Protective Effect of Ginsenoside against Acute Renal Failure and Expression of TH in the Locus Coeruleus

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Summary

Acute renal failure (ARF) is mainly characterized by acute tubular necrosis. No significant change was found for mortality rates over the past few decades despite significant advances in supportive care. In recent years, great efforts have been focused on traditional and herbal medicine, which is much less toxic than those agents conventionally used and nowadays considered as a novel therapeutic agent for ARF. However, the effect of ginsenoside (GS) administrated orally on ARF has not been reported yet and little is known about its cellular and molecular mechanism. The purpose of the study is to investigate the protective effect of ginsenoside on ARF rats and the changes of TH-immunoreactivity (TH-IR) and the mediation of mitogen-activated protein kinases (MAPK) in the locus coeruleus. In our assay, glycerol-induced acute renal failure in rats was employed to study the protective effects of ginsenoside. Our results indicated that the treatment of ginsenosides in ARF rats for 48h significantly reduced the serum BUN, Cre level, and lipid peroxidant, restored the GSH level and the renal normal morphology. Immunohistochemistry showed that an obvious increase of TH-IR was further enhanced in ARF+GS group. The same result was also observed in the changes of p-ERK1/2-IR in the locus coeruleus. Our results suggest that ginsenoside administrated orally may have a strong renal protective effect against glycerol-induced ARF, and ginsenoside can also activate the brain

catecholaminergic neurons in the locus coeruleus. Whether there is a correlation between the renal protective effect of GS against ARF and the activation of TH in the LC is a focal point in our lab in the future.

Key words

Ginsenoside · Acute renal failure · Locus Coeruleus ·Tyrosine hydroxylase · Protective effect

Introduction

Acute renal failure (ARF) is frequent in hospitalized critically ill patients and mortality associated with ARF is largely unchanged over many decades. ARF is mainly characterized by acute tubular necrosis. Progress in elucidation of the pathophysiology has led to development and testing of many therapeutic drugs and other interventions in animal and human forms of acute tubular necrosis (Kellum 2004, Lameire *et al.*2003). Renal replacement therapy has promising features in treating with ARF, especially before complication. However, it was reported that incidence was rising over the past two decades (Hou *et al.*1983, Nash *et al.*2002) and case fatality rates exceeded 50% among those who required dialytic support（Metnitz *et al.*2002，Mehta *et al.*2004）. Therefore, mortality rates have changed little over the past few decades despite significant advances in supportive care. Therefore, preventions of the occurrence and progression of ARF has become a very important issue. In recent years, great efforts have been focused on traditional and herbal medicine without toxic effects to provide a novel therapeutic agent for ARF.

Panax ginseng C.A. Meyer is a well-known folk medicine in the Far East countries and it has served as an important component of many Chinese prescriptions since thousands of years（Wen *et al.*1996）. Ginsenosides (GS), the principal active ingredients of ginseng, has a wide

range of pharmacological and physiological actions, such as antiaging, immunoenhancement, antistress and antitumor（Sugaya *et al.*1988，Hasegawa *et al.*2002, Kaneko *et al.*2004）. Moreover, it has been reported that sun ginseng (SG, heat-processed Panax ginseng C. A. MEYER at 120°C) showed strong protective effect against diabetic renal damage（Kang *et al.*2006）. However, the effect of GS administrated orally on ARF has not been reported yet and little is known about its cellular and molecular mechanism.

Locus coeruleus (LC), an important integrated site in the pons, regulates sympathetic nerve activity, fluid balance and arginine vasopressin (AVP) release. Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, is highly expressed in locus coeruleus in the brain. Exposure to many types of physiological, social or pharmacological stressors, such as cold, restraint, footshock, isolation, forced walking and chronic social stress increases TH mRNA in the LC （Angulo *et al.* 1991，Watanabe *et al.*1995，Rusnák *et al.*1998）. It is reported that the increase in the expression of TH in the LC coincides with increased transcription（Chang *et al.*2000）. Similarly, the TH gene is often associated with induction of the AP-1 transcription factor c-Fos（Sun *et al.*2003）. The mitogen-activated protein kinases (MAPK) function in the intracellular signaling pathways, transferring extracellular stimuli into intracellular transcriptional responses, play an important role in the activation of several transcription factors （Karin *et al.* 1995）. The extracellular signal-regulated kinases (ERK), is one of the four subfamilies of MAP kinases. In particular, ERK stimulates c-fos expression（Whitmarsh *et al.*1996）, indicating that MAPK may facilitate the formation of c-fos/c-Jun hetero dimmers, controlling the regulation of AP-1 activity. We have hypothesized that MAPK might be involved in the differential regulation of TH induction in the brain catecholaminergic neurons.

