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Original Research Article

Gomisin A inhibits hypoxia/reoxygenation-induced myocardial cell injury by modulating TLR4–NF-κB pathway

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Abstract

Purpose: *To determine the impact of Gomisin A (GomA) on myocardial cell damage caused by hypoxia/reoxygenation (H/R).*
Methods: *Various methods, including MTT, western blot and flow cytometry were used to to assess the viability of H9c2 cardiomyocytes and cell apoptosis. Expression levels of various enzymes and cytokines, including superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). Western blot was used to evaluate the expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and Toll-like receptor 4 (TLR4), as well as the phosphorylation of p65 and nuclear factor kappa B (NF-κB) inhibitor alpha (IκBα).*
Results: *Low concentrations of GomA had no toxic effect on H9c2 cells. In H/R-stressed H9c2 cells, GomA increased cell viability and reduced cell apoptosis, suggesting that GomA inhibited H/R-induced cell apoptosis. Additionally, GomA alleviated H/R-induced oxidative stress and inflammation by increasing the expression of SOD and CAT, while decreasing the expression of MDA, iNOS, COX-2, TNF-α, IL-1β, and IL-6. GomA also suppressed the H/R-induced TLR4–NF-κB pathway by reducing the expression of TLR4 and the phosphorylation of p65 and IκBα.*
Conclusion: *These results indicate that GomA is a potential candidate for the treatment of myocardial ischemia/reperfusion (MI/R) injury by increasing cell viability, reducing cell apoptosis, alleviating oxidative stress, and reducing inflammation by inhibiting TLR4–NF-κB pathway.*

Keywords: *Cell injury, Gomisin A, Myocardial cells, NF-Kb, TLR4, Inflammatory cytokines, Hypoxia-reperfusion*

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INTRODUCTION

Ventricular cell death in response to MI/R is a major contributor to mortality in coronary heart disease patients. MI/R triggers a complex series of inflammatory responses that cause more severe injury, cardiac arrhythmias, and cell death in the affected tissue [1]. Despite the numerous studies on the subject, the pathogenesis of MI/R injury remains complex and multifactorial, involving factors such as reactive oxygen species accumulation, inflammatory cytokine influx, calcium overload, and mitochondrial permeability transition pore opening [2]. To date, clinical

outcomes remain unsatisfactory, and there is a need to develop more effective therapies.

GomA, a lignan compound extracted from Schisandrae Fructus, possesses various biological properties including hepatoprotection [3], antitumor activity [4], anti-obesity, and anti-oxidative stress activities [5]. It has been shown to block the activation of NF-κB–mitogen-activated protein kinase (MAPK) pathway and suppress lipopolysaccharide-induced inflammation in N9 microglia [6]. Additionally, GomA has been shown to inhibit oxidative stress in osteoblasts exposed to high glucose [5]. Despite its many pharmacological properties, the full potential of GomA is yet to be fully understood, and its ability to mitigate H/R-induced myocardial injury requires further investigation.

This study aims to investigate the effects of GomA treatment on cell viability, apoptosis, oxidative stress, and inflammation in a H/R-induced injury model, and to identify the underlying pathways.

EXPERIMENTAL

Cell culture

The following experiment was performed to evaluate the effect of GomA on H9c2 cardiomyocytes. The cardiomyocytes were obtained from embryonic rat heart tissue and cultured in Dulbecco's modified Eagle medium containing fetal bovine serum (10 %, 37 °C, 5 % CO_2).

H/R-induced injury model

In order to create a hypoxia/reoxygenation (H/R)-induced injury model, cardiomyocytes were placed in a hypoxic incubator (94 % N_2, 5 % CO_2, and 1 % O_2; 8h), and then transferred to a reoxygenation incubator (95 % air and 5 % CO_2; 24h). After that, the cells were treated respectively with different concentrations of GomA (0, 5, 10 or 20 μM; 3 h). A control group of cells were also cultured under normal conditions (37 °C, 5 % CO_2) without being exposed to hypoxia.

MTT assay

To assess cell viability, MTT assay was performed. H/R-treated H9c2 cells were seeded (96-well plate), and treated with different concentrations of GomA (0, 5, 10 or 20 μM; 3 h). MTT solution was then added to cells at a final concentration (0.5 mg/mL) and incubated (4 h). After adding MTT solution (0.5 mg/mL) and incubating for 4 hours, the cells were washed three times and incubated with Formazan solvent for another 3 hours to solubilize formazan. The absorbance (570 nm) was measured using a microplate reader to determine the proportion of viable cells.

