Neuroprotective effects of *Fructus Chebulae* extracts on experimental models of cerebral ischemia

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**Abstract**

**OBJECTIVE:** To investigate the neuroprotective effects of *Fructus Chebulae* extract using both in vivo and in vitro models of cerebral ischemia.

**METHODS:** As an in vitro model, oxygen glucose deprivation followed by reoxygenation (OGD-R) and hydrogen peroxide (H$_2$O$_2$) induced cellular damage in rat pheochromocytoma (PC12) cells was used to investigate the neuroprotective effects of extract of *Fructus Chebulae*. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to calculate cell survival. For in vivo, occlusion of left middle cerebral artery on rats was carried out as a focal cerebral ischemic model.

**RESULTS:** *Fructus Chebulae* extract increases the PC12 cell survival against OGD-R and H$_2$O$_2$ by 68% and 91.4% respectively. *Fructus Chebulae* also decreases the cerebral infarct volume by 39% and extent of hemisphere swelling from 17% in control group to 10% in *Fructus Chebulae* treated group.

**CONCLUSION:** *Fructus Chebulae*, as a traditional medicine, can rescue the neuronal cell death against ischemia related damage. The possible mechanism for the neuroprotection might be the inhibition of oxidative damages occurring after acute phase of cerebral ischemia.

**Key words:** *Fructus Chebulae*; Pheochromocytoma; Neuroprotection; Brain ischemia; Middle cerebral artery

**INTRODUCTION**

Cerebral ischemic stroke is recognized as the most common reason of disability in old age and is the common cause of death worldwide. A number of neuroprotective compounds, such as calcium channel inhibitors, antagonists of glutamate receptor, free radical scavenging agents, anti-inflammatory agents, nitric oxide synthase inhibitors etc., have been suggested for their therapeutic potential in stroke patients. However, though these drugs have been found to be effective in experimental studies, they have shown mixed efficacy in human clinical trials. Currently practiced pharmacological therapy for stroke patient is ineffective and that leads to the search for newer pharmacologic approaches. Because of the absence of effective and commonly applicable pharmacological agent for stroke treatment, traditional medicines, mainly of herbal origin, are attracting attention. In this regards, we have been screening several medicinal plants for their possible protec-
tive effects in cerebral ischemia/stroke, and among such plants, Fructus Chebulae, a well-known medicinal plant in Ayurveda, showed the promising effects. In Ayurveda, Fructus Chebulae is commonly used as cardiotonic, digestive, diuretic, antitussive, antidiabetic, and laxative. Fructus Chebulae was previously reported to have antioxidant, anticancer, anti-inflammatory, anti-hyperglycemic, anti-ulcer, hepatoprotective, cytoprotective, and cardio-protective activities. Major phytochemicals reported from fruit are chebulic acid, chebulagic acid, ellagic acid, quercetin, gallic acid, corilagin, rutin, casuarinin, tannic acid, etc. Recently, Fructus Chebulae and its phytochemicals, have been reported to possess strong antioxidant and anti-inflammatory effects. In spite of the abundant evidence supporting the antioxidant and anti-inflammatory effects of Fructus Chebulae, and its active polyphenols; few in vitro researches have been reported about the neuroprotective effect of Fructus Chebulae. However, the neuroprotective potentials of Fructus Chebulae extract using in vivo ischemic models have not been reported yet. In present study, the neuroprotective activity of Fructus Chebulae extracts was investigated in experimental ischemic models. We used rat pheochromocytoma (PC12) cell line treated with oxygen glucose deprivation followed by re-oxygenation (OGD-R) and hydrogen peroxide (H2O2) as an in vitro model. For in vivo, occlusion of middle cerebral artery (MCAs) induced focal cerebral ischemia models in rats was used.

MATERIALS AND METHODS

Extraction and sample preparation
After proper identification by the experts and literature comparison, Fructus Chebulae were purchased from National Exports Private Limited (Kathmandu, Nepal). A herbarium and voucher specimen (HP203) was preserved in the College of Korean Medicine, Kyung Hee University, Seoul, South Korea. Total 100 g of Fructus Chebulae was grinded and powder (100 g) was extracted using 70% methanol (1000 mL) under reflux conditions (Sigma Chemical Co., St. Louis, MO, USA). The organic solvent of extracts was then evaporated and lyophilized until complete dryness. The yield value of extract was found as 36.48%.

