Protective Effect of Ginsenoside against Acute Renal Failure and Expression of Tyrosine Hydroxylase 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 effort has been focused on traditional and herbal medicine, which is much less toxic than those agents conventionally used and which is nowadays considered as a novel therapeutic agent for ARF. However, the effect of ginsenosides (GS) administered 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 in rats with ARF on the changes of tyrosine hydroxylase immunoreactivity (TH-IR) as well as on the involvement 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 ARF rats with ginsenosides for 48 h significantly reduced the serum blood urea nitrogen, creatinine level, and lipid peroxidation, restored the GSH level and the normal renal morphology. Immunohistochemistry showed that an obvious increase of TH-IR was further enhanced in ARF+GS group. The same effect was also observed in the changes of p-ERK1/2-IR in the locus coeruleus. Our results suggest that ginsenoside administered 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. Our future attention will be focused to the question whether there is a correlation between the renal protective effect of ginsenosides against acute renal failure and the activation of tyrosine hydroxylase in the locus coeruleus.

Key words

Ginsenoside • Acute renal failure • Locus coeruleus • Tyrosine hydroxylase • Protective effect

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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 ARF pathophysiology has led to the 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 of ARF, especially before complications. However, it was reported that the incidence of ARF was rising over the past two decades (Hou *et al.* 1983, Nash *et al.* 2002) and mortality exceeded 50 % among those who required dialysis 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 effort has been focused on traditional and herbal medicine without toxic effects to provide a novel therapeutic agent for ARF.

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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, have 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* 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 integrative 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 was 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 the induction of AP-1 transcription factor c-Fos (Sun *et al.* 2003). The mitogen-activated protein kinases (MAPK) are involved in the intracellular signaling pathways, transferring extracellular stimuli into intracellular transcriptional responses. They play an important role in the activation of several transcription factors (Karin *et al*. 1995). The extracellular signal-regulated kinases (ERK) represent one of the four subfamilies of MAP kinases. In particular, ERK stimulate 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 *in vivo* study 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 the 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, as well as their interaction in glycerol-induced ARF rats remains unclear.

Based on these findings, it could be speculated that ginsenosides might have protective effects and there might be some interactions between catecholaminergic neurons and MAPK signal pathway in the LC of glycerol-induced ARF rats. Therefore, we examined 1) the changes of blood urea nitrogen and serum creatinine in ARF rats treated with ginsenosides for 48 h, 2) the changes of malondialdehyde and the reduced glutathione in renal cortex homogenate of ARF rats treated with ginsenoside for 48 h, 3) the changes of tyrosine hydroxylase-immunoreactivity in the LC of glycerol-induced ARF rats treated with ginsenoside for 48 h, and 4) the changes of phospho-ERK1/2-immunoreactivity (p-ERK1/2-IR) in the LC of glycerol-induced ARF rats treated with ginsenoside for 48 h.

Methods

Animals

Healthy male Sprague-Dawley rats (Dalian Medical University Animal Center, China) weighing 180-220 g were kept on a 12 h/12 h 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.

Animal treatment

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

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 being deprived of water for 16 h, ARF+NS group and ARF+GS group were given intramuscularly 10 ml/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 25 mg GS in 2 ml NS using a stomach tube, at the same

time, ARF+NS group was given 2 ml NS. The treatment of the other two groups were analogous. They were treated with GS or NS for two consecutive days (once per 6 h, 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 48 h, 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 412 nm.

Renal histology

Forty-eight hours after the administration of ginsenoside or physiological saline, 40 rats were deeply anesthetized with 4 % chloral hydrate (400 mg/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 3 days.

Kidney was washed by pure water for 12 h and then was embedded in paraffin and used for histological examination. Sections (4 μm thick) 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 to –10.04 mm) was based on the atlas by Paxinos and Watson. The sections above were rinsed three times in PBS for 10 min and then incubated with 0.2 % Triton for 5 min. Then 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 with 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 20 min. Subsequently, the sections were rinsed three times in PBS for 10 min, and further incubated with 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 (High-resolution pathological image analysis system) 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

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. On the contrary, glycerol-induced ARF rats treated with ginsenoside for 48 h (ARF+GS group) showed a significant decrease in BUN and Cre (Fig. 1, $P<0.05$).

