Lutein protects against severe traumatic brain injury through anti-inflammation and antioxidative effects via ICAM-1/Nrf-2

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Abstract. Many studies have reported that lutein may exert its biological activities, including anti-inflammation, anti-oxidase and anti-apoptosis, through effects on reactive oxygen species (ROS). Thus, lutein may prevent the damaging activities of ROS in cells. The current study investigated the effect of lutein against severe traumatic brain injury (STBI) and examined the mechanism of this protective effect. Sprague-Dawley rats were randomly divided into 5 groups: Control group, STBI model group, 40 mg/kg lutein-treated group, 80 mg/kg lutein-treated group and 160 mg/kg lutein-treated group. In this study, lutein protects against STBI, suppressed, interleukin (IL)-1β, IL-6 and monocyte chemoattractant protein-1 expression, reduced serum ROS levels, and reduced superoxide dismutase and glutathione peroxidase activities in STBI rats. Treatment with lutein effectively downregulated the expression of NF-κB p65 and cyclooxygenase-2, intercellular adhesion molecule (ICAM)-1 protein, and upregulated nuclear factor erythroid 2 like 2 (Nrf-2) and endothelin-1 protein levels in STBI rats. These findings demonstrated that lutein protects against STBI, has anti-inflammation and antioxidative effects and alters ICAM-1/Nrf-2 expression, which may be a novel therapeutic for STBI the clinic.

Introduction

The current mechanisms known to be involved in severe traumatic brain injury (STBI) include inflammatory responses, disorder of energy metabolism, lipid peroxide responses caused by oxidative stress, toxic effects of excitatory amino acid and cell apoptosis (1). The inflammatory response is a complex cellular and molecular response, which is predominantly mediated by inflammatory cells and inflammatory mediators (2). Increasing evidence indicates that an excessive inflammatory response in local cerebral tissues following cerebral ischemia-reperfusion is a major cause of reperfusion injury. Inflammatory responses have an essential role in the pathogenesis of STBI (3). In addition, the inflammatory response is potentially causes the secondary damage of cerebral tissues following cerebral ischemia.

Oxidative stress is cause of cellular damage triggered by imbalance between oxidation levels and antioxidant functions (4). The increase of free radicals, such as reactive oxygen species (ROS), is the primary cause of oxidative stress. As a second messenger molecule, ROS can alter the expression of genes or proteins to affect intracellular signaling cascades (5). ROS are involved in normal physiological functions; however, they can also oxidize cellular components, resulting in cellular damage. Neurons have high metabolic activity, with high oxygen consumption and relatively low endogenous antioxidant activity, and thus, are particularly sensitive to oxidative stress. In addition, the brain is rich in lipids (6). Decomposition of ROS produces hydrogen peroxide and free radicals, which trigger lipid peroxidation of neuronal membranes. As a result, the central nervous system is highly susceptible to oxidative stress injury (4).

As a natural carotenoid, lutein (Fig. 1) is present in various fruits, vegetables and flowers. It is highly concentrated in leafy vegetables, including *Brassica oleracea* and spinach, and flowers, such as *Calendula officinalis*. Studies have suggested that natural lutein exists in plants and it is a favorable antioxidant (7,8). Lutein prevents damage to biological membranes caused by free radicals. Due to its antioxidant functions, it has been widely employed in treating human diseases including diabetic retinopathy, fertilization and ischemia/reperfusion injury (7). However, the risks caused by inappropriate supplementation of antioxidant agents have not been investigated fully. Under oxidative stress conditions, antioxidant agents may potentially cause pro-oxidative effects (9). Du *et al* (8) previously reported that lutein prevents alcohol-induced liver disease through antioxidative stress and anti-inflammatory effects, and increased Nrf-2 expression levels. Wang *et al* (10) reported that lutein prevents hyperhomo-cysteinemia via suppression of oxidative stress by NF-κB p65 and ICAM-1/ET-1. Therefore, as lutein regulates Nrf-2 and ICAM-1 expression, the current study aimed to investigate the effect of lutein and how it protects against STBI and the possible underlying mechanism.

