Ginsenoside Rc Protects Neurons From Oxygen-glucose Deprivation Injuries and Its Mechanistic Investigation via a Network Pharmacology-based Analysis

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Abstract

Background: Ginsenoside Rc (Rc) is one of the major active components of Panax ginseng Meyer. Studies have shown that Rc has remarkable effect in protection of nervous system. However, the potential molecular mechanism of its neuroprotective effect remains unclear. Our study aim to investigate the neuroprotective effect of Rc on neuron damage and explore the potential mechanism on its regulation of TNF-α and DRP-1.

Methods: Oxygen-glucose deprivation reperfusion (OGD/R) cell neuron damage modle was induced by $Na_2S_2O_4$ and EBSS solution. After preventive administration, cell viability and cell toxicity were detected to evaluate the putative neuroprotective properties of Rc. Network pharmacology and molecular docking simulation studies were performed to predict the potential targets and pharmacological mechanism. Furthermore, the prediction was validated via western blot assay and specific antagonist.

Results: In OGD/R injured cells, Rc significantly improved cell viability (Rc middle dose vs. OGD/R model: 67.3±2.33% vs. 55.7±1.14%, $P<0.05$) and obviously decreased cell toxicity (Rc middle dose vs. OGD/R model: 147±39.7% vs. 232±29.4%, $P<0.01$). Analysis of network pharmacology and molecular docking indicated that the key targets of Rc are TNF-α and DRP-1. Subsequently molecular biological studies showed a significant increase on expression of TNF-α and DRP-1 in model group. Conversely, administration of Rc reversed the alteration significantly and presented a dose dependence. By adding antagonist, we validated that Rc had an indirect regulation on TNF-α and DRP-1.

Conclusions: Rc possess protective properties against OGD-induced neuron damage by regulating the expression of TNF-α and DRP-1.

Background

Stroke is a brain injured disease with high incidence, death, and disability rates. The global burden of disease in 2016 showed that stroke is the leading cause of life years loss in China[1,2]. In clinic, ischemic stroke (IS) accounts more than 85% of stroke. For the past two decades, the mainstay of acute ischemic stroke (AIS) management has been attempted reperfusion of ischemic tissue with intravenous thrombolysis[3]. Until now, alteplase, one of the recombinant tissue plasminogen activator, is the only fibrinolytic agent with Food and Drug Administration (FDA)-approved for AIS treatment. However, there were less than 3% patients who can benefit from intravenous thrombolysis because of the strict therapeutic time window limit and fatal side effects[4]. Besides, fibrinolytic therapy may cause ischemia-reperfusion (I/R) injury, which results in high morbidity and high mortality[5]. Currently, neuroprotective therapies have shown the potential to prolong the therapeutic window for reperfusion prior to endovascular interventions[6]. However, neuroprotection for stroke has shown great promise but has had little translational success. Therefore, the development of a clinically effective and safe neuroprotective drug for the treatment of ischemic stroke remains an urgent unmet need.

Ginsenoside Rc (Rc) is a major natural product isolated from Panax ginseng Meyer, which is widely used as both a preventive and therapeutic treatment against various diseases. Rc has exhibited effects on protection of central nervous system[7,8], prevention of diabetes[9], anti-inflammatory[10], anti-oxidation[11] and anti-adipogenesis[12]. In addition, Rc can be absorbed into blood and brain tissue rapidly after administration[13]. Researches have shown that ginsenosides are effective in treating cerebral I/R and other nervous system diseases[14]. Resently, some ginsenosides are regarded as neuroprotective agents to attenuate IS damages[15,16]. However, although the pharmacological activities of Rc have been well studied, it remains not clear whether RC has neuroprotective effect in IS. Moreover, the specific mechanisms have not been fully elucidated. From a therapeutic perspective, it is important to understand the functional mechanisms of RC to be developed into clinically effective and safe neuroprotective drug.