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 48 h (ARF+GS group) significantly reduced the MDA level and restored the GSH level as compared to ARF+NS group (Fig. 2, $P<0.05$).

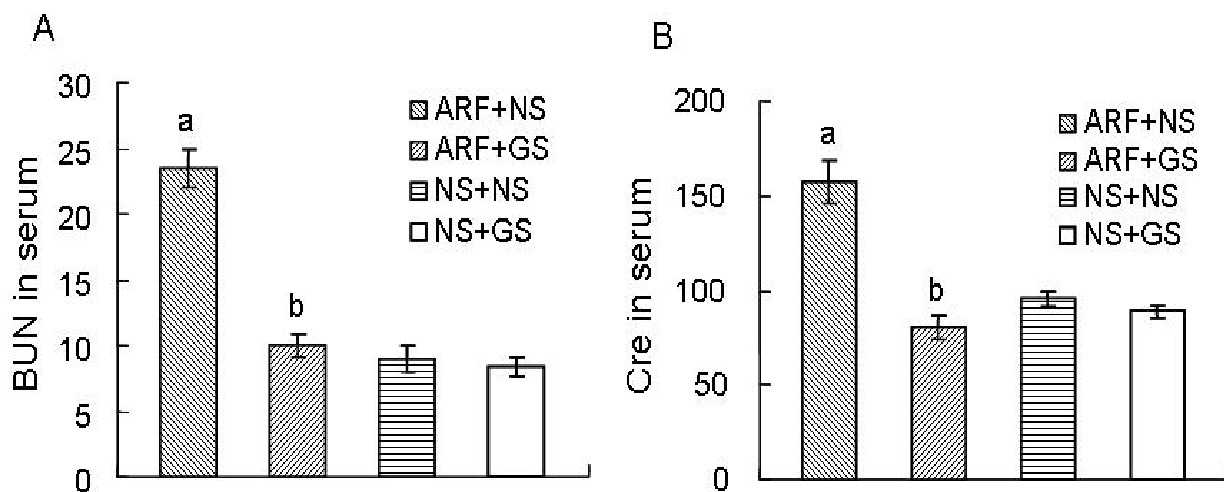

Fig. 1. Changes of BUN and Cre in serum in the ARF rats treated with GS for 48 h. **A.** BUN in serum **B.** Cre in serum. Data are mean ± S.E.M. n=10 ([a] P<0.05 ARF+NS group vs. NS+NS group, [b] P<0.05 ARF+GS group vs. ARF+NS group).