A recent study in vivo indicated that the phenotypic differentiation of LC noradrenergic neurons mediated by brain-derived neurotrophic factor (BDNF) was enhanced by corticotrophin-releasing factor(CRF) through the activation of a cAMP-dependent signaling pathway that involved activation of ERK1/2（Traver *et al.*2006）. Previous in vitro experiment suggested that ERK signaling pathway in the kidney was strongly related to the renal function and renal cell regeneration after the glycerol injection（Ishizuka *et al.*1999）. However, the roles of brain catecholaminergic neural pathway and brain MAPK signal pathway, and their interaction in glycerol-induced ARF rats remains unclear.

Based on these findings, it could be speculated that gisenosides might have protective effect and there might be an interaction between catecholaminergic neurons and MAPK signal pathway in the LC in glycerol-induced ARF rats to some extent. Therefore, we herein examines 1) the changes of blood urea nitrogen (BUN) and serum creatinine (Cre) in serum in the ARF rats treated with ginsenoside for 48h, 2) the changes of malondialdehyde (MDA) and the reduced glutathione (GSH) in renal cortex homogenate in the ARF rats treated with ginsenoside for 48h, 3) the changes of tyrosine hydroxylase-immunoreactivity (TH-IR) in the LC in glycerol-induced ARF rats treated with ginsenoside for 48h, 4) the changes of phospho-ERK1/2-immunoreactivity (p-ERK1/2-IR) in the LC in glycerol-induced ARF rats treated with ginsenoside for 48h.

Methods

Animals

Healthy male Sprague-Dawley rats (Dalian Medical University Animal Center, China) weighing 180~220g were kept on a 12h/12h light/dark schedule with a free access to standard

laboratory food and water at room temperature. During this time, rats were handled daily to avoid stress-induced expression on the day of the experiment.

Animals' Treatment

Eighty male rats were used in this study. After several days of adaptation, they were deprived of water from 4 p.m. to 8 a.m. for 16h.

Forty rats for the experiment in vivo were divided randomly into four groups(n=10 per group): acute renal failure (ARF) + physiological saline(NS) group, ARF + ginsenoside (GS) group, NS+NS group and NS+GS group. After deprived of water for 16h, ARF+NS group and ARF+GS group were administrated intramuscularly with 10ml/kg b.w. of 50% (vol/vol) glycerol solution distributed equally in both hind limbs. As soon as the model was established, ARF+GS group was given 25mg GS in 2ml NS using a stomach tube, at the same time, ARF+NS group was given 2ml NS. The treatment of the other two groups were similar to them. They were treated with GS or NS for two consecutive days (once per 6h, twice per day).

Another 24 rats for immunohistochemistry were also divided into 4 groups and treated as described in the experiment in vivo.

Experiment in vivo

After treatment with ginsenoside or physiological saline for 48h, blood samples for urea nitrogen and creatinine determination were taken from the angulus oculi medialis. Subsequently kidney was removed, then renal cortex was homogenized with ice-cold physiological saline.

Blood urea nitrogen (BUN) was assayed by the Fearon method and serum creatinine

(Cre) by the Jaffe method using standard diagnostic kits.

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) using commercial reagents (Nanjing Jiancheng Bioengineering Institute, P.R. China).

The reduced glutathione (GSH) was measured by the method of Jollow using commercial reagents (Nanjing Jiancheng Bioengineering Institute, P.R. China). It was observed by measuring the absorbance at 412nm.

Renal histology

Forty-eight hours after the administration of ginsenoside or physiological saline, 40 rats were deeply anesthetized with 4% chloral hydrate (400mg/kg b.w., i.p.) and perfused transcardially with 1 % and 4 % paraformaldehyde for the fixation of the brain and kidney tissue. Brain and kidney tissue were removed, post-fixed in 4 % paraformaldehyde and immersed into a phosphate buffer saline (PBS) containing 30 % sucrose for three days.

Kindey was washed by pure water for 12h and then was embedded in paraffin and used for histological examination. 4 μm thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin. The renal sections were examined in blind fashion for hemorrhagic and hyaline casts, tubular necrosis and apical blebbing in all treatments.

