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Protective Effect of Silibinin during Cerebral Ischemia is correlated with Altering Oxidative Stress, Transition Metal, and Antioxidant

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Abstract Cerebral ischemia may elevate oxidative stress due to a large amount of the generated reactive oxygen species. Silibinin is an active component of flavonoids possessing anti-inflammatory and anti-oxidative properties. Transition metal of iron (Fe), copper (Cu), and antioxidant of superoxide dismutase (SOD) and catalase (CAT) are required for the brain functions. This study was to elucidate whether protective effect of silibinin on the ischemic brain cortex is correlated with altering the level of transition metal, oxidative stress, and antioxidant activity. Rats were divided into four groups and the prevention subject was administered with silibinin (100 mg/kg) once daily for consecutive 10 days before cerebral ischemia. Cerebral ischemia was induced by ligation of right middle cerebral artery plus right common carotid artery for 1 hour. The right brain cortex tissue was homogenate and the supernatant was harvested for further biochemical analysis. The obtained results showed that level of malondialdehyde (MDA), Fe, and Cu was significantly decreased but the antioxidant activity of SOD and CAT was markedly increased in the prevention subject as compared to the ischemic group. In summary, our experimental findings demonstrate that neuroprotection of silibininon the ischemic brain cortex is correlated with altering the oxidative stress, transition metal level, and antioxidant activity.

Keywords ferrite nanoparticles, magnetic drug delivery system

Introduction

Silibinin is a natural flavonoid compound mainly existed in the plants [1-3]. Previous study has showed that silibinin exerts beneficial effect on hepato-protection, renal-protection, cardiac-protection, and neuroprotection mainly due to its antioxidant property [4-6]. Cerebral ischemia may generate toxic reactive oxygen species (ROS) and promotes further oxidative brain injury [1]. Lipid peroxidation is a complex ROS-mediated reaction induced by degradation of the cellular component of polyunsaturated fatty acid (PUFA) in cells [7, 8]. Therefore, increased lipid peroxidation level, as reflected by an elevated malondialdehyde (MDA) level, is representative of enhanced oxidative stress and tissue injury [7, 8]. Multiple evidences have revealed that proper transition metal level of iron (Fe) and copper (Cu) is required for the brain for maintaining normal physiological functions [9]. In contrast, increase of both elements has been published to be deleterious to the health due to the generation of toxic ROS [9].

Maintaining a delicate balance between ROS level and antioxidant activity in the brain tissue is crucial for preventing ROS attack for the neuronal cells [10, 11]. Superoxide dismutase (SOD) and catalase (CAT) are two major antioxidant existed in the brain. The antioxidant of SOD is to catalyze the formation of toxic forms of superoxide radicals into non-toxic oxygen and hydrogen peroxide. Enzyme function of CAT is to convert toxic hydrogen peroxide into water and oxygen. It has been well-realized that declined antioxidant activity is implicated with increased oxidative tissue injury [10, 11].

There is no data available on exploring whether neuroprotective effect of silibinin on the ischemic brain cortex is correlated with the alteration of the level of oxidative stress, transition metal, and antioxidant activity so far.

Materials and Methods

Animal Treatment and Cerebral Ischemic Surgery

Forty male Sprague-Dawley rats, weighing from 250-300 g were encompassed in this experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Central Taiwan University of Science and Technology. Experimentally, rats were kept in stainless-steel mesh cages, housed under controlled conditions (22 ± 2°C, 50 ± 20% relative humidity, 12-h light-dark cycle) with diet and water for one week after purchase. The rats were randomly divided into four groups of 10 each as followed: control (treatment of rats with normal saline), ligation (ligation of right middle cerebral artery plus right common carotid artery for 1 hour), silibinin (intraperitoneally injected with 100 mg/Kg of silibinin once daily for 10 days purchased from Sigma-Aldrich, Germany), and prevention (intraperitoneally injected with 100 mg/Kg of silibinin once daily for 10 days before ligation). The right brain cortex tissues were harvested, homogenized, and centrifuged at 4°C for 10 min at the speed of 650 g, and the supernatants were collected for biochemical assay.