Network pharmacology is a prospective strategy to explore the multi-targets regulation networks of chemicals with combining systematic methods[17]. Until now, it has been reported in lots of researches on exploring the molecular mechanisms of effective components from TCM herbs[18]. Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine (TCMIP) is an intelligent data mining platform which has been extensively used to explain the functional mechanisms of TCM[19,20]. Besides, molecular docking is a computational method for predicting the placement of ligands in the binding sites of their receptors. It has been widely used in drug discovery[21]. Oxygen-glucose deprivation(OGD/R) cell modelhas been well recognized for stroke in vitro. Therefore, this study aimed to investigate the neuroprotective potential of Rc and explore the mechanism for treatment on IS via methods of network pharmacology, including target prediction of chemical, topological feature analysis, and molecular docking. Then the putative potential targets of Rc in OGD/R injury will be validated by molecular biology approach.

Materials And Methods

Chemicals and reagents

Ginsenoside Rc (purity,≥98%) was obtained from Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China). Cell Counting Kit-8 (CCK-8) was from Dojindo Institute (Kumamoto, Japan). Lactate Dehydrogenase (LDH) Activity Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Bicinchoninic Acid (BCA) Kit for Protein Determination was purchased from NanJing KeyGen Biotech Co.,Ltd. (Nanjing, China). Cell lysis buffer was obtained from Promega Corporation (Madison, WI, USA).

Cell culture

PC12 cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose Dulbecco's modifed Eagle's medium (DMEM; GIBCO, Waltham, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; GIBCO,

Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37˚C in a humidifed atmosphere with 5% CO_2.

Cell treatment and OGD/R model

Cells were seeded in 96-well plate at a density of 6×10^3 cells/well and incubated for 24 hours. Thereafter, Nimodipine (positive drug) group was treated with 5μmol/L Nimodipine. Treatment groups were treated with 1000, 500, and 100μmol/L Rc, respectively. While control group and model group were just cultured in normal medium. After culturing for 24 hours, Earle's Balanced Salts (EBSS) with 5 mmol/L $Na_2S_2O_4$ was added into groups except control group. After culturing for 80 minutes, the medium was replaced by normal DMEM with glucose and cells were cultured for 24 hours for reoxygenation under normoxic condition.

Preparation of cell lysates

Adherent cells were harvested using a cell scraper, washed twice with PBS and maintained on ice for 5 min. Following centrifugation at 14, 000 rpm for 15 min at 4°C, the cell pellets were dissolved in cell lysis buffer containing 1μL PMSF and Cocktail (Sigma-Aldrich; Merck KGaA), and maintained on ice for 30 min. Following centrifugation at 10,000 rpm for 5 min at 4˚C, the supernatants were collected. Protein concentration in cell lysates was determined by the BCA kit assay according to the manufacturer's instructions.

Cell viability and toxicity assay.

In order to investigate the putative protective properties of Rc in OGD on PC12 cells, cell viability was evaluated using a CCK-8 assay, and cell toxicity was determined by LDH assay. After treatment, CCK-8 assay and LDH assay were performed according to the manufacturer's instructions as described previously[22].

Target prediction

Identifcation of targets of Rc is a key step in understanding its mechanisms. In this study, the methods,includingTCMIP[19] (http://www.tcmip.cn/TCMIP/index.php/Home/Login/login.html),was used to derive molecular target information for target prediction. TCMIP is a new powerful platform for predicting targets of actual bioactive ingredients that based on similarity ensemble analysis.

Functional analysis of the putative targets by STRING.

The STRING database (https://string-db.org/), including data on interacting proteins or genes in humans, was used to determine protein-protein interactions (PPI). Interactions among the putative targets were identified using a threshold score of 0.7. To mine the critical targets related to ischemic stroke, a target-function network of ischemic stroke was constructed, with the target-function relation based on the network topological analysis by Cytoscape 3.7.1.

Molecular docking of Rc.