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Materials and methods

Animals, experimental treatment and groupings. Adult male Sprague-Dawley rats (250-300 g, 8-10 weeks, n=10) were obtained from the Animal Experimental Center of Shantou University Medical College (Shantou, China) and housed at 24-25˚C and exposed to a 12 h light-dark cycle. The experimental procedures were conformed to the guidelines and approved by the institutional Animal Care Committee of the First Affiliated Hospital of Shantou University Medical College. Sprague-Dawley rats were randomly divided into five groups (10 rats per group): Control group, STBI model group, 40 mg/kg lutein-treated group, 80 mg/kg lutein-treated group and 160 mg/kg lutein-treated group. Lutein diets were administrated for 5 weeks before the surgery. Rats were anesthetized with 90 mg/kg ketamine and 10 mg/kg of xylazine intraperitoneally. After anesthesia, the cortex of rats were exposed using a TBI-0200 TBI Model system (Precision Systems and Instrumentation, LLC, Fairfax, VA, USA). A 3 mm diameter circular craniotomy was enforced using a burr drill at the midsagittal suture. The impacting shaft was lengthened, and the impact tip was massed. In the control group, rats were subjected to anesthesia and operation.

Skilled forelimb reaching test and contusion volume measurement. After treatment with lutein at 5 weeks, first reach success was defined as the rat firmly grasping the pellet and placing it into its mouth. The forelimb reach testing session consisted of 20 reaching opportunities. A maximal time limit of 5 min per testing session was set. This test was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of inflammation, ROS and oxidative stress. Rats were anesthetized with 35 mg/kg pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 200 μl blood was collected after treatment with lutein at 5 weeks and centrifuged at 10,000 x g for 10 min at 4˚C. The levels of tumor necrosis factor-α (TNF-α, cat. no. RTA00, interleukin-1β (IL-1β, cat. no. RLB0, interleukin-6 (IL-6, cat. no. R6000B), monocyte chemoattractant protein-1 (MCP-1, cat. no. MJE00 and superoxide dismutase (SOD, cat. no. DYC3419-2 (all purchased from R&D Systems China Co., Ltd., Shanghai, China), and glutathione (GSH; cat. no. A006-2; Nanjing Institute of Biological Engineering), were measured using ELISA kits. To determine ROS levels, tissue hippocampus homogenates was collected after treatment with lutein at 5 weeks were incubated with 2',7'-dichloroflorescein diacetate (Beyotime Institute of Biotechnology, Haimen, China) at 37˚C for 15 min. The homogenates were centrifuged at 10,000 x g for 15 min at 4˚C, resuspended in phosphate buffered saline and incubated for 1 h at 37˚C. ROS level was measured spectrofluorimetrically at 485 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blotting. The tissue hippocampus homogenate was lysed in radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 10,000 x g for 15 min at 4˚C. Protein concentration was determinate using the bicinchoninic acid method (Beyotime Institute of Biotechnology). Protein (50 μg) was separated by 8-12% SDS-PAGE gels and then electrophoretically transferred onto PVDF membranes. PVDF membranes were blocked with 5% non-fat dried milk for 1 h at 37˚C and incubated with nuclear factor-κB (NF-κB) p65 (cat. no. 8242, 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), cyclooxygenase-2 (COX-2; cat. no. 12282, 1:4,000; Cell Signaling Technology, Inc.), ICAM-1 (cat. no. 4915, 1:3,000; Cell Signaling Technology, Inc.), Nrf-2 (1:3,000; cat. no. 57736, Cell Signaling Technology, Inc.), endothelin-1 (ET-1; cat. no. ab2786, 1:2,000; Abcam) and GAPDH (cat. no. A01020, 1:10,000; Amyjet Scientific, Inc., Wuhan, China) at 4˚C overnight. The membrane was incubated with secondary peroxidase-conjugated goat anti-mouse or-rabbit IgG (cat. no. A21020, 1:5,000; Amyjet Scientific, Inc.) for 1 h and was visualized with an enhanced chemiluminescence system (Beyotime Institute of Biotechnology). The densitometry of western blot bands was performed using optical density scanning and ImageJ software version 3.0 (National Institutes of Health). Experiments were performed three times.

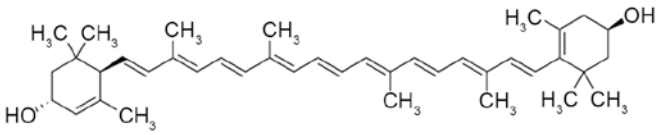

Figure 1. Chemical structure of lutein.