Western blot

Total protein was extracted from H9c2 cells, and treated with GomA using a RIPA lysis buffer from Thermo Scientific (Cat no. 89901, Carlsbad, California, USA). The proteins were then separated by SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane. The membrane was incubated (4 ℃; 12 h) with various primary antibodies including anti-Bcl2 (Cat. no. ab196495, 1:2000), anti-Bax (Cat. no. ab32503, 1:3000), anti-iNOS (Cat. no. ab178945, Abcam; 1:2000), and anti-TLR4 (Cat. no. ab217274, 1:3000) from Abcam (Cambridge, MA, USA), anti–COX-2 (Cat. no. 12282, 1:2000), anti-phosphor(p)-p65 (Cat. no. 3033, 1:2000), anti-p65 (Cat. no. 8242, 1:3000), anti-p-IκBα (Cat. no. 2859, 1:1000), and anti- IκBα (Cat. No. 4812, 1:2000) from Cell Signaling Technology (Denver, USA).

In addition, GAPDH (Cat. no. 10494-1-AP, ProteinTech Group, Chicago, IL, USA; 1:8000) was used as a control. The secondary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat. no. B900210, ProteinTech Group; 1:5000) was used to detect the target bands, which were then visualized using the ECL Western Blotting Detection Kit from Solarbio Life Sciences (Beijing, China). The Western blot signals were quantified and normalized against the intensity of the corresponding GAPDH band using ImageJ software.

Flow cytometry

Apoptosis in the H9c2 cells treated with different concentrations of GomA was measured using an apoptosis detection kit from BD Biosciences (Franklin Lakes, NJ, USA). H/R-treated H9c2 cells under 0, 5, 10, or 20 μM GomA were stained with FITC and PI and analyzed by cell sorting, using a FACS Calibur from BD Biosciences. Data were further analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Enzyme-linked immunosorbent assay (ELISA)

Expression levels of MDA, SOD, and CAT were evaluated using ELISA kits (MDA: Cat. o. ab238537, Abcam, Cat. no. EIASODC,

ThermoFisher Scientific, Waltham, MA, USA; CAT: Cat. no. MBS726781, MyBioSource Inc., San Diego, CA, USA). The concentrations of TNF-α, IL-1β, and IL-6 were measured in cell culture supernatants using their respective ELISA kits from Solarbio Life Sciences (TNF-α: SEKR-0009; IL-1β: SEKR-0002; IL-6: SEKR-0005). The ELISA procedure involved incubating (2 h; ELISA well) the samples (100 µL) with antibody solution (100 µL), washing (5 times) the wells, incubating (1 h) with working enzymatic reagent, washing the wells (5 times), and incubating with tetramethylbenzidine reagent (100 µL; 30 min). The absorbance value was read at 450 nm (wavelength correction of 570 nm) after the reaction was terminated with stop solution (50 µL).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0. Data were presented as the mean ± standard error from three biological replicates. Differences between the two groups were compared using unpaired t-tests. $P < 0.05$, or $p < 0.01$ was considered statistically significant, as applicable.

RESULTS

GomA treatment enhanced cell viability in H/R-treated H9c2 cells

The structure of GomA was shown in Figure 1 A. The cytotoxicity of GomA was assessed using the MTT assay, and it was discovered that GomA treatments at concentrations of 1, 5, 10, 20, or 50 µM had no apparent cytotoxic effects on H9c2 cells (Figure 1 B).

Next, the impact of GomA on H9c2 cell viability was evaluated under H/R conditions. H/R conditions resulted in a decrease in cell viability to approximately 60 %, and 5 µM GomA treatment increased cell viability to 70 %, and greater increases were observed with 10, 20, or 50 µM GomA, with cell viability greater than 80 % (Figure 1 C). These results suggested that GomA treatment increased cell viability in H9c2 cells subjected to H/R stress.