Molecular docking was analyzed using the SYBYL-X 2.1.1 software (Certara, L. P.). The scoring function total-Score equal to 5 was used as a threshold to evaluate the interaction between ingredients and targets. The structure of disease targets employed in the analysis of docking was obtained from the Protein Data Bank (PDB, http://www.rcsb.org). The co-crystalized ligand and water molecules were removed from the structure, while H atoms were added and side chains were fixed during protein preparation. The Surflex-Dock (SFXC) docking mode was used, and the procedure was conducted as previously described. Total Surflex-Dock scores represent binding affinities.

Western blot analysis.

Cellular lysates (protein content, 20 μg/lane) were separated using SDS PAGE on a 10% (w/v) gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore; USA). The membranes, after blocking with 5% nonfat milk (Sigma-Aldrich; Merck KGaA) for 1 hour at room temperature, were probed with the primary antibodies at 4˚C overnight, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (TNF-α Rabbit Antibody, ab11564; DRP-1 Rabbit Antibody, ab184247; Abcam, UK) at room temperature for 2 hours. Protein bands were visualized by using the ECL western detection reagent. GAPDH was used as the loading control. Densities of the protein bands were determined using ImageJ2x software.

Statistical analysis.

Data are expressed as the mean ± standard deviation. The statistical signifcance of the differences between groups was assessed using One-way analysis of variance. Graphpad Prism version 8.0.1 for Windows was used to perform the statistical analyses. $P<0.05$ was considered to indicate a statistically signifcant difference.

Results

Effects of ginsenoside Rc on cell viability and toxicity in OGD/R injured cells.

To evaluate the putative neuroprotective properties of Rc in OGD/R injured cells, the cell viability was detected by CCK-8 assay and the cytotoxicity was determined by LDH leakage. As shown in Fig.1A , compared with control group, the cell viability in model group（55.7±1.14%）indicated impairment ($^{\#\#}P<0.01$). Compared with model group, the cell viability in various concentrations of Rc groups（high-dose group: 67.1±2.83%; middle-dose group: 67.3±2.33%; low-dose group: 66.5±4.76%） were significantly improved (*P<0. 05). Furthermore, the LDH leakage in OGD/R injured cells was determined as shown in Fig.1B. The LDH leakage was obviously enhanced in model group (232±29 .4%), as compared with control group (179±51.2%; $^{\#\#}P<0. 01$). Rc treatment could inhibit the LDH release （high-dose group: 180±37.4%; middle-dose group: 147±39.7%; low-dose group: 166±34.1%） compared to model group

(**P<0. 01). The result showed that Rc improved cell viability and reduced the toxicity of LDH leakage in OGD/R injured cells.

Target prediction.

Eighteen potential targets of Rc were predited by TCMIP database. Then protein-protein interactions of these targets were determined by The STRING database as shown in Fig.2B. As revealed in Fig.2C, the enrichment of GO was performed by STRING. The enrichment of functional pathway was found out that the top 10 of pathway, such as TNF signaling pathway, is illustrated in Fig.2D. The network topological analysis by Cytoscape demonstrated that targets with the top five scores of degree were TNF. The key pathway of Rc maybe the TNF signal pathway.

The targets were analysed in Cytoscape and ranked by degree as shown in Table 1. The top 4 were TNF/CASPA3/NFKB1/IL6.

Table 1. The results of network topological analysis by Cytoscape

Name	Degree	BetweennessCentrality	ClosenessCentrality
TNF	10	0.18861111	0.69565217
CASP3	10	0.18680556	0.72727273
NFKB1	10	0.13583333	0.72727273
IL6	9	0.10513889	0.69565217
IL1B	8	0.06305556	0.61538462
NR3C1	8	0.07291667	0.61538462
NFKB2	7	0.00333333	0.59259259
RIPK3	6	0.02902778	0.57142857
ROCK1	5	0.15833333	0.55172414
VDR	4	0.04444444	0.51612903

Key targets of the Rc in TNF signal pathway

To identify the key targets of Rc, the targets in TNF pathway related to ischemic stroke were imported into SYBYL to find out key target. The results of molecular docking are shown in Fig.3A. As depicted in Fig.3A, RC is bound to residues outside the active pocket of TNF (key residues including ARG201/ASN200/ASP254/ASN182/ARG143/ASP294/ARG295)by hydrogen bonds. The overall spatial

structure indicates that the TNF/Rc complexes are stable, indicating the interactions of Rc with their targets may have an active functional role.