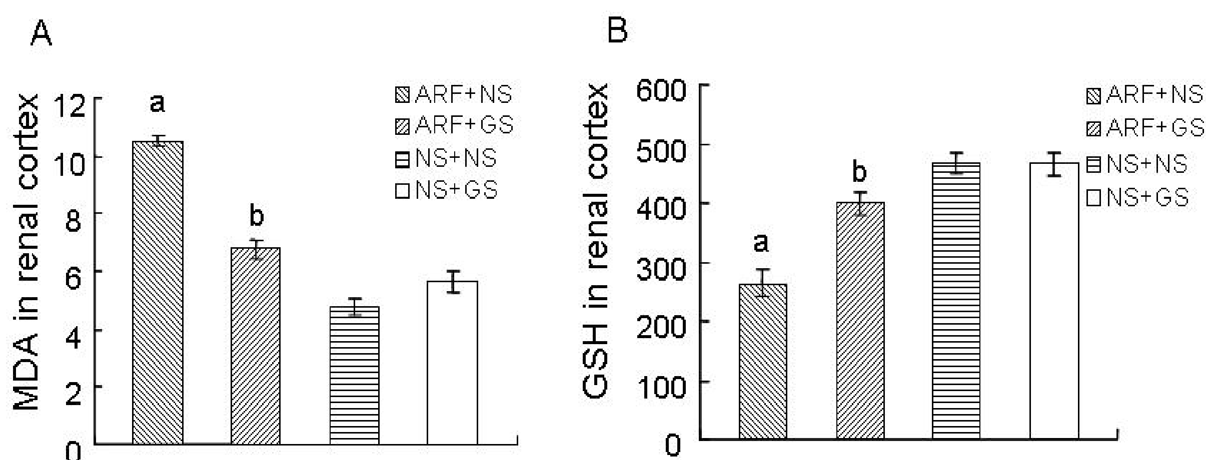

Fig. 2. Changes of MDA and GSH in renal cortex homogenate in the ARF rats treated with GS for 48 h. **A.** MDA in renal cortex **B.** GSH in renal cortex. Data are mean ± S.E.M. n=10 ([a] P<0.05 ARF+NS group vs. NS+NS group, [b] P<0.05 ARF+GS group vs. ARF+NS group).

Effect of GS on glycerol-induced changes in renal morphology

The histopathological changes were showed in Figure 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). Kidney sections of GS-treated rats preserved the normal morphology of the kidney (Fig. 3B).

Effect of GS on the changes of TH-IR in the LC of 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 (Fig. 5).

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 of ARF+NS group (Fig. 6A, P<0.05); but p-ERK-IR 1/2 was further enhanced in ARF+GS group compared with ARF+NS group (Fig. 6B, P<0.05 (Fig. 7).

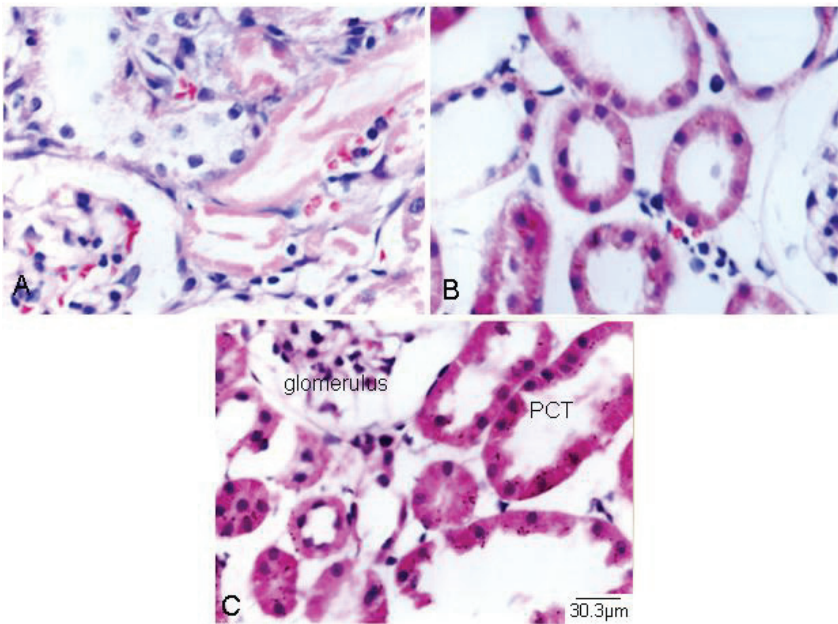

Fig. 3. Changes of necrosis degree in PCT in the ARF rats treated with GS for 48 h (HE staining). **A.** ARF+NS group; **B.** ARF+GS group; **C.** NS+NS group. Bar indicates 30.3 μm.