Statistical analysis. All values are presented as the mean ± standard deviation and statistically analyzed using one-way analysis of variance followed by Student-Newman-Keuls multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of lutein on skilled motor function and contusion volume. The present study demonstrated that the skilled motor functions of STBI model group rats were significantly reduced compared with the control group (Fig. 2A). Compared with the STBI model group, treatment with lutein significantly increased the inhibition of skilled motor function of STBI rat (Fig. 2A). Additionally, the contusion volume in STBI model rats was significantly increased compared with the control group (Fig. 2B). Treatment with lutein significantly inhibited the increase in contusion volume compared with the STBI model group (Fig. 2B).

Anti-inflammatory effect of lutein in STBI. There were significant increases in the TNF-α, IL-1β, IL-6 and MCP-1 levels in STBI model rats compared with the control group (Fig. 3). However, lutein significantly decreased the induced TNF-α, IL-1β, IL-6 and MCP-1 levels in STBI rats compared with the untreated STBI model group (Fig. 3).

Effect lutein on ROS level in STBI. Fig. 4 demonstrated a significant increase in ROS level in STBI model rats compared with the control group. Additionally, compared with the STBI model group, lutein treatment significantly inhibited the increase of ROS levels caused by STBI (Fig. 4).

Effect of lutein on oxidative stress in STBI. Furthermore, an inhibition of SOD and GPx in STBI model rats was observed

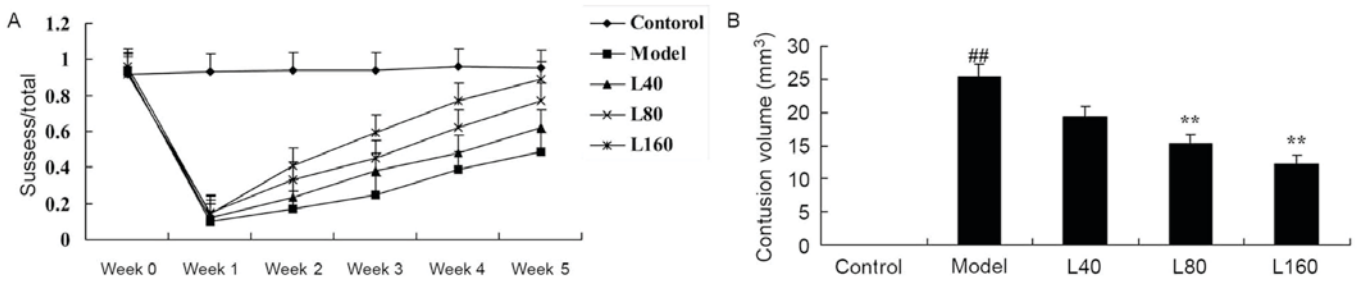

Figure 2. Effect of lutein on skilled motor function and contusion volume. (A) Skilled motor function (forelimb reaching test) and (B) contusion volume were measured in STBI model rats treated with lutein. ##P<0.01 vs. control group; **P<0.01 vs. model group. Model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury. There were six mice in each group.

