

Glibenclamide exacerbates adriamycin-induced cardiotoxicity by activating oxidative stress-induced endoplasmic reticulum stress in rats

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Abstract. Adriamycin (ADR) is a chemotherapeutic drug used to treat tumors in a clinical setting. However, its use is limited by a side effect of cardiotoxicity. Glibenclamide (Gli), an inhibitor of mitochondrial ATP-dependent potassium (K-ATP) channels, blocks the cardioprotective effects of mitochondrial K-ATP channel openers and induces apoptosis in rodent pancreatic islet β -cell lines. However, little is known about the role of Gli in ADR-induced cardiotoxicity. The present study was designed to investigate the impact of Gli on ADR-induced cardiotoxicity in rats. A total of 60 male Sprague-Dawley rats were divided into the following 4 groups: i) Control; ii) Gli; iii) ADR; and iv) Gli+ADR ($n=15$ in each). The rats in the ADR and Gli+ADR groups were treated with ADR (intraperitoneal, 2.5 mg/kg/week) for 6 weeks. The rats in the Gli and Gli+ADR groups received Gli at a dose of 12 mg/kg/day via gastric lavage for 30 days from the eighth week of the study. Following the completion of Gli treatment, cardiac function was assessed by echocardiography, and the rats were sacrificed. The hearts were subsequently harvested for analysis. The rats in the ADR group demonstrated significantly impaired cardiac function and increased levels of oxidative stress, endoplasmic reticulum stress (ERS) and apoptosis in the heart compared with rats in the control and Gli groups (without ADR treatment). These abnormalities were exacerbated by Gli in the Gli+ADR group. Gli treatment decreased cardiac function and significantly increased oxidative stress, ERS and apoptosis levels in myocardial tissues in rats treated with ADR. The

findings indicated that Gli triggers oxidative stress-induced ERS, and thus exacerbates ADR-induced cardiotoxicity in rats.

Introduction

Adriamycin (ADR)-containing chemotherapy is known to cause dose-dependent and irreversible cardiac damage, which manifests clinically as a decrease in the left ventricular ejection fraction (EF) and heart functional deterioration (1). Patients with ADR-induced cardiomyopathy have a 1-year survival rate of no more than 50% (2). Limiting the use of ADR and discontinuing the drug are the only clinically accepted methods of preventing ADR-induced cardiomyopathy (3); thus, treating cancer with ADR is challenging. The pathogenesis of ADR-induced cardiotoxicity is thought to be driven by the generation of reactive oxygen species (ROS) (4), as well as fibrosis, calcium overload and apoptosis (5).

Endoplasmic reticulum stress (ERS) is known to be involved in the development of many diseases (6,7) and is characterized by the abnormal accumulation of unfolded and misfolded proteins (8). ERS can be activated by external and internal stimuli, such as hypoxia, oxidative stress, inflammation and toxic compounds (9). In particular, oxidative stress is believed to be closely related to ERS in the pathogenesis of numerous diseases. Moderate ERS plays an important role in maintaining endoplasmic reticulum (ER) function and homeostasis by enhancing protein folding capacity, while excessive ERS leads to cell injury and apoptosis (9).

It is well known that adenosine triphosphate-sensitive potassium channels (K-ATP) are distributed in various tissues throughout the body, including cardiac muscle, skeletal muscle, smooth muscle and the brain (10). Channel opening plays a cytoprotective role under various pathophysiological conditions (11). Glibenclamide (Gli), a K-ATP channel blocker, has been shown to induce apoptosis and loss of function in pancreatic β -cell lines (12) by activating ERS. Gli also impairs the protective effects of ischaemic pre-conditioning and K-ATP channel opening in the heart. However, little is known about the role of Gli in ADR-induced cardiotoxicity. Thus, we sought to investigate the impact of Gli on ADR-induced cardiotoxicity in rats and the related mechanisms.

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Materials and methods

Animals. All procedures involving animals were approved by the Ethics Committee for Animal Research of Wuhan University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and the National Research Council. All animals were acclimated to the laboratory for at least one week before the experiments.

