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Effects of Alpha-Mangostin Encapsulated in Nanostructured Lipid Carriers in Mice with Cerebral Ischemia Reperfusion Injury

(Kesan Alfa-Mangostin yang Dikapsulkan dalam Pembawa Lipid Berstruktur Nano pada Tikus dengan Kecederaan Reperfusi Iskemia Serebrum)

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ABSTRACT

Cerebral ischemia reperfusion injury (CIRI) is a phenomenon in which the cerebral blood supply is restored after a period of ischemia, resulting in irreversible damage to brain tissue. Oxidative stress plays a crucial role in the development of CIRI, therefore, targeting oxidative stress might be an effective strategy for CIRI prevention and treatment. Many therapeutic substances possess antioxidant and protective properties against neurodegenerative disorders but lack of in vivo *application due to their solubility, and bioavailability. We investigated the effects of alpha-mangostin (*αM*) encapsulated in nanostructured lipid carriers (*αM-*NLC) on CIRI in mice. Forty male ICR mice were randomly divided into four groups: Sham, ischemia reperfusion (IR), ischemia reperfusion with 25 mg/kg of αM (IR+*αM*), and ischemia reperfusion with 25 mg/kg of αM-NLC (IR+*αM-*NLC). After 6 days of oral administrations, IR was delivered using 30 min of bilateral common carotid artery occlusion, followed by 45 min of reperfusion. Cerebral infarction volume, hippocampal neuronal and corpus callosum (CC) white matter damage, malondialdehyde (MDA) level, and catalase (CAT) activity were evaluated. Our results indicated that αM and αM-NLC prevent lipid peroxidation as well as hippocampal CA1, CA3, and CC damage ($p<0.05$). Only αM-NLC prevented cerebral infarction and enhanced CAT activity ($p<0.05$). We therefore conclude that* αM and αM-*NLC have neuroprotective effects against CIRI, and NLC increases therapeutic efficacy of αM against CIRI.*

Keywords: Alpha-mangostin; cerebral ischemia reperfusion injury; nanostructured lipid carrier; neurodegeneration; oxidative stress

ABSTRAK

Kecederaan reperfusi iskemia serebrum (CIRI) adalah fenomena bekalan darah serebrum dipulihkan setelah tempoh iskemia dan mengakibatkan kerosakan yang tidak dapat dipulihkan pada tisu otak. Tekanan oksidatif memainkan peranan penting dalam perkembangan CIRI, oleh itu, pensasaran tekanan oksidatif mungkin merupakan strategi yang berkesan bagi pencegahan dan rawatan CIRI. Banyak bahan terapeutik memiliki sifat antioksidan dan pelindung terhadap gangguan neurodegeneratif tetapi kekurangan aplikasi in vivo *disebabkan keterlarutan dan ketersediaan bio. Kami mengkaji kesan alfa-mangostin (αM) yang dikapsulkan dalam pembawa lipid berstruktur nano* (αM-*NLC) pada CIRI atas tikus. Empat puluh tikus ICR jantan dibahagikan secara rawak kepada empat kumpulan: Sham, reperfusi iskemia (IR), reperfusi iskemia dengan 25 mg/kg αM* (*IR*+αM) *dan reperfusi iskemia dengan 25 mg/kg of αM-NLC* (*IR*+αM-*NLC*). *Selepas pemberian oral selama 6 hari, IR dihantar dengan menggunakan oklusi arteri karotid dua hala selama 30 min, diikuti dengan reperfusi selama 45 min. Isipadu infarksi serebrum, kerosakan bahan putih hipokampus neuron dan korpus kalosum (CC), tahap malondialdehid (MDA) dan aktiviti pemangkin (CAT) dinilai. Hasil kajian kami menunjukkan bahawa αM dan αM-NLC mencegah peroksidasi lipid serta kerosakan hipokampus CA1, CA3, dan CC* (*$p<0.05$*). *Hanya αM-NLC yang menghalang infraksi serebrum dan meningkatkan aktiviti pemangkin CAT* (*$p<0.05$*). *Oleh itu, kami menyimpulkan bahawa αM dan αM-NLC mempunyai kesan neuropelindung terhadap CIRI dan NLC meningkatkan keberkesanan terapeutik αM terhadap CIRI.*