Immunohistochemistry analysis

When the brain tissues were submerged, 50 μm thick coronal brain sections were sliced on a vibratome. The identification of locus coeruleus (bregma –9.68 mm ~ –10.04mm) was based on the atlas by Paxinos and Watson. The sections above were rinsed three times in PBS 10 min

and then incubated with 0.2 % Triton for 5min. The sections were rinsed three times in PBS 10 min and incubated with bovine serum albumin (2 % BSA) (Sigma Co., USA) for 1 h. Thereafter the sections were incubated in the primary antibody (TH-Ab, 1:1000, Sigma Co., USA; p-ERK1/2-Ab, 1:100, Boster Company, P.R. China) overnight at 4 °C. The sections were then rinsed three times in PBS for 10 min and incubated with 2 % BSA for 20min. Subsequently, the sections were rinsed three times in PBS for 10 min, and further incubated in the biotinylated-second antibody (Boster Company, P.R. China) at room temperature for 2 h. Finally, the sections were again rinsed three times in PBS for 10 min and incubated with the avidin-biotin complex ABC (Boster Company, P.R. China) at room temperature for 2 h. Diaminobenzidine (DAB, Sigma Co., USA) was used for signal detection. The control sections were incubated with PBS instead of primary antibody. The HPIAS series colorful pathology photographic system was used to analyze TH-IR and p-ERK1/2-IR positive neurons. The brain sections were observed in a 20 × magnification. The number and optical density of TH-IR and p-ERK1/2-IR positive neurons were calculated per area and per group.

Statistical analysis

All data were expressed as mean ± S.E.M. Statistical evaluation was done using ANOVA with *post hoc* test of LSD in Equal Variances Assumed. In all comparisons, statistical significance was set at $P<0.05$.

Results

1. Effect of GS on glycerol-induced renal dysfunction.

Glycerol administration resulted in a significant increase in BUN and Cre levels as compared to those in NS+NS group. However, Glycerol-induced ARF rats treated with ginsenoside for 48h (ARF+GS group) showed a significant decrease in BUN and Cre (Fig.1, $P < 0.05$).

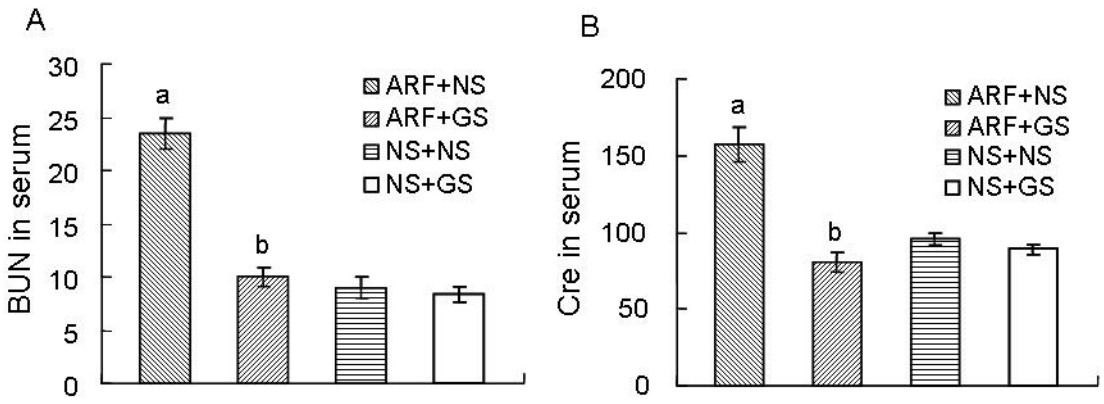

Fig. 1. Changes of BUN and Cre in serum in the ARF rats treated with GS for 48h. A: BUN in serum B: Cre in serum. Data are mean ± S.E.M. n=10 ($^{a}P<0.05$ ARF+NS group vs. NS+NS group, $^{b}P<0.05$ ARF+GS group vs. ARF+NS group).

2. Effect of GS on renal MDA and GSH level

Glycerol-treated rats (ARF+NS group) showed a significant lipid peroxidation as indicated by a marked increase of renal MDA level and decreased GSH level as compared to that in NS+NS group. However, treatment with GS for 48h (ARF+GS group) significantly reduced the MDA level and restored the GSH level as compared to ARF+NS group (Fig.2, $P < 0.05$).

