Wogonin Alleviates Cisplatin-induced Cardiotoxicity in Mice Via Inhibiting Gasdermin D-mediated Pyroptosis

Jiajun Xu, MD, Bin Zhang, MD, Zhenliang Chu, MD, Fenfen Jiang, MD, and Jibo Han, MD

Abstract: Cardiotoxicity has been well documented as a side effect of cisplatin (CDDP) treatment. The inflammatory response plays a crucial role in the pathological process of CDDP-induced cardiotoxicity. Wogonin is a natural flavonoid compound that possesses cardioprotective and anti-inflammatory qualities. Knowledge of the pharmacological effect and mechanism of wogonin could reveal an efficient way to identify therapeutic strategies. In this study, the potential of wogonin to antagonize CDDP-induced cardiotoxicity was evaluated in C57BL/6 mice in vivo and in H9c2 cells in vitro. The results showed that wogonin protected against CDDP-induced cardiac dysfunction, myocardial injury, and pyroptosis in vivo. Using a Gasdermin D expression plasmid, we revealed that wogonin dramatically reduced CDDP-induced pyroptosis by modulating the Gasdermin D protein in H9c2 cells. In conclusion, wogonin has great potential in attenuating CDDP-induced cardiotoxicity. In addition, greater emphasis should be placed on the antipyroptotic effects of wogonin for the treatment of other diseases.

Key Words: wogonin, cisplatin, cardiotoxicity, GSDMD, pyroptosis

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INTRODUCTION

Conventional cancer chemotherapy-induced cardiac dysfunction has become a global problem.[1] Multiple types of anticancer chemotherapeutic agents have exhibited high rates of cardiotoxicity, one of which is the platinum-like chemotherapy drug cisplatin (cis-diamminedichloroplatinum, CDDP).[1] CDDP-induced cardiotoxicity manifests as irreversible degenerative cardiomyopathy, arrhythmias, congestive heart failure, and abrupt cardiac death.[2,3] The underlying mechanisms by which CDDP induces cardiotoxicity include inflammation, mitochondrial damage, oxidative stress, and apoptosis,[2–4] but the proposed molecular mechanisms remain unclear. Therefore, revealing the molecular disorders associated with CDDP cardiotoxicity will be helpful in identifying new drugs to prevent tumor patients from developing cardiovascular complications.

Inflammation, apoptosis, and necrosis are involved and play vital roles in CDDP-induced cardiotoxicity.[4,5] Gasdermin D (GSDMD) is known as the key protein in pyroptosis, which is a proinflammatory programmed cell death.[6] After the cleavage of caspase-1/4/11, GSDMD aggregates to form pores in the cell membrane.[6,7] These pores destroy the integrity of the cell membrane and cause cell swelling and rupture. This process is accompanied by the maturation and release of intracellular interleukin (IL)-1β and IL-18, which consequently boost the inflammatory response.[8] Many proinflammatory factors are involved in CDDP- induced cardiotoxicity including IL-1β.[9] Thus, we hypothesized that CDDP treatment may induce cardiomyopathy through pyroptosis and its key protein GSDMD.

The application of anti-inflammatory drugs combined with conventional chemotherapeutic agents is a potential strategy for treating cancer patients. Wogonin (5,7-dihydroxy-8-methoxyflavone, Wog) is a flavonoid derivative of *S. baicalensis* Georg.[10] Wogonin has a wide range of pharmacological activities, including anti-inflammatory, antioxidant, anticancer, and cardioprotective activities.[11–14]

With a focus on the medical potential of natural products, we examined the protective effect of wogonin on CDDP-induced cardiotoxicity and conducted in-depth research on the mechanism underlying this effect. Our results showed that wogonin could reduce CDDP-induced cardiac dysfunction, myocardial injury, and myocardial pyroptosis in mice. The cardioprotective effect of wogonin was closely associated with the inhibition of GSDMD.

MATERIALS AND METHODS

Materials

CDDP was purchased from MedChemExpress (Monmouth Junction, NJ). CDDP was dissolved in saline for in vivo experiments and in DMF for in vitro experiments. Wogonin was obtained from Alexis Biochemicals (San Diego, CA), dissolved in a 1% sodium carboxymethyl cellulose solution for in vivo studies and in dimethyl sulfoxide for in vitro experiments. The chemical structure of wogonin is shown in Figure 1A.

