Integrated chemical profiling, network pharmacology and pharmacological evaluation to explore the potential mechanism of Xinbao pill against myocardial ischaemia–reperfusion injury

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ABSTRACT
Context: Xinbao pill (XBW), a traditional Chinese herbal formula, is widely used in clinical treatment for cardiovascular diseases; however, the therapeutic effect of XBW on myocardial ischaemia–reperfusion injury (MI/RI) is unclear.
Objective: This study evaluates the cardioprotective effect and molecular mechanism of XBW against MI/RI.
Materials and methods: A phytochemistry-based network pharmacology analysis was used to uncover the mechanism of XBW against MI/RI. Ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry method was used to identify chemicals. MI/RI-related targets of XBW were predicted using TargetNet database, OMIC database, etc. Sprague-Dawley (SD) rats under anterior descending artery ligation model were divided into Sham, MI/RI and XBW (180 mg/kg, intragastric administration). After 30 min ischaemia and 24 h reperfusion, heart tissues were collected for measurement of myocardial infarct size. After oxygen glucose deprivation for 6 h, H9c2 cells were treated with XBW (60, 240 and 720 μg/mL) and diazoxide (100 μM) for 18 h of reperfusion.
Results: Thirty-seven chemicals were identified in XBW; 50 MI/RI-related targets of XBW were predicted using indicated databases. XBW significantly reduced infarct size and creatine kinase MB (CK-MB) level after MI/RI; XBW protected H9c2 cells against OGD/R injury. Gene ontology (GO) and KEGG pathway enrichment analyses by String database showed that the cardioprotective effect of XBW was associated with autophagy and apoptosis signalling pathways. Experimental investigation also verified that XBW suppressed apoptosis, autophagy and endoplasmic reticulum (ER) stress.
Conclusions: XBW showed therapeutic effects against MI/RI mainly via attenuating apoptosis though suppressing excessive autophagy and ER stress.

Introduction
Myocardial ischaemia–reperfusion injury (MI/RI) is a difficult problem after percutaneous coronary intervention (PCI) or thrombolytic therapy, which seriously affects the patients’ quality of life. It is reported that about 10% patients with myocardial infarction die from lethal reperfusion injury (Yellow and Hausenloy 2007). Current mechanism studies show that MI/RI is associated with oxidative stress, inflammation, cardiomyocyte apoptosis, calcium overload or complement activation (Thind et al. 2015). Various pharmacological agents have been developed to reduce MI/RI based on them, but the effect is not ideal. It still lacks in effective and safe approaches for preventing MI/RI (Hausenloy and Yellow 2013), 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 consists of Datura metel L. (Yangjinhua), Cornus cervi pantotrichum (CCP, Lurong), Aconitum carmichaelii (Fuzi), Panax ginseng C.A.Mey., Panax notoginseng (Burk.) F.H.Chen., Cinnamomum cassia Presl (Rougui), moschus (Shexiang), Borneolum syntheticum (Bingpian) and Venenum Bufonis (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 traditional Chinese medicine (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 ischaemic heart disease and ischaemic
changes of electrocardiogram (He et al. 2020). Pharmacological studies showed that XBW and its components have a definite cardioprotection. For example, XBW suppressed cardiac hypertrophy via regulation of PI3K/Akt/GSK3β signalling pathway (He et al. 2020). XBW also attenuated chronic heart failure in a rat model (Zhao et al. 2010; Li et al. 2018). Ginsenoside Rg1, Rb1 (Zheng et al. 2017), Rg3 (Zhang et al. 2016), Rd (Zeng et al. 2015) significantly reduced myocardial infarct size and improved cardiac function in I/R injured model through suppressing oxidative stress, apoptosis and inflammation. Notoginsenoside R1 pre-treatment ameliorated myocardial injury induced by I/R via inhibiting ROCK and promoting mitochondrial ATP synthase δ-subunits (Tong et al. 2019). 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 MI/RI.

This study used ultra performance liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) to identify the chemical profile of XBW, and the database to find out the predicted targets 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 (Figure 1).