A total of 60 male Sprague-Dawley (SD) rats (150-180 g) were purchased from the Experimental Animal Center of Wuhan University and were randomly divided into the following four groups: i) Control; ii) Gli (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); iii) ADR (Actavis Italy S.p.A., Nerviano, MI, Italy); and iv) Gli+ADR (n=15 in each group). The rats in the ADR and Gli+ADR groups were treated with ADR at a dose of 2.5 mg/kg/week via intraperitoneal injection for 6 weeks, while the rats in the control and the Gli groups were treated with normal saline (via intraperitoneal injection) at the same dose with ADR for 6 weeks. The rats in the Gli group and the Gli+ADR group received Gli at a dose of 12 mg/kg/day via gastric lavage for 30 days from the eighth week of the study, while the rats in the control and the Gli groups were treated with solvent (via gastric lavage) at the same dose with Gli for 30 days. The doses of ADR and Gli were selected on the basis of the previous studies (13,14). Upon completion of the 30-day Gli or solvent treatment period, cardiac function was assessed by echocardiography. The rats were subsequently sacrificed using an overdose of anesthesia (100 mg/kg pentobarbital), and the cardiac tissues harvested. The cardiac tissues used for haematoxylin & eosin (H&E) and TUNEL assay were saved in formalin, and those for western blotting and RT-qPCR were saved in -80°C. However, the cardiac tissues used for superoxide dismutase (SOD) and malondialdehyde (MDA) measurements must be tested as soon as possible after extracted from the rats.

Echocardiography. Echocardiography was performed using a high-resolution ultrasound imaging system equipped with a 7V3 probe with a frequency of 6.0 MHz (Acuson Sequoia 512; Siemens Medical Solutions, Mountain View, CA, USA). Data pertaining to the following parameters were recorded: EF%, fractional shortening % (FS%), left ventricular internal dimension diastolic (LVIDD), left ventricular internal dimension systole (LVIDS), left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV). Data pertaining to the FS%, LVIDD and LVIDS were recorded from parasternal long-axis M-mode images in accordance with the American Society of Echocardiography guidelines. The data represent the average measurements from three to five consecutive cardiac cycles. The LVEDV and LVESV were calculated from bi-dimensional long-axis parasternal views by the single-plane area-length method. The EF% was calculated as follows: EF%=(LVEDV-LVESV)/LVEDV x 100%.

Histological examination and TdT-mediated dUTP nick end labeling (TUNEL) assay. Myocardial tissues removed from the middle portion of each heart were fixed in 10% buffered formalin for 24 h, embedded in paraffin and sliced into

5 µm-thick sections, which were then stained with H&E and visualized by light microscopy for heart size assessments.

The cardiomyocyte apoptosis rate was assessed by the TUNEL assay. The steps are as below: Sections (3 µm) from formalin-fixed paraffin-embedded myocardial tissues were deparaffinized with xylene and dehydrated with ethanol. The slides were then rinsed twice with PBS and treated with proteinase K (151 g/ml in 10 mMTris/HCl, pH 7.4-8.0) for 15 min at 37°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature. The tissue sections were then analyzed with an *in situ* cell death detection kit (POD; Roche Diagnostics GmbH, Mannheim, Germany), in accordance with the manufacturer's instructions. The reactions were visualized with fluorescence microscopy and measured with a quantitative digital image analysis system (Image-Pro Plus 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

SOD activity and MDA content measurements. SOD activity in myocardial tissue was detected using the xanthine oxidase (XO) technique. This procedure depends on the inhibition of nitrite (NIT) reduction by the superoxide anion, which is generated by the combination of xanthine and XO. An SOD assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to assess SOD activity. One unit of SOD decreased the rate of NIT reduction by 50%. SOD activity in the myocardial tissue homogenate was expressed as U/mg protein.

MDA content in myocardial tissue was assayed by the thiobarbituric acid (TBA) method. This method is based on the theory that at high temperature (90-100°C) and under acidic conditions, MDA reacts with TBA to form TBARS, the production of which was measured at 532 nm by a spectrophotometer. An MDA assay kit (Nanjing Jiancheng Bioengineering Institute) was used to assess MDA concentrations. MDA content in the myocardial tissue homogenate was expressed as nmol/mg protein.