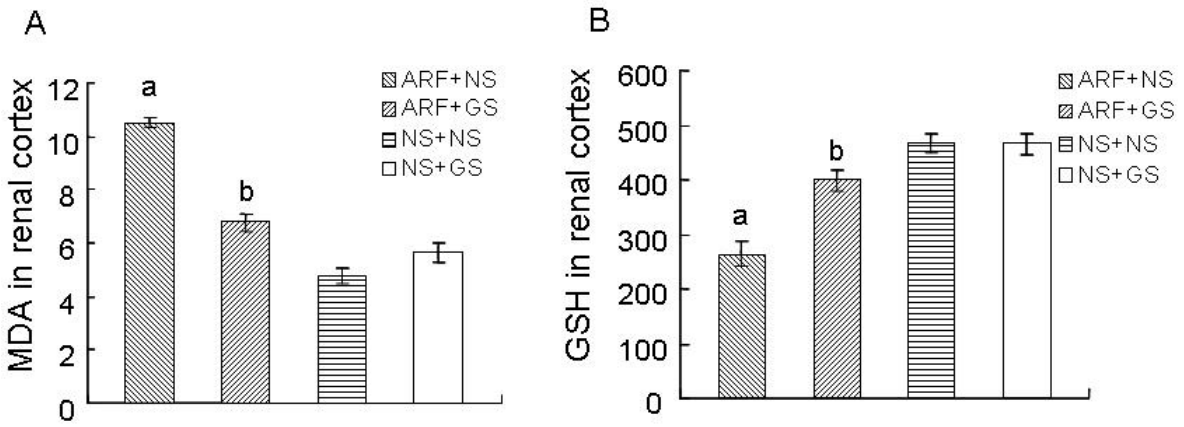

Fig. 2. Changes of MDA and GSH in renal cortex homogenate in the ARF rats treated with GS for 48h. A: MDA in renal cortex B: GSH in renal cortex. Data are mean ± S.E.M. n=10 ($^{a}P<0.05$ ARF+NS group vs. NS+NS group, $^{b}P<0.05$ ARF+GS group vs. ARF+NS group).

3. Effect of GS on glycerol-induced changes in renal morphology

The histopathological changes were showed in Fig. 3. The NS+NS group did not show any morphological changes (Fig.3C). By contrast, the kidneys of rats treated with glycerol showed marked histological changes in cortex. The renal sections showed severe apical blebbing, hyaline casts and tubular necrosis (Fig.3A) . GS treated kidney sections preserved the normal morphology of the kidney (Fig.3B).

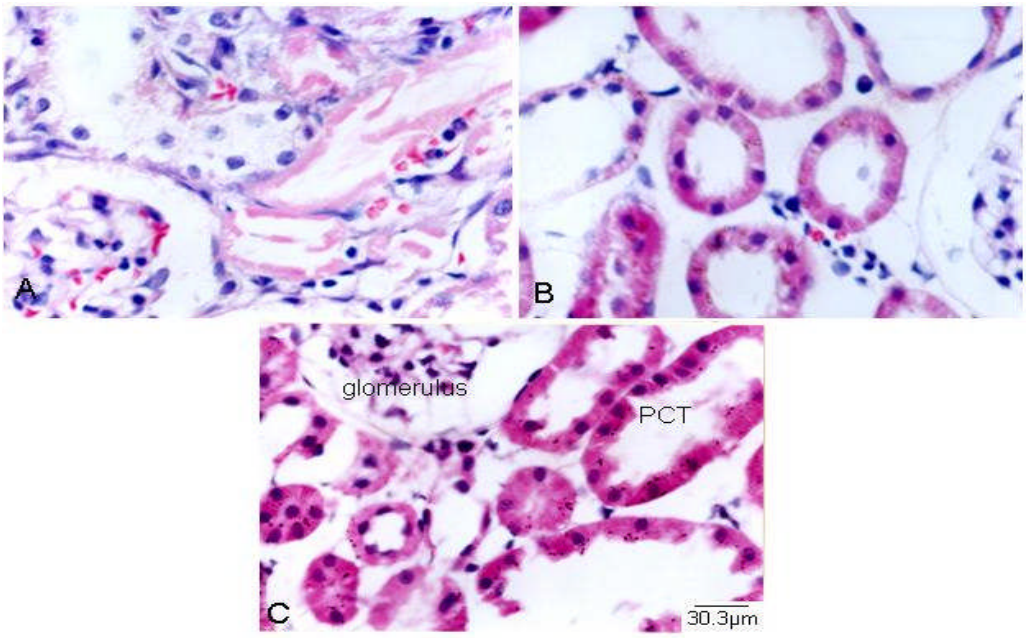

Fig. 3. Changes of necrosis degree in PCT in the ARF rats treated with GS for 48h.（HE staining）. A: ARF+NS group; B: ARF+GS group； C: NS+NS group**.** Bar indicates 30.3 μm.

4. Effect of GS on the changes of TH-IR in the LC in ARF rats induced by glycerol

In the pons of NS+NS group, TH-IR positive neurons were distributed predominantly in the LC (Fig. 4C). The most striking difference in TH-IR positive neurons in ARF+NS group compared with the NS + NS group was a significant increase in optical density and number of TH-IR staining neurons in the LC. This was illustrated by comparing Figure 4A (ARF+NS group) with Figure 4C (NS+NS group). There was a further increase of optical density and number of TH-IR positive neurons in the LC of ARF+GS group (Fig. 4B) when compared with ARF+NS group. TH-IR in the LC of NS+GS group was slightly increased, compared with that of NS+NS group. The data on TH-IR in the LC were summarized in Figure 5.