Animal Experiments

Four-week-old male C57BL/6 mice weighing 18–22 gm were obtained from Beijing Vital River Laboratory Animal Technology Co Ltd (Beijing, China). All animal care and experimental procedures were performed in accordance with the directives outlined in the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health). Animal care and experimental protocols were approved by the Committee on Animal Care of the Second Affiliated Hospital of Jiaxing University (Jiaxing, China; approval no. JXEY-2019GZ006).

The dose of CDDP and treatment time were chosen according to previous studies.[2,5,9,15,16] CDDP (6 mg/kg) in 0.9% normal saline was administered by intraperitoneal injection on the 1st, 8th, and 15th days as previously reported.[5] Mice were randomly divided into 3 groups: the control group (Ctrl, n = 6), the CDDP group (CDDP, n = 6), and the CDDP + Wogonin group [CDDP + Wog, n = 6, intragastric administration of Wog (10 mg kg^{-1} day^{-1})[17] plus CDDP]. On the 22nd day, transthoracic echocardiography was performed on the mice to examine cardiac function, and then all animals were sacrificed under sodium pentobarbital anesthesia. Blood samples and heart tissues were collected.

Cardiac Function

Cardiac function was examined in anaesthetized mice noninvasively by transthoracic echocardiography (VisualSonics, Toronto, Canada) one day before sacrifice. Echocardiography was performed by a SONOS 5500 ultrasound (Philips Electronics, Amsterdam, Netherlands) with a 15-MHz linear array ultrasound transducer. The left ventricular internal dimension in diastole (LVIDs), left ventricular internal dimension in systole (LVIDd), interventricular septal thickness at end diastolic (IVSd), interventricular septal thickness at end systolic (IVSs), left ventricular posterior wall thickness at end diastolic (LVPWd), and left ventricular posterior wall thickness at end systolic (LVPWs) were assessed from M-mode images. The ejection fraction (EF) was calculated from the LV end diastolic volume (LVEDV) and LV end-systolic volume (LVESV) using the equation (LVEDV − LVESV)/LVEDV × 100%. Fractional shortening (FS) was calculated using the equation FS = [(LVIDd − LVIDs)/LVIDd] × 100%.

Pathological Staining

The cardiac tissues were collected, fixed in 4% paraformaldehyde, and embedded in paraffin. The sections (5 μm) were stained with hematoxylin and eosin (H&E) for examination by light microscopy (Leica, Germany). Paraffin sections were also used for immunohistochemical analysis of GSDMD (Santa Cruz, cat. no. 393581, 1:200) and IL-1β (Abcam, cat. no. 9722, 1:200) using routine techniques. Immunoreactivity was detected by diaminobenzidine. Cardiac tissue sections were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) according to the manufacturer's operating procedures (Yeasen, Shanghai, China). Images were taken with a fluorescence microscope (Leica, Germany).

Inflammatory Caspase Activity Assay

Caspase-1 and Caspase-11 activities were determined by assay kits (Beyotime, Shanghai, China) according to the manufacturer's protocol. The inflammatory caspase activities were normalized to the protein concentration of the corresponding cell lysate and are expressed as enzymatic units per microgram of protein.

Determination of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) levels were measured by an assay kit (Beyotime, Shanghai, China). Heart tissue and serum were incubated with LDH working reagent to measure LDH levels according to the manufacturer's instructions. Media from H9c2 cells were incubated with LDH working reagent to measure LDH release.
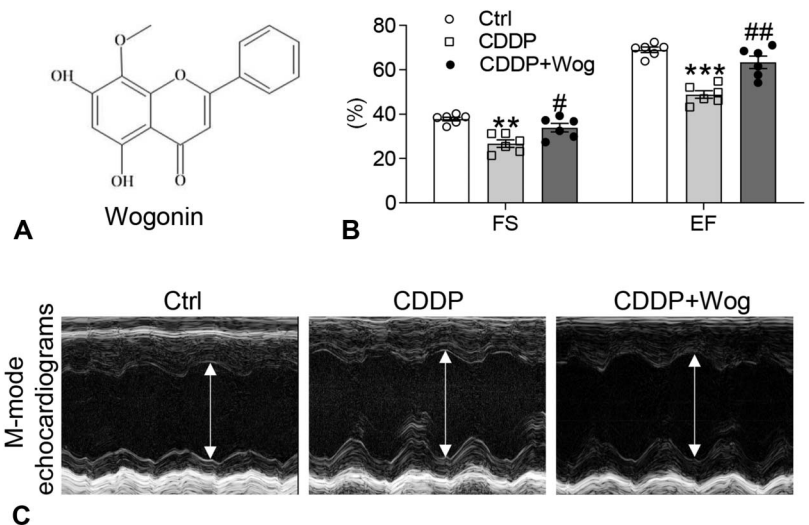
FIGURE 1. Wogonin prevents CDDP-induced cardiac dysfunction in vivo. A, Chemical structure of wogonin. B, Echocardiographic data showing the effects of wogonin on cardiac dysfunction induced by CDDP. C, Representative images of the M-mode of the left ventricle (n = 6 in each group; *, vs. the Ctrl group; #, vs. the CDDP group; #P < 0.05, ** and ##P < 0.01, ***P < 0.001).