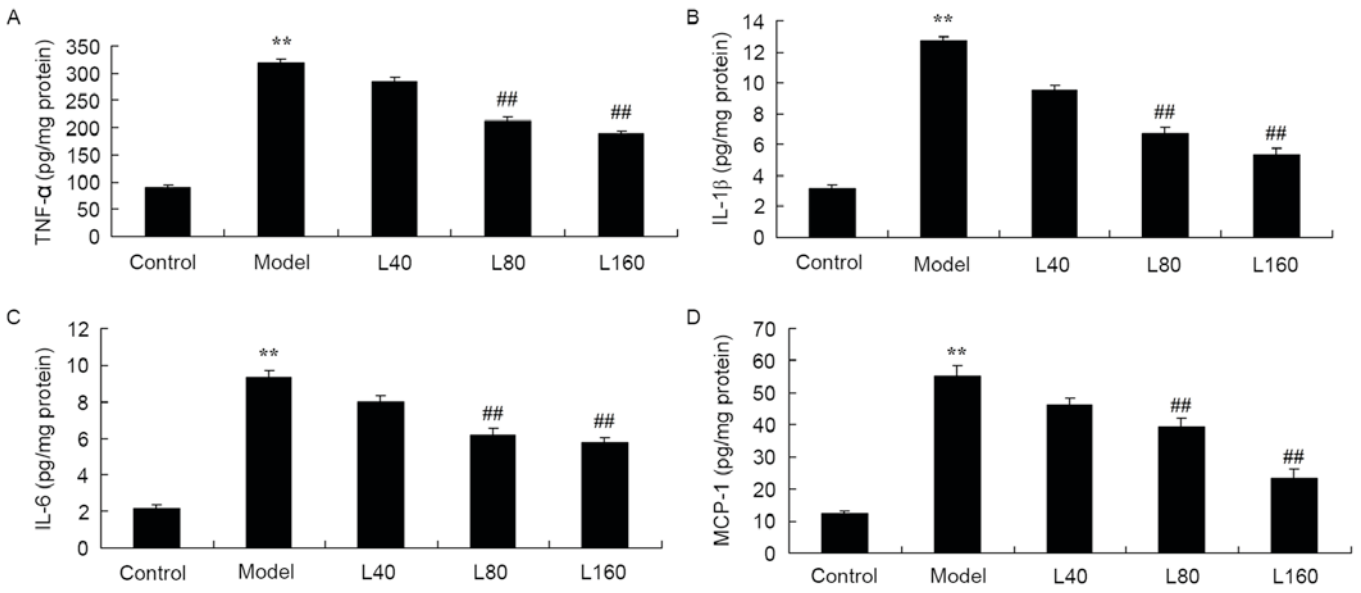

Figure 3. Anti-inflammatory effect of lutein in STBI. The effect of lutein on (A) TNF-α, (B) IL-1β, (C) IL-6 (D) and MCP-1 levels in the STBI model. **P<0.01 vs. control group; ##P<0.01 vs. model group. Model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury. There were six mice in each group.

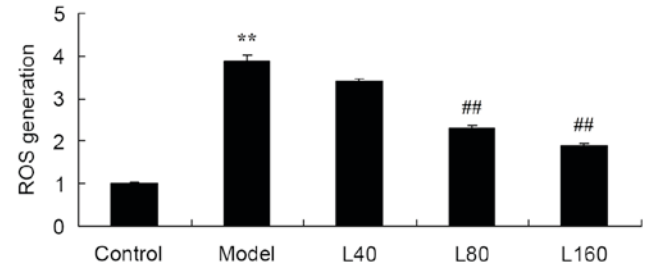

Figure 4. Effect lutein on ROS level in the STBI model. **P<0.01 vs. control group; ##P<0.01 vs. model group. ROS, reactive oxygen species; model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury. There were six mice in each group.

compared with the control group (Fig. 5). However, pre-treatment with lutein significantly alleviated the inhibition of SOD and GPx compared with untreated STBI model rats (Fig. 5).

Effect of lutein on NF-κB p65, COX-2 and ICAM-1 protein expression in STBI. As demonstrated in Fig. 6, the *NF-κB p65, COX-2 and ICAM-1* protein expression in STBI model rats was significantly increased compared with the control group. Treatment with lutein significantly suppressed the activation of *NF-κB p65, COX-2 and ICAM-1* protein expression compared with untreated STBI model rats (Fig. 6).

Effect of lutein on Nrf-2 and ET-1 protein expression in STBI. As presented in Fig. 7, there was a significant inhibition of Nrf-2 and ET-1 protein expression in STBI model rats compared with control rats. Lutein treatment significantly reduced the inhibition of Nrf-2 and ET-*1* protein expression in STBI model rats compared with untreated STBI model group rats (Fig. 7).

Discussion

ischemic cerebral infarction leads to neurocyte death in penumbra surrounding infarction regions (11). Thrombolytic therapy during the early stages can recover blood flow in penumbra as soon as possible, which regains oxygen and nutrient supply, and reduces nerve cell damage (12). With the progression of thrombolytic therapy, the effects of reperfusion injury following restoration of blood circulation is gaining attention (13). STBI is one of the major causes of cerebral

