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SHORT COMMUNICATION

Ginsenoside Rg1 protects against transient focal cerebral ischemic injury and suppresses its systemic metabolic changes in cerabral injury rats

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KEY WORDS

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Abstract Ginsenoside Rg_1 (GR), a major bioactive compound of traditional Chinese medicine, such as Panax ginseng or Radix Notoginseng, has been shown to exert neuroprotective effects against ischemic stroke. However, pharmacokinetic studies have suggested that GR could not be efficiently transported through the blood–brain barrier. The mechanism by which GR attenuates cerebral ischemic injury *in vivo* remains largely unknown. Therefore, this study explored potential neuro-protective effects of GR through its systemic metabolic regulating mechanism by using mass spectrometry–based metabolomic profiling. Rats with middle cerebral artery occlusion (MCAO) were treated with GR intravenously. Their metabolic profiles in serum were measured by gas chromatography coupled with mass spectrometry on 1 and 3 days after MCAO. GR exhibited a potent neuro-protective effect by significantly decreasing the neurological scores and infarct volume in the MCAO rats. Moreover, 18 differential metabolites were tentatively identified, all of which appeared to correlate well with these disease indices. Our findings suggested that GR carries a therapeutic potential in stroke possibly through a feed-back mechanism by regulating systematic metabolic mediation.

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1. Introduction

Ischemic stroke, a complex devastating disease, is caused by a drastic disruption of cerebral blood flow that triggers a multistep pathophysiological ischemic cascade sufficient to cause metabolic disorder or functional deficit. Benchmark studies have reported quite a few neuro-protective agents with moderate efficacy in stroke management. However, the treatment of stroke remains considerably unsatisfactory as the performance of almost all neuro-protective agents in the clinical treatment of patients with stroke had been disappointing[1–3]. Therefore, new pharmacological strategies for stroke management are needed to improve its outcome and elucidate the physiological mechanism of this disease.

Ginsenoside Rg_1 (GR; Fig. 1), a panaxatriol saponin, is the major active compound in Panax ginseng and Radix Notoginseng[4], which are two distinguished herbs that have been used for thousands of years in clinical treatment in China. Research has shown that GR exerts neurotrophic and neuro-protective effects on ischemic stroke[5], and its possible mechanism has been attributed to its anti-oxidative effects on neurons, anti-edema properties, and regulation of various signaling pathways[6,7]. However, pharmacokinetic studies have demonstrated that GR could neither be efficiently transported through the blood-brain barrier nor be efficiently distributed to reach its effective concentration in brain tissue[8]. Therefore, the mechanism by which GR affects cerebral ischemic injury *in vivo* remains largely unknown and warrants further investigation.

Metabolomics has been used for identifying potential diagnostic biomarkers, and elucidating mechanism for complex disease and toxicity associated with the treatment[9–11]. Metabolomics can comprehensively measure the metabolites present in the circulation, which could reflect the balance between blood and the internal environment of brain tissue. Yang et al.[12] reported that their comprehensive analysis of a wide range of metabolites in various physiological states apparently showed promising perspectives in complex disease, whereas Jiang et al.[13] suggested that metabolomics might effectively aid in the diagnosis of ischemic stroke and prognostic estimation.

We hypothesized that there is a systemic feed-back regulating mechanism, through which GR indirectly exerts its effects on neurons. The results of this study further explain the neuro-protective mechanism of GR on cerebral ischemic injury. In addition, with the help of potential systemic metabolites in the circulation screened and identified using gas chromatography coupled with mass spectrometry (GC-MS) based on a metabolomic approach, the present study was also to explore potential nontargeted biomarkers for the diagnosis of ischemic stroke.

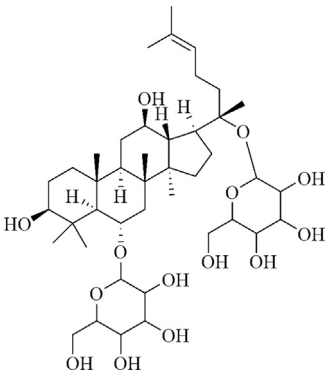

Figure 1 Chemical structure of GR.

2. Materials and methods

2.1. Animal care

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Capital Medical University (Beijing, China) and conformed to internationally accepted standards. Sixty male Sprague-Dawley rats (280–320 g) were supplied by the Vital River Laboratories (Beijing, China) and housed under controlled conditions with a 12-h light/12-h dark photoperiod, at 21 ± 2 °C, and with a relative humidity of $60\% \pm 5\%$ for at least 1 week before the experiment. They were allowed free access to a standard rodent diet and tap water.