OGD-R induced cell injury
OGD-R experiment was performed as described previously. Briefly, PC12 cells at a density of 1.5 × 10^5 cells/well were seeded and cultured at 37°C. Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1 x 10^5 U/L penicillin and 100 mg/L streptomycin (Gibco BRL, Grand Island, NY, USA) in a 5% CO2 incubator was used as culture medium. OGD-R was performed at 24 h after the cell seeding. PC12 cells were first washed with phosphate buffer saline (PBS) followed by glucose-free DMEM. Then fresh glucose free DMEM was placed in cultures and kept in a hypoxic chamber (Forma Science, UK) containing 5% CO2 and 95% N2 for 4 h. After 4 h, fresh glucose solution was added, and the cells were incubated in a 5% CO2 incubator at 37°C for 24 h. PC 12 cells were treated with different concentrations of Fructus Chebulae extracts (at 0.1, 1, 10 μg/mL) 30 min before and during OGD period. Serum free glucose DMEM of same amount was used for the control group. For positive control, Baicalein, a well-known neuroprotective and antioxidative compound was used.

H2O2 induced cell injury
Prior to each experiment, H2O2 was diluted from stock solution (30%) using DMEM. For H2O2 induced cell injury, cultured PC12 cells were first washed with FBS free DMEM and treated with 200 μM H2O2 solution and incubated 24 h. Different concentrations of Fructus Chebulae extracts were treated 2 h before and during H2O2 exposure period. Control group were not treated with H2O2, however incubated under the same conditions.

Animals and MCAo surgery
All animal handling and surgical procedures were performed in compliance with animal welfare guidelines of Korean Academy of Medical Sciences and the National Institutes of Health (NIH). Male Sprague-Dawley rats [(300 ± 5) g, 8 weeks], were purchased from Samtako, Gyeonggi-do, Korea. Before experimental procedure, rats were acclimatized in controlled conditions of temperature (22°C ± 2°C), relative humidity, and a 12 h light/dark cycle, with free access of food and water.

Rats were ventrally fixed in operating frame and anesthetized with isoflurane in N2O:O2 (3:1). The body temperature was controlled and maintained at 37.0 ± 0.5°C during the experimental procedure with rectal thermistor connected with a heating blanket (Harvard Apparatus, Holliston, MA, USA). Focal cerebral ischemia was induced using the modified methods of intraluminal suture occlusion. After 90 min of MCAo period, the suture was carefully removed and reperfusion was allowed for 24 h. Body temperature was maintained at 37.0°C ± 0.5°C, till the 6.5 h after occlusion. For the sham operated group, similar surgical procedure was done but the MCA was not occluded.

Drug treatment
Animals were randomly divided into 4 different groups; sham, control, and 2 extract treated groups (300 and 500 mg/kg). Different concentration of Fructus Chebulae extracts was dissolved in 5% dimethylsulfoxide (DMSO) and orally administered immediately after MCA occlusion (0 min). The doses of Fructus Chebulae extracts (determined based on the previous experi-
Extent of Edema = \frac{\text{Volume of IH} - \text{volume of CH}}{\text{Volume of CH}} \times 100\%

Where IH is ipsilateral (infarct) hemisphere and CH is contralateral (unaffected) hemisphere

**RESULTS**

**Fructus Chebulae extract protects PC12 cells from OGD-R induced damage**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays was used to determine the survival of cells after treatment of *Fructus Chebulae* extract. At different concentration (0.1-10 μg/mL), *Fructus Chebulae* didn’t show the cytotoxic effects on cultured PC12 cells indicating that it is safe for cellular experiment. To study the protective effect of *Fructus Chebulae* extract after OGD-R induced damage, *Fructus Chebulae* extracts were treated 30 min before and during 4 h of OGD period. Cell density in OGD group was significantly reduced (50.1%±1.1%) whereas in *Fructus Chebulae* extract treated groups (at 0.1, 1, 10 μg/mL) cell survival was found as 62.5%±2.2%, 68.1%±2.7% and 55.8%±1.9% respectively, all P<0.05 as compared to that of control group (set as 100%). Baicalein, used as positive control, at 0.27 μg/mL (1 μM) showed 74.5%±2.9% cell viability (Figure 1).