GomA treatment decreased H/R-induced apoptosis in H9c2 cells

Western blot analysis was used to determine cell apoptosis by measuring the expression levels of the anti-apoptotic protein Bcl2, and the pro-apoptotic protein Bax. Bcl2 expression levels were significantly reduced in H/R-stressed H9c2 cells. H/R-stressed H9c2 cells with 10 or 20 µM GomA significantly increased Bcl2 expression levels compared with untreated cells. Bax expression was increased under H/R conditions, but decreased significantly in H/R-stressed H9c2 cells that were treated with 5, 10, or 20 µM GomA (Figure 2 A). Flow cytometry was also performed to evaluate cell apoptosis, which in the H/R group, was elevated by 23.4 % compared with that in the control group. Also, compared to the control group, H/R-stressed H9c2 cells treated with 10 and 20 µM GomA showed only 17 % and 12.5 % increase, respectively, which was significantly lower than the apoptotic rate observed in the H/R group (Figure 2 B). These findings suggested that GomA inhibited H/R-induced apoptosis in H9c2 cells.

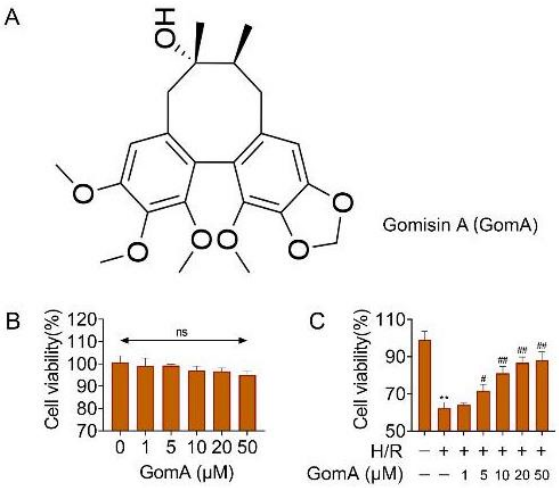

Figure 1: GomA treatment enhanced cell viability in H/R-stressed H9c2 cells. (A): Chemical structure of GomA; (B): GomA treatment at concentrations below 50 µM induced no obvious cytotoxic effects in H9c2 cells; (C): The effects of GomA treatment on the viability in H/R-stressed H9c2 cells. * comparison to blank group; # comparison to the group under H/R induction without GomA treatment. * (#) $p < 0.05$, ** (##) $p < 0.01$

GomA treatment decreased H/R-induced oxidative stress in H9c2 cells

To investigate the effects of GomA treatment on oxidative stress, the expression levels of three widely accepted oxidative stress markers (SOD, MDA, and CAT) were measured [7-9]. ELISA assay showed that SOD expression was drastically downregulated under H/R stress, however, its expression was increased significantly in H/R-stressed H9c2 cells treated with 10 and 20 µM GomA. MDA expression increased significantly under H/R conditions, and 10 and 20 µM GomA showed significant reduction of MDA expression in H/R-stressed H9c2 cells. CAT expression declined under H/R

conditions, but its expression was upregulated significantly in H/R-stressed H9c2 cells treated with 10 and 20 µM GomA (Figure 3 A). iNOS and COX-2 expression levels were elevated significantly under H/R conditions. However, GomA treatment significantly decreased iNOS and COX-2 expression levels in H/R-stressed H9c2 cells (Figure 3 B). The data indicates that GomA alleviated H/R-induced oxidative stress.

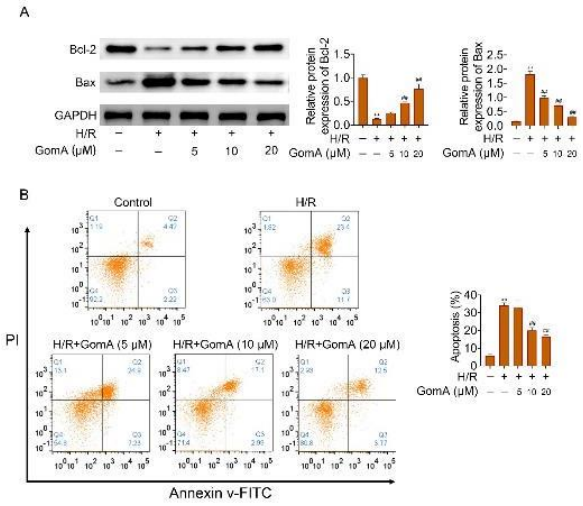

Figure 2: GomA treatment decreased H/R-induced apoptosis in H9c2 cells. (A): Treatment with 10 or 20 µM GomA significantly increased Bcl2 expression, while Bax expression decreased significantly under treatment of GomA at 5, 10, or 20 µM in H/R-stressed H9c2 cells; (B): 10 or 20 µM GomA caused significantly decreased cell apoptosis in H/R-stressed H9c2 cells