2.2. Chemicals and reagents

GR was purchased from Ronghe Technology Co., Ltd. ($\geq 98\%$ purity, Shanghai, China). *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane were purchased from Sigma-Aldrich (St. Louis, USA). Ribitol (internal standard) was purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China), and 2,3,5-triphenyltetrazolium chloride was obtained from Sigma-Aldrich.

2.3. Transient focal cerebral ischemia

Middle cerebral artery occlusion (MCAO) was induced using the intraluminal suture method[14,15]. Briefly, rats were anesthetized using chloral hydrate (400 mg/kg); a 2.0 cm skin incision was made in the ventral neck muscles. A 3–0 MCAO monofilament (Beijing Sunbio Biotech Co., Ltd.) was advanced to block the origin of the MCA. Regional cerebral blood flow (rCBF) was monitored by a laser Doppler computerized main unit (Perimed AB, Sweden). The MCAO was set up with rCBF sharp decreasing to 20% of baseline level before ischemia; otherwise, animals were excluded. Reperfusion was performed by withdrawing the monofilament after 120 min ischemia, and then wounds were closed. During the surgery and reperfusion, rectal temperature was maintained at 37 ± 0.5 °C by means of a heating blanket. The mortality of the MCAO model is around 15%. A similar procedure without MCAO was conducted in control rats.

2.4. Animal treatments

After MCAO, rats were randomly divided into the following groups: sham operation (control); MCAO treated with water (model); MCAO treated with 30 mg/kg GR per day (GR 30 mg/kg); and MCAO treated with 60 mg/kg GR per day (GR 60 mg/kg). After reperfusion for 2 h, rats were treated with GR intravenously twice daily for 3 days. The control and model groups were treated with an equal volume of saline.

2.5. Neurological function evaluation

The neurological function of the conscious rats was blindly evaluated 1 day and 3 day after MCAO. A five-point scale reported previously[16] was carried out to evaluated the neurological deficit scores (score 0, no deficit being observed; score 1, failing to fully extend left forepaw; score 2, circling to the left side; score 3, falling to the left; score 4, with no walking and consciousness being depressed) by an observer who was blind to the group.

