Identifying the Chemical Profile and Pharmacological Mechanism of Xinbao Pill against Myocardial Ischemia-Reperfusion Injury Using Ultra-Fast Liquid Chromatography-Quadrupole-Time-Of-Flight Tandem Mass Spectrometry Coupled with Network Pharmacology-Based Investigation

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Research

Keywords: Myocardial ischemia-reperfusion injury, Xinbao pill, chemical profile, network pharmacology, autophagy, ER stress

Posted Date: June 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-600431/v1>

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Abstract

Background: Xinbao Pill (Xin-Bao-Wan, XBW) is a traditional Chinese herbal formula, which is widely used in clinical treatment for cardiovascular diseases; however, the therapeutic effect of XBW on myocardial ischemia-reperfusion injury (MI/RI) is unclear. In this study, we evaluated the cardioprotective effect and molecular mechanism of XBW against MI/RI.

Methods: A phytochemistry-based network pharmacology analysis was used to uncover the mechanism of XBW against MI/RI. Firstly, ultraperformance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) method was used to identify chemicals in XBW. MI/RI-related targets of XBW were predicted using TargetNet database, OMIC database and reported literatures. GO and KEGG pathway enrichment analyses were performed using String database. Left anterior descending artery (LAD) ligation-induced MI/RI rat model and oxygen glucose deprivation-reperfusion (OGD/R)-induced H9c2 cell model were used to evaluate the cardioprotective effect and potential mechanism of XBW.

Results: 37 chemicals were identified in XBW by using UHPLC-QTOF-MS; 50 MI/RI-related targets of XBW were predicted using TargetNet database, OMIC database and reported literatures. *In vivo* study, XBW (180 mg/kg) significantly reduced myocardial infarct size and creatine kinase MB (CK-MB) level induced by MI/RI in rat model; *in vitro* study, XBW protected H9c2 cells against OGD/R injury in a dose-dependent manner. GO and KEGG pathway enrichment analyses showed that the cardioprotective effect of XBW was associated with 5 significant pathways including autophagy, apoptosis, HIF-1 signaling pathway, PI3K-Akt signaling pathway, FoxO signaling pathway. Experimental investigation also verified that XBW could suppress apoptosis, autophagy and endoplasmic reticulum (ER) stress.

Conclusion: XBW showed therapeutic effects against MI/RI mainly via attenuating apoptosis though suppressing excessive autophagy and ER stress.

Background

Myocardial ischemia-reperfusion injury (MI/RI) is a difficult problem after percutaneous coronary intervention (PCI) or thrombolytic therapy, which seriously affect the patients' quality of life. It is reported that about 10% patients with myocardial infarction die from lethal reperfusion injury (1). Current mechanism studies show that MI/RI is associated with oxidative stress, inflammation, cardiomyocyte apoptosis, calcium overload or complement activation (2). Various pharmacological agents were developed to reduced MI/RI based on them, but the effect is not ideal. It still lacks in effective and safe approaches for preventing MI/RI (3), which force us to explore some promising therapies.

Xinbao Pill (Xin-Bao-Wan, XBW) is a traditional Chinese herbal formula developed by Minghan Weng, a researcher from Guangdong Institute of Materia Medica. It is consists of *Datura metel* L. (Yangjinhua), *Cornu cervi pantotrichum* (CCP, Lurong), *Aconitum carmichaelii Debeaux* (Fuzi), *Panax ginseng* C.A.Mey., *Panax notoginseng* (Burkill) F.H.Chen., *Cinnamomum cassia* (L.) J.Presl (Rougui), moschus (Shexiang),

Borneolum syntheticum (Bingpian) and Chinese toad (*Bufo gargarizans*, Chansu). Xinbao pill has the effects of warming and tonifying heart and kidney; replenishing Qi and assisting Yang; promoting blood circulation to remove obstructions from meridians. In TCM clinic, Xinbao pill is used to treat chronic cardiac insufficiency caused by heart and kidney Yang deficiency; heart pulse stasis, bradycardia and sinus syndrome caused by sinus insufficiency; angina pectoris caused by ischemic heart disease and ischemic changes of electrocardiogram (4). Pharmacological studies showed that XBW and its components have a definite cardioprotection. For example, XBW suppressed cardiac hypertrophy via regulation of PI3K/Akt/GSK3 β signaling pathway (4). XBW also attenuated chronic heart failure in a rat model (5, 6). Ginsenoside Rg1, Rb1 (7), Rg3 (8), Rd (9) significantly reduced myocardial infarct size and improved cardiac function in I/R injured model through suppressing oxidative stress, apoptosis and inflammation. Notoginsenoside R1 pretreatment ameliorated myocardial injury induced by I/R via inhibiting ROCK and promoting mitochondrial ATP synthase δ -subunits (10). However, as a Chinese traditional formula, only few studies have illustrated the cardioprotection of XBW against MI/RI. Therefore, it is meaningful to clarify the underlying mechanism of XBW against MIRI.