Kata kunci: *Alfa-mangostin; kecederaan iskemia reperfusi serebrum; neurodegenerasi; pembawa lipid berstruktur nano; tekanan oksidatif*

Introduction

Cerebral ischemia reperfusion injury (CIRI) is a common feature of ischemic stroke, which occurs when the restoration of blood flow to ischemic tissue (Liang et al. 2015). Ischemic stroke is the second leading cause of death worldwide and imposes significant physical and mental disability burdens (Donkor 2018). Vulnerable brain areas affected by ischemia include the cerebral cortex, hippocampus, basal ganglia, thalamus, and deep white matter areas. Damage can occur *via* a variety of pathomechanisms, including excitotoxicity, acidosis, intracellular calcium overload, inflammation, necrosis, and apoptosis (Lee et al. 2000). Therapeutic approaches for ischemia include therapeutic approaches for ischemia include the restoration of blood flow as soon as, but early stage blood flow restoration can exacerbate the extent of tissue injury because the reintroduction of oxygen induces excessive production of reactive oxygen species (ROS), which attract cellular components, inducing oxidative stress and, apoptotic cell death (Poellmann et al. 2018). Oxidative stress results from an imbalance between ROS generation and antioxidant defense systems (Sun et al. 2018) and plays a critical role in the pathogenesis of CIRI (Kishimoto et al. 2019). Brain tissue is highly susceptible to oxidative damage because it has low antioxidant activity levels and high levels of polyunsaturated fatty acids, which are the targets of ROS attraction (Kalogeris et al. 2012). Therefore, increasing antioxidant levels in the brain is one strategy for delaying or preventing brain tissue damage in CIRI. Previous studies have explored the administration of exogenous antioxidants to prevent or ameliorate the harmful effects of ischemia reperfusion (IR) injury in animal models (Tapeinos et al. 2017; Wicha et al. 2017).

Alpha-mangostin (αM), a novel polyphenolic xanthone derivative found in mangosteen pericarp, possesses a variety of pharmacological properties such as antioxidant, anticancer, and cytotoxicity activities (Ghasemzadeh et al. 2018). Evidence from *in vivo* and *in vitro* studies indicates that αM has a protective effect on oxidative stress, attenuates inflammatory responses, and improves mitochondrial dysfunction in neurodegenerative conditions (Catorce et al. 2016; Márquez-Valadez et al. 2009; Wang et al. 2016; Yao et al. 2016). In addition, pharmacokinetic studies have reported that αM has high tissue distribution except in the brain as well as low oral bioavailability due to its poor water solubility which impedes it from crossing the blood-brain barrier. Moreover, αM is also absorbed rapidly in the gastrointestinal tract and excreted in the liver and kidneys (Choi et al. 2014; Zhang et al. 2016). To improve the neuroprotective efficacy of αM, nanostructured lipid carriers (NLC) have been introduced (Poellmann et al. 2018). Previous studies have reported that αM loaded nanoparticles can improve the therapeutic efficacy of αM in Alzheimer's disease *via* a variety beneficial effects: improved biodistribution, enhanced brain clearance of amyloid beta, attenuated neuroinflammation response, and ameliorated neurological change compared with free αM (Yao et al. 2016).

Nanoparticle delivery vehicles are colloid materials that include particulate substances that are approximately 1-500 nm in diameter (Poellmann et al. 2018). Medical nanoparticle applications include packing drugs, delivering drugs to target organs, maintaining pharmacological properties, increasing stability, and improving bioavailability (Masserini 2013). In this study, to encapsulate αM, we used NLC, which are lipid nanoparticles (average diameter $\leq$ 100 nm) that have advantages over other nanoparticles, including high drug loading, enhanced stability, and the ability to prevent drug expulsion during storage (Tapeinos et al. 2017; Yostawonkul et al. 2017). Therefore, we aimed to investigate the neuroprotective effect of free αM and NLC-encapsulated αM in mice model of CIRI using histopathological and biochemical evaluations.