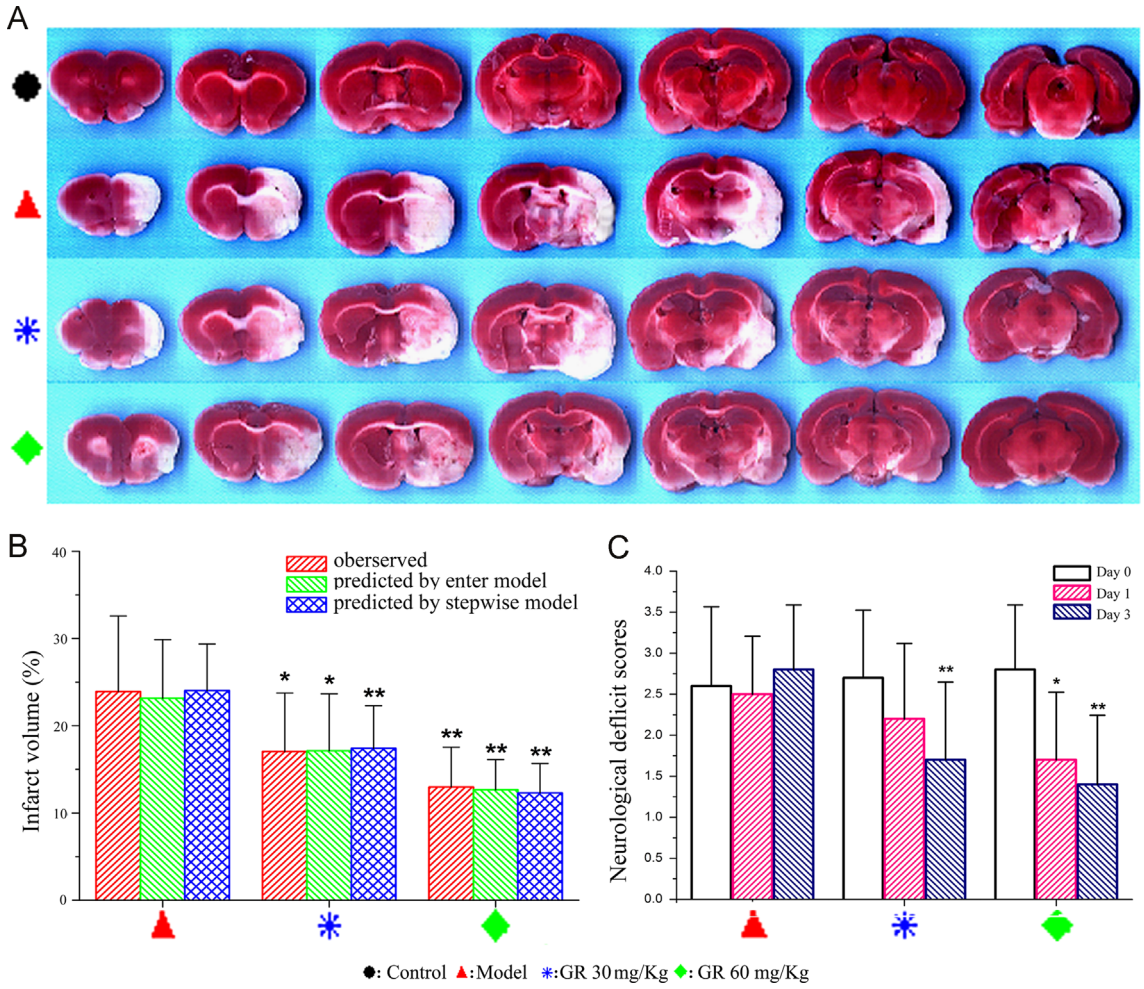

Figure 2 Protective effect of GR on cerebral ischemic injury. (A) Infarct images observed using 2,3,5-triphenyltetrazolium chloride staining on day 3 after MCAO ($n=10$). (B) Significant reduction in infarct volume in the GR 30 mg/kg treatment group and 60 mg/kg treatment group compared with the model group consistent with the results predicted by the enter regression and stepwise models. (C) Neurological scores at 24 h after MCAO, the scores of control group was zero, data not shown. $^{*}P<0.05$; $^{**}P<0.01$.

2.6. *Infarct volume measurement*

The animals were euthanized on day 3 after MCAO. Their brains were collected and sliced into seven coronal sections with 2-mm thickness each stained with 2% solution of 2,3,5-triphenyltetrazolium chloride in saline at 37 °C for 30 min[14,15].

2.7. *Sample collection and preparation*

On day 1 and day 3 after MCAO, the rats were anesthetized by ether inhalation after their treatment. Their blood was collected from orbital venous and centrifuged at 1500 × *g* for 10 min. Serum was stored at −80 °C until use.

Thawed serum samples (100 μL) were spiked with the internal standard (5 μL of 1 mg/mL ribitol) and mixed with acetone (200 μL) by vortexing for 1 min. After incubation on ice for 10 min, the mixture was centrifuged at 4 °C for 10 min at 10,000 × *g*. The supernatant (200 μL) was transferred to a GC vial and evaporated to dryness under vacuum at 35 °C for approximately 4 h in a concentrator (Thermo Speedvac Concentrator, USA). Derivatization was performed using methoxyamine pyridine (35 μL; 15 mg/mL) at 70 °C for 1 h, followed by *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (35 μL) with 1% trimethylchlorosilane as the catalyst at room temperature (25 °C) for 1 h. Finally, *n*-heptane (100 μL) was added to dilute the solution after the derivatization, and the supernatant was used for GC-MS analysis.

2.8. *GC-MS analysis*

The system consisted of an Agilent 7683 Series mass spectrometer (Electron ionization mass spectra were recorded at 70 eV and 2 scans/s. Full-scan mass spectra were acquired from 60 to 600 *m/z* at the scan rate of five times per second) coupled to an Agilent 6890 gas chromatograph system (Agilent Technologies, Atlanta, USA). One microliter of sample solution was injected in splitless mode to a ZB-5MS column (30 m × 250 μm × 0.25 μm) with helium as the carrier gas at the flow rate of 0.8 mL/min. The injector temperature was set at 270 °C. The temperature of the ion source was adjusted to 230 °C, whereas that of the quadrupole was set at 150 °C. The initial temperature of the column was maintained at 70 °C for 5 min. It was then increased at the rate of 10 °C/min to 90 °C where it was held for 1 min, at 5 °C/min to 140 °C for 2 min, at 12 °C/min to 182 °C for 2 min, and then at 2 °C/min to 225 °C for 3 min. Subsequently, the temperature was increased to 300 °C at the rate of 15 °C/min and held for 5 min. The peaks of interest were identified by searching the NIST