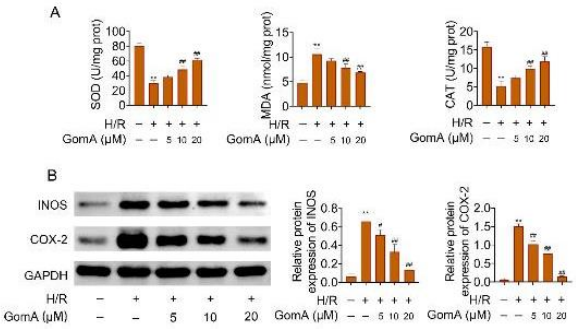

Figure 3: GomA treatment decreased H/R-induced oxidative stress in H9c2 cells. (A): SOD and CAT levels increased whereas, MDA concentration declined significantly with 10 or 20 µM GomA treatment in H/R-stressed H9c2 cells; (B): GomA treatment significantly reduced iNOS and COX-2 expression levels in H/R-stressed H9c2 cells

GomA treatment repressed H/R-induced inflammation in H9c2 cells

Three inflammation-related molecules (TNF-α, IL-1β, and IL-6) were measured using ELISA assay. Increased expression levels of TNF-α, IL-1β, and IL-6 were observed under H/R conditions, indicating the induction of inflammation. GomA treatment at 5, 10, or 20 µM decreased these levels in H/R-stressed H9c2 cells. IL-6 expression level decreased significantly with 10 and 20 µM GomA treatment in H/R-stressed H9c2 cells, but no change in IL-6 levels was caused by 5 µM GomA treatment in H/R-stressed H9c2 cells (Figure 4). These results suggested that GomA treatment suppressed H/R-induced inflammation in H9c2 cells.

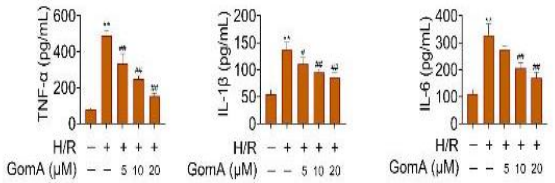

Figure 4: GomA treatment inhibited H/R-induced inflammation in H9c2 cells. Treatment with 5, 10, or 20 µM GomA reduced TNF-α and IL-1β expression levels in H/R-stressed H9c2 cells. IL-6 expression decreased significantly with 10 or 20 µM GomA treatment in H/R-stressed H9c2 cells

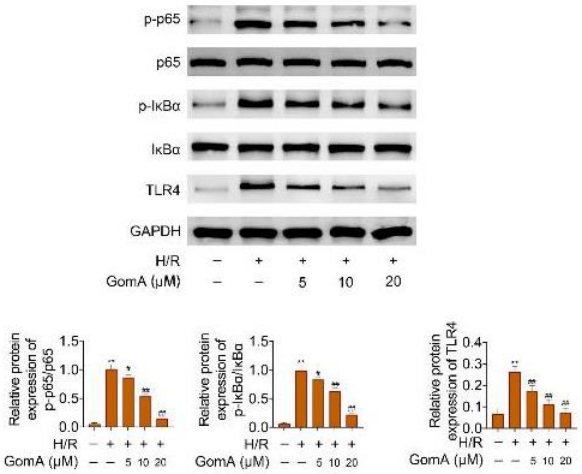

Figure 5: GomA treatment inhibited H/R-induced activation of the TLR4–NF-κB pathway in H9c2 cells. GomA treatment significantly reduced TLR4 expression levels and phosphorylation of p65 and IkBα in H/R-stressed H9c2 cells

GomA treatment inhibited the activation of the H/R-induced TLR4–NF-κB pathway in H9c2 cells

H/R conditions increased TLR4 expression, but significantly decreased by approximately 15, 50, and 80 %, following treatment with 5, 10, and 20 µM GomA, respectively. The phosphorylations of p65 and IκBα were elevated under H/R conditions, whereas phosphorylations of p65 reduced by approximately 20, 40, and 75 % following treatment with 5, 10, and 20 µM GomA,

respectively. Besides, the phosphorylation of IκBα decreased by approximately 40, 50, and 80 %, following treatment with 5, 10, and 20 μM GomA in H/R-stressed H9c2 cells, respectively (Figure 5). The data indicates that GomA inhibited H/R-induced activation of the TLR4–NF-κB pathway.