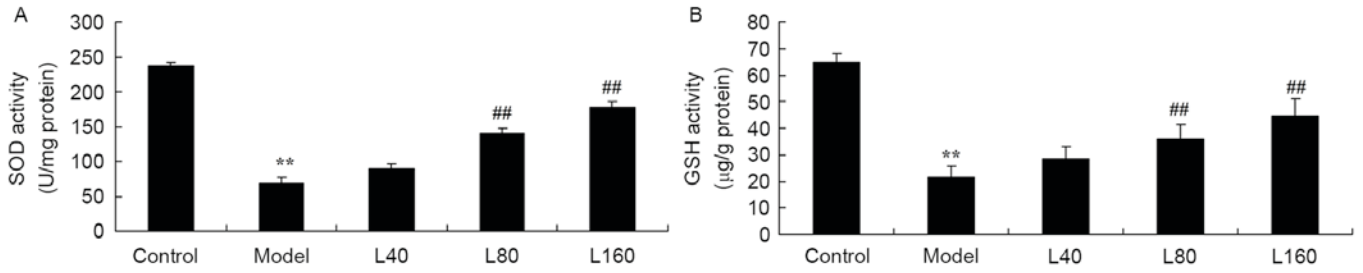

Figure 5. Effect lutein on oxidative stress in STBI. (A) SOD and (B) GSH activities in were measured in STBI model rats. **P<0.01 vs. control group; ##P<0.01 vs. model group. SOD, superoxide dismutase; GPx, glutathione peroxidase; model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury. There were six mice in each group.

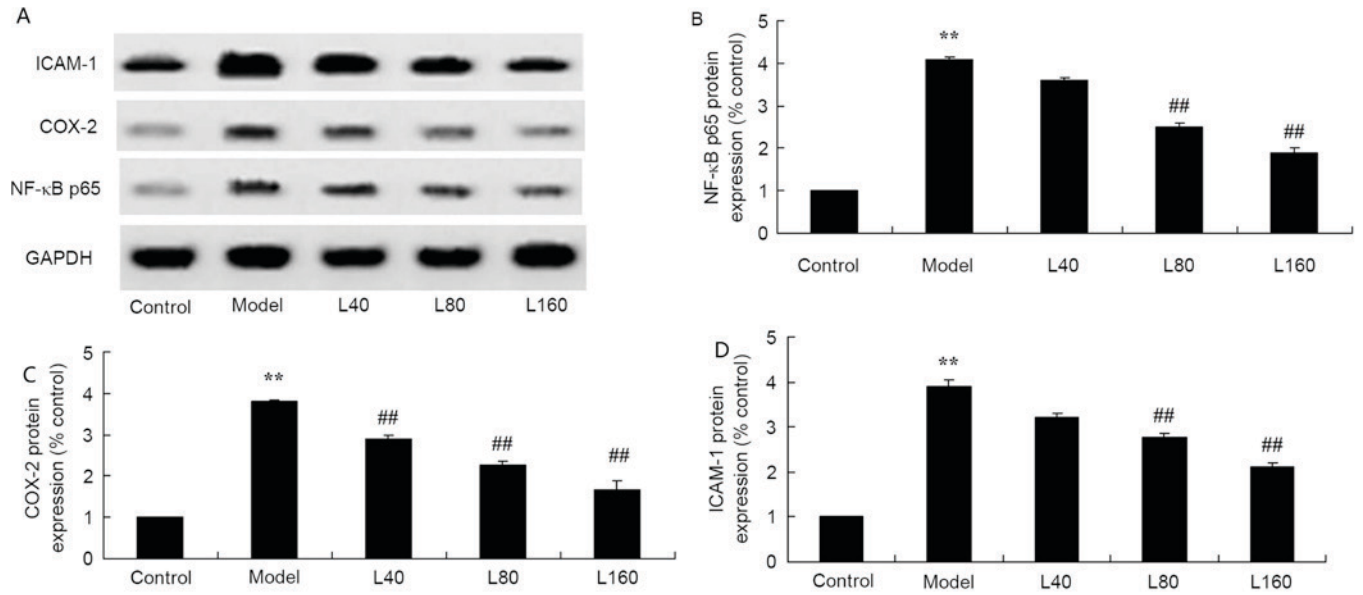

Figure 6. Effect of lutein on NF-κB p65, COX-2 and ICAM-1 protein expression in STBI. Effect of lutein on NF-κB p65, COX-2 and ICAM-1 protein expression by (A) western blotting. Densitometric analysis of (B) NF-κB p65, (C) COX-2 and (D) ICAM-1. **P<0.01 vs. control group; ##P<0.01 vs. model group. NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2; ICAM-1, intercellular adhesion molecule-1; model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury. There were six mice in each group.