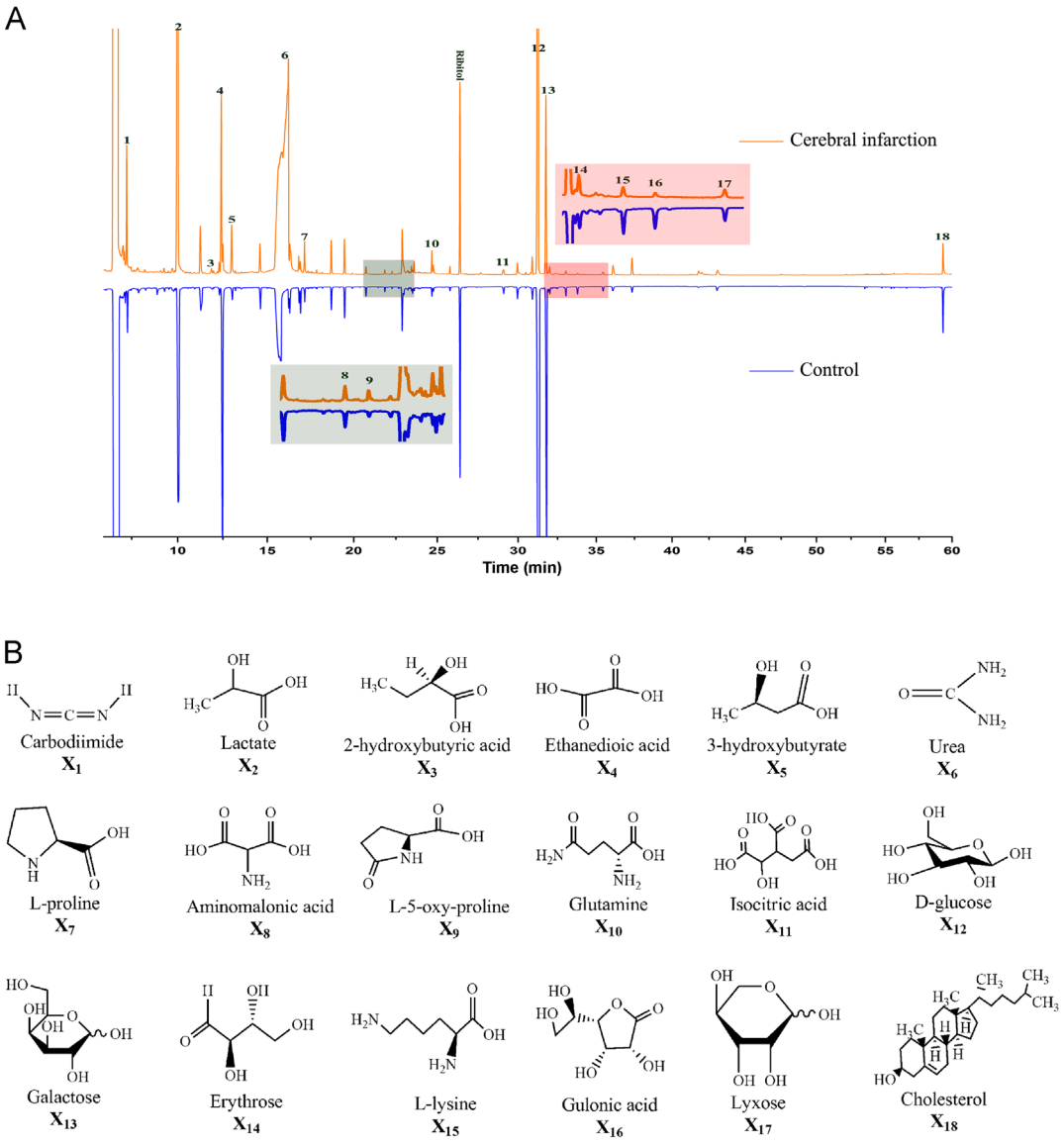

Figure 3 Metabolic profiles in the circulation of MCAO and control rats ($n = 10$). (A) Typical total ion current chromatograms of rat serum from the control and model groups. The numbered peaks represent the metabolites in panel B. (B) Structural information on the differential metabolites identified between the control and model groups.

v1.0.0.12 mass spectra library and compared with the peaks of internal standards.

2.9. *Statistical analysis*

Statistical analysis was performed in SPSS version 11.5 statistical software (SPSS Inc.). Data were expressed as mean ± SEM. For a normal distribution, student's *t* test was used to compare between two groups, whereas one-way ANOVA was conducted for comparisons of multiple groups, followed by Fisher's least significant difference test; while non-parameter analysis was used for non-normal distribution. Multiple linear regression analysis was performed to determine the relationship between infarct volume and the metabolites during ischemia, and the predicted and observed infarct volumes in the control group were then compared using analysis of covariance. In addition, principal component analysis (PCA) was performed with SIMCA-P 11.5 (Umetrics AB, Umea, Sweden).

