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Research Article

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The Neuroprotective Mechanism of Antrodia Camphorata on the Ischemic Cerebral Cortex is Correlated to the Alterations of Lipid Peroxidation Status and Essential Element Concentration

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Abstract Antrodia camphorata (AC) has been suggested as an alternative Chinese medicine commonly used to protect and treat liver diseases due to its beneficial functions of anti-inflammation and anti-oxidation. Essential element of magnesium (Mg), zinc (Zn), iron (Fe), and copper (Cu) is required for brain functions. Our study was to explore whether antrodia camphorata is able to protect the ischemic brain cortex from oxidative injury and this neuroprotection is correlated with the disturbance of lipid peroxidation and essential elements. Forty male Sprague-Dawley rats were randomly divided into four groups as follows: control, ligation, AC, and prevention. The control and ligation rats were intraperitoneally injected with physiological saline and the AC and prevention rats were given with AC (100mg/Kg) once a day for 7 consecutive days. Cerebral ischemic injury was induced by ligation of the right middle cerebral artery (RMCA) and right common carotid artery (RCMCA) for 1 hour followed by harvesting and homogenizing cerebral cortex tissues to analyze the levels of MDA, Mg, Zn, Fe, Cu, and the Zn/Cu ratio. Experimental observations indicate that ischemic stroke obviously increased the levels of MDA, Fe, and Cu but decreased Zn/Cu ratio, Mg, and Zn concentrations. However, pretreating of rats with AC before ischemic insult markedly reversed all experimental results. Accordingly, we suggest that neuroprotection of AC on the ischemic cerebral cortex is highly associated not only with declining the level of lipid peroxidation, Fe, and Cu but also with enhancing the levels of Mg, Zn, and the Zn/Cu ratio.

Keywords Antrodia camphorata · ischemic stroke · lipid peroxidation · Zn/Cu ratio · essential element.

1. Introduction

It is also recognized that ischemic stroke is the major type of stroke and mainly results in disability and mortality in older people worldwide [1-4]. The occurrence of ischemic stroke not only generates a large amount of toxic reactive oxygen species (ROS) but also elevates oxidative stress and injury in the affected brain via deleterious lipid peroxidation [3-4]. In general, the reactive processes of adverse lipid peroxidation consist of three steps including initiation, propagation, and termination, and eventually in producing a variety of chemical products [3-4]. Among

them, the MDA is not only the most mutagenic product but also its chemical property is very stable. In this regard, MDA has been used for many years as a convenient biomarker to evaluate the intensity of lipid peroxidation [3-4].

Antrodia camphorata is one of the traditional Chinese medicines commonly used as complementary and alternative medicines in Asia countries for treating chemical intoxication, liver disease, and diarrhea [2-4]. The former investigation has proposed that antrodia camphorata induces apoptosis in human promyelocytic leukemia (HL-60) cells [5]. Meanwhile, antrodia camphorata exerts a powerful free radical scavenging ability so as to decline free radical-mediated hepatic devastation such as toxic chemical intoxication [6].

A proper essential element level is required for the brain to maintain normal physiological functions. Magnesium (Mg), zinc (Zn), iron (Fe), and copper (Cu) are essential for brain tissues [7-10]. Much attraction has recently been paid to the antioxidant-related elements of Mg and Zn because they possess innate antioxidant and anti-inflammatory properties [7, 8]. On the other hand, increasing evidence has proposed that the essential elements Fe and Cu are important for brain metabolism. However, an overload of both levels is harmful to the brain owing to the generation of toxic hydroxyl radical (OH•) [9, 10]. The aim of this study was to explore whether antrodia camphorata is capable of protecting the ischemic brain cortex from oxidative injury and whether this protective effect is correlated with the changes in lipid peroxidation status as well as essential element levels.