In this study, we first used the ultra-fast liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) to identify the chemical profile of XBW, and used the database to find out the predicted target of the chemicals, then built a compound-target-disease network. Finally, we systematically investigated the cardioprotective effect of XBW against MI/RI *in vitro* and *in vivo* model and verified predicted pathway by pharmacological assays (Fig. 1).

Material And Methods

Drugs and reagents

Xinbao Pills were supplied by Guangdong Xinbao Pharm-tech Co., Ltd (Guangzhou, China). Rabbit anti-Bcl2 antibody (1:1000; Cat. No. #A00040-1) and rabbit anti-Bax antibody (1:1000; Cat. No. #BM3964) were purchased from Boster Biological Technology co. Ltd (CA, USA). Rabbit anti-Beclin-1 (1:1000; Cat. No. #3495), rabbit anti-LC3II antibody (1:1000; Cat. No. #2775), rabbit anti-Caspase-3 antibody (1:1000; Cat. No. #9662), goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000, Cat. No. #7074 or #7076) were from Cell Signaling Technology (Boston, MA, USA). Rabbit anti- β -tubulin antibody (1:1000, Cat. No. #bs0210R) was bought from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Mouse anti-GAPDH antibody (1:1000; Cat. No. #GB12002) and mouse anti- β -actin antibody (1:1000, Cat. No. #GB12001) were purchased from was from Servicebio (Wuhan, China). Diazoxide (Cat. No. #D9035), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat. No. #M5655) and triphenyltetrazolium chloride (TTC, Cat. No. #T8877) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Ultraperformanc liquid chromatography- Quadrupole-Time-of-Flight Tandem Mass Spectrometry (UPLC-Q-TOF-MS) analysis

Xinbao pills were firstly broken into powder. A total of 1.0 g of powder was weighted and extracted with 30 mL methanol solution by ultrasonic extraction for 30 min twice. Extracted solutions were combined and concentrated. After that, it was prepared into 10 mg/mL stock solution and filtered by 0.45 µm micropore fil for analysis. UPLC-Q-TOF-MS analysis were performed with Waters Acquity UPLC I-class and a Xevo G2-S Q ToF time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA). Waters BEH C18 column (2.1 x 100 mm, 1.7 µm) was used for separation. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The gradient was used as follows: 0.00-0.5 min, 5% B; 0.5-1.0 min, 5%-15% B; 1.0-4.0 min, 15%-25% B; 4.0-6.0 min, 25%-35% B; 6.0-8.0 min, 35%-45% B; 8.0-9.0 min, 45%-75% B; 9.0-11.0 min, 75%-90% B; 11.0-13.0 min, 90%-95% B; 13.0-15.0 min, 95% B; 15.0-15.1 min, 95%-5% B; 15.1-17.0 min, 5% B. The column oven was set at 45°C, the flow rate was 0.4 mL/min and the injection volume was 0.5 µL. Optimal MS parameters were set as follows: the ion source temperature (120°C); the capillary voltage (2.0 kV); the cone voltage (40 V); the desolvation gas temperature (400°C); the desolvation gas flow (800 L/hr). Masslynx 4.1 software was used to analyse the data and the Waters UNIFI Scientific Information System was used to process the structure of the chemical compositions.

Cells and treatment

Rat cardiomyocyte H9c2 cells were purchased from the American Type Culture Collection (ATCC, USA). H9c2 cells were cultured in DMEM including 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C, 5% CO₂. For treatment, H9c2 cells were treated with indicated drugs (XBW (0, 10, 60, 240, 720 µg/mL), diazoxide (100 µM)) and then subjected to OGD condition (After medium was washed by PBS and replaced with DMEM without glucose and FBS, cells were put into a chamber saturated with 95% N₂ and 5% CO₂) for 6 h and reperfusion for another 18 h. For drug preparation, powdered XBW was extracted with ethanol by ultrasonic extraction for 30 min twice. After concentration, extracts were dissolved in DMEM containing 0.05% ethanol and filtered by 0.22 µm to prepare different doses given drugs.