TABLE 1. Sequences of Primers for Real-time qPCR Assay Used in the Study

Gene	Species	FW	RW
NLRP3	Mouse	ATTACCCGCCCGAGAAAGG	CATGAGTGTGGCTAGATCCAAG
IL-18	Mouse	CATGTACAAAGACAGTGAAGTAAGAGG	TTTCAGGTGGATCCATTTCC
IL-1β	Mouse	ACTCCTTAGTCCTCGGCCA	CCATCAGAGGCAAGGAGGAA
β-actin	Mouse	CCGTGAAAAGATGACCCAGA	TACGACCAGAGGCATACAG

Determination of Serum Creatine Kinase-muscle/Brain

Serum was collected, and the level of creatine kinase-muscle/brain (CK-MB) was measured by the corresponding kit, which was purchased from Jiangsu Nanjing Jiancheng Co, Ltd (Nanjing, China).

Cell Culture

H9c2 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco, Eggenstein, Germany) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin.

GSDMD overexpression in cells was achieved using GSDMD plasmids (GenePharma Co, Ltd, Shanghai, China). Transfection of H9c2 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Overexpression was verified by Western blotting.

The CCK-8 assay was used to assess the viability of H9c2 cells according to the manufacturer's instructions (Nuoyang Biotechnology Co, Ltd, HangZhou, China).

Determination of Cytokine Levels

IL-18 and IL-1β protein levels in H9c2 cell media were determined using cytokine-specific ELISA kits (eBiosciences Inc, CA).

Western Blotting

The general procedure for Western blotting was described in our previous publication.[18] Antibodies against GSDMD (cat. no. 393581, 1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Caspase-1 (cat.no. 22915-1-AP, 1:1000) were obtained from Proteintech (Rosemont). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (cat. no. 5174, 1:1000) and the horseradish peroxidase-conjugated secondary antibody were obtained from Cell Signalling Technology (Danvers, MA).

Real-Time Quantitative PCR

The general procedure for real-time quantitative polymerase chain reaction (RT-qPCR) was described in our previous publication.[18] Primers for genes including NLRP3, IL-18, IL-1β, and β-actin were synthesized by Invitrogen (Shanghai, China). The primer sequences used are shown in Table 1. Target mRNA was normalized to β-actin.

Statistical Analysis

All data are expressed as the means ± SEM. Statistical analyses were performed using GraphPad Pro Prism 8.0 (GraphPad, San Diego, CA). Student's t test or one-way analysis of variance followed by the multiple comparisons test with Bonferroni correction were used to analyze the differences between sets of data. We used one-way analysis of variance followed by multiple comparisons test with Bonferroni correction when comparing data from more than 2 groups and Student's t test when comparing data from 2 groups. A P value <0.05 was considered significant.