Western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cardiac tissues were lysed in RIPA lysis buffer, and the protein concentration was determined with a BCA protein assay kit. The protein extracts (30 µg per lane) were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes, which were probed with various primary antibodies. After incubating with the appropriate secondary antibodies for 1 h at room temperature, the membranes were treated with ECL reagents (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the signals were visualized with an Odyssey Imaging System. The expression levels of specific protein were normalized to those of GAPDH on the same PVDF membrane. The following primary antibodies were used for the experiment: Anti-GAPDH antibody; anti-Bax antibody (Epitomics, Burlingame, CA, USA); anti-Bcl-2 antibody; anti-glucose-regulated protein 78 (GRP78) antibody; anti-C/EBP homologous protein (CHOP) antibody, anti-phosphorylated eukaryotic translational initiation factor 2α (p-eIF2α) antibody, anti-activating transcription factor 6α (ATF6α) antibody and anti-X-box-binding protein 1 (XBPI) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA).

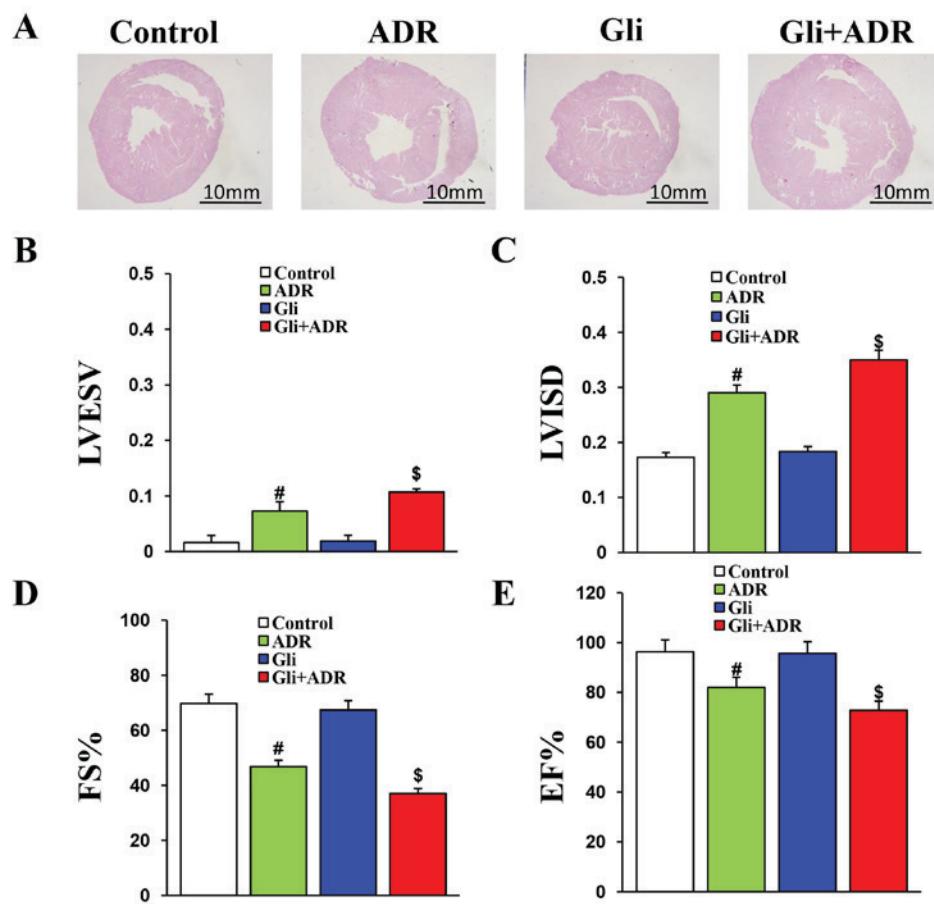


Figure 1. Gli exacerbates ADR-induced impairments in cardiac function of rats. (A) Histological analysis of heart sections from the four groups (scale bar, 20 mm). The (B) LVESV, (C) LVISD, (D) FS% and (E) EF% data for the four groups. [#]P<0.05 vs. Control and Gli groups, ^{\$}P<0.05 vs. ADR group. ADR, adriamycin; Gli, glibenclamide; LVESV, left ventricular end systolic volume; LVISD, left ventricular internal dimension systole; FS, fractional shortening; and EF, ejection fraction.