Referring to literature on ischemic stroke, TNF signal pathway (As shown in Fig.3B), including TNF, DRP1, play important role in necroptosis.

Effect of ginsnoside Rc on the expression of TNF-α/DRP-1 in OGD/R injured cells

To explore the effect on TNF and DRP-1 by RC, as shown in Fig.4, the result revealed that Rc decreased the protein level of TNF-α and DRP-1 in OGD/R injured cells. There was a significant increase in expression of TNF-α and DRP-1 of model group compared with control group (**P<0.01). Conversely, administration of different concentrations of Rc resulted in an extremely significant decrease in protein level of TNF-α and DRP-1 with a certain dose dependence compared with model group (**P<0.01).

Effects on cell survival rate induced by ginsnoside Rc and EvP4593/Mdivi-1

To further determine the correlation between Rc and TNF-α/DRP-1, cells were treated by EvP4593 (antagonist of TNF-α) and Mdivi-1 (antagonist of DRP-1), respectively. Cell survival rate was detected by CCK-8 assay. The result was shown in Fig.4D. Compared with Rc group (65.87±9.67%), the survival rate of Rc+EvP4593 (57.42±5.22%) was tended to decrease. Compared with model group, the survival rate of EvP4593 group showed no significant difference. The result indicated that Rc may had an indirect effect on TNF-α. Besides, the survival rate of Rc+Mdivi-1 showed no significant difference with Rc group. There was also no significant difference between model group and Mdivi-1 group, which indicated that Rc may also had an indirect effect on DRP-1.

Discussion

Ischemic stroke is a complex multifactorial disease caused by infarction and result in the loss of neurologic function. For decades, the most effective way to treat cerebral infarction is fibrinolytic therapy[23]. However, there isn’t any effective treatment for stroke caused by neuronal damage and death. In this study, we investigated the putative neuroprotective properties of Rc and the molecular machanism, which showed a positive significance for development of neuroprotective drugs for the treatment of cerebral I/R injury to reduce safety concerns caused by antithrombotic drugs in IS.

In our study, we found that pretreatment of Rc enhanced the cell viability and reduced the cell toxicity on OGD/R injured cells (Fig.1), which provided evidence for Rc as one of the active components of P. ginseng.Then, network pharmacology and molecular docking were applied to predict the related targets and corresponding mechanisms of Rc in treatment of IS. A protein-protein interaction network based on Rc was constructed. Bynetwork topological analysis, we revealed and highlighted that Rc were involved in TNF signal pathway (Fig.2). Analysis of molecular docking and literature on IS indicated that TNF signal pathway (Fig.3), including TNF, DRP-1, play important role in IS. The correlation between Rc and predicted targets was explored via western blots. Results showed that Rc reversed the expression of TNF-α and

DRP-1 on OGD/R injured cells when compared with model group (Fig.4). To further explore whether Rc possess neuroprotective effect by directly targeting TNF-α and DRP-1, the antagonists were added as interfering agent respectively. The result showed that Rc had an indirect effect on TNF-α and DRP-1 (Fig.4). In terms of experimental method, network pharmacology and molecular docking are not enough to judge the targets of Rc in IS. So cell experiments were combined in this study for illustration. This work may has practical significance for rapidly discovery on related targets and corresponding mechanisms of monomer drugs.