Cell Viability

The cell viability was measured by MTT assay. In brief, after drug treatment, 0.5 mg/mL MTT solution was added to 96-well plate and incubated at 37 °C for 4 h. When removal of the supernatant, 150 µL of DMSO was used to dissolve the formazan products. The absorption was determined by a microplate reader at 490 nm (Thermo Fisher Scientific, Waltham, CA, USA).

Western blot analysis

Briefly, H9c2 cells and cardiac tissue were extracted by 1 X RIPA buffer (Solarbio Life Sciences, Beijing, China) containing protease and phosphatase inhibitors. The proteins were loaded to 10% SDS-polyacrylamide gels for electrophoresis and transferred to a polyvinylidenedifluoride (PVDF) membrane. After blotting in 5% nonfat milk for 1.0 h, the membranes were incubated with specific primary antibodies (Bcl2, Caspase-3, Bax, Baclin-1, LC3II, BIP, GAPDH, β-tubulin, β-actin) overnight and goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT. After

washing with 1 X TBST, the bands were detected with enhanced chemiluminescence (ECL) detection reagents from Absin Bioscience Inc (Shanghai, China).

Animal experiments

The experimental procedures and protocols were approved by the Committee on Ethical USE of Animals of Guangzhou University of Chinese Medicine (No.IITCM-20180306). SD male rats (weighting 250–280 g) were obtained from animal laboratory center of Southern Medical University and divided into three groups: Sham group (n = 6), MI/R model group (MI/R + Saline, n = 6), XBW group (MI/R + XBW, n = 6). In brief, SD rats were anesthetized by intraperitoneal injection of 40 mg/kg 2% pentobarbital sodium. After anesthesia, rats were fixed and plugged into a ventilator. After opening the chest between the 3rd and 4th rib, the left anterior descending (LAD) artery was ligated with a 6 – 0 silk suture and PE10 tube for 30 min, and then reperfusion for 24 h. Sham group only received left thoracotomy without ligation. The MI/R + XBW group received 180 mg/kg XBW by intragastric administration at 1 h before MI/R. Powdered XBW was dissolved in 0.9% saline, and prepared the given solution according to the weight of rats. After 24 h of reperfusion, heart tissues were cut into 2 mm sections, and incubated in 1% TTC solution at 37 °C for 15 min, then fixed in 4% PFA overnight. The images of these sections were captured, and the infarct size was analyzed and calculated by image J.

H&E staining

Hearts were fixed in the 4% PFA solution, dehydrated by gradient alcohol, and embedded with paraffin. 5 µm of paraffin slices were rehydrated by gradient alcohol, and stained with hematoxylin and eosin. Then, the slices were washed with water, dehydrated by 80%, 90% and 100% ethanol, and sealed with neutral gum. Finally, the slices were observed and photographed under light microscope.

Predicting targets of compounds in XBW

The mol2 format files of UPLC-Q-TOF-MS-identified chemicals were downloaded from Pubchem database and uploaded to TargetNet webserver (<http://targetnet.scbdd.com>). In practice, predict protein targets with the score of > 0.90 were selected. In addition, the targets of chemicals reported in literatures were also collected.

Collecting MI/RI-associated targets

The targets related to MI/RI were selected from OMIM database (<https://omim.org/>) and the literatures using “myocardial ischemia-reperfusion injury” as the keywords.

Gene Ontology (GO) and pathway enrichment of potential targets

The Gene Ontology (GO) biological process (BP), molecular function (MF) and cellular component (CC), Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway were analyzed by using the String Database (<https://string-db.org/cgi/input.pl>). Only the false discovery rate (FDR) ≤ 0.05 were selected.

Statistics analysis

The results were expressed as means $\pm$ SD or means $\pm$ SEM from no less than three independent experiments. Statistical analysis was performed by one-way ANOVA with GraphPad Prism software (La Jolla, CA, USA). And A p -value < 0.05 was considered as statistical significance.