DISCUSSION

There is an urgent need for the development of effective clinical treatments for ventricular cell injury and cell death caused by MI/RI [1]. GomA has been reported to inhibit cell survival, mediated by AMPK and ERK–JNK, indicating the inhibitory role of GomA in the cell viability of metastatic melanoma [4]. GomA also promotes cell viability in stress-induced premature senescence [12]. The present study attempted to examine the effects of GomA treatment on cell viability in a rat myocardial H9c2 cell model. The results revealed that GomA alleviated cell damage and enhanced cell viability in HR-treated H9c2 cells, thus indicating the potential benefits of GomA treatment for MI/RI.

To further determine the mechanism by which GomA enhanced cell viability in HR-treated H9c2 cells, cell apoptosis was analyzed. GomA exerted anti-apoptotic activities as well as properties in liver failure models induced by galactosamine and lipopolysaccharide [13]. In melanoma cells with metastasis, GomA has been found to induce caspase-mediated apoptosis [4]. Although the Gomisin variants, Gomisin J and Gomisin N, have been associated with apoptosis-inducing activities, anti-apoptotic activity has been reported in all reports of GomA to date [14].

Consistently, GomA attenuated H9c2 cell apoptosis and enhance cell viability in a generated H/R-induced injury model. Previous studies have reported that GomA decreased oxidative stress in ovarian cancer cells, thus enhancing the antitumor effect [15]. GomA also inhibited the formation of reactive oxygen species under conditions of stress-induced premature senescence [12]. Under conditions of high glucose–mediated oxidative stress, GomA enhanced the expression of heme oxygenase-1, increased antioxidant enzymatic activity, and augmented mitochondrial biogenesis, thus reducing oxidative stress [5]. These findings concluded that GomA treatment lead to a decrease in oxidative stress.

In this section, five genes were used as the indicators for oxidative stress. SOD is an oxidative stress biomarker monitored in patients with diabetes [8]. MDA is considered a biomarker for nutrition-associated oxidative stress [7]. CAT mitigates oxidative stress by destroying cellular hydrogen peroxide, and is considered as a crucial antioxidant enzyme [9]. iNOS gene transcription is promoted under oxidative stress conditions [10]. COX-2–dependent vasoconstriction is increased by the enhanced generation of reactive oxygen species [11]. It is reported that GomA treatment reduces LPS-induced iNOS and COX-2 expression [16]. Therefore, these observed changes in the expression of these target genes indicated the ability of GomA to regulate oxidative stress.

Studies have indicated that GomA alleviates oxidative stress and inflammatory activities induced by carbon tetrachloride in rats [3]. GomA was found to suppress lipopolysaccharide-induced inflammation by inhibiting the activation of the NF-κB–MAPK pathway [6]. Therefore, it was important to examine the inflammatory response in the H/R-induced injury model. Consistent with previous findings, the results of this present study showed that GomA inhibited inflammation. The treatment of GomA regulated the expression of TLR4 and the phosphorylations of p65 and IκBα, which are key components of the inflammatory pathway. There is ample evidence that Gomisin is capable of inhibiting the activation of the TLR4–NF-κB pathway. For instance, *Schisandra chinensis* extract has been shown to improve MI/RI by regulating the TLR4–NF-κB–MyD88 pathway [17]. Therefore, it can be concluded that GomA suppressed inflammation by inhibiting the activation of the TLR4–NF-κB pathway.

CONCLUSION

The findings of this study revealed that GomA enhances cell viability, reduces cell apoptosis, and combats oxidative stress in H/R-stressed H9c2 cells. GomA also suppresses inflammation by inhibiting the activation of TLR4–NF-κB pathway. These findings suggest that GomA is a promising therapeutic option for the treatment of MI/RI.

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tianbao Chen and Chengbo Chen designed and performed all study experiments, supervised data collection, analyzed the data, and interpreted the data. Tianbao Chen, Chengbo Chen, Xiaojun Ji, and Yuanfang Zhu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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