The tumor necrosis factor (TNF) superfamily of cytokines activate signaling pathways plays an important role in cell survival, death, and differentiation. A network pharmacology research had indicated that the treatment of compounds on stroke may related to TNF signaling pathway and TNF-α may be one of the molecular markers for stroke[24]. TNF-α is a pleiotropic inflammatory cytokine cytokine with various biological functions, which is mainly produced by macrophages and monocyte. TNF-α participates in several immunity disease and inflammatory disease[25], and it is a crucial determinant of inflammatory reaction in stroke[26]. Studies have shown that the expression of TNF-α after cerebral I/R has neurotoxic effect on nervous system[27]. Moreover, it was suggested that TNF-α may play a neurorotective role in stroke by downregulating apoptosis[28]. In addition, we found that stroke may be related to NFκB1 and CASP3 (table 1), which is consistant with the existing research finding[29]. It is well known that TNF-α plays a critical role on the activation of NFκB and caspase-3[30,31], which also provided evidence to illustrate TNF-α as a key target.

Dynamin-related protein-1 (DRP-1) is the dynamin of mitochondrial fission[32]. The imbalance of mitochondrial fusion/division has great influence on I/R injuries[33]. Previous studies had shown that TNF-α and DRP-1 played a regulatory role in amelioration of cerebral ischemic injury and neuroinflammation[34]. Moreover, TNF-α is the predominant inducer of DRP1 S616 phosphorylation during sepsis[35]. DRP-1 had neuroprotective effect in OGD-induced hippocampal neurons[36]. The overexpression of DRP-1 in nerve cells will eventually lead to cell apoptosis and mitochondrial lysis through activation of caspase system and damage of important proteins and organelles in mitochondria[37]. Our study document and validation suggested that Rc ameliorates neuron damage on oxygen-glucose deprivation associated with regulating TNF-α and DRP-1. The neuroprotective effects of Rc may be related to inflammatory response and necroptosis during nerve cell injury.

Conclusions

This study identified the neuroprotective activity of ginsenoside Rc mediated by its regulation on TNF-α and DRP-1. Rc may be involved in the pathological process of cell necroptosis and inflammatory. This work provide a step forward in the understanding of the neuroprotective effect and underlying mechanism of Rc on OGD induced neuron damage. However, the current study is performed based on in vitro experiments and the conclusions remain to beconfrmed by in vivo experiments.

List Of Abbreviations

Rc	Ginsenoside Rc
IS	ischemic stroke
I/R	ischemia-reperfusion
TCMIP	Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine
OGD/R	oxygen-glucose deprivation reperfusion
TNF-α	Tumor necrosis factor-α
Drp-1	Dynamin-related protein-1

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This manuscript is approved by all authors for publication.

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Mingmin Huang and Shaoru Chen contributed equally to this manuscript.

MX and SW conceived and designed the idea; KL performed the experiments; MH and SC analyzed the data; KZ and QL performed network pharmacology analysis; MX and MH wrote the paper. All authors read and approved the final manuscript.

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Figures

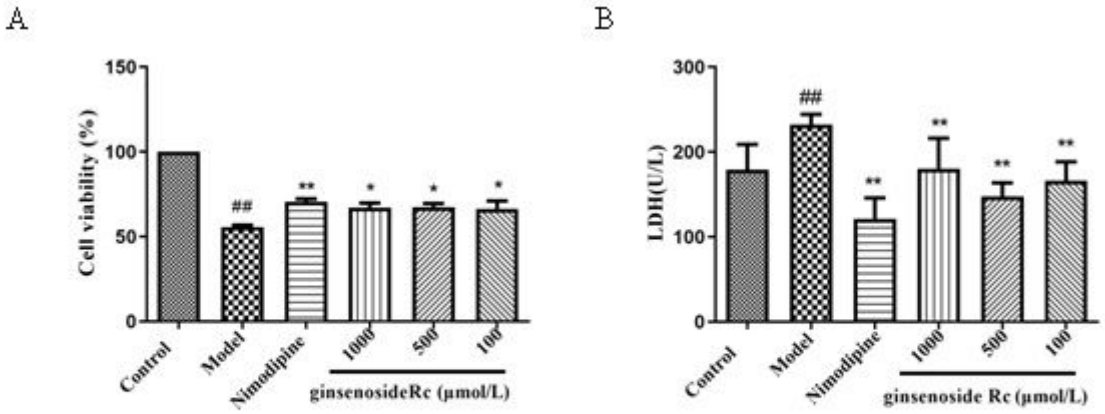

Figure 1.