2. Materials and Methods

Animal preparation and the harvest of brain cortex tissues

Experimentally, forty male Sprague-Dawley rats, weighing from 200-220 g, were encompassed in this study. All rats were housed under controlled conditions in the animal room (22 ± 2°C, 50 ± 20% relative humidity, 12-h light-dark cycle). Three days after caging, rats were randomly divided into four groups of 10 each as follows: control (treated with physiological saline); ligation (physiological saline was administered before ligation of right middle cerebral artery (RMCA) plus right common carotid artery (RCCA) for 1 hour); AC (intraperitoneally injected rats with AC at dosage of 100mg/Kg once a day for consecutive 7 days); and prevention (pretreatment of rats with AC at dosage of 100mg/Kg once a day for 7 days followed by ligation of the RMCA plus the RCCA for 1 hour). On day 8, all rats were anesthetized and the brain cortex tissues were collected for biochemical analysis. Experimentally, all animal used protocol that listed and mentioned above has been approved in advance by the Institutional Animal Care and Use Committee (IACUC) of Central Taiwan University of Science and Technology.

The malondialdehyde (MDA) level analysis

The malondialdehyde (MDA) is the end-product of lipid peroxidation. In general, 0.2 g of the brain cortex tissue was pipetted into Pyrex tube contains 4.8 ml cold H_3PO_4 solution (1% w/v) followed by adding 1 ml of the TBA reagent into tube and boiled at 100 ℃ for 1 hour following by adding 4 ml of the butanol solution into the tube and centrifuged at 1600 g for 5 minutes. Finally, the supernatant was collected and the MDA level was assayed using spectrophotometry (U-1900, Hitachi, Japan) at the wavelength of 532 nm. The chemical reagent of 1, 1, 3, 3-tetraethoxypropane was applied to act as a standard solution to interact with thiobarbituric acid (TBA) reactive substance. The analyzing principle of this detective method is based on determining the intensity of the pink color which is produced by the interaction of TBA with the lipid peroxidation product of the MDA.

Determination of the essential element concentration in cerebral cortex tissues

Experimentally, 0.2g of the obtained brain cortex tissues was used for the analysis of the essential element concentration of Mg, Zn, Fe, and Cu. In brief, all containers that used in measuring the essential element were soaked with the concentration of 50% nitric acid, rinsed with ultrapure water followed by drying in an oven at the temperature of 50℃ for later experimental used. The standard solutions of both four elements were dissolved in the concentration of 0.1 mol/L nitric acid solution purchased from Merck, Germany. The concentration of Mg, Zn, Fe, and Cu in the brain cortex tissues was measured by SavantAA Z graphite furnace atomic absorption spectrophotometer (GBC Scientific Equipment Pty Ltd., Melbourne, Australia) with PAL4000 auto-sampler and longitudinal Zeeman Effect background correction system.

Statistical Analysis

All data were expressed as mean ± S.D. The experimental results were analyzed by the statistical method of Kruskal-Wallis one-way analysis of variance (ANOVA). Once the analyzed values showed significant differences among the groups, each group was compared using the Fisher's Least Significant Difference (FLSD) test. The statistical differences were significantly considered at a *P*-value of less than 0.05. a: $P < 0.05$, *vs*. control group; b: $P < 0.05$, *vs*. ligation subject.

3. Results & Discussion

The malondialdehyde (MDA) Levels in the cerebral cortex tissues

In the current study, the value of the MDA in the group of control, ligation, AC, and prevention was 12.16 ± 0.28, 16.59 ± 0.65, 10.71 ± 0.47, and 13.06 ± 0.10 μmol/g protein, respectively (Figure 1). Obviously, cerebral ischemic lesion leads to an elevated MDA level but pretreating rats with AC before ischemic insult significantly ($p < 0.05$) declined the MDA concentration as listed in Figure 1.