3. Results

3.1. *Protective effect of GR on cerebral infarction*

Neurological deficit scores and infarct volume with or without GR treatment were measured to examine the influence of GR on cerebral ischemic injury. The animals were divided into four groups: control, model, GR of 30 mg/kg, and GR of 60 mg/kg. A remarkably decreased pale-colored region was observed in the GR-treated groups compared with the model group (Fig. 2A). The cerebral infarct volumes were significantly reduced after GR treatment compared with model treatment, which was consistent with the results predicted by the enter regression and stepwise models (Fig. 2B). Neurological functional

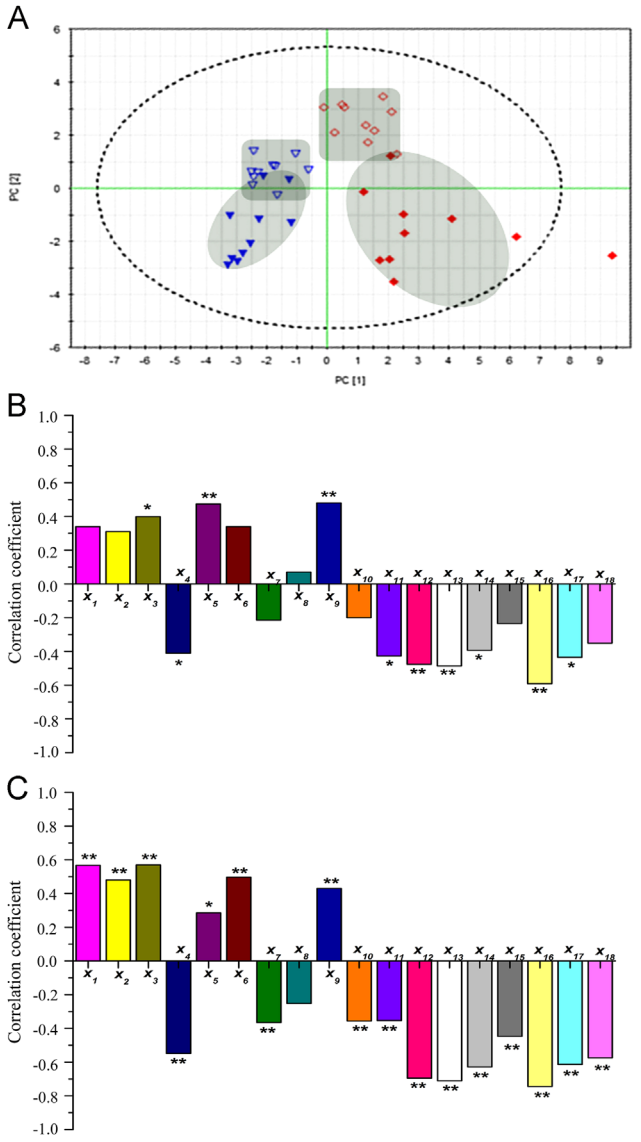

Figure 4 Correlation between differential metabolites and cerebral infarct injury ($n=10$). (A) Score plots of the PCA map for the control group (▾) and model group (♦) on day 1 (open symbols) and day 3 (filled symbols). (B) Pearson's correlation coefficients of the 18 differential metabolites and neurological deficit scores on day 1. (C) Pearson's correlation coefficients of the 18 differential metabolites and infarct volume on day 3. **X_1, X_2, X_3, …, X_{18}** represent the corresponding metabolites identified in Fig. 2. $^{*}P<0.05$; $^{**}P<0.01$.

deficits were observed on day 1 and day 3 after MCAO in rats, which could be prevented by GR treatment (Fig. 2C). Compared with the model group, significant reduction in the neurological deficit scores were observed in the GR 60 mg/kg group on day 1, and both GR treatment group on day 3. These results suggested that GR could prevent cerebral ischemic injury with intravenously treatment.

3.2. *Serum metabolic profiles of control and MCAO rats*

Representative total ion current chromatograms of sera from the control and model (cerebral infarction) groups are shown in Fig. 3A. Forty-five endogenous metabolites in serum were identified by the NIST mass spectra library. According to the peak area ratios of the metabolites to the internal standard (ribitol), 18 metabolites were changed significantly ($P<0.05$) compared with the control group on day 1 or day 3. The chemical structures of these 18 differential metabolites are shown in Fig. 3B.