For RT-qPCR, total RNA was extracted from ventricular tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and then first-strand cDNA was synthesized from the RNA using a Transcripter First-strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). RT-qPCR was performed using SYBR-Green PCR Master Mix (Roche Diagnostics) to determine the expression levels of the genes of interest, and the results were normalized against the expression levels of GAPDH. The following primers were used for the experiment: Bax: Forward, 5'-TAGCAAACCTGGTGCTCAAGG-3'; and reverse, 5'-TCTTGGATCCAGACAAGCAG-3'. Bcl-2: Forward, 5'-AGCATGCGACCTCTGTTGA-3'; and reverse, 5'-TCACTTGTGGCCCAGGTATG-3'.

Statistical analysis. All the statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Inter-group comparisons were analyzed by one-way ANOVA. The data were expressed as the mean \pm standard deviation. All P-values were two-sided, and P<0.05 was considered to indicate a statistically significant difference.

Results

Mortality of rats. Out of 60 rats, 48 completed the study. The mortality of ADR group and Gli+ADR group were 33.3 and

53.3% at the end of the interventions, while no deaths were encountered in other groups.

Gli exacerbates ADR induced impairments in cardiac function in rats. The H&E staining results indicated that the increases in heart cross-sectional size induced by ADR were exacerbated by Gli in the rats in the Gli+ADR group (no data were obtained) (Fig. 1A). Echocardiography was performed to measure relative cardiac functional parameters in each rat. The results consistently indicated that Gli aggravates ADR-induced impairments in cardiac function. The LVESV and LVISD in the Gli+ADR group were significantly larger than those in the ADR group (Fig. 1B and C), while the FS and EF% in the Gli+ADR group were obviously lower than those in the ADR group (Fig. 1D and E). However, there were no significant differences in LVIDD and LVEVD among the four groups (data not shown).

Effect of Gli on oxidative stress in ADR-treated rats. SOD is the major defense against ROS production in cells while MDA is the product of the effects of ROS on cell membrane lipid. Thus, SOD and MDA are used to evaluate oxidative stress. In this study, ADR elicited a significant decrease in SOD levels in rat cardiac tissues in the ADR group compared with those in the control group. Gli treatment decreased SOD levels further in the Gli+ADR group (Fig. 2A). However, ADR increased

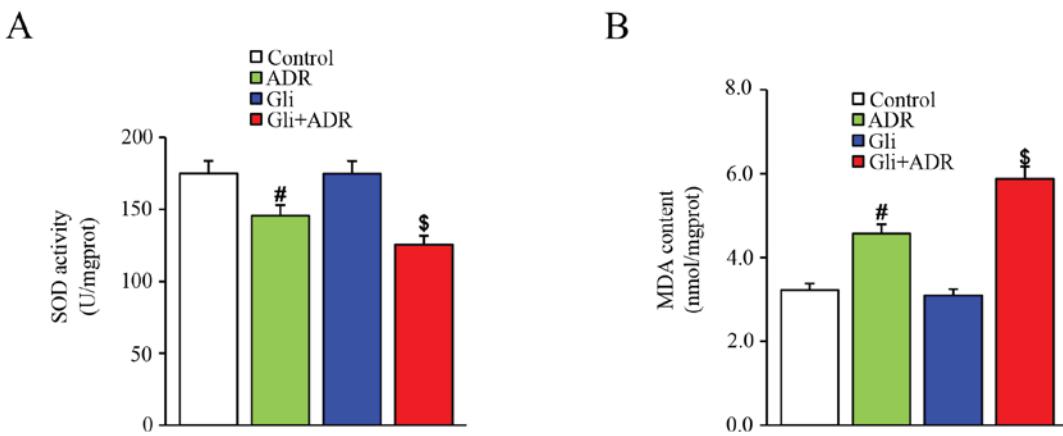


Figure 2. Effects of ADR and Gli on the expression levels of oxidative stress-related molecules. (A) SOD activity and (B) MDA content in myocardial tissue. [#]P<0.05 vs. Control and Gli groups, ^{\$}P<0.05 vs. ADR group. ADR, adriamycin; Gli, glibenclamide; SOD, superoxide dismutase; and MDA, malondialdehyde.