Materials 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. (Pleasanton, CA). 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). 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 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).

Ultra performance liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry analysis

Xinbao pills were first broken into powder. A total of 1.0 g of powder was weighted and extracted with 30 mL of 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 film for analysis. UPLC-Q-TOF-MS analysis was performed with Waters Acquity UPLC I-class and a Xevo G2-S Q Tof time-of-flight mass spectrometer (Waters Corporation, Milford, MA). Waters BEH C18 column (2.1 × 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%; 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/h). 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, Manassas, VA). H9c2 cells were cultured in DMEM including 10% foetal 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 and 720 μg/mL), diazoxide (100 μM)) and then subjected to OGD condition (after medium was washed by phosphate buffer solution (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 0.5% ethanol and filtered by 0.22 μm to prepare different doses given drugs.

Cell viability
The cell viability was measured by the 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 removing the supernatant, 150 μL of dimethyl sulphoxide (DMSO) was used to dissolve the formazan products. The absorption was determined by a microplate reader at 490 nm (Thermo Fisher Scientific, Waltham, CA).

Western blot analysis
Briefly, H9c2 cells and cardiac tissue were extracted by 1× 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 polyvinylidene difluoride (PVDF) membrane. After blotting in 5% non-fat 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-HRP-conjugated secondary antibodies for 1 h at room temperature (RT). After washing with 1× 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).
Table 1. Characterization of the chemical constituents in XBW by UHPLC-Q-TOF-MS.