Materials and Methods

Animals

Eleven-week old ICR mice (*Mus musculus*) weighing 40±2 g were obtained from the National Laboratory Center of Mahidol University (Nakhon Pathom, Thailand). All animals received a standard diet (mouse diet food No. 082G) and reverse osmosis (RO) water, were treated humanely, and were maintained at 23±2 °C with a 12 h-light/12 h-dark cycle. The experimental procedure was approved by the Animal Ethics Committee, Faculty of Science, Kasetsart University (ID#ACKU61-SCI-011).

Drugs and Chemicals

NLC-encapsulated αM (αM-NLC) and free αM were procured from the National Nanotechnology Centre (NANOTEC), Pathum Thani, Thailand.

Experimental Procedure

Forty mice were randomly divided into 4 groups (n=10): Sham (10% Tween 80 used as a vehicle), IR (10% Tween 80), IR+αM group (αM 25 mg/kg dissolved in 10% Tween 80 with IR induction), and IR+αM-NLC (αM-NLC 25 mg/

kg with IR induction). Oral administration of the vehicle, αM, and αM-NLC was performed daily for 6 days prior to IR induction.

CEREBRAL ISCHEMIA REPERFUSION INJURY INDUCTION

After 2 h of fasting, all mice were anesthetized *via* intraperitoneal injection of 45 mg/kg of sodium pentobarbital and 0.60 mg/kg of atropine. CIRI was induced in the IR, IR+αM, and IR+αM-NLC groups using 30 min of bilateral common carotid artery occlusion, followed by 45 min of reperfusion (Sakamula & Thong-Asa 2018; Sakamula et al. 2019). Mice in the Sham group only underwent surgical exposure of the bilateral common carotid artery rather than occlusion. After completion of IR procedures, all mice were decapitated, and their brains were collected quickly to evaluate brain infarction volume and perform histopathological and biochemical evaluations.

CEREBRAL INFARCTION VOLUME

Brains were cut into serial sections (2 mm thick in an acrylic brain block) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 10 min, then stored in 10% neutral buffed formalin for 24 h. Brain images were captured and analyzed for infarction volume using Image J system. The red regions represent the normal tissue, and the pale region represents the infarct area (Sakamula et al. 2019).

HISTOPATHOLOGICAL ANALYSIS

After infarction volume evaluation, the brain sections were hydrated in a subsequently diluted concentration of ethanol, cleared in xylene, embedded in paraffin, and sliced into 5 μm thick sections. Hippocampal cell death was observed in the CA1, CA3, and DG subregions using 0.1% cresyl violet staining. Dead cells are indicated by dark crests of violet stain, the disappearance of the nucleus, and nucleoli with vacuoles around the cells (Thong-Asa et al. 2017). Total viable cells and dead cells were counted in 5 slices of dorsal hippocampus which start from the bregma -1.34 to -2.00 mm (Paxinos & Franklin 2008). The result is represented as the percentage of dead cells. White matter density of the corpus callosum (CC) was observed using 0.1% luxol fast blue staining. Image J system was used for CC white matter density measurement.

BIOCHEMICAL EVALUATION

Brains were washed in 0.9% cold normal saline and homogenized with phosphate buffer saline (PBS) (50 mM, pH 7.4). Homogenized brain tissue was separated into 2 parts; the first was used to evaluate malondialdehyde (MDA) levels, and the was further centrifuged at 10,000 rpm at 4 °C for 10 min for supernatant evaluation of catalase (CAT) activity.

MALONDIALDEHYDE ASSAY

MDA is lipid peroxidation product used as an oxidant indicator. Brain homogenate was incubated in 4% sodium dodecyl sulfate (SDS), 20% acetic acid and 0.5% thiobarbituric acid (TBA) and heated at 95 °C for 1 h. After cooling and centrifugation (10,000 rpm at 4 °C for 10 min), the supernatant was read at 532 nm using a microplate reader. MDA levels were determined using a standard curve ($y=0.0058x+0.0305$, $R^2=0.9963$), and the values were expressed as μmol/mg of tissue protein (Badmus et al. 2011).

CATALASE ASSAY

The volume of the 50 μL supernatant was increased to 3 mL using PBS (50 mM, pH 7.4) containing H_2O_2 and read at 240 nm for 3 min (30 s intervals). CAT activity was expressed as U/mg of protein using the extinction coefficient of H_2O_2, which is 0.0436 cm^{-1} (Hadwan & Abed 2016).