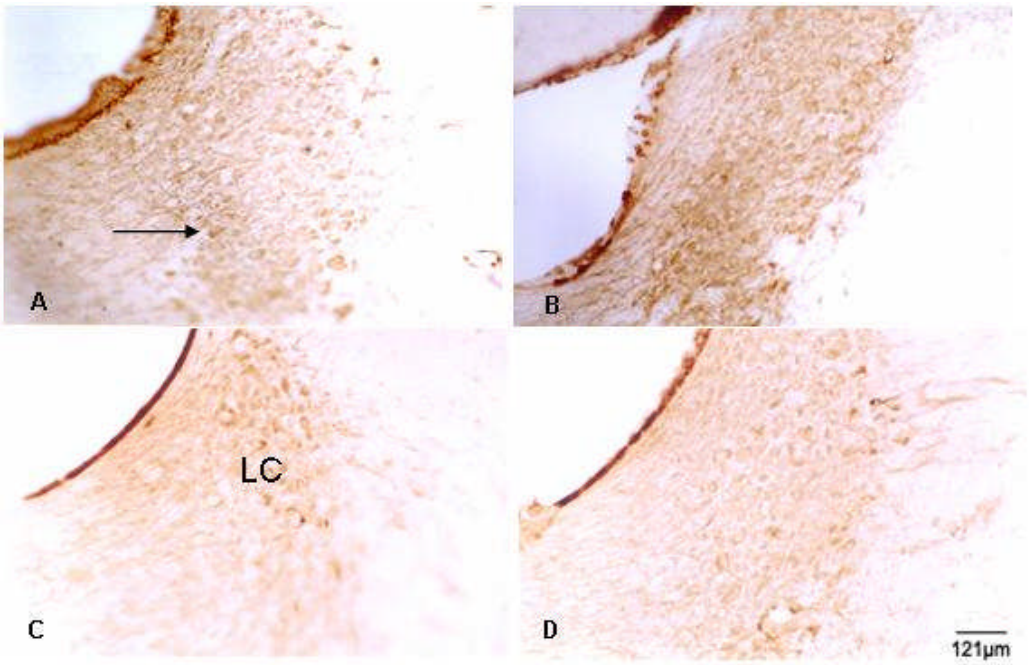

Fig.4. Change of TH-IR in Locus coeruleus in the ARF rats treated with GS for 48h. A: ARF+NS group; B: ARF+GS group; C: NS+NS group; D: NS+GS group. Bar indicates 121 μm, arrow points to TH-IR positive neurons.

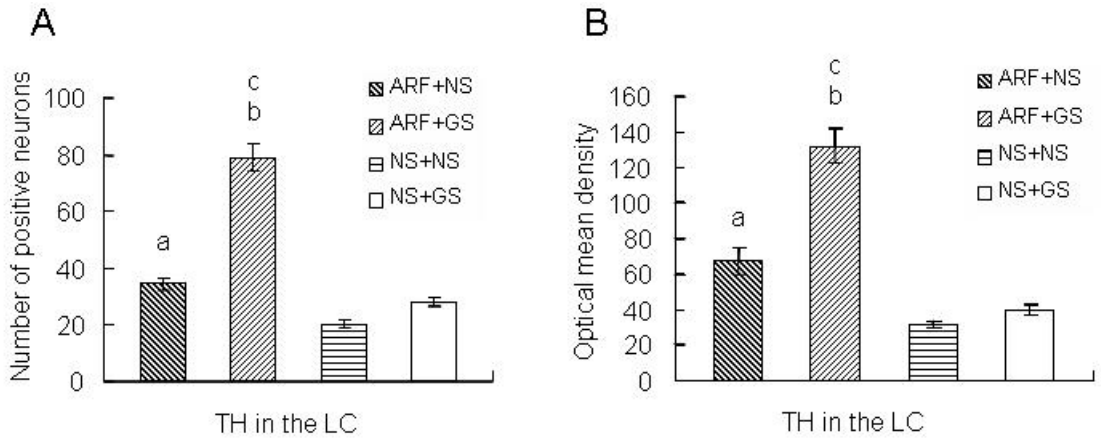

Fig.5. Quantitative analysis of TH-IR positive neurons and optical mean density of TH-IR in Locus coeruleus in the ARF rats treated with GS for 48h. A: the number of TH-IR positive neurons. B: optical density of TH-IR positive neurons. Data are mean ± S.E.M. n=6 (^{a} P<0.05 ARF+NS group vs. NS+NS group, ^{b} P<0.05 ARF+GS group vs. ARF+NS group, ^{c} P<0.05

ARF+GS group vs. NS+NS group).

6. Effect of GS on the changes of p-ERK1/2-IR in the LC in ARF rats induced by glycerol

Immunohistochemistry also showed an obvious increase of ERK-IR in the LC In ARF+NS group(Fig. 6A, $P < 0.05$); but p-ERK-IR 1/2 was further enhanced in ARF+GS group, compared with that in ARF+NS group(Fig. 6B, $P < 0.05$). The data on p-ERK1/2-IR in the LC were summarized in Figure 7.