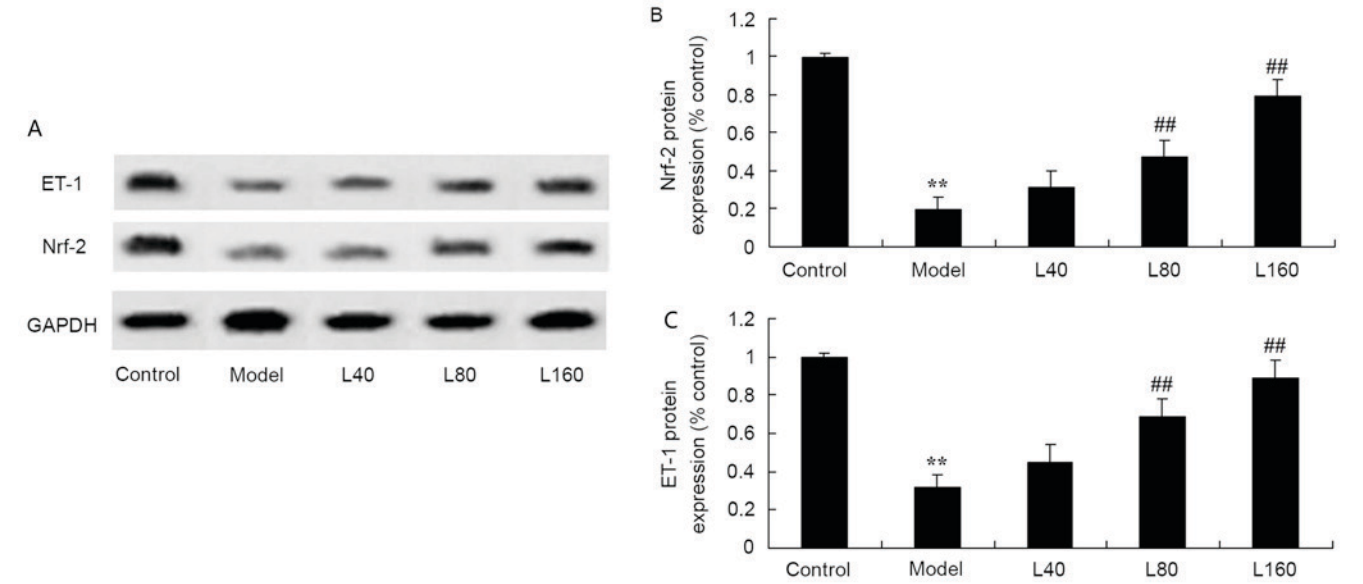

Figure 7. Effect of lutein on Nrf-2 and ET-1 protein expression in STBI. Effect of lutein on Nrf-2 and ET-1 protein expression by (A) western blotting. Densitometric analysis of (B) Nrf-2 and (C) ET-1. **P<0.01 vs. control group; ##P<0.01 vs. model group. Nrf-2, nuclear factor erythroid 2 like 2; ET-1, endothelin-1; model, STBI model group; L40, 40 mg/kg lutein group; L80, 80 mg/kg lutein group; L160, 160 mg/kg lutein group; STBI, severe traumatic brain injury.

ischemic disease, however, the mechanisms of STBI are not yet and there is a lack of effective interventions to treat STBI (14). The current study demonstrated that lutein significantly reduced contusion volume and increased the inhibited skilled motor functions caused by STBI in a rat model.

STBI directly damage the brain. Injured neurons and local inflammatory cells release inflammatory cytokines, chemotactic factors (such as MCP-1), neurotransmitters and oxygen radicals (15). These promote the expression of adhesion molecules and cause damage to the vascular endothelium (15). Additionally, STBI increases the expression of matrix metalloproteinases, damages the blood brain barrier, the inflammatory response is extended and neuron damage is worsened (16). Due to the destruction of the blood brain barrier or restoration of blood flow following reperfusion, inflammatory signals can enter the periphery through the blood and trigger a systemic immune response (17). When an inflammatory reaction occurs, a damage repair regulatory response may be initiated to repair tissue damage or necrosis and promote the regeneration of nerve and blood vessels (18). The current study demonstrated that lutein significantly decreased the expression of TNF-α, IL-1β, IL-6 and MCP-1 activities in STBI rat. Cheng *et al* (19) previously reported that lutein protects against ischemia-reperfusion injury by modulating oxidative stress and inflammation, and suppression of NF-κB and COX-2 expression.