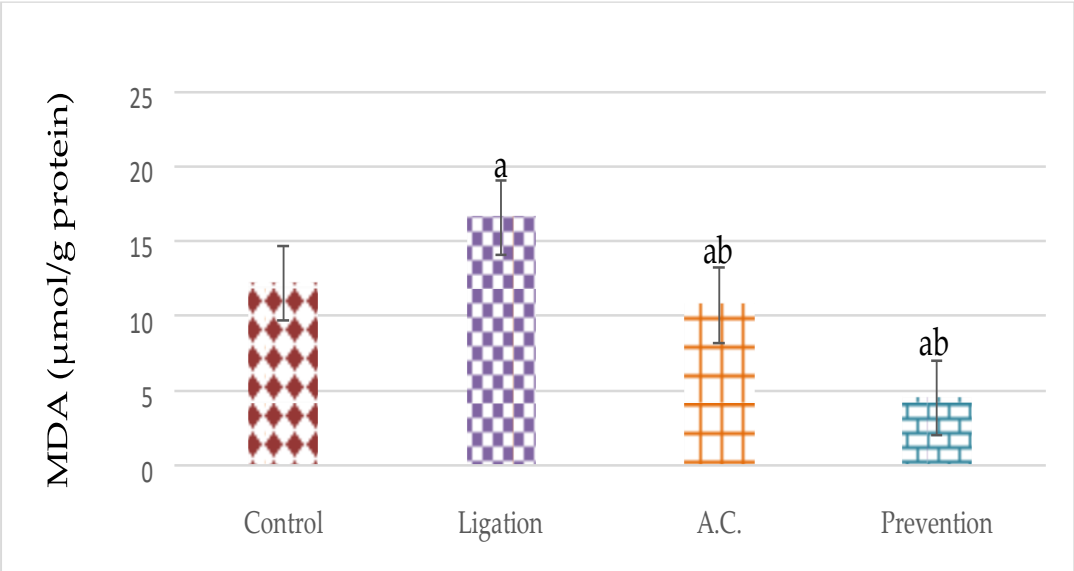

Figure 1: The MDA level in cerebral cortex tissues. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from prevention group

The essential element concentration in brain cortex tissues

The Mg concentration in the group of control, ligation, AC, and prevention was 712 ± 27, 616 ± 41, 808 ± 39, and 750 ± 33 μg/g, respectively. Cerebral ischemia results in a decreased Mg level but pretreatment with AC prior to ischemia markedly increases the Mg concentration (Figure 2).

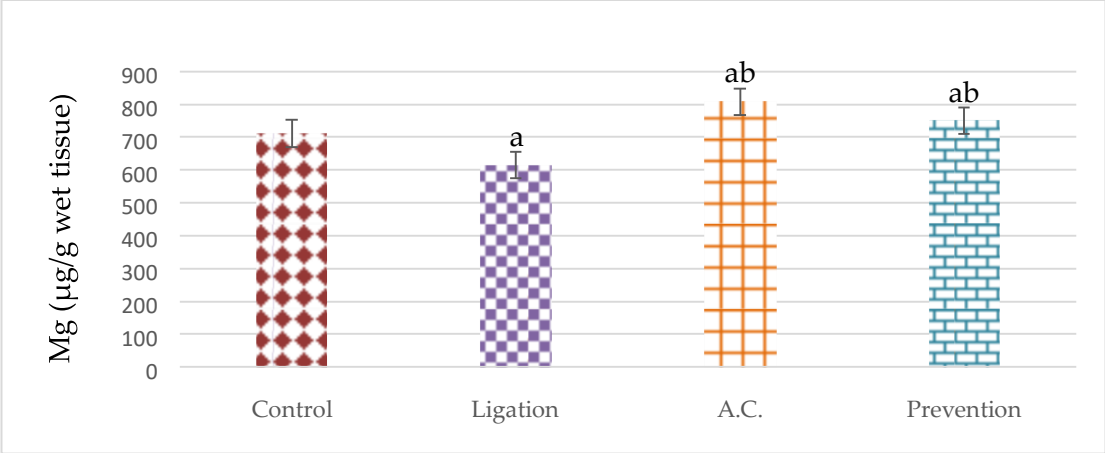

Figure 2: The Mg level in cerebral cortex tissues. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from prevention group.