Results

Chemical Profile of XBW by UPLC-QTOF-MS

A specific UPLC-QTOF-MS method was used to identify the chemicals in XBW. As shown in Figure 2 and Table 1, a total of 37 compounds were identified by comparing their retention time with that of reference compounds or comparing their retention behaviors, empirical molecular formula and proposed fragmentations with that in literatures (11-25), including ginsenoside Rg1-3, Rb1-3, Re, Ro, gypenoside XVII, aconine, mesaconine, isotalatizidine, scopolamine, arenobufagin, *ect*. The chemical components were derived from Fuzi, *Panax ginseng*, *Panax notoginseng*, *Flos Daturae*, CCP, and toad (Chansu) in XBW.

Effect of XBW against myocardial ischemia-reperfusion injury in rat model *in vivo* and oxygen glucose deprivation-reperfusion (OGD/R) cell model *in vitro*

LAD ligation-induced MI/RI rat model was used to investigate the cardioprotective effect of XBW. As shown in Figure 3A and 3B, XBW administration significantly reduced infarct size. CK-MB is one of biomarker in serum for MI/RI, and XBW administration decreased the level of CK-MB induced by MI/RI (Figure 3C). Echocardiography in Figure 3D exhibited that XBW improved cardiac function. H&E staining showed that XBW administration attenuated inflammatory cell infiltration, and disordered myocardial fiber induced by MI/RI (Figure 3E). *In vitro* study, XBW protected H9c2 cell against OGD/R injury in a dose-dependent manner (Figure 4A-B). These results suggested that XBW ameliorated MI/RI.

Target Identification and Network Analysis

Using TargetNet database and literatures reported targets, we obtained 246 targets of 37 compounds in XBW (Figure 5A). The network contained 283 nodes and 462 edges, and its average number of neighbors was 3.216. After crossing with MI/RI targets, 50 targets were identified and a compound-target network was constructed in Figure 5B and 5C.

After GO enrichment analysis of the targets by the String database, the top 15 enrichment results listed in biological processes (BP), molecular functions (MF) and cellular components (CC) are shown in Figure 6A, which indicated that XBW may regulate the apoptosis and stress response of cardiomyocytes via protein binding, enzyme binding, transcription factor binding, protein kinase binding, extracellular space, CHOP-ATF4 complex, *etc* to attenuate MI/RI. To clarify the underlying pathways of XBW on MI/RI, KEGG pathway analysis was performed in Figure 6B, which exhibited the top 20 related signaling pathways excluding the specific cancer related pathways. HIF-1 signaling pathway, PI3K-Akt signaling pathway, autophagy, FoxO signaling pathway, apoptosis, *etc*. Based on the protein-protein interactions (PPI)

analysis (Figure 6C), CASP3, MTOR (apamycin), SIRT1, HIF1A, ATF4, GRP78 (BIP, glucose regulated protein 78) and ATG7 were identified with high-degree targets, which played important roles in apoptosis, autophagy and endoplasmic reticulum (ER) stress.

Experimental validations of the molecular mechanisms of XBW against MIRI.

Cardiomyocyte apoptosis is a key factor in the pathological process of MI/RI. As shown in Figure 7A, after 24 h reperfusion, TUNEL staining showed the percentage of apoptosis-positive cells in MI/RI group increased, while decreased in XBW treatment group. In addition, the apoptosis related proteins were detected. XBW increased the ratio of Bcl2/Bax expression and decreased caspase 3 expression (Figure 7B-D).

During MI/RI, autophagy was also activated following MI/RI in cardiomyocytes to involve in the apoptosis of myocardial cells (26, 27). Bcln-1 and LC3II expressions were detected (Figure 8A-C). The expressions of Bcln-1 and LC3II in cardiac tissue were markedly increased in MI/RI group, while significantly decreased in XBW administration group. *In vitro* study, XBW could also decrease Beclin-1 expression induced by OGD/R in H9c2 cells (Figure 8D and 8E). Cardiomyocytes open the unfolded protein response triggered by ER stress as a defensive mechanism at early stage of MI/RI, however, excessive ER stress induced cell apoptosis or even death. As shown in Figure 8F and 8G, the expression of BIP, a marker of ER stress, was significantly increased in MI/RI group compared with that in the Sham group, however, XBW administration reduced the elevation of BIP expression. Taken together, these data demonstrated that the cardioprotective effect of XBW against MI/RI was associated with the attenuation of ER stress and autophagy.