RESULTS

Wogonin Prevents CDDP-induced Cardiac Dysfunction In Vivo

We first investigated the protective effects of wogonin on CDDP-induced cardiac dysfunction by transthoracic echocardiography. Based on a previous study that showed that oral administration of 10 mg/kg wogonin in mice protects against isoprenaline-induced myocardial hypertrophy,[17] we also selected 10 mg/kg for our model. We found that the CDDP-induced depression of EF and FS were prevented in mice treated with wogonin (Fig. 1B). Furthermore, the M-mode echocardiographic results showed that other cardiac functional deficits, such as LVIDd, LVIDs, IVSd, and IVSs, were also reversed in wogonin-treated mice (Fig. 1C and Table 2). These findings indicate that wogonin protects against functional cardiac abnormalities induced by CDDP in vivo.
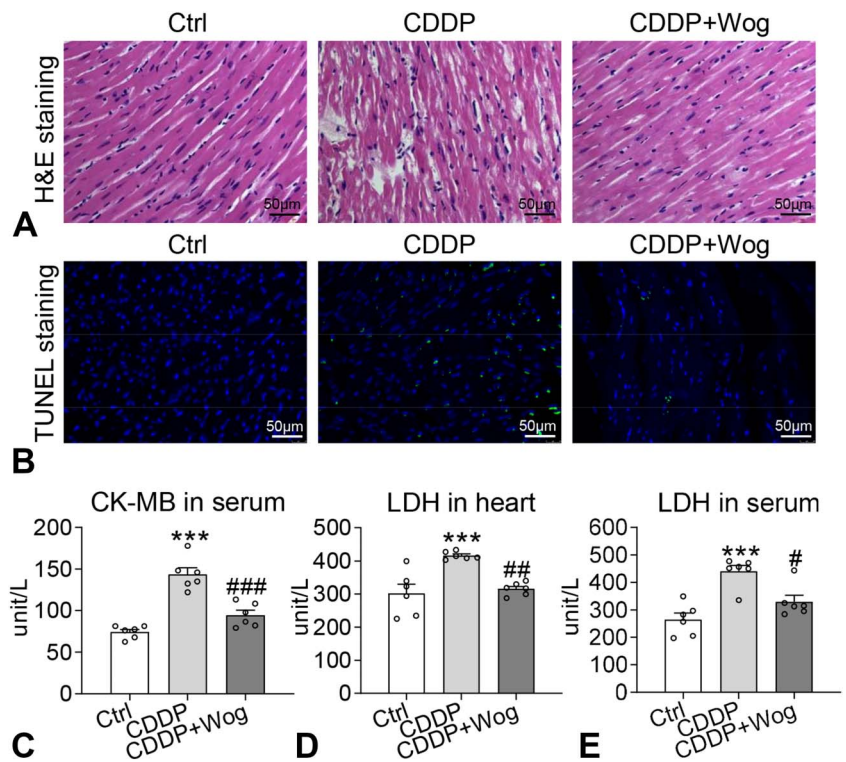
Wogonin Protects Against CDDP-induced Myocardial Injury In Vivo

We then stained mouse heart tissues with H&E to measure myocardial disorder. Our results showed increased myocardial disorder in CDDP-induced mice, but not in wogonin-treated mice (Fig. 2A). Pyroptosis shares some characteristics

TABLE 2. Biometric and Echocardiographic Parameters of CDDP Mice

Parameter[a]	Ctrl	CDDP	CDDP + Wog
IVSd, mm	0.71 ± 0.03	0.84 ± 0.03**	0.73 ± 0.01#
IVSs, mm	1.09 ± 0.02	1.25 ± 0.05*	1.10 ± 0.03#
LVPWd, mm	0.73 ± 0.02	0.84 ± 0.05**	0.76 ± 0.02
LVPWs, mm	1.11 ± 0.03	1.39 ± 0.09	1.18 ± 0.02
LVIDd, mm	3.15 ± 0.04	3.91 ± 0.06***	3.38 ± 0.11##
LVIDs, mm	2.03 ± 0.06	2.79 ± 0.20**	2.28 ± 0.08#

[a]Values shown as mean ± SEM, n = 6 in each group; *, versus Ctrl group; * and #, versus CDDP group; #$P < 0.05$, ** and ##$P < 0.01$, ***$P < 0.001$.

FIGURE 2. Wogonin protects against CDDP-induced myocardial injury in vivo. A, Representative images of H&E staining (×400 magnification). B, Representative images of TUNEL staining (Green: TUNEL-positive cells, DAPI: nucleus; ×400 magnification). C, Levels of serum CK-MB in CDDP-induced mice and mice treated with wogonin. D, LDH levels in the heart tissue of CDDP-induced mice and mice treated with wogonin. E, LDH levels in serum (n = 6 in each group; *, vs. the Ctrl group; #, vs. the CDDP group; ##$P < 0.01$, *** and ###$P < 0.001$).