| No. | Chemical name | Pubchem CID | Cas no. | Formula | 2D structure | Molecular weight (Da) | ESI+ (m/z) | ESI– (m/z) | Fragmentations (m/z) | RT (min) | Chinese Medicine | Ref.
|-----|---------------|-------------|---------|---------|--------------|----------------------|-----------|-----------|---------------------|----------|-----------------|-------|
| 1   | Adenosine    | 60961       | 58-61-7 | C_{10}H_{13}N_{5}O_{4} | ![2D structure](image1) | 267.0967 | [M + H]^+ : 268.1047 | [M–H]^– : 266.1047 | 136.0909, [M + H–C_{2}H_{4}O]^{+} | 0.80 | CCP | Shen et al. (2019) |
| 2   | Karakolidine | 101308844   | 41655-13-4 | C_{22}H_{35}NO_{5} | ![2D structure](image2) | 393.2515 | [M + H]^+ : 394.2594 | – | 376.2474 [M + H–H_{2}O]^+; 317.1796 [M + H–H_{2}O–C_{2}H_{4}N]^{+} | 1.58 | Fuzi | Zhang et al. (2019) |
| 3   | Mesaconine   | 101671037   | 6792-09-2 | C_{24}H_{39}NO_{9} | ![2D structure](image3) | 485.2625 | [M + H]^+ : 486.2716 | – | 468.2616 [M + H–H_{2}O]^+; 454.2431 [M + H–H_{2}O–C_{2}H_{4}]^{+}; 424.2740 [M + H–H_{2}O–C_{2}H_{4}–CH_{2}O]^{+} | 1.80 | Fuzi | Zhang et al. (2019) |
| 4   | Isotalatizidine | 11452543   | 763-368-3 | C_{22}H_{31}NO_{5} | ![2D structure](image4) | 408.2730 | [M + H]^+ : 408.2730 | – | 390.2634 [M + H–H_{2}O]^+; 378.2625 [M + H–CH_{2}O]^{+}; 360.2511 [M + H–H_{2}O–CH_{2}O]^{+} | 1.91 | Fuzi | Zhang et al. (2019) |
| 5   | Aconine      | 417761      | 509-20-6 | C_{25}H_{41}NO_{9} | ![2D structure](image5) | 499.2781 | [M + H]^+ : 500.2869 | – | 468.2616 [M + H–H_{2}O–CH_{2}O]^{+}; 454.2790 [M + H–H_{2}O–C_{2}H_{4}]^{+}; 438.2846 [M + H–H_{2}O–C_{2}H_{4}–CH_{2}O]^{+}; 408.2730 [M + H–H_{2}O–C_{2}H_{4}–CH_{2}O–C_{2}H_{4}O]^{+}; 378.2625 [M + H–H_{2}O–C_{2}H_{4}–CH_{2}O–C_{2}H_{4}O–C_{2}H_{4}O]^{+} | 1.97 | Fuzi | Zhang et al. (2019) |
| 6   | Songorine    | 139291804   | 509-24-0 | C_{22}H_{31}NO_{5} | ![2D structure](image6) | 357.2304 | [M + H]^+ : 358.2383 | – | 340.2252 [M + H–H_{2}O]^{+}; 330.2275 [M + H–C_{2}H_{4}]^{+} | 2.04 | Fuzi | Zhang et al. (2019) |
| 7   | Scopolamine  | 300322      | 51-34-3 | C_{15}H_{19}NO_{4} | ![2D structure](image7) | 303.1471 | [M + H]^+ : 304.1531 | – | 156.1013 [M + H–C_{2}H_{4}O]^{+}; 138.0909 [M + H–C_{2}H_{4}O–H_{2}O]^{+} | 2.13 | Datura metel L. | Cirilli et al. (2018) |
| No. | Chemical name         | Pubchem CID | CAS no. | Formula        | Molecular weight (Da) | ESI+ (m/z)       | ESI– (m/z)       | Fragmentations (m/z) | RT (min) | Chinese Medicine | Ref.                  |
|-----|-----------------------|-------------|---------|----------------|-----------------------|-----------------|-----------------|---------------------|-----------|-------------------|-----------------------|
| 8   | Hypaconine            | 101671038   | 63238-68-6 | C_{20}H_{22}NO_{8} | 469.2676              | [M + H]^+ : 470.2740 | 438.2520        | [M + H-CH(OH)]^+ | 2.23      | Fuzi              | Zhang et al. (2019)  |
| 9   | Fuziline              | 131675180   | 80665-72-1 | C_{20}H_{22}NO_{7} | 453.2726              | [M + H]^+ : 454.2824 | 438.2520        | [M + HCOO]^– : 498.2682 | 2.26      | Fuzi              | Zhang et al. (2019)  |
| 10  | Neoline               | 12313185    | 466-26-2  | C_{20}H_{22}NO_{6} | 437.2777              | [M + H]^+ : 438.2846 | 420.2758        | [M + H-H_{2}O]^+ : 388.249 | 2.41      | Fuzi              | Zhang et al. (2019)  |
|     | 10-Hydroxynorine      | 138114026   | 132362-42-6 | C_{20}H_{22}NO_{7} | 453.2726              | [M + H]^+ : 454.2824 | 438.2468        | [M + H-H_{2}O]^+ : 406.2579 | 2.52      | Fuzi              | Takayama et al. (1990) |
| 12  | 3-Deoxyaconine        | 132580133   | 5877-69-0 | C_{20}H_{22}NO_{6} | 483.2832              | [M + H]^+ : 484.2882 | 452.2610        | [M + H-C_{2}H_{4}]^+ | 2.59      | Fuzi              | Wang et al. (2014)   |
| 13  | Atropine              | 174174      | 51-55-8  | C_{17}H_{23}NO_{3} | 289.1678              | [M + H]^+ : 290.1751 | 260.1735        | [M + H-C_{2}H_{4}]^+ | 2.66      | Datura metel L.    | Boermans et al. (2006) |
| 14  | Talatisamine          | 159891      | 20501-56-8 | C_{20}H_{22}NO_{5} | 421.2828              | [M + H]^+ : 422.2917 | 390.2634        | [M + H-C_{2}H_{4}OH]^+ : 372.2533 | 2.74      | Fuzi              | Zhang et al. (2019)  |
| No. | Chemical name                  | Pubchem CID | Cas no.       | Formula      | RT (min) | Chinese Medicine | Ref.          |
|-----|--------------------------------|-------------|---------------|--------------|----------|-----------------|---------------|
| 15  | 14-Acetyltalatizamine           | 156166      | 71239-55-9    | C_{26}H_{41}NO_{6}       | 3.64     | Fuzi            | Zhang et al. (2019) |
| 16  | 14-Benzoyl-10-hydroxymesaconine | 70692815    |               | C_{31}H_{43}NO_{11}      | 3.79     | Fuzi            | Wu et al. (2012)  |
| 17  | Ginsenoside M6A                 | 90478300    | 93376-72-8    | C_{49}H_{82}O_{19}       | 4.47     | Panax ginseng C.A.Mey. | Li et al. (2019) |
| 18  | Notoginsenoside R1              | 441934      | 80418-24-2    | C_{47}H_{80}O_{18}       | 4.65     | Panax notoginseng | Chen et al. (2018) |
| 19  | Benzoylmesaconine               | 24832659    | 63238-67-5    | C_{31}H_{43}NO_{10}      | 4.86     | Fuzi            | Zhang et al. (2019) |
| 20  | Ginsenoside Rg1                 | 441923      | 22427-39-0    | C_{42}H_{72}O_{14}       | 5.03a    | Panax ginseng C.A.Mey. | Chen et al. (2018) |