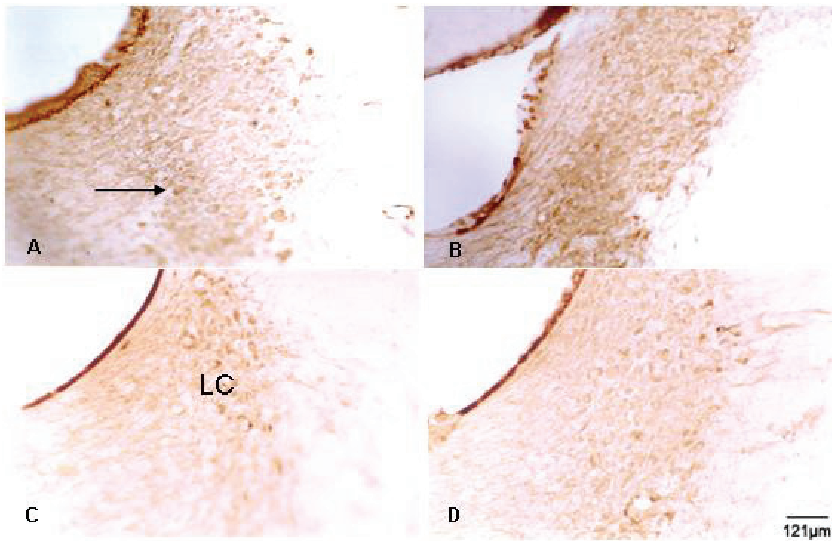

Fig. 4. Change of TH-IR in Locus coeruleus in the ARF rats treated with GS for 48 h. **A.** ARF+NS group; **B.** ARF+GS group; **C.** NS+NS group; **D.** NS+GS group. Bar indicates 121 μm, arrow points to TH-IR positive neurons.

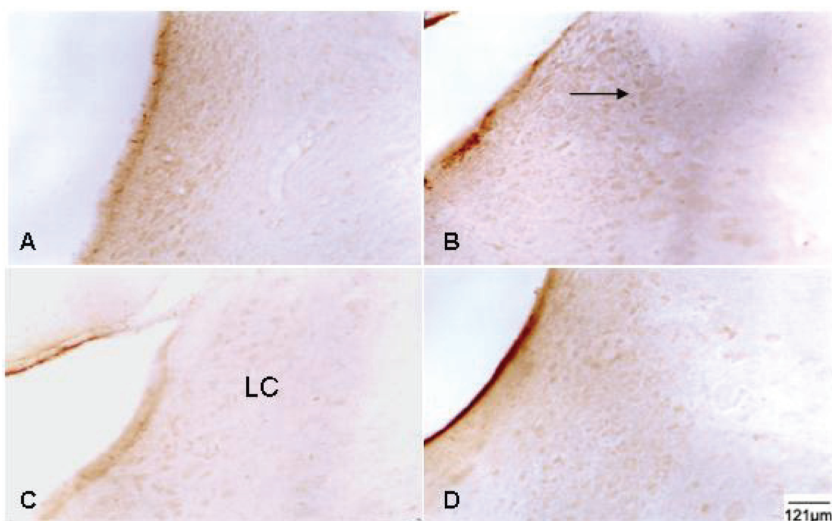

Fig. 6. Change of p-ERK1/2-IR in Locus coeruleus in the ARF rats treated with GS for 48 h. **A:** ARF+NS group; **B:** ARF+GS group; **C:** NS+NS group; **D:** NS+GS group. Bar indicates 121 μm, arrow points to p-ERK1/2-IR positive neurons.