with apoptosis, such as DNA fragmentation. TUNEL staining of heart tissues from CDDP-induced mice showed increased TUNEL-positive apoptotic cells, indicating increased cell death with nuclear DNA damage (Fig. 2B). However, treatment with wogonin alleviated this abnormality. These results were consistent with serum CK-MB levels, which were significantly elevated in CDDP-induced mice and dampened in the wogonin group (Fig. 2C). CDDP-induced mice also showed increased LDH levels in heart tissue, indicating cell membrane permeability and myocardial injury, and this effect was reversed in wogonin-treated mice (Fig. 2D). Similar trends in serum LDH levels were observed (Fig. 2E). These findings indicate that wogonin prevents CDDP-induced myocardial injury in vivo.
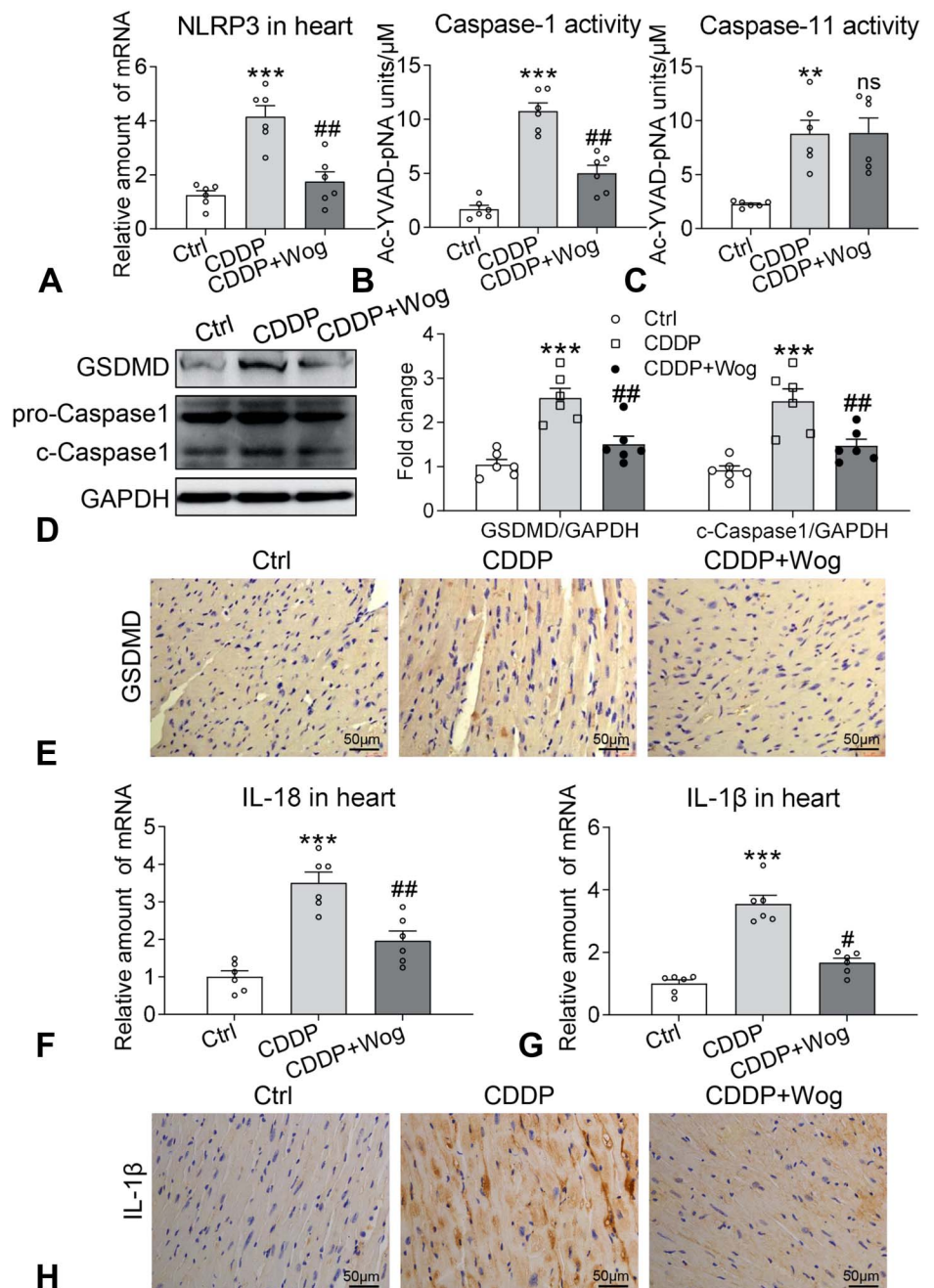
Wogonin Alleviates CDDP-induced Myocardial Pyroptosis In Vivo