As shown in Figure 3, the Zn level in the control, ligation, AC, and prevention groups was 88.56 ± 7.24, 61.06 ± 4.17, 100.65 ± 8.19, and 78.37 ± 5.00 µg/g, respectively. A decreased Zn concentration was found in the situation of cerebral ischemia. However, pretreatment of rats with AC prior to ischemia markedly increases the Zn concentration.

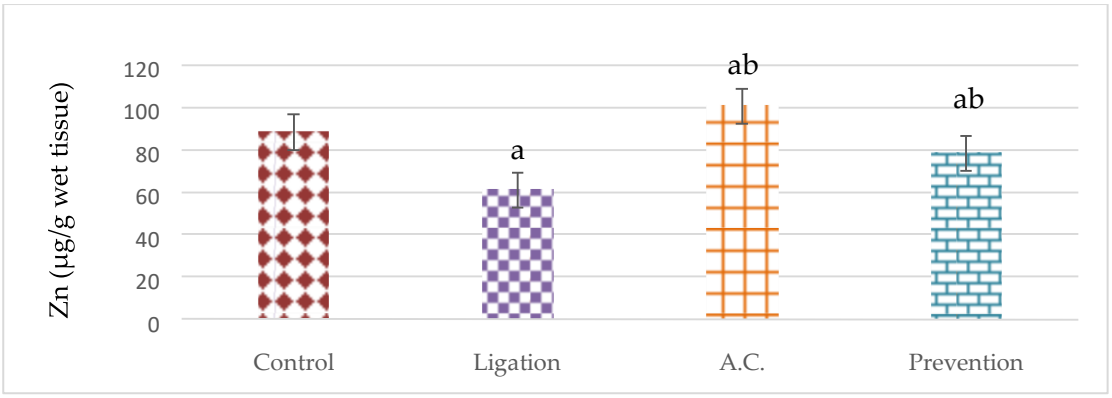

Figure 3: The Zn level in cerebral cortex tissues. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from prevention group.

For Fe level analysis, value of the Fe level in the control, ligation, AC, and prevention groups was 57.58 ± 4.64, 91.82 ± 6.89, 47.72 ± 4.73, and 67.34 ± 6.92 µg/g, respectively. Interestingly, cerebral ischemia results in an enhanced Fe level but pretreating with AC before ischemic injury significantly decreased the Fe concentration (Figure 4).

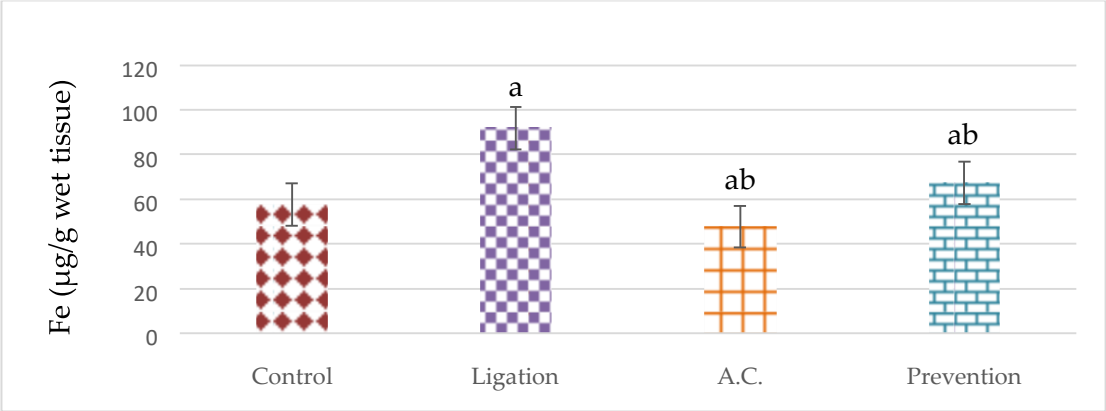

Figure 4: The Fe level in cerebral cortex tissues. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference (p <0.05) from control subjects. b: Significant difference (p <0.05) from prevention group.