STATISTICAL ANALYSIS

All data were expressed as mean ± standard error (SE). All statistical analyses were performed using the Statistical Package for Social Sciences, version 16.0 (SPSS, Inc., Chicago, IL). Groups were compared using a one-way ANOVA with Fisher's PLSD post hoc test. Differences were considered statistically significant at $p<0.05$.

Results and Discussion

NLC is used as a delivery system to facilitate high drug loading, increase loading efficiency, increase stability, prolong half-life, enhance oral bioavailability, provide controlled release of encapsulated materials, exhibit low toxicity, and can cross the blood-brain barrier (Fang et al. 2012; Tamjidi et al. 2013). They are used in therapies for brain diseases such as neurodegenerative disorder and brain tumors (Liu et al. 2010; Mu et al. 2011). In the present study, we applied NLC to αM and found that free-αM and αM-NLC exert protective effects against oxidative stress, hippocampal damage, and white matter damage in CIRI mice. Importantly, however, we found that αM-NLC prevented cerebral infarction and hippocampal CA3 cell death more effectively and enhanced CAT activity. Thus,

the present study's results support the benefits of NLC use. For instance, the percentage of cerebral infarction was significantly increased in the IR group compared to Sham group ($p<0.05$), whereas in the IR+αM-NCL group, it decreased significantly compared to the IR group ($p<0.05$), but not the IR+αM group ($p>0.05$, Figure 1). This indicates that free αM did not prevent cerebral infarction, but αM-NLC increased the efficacy of cerebral infarction prevention in CIRI mice. The advantage of αM-NLC may involve prohibiting the effects of ATP and phosphocreatine discretions (Buelna-Chontal et al. 2011).

We selected 25 mg/kg as the lowest active dose used *in vivo* because a prior study demonstrated its preventive effects for animal models of degenerative disease by significantly reducing MDA and significantly increasing superoxide dismutase (SOD), CAT, and GSH (Kumar et al. 2016). Our results indicate that the MDA level in the IR group increased significantly compared to that of the Sham group ($p<0.05$). The MDA levels of the IR+αM and IR+αM-NLC groups decreased significantly compared to those of the IR group ($p<0.05$), which also indicates ameliorative effects of αM and αM-NLC on brain tissue lipid peroxidation. Although IR induction did not significantly reduce CAT activity ($p>0.05$), αM-NLC enhanced CAT activity significantly compared to the IR group ($p<0.05$, Figure 2). Alpha-mangostin is known as an antioxidant, the previous studies proved that αM has a protective effect in models of cardiac ischemia reperfusion injury by reducing oxidative stress resulting from decreased MDA levels and increased enzymatic activities of antioxidants such as SOD and CAT (Buelna-Chontal et al. 2011; Sampath & Vijayaraghavan 2007). In our study, which is the first to investigate the effect of αM in the brain tissue of CIRI mice, we found that αM had the potential to protect brain tissue by decreasing MDA levels but not effect on CAT activity. During the CIRI process, ROS production increased remarkably, resulting in lipid peroxidation, which affects lipid membranes. MDA is a well-known marker of oxidative stress, and it is one of the final product of lipid peroxidation (Gonzalez-Montero et al. 2018; Sun et al. 2018). In addition to CIRI induced lipid peroxidation, it has been reported along with the decrease of antioxidant enzymes such as SOD and glutathione peroxidase (Wicha et al. 2017), but the CAT enzyme is controversial. Many studies have reported that CIRI reduces CAT activity, but a few studies have suggested that CAT activity might increase in ischemic brain tissue (Lee & Won 2014). Our results showed that only CAT activity tend to decrease after IR induction and may be associated with a mild degree of IR (Yan et al. 2011).

A previous study claimed that αM affects CAT activity in heart and kidney tissue by inducing CAT expression against toxicity (Perez-Rojas et al. 2009), but in our study, αM did not increase CAT in the brain tissue due to the mild degree of IR; for this reason, it also did not decrease CAT. In the present study, we found that αM-NLC enhanced CAT activity compared to free αM. Therefore, NLC use may improve αM's pharmacokinetic properties in brain tissue, as it enhanced CAT activity in the present study.