Determination of the Malondialdehyde (MDA) Content in Brain Cortex Tissues

Malondialdehyde (MDA), the end-product of lipid peroxidation, was measured for evaluating the intensity of oxidative tissue injury. In general, the reagent of thiobarbituric acid (TBA) was purchased from E. Merck (Germany). The analytical principle of the MDA level is that the reagent of 1, 1, 3, 3-tetraethoxypropane (TEP) was applied as a standard solution in the reaction with thiobarbituric acid-reactive substance (TBARS). Experimentally, the analytical principle is based on determining the pink color that is produced by the interaction of TBA with the MDA. The MDA level was measured by means of spectrophotometer (U-1900, Hitachi, Japan) and detects its absorption at the wavelength of 532 nm.

Measurement of Transition Metal of Fe and Cu in Brain Cortex Tissues

Experimentally, 0.2 g of the brain cortex was digested with an ultra-pure grade solution of nitric acid. All containers used were soaked with 50% nitric acid and rinsed with ultra-pure deionized water (Millipore, USA). The standard solution (1000 μg/ml) of Fe and Cu was purchased from Merck (Germany). The transition metal level of Fe and Cu was assayed at the wavelength of 248.3 and 324.7 nm respectively by SavantAA Z graphite furnace atomic absorption spectrophotometer (GBC Scientific Equipment Pty Ltd., Melbourne, Australia).

Determination of Enzyme Activity of SOD and CAT in Brain Cortex Tissues

Experimentally, 0.2 g of the brain cortex was homogenized and the supernatants were collected. The CAT activity was measured according to the commercial kit (Cayman Chemical Company, USA). The analytical principle is based on the reaction of the enzyme with methanol in the presence of hydrogen peroxide. The formaldehyde produced is measured with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole as the chromogen. The SOD activity was measured according to the commercial kit (Cayman Chemical Company, USA). Experimentally, both enzyme activities were assayed via spectrophotometry (Thermo Scientific Multiskan Spectrum, USA).

Determination of the Protein Level in the Homogenates of Brain Cortex

The protein level was assayed by the commercial kit of Bio Chain, USA, based on the improved method of Coomassie Blue G. In general, the dye can form a blue complex which is specifically with the protein of brain cortex and followed by detected by means of the instrument of spectrophotometer (Thermo Scientific Multiskan Spectrum, USA) at the wavelength of 595 nm experimentally. All the experimental data were expressed as mean ± S.D. The obtained data were analyzed by one-way analysis of variance (One-way ANOVA) test. Once statistical data showed significant differences among the group means, each group was compared using the statistical method of least significant difference (LSD) test. Experimentally, the statistical differences were significantly considered at a p value less than 0.05.

Results

Malondialdehyde (MDA) Level in Brain Cortex Tissues

As shown in Figure 1, the MDA level in the group of control, ligation, silibinin, and prevention was 15.76±1, 20.89±3.21, 10.03±2.14, and 16.89±1.42 nmol/g protein. respectively. The MDA level was significantly reduced in the prevention group as compared with the ligation subject.