NF-κB is a transcription factor involved various signal transduction pathways (16). It is a key protein involved in regulating the expression of inflammatory factors during cerebral ischemia-reperfusion. Various studies have confirmed that cerebral ischemia-reperfusion induces the activation of NF-κB. After cerebral ischemia-reperfusion, activated NF-κB dissociates for IκB and phosphorylated IκB is subsequently degraded (20,21). Activated NF-κB translocates from the cytoplasm to the cell nucleus. When the expression level of NF-κB is increased that expression of ICAM-1 and TNF-α are also subsequently increased (22). This indicates that cerebral ischemia can activate NF-κB causing increased expression of regulated target genes, including ICAM-1 and TNF-α. This increased the inflammatory response further, which worsens STBI (23). The current study demonstrated the lutein treatment significantly suppressed NF-κB protein expression in STBI model rats. Cheng *et al* (19) previously demonstrated that lutein protects against ischemia-reperfusion injury by modulating oxidative stress and inflammation, and suppressing of NF-κB and COX-2 expression.

When ischemia-reperfusion occurs at acute stage of ischemic cerebrovascular disease, high levels of ROS are released, which act on the cytomembrane surface of blood vessels in the brain (24). Mitochondria are destroyed, which promotes more ROS to be released. Hypoxia-ischemia of cerebral cells reduces energy production, resulting in the activity of phospholipase A2, break down of membrane phospholipids and the release of arachidonic acid (25). When reperfusion occurs, epoxidase and lipoxygenase degrade arachidonic acid and generate superoxide radicals (26). The destruction of mitochondria and the electron transport chain can also promote the formation of superoxide radicals and aggravate cerebral damages (6). The current study demonstrated that lutein treatment significantly inhibited the activation of ROS level and reduced the inhibition of SOD and GPx caused by STBI in model rats. Li *et al* (7) suggested that lutein protects against hepatotoxicity induced by arsenic via suppression of ROS and antioxidant Nrf2 signaling.

Under normal conditions, the majority of cells do not express COX-2, and there is low COX-2 expresses in cerebral tissues. However, COX-2 expression is induced by inflammation (27). Various inflammatory stimulating factors, including lipopolysaccharide, IL-1, TNF, epidermal growth factors and platelet-activating factors, can induce expression of COX-2, resulting in the increase of prostaglandin contents (28). Significant and continuous increase of COX-2 expression in cerebral tissues after cerebral ischemia is a key factor that causes and promotes inflammatory responses during cerebral damages (29). Additionally, COX-2 is also a key factor that mediates cytotoxicity caused by inflammation in ischemic brain injury (30). Studies suggested that COX-2 is involved in the development of cerebral damage following ischemia and is associated with prognosis following cerebral ischemia (28,31). In the current study, lutein treatment significantly suppressed COX-2 protein expression in STBI rats. Cheng *et al* (19) previously reported that lutein protects against ischemia/reperfusion injury by modulating oxidative stress and inflammation, and suppression of COX-2 expression.

The kelch like ECH associated protein 1 (Keap1)-Nrf2-antioxidant responsive element system is now considered to be the major defense mechanism that is induced to protect against oxidative stress. Keap1 negatively regulates Nrf2, which is a transcription factor that regulates the expression of genes with antioxidant functions (32). Under normal conditions, Keap1 and Nrf2 bind together. They are subsequently rapidly degraded in the cytoplasm through the ubiquitin proteasome system, blocking the transcription of antioxidant target genes mediated by Nrf2 (33). Under oxidative stress, activities of Keap1 are inhibited and Nrf2 is released and translocates to the nucleus, thus, inducing the expression of downstream antioxidant genes (23). Peroxiredoxins can scavenge excessive ROS through a series of synergistic effects and have an essential role in maintaining the normal cellular redox state and reducing oxidative stress (34). In the current study, lutein significantly downregulated the expression of ICAM-1 and counteracted the inhibition of Nrf-2 and ET-1 protein expression induced by STBI. Du *et al* (8) previously reported that lutein prevents alcohol-induced liver disease through antioxidative stress and anti-inflammatory effects, and increased Nrf-2 expression levels. Wang *et al* (10) reported that lutein prevents hyperhomo-cysteinemia through suppression of oxidative stress by NF-κB p65 and ICAM-1/ET-1.

In conclusion, the current study identified the lutein rescued the inhibition of skilled motor functions caused by STBI and reduced the contusion volume in STBI rats. Additionally, lutein altered the expression of antioxidative and inflammation-associated protein. The protective effect of lutein in this STBI model may be mediated via effects on an NF-κB p65/ICAM-1/Nrf-2 signaling pathway, which suggested that lutein may be developed as drug for the treatment of STBI in the clinic.

Acknowledgements

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