We also demonstrated the beneficial effect of NLC in vulnerable brain areas. The percentage of hippocampal cell death in CA1 and CA3 increased significantly in IR group compared with the Sham group ($p<0.05$), but not in DG ($p>0.05$, Figure 3). The IR + αM and IR + αM-NLC groups showed a significant reduction of CA1 and CA3 neuronal death when compared to the IR group ($p<0.05$). It is interesting that the percentage of dead cells in CA3 of the IR + αM group was significantly higher than in the Sham group ($p<0.05$) but not the IR + αM-NLC group. This result indicates that NLC can increase αM's efficacy in preventing CA3 damage. The hippocampus plays an important role in learning and memory, and this brain area is sensitive to ischemia (Aboutaleb et al. 2016). The mechanism of hippocampal injury and death is associated with excitotoxicity because the hippocampus has many glutamate NMDA receptors (N-methyl-D-aspartate receptors) and easily activates excitotoxicity (Jia et al. 2009). In our study, CIRI induced hippocampal cell death in CA1 and CA3, but it did not affect DG, which a has higher expression of glia glutamate transporter-1 (GLT-1) and a function for removing glutamate from extracellular spaces and maintaining extracellular glutamate in the brain below neurotoxic levels (Rao et al. 2001; Rothstein et al. 1996). Although, as previously described, CA1 is more susceptible than CA3 to global ischemic conditions, recently detailed that Zn^{2+} mobilization excitotoxic induced neuronal death may differ in these two areas during oxygen and glucose deprivation in the ischemic period. CA1 showed 10-30 min of rising of cytosolic Zn^{2+} levels, followed by 40-60 min of recovery, but the recovery period for cytosolic Zn^{2+} increase after oxygen and glucose deprivation did not occur in CA3 (Medvedeva et al. 2017). This may indicate that CA3 is more susceptible to IR, in which ischemia results in oxygen and glucose deprivation induced excitotoxicity is followed by reperfusion with ROS augmentation, causing injury exacerbation. NLC might increase αM's ability to improve excitotoxicity and Zn^{2+} mobilization in CA3 during the ischemic period, and it might enhance αM's efficacy in free radical scavenging during reperfusion. As a result, in the present study, the

αM-NLC group maintained CA3 viability and did not differ from the Sham group in this regard. However, further studies are necessary to determine the precise mechanisms. Alpha-mangostin exhibited a protective effect not just in vulnerable hippocampal areas, but in the white matter as well. CC density decreased significantly in the IR group compared to the Sham group ($p<0.05$), but in the IR+αM and IR+αM-NLC groups, decrease in the percentage of CC density was mitigated significantly compared with the IR group ($p<0.05$, Figure 4). This indicates that αM and αM-NLC effectively prevented white matter damage in CIRI mice. The CC is the largest white matter structure in the brain and is exquisitely vulnerable to ischemia (Liang et al. 2015). In addition, oligodendrocytes in white matter are highly sensitive to ischemia-induced oxidative stress, excitotoxicity, and inflammation, which results in white matter density reduction after ischemia (Wang et al. 2016). It is likely that αM exerts its protective effect against brain tissue damage through its properties as a free radical scavenger (Márquez-Valadez et al. 2009).