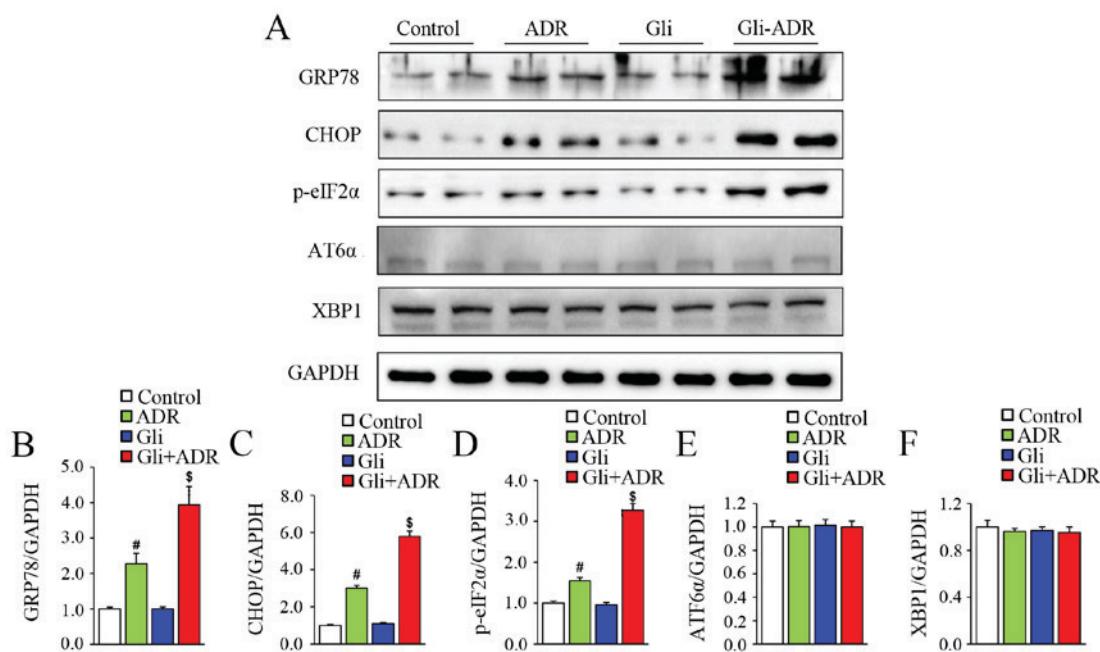


Figure 3. Effects of Gli on ERS-related biomarker expression in ADR-treated rats. (A) Western blotting results for the protein expression levels of GRP78, CHOP, p-eIF2 α , ATF6 α and XBP1 in myocardial tissue and the quantified expression levels of (B) GRP78, (C) CHOP, (D) p-eIF2 α , (E) ATF6 α and (F) XBP1. [#]P<0.05 vs. Control and Gli groups, ^{\$}P<0.05 vs. ADR group. ADR, adriamycin; Gli, glibenclamide; GRP78, glucose-regulated protein 78; p-eIF2 α , phosphorylated eukaryotic translational initiation factor 2 α ; CHOP, C/EBP homologous protein; ATF6 α , activating transcription factor 6 α ; and XBP1, X-box-binding protein-1.

MDA levels in rat cardiac tissues in the ADR group compared with those in the control group, a change that was markedly exacerbated by Gli in the Gli+ADR group (Fig. 2B).

The effect of Gli on ERS in ADR-treated rats. To assess the effect of Gli on ERS, we measured the expression levels of ERS-related biomarkers by western blotting. The protein levels of GRP78, CHOP and p-eIF2 α in the rats of ADR group were significantly higher than those in the control group, but were remarkably lower than those in the Gli+ADR group (Fig. 3A-D). However, there were no significant differences in ATF6 α and XBP1 protein expression levels among the four groups (Fig. 3A, E and F). Therefore, Gli exacerbates ADR-induced cardiotoxicity by activating ERS.

Gli exacerbates ADR-induced myocardial cell apoptosis in rats. Apoptosis is a type of terminal pathological changes that occurs in ADR-induced cardiotoxicity. To assess the effect of Gli on myocardial cell apoptosis, we assessed the expression levels of apoptosis-related markers. TUNEL staining showed that ADR increased the cardiomyocyte apoptosis rate in the ADR group compared with the control group and that Gli increased the apoptosis rate further in the Gli+ADR group (Fig. 4, scale bar, 50 μm).