Discussion

Myocardial ischemia-reperfusion injury is a difficult clinical problem myocardial infarction therapy, however, current medications for treating MIRI are not ideal (2). The traditional Chinese medicine exhibits unique advantages in the treatment of cardiovascular diseases, based on multiple components and multiple targets (28). XBW is a patented traditional Chinese herbal formula, which has been listed in China for more than 30 years. It is used for treating ischemic heart disease and chronic heart failure (4). However, there is a lack of evidences for the material basis and underlying mechanism of XBW against MI/RI. In the current study, we integrated chemical profile, network pharmacology, pharmacology and molecular cell biology to investigate the cardioprotective effect and mechanism of XBW against MI/IR.

XBW has been used to treating coronary heart disease and chronic heart failure (5). In our study, an *in vivo* MI/RI rat model was used by performing LAD. The results showed that XBW administration remarkably decreased MI/RI-induced myocardial infarct size and improved cardiac LV function. Moreover, the *in vitro* results revealed that XBW could also reduce OGD/R-induced cell injury. XBW is composed of nine Chinese medicine. We used UPLC-Q-TOF-MS/MS method to identify 37 chemical constitutes in Table 1, which provided the information of material basis. Importantly, most of these components were from *Panax ginseng* C.A.Mey and Fuzi, which are monarch drugs in XBW. For example, *Panax ginseng*

has the effects of invigorating Qi, promoting tissue regeneration and enhancing human body resistances (29). Fuzi possesses the effects of causing restoration from collapse, reinforcing fire and Yang, and is used to treat acute myocardial infarction and chronic heart failure (30). Consistently, reports have also showed that *Panax ginseng*, ginsenoside Rg3 and notoginsenoside R1 can alleviate MI/RI by suppressing oxidative stress, apoptosis, inflammation and regulating myocardial energy metabolism (8, 10, 31). Fuzi and its alkaloids can improve inotropic effect, left ventricular diastolic function (32), and energy metabolism (33), scavenge hydroxyl radicals and suppress lipid peroxidation to show the cardioprotective effects (34). Moreover, network pharmacological analysis of XBW identified that ginsenoside Rg3, Rg1, Rb3, arenobufagin, and notoginsenoside R1 had high degrees, which may be the active compounds of XBW. Thus, the components of XBW are complicated and their pharmacological effects need to be verified in the future work.

After screening with MI/RI-related proteins, 50 putative targets of XBW were collected. Among them, CASP3 (caspase 3) activation is a biochemical hallmark of apoptosis (35); BCL2 (B cell lymphoma-2) plays an important role in the negative regulation of apoptosis (36); GRP78 (BIP) is an ER homeostasis marker and upregulated during MI/IR (37); Eukaryotic initiation factor 2 alpha (eIF2 α) and activating transcription factor-4 (ATF4) can mediate myocardial ER stress (38). mTOR and Beclin-1 are two key autophagy-related proteins in MI/R injury (39). The results illustrated that XBW might regulate above proteins to show cardioprotective effect. GO enrichment analysis showed that XBW can treating MI/RI by regulation of cell death, apoptosis process, and response to stress. KEGG enrichment analysis demonstrated that apoptosis, autophagy, HIF-1 signaling pathway, PI3K/Akt signaling pathway, and FoxO signaling pathway were involved in XBW for treating MI/RI. To further validate the prediction and analysis, we investigated the key potential mechanism of XBW against MI/RI *in vitro* and *in vivo*.

Reducing cardiomyocyte death and infarct size is necessary to MI/RI. Myocardial apoptosis is a key factor for the most of cell death during cardiac pathological processes of MI/RI, while blocking the apoptosis-related signaling pathways helps prevent myocardial injury (40, 41). Anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are involved in the stage of apoptosis. In the present study, XBW suppressed myocardial apoptosis with the decreased TUNEL positive cells and the increased ratio of Bcl2/Bax expression. Therefore, XBW has the potential effects for attenuating myocardial apoptosis for the patients with MI/RI.