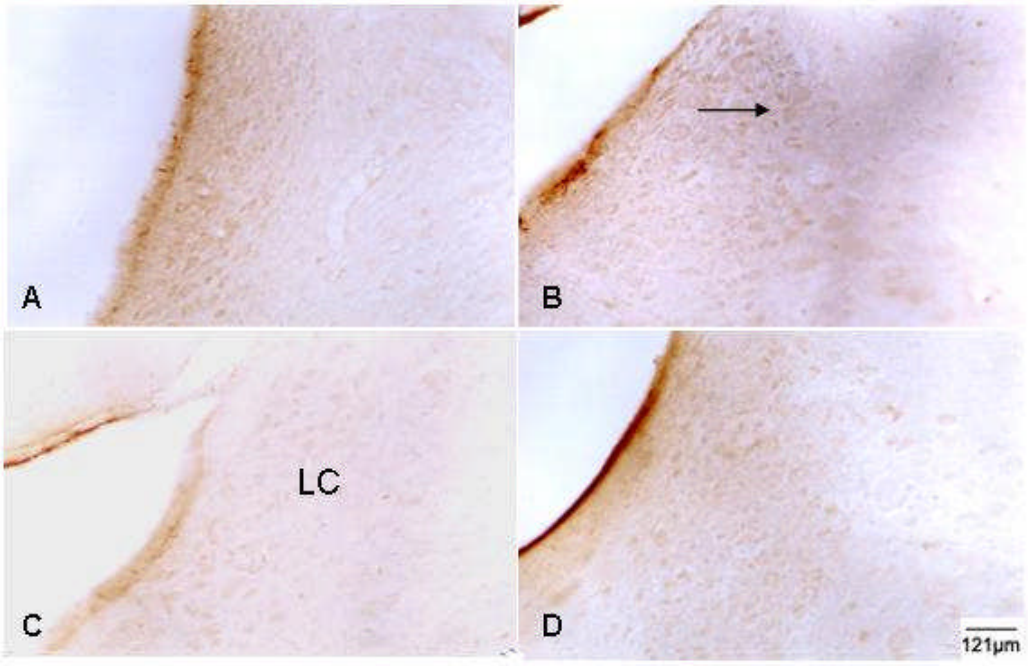

Fig.6. Change of p-ERK1/2-IR in Locus coeruleus in the ARF rats treated with GS for 48h. A: ARF+NS group; B: ARF+GS group；C: NS+NS group；D: NS+GS group. Bar indicates 121 μm, arrow points to p-ERK1/2-IR positive neurons.

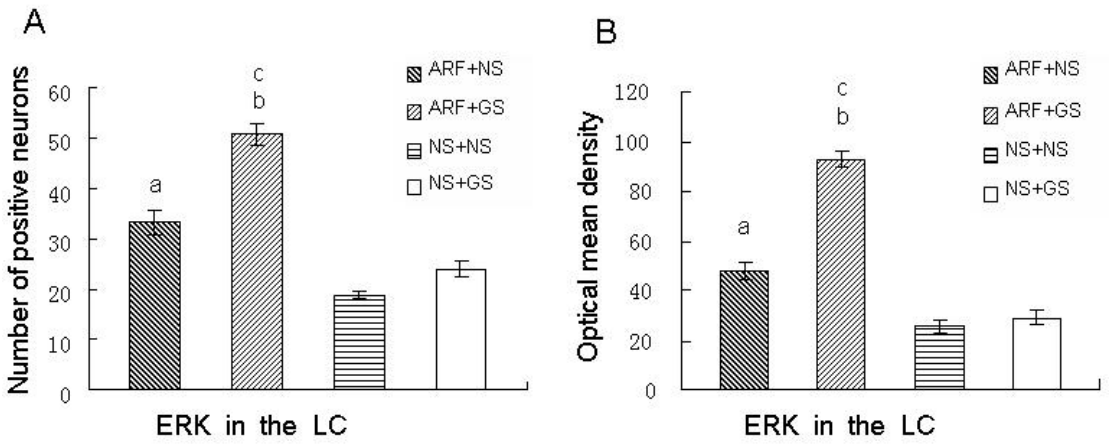

Fig.7. Quantitative analysis of p-ERK1/2-IR positive neurons and optical mean density of p-ERK1/2-IR in Locus coeruleus in the ARF rats treated with GS for 48h. A: the number of p-ERK1/2-IR positive neurons. B: optical density of p-ERK1/2-IR positive neurons. Data are mean ± S.E.M. n=6 (^{a} P<0.05 ARF+NS group vs. NS+NS group, ^{b} P<0.05 ARF+GS group vs. ARF+NS group, ^{c} P<0.05 ARF+GS group vs. NS+NS group).