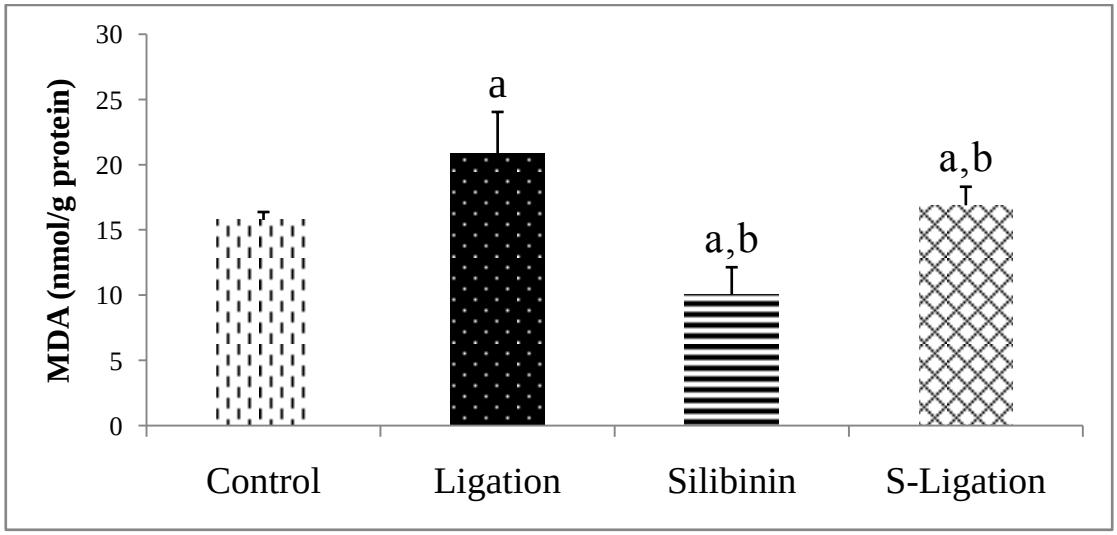

Figure 1: Profiles of MDA level in ebrain cortex. Data were expressed as mean ± S.D. One-way ANOVA followed by Least Significant Difference was used presently. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from ligation group.

Transition Metal Level of Fe and Cu in the Brain Cortex Tissues

As listed in Figure 2, the Fe level in the group of control, ligation, silibinin, and prevention was 42.98±2.30, 80.04±9.23, 48.90±5.38, and 55.46±3.84 μg/g, respectively. Obviously, the Fe level was declined in the prevention group as compared with the ligation subject. Likewise, Figure 3 showed that the Cu concentration in the group of control, ligation, silibinin, and prevention was 2.64±0.41, 5.84±0.16, 3.15±0.10, and 4.33±0.83 μg/g, respectively. Compared with the ligation subject, the Cu level was obviously declined in the prevention group.

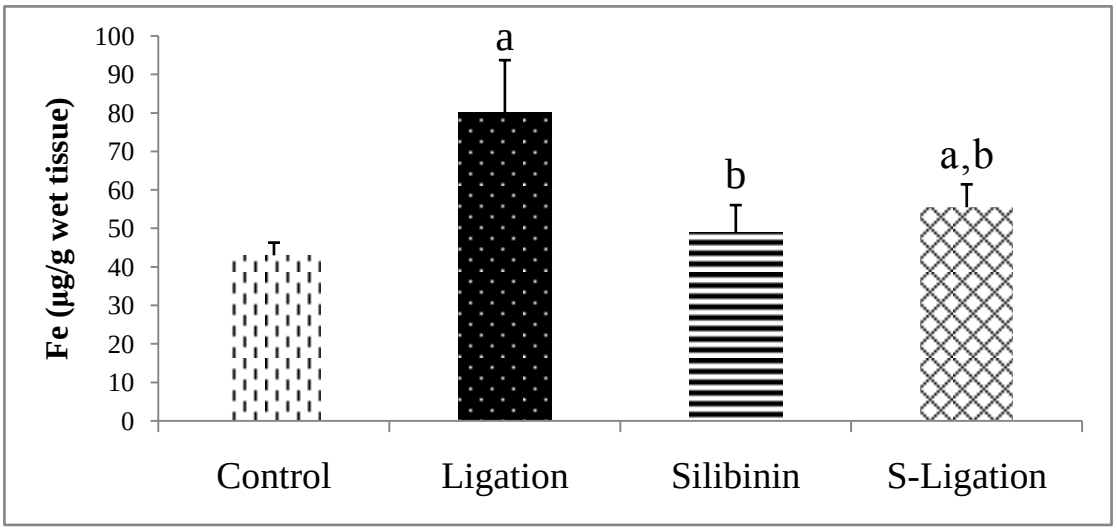

Figure 2: Profiles of Fe level in brain cortex. Data were expressed as mean ± S.D. One-way ANOVA followed by Least Significant Difference was used presently. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from ligation group.