Figure 1

Effects of ginsenoside Rc in OGD/R injured cells($\bar{x}$±s, n=6). (A) RC improved cell viability in OGD/R injured cells. (B) RC decreased LDH release in OGD/R injured cells. ##P<0.01 vs control group. **P<0.01, *P<0.05 vs model group.

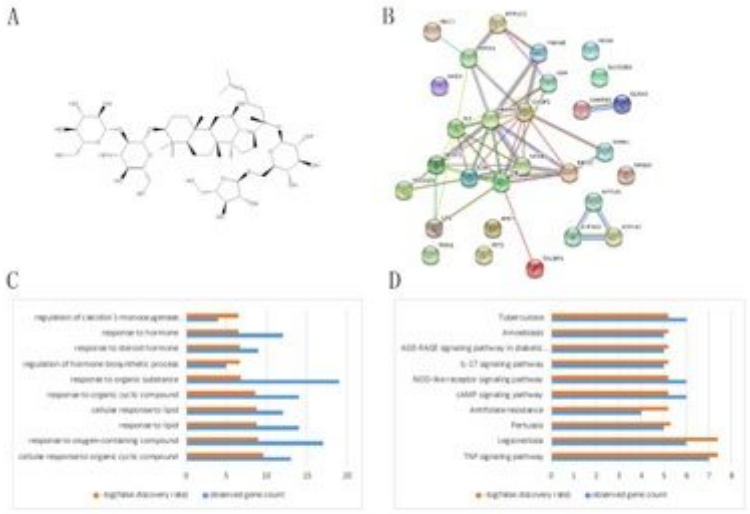

Figure.2

Figure 2

Target prediction of RC. (A)The structure of RC. (B)Protein-protein interactions of these targets related to RC. (C) The enrichment of GO related to RC. (D) The enrichment of functional pathway related to RC(orange pillars represent the false discovery rate, blue pillars represent the count of targets).

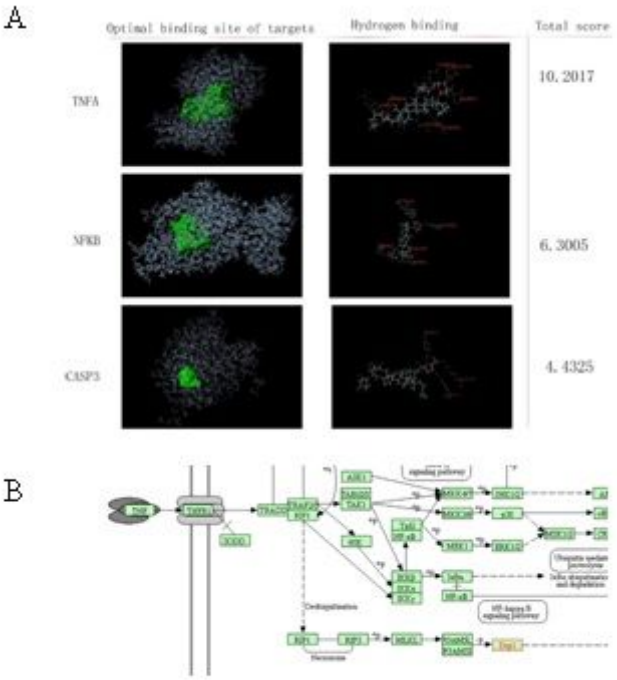

Figure.3

Figure 3

Key targets of the Rc in TNF signal pathway. (A) Molecular docking of RC with TNFA, NFKB, and CAP3. (B) TNF signal pathway.