(continued)
| No. | Chemical name          | Pubchem CID | Cas no. | Formula         | 2D structure | Molecular weight (Da) | ESI+ (m/z)    | ESI– (m/z)    | Fragmentations (m/z) | RT (min) | Chinese Medicine | Ref.                |
|-----|------------------------|-------------|---------|-----------------|--------------|-----------------------|--------------|--------------|---------------------|----------|------------------|---------------------|
| 21  | Ginsenoside Re         | 441921      | 52286-59-6 | C_{48}H_{62}O_{18} | ![Structure](https://via.placeholder.com/50) | 946.5501   | [M + Na]^+; 969.5352 | [M–H]^–; 945.5521 | 794.487 [M-H-Rha]^–; 637.4375 [M-H-Rha-glu]^– | 5.03b    | Panax ginseng C.A.Mey. | Chen DX et al. (2015) |
| 22  | Areobufagin            | 12305198    | 464-74-4 | C_{24}H_{32}O_{6} | ![Structure](https://via.placeholder.com/50) | 416.2199   | [M + H]^+; 417.2274 |              | 399.2154 [M+ H-H_2O]^+; 371.2236 [M+ H-H_2O-H_2O]^+ | 5.26     | Chansu           | Wei et al. (2020)    |
| 23  | Benzoylaconine         | 20055771    | 466-24-0 | C_{32}H_{45}NO_{10} | ![Structure](https://via.placeholder.com/50) | 603.3043   | [M + H]^+; 604.3115 |              | 586.3028 [M+ H-H_2O]^+; 572.2827 [M+ H-C_6H_5OH]^+; 554.2757 [M+ H-C_6H_5OH-H_2O]^+; 522.2489 [M+ H-C_6H_5OH-C_6H_5OH]^+ | 5.39     | Fuzi             | Zhang et al. (2019)  |
| 24  | Benzoylhyperaconine    | 78358526    | 63238-66-4 | C_{31}H_{43}NO_{9} | ![Structure](https://via.placeholder.com/50) | 573.2938   | [M + H]^+; 574.3012 | [M + HCOO]^–; 542.2737 [M+ H-C_6H_5OH]^+; 510.2493 [M+ H-C_6H_5OH-C_6H_5OH]^+ | 5.74     | Fuzi             | Zhang et al. (2019)  |
| 25  | Ginsenoside Ra3        | 73157064    | 90985-77-6 | C_{59}H_{100}O_{27} | ![Structure](https://via.placeholder.com/50) | 1240.6451  | [M–2H]/2–; 619.3145 | 1107.6091 [M–H-xyl]^–; 945.5438 [M–H-xyl-glu]^–; 783.4946 [M–H-xyl-glu-glu]^– | 6.59     | Panax ginseng C.A.Mey. | Chen YJ et al. (2015) |
| 26  | Ginsenoside F3         | 46887678    | 62025-50-7 | C_{49}H_{38}O_{3} | ![Structure](https://via.placeholder.com/50) | 770.4816   | [M–H]^–; 769.4797; 637.4350 [M–H-glu]^–; 475.3797 [M–H-glu-glu]^– | 7.00     | Panax ginseng C.A.Mey. | Du et al. (2018)     |