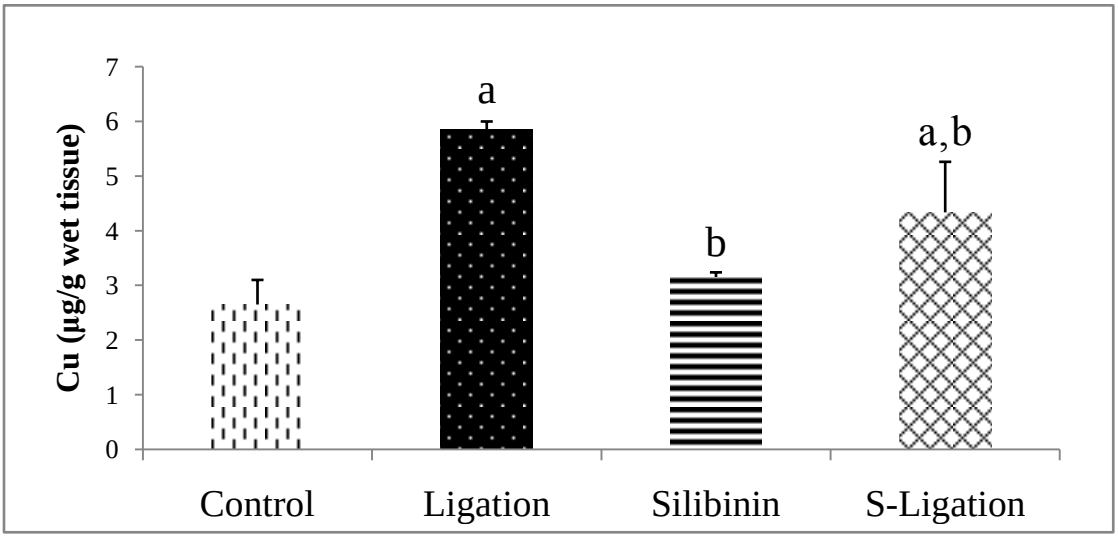

Figure 3: Profiles of Cu level in brain cortex. Data were expressed as mean ± S.D. One-way ANOVA followed by Least Significant Difference was used presently. a: Significant difference ($p < 0.05$) from control subjects. b: Significant difference ($p < 0.05$) from ligation group.

Antioxidant Activity of SOD and CAT in Brain Cortex Tissues

Value of SOD (Figure 4) in the subject of control, ligation, silibinin, and prevention was 1.82±0.04, 1.53±0.03, 1.79±0.08, and 1.57±0.04 U/g protein, respectively. Compared with the ligation subject, the SOD activity was significantly enhanced in the prevention group. Figure 5 indicated that the antioxidant activity of CAT in the four groups of control, ligation, silibinin, and prevention was 7.72±0.14, 5.84±0.21, 10.09±0.24, and 8.82±0.25 μmol/g protein, respectively. Specifically, both enzyme activities were markedly elevated in the prevention rats as compared with the ligation subject.