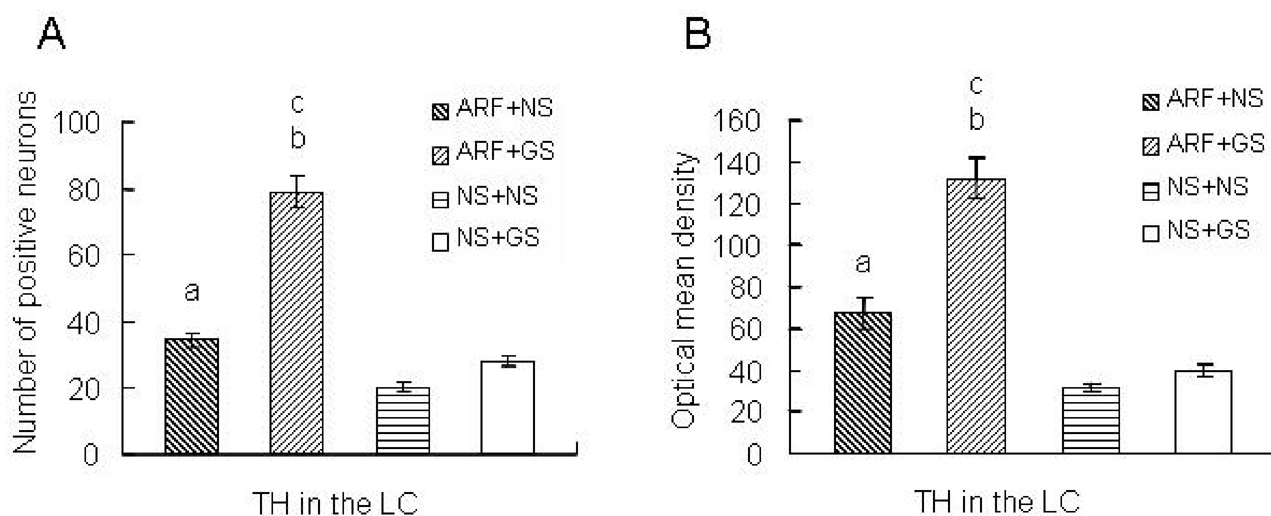

Fig. 5. Quantitative analysis of TH-IR positive neurons and optical mean density of TH-IR in locus coeruleus in the ARF rats treated with GS for 48 h. **A.** the number of TH-IR positive neurons. **B.** optical density of TH-IR positive neurons. Data are mean ± S.E.M. n=6 ([a] P<0.05 ARF+NS group vs. 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).

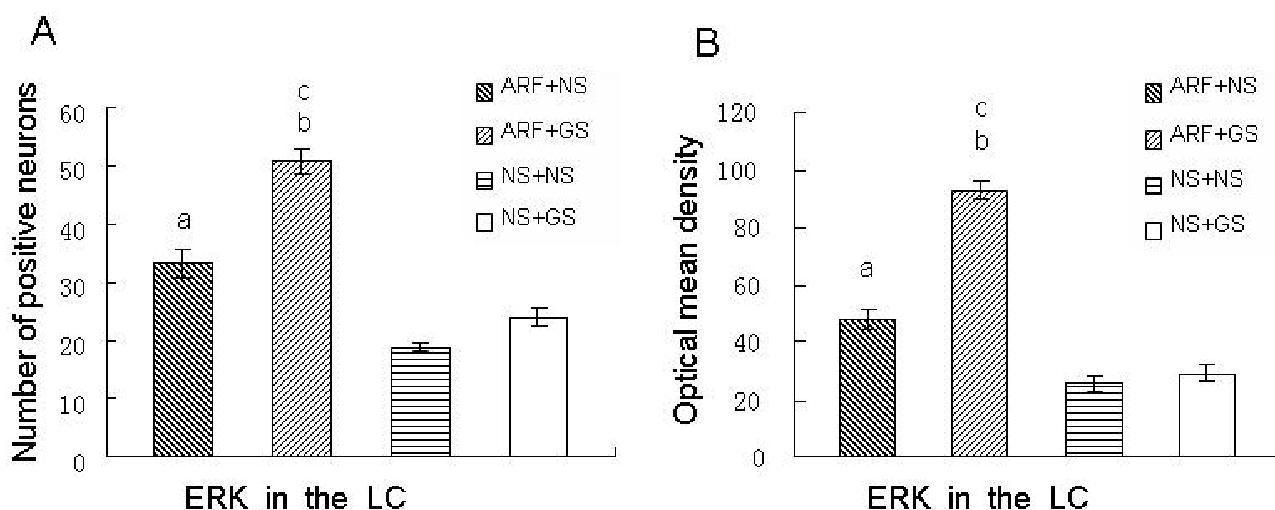

Fig. 7. Quantitative analysis of p-ERK1/2-IR positive neurons and optical mean density of p-ERK1/2-IR in locus coeruleus in the ARF rats treated with GS for 48 h. **A.** the number of p-ERK1/2-IR positive neurons. **B.** optical density of p-ERK1/2-IR positive neurons. Data are mean ± S.E.M. n=6 ([a] 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).