An emerging number of evidences have indicated that ER stress is involved in the development and pathogenesis of MI/RI (42). During MI/RI, the balance of the homeostasis for the endoplasmic reticulum is broken, subsequently unfolded or misfolded proteins are accumulating in myocardial cells, and eventually triggering ER stress (43). At the early stage, a certain degree of ER stress helps self-repair injured cells, however, if ER stress is excessive, it will provoke the apoptotic signaling pathway activation (44). GRP78 (BIP) is a calcium ion-binding molecular chaperone in the endoplasmic reticulum. When undergoing ER stress, GRP78 and endoplasmic reticulum cross to activate the downstream CHOP-associated apoptotic signaling pathways (45). In our study, XBW treatment decreased the expression of BIP induced by MI/RI *in vivo* or OGD/R injury *in vitro*. Autophagy has a dual function in MI/RI (46).

Several studies showed that reduction of autophagy clearance in myocardial cells during MI/RI threatens cell survival (47, 48). Promoting autophagy moderately may protect cell and mitochondrial injury in MI/RI (26, 27). However, at the late stage of MI/RI, it induces excessive activation of autophagy, resulting in cytotoxic cell death (49–51). Thus, prevention of excessive autophagy activated during MI/RI may be benefit to reduce cardiomyocyte death and improve cardiac function. In present study, XBW treatment inhibited MI/RI-induced beclin-1 and LC3II expressions to inactivate excessive autophagy. These results fully demonstrated that XBW protected heart against MI/RI through the multicomponent, multitarget and multipathway. The pathogenesis of MI/RI is complex, and XBW has potential clinical application value for the prevention and treatment of multiple pathways.

However, there are still several limitations in our study to solve in the future work. By using UPLC-Q-TOF-MS/MS method, 37 major compounds from six medicinal materials, but the volatile constituents in the left three ones including *Cinnamomum cassia* (L.) J.Presl (Rougui), *moschus* (Shexiang) and *Borneolum syntheticum* (Bingpian) had not been detected, which needs QC-MS analysis in our later work to rich material basis of XBW. In addition, the present study only evaluated the overall efficacy and mechanism of XBW, but the effects and underlying mechanism of the identified active compounds, such as ginsenoside Rg3, Rg1, Rb3, etc, have to be further verified.

Conclusion

In the present study, we revealed the therapeutic effect and underlying mechanism of XBW against MI/IR based on chemical profile, network pharmacology and experimental support. 37 chemical constituents in XBW were identified, 50 potential MI/RI targets, and 5 significant pathways were achieved by network pharmacology analysis. Collectively, our results demonstrated that XBW ameliorated the apoptosis of cardiomyocytes in MI/RI by suppressing autophagy and ER stress (Fig. 9).

Abbreviations

ATF4, Activating transcription factor-4; BCL2, B cell lymphoma-2; BP, Biological process; CASP3, Caspase 3; CC, Cellular component; CK-MB, Creatine kinase MB; ECL, Enhanced chemiluminescence; eIF2 α , Eukaryotic initiation factor 2 alpha; ER, Endoplasmic reticulum; FBS, Fetal bovine serum; FDR, False discovery rate; GO, Gene Ontology; HRP, Horseradish peroxidase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAD, Left anterior descending; MI/RI, Myocardial ischemia-reperfusion injury ; MF, Molecular function; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCI, Percutaneous coronary intervention; PPI, Protein-protein interactions; PS, Penicillin/streptomycin; PVDF, Polyvinylidenedifluoride; TTC, Triphenyltetrazolium chloride; UHPLC-QTOF-MS, Ultraperformance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; XBW, Xinbao Pill

Declarations

Acknowledgements

The authors are grateful for the support from Molecular Pharmacology Laboratory in School of Pharmaceutical Sciences, GZUCM.

Authors' contributions

YC and ZL designed the experiments, conducted and revised the manuscript. YY, TC, JL and SC performed the experiments and wrote the manuscript. RC, LW, JH, QL and XQ collected and analyzed partially the data. All authors reviewed and revised the manuscript.

Funding

This work was supported by Key-Area Research and Development Program of Guangdong Province (No. 2020B1111100004), Science and Technology Program of Guangzhou (No. 2021-02-01-11-3016-0014), the National Natural Science Foundation of China (No. 81803765, 82074053), the Natural Science Foundation of Guangdong Province (No. 1914050000567), the Special Innovation Projects of Universities in Guangdong Province (No. 2019KTSCX027), Guangdong Key Laboratory for translational Cancer research of Chinese Medicine (No. 2018B030322011), State Commission of Science Technology of China (No.2017YFE0119900).