Pyroptosis is a proinflammatory form of programmed cell death. CDDP induces NLRP3/Caspase-1/GSDMD signalling and pyroptosis in acute renal injury.[19,20] Caspase-11 promotes CDDP-induced renal tubular apoptosis.[21] We assessed whether pyroptosis was involved in the effects of CDDP and wogonin. CDDP stimulation increased the mRNA level of NLRP3 (Fig. 3A) and the activities of Caspase-1 (Fig. 3B) and Caspase-11 (Fig. 3C), suggesting that pyroptotic inflammasomes and inflammatory caspases were activated in cardiac myocytes exposed to CDDP. Wogonin decreased the induction of NLRP3 (Fig. 3A) and Caspase-1 (Fig. 3B), but it did not inhibit the activity of caspase-11 (Fig. 3C). Recent studies have verified that GSDMD is the executioner of pyroptotic cell death and is

cleaved by caspase-1/4/11.[22,23] The expression of GSDMD and c-Caspase1 was induced in the heart tissue of mice after CDDP stimulation, and wogonin treatment repressed the induction of GSDMD and c-Caspase1 (Fig. 3D). Immunohistochemical analysis of GSDMD in heart tissues was consistent with the Western blot analysis (Fig. 3E). Activated GSDMD assembles pores in the plasma membrane, causing cell swelling and the extensive release of proinflammatory substances such as IL-18 and IL-1β.[23] IL-18 and IL-1β mRNA levels were upregulated in CDDP-induced mice (Figs. 3F–G). Immunohistochemical analysis of IL-1β in heart tissues was consistent with the above results (Fig. 3H). Treatment with wogonin in the CDDP group reduced IL-18 and IL-1β levels. These findings suggest that wogonin reduces CDDP-induced myocardial pyroptosis via the NLRP3/Caspase-1/GSDMD signalling pathway.

The Antipyroptotic Effects of Wogonin Involve Modulating GSDMD

To confirm the involvement of GSDMD in the antipyroptotic effects of wogonin on CDDP-challenged H9c2 cells, we transfected H9c2 cells with GSDMD-expressing plasmids (Fig. 4A). Compared with the negative control, H9c2 cells transfected with specific plasmids exhibited increased protein levels by more than 3-fold. We determined the effect of CDDP on cell viability, and 5 μM CDDP significantly inhibited H9c2 cell proliferation. Thus, we selected 5 μM CDDP for our in vitro study (Fig. 4B). We measured IL-18 and IL-1β levels in the media of H9c2 cells. Both assays showed increased IL-18 and IL-1β levels in CDDP-stimulated H9c2 cells, whereas wogonin treatment

FIGURE 3. Wogonin alleviates CDDP-induced myocardial pyroptosis in vivo. A, mRNA levels of NLRP3 in heart tissues. B and C, Cell lysates were collected and subjected to Caspase-1 or Caspase-11 enzymatic assays. Ac-YVAD-pNA is the substrate of caspase in the assay kit. D, Western blot analysis of caspase1 and GSDMD expression in heart tissues. Densitometric analysis is shown in the right panel. E, Representative immunohistochemical images of GSDMD (brown) in heart tissues. F–G, mRNA levels of IL-18 and IL-1β in the heart tissues of mice were determined by RT-qPCR. H, Representative immunohistochemical images of IL-1β (brown) in heart tissues (n = 6 in each group; *, vs. the Ctrl group; #, vs. the CDDP group; #P < 0.05, ##P < 0.01, ***P < 0.001).

attenuated the release of these cytokines (Figs. 4C, D). However, wogonin pretreatment of cells transfected with GSDMD-expressing plasmids resulted in smaller reductions in the levels of IL-18 (Fig. 4C) and IL-1β (Fig. 4D) than in the CDDP+ wogonin group. LDH release by H9c2 cells was analyzed and confirmed that GSDMD overexpression prevented the antipyroptotic effect of wogonin (Fig. 4E). In summary, the decreased wogonin activity after GSDMD overexpression suggests that wogonin engages GSDMD to exert its antipyroptotic effect on CDDP-stimulated H9c2 cells.