Similarly, level of the essential element Cu in the group of control, ligation, AC, and prevention was 3.79 ± 0.32, 6.76 ± 0.28, 3.42 ± 0.31, and 4.57 ± 0.61 μg/g, respectively. A higher Cu concentration was observed in the ischemic subject as compared to the control group (P < 0.05). Clearly, pretreating rats with AC before ischemia significantly declined the Cu levels as showed in Figure 5.

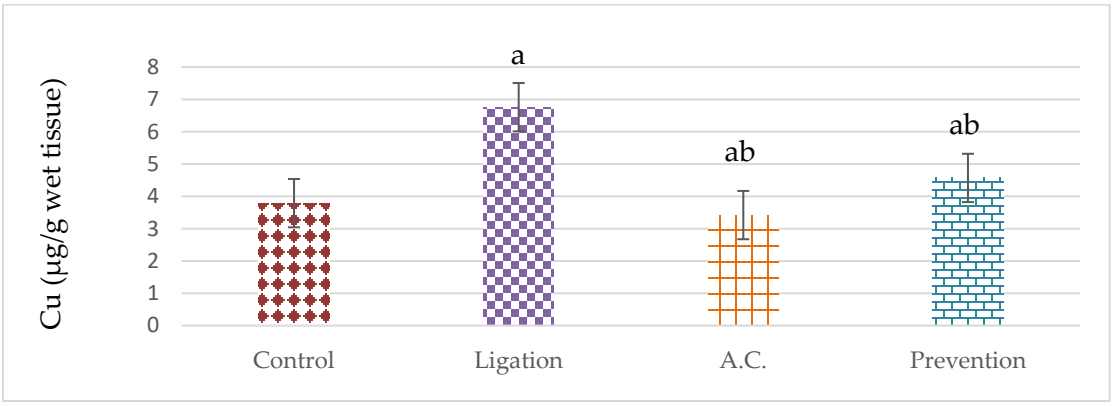

Figure 5: The Cu level in cerebral cortex tissues. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference (p <0.05) from control subjects. b: Significant difference (p <0.05) from prevention group.

Finally, the Zn/Cu ratio in the group of control, ligation, AC, and prevention was 22.37 ± 1.83, 9.01 ± 0.72, 28.50 ± 2.30, and 16.80 ± 1.36 μg/g, respectively. A declined Zn/Cu ratio was observed in the ligation group but interestingly, pretreatment of rats with AC before ischemic surgery obtained an increased Zn/Cu ratio as listed in Figure 6.

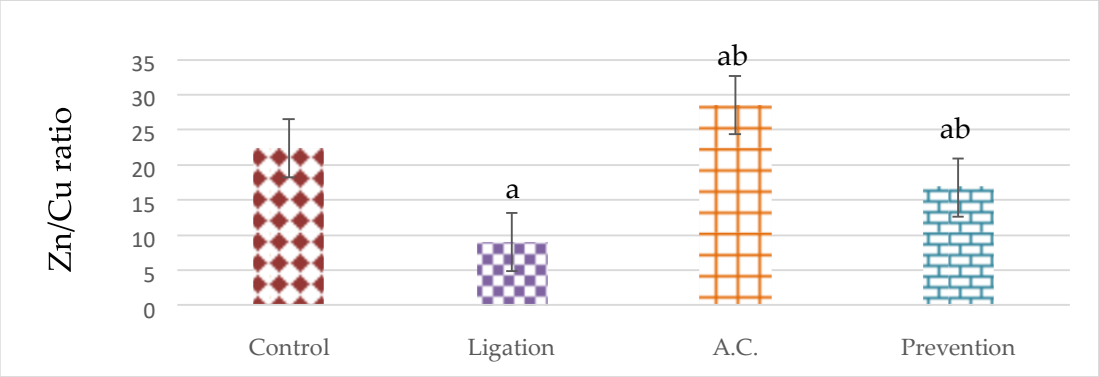

Figure 6: The Zn/Cu ratio in cerebral cortex. Data were expressed as mean ± S.D. The statistical method of One-way ANOVA followed by Least Significant Difference was used. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from prevention group.