Conflict of interests

TC, RC, LW, QL and XQ were employed by Guangdong Xinbao Pharm-tech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

All datasets presented in this work are included in the article.

Ethics approval and consent to participate

The animal study was approved by the Committee on Ethical USE of Animals of Guangzhou University of Chinese Medicine (No.IITCM-20180306).

Consent for publication

All authors have read the manuscript and consent for publication.

Competing interests

TC, RC, LW, QL and XQ were employed by Guangdong Xinbao Pharm-tech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Due to technical limitations, Table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Flowchart showing the network pharmacological and experimental studies for the investigation of the cardioprotection of Xinbao pill against myocardial ischemia-reperfusion

Figure 2

Identification of chemical constituents from XBW extracts (A) positive ionization mode; (B) negative ionization mode.

Figure 3

XBW administration attenuated MI/RI in LAD-induced rat model. (A) Representative images of TTC staining for the myocardial infarct size. (B) XBW decreased myocardial infarct size. $n = 6$, $*p < 0.05$, MI/R group vs MI/R +XBW group. (C) XBW reduced serum CK-MB level in rats. $n = 6$, CK-MB level in serum was measured by ELISA. Results were expressed as the mean $\pm$ SEM. $##p < 0.01$, MI/R group vs Sham group; $*p < 0.05$, MI/R group vs MI/R +XBW group. (D) Representative images of M-mode echocardiography from each group. (E) Effects of XBW on cell morphology and HE staining.

Image not available with this version

Figure 4

XBW protected H9c2 cells against OGD/R injury. (A) The cytotoxicity of XBW on H9c2 cells. H9c2 cells were treated with indicated doses of XBW (0, 4, 8, 16, 32, 64, 128, 256, 512, 1024 $\mu\text{g/mL}$) for 48 h and determined by the MTT assay. (B) The effects of XBW on OGD/R-induced H9c2 cells. H9c2 cells were treated with indicated doses of XBW (0, 10, 60, 240, 720 $\mu\text{g/mL}$) and diazoxide (100 μM), and followed by 6 h ODG condition and 18 h reperfusion. Cell viability was measured by the MTT assay. Results were expressed as mean $\pm$ standard SD, n = 4. ### p<0.001, OGD/R group vs Ctrl group; *p <0.05, XBW group vs OGD/R group.

A

B

Figure 5

Network of compound and disease targets. (A) Compound-Target network. Network of 37 compounds from XBW and 246 putative targets. (B) The common targets of compound targets and MI/RI targets.

Figure 7

XBW suppressed myocardial apoptosis in rat with MI/R. (A) TUNEL staining for apoptosis cells. (B-D) The effect of XBW on apoptosis-related protein expressions. All results are expressed as the mean $\pm$ SEM. $n = 5\sim 6$, # $p < 0.05$, Sham vs MI/R group; * $p < 0.05$, MI/R+XBW vs MI/R group.

A

B

C

D

E

F

G

Figure 8

XBW prevented excessive autophagy and ER stress under MI/RI in vivo and in vitro models. (A-C) The expressions of Beclin-1 and LC3II in heart tissue. After MI/RI, the heart tissues in each group were collected and subjected to Western blotting analysis. $n = 5$, ## $p < 0.01$, ### $p < 0.001$, Sham vs MI/R group; * $P < 0.05$, ** $P < 0.001$, MI/R+XBW vs MI/R group. (D and E) The expression of Beclin-1 in H9c2 cells. H9c2 cells were treated with indicated doses of XBW (0, 30, 60, 240 μg/mL), and followed by 6 h ODG condition

and 18 h reperfusion. Protein of H9c2 cells were isolated and subjected to Western blotting. n = 3, ## p<0.01, ### p<0.001, Ctrl vs OGD/R; *p<0.05,**p<0.001, OGD/R+XBW vs OGD/R. (F and G) The expression of BIP in heart tissue. After MI/RI, the heart tissues in each group were collected and subjected to Western blotting analysis. n = 5, ## p<0.01, Sham vs MI/R group; *P<0.05, MI/R+XBW vs MI/R group.

Figure 9

Overview of potential mechanism of XBW against myocardial ischemia-reperfusion injury.

Supplementary Files

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- [Table1.docx](#)