DISCUSSION

This study revealed that wogonin, a flavonoid derivative of *S. baicalensis* Georg, protects against CDDP-induced cardiac dysfunction, myocardial injury, and myocardial pyroptosis in mice. In addition, wogonin engages GSDMD, the key pyroptosis protein, and inhibits NLRP3/Caspase-1/ GSDMD signalling in cardiomyocytes, which may inhibit the process by which GSDMD forms pores in the cell membrane. Furthermore, wogonin suppresses pyroptosis accompanied by the release of IL-18, IL-1β, and LDH and ameliorates the myocardial injury induced by CDDP (Fig. 4F).
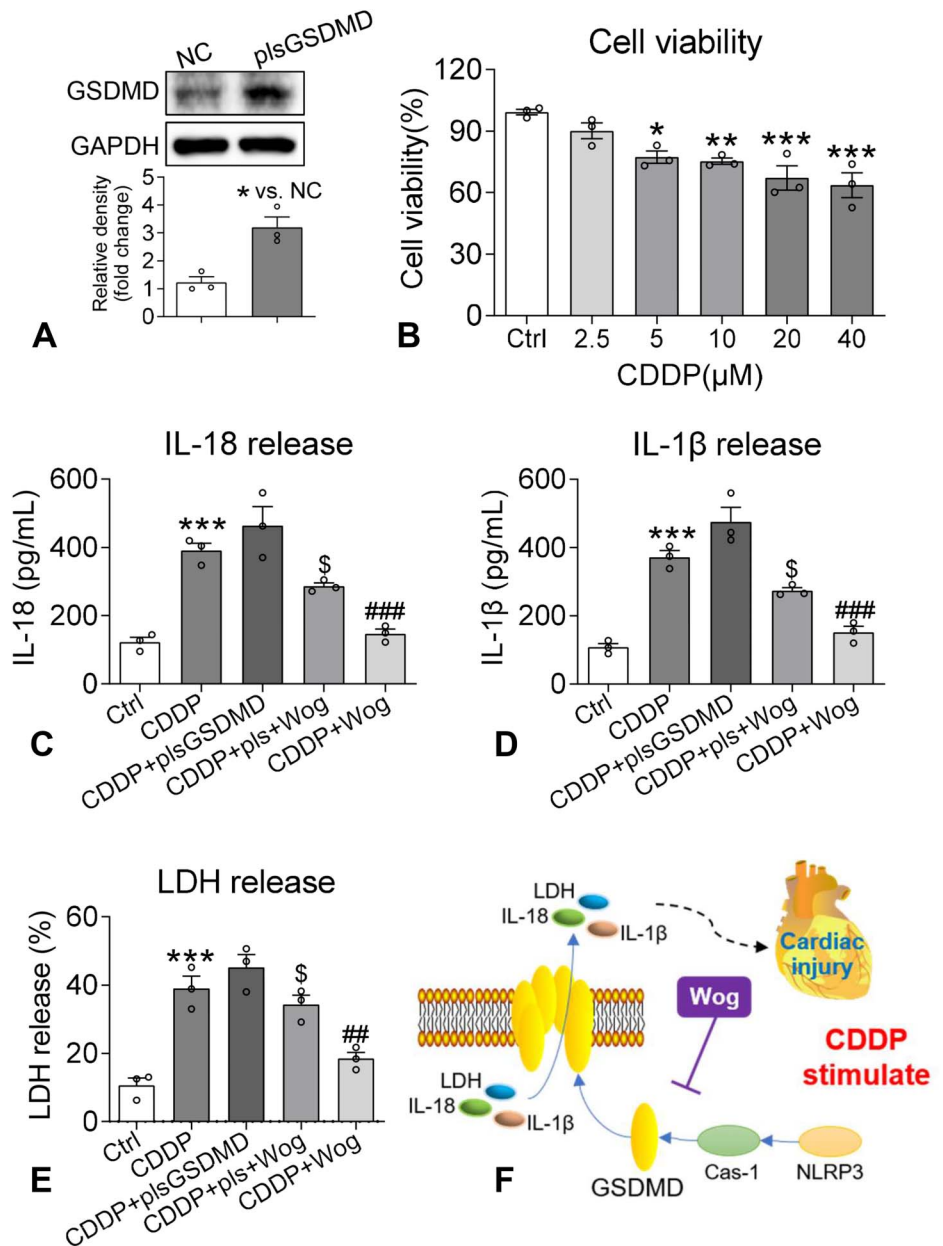
FIGURE 4. The antipyroptotic effects of wogonin involve modulating GSDMD. A, H9c2 cells were transfected with GSDMD-expressing plasmid (plsGSDMD) or empty plasmid (negative control, NC). Western blotting was performed to assess the protein levels of GSDMD. Densitometric analysis is shown in lower panel. B, H9c2 cells were treated with CDDP at the indicated concentrations for 72 hours, and then cell viability was analyzed by CCK-8 assays. H9c2 cells were pretreated with wogonin (20 μM) for 1 hour and then incubated with CDDP (5 μM) for the indicated times. C–D, After exposure to CDDP for 24 hours, IL-18 and IL-1β release from GSDMD-overexpressing H9c2 cells in the presence or absence of wogonin was measured by ELISA. E, After exposure to CDDP for 24 hours, LDH release from GSDMD-overexpressing H9c2 cells in the presence or absence of wogonin was measured by an assay kit. F, Schematic illustration of the role of wogonin in GSDMD-mediated pyroptosis in CDDP-induced myocardial injury (n = 3 in each group; *, vs. the Ctrl group; #, vs. the CDDP group; $, vs. the CDDP + wogonin group; * and $P < 0.05, ** and ##P < 0.01, *** and ###P < 0.001).