Ischemic stroke mainly suffer from dis-convenience in elder populations [11]. The etiology of ischemic stroke is the disruption of blood supply. Thereby, deficiency of energy and oxygen leads to an increased reactive oxygen species (ROS) levels including superoxide radicals, hydrogen peroxide, and hydroxyl radicals [12]. Under this situation, ROS attacks the component of polyunsaturated fatty acid in brain tissues and eventually, brain tissues death [13].

Antrodia camphorata (AC) has been proposed as an alternative Chinese medicine that commonly applied to protect and treat a variety of liver diseases based on its beneficial effects in anti-oxidation and anti-inflammation [2]. Moreover, AC has been applied to treat some diseases such as liver disease, abdominal pain, diarrhea, and hypertension [2-4]. Other investigation has proposed that AC can obviously decline the occurrence of apoptosis in human promyelocytic leukemia (HL-60) cells [5]. Further, AC exerts free radical scavenging capacity so as to decline chemical-mediated hepatic injury [6]. Former in vivo experiment states their finding that AC exerts antioxidant efficacy so as to against H_2O_2-induced cytotoxicity in HepG2 in carbon tetrachloride (CCl_4)-induced hepatotoxicity [14]. Our present finding suggests that cerebral ischemic lesion leads to an elevated MDA level and this effect is thinkable to be correlated with an increased ROS levels. However, pretreatment of rats with AC for 7 days before ischemic insult significantly ($p < 0.05$) decreased the MDA concentration on the ischemic cerebral cortex (Figure 1). As mentioned above, it is obvious to note here that AC possesses the ability to reduce cerebral ischemia-generated toxic ROS levels. Under this circumstance, the ROS-mediated adverse lipid peroxidation effect is decreased.

Essential element of magnesium (Mg) and zinc (Zn) is required for the brain cells for normal metabolism [7, 8]. Study has indicated that Mg and Zn possess antioxidant and anti-inflammatory effects. Previous study has shown that Mg possesses profound beneficial effects for living organisms not only in declining inflammatory effect, maintaining cell membrane integrity, but also in attenuating lipid peroxidation [7, 8]. In vivo study has suggested that PQ-induced oxidative lung injury can be improved by treating rats with magnesium isoglycyrrhizinate [15]. Further clinical investigation has revealed that intravenous administration of acute stroke patients with magnesium sulfate obviously ameliorates brain damage [16]. Likewise, investigation suggested that supplementation of fetal mice with magnesium sulfate not only can decline inflammatory effect but also can mitigate the MDA concentration due to the attenuated lipid peroxidation effect in the brain [17, 18]. Additionally, restriction of the dietary Mg intake increases PQ toxicity in rats [19]. Conversely, reduced Mg level not only exacerbates the oxidative lesion but also increases PQ toxicity in an animal model [20]. Preceding study has proposed that essential element Zn can attenuate ROS-induced lesion due to its innate antioxidant and anti-inflammatory effects [21]. In turn, reduction of the Zn concentration is correlated to a decreased protective ability [8]. Animal study has demonstrated that depletion of the Zn concentration is associated with an increased inflammatory effect as well as an enhanced lipid peroxidation

found in rat model [22]. Other cellular investigation has shown that deficiency in Zn concentration results in AP-1 activation in 3T3 cells due to an elevated ROS level [23]. Additionally, clinical study indicates that reduction in Zn level is correlated to the etiology of patients with liver cirrhosis and liver hepatitis [24]. Our experimental results suggest that a decreased Mg and Zn levels were observed during cerebral ischemic lesion but pretreatment of rats with AC for 7 days before ischemia significantly ($p < 0.05$) increased both elements concentration on the ischemic cerebral cortex as listed in Figure 2 and 3. Collect, it is of note that antrodia camphorata can enhance the Mg and Zn so as to decline cerebral ischemia-induced toxic ROS levels. Given this fact, ROS-mediated adverse lipid peroxidation effect is attenuated.