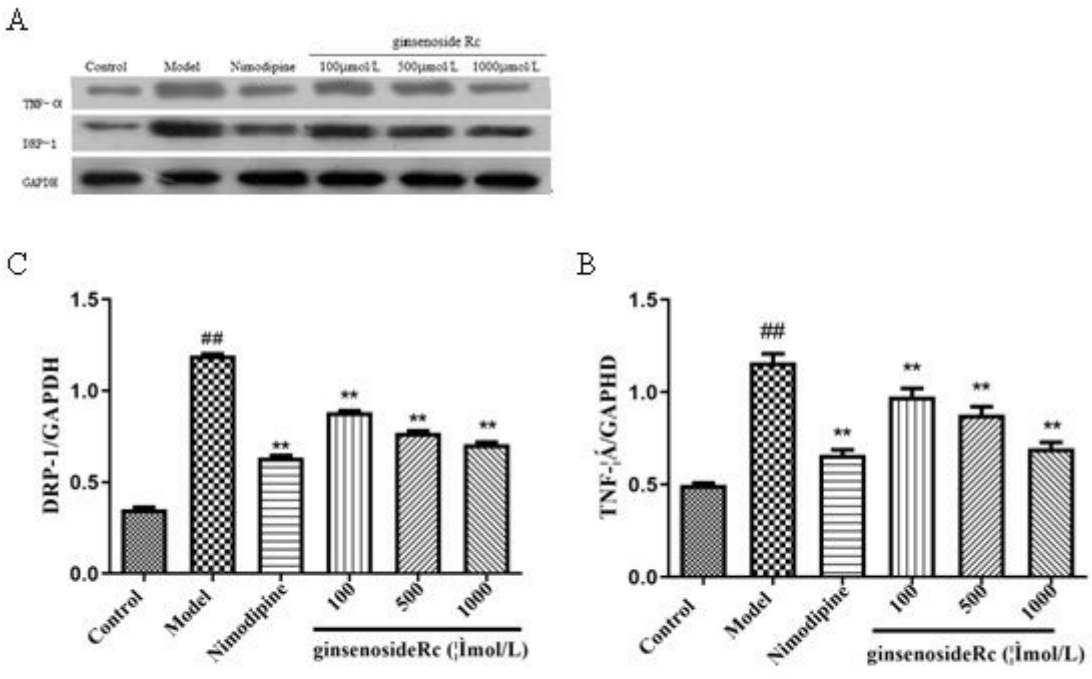

Figure 4.

Figure 4

Protective effects of ginsenoside Rc on the protein level of TNF-α/DRP-1 in OGD/R injury cells ($\bar{x}$±s, n=3). ##P<0.01 vs control group. **P<0.01, *P<0.05 vs model group.

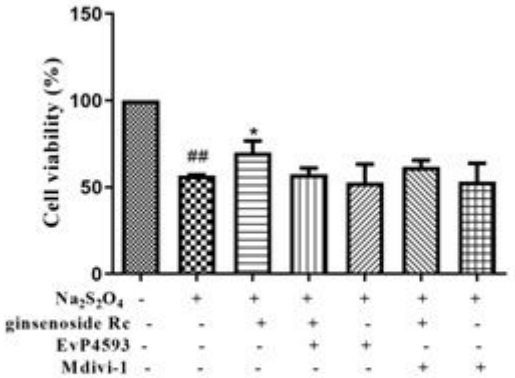

Figure 5.

Figure 5

Effects on cell survival rate induced by ginsnoside Rc and EvP4593/Mdivi-1 in OGD/R injured cells($\bar{x}$±s, n=6). ##P<0. 01 vs control group. **P<0.01, *P<0.05 vs model group.