**Figure 1 Protective effect of Fructus Chebulae extracts in PC12 cells exposed to OGD-R**

A: control; B: OGD-R; C: *Fructus Chebulae* (0.1 μg/mL); D: *Fructus Chebulae* (1 μg/mL); E: *Fructus Chebulae* (10 μg/mL); F: Baicalein (0.27 μg/mL). PC12 cells were treated with OGD for 4 h and re-oxygenated for 24 h. PC12 cells were treated with *Fructus Chebulae* extracts at different concentrations at 30 min before and 4 h of OGD period. Cell viability in control group was set as 100%, and cell densities in other experimental groups were shown as percentage of control. OGD-R: reoxygenation. *P<0.01, †P<0.001, and ‡P<0.05 as compared to the OGD-R group.

**Fructus Chebulae extract protects PC12 cells from H₂O₂ induced oxidative damage**

We next determined the protective effect of *Fructus Chebulae* extract on H₂O₂ induced oxidative damage in PC12 cells. H₂O₂ showed the concentration and time-dependent toxicity in PC12 cells (data not shown). Concentration of H₂O₂ used was 200 μM for and it was determined based on the toxicity study. H₂O₂ treated group showed the cell viability of 71.4%±1.3% whereas that in *Fructus Chebulae* extract treated groups were 81.6%±3.8% (0.01 μg/mL), 91.3%±3.7% (0.1 μg/mL), and 86.8%±4.2% (1 μg/mL) respectively, all P<0.05 as compared to that of control group (set as 100%). Baicalein, used as positive control, treated cells showed 83.8%±2.5% (0.27 μg/mL) cell viability against H₂O₂ (Figure 2).

**Fructus Chebulae extracts reduce the infarct volumes and cerebrovascular edema in rats**

*Fructus Chebulae* extracts significantly reduced the brain infarct volume after 90 min of MCAo followed by 24 h reperfusion. The representative TTC stained brain
section photographs are shown in Figure 3A. The infarct volume of rats, consuming 500 mg/kg of Fructus Chebulae extracts (207.105 mm\(^3\)) was found to be significantly lower than that in control (vehicle) treated group (388.932 mm\(^3\)) (\(P < 0.05\)). The extracts at 500 mg/kg showed the 39% reduction of infarct volume, whereas that of 300 mg/kg was 32% (Figure 3B). Effect of Fructus Chebulae on extent of hemisphere swelling are shown in Figure 3C.

DISCUSSION

As in the ischemic conditions, OGD-R increases the formation of free radicals, produce from different sources such as inflammatory cytokines, glutamate toxicity etc.\(^{23,24}\) Overproduction of free radicals in the cells are believed to be the key mediators of tissue damage after ischemia-reperfusion in animal models\(^{25}\) and as OGD-R mimics the tissue ischemia similar cellular events occur in PC12 cells as well. Cellular damage after ischemia reperfusion is a very complex pathophysiological condition, which is associated with impairment of several cellular and vascular responses. Oxidative stress induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) play the crucial role to initiate a wide ranges of intracellular signaling processes that results in excessive cytokine and chemokine response which ultimately leads to the cellular death.\(^{26}\) Once the ROS and RNS are generated in the cells, various oxidative cascades take place simultaneously, including free radical production, excitotoxicity, enzymatic alterations, sudden start and aggravation of mitochondrial respiratory chains and stimulation of the inflammatory processes.\(^{27}\) Among such oxidative cascades, damage due to free radicals and inflammation are the key factor for the cellular damage. Therefore, phytochemicals having strong anti-inflammatory, anti-oxidant, anti-glutamate properties can antagonize the cytotoxic effects of free radicals and protect the cells against hypoxia induced damage.\(^{24,28-30}\)