A growing amount of investigations have demonstrated that iron (Fe) and copper (Cu) is essential for the neuronal cells for maintaining cellular functions and conversely, perturbation of both levels has been reported to be detrimental to the brain [9, 10]. Fe is a fundamental and ubiquitous element [9]. Under normal circumstance, Fe is tightly controlled due to its innate oxidative property in nature [25]. Investigation has revealed that Fe overload can spontaneously interact with the toxic ROS via Fenton-reaction, a pathway for generating toxic hydroxyl radicals [9, 23]. In addition, the generated hydroxyl radicals can automatically react with the component of poly-unsaturated fatty acid (PUFA) and eventually initiating adverse lipid peroxidation [9, 23]. Our experimental result showed that ischemic stroke results in an increased Fe pattern in the ischemic brain cortex but interestingly, pretreating rats with AC significantly decreased the Fe concentration. Here we speculate that AC can effectively decline the Fe level. As a result, Fe-mediated Fenton-reaction is reduced and further deleterious lipid peroxidation was decreased in the ischemic brain cortex. The essential element copper (Cu) plays an indispensable role and involves in a variety of biological roles in acting as the component of bone tissue, skin pigments, liver enzymes, myelin maintenance in the nervous system as well as hemoglobin synthesis [26, 27]. Like Fe element, Cu level is tightly controlled within cells to keep the mount needed for the brain cell metabolism to avoid toxic concentration [28]. Former research has reported that due to its active chemical property, Cu overload can spontaneously interact with the ROS, generating harmful hydroxyl radicals via the pathway of Fenton-reaction [26, 27]. Moreover, it has been documented that a variety of human disorders is correlated to Cu overloaded including ischemic stroke and liver diseases [26, 27]. Similar to the previous finding, our present result reveals that ischemic stroke significantly increased the Cu levels was in the ischemic cerebral cortex but interestingly, pretreatment of rats with AC prior to ischemic injury markedly declined the Cu concentration as compared to the ischemic subject. Altogether, our experimental finding demonstrates that neuroprotection of AC during cerebral ischemia, at least in part, is responsible for reducing the Cu level. Under this condition, Cu-mediated Fenton reaction and further lipid peroxidation effect, as reflected by a decreased MDA level are significantly reduced in the ischemic cerebral cortex.

Recent study proposed that the Zn/Cu ratio is a useful biomarker for evaluating the situations of oxidative stress and inflammation [29]. Clinical investigation reveals a negative correlation between lower Zn/Cu ratio and higher larger brain infarct volume in stroke patients [29]. On the other hand, a significant association between lower Zn/Cu ratio and enhanced inflammatory cytokine of C-reactive protein (CRP) was observed in men with cerebral ischemic injury [30]. In this study, we found that ischemic stroke shows lower Zn/Cu ratio but crucially, pretreatment of experimental rats with AC for 7 days before ischemic insult obviously increases the Zn/Cu ratio. Based on our finding, we conclude that neuroprotection of AC on the ischemic cerebral cortex is correlated with increasing the Zn concentration but decreasing the Cu level. Consequently, a higher Zn/Cu ratio was observed in the prevention group, and this phenomenon, at least in part, is recognized to effectively ameliorate cerebral ischemia-induced oxidative damage.

3. Conclusion

Our experimental findings suggest that ischemic stroke-induced oxidative injury not only significantly decreases the concentration of Mg, Zn, and Zn/Cu ratio but also increases the essential element levels of Fe and Cu in the ischemic cerebral cortex. As a consequence, enhanced oxidative stress results in an elevated lipid peroxidation status. However, due to its innate beneficial efficacies of anti-inflammation and anti-oxidation, pretreatment of rats with AC before ischemia obviously reverses all biochemical results. Altogether, it seems likely that AC possesses

clinically medicinal potential in preventing and alleviating ROS-mediated oxidative damage for cerebral ischemic patients.

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