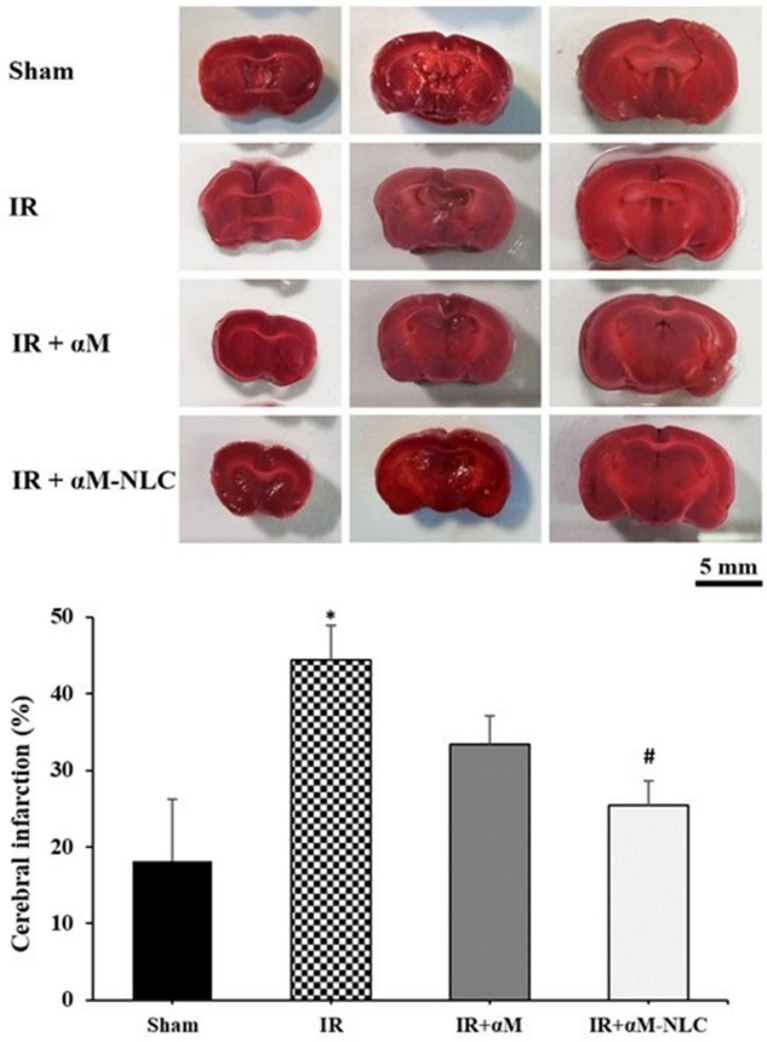

FIGURE 1. Photomicrograph of the ttc stained brain sections. The scale bar indicates a distance of 2.5 mm. Histogram of the percentage of cerebral infarction volume. Values expressed as mean ± SEM. *indicates a significant difference compares to the Sham group. #indicates a significant difference compared to the IR group

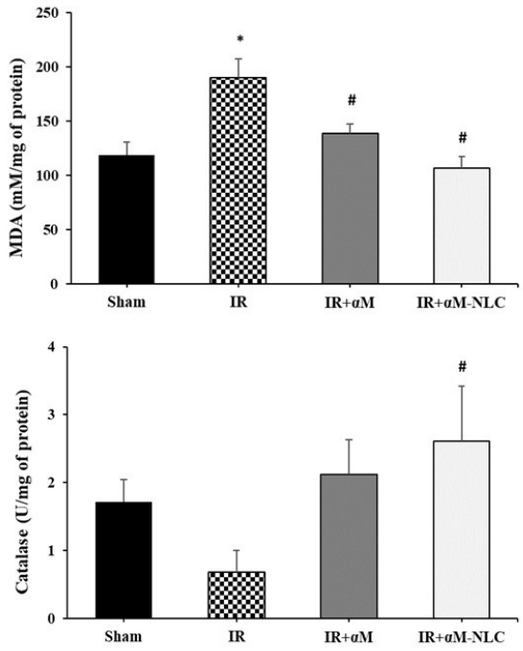

FIGURE 2. Histogram of the brain tissue's oxidative status. MDA and catalase. Values expressed as mean ± SEM. *indicates a significant difference compared to the Sham group. #indicates a significant difference compared to the IR group

