with the vehicle group at 72 hours. All the treatments had reduced infarct volumes at 72 hours compared to the vehicle group while the significant differences were observed with melatonin and melatonin plus calpeptin treatments. This 72 hour-lasting beneficial effect was more profound in melatonin group than that of calpeptin group.

After reperfusion, the neurological deficit reached its peak at 24 hours and gradually declined at 48 and 72 hours. At 24 hours of reperfusion, melatonin and melatonin plus calpeptin but not calpeptin significantly reduced neurological deficit. At 72 hours of reperfusion, only melatonin but not the other two treatments achieved a significant benefit compared to the vehicle treatment, indicating a lasting benefit of melatonin in reducing neurological deficit. However, only the treatment with the combination ameliorated neuronal degeneration at 72 hours of reperfusion. All the treatments did not significantly impact surviving neurons and macrophage/activated microglia at 72 hours of reperfusion.

The post-reperfusion intervention is more relevant to the clinical setting of acute treatment of ischemic stroke especially following chemical thrombolysis or mechanical thrombectomy. Although the neuroprotective potential of melatonin via an IP or IV injection has been extensively evaluated in many experimental stroke models involving different mammalian species (9, 41), beneficial effects of ICV administration of melatonin has not been previously reported. As ICV administration is appropriate for calpeptin (10), the same route is also used for melatonin in this study. Melatonin post-reperfusion treatment seemed not as effective as its given prior to ischemia or before reperfusion. Studies have reported that time of the first dose of melatonin is a crucial factor for its neuroprotective effects (9, 66). Melatonin treatment is most effective with the first dose given within 1 hour of reperfusion, and additional doses barely provide further benefits (9). Similar findings have been observed in myocardial ischemia-reperfusion injury in which melatonin given before ischemia is much more effective than when it is given at the time of reperfusion (67). Although the ICV dose is about 1/100 of that for IP injection (45), the beneficial effects of a single post-reperfusion ICV injection of melatonin in ameliorating injury are emerging at 24 hours and become evident at 72 hours. The underlying neuroprotective mechanisms are partly mediated via early suppression of both neuronal degeneration and caspase 3 activation.

In a previous study, calpeptin at much higher dose of 180–200 μg/kg was given 30 minutes before an 120-minute MCAO rat model. The results showed that the infarct volume, neurological deficit, caspase 3 (using immunohistochemistry) and neuronal apoptosis (using TUNEL assay) in hippocampal CA1 region were significantly reduced at 12, 24 and 48 hours (10). The present post-reperfusion ICV administration of calpeptin at much lower doses also achieved some benefits at 24 hours, but the benefits failed to reach statistical significance at 72 hours. The underlying neuroprotective mechanism is partly mediated via early suppression of neuronal degeneration.

A combination of melatonin and calpeptin achieved better protection than each alone at 24 hours post-reperfusion. At 72 hours, the combination and melatonin alone showed similar protective effects which were better than that of calpeptin alone. As to the suppression of neuronal degeneration, the combination seemed to have more long-lasting effect than the others. Even though melatonin attenuated reperfusion-induced endogenous calpain upregulation (47), it appeared that the combination potentiated the neuroprotective benefit of melatonin. In the clinical context, neuroprotective acute reperfusion treatment can reduce reperfusion injury and even hemorrhagic transformation risk. A more focused delivery of neuroprotective drugs can be made via endovascular route following successful mechanical thrombectomy (i.e., busting of the clot). Further studies should be conducted to evaluate the benefit of post-ischemic administration of different combinations of melatonin and calpeptin after relatively longer periods of reperfusion.

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AUTHORSHIP

YF and RTFC were involved in development of the hypotheses and experimental design. YF conducted the majority of the experiments. YF, QX and RTFC performed the data analyses. YF and QX drafted the manuscript. RTFC critically revised the manuscript. YF, QX and RTFC approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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