Disscussion

Hypertonic glycerol injection in rats is one of the most widely used model of experimental acute renal failure(ARF). It is known as animal model of rhabdomyolysis（Abul-ezz *et al.*1991）. A number of studies have shown that rhabdomyolysis-induced myoglobinuric acute renal failure acounts for about 10%~40% of all cases of acute renal failure（Chander *et al.*2005）. It has been reported that myoglobinuric acute renal failure has three pathogenic mechanisms: tubular obstruction, renal vasoconstriction and oxidative stress （Polo-Romero *et al.*2004）. The latter is generated through the iron released from the group hemo of the myoglobin. Iron induces the formation of high-activity oxygen free radicals that increase oxidative stress and provoke

lipid peroxidation and cellular death（Polo-Romero *et al.*2004,Vlahović *et al.*2007）.

Ginsenosides are triterpenes saponins considered to be the main bioactive principles of the most important oriental herbal medicine “ginseng” derived from the roots and rhizomes of different *Panax* species (Araliaceae).Up to now more than 80 ginsenosides have been isolated from *Panax* species（Fuzzati, 2004）. Based on their structural differences, they can be classified into three categories: the panaxadiol group (e.g., Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Rs1), the panaxatriol group (e.g., Re, Rf, Rg1, Rg2, Rh1), and the oleanolic acid group (e.g., Ro)（Tachikawa *et al*.1999）. The ginsenoside content of ginseng is varying depending on the Panax species, the plant age, the part of the plant, the preservation method, the season of harvest, and the extraction method.

In the present study, 48h after glycerol administration the level of BUN and Cre significantly increased. The data are consistent with previous reports（Chander *et al.*2005）. These results indicated that renal function in ARF rats was damaged severely. However, after oral administration of ginsenosides for 48h, the level of BUN and Cre markedly decreased. These results suggested that ginsenosides could obviously improve renal function of ARF rats. The morphology of glycerol-induced ARF was acute tubular necrosis, particularly in the proximal tubule. In the present study, we observed that ARF rats showed a severe acute tubular necrosis. Furthermore, we observed that administration of ginsenosides in ARF rats relieved the severity of acute tubular necrosis. These evidences indicated that structural changes was taken place in glycerol-induced ARF rats. However, oral administration of ginsenosides decreased the severity of acute tubular necrosis, restored the renal morphology of ARF rats. These results above suggested that ginsenosides played an important part in renal protecting effect against ARF.

In glycerol-induced ARF model, reactive oxygen metabolites were proved to be the key

mediators of tissue injury. Previous studies demonstrated the role of hydroxyl radical enhanced generation of hydrogen peroxide and ponited out mitochondrial as a critical site of heme-induced free radical formation (Guidet *et al.*1989). It was reported that ginsenosides could alleviate oxidative stress by scavenging of free radicals, inhibiting of nitric oxide (NO) production which usually accompanied glutamate excitotoxicity, inducing superoxide dismutase (SOD1) and catalase genes and reducing lipid peroxidation （Kim *et al.*1998）.

The malodialdehyde (MDA) content, a measure of lipid peroxidation, its production is paralleled with the degree of oxidative stress. Therefore, the assay of MDA could be a marker of cell damage. In the present study, we observed that the level of MDA in renal cortex homogerate in ARF rats significantly increased, indicating that reactive oxidative species was generated in the model and oxidative damage was enhanced. It was consistent with previous reference （Chander *et al.*2005）. In addition, we also demonstrated that the level of MDA in ARF+GS group significantly decreased. Our results indicated that ginsenosides, as a inhibitor of lipid peroxidation resulting from oxidative stress, played a crucial role in protection against damage associated with rhabdomyolysis.

Glutathione (GSH), a tripeptide, occurs in high concentrations in virtually all mammalian cells and is the most prevalent intracellular thiol. GSH has many diverse functions, one of which is the protection against oxidative damage. The importance of GSH in protecting cells against oxidative injury has been delineated in numerous in vitro studies where depletion of GSH resulted in markedly enhanced toxicity（Suttorp *et al.*1986）and increased nonprotein sulfhydry content provided protection. In our study, we observed that the level of GSH in renal cortex homogenate in ARF rats markedly decreased, but obviously increased after treated with ginsenosides for 48h, indicating that intramuscular injection of glycerol produced significant

depletion of renal GSH. However, oral administration of ginsenosides may alleviate oxidative stress through the increase of GSH. Taken together, ginsenosides possessed the properties of oxidative free radical scavenger and opposed lipid peroxidation production such as MDA, etc.