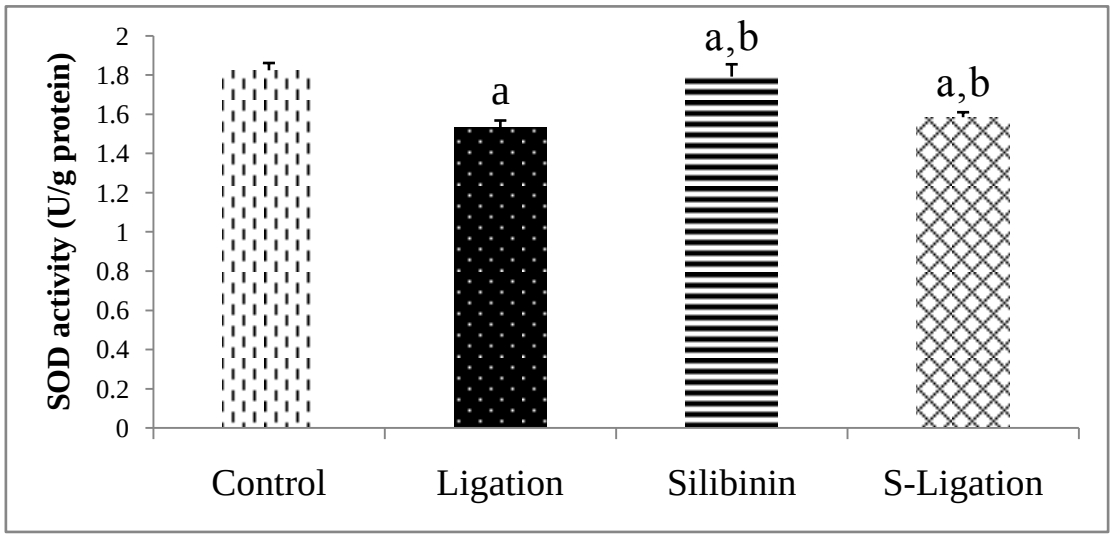

Figure 4: Profiles of SOD level in brain cortex. Data were expressed as mean ± S.D. One-way ANOVA followed by Least Significant Difference was used presently. a: Significant difference (p <0.05) from control subjects. b: Significant difference (p <0.05) from ligation group.

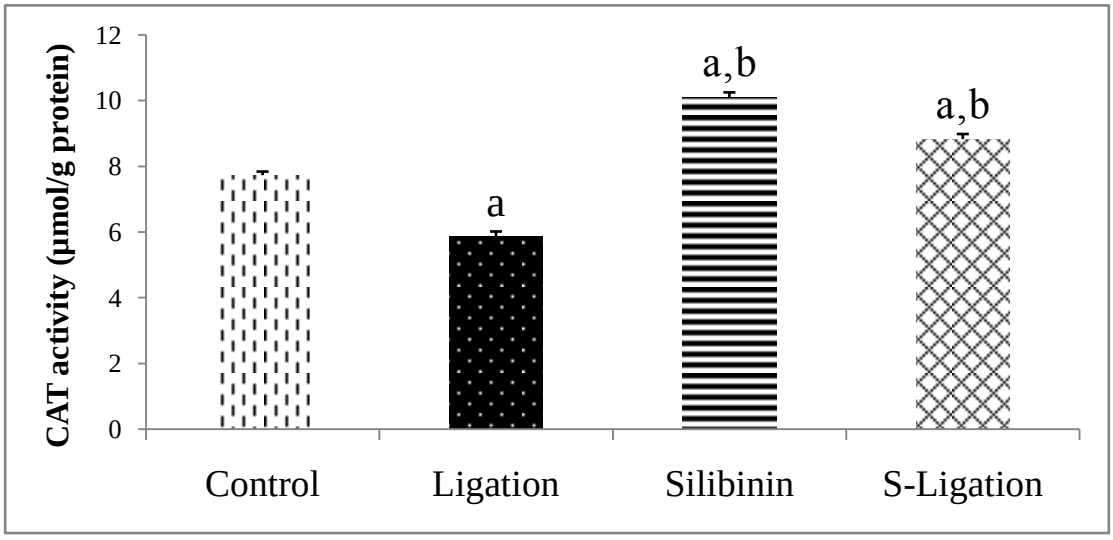

Figure 5: Profiles of CAT level in brain cortex. Data were expressed as mean ± S.D. One-way ANOVA followed by Least Significant Difference was used presently. a: Significant difference (p <0.05) from control subjects. b: Significant difference (p <0.05) from ligation group.