In this study, Fructus Chebulae extract significantly protected the OGD-R exposed PC12 cells. The 4 h of OGD exposure and 24 h of re-oxygenation leads to the significant damage in PC12 cells compared to that of control cells. Treatment of PC12 cells with Fructus Chebulae extract at different concentration, 30 min before followed by 4 h during the OGD period, significantly increases the cell survival. OGD facilitates the cytotoxicity in the cells by activating the cascades of oxidative events such as nuclear factor-\(\kappa B\), lipoxygenase, IkB kinase, suggesting that OGD-R acts as the major source of free radical in ischemic conditions.\(^{31,32}\) In present study, Fructus Chebulae extract significantly sequences...
tered the DPPH free radicals and the strong antioxidant efficacy of *Fructus Chebulae* extract might acts as the key role for ROS sequestering, which are in the ischemic conditions, like OGD-R and protects the cells from damage. *Fructus Chebulae* extract significantly protects (91.52%) the PC12 cell from H$_2$O$_2$ induced oxidative damage, and the protective effect of extracts was found to be higher than that of potent antioxidant compound, baicalein (84.02%). H$_2$O$_2$ itself is not a free radical and has a limited reactivity; however, it is believed to be the major precursor of the highly reactive hydroxyl radical. Previous studies have reported the intimate association between neurodegenerative disease and H$_2$O$_2$, and it has been advised that levels H$_2$O$_2$ is increased during various pathological conditions, such as ischemia. Reactive oxygen species, such as H$_2$O$_2$, readily damage several biomolecules that can eventually lead to apoptotic or necrotic cell death. In this study, the protective effect of *Fructus Chebulae* extract was not concentration dependent. Interestingly, the cell viability in *Fructus Chebulae* extract treated group was higher in less concentration of *Fructus Chebulae* extract and lower in higher concentration. Gallic acid, quercetin, and ellagic acids are the major polyphenolic compounds from *Fructus Chebulae* and they are well-known antioxidant. These polyphenols are previously reported to have pro-oxidant activity at higher concentrations and such properties of *Fructus Chebulae* polyphenols might explain the lesser protective effect of *Fructus Chebulae* extract at higher concentration. After MCAo induced focal cerebral ischemia, the acute phase damage occurs in ischemic core that consists of the parietal somato-sensory cortex and lateral portion of the caudate putamen. The region around the ischemic core, penumbra, undergoes slow tissue damage after several hours of ischemic induction. The penumbral tissue damage can be protected by preventing the cell death processes using neuroprotective agents. The neuroprotection in ischemic core is almost impossible and penumbra is the target of therapeutic efforts in the acute phase of ischemia or stroke. *Fructus Chebulae* treatment (500 mg/kg) reduced the brain damage in penumbra, and the infarct area was limited to part of the caudate putamen and parietal cortex, which is believed to be the ischemic core. Therefore, these results suggest that *Fructus Chebulae* can protect neuronal cells in the penumbra region and it can act as a potential neuroprotective agent in stroke. As shown in figure 3C, the extent of hemispheric swelling in extracts treated group at higher dose (500 mg/kg), was significantly lower than that of vehicle treated group. Generally, cerebral edema is determined through calculating the water content by subtracting the weight of dry brain from the weight of wet brain. However, in this study, we relatively calculated the extent of edema to measure the hemisphere swelling index as described. Previous studies established the correlations between neurological deficit after MCA occlusion and the extent of cerebral edema as estimated by hemispheric enlargement. Cerebrovascular edema after ischemic insult appears to contribute severely to morbidity and mortality in stroke patient. Following cerebral ischemia/stroke swelling of damaged cells, leaking of injured blood vessels occurs that subsequently block the absorption pathways forcing fluid to enter brain tissues. Cytotoxic edema which is developed in response to the occlusion of large MCA is a major clinical problem and it has been associated with about 80% mortality rate in stroke patient. Occlusion of cerebral vessels initiates a sequence of events involving cell swelling, followed by blood brain barrier leakage and tissue hemorrhage. Activated microglia play pivotal role to produce various pro-inflammatory cytokines such as IL-6, TNFα, IL-1β, and also anti-inflammatory cytokines such as IL-10, IL-4, and TGFβ, contributing the cerebrovascular edema. *Fructus Chebulae* is reported to have strong inhibitory effect against inflammatory chemokine, IL-8 and NF-κB, mediator of cellular inflammation suggesting that it can prevent the secretion of inflammatory mediators through the activated microglia after stroke. In our study, the hemisphere swelling index in rats treated with 500 mg/kg of *Fructus Chebulae* extracts was significantly lower than that in vehicle treated group. In summary, *Fructus Chebulae* extract showed the significant protective effect against *in vitro* OGD-R and H$_2$O$_2$ induced cell death. It also has protective effect against neuronal cell death on MCAo/reperfusion induced cerebral ischemic injury, suggesting that it can act the potential herbal medicine, to treat cerebral ischemia and related diseases.

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