To confirm the above findings, we performed western blot analysis and RT-qPCR to assess the protein and mRNA expression levels of apoptosis-related biomarkers, such as Bax and Bcl-2, respectively. As shown in Fig. 5A, Bax mRNA expression levels in myocardial tissues in the ADR group were

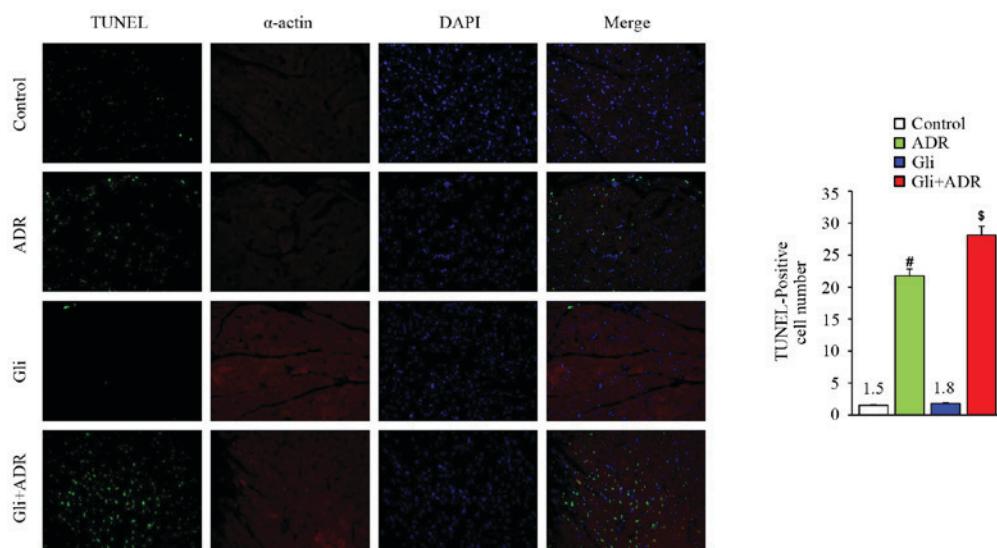


Figure 4. TUNEL staining results. $^{\#}$ P<0.05 vs. Control and Gli groups, $^{\$}$ P<0.05 vs. ADR group. ADR, adriamycin; Gli, glibenclamide.