3.3. *Association between differential metabolites expression and cerebral infarct injury*

PCA was carried out to evaluate the capability of the GC-MS-based metabolomic approach to differentiate the model group from the control group. The PCA score plots (Fig. 4A) showed marked separation between these groups on days 1 and 3, when time-dependent profile separations could also be observed in the model group.

The correlation coefficients between neurological deficit scores (Fig. 4B) and infarct volume (Fig. 4C) with the 18 differential metabolites were listed. Of these metabolites, $\mathbf{X_3}$ and $\mathbf{X_7}$ were found to positive and negative correlate with neurological scores on day 1, respectively, whereas $\mathbf{X_6}$ and $\mathbf{X_{12}}$ were found to positive and negative correlate with infarct volume on day 3, respectively.

3.4. *Prediction of infarct volume using metabolic profiling*

Multiple linear regression analysis was performed to build a prediction model of infarct volume based on the differential metabolites. As shown in Fig. 5A, a linear regression model exhibited a significantly high R^2 value (0.871; $P<0.05$) using the enter selection method with all factors entered. The enter model was further validated by covariance analysis of predicted and observed infarct volumes in MCAO rats ($P<0.05$) (Fig. 5B), indicating that the infarct volume might be predicted using this model with these metabolites.

A stepwise regression selection procedure was performed to build a compact model (Fig. 5C) with two factors finally entered ($R^2=0.678$; $P<0.05$) to avoid high R^2 values using the enter procedure due to overfitting with a high number of factors. As shown in Fig. 5D, the model was validated by covariance analysis using the infarct volume in MCAO rats as the test set ($P<0.05$). These results suggested that metabolite $\mathbf{X_4}$ (Ethanedioic acid) and $\mathbf{X_{18}}$ (Cholesterol) are potential major biomarkers for the prediction of infarct volume and that all the metabolites might be systemic nontargeted biomarkers for the prediction of cerebral ischemic injury.

3.5. *Intervention of GR in the differential metabolites in the circulation*

Differential metabolites, which have been previously confirmed to be correlated with the evolution of ischemic injury, were used to investigate the potential neuro-protective mechanism of GR. Good classifications were observed in the PCA score plots for days 1 and 3 ($n=10$) (Fig. 6A). The model group was distributed on the bottom right side, well separated from the control group on the top left side. Sample plots from the model+GR-treated groups were moved from the bottom right side to the top left side in a time- and dose-dependent fashion, indicating that the differential metabolites in the circulation after MCAO could be regulated back to normal levels.

As shown in Fig. 6B, changes in the percentages of relative peak area ratios in the control group were calculated and compared

with those in the model group to evaluate the recovery condition of each metabolite after GR treatment. With GR 30 mg/kg treatment, $\mathbf{X_3}$ and $\mathbf{X_8}$ metabolites were significantly regulated back to their normal status on days 1 and 3, respectively. Meanwhile, with GR 60 mg/kg treatment, $\mathbf{X_6}$ and $\mathbf{X_{16}}$ metabolites were regulated significantly back on days 1 and 3, respectively.

4. Discussion

Stroke is the leading cause of disability and second major cause of death in China[17,18]. However, until now, its treatment remains considerably unsatisfactory. Traditional Chinese medicines have been practiced in stroke prevention and management for thousands of years[19]. GR, an active constituent of protopanaxatriol found in traditional Chinese medicine agents, such as Ginseng and Radix Notoginseng, is known to have potent neuro-protective effects against brain injury[20]. Most experimental studies have focused on neurons as neuro-protective targets of GR[5,21,22]. However, research has also demonstrated that GR is poorly distributed to the brain because it could not be efficiently transported through the blood-brain barrier and reach the effective concentration in brain tissue[8]. Accordingly, whether GR could be effectively distributed into brain tissue and directly carry out potent neuro-protective effects on neurons remains unclear. We thus hypothesized that the neuro-protective effects of GR could be attributed to a potential systemic regulating mechanism. Following cerebral infarction, the microenvironment of blood circulation is disturbed by central nervous system disorders, which might react by affecting the injury course of brain tissue. Then small molecule metabolites transported through the blood-brain barrier, the degradation of the micro-environment of the brain enhances cerebral tissue damage, which showed that the metabolites were significantly correlated with cerebral damages[23].