Discussion

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 heme group 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 triterpene 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, 48 h after glycerol administration the levels of BUN and Cre were significantly increased. Our data are consistent with previous reports (Chander *et al.* 2005). These results indicated that renal function was severely damaged in ARF rats. However, after oral administration of ginsenosides for 48 h, the level of BUN and Cre were not increased. These results suggested that ginsenosides could obviously protect 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 the administration of ginsenosides in ARF rats relieved the severity of acute tubular necrosis. These evidences indicated that structural changes occurred in glycerol-induced ARF rats. However, oral administration of ginsenosides decreased the severity of acute tubular necrosis, and restored the renal morphology of ARF rats. The above results 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 pointed out mitochondria 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 free radicals, inhibiting of nitric oxide (NO) production which usually accompanied glutamate excitotoxicity, inducing of superoxide dismutase (SOD1) and catalase genes and reducing lipid peroxidation (Kim *et al*. 1998).

The malodialdehyde (MDA) content, a measure of lipid peroxidation, 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 MDA level in renal cortex homogenate from ARF rats was significantly increased, indicating that reactive oxidative species were generated in this model in which oxidative damage was enhanced. This was consistent with previous findings (Chander *et al.* 2005). In addition, we also demonstrated that the level of MDA in ARF+GS group was significantly decreased. Our results indicated that ginsenosides, as inhibitors of lipid peroxidation resulting from oxidative stress, played a crucial role in the protection against the damage associated with rhabdomyolysis.

Glutathione (GSH), a tripeptide is present in high concentrations in virtually all mammalian cells and is the most prevalent intracellular thiol. GSH has many diverse functions, one of them being the protection against oxidative damage. The importance of GSH in protecting cells against oxidative injury has been noted in numerous *in vitro* studies in which the depletion of GSH resulted in markedly enhanced toxicity (Suttorp *et al.* 1986) and increased non-protein sulfhydryl content providing a protection. We observed that the level of GSH in renal cortex homogenate of ARF rats was markedly decreased, but it obviously increased after the treatment with ginsenosides for 48 h, 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, opposing lipid peroxidation production such as MDA.

The kidney is an 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). Locus coeruleus, a pontine nucleus with high density of norepinephrine-containing neurons, also plays a role in neural regulation of cardiovascular functions, specially modulating the baroreflex (Chan *et al*. 1992).

It has been established that transient renal ischemia induced an increase in electrical activity of renal afferent nerve and neurons in rostroventrolateral medulla (RVLM), and enhanced Fos expression in

RVLM neurons (Ding *et al.* 2001). These findings implied that renal ischemia might activate the brainstem nuclei. There was also evidence that renal ischemia could induce changes in Fos expression associated with the activation of the catecholamine-containing neurons in brainstem nuclei (demonstrated by double immunohistochemistry). This implies that catecholaminergic neurons in the brainstem nuclei could be activated by oxidative stress or renal ischemia. Recent findings showed that ginsenosides might act on the central and/or 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 (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) - 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 *in vivo* ginsenosides could activate catecholaminergic neurons in the LC to some extent.

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 that renal oxidative stress could enhance the expression of TH in the LC. Therefore, we hypothesized that it might be a compensatory mechanism of catecholaminergic neurons in ARF rats to renal vasoconstriction or oxidative stress. Furthermore, we also observed that the expression of TH was considerably upregulated in ARF rats treated with ginsenosides for 48 h compared with that in ARF+NS Group. This indicates that ginsenosides can significantly activate the catecholaminergic neurons in the LC of rats with ARF. This might be one of the effects of the ginsenosides on central nervous system in glycerol-induced ARF rats.

Other investigators have reported that several physiologically stressful stimuli, including seizure induction, ischemic insult, formalin injection and electroconvulsive shock, 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 of ARF rats was significantly increased. It indicates 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 48 h enhanced the expression of p-ERK1/2 as well as the expression of TH. Combining reported references with our results, we hypothesize 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 ½-IR in the LC. We suggest that the latter should be a compensatory mechanism of central nervous system in ARF rats. Our study also shows that oral administration of ginsenosides has strong renal protective effect: restores renal histological changes, increases renal antioxidative activity by some humoral mechanisms, and improves renal function in glycerol-induced ARF rats. This study suggests a new way to be considered for 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 for the future.

Conflict of Interest

There is no conflict of interest.

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