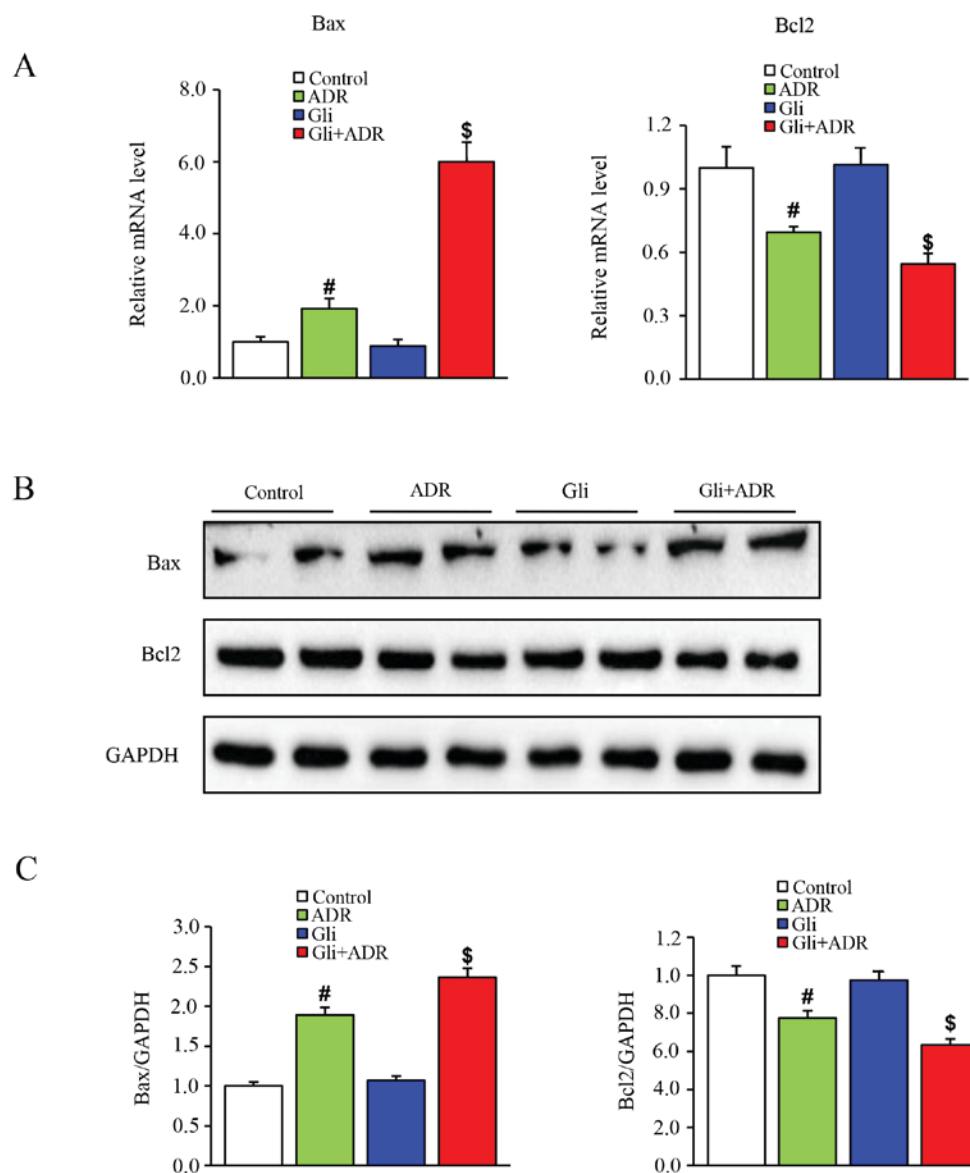


Figure 5. Effects of Gli on apoptosis-related biomarker expression in ADR-treated rats. (A) The quantified mRNA expression levels of Bax and Bcl-2 in myocardial tissue. (B) Western blotting results for the expression levels of Bax and Bcl-2 in myocardial tissue and (C) the quantified expression levels of Bax and Bcl-2. $^{\#}$ P<0.05 vs. Control and Gli groups, $^{\$}$ P<0.05 vs. ADR group. ADR, adriamycin; Gli, glibenclamide;

significantly higher than those in the control group but were significantly higher than those in the Gli+ADR group. By contrast, Bcl-2 mRNA expression levels in myocardial tissues in the ADR group were significantly lower than those in the control group and were significantly higher than those in the Gli+ADR group.

The western blot analysis results were consistent with the RT-qPCR results. As shown in Fig. 5B and C, Gli elicited a significant increase in Bax protein expression levels and a significant decrease in Bcl-2 protein expression levels in the Gli+ADR group compared with the ADR and control groups.

Discussion

A previous study showed that Gli (a K-ATP channel blocker) offsets the cardioprotective effect of nicorandil (a K-ATP channel opener) in ADR-treated rats (15). In the present study, the highest mortality was observed in the Gli+ADR group, which was followed by the ADR group, indicating that Gli could increase mortality induced by the ADR. Besides, we also observed that the cross-sectional sizes of rat hearts from the Gli+ADR group were larger than those from the ADR and control groups (no data were obtained). Furthermore, more serious heart functional deterioration occurred in the rats of the Gli+ADR group than in the rats of the ADR group, as demonstrated by echocardiography. Therefore, our findings were consistent with those of previous reports.

Oxidative stress is a form of cellular stress and damage caused by an imbalance between ROS generation and anti-oxidant defense mechanisms (16). Oxidative stress generally arises because of the excessive accumulation of ROS, which overpowers the antioxidant defences of the body and induces an oxidative reaction (16). A limited number of ROS are normally produced by cellular metabolic processes (17). However, excessive accumulation of ROS or persistent exposure to ROS can lead to the development of many diseases (18). Previous research has indicated that oxidative stress is the major mechanism underlying ADR-induced cardiotoxicity (19). In this study, we observed that ADR reduced SOD levels and increased MDA levels in rat cardiac tissues in the ADR group, findings that were consistent with those of the above mentioned studies. Gli reduced SOD levels and increased MDA levels further in rat cardiac tissues in the Gli+ADR group. Thus, we concluded that Gli promotes ROS generation under ADR stimulation.