It has been shown that many small molecule metabolites in the circulation system may contribute to cerebral ischemic disorders[13]. In this study, GC-MS-based metabolomic profiling was performed to screen differential metabolites in the circulation after MCAO. Eighteen metabolites in serum were tentatively identified. With PCA, these metabolites can clearly distinguish MCAO rats from the controls. Meanwhile, the multivariate linear regression model showed that these metabolites significantly correlated with the neurological deficit scores on day 1 and with the infarct volume on

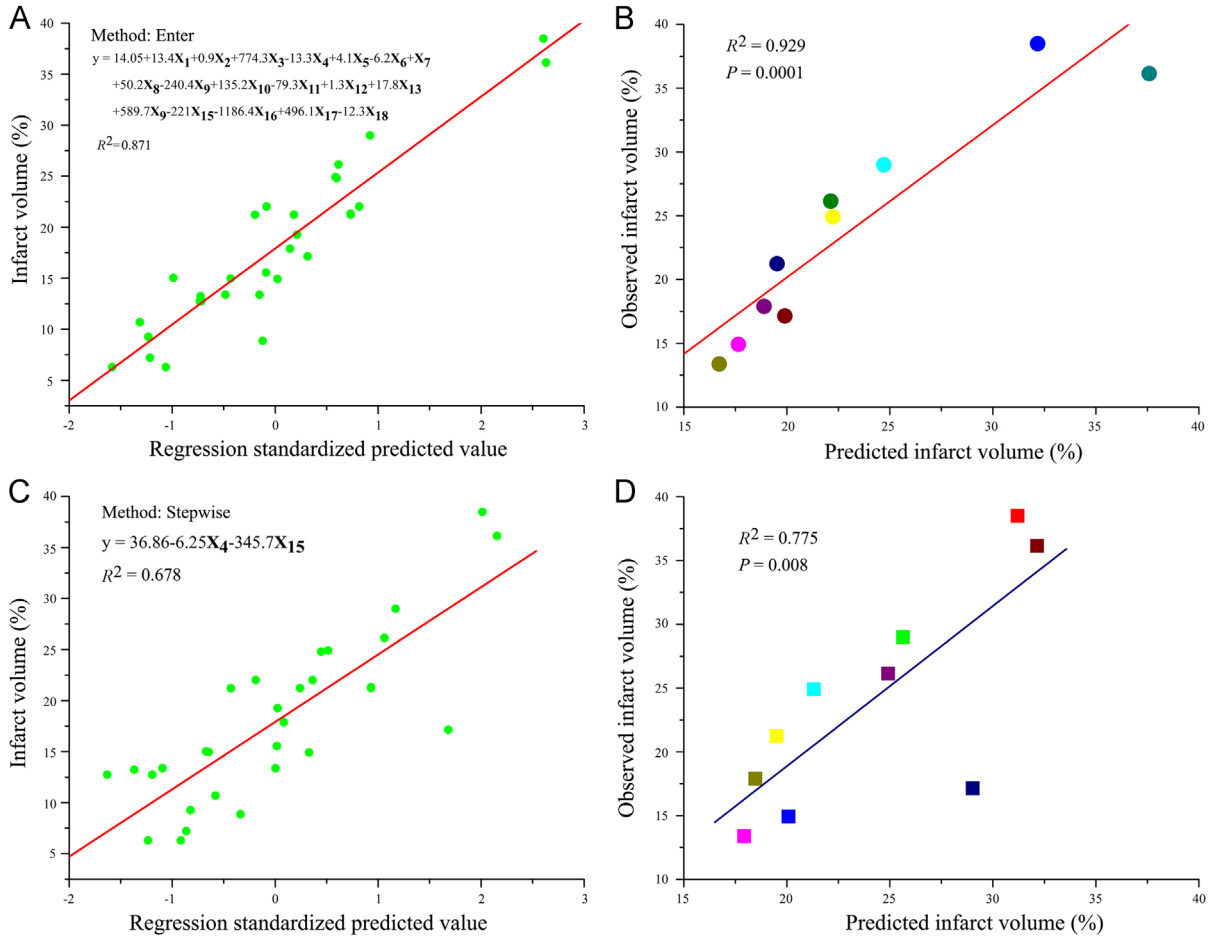

Figure 5 Prediction model for cerebral infarction built based on the differential metabolites using multiple regression analysis. (A) Model built using the enter selection method ($n=30$). (B) Results of the covariance analysis of predicted and observed infarct volumes in MCAO rats, with the predicted infarct volume calculated using the equation in panel A ($n=10$). (C) Model built using the stepwise selection method ($n=30$). (D) Results of the covariance analysis of predicted and observed infarct volumes in MCAO rats, with the predicted infarct volume calculated using the equation in panel C ($n=10$).