The kidney is a sensory organ richly innervated with both mechanosensitive and chemosensitive afferent nerve fibers, and renal afferent nerves project directly to a number of areas in the central nervous system contributing to arterial pressure regulation(Ciriello *et al.*1983). It has been established that alterations in renal sympathetic nerve discharge with changes in neurotransmitter release could directly influence renal tubular transport function as well as renin secretion（DiBona 2005）.

It has been reported that renal oxidative stress mediates the stimulation of sympathetic nerve activity in the phenol renal injury model of hypertension（Ye *et al*.2006）. The LC, a pontine nucleus with high density of norepinephrine-containing neurons, also play a role in neural regulation of cardiovascular functions, specially modulating the baroreceptor reflex（Chan *et al*.1992）.

It has been established that renal transient ischemia induced an increase in electrical activity of renal afferent nerve and neurons in rostal ventrolateral medulla (RVLM), enhanced Fos expression in RVLM neurons（Ding *et al.*2001）. This findings implied that renal ischemia might activate the brainstem nuclei. There were also evidences that renal ischemia could induce Fos expression change associated with the activation of the catecholamine-containing neurons in brainstem nuclei by double immunohistochemistry. All above implied that neurons in the brainstem nuclei, such as catecholaminergic neurons, could be activated by oxidative stress or renal ischemia. Recent findings showed that ginsenosides were responsible for its effects on the central nervous system and the peripheral nervous system（Nah *et al*. 2007）. It was also reported

that pretreatment with ginsenoside Rg1 prevented the loss of TH-positive neurons in substantia nigra in MPTP-induced Parkinson mouse（Van Kampen *et al.*2003）.

In the present study, we observed that the rats treated with GS (NS+GS) showed a slight increase of TH-IR in the LC, compared with that of NS+NS group. This result implied that ginsenosides could activate catecholaminergic neurons in the LC to some extent in vivo.

We observed that glycerol-induced ARF rats showed an obvious increase of TH-IR in the LC. Our results indicated that catecholaminergic neurons in the LC were excited after intramuscular injection of glycerol. This finding suggested renal oxidative stress could enhance the expression of TH in the LC. Therefore, we hypothesized that it might be a compensation mechanism of catecholaminergic neurons in ARF rats to renal vasoconstriction, oxidative stress, increase of free radicals. Furthermore, we also observed that the expression of TH was increasingly upregulated in ARF rats treated with ginsenosides for 48h compared with that in ARF+NS Group. This evidence indicated that ginsenosides could significantly activate the catecholaminergic neurons in the LC in rats with ARF. This might be one of the effects of the ginsenosides on central nervous system in glycerol-ARF rats.

Other investigators have reported that several physiologically stressful stimuli, including seizure induction, ischemic insult, formalin injection and electroconvulsive shock (ECS), could activate MAPK signal in various brain regions（Imbe *et al.*2004）. Previous study have indicated that p-ERK1/2 may activate c-fos, leading to the AP-1 activation and specific TH induction in the LC in response to stress（Shimizu *et al.*2004）. Similarly, it has been reported that phosphorylation of both ERK1 and ERK2 was increased markedly by repeated stress. Immunohistochemistry indicated that phospho-ERK 1/2 was almost exclusively localized in the TH-positive cells of the LC following repeated stress（Hebert *et al.*2005）. The results of the

present study also showed that p-ERK1/2-IR in the LC in ARF rats was significantly increased. It indicated that MAPK signal pathway in the LC was excited after intramuscular injection of glycerol and might be involved in the regulation of catecholaminergic neurons in the LC. In addition, we also observed that glycerol-induced ARF rats treated with ginsenosides for 48h enhanced the expression of p-ERK1/2, as well as the expression of TH. Combining reported references with our results, we hypothesized that oral administration of ginsenosides might increasingly upregulate MAPK signal pathway in ARF rats, activating some related transcription factors and then regulating the catecholaminergic neurons activity. This might be one of the central mechanisms of ginsenosides against ARF.

On the basis of the above results, we propose that glycerol-induced ARF causes the changes not only in the kidney such as acute tubular necrosis and oxidative stress but also in central nervous system such as the upregulation of the TH-IR and phospho-ERK 1/2-IR in the LC. We suggest that the latter should be a compensation mechanism of central nervous system in ARF rats. This study also shows that oral administration of ginsenosides have strong renal protective effect: restore renal histological changes, increase renal antioxidative activity by some humoral mechanisms, improving renal function in glycerol-induced ARF rats. This study provides us information to develop a new way to consider the treatment of rhabdomyolysis-induced myoglobinuric acute renal failure. In addition, the possible relationship between the activation of catecholaminergic neurons in the LC and the renal protective effect of ginsenosides against ARF will be a focal point in our lab in the future.

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