Discussion

Our experimental findings clearly suggest that pretreatment of rats with silibinin before cerebral ischemic insult not only enhances antioxidant activity of SOD and CAT but also declines the transition metal level of Fe and Cu on the ischemic brain cortex. Silibinin possesses an active component of flavonoids that can exert the property of anti-inflammation and anti-oxidation [12-14]. In addition, a variety of studies have indicated that silibinin has been received a tremendous amount of attention as herbal remedy for the treatment of a variety of human disorders such as protecting arsenic-induced hepatic dysfunction in rats, attenuating methotrexate-induced pulmonary injury by targeting oxidative stress, suppressing astroglial activation in a mouse model of acute Parkinson's disease by modulating the ERK and JNK signaling pathways, and preventing the amyloid beta peptide-induced memory impairment and oxidative stress in mice [12-16]. As mention above, it is of note that all of the beneficial effects

provide by silibinin is predominantly based on its antioxidant and anti-inflammatory ability. Despite the above-mentioned studies, there are no reports on exploring the precise role of silibinin on influencing the transition metal level of Fe and Cu during cerebral ischemic insult to date.

Cerebral ischemia mainly occurs in older population worldwide and is the fourth leading cause of death in Taiwan. Cerebral ischemic insult can enhance oxidative stress on the ischemic area via generating toxic reactive oxygen species (ROS). Moreover, these generated ROS results from ischemic event can directly damage to the cellular components of the brain tissues including cell membrane, protein, and nucleic acid [22]. Among them, much attention has predominantly focused on the influence of lipid peroxidation effect [22]. Lipid peroxidation is conducted by the degradation of the cellular component of polyunsaturated fatty acid (PUFA) [7, 8]. In general, the chemical processes of lipid peroxidation consist of three stages including initiation, propagation, and termination. At the end stage of lipid peroxidation, a variety of products such as malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal were generated. Among them, MDA has specifically been reported the most stable and mutagenic product [7, 8]. In this regard, determination of the MDA concentration has been widely applied to act as a convenient biomarker for evaluating the intensity of oxidative stress and oxidative injury [7, 8]. Our current result showed that pretreatment of rats with silibinin before ischemic insult significantly reduced the MDA levels in the ischemic brain cortex as compared to the ischemic subject as listed in Figure 1. In vivo studies have clearly demonstrated that silibinin can effectively attenuate lipid peroxidation on cells as reflected by a decreased MDA concentration [7, 8]. Our present finding is consistent with the former investigation and suggested that neuroprotection of silibinin during cerebral ischemia is highly associated with attenuating ROS-mediated lipid peroxidation on the ischemic brain cortex [1, 3, 5, 7, 8].

A growing amount of studies have indicated that appropriate transition metal of Fe and Cu is essential for the neuronal cells for maintaining their physiological functions [9, 23, 24]. Instead, perturbation of both transition metal levels has been evidenced to be detrimental to all living organisms [9]. Fe is a fundamental and ubiquitous element existed in most known life forms [9]. Under normal situations, Fe levels are tightly controlled within the cells due to its actively oxidative property in nature [23]. Previous investigation has proposed that excessive Fe levels can interact with the toxic ROS via adverse Fenton-reaction, a pathway for generating the source of toxic hydroxyl radicals [9, 23]. In addition to this, the generated hydroxyl radicals can interact with the component of PUFA and eventually result in further harmful lipid peroxidation on cells [9, 23]. Obviously, our experimental result showed that an increased Fe pattern was found in the ischemic brain cortex but interestingly, pretreatment of rats with silibinin significantly declined the Fe level. Thereby, it is reasonable to conclude our present finding here that silibinin can markedly reduce the Fe concentration and thereby seems may decline the Fenton-reaction. Under this situation, further deleterious lipid peroxidation effect was reduced on the ischemic brain cortex. As a result, further oxidative brain lesion was reduced.