The ER is an important cellular organelle in eukaryotes and participates in the regulation of protein biosynthesis, folding, transport and modification (20). However, ER-mediated protein folding is highly and acutely sensitive to intracellular and extracellular stimuli, such as disruptions of redox homeostasis, ER calcium ions, changes in energy storage, elevations in mRNA translation and inflammation (21,22). These phenomena can lead to protein misfolding. A few misfolded proteins can normally be found in the ER; however, excessive protein misfolding in the ER can lead to cellular stress known as ERS (23). Previous studies have shown that ERS is closely associated with oxidative stress (18,24) and participates in the pathogenesis of numerous diseases. Oxidative stress can cause imbalances in reduction-oxidation (redox) and can activate ERS by decreasing the efficiency of protein-folding

pathways and by promoting protein misfolding (24). GRP78, an ER chaperone, is an indicator of ERS and a marker of ERS activation (9). Under normal conditions, GRP78 is bound to the unfolded protein response (UPR, an appropriate adaptive response to ERS that relieves ERS by attenuating protein translation and degrading misfolded or unfolded protein) signal transducers, such as ATF6, IRE1 and PERK. In the ATF6 pathway, ATF6 α releases from GRP78 and transfers to the nucleus to stimulate the expression of genes related to the UPR (25). In the IRE1 pathway, IRE1 cleaves and releases XBP1 mRNA, which is translated into the active XBP1 protein. The activated XBP1 protein then binds to several UPR-related transcription factors, which leads to target gene up-regulation (26). In the PERK pathway, PERK phosphorylates eIF2 α and abrogates protein synthesis, thereby reducing the ER workload to relieve ERS (27). However, phosphorylated-eIF2 α (p-eIF2 α) also triggers the synthesis of CHOP (28), which is considered as a vital event in ERS-induced apoptosis. CHOP has been demonstrated to contribute to apoptosis induced by cytokines by activating mitochondrial apoptosis pathways (29). In our study, we observed that GRP78, CHOP and p-eIF2 α protein expression levels were significantly higher in the rats in the ADR group compared with control group, but were remarkably lower in the rats in the ADR group than in those in the Gli+ADR group. Thus, ERS was activated in rat cardiac tissues damaged by ADR. Gli exacerbates ERS activation by activating oxidative stress. However, we observed no significant differences in ATF6 α and XBP1 protein expression levels among the four groups, perhaps because the ATF6 and IRE1 pathways were not activated effectively, and the UPR failed to relieve ERS.

As a terminal pathophysiological process in cells, apoptosis has been shown to be induced by a variety of stressors, such as biomechanical stress, oxidative stress and ERS (30). A previous study reported that ADR induces myocardial cell apoptosis (31). Consistent with the results of the above report, our results indicated that the cardiomyocyte apoptosis rate and Bax protein and mRNA expression levels were significantly increased, while Bcl-2 protein and mRNA expression levels were markedly decreased in the ADR group compared with the control group. However, after treated with Gli, the above changes we detected in the Gli+ADR group were exacerbated further. Therefore, Gli exacerbates myocardial cell apoptosis in rats treated with ADR by activating oxidative stress-induced ERS.

Gli, a type of sulfonylurea, has been widely used to treat type 2 diabetes since the early 1950s by stimulating the release of insulin from pancreatic β -cells and by reducing blood glucose levels (32). Our findings indicated that Gli exacerbates ADR-induced cardiotoxicity by activating oxidative stress-induced ERS. However, there are limitations in this study, including that the baseline cardiac function of rats was not assessed and there wasn't another group with Gli at another dose. These limitations may affect the difference during the four groups and let us hard to know the relationship between the dose of Gli and the effects of Gli on ADR-induced cardiotoxicity. Despite above limitations, our results suggest that we should not use sulfonylurea (Gli) and ADR simultaneously when treating patients with malignant tumours and type 2 diabetes.

Acknowledgements

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