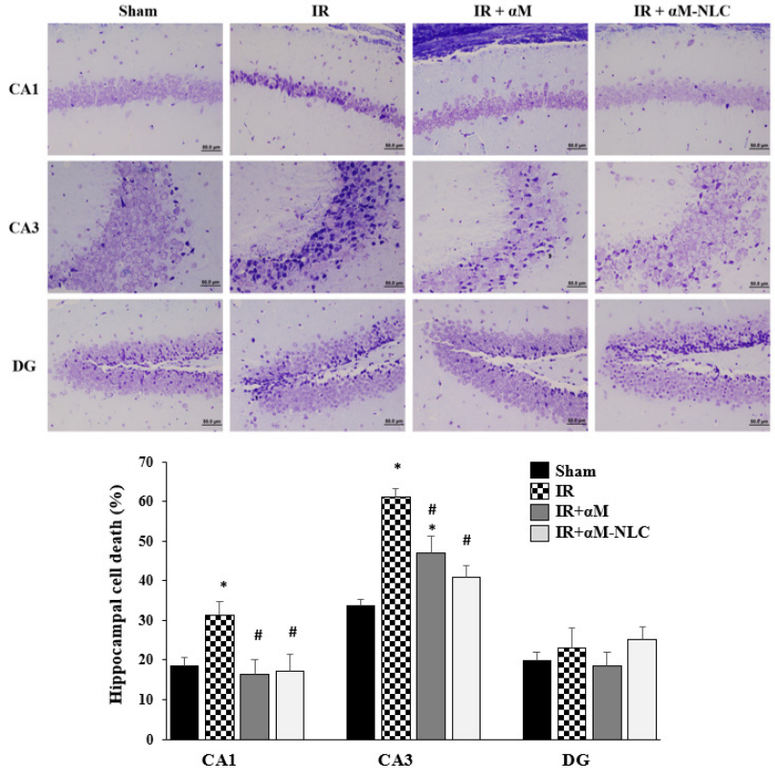

FIGURE 3. Photomicrograph of the dorsal hippocampal subregions CA1, CA3, and DG with 0.1% cresyl violet staining. Captured at 200× magnification; scale bar indicates a distance of 50 µm. Histogram of the percentage of hippocampal cell death. Values expressed as mean ± SEM. *indicates a significant difference compared to the Sham group. #indicates a significant difference compared to the IR group

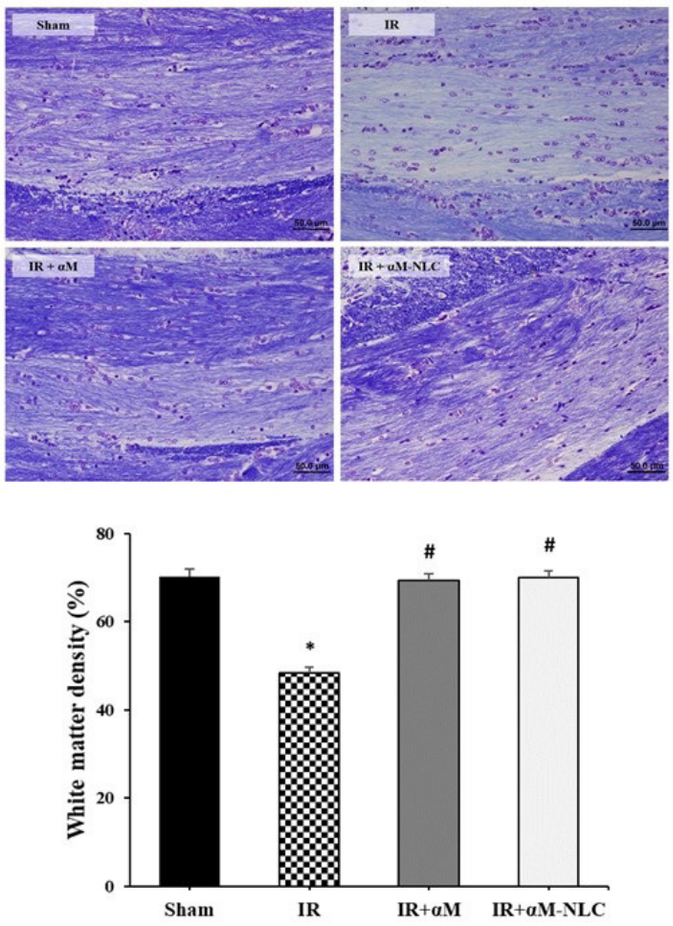

FIGURE 4. Photomicrograph of CC white matter density with 0.1% luxol fast blue staining. Captured at 200× of magnification; scale bar indicates a distance of 50 µm. Histogram of the percentage of white matter density. Values expressed as mean ± SEM. *indicates a significant difference compared to the Sham group. #indicates a significant difference compared to the IR group

CONCLUSION

Pretreatment with αM 25 mg/kg attenuated hippocampal CA1 and CA3 degeneration, CC white matter damage, and MDA lipid peroxidation, whereas NLC improved the efficacy of αM by ameliorating cerebral infarction and hippocampal CA3 degeneration and enhancing CAT activity in CIRI mice. Therefore, αM may be a promising potential protective agent for use in CIRI, and NLC could improve αM's therapeutic efficacy.

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