CDDP, a platinum-like chemotherapy drug, is widely used to treat human cancers and is accompanied by severe side effects, including cardiotoxicity, nephrotoxicity, and hepatotoxicity. CDDP-induced cardiotoxicity mainly manifests as irreversible cardiomyopathy. Once cardiotoxicity occurs, cardiotoxic events can ultimately result in congestive heart failure and abrupt cardiac death.[2,3] Therefore, it is necessary to identify cardioprotective agents to combat CDDP-induced cardiotoxicity.

Wogonin is a flavonoid that has the potential to inhibit inflammation and exert cardioprotection. It has been reported that wogonin can relieve diabetic cardiomyopathy via its anti-inflammatory properties.[11] In this study, we found that wogonin could reduce CDDP-induced cardiac dysfunction and other cardiac functional deficits, such as LVIDd, LVIDs, IVSd, and

IVSs, in vivo (Figs. 1B, C and Table 2). In addition, wogonin attenuated myocardial disorder and the number of TUNEL-positive apoptotic cells and increased serum CK-MB levels caused by CDDP (Figs. 2A–C). Pyroptosis is a proinflammatory form of programmed cell death that shares some characteristics with apoptosis, including DNA fragmentation, nuclear condensation, caspase dependence, and positive Annexin V staining.[8] TUNEL staining was mainly used to detect DNA fragmentation. These findings indicated that wogonin had a protective effect against CDDP-induced myocardial damage.

The critical underlying mechanisms by which CDDP induces cardiotoxicity include cell apoptosis, necrosis, and inflammation.[2–4] Many proinflammatory factors are involved in CDDP-induced cardiotoxicity, such as IL-1β.[9] Notably,

alleviating the inflammatory response protects cardiomyocytes against cardiac injury caused by CDDP.[3–5] Pyroptosis is a proinflammatory form of programmed cell death that is different from apoptosis and necroptosis. GSDMD is the key executioner of pyroptotic cell death and controls the release of proinflammatory cytokines such as IL-1β.[6–8] Thus, we hypothesized that CDDP treatment could induce cardiomyopathy through pyroptosis via the key protein GSDMD. Here, we demonstrated that CDDP-induced myocardial injury increased the expression of GSDMD (Figs. 3D, E) and promoted LDH (Figs. 2D, E), IL-18 (Fig. 3F), and IL-1β (Figs. 3G, H) release in vivo, indicating that targeting GSDMD-mediated pyroptosis could be a therapeutic strategy to protect the myocardium against CDDP-induced injury. Furthermore, wogonin treatment reduced the expression of GSDMD-N and the release of LDH, IL-18, and IL-1β. Pyroptosis is controlled by inflammatory caspases, such as caspase1 and caspase11. CDDP induces NLRP3/Caspase-1/GSDMD signalling and pyroptosis in acute renal injury.[19,20] Caspase-11 promotes CDDP-induced renal tubular apoptosis.[21] CDDP treatment increased the mRNA level of NLRP3 (Fig. 3A) and the activities of Caspase1 (Fig. 3B) and Caspase11 (Fig. 3C), suggesting that pyroptotic inflammasomes and inflammatory caspases were activated in cardiomyocytes exposed to CDDP.

GSDMD is the key executioner of pyroptotic cell death.[22,23] Here, to further determine the role of GSDMD in the anti-pyroptotic effects of wogonin, GSDMD-expressing plasmids were transfected into H9c2 cells (Fig. 4A). Wogonin was not able to inhibit the levels of IL-18 (Fig. 4C), IL-1β (Fig. 4D), and LDH (Fig. 4E) in cells transfected with GSDMD-expressing plasmids compared with those in the CDDP + wogonin group. These results suggested that wogonin could inhibit pyroptosis induced by CDDP by blocking the GSDMD protein.

In the present study, we have shown that wogonin, a natural compound, attenuates cardiac injury induced by CDDP by inhibiting GSDMD-mediated pyroptosis. Moreover, our results clearly indicate that targeting GSDMD might be a new strategy for treating myocardial damage induced by CDDP. These findings provide support for the potential use and future research of wogonin for improving cardiac dysfunction and treating chemotherapy-induced cardiotoxicity.

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