Cu is an essential trace element and plays an indispensable role in the physiology of all living organisms. Basically, the transition metal Cu is involved in a variety of biological and physiological roles such as acting as the component of liver enzymes, melanin, skin pigments, bone tissue formation, myelin maintenance in the nervous system, and the synthesis of hemoglobin [25, 26]. Similar to the Fe metal, cellular Cu level is in fact tightly regulated within the cells to keep the mount needed for the brain cell metabolism while avoiding toxic concentration [24]. Likewise, previous research has demonstrated that Cu overload can spontaneously interact with the ROS, generating harmful hydroxyl radicals via the chemical pathway of Fenton-reaction [25, 26]. In addition, a variety of human disorders has been reported in responsible for increased Cu level such as liver disease and cerebral ischemia [25, 26]. Our current experimental result clearly showed that Cu concentration was significantly increased on the ischemic brain cortex but specifically, silibinin obviously reduced the Cu level as compared to the ischemic subject. As mentioned above, it is also realized that cerebral ischemia may result in Cu overload but significantly, pretreatment of rats with silibinin declines the Cu level on the ischemic brain. Our present finding convincingly suggests that neuroprotection of silibinin during cerebral ischemia is correlated with declining the Cu level on the ischemic brain cortex. This positive response provides by silibinin is also believed not only in correlating with reducing lipid peroxidation effect as reflected by an increased MDA level but also in declining further deleterious oxidative brain lesion.

Basically, proper antioxidant defensive system is closely responsible for the brain health. Dynamic equilibrium between pro-oxidant and antioxidant is therefore essential and crucial for the brain tissues to attenuate oxidative attack produced by normal brain tissue metabolism. Among them, superoxide dismutase (SOD) and catalase (CAT) are two major antioxidant defensive enzymes existed in the brain tissues [27, 28]. The biological function of SOD is to decompose superoxide radicals into oxygen and hydrogen peroxide (H_2O_2). On the other hand, the generated H_2O_2 can be converted into water and oxygen by the antioxidant CAT enzyme. Previous investigation has revealed that SOD is the first-line defensive enzyme in scavenging superoxide radical in the brain tissues [22, 29, 30]. Furthermore, it has been documented that silibinin can significantly protect liver cells from apoptosis and death via increasing the SOD activity [15, 16]. Similarly, our current result indicated that cerebral ischemic event markedly declines SOD activity but interestingly, this adverse phenomenon can be reversed by means of pretreating of rats with silibinin prior to cerebral ischemia. Obviously, the trend of our experimental observation is in accordance with the preceding studies [22, 24, 27, 28], and the interpretation of our observation is that pretreatment of rats with silibinin can significantly enhance the SOD activity; increased SOD activity also can specifically scavenge the toxic superoxide radicals and given the fact, further oxidative brain injury was reduced in the prevention subject as reflected by a decreased MDA level as compared to the ischemic group.

Antioxidant enzyme of CAT has been evidenced can exert its specific ability in conversing the harmful H_2O_2 into the non-toxic oxygen and water [1, 10, 27-30]. Preceding in vivo study has revealed the fact that silibinin can elevate intracellular CAT activity [27, 28]. Similarly, the trend of our present result was in accordance with the former investigation and indicated that pretreatment of rats with silibinin before ischemic insult significantly increases the CAT activity on the ischemic brain cortex. Based on our present finding and we suggest that silibinin indeed possesses the ability to increase the antioxidant CAT activity on the ischemic brain. This positive effect is also important for the ischemic brain to eliminate toxic hydrogen peroxide (H_2O_2) conducted by the ischemic brain tissues. Under this circumstance, ROS-mediated lipid peroxidation and further oxidative brain tissue damage are declined in the prevention group as compared to the ischemic subject.

Conclusion

Taking together, our experimental evidence obviously demonstrate that pretreatment of rats with silibinin before cerebral ischemic insult not only significantly declines the oxidative stress and the transition metal concentration of Fe and Cu, but also markedly increases the antioxidant enzyme activity of SOD and CAT on the ischemic brain cortex. In addition to this, it is important to emphasize the positive effect of silibinin here that silibinin seems may possess the medicinal potential in preventing and even in treating cerebral ischemic injury shortly.

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