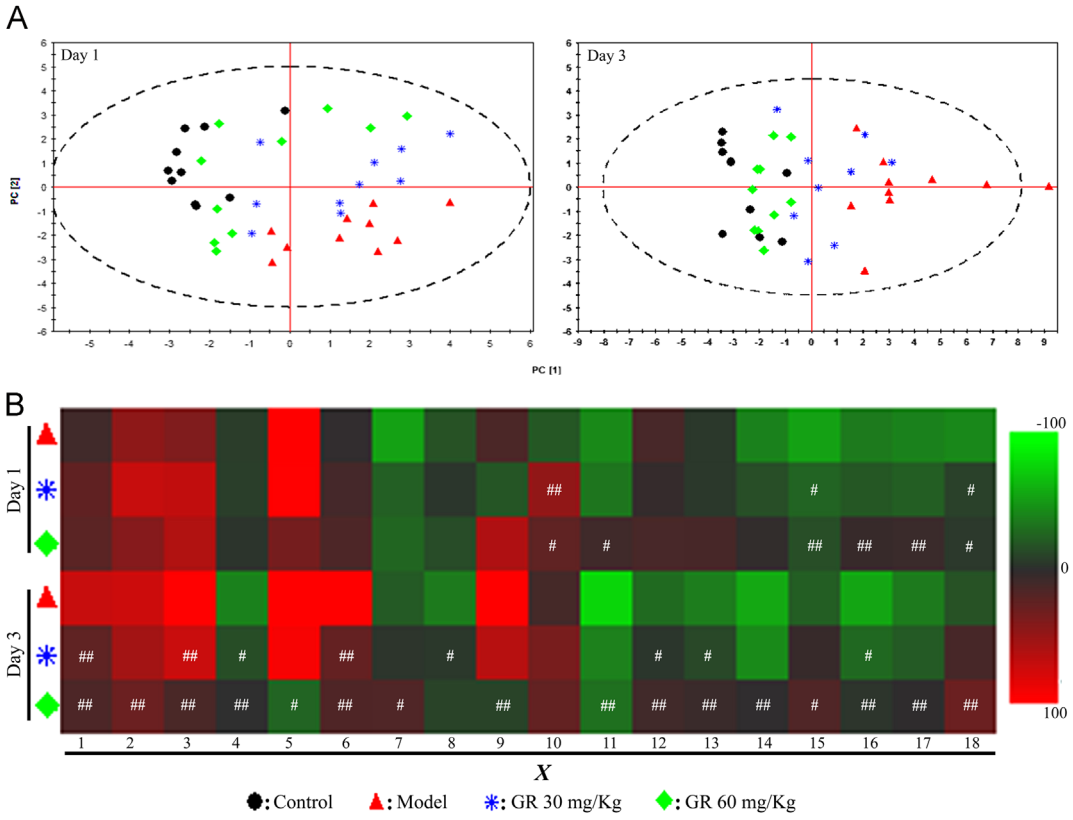

Figure 6 Intervention of GR in the differential metabolites in the circulation after MCAO ($n=10$). (A) PCA score plots of differential metabolites in serum on days 1 and 3. (B) Heat maps indicating the recovery condition of the differential metabolites after GR treatment on days 1 and 3. The color intensity represents changes in the percentages of relative peak area ratios compared with the control. Green indicates decreased expression, whereas red signifies increased expression. $^{\#}P<0.05$; $^{\#\#}P<0.01$, compared with the model group.

day 3, implying that these metabolites correlated with the cerebral injury process. Therefore, these differential metabolites might be a potential systemic biomarker for cerebral ischemic injury prediction and potential systemic targets for cerebral injury regulation.

This study has provided preliminary evidence that GR could protect against cerebral ischemic injury, including the reductions in neurological deficit scores and infarct volume. We found that the differential metabolites in the circulation after MCAO might be regulated back to their normal levels after GR treatment. Furthermore, the infarct volumes predicted by the enter and stepwise prediction models were consistent with the observed values. These findings suggested that the neuro-protective effects of GR are correlated with regulating the imbalance of metabolites to a normal status in the circulation. However, further studies are needed in order to determine whether the changes in serum metabolite levels are mechanistically linked to the neurological changes.

In conclusion, by using an integrated analytical approach based on GC-MS, this study found preliminary data in support of the idea that the neuro-protective effects of GR on cerebral ischemic injury might be attributed to a potential indirect systemic regulating mechanism, associated with anaerobic glycolysis, the tricarboxylic acid cycle, the urea cycle and the amino acid metabolism. The differential metabolites detected in this study might be a potential systemic biomarker for the prediction of cerebral infarct injury. These findings may eventually enhance the current understanding the pharmacological targets and mechanism of GR on cerebral infarction. Further research will be carried out to support the above-described hypothesis.

Acknowledgments

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