(continued)
| No. | Chemical name | Pubchem CID | Cas no. | Formula | Molecular weight (Da) | ESI+ (m/z) | ESI– (m/z) | Fragmentations (m/z) | RT (min) | Chinese Medicine | Ref. |
|-----|---------------|-------------|---------|---------|----------------------|-----------|-----------|--------------------|---------|-----------------|------|
| 27  | Ginsenoside Rb1 | 9886279 | 41753-43-9 | C$_{54}$H$_{92}$O$_{23}$ | 1108.6029 | [M+Na]$^+$: 1139.5986 | [M-H]: 1107.6023; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 589.5458 | 7.07 | Panax ginseng C.A.Mey. | Chen DX et al. (2015) |
| 28  | Ginsenoside Rb2 | 432450 | 11021-13-9 | C$_{53}$H$_{90}$O$_{22}$ | 1078.5924 | [M-H]: 1077.5958; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 821.4415 | [M-H-Ara]: 945.5458; [M-H-Ara-glu]: 783.4946; [M-H-Ara-glu-glu]: 621.4415 | 7.27 | Panax ginseng C.A.Mey. | Chen DX et al. (2015) |
| 29  | Ginsenoside Ro | 11815-42 | 34367-04-9 | C$_{48}$H$_{76}$O$_{19}$ | 956.4981 | [M-H]: 955.4960 | [M-H-glu]: 793.4425; [M-H-glu-glu]: 621.4415; [M-H-glu-glu-glu]: 450.4070 | 7.34 | Panax ginseng C.A.Mey. | Du et al. (2018) |
| 30  | Ginsenoside Rb3 | 12912363 | 68406-26-8 | C$_{53}$H$_{90}$O$_{22}$ | 1078.5924 | [M-H]: 1077.5958; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 821.4415 | [M-H-xyl]: 945.5458; [M-H-xyl-glu]: 783.4946; [M-H-xyl-glu-glu]: 621.4415 | 7.48a | Panax ginseng C.A.Mey. | Chen YJ et al. (2015) |
| 31  | Ginsenoside Rc | 12855809 | 11021-14-0 | C$_{53}$H$_{90}$O$_{22}$ | 1078.5924 | [M-H]: 1077.5958; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 821.4415 | [M-H-Xylofuranose]: 945.5458; [M-H-Xylofuranose-glu]: 783.4946; [M-H-Xylofuranose-glu-glu]: 621.4415 | 7.48b | Panax ginseng C.A.Mey. | Chen et al. (2018) |
| 32  | Ginsenoside b1 | 71587485 | 132929-86-3 | C$_{56}$H$_{94}$O$_{24}$ | 1150.6135 | [M-H]: 1149.6147; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 821.4415 | [M-H-Ac]: 1107.6023; [M-H-Ac-H$_2$O]: 945.5458; [M-H-Ac-CH$_2$O]: 783.4946; [M-H-Ac-glu]: 621.4415; [M-H-Ac-glu-glu]: 450.4070 | 7.70 | Panax ginseng C.A.Mey. |
| 33  | Ginsenoside Rd | 24721561 | 52705-93-8 | C$_{54}$H$_{92}$O$_{23}$ | 946.5501 | [M-H]: 945.5458; [M+HCOO]$^-$: 821.4415; [M-H$_2$O]$^-$: 821.4415 | [M-H-glu]: 783.4946; [M-H-glu-glu]: 621.4415; [M-H-glu-glu-glu]: 450.4070 | 8.00 | Panax ginseng C.A.Mey. | Chen et al. (2018) |
| No. | Chemical name        | Pubchem CID | CAS no.   | Formula       | 2D structure | Molecular weight (Da) | ESI+ (m/z) | ESI− (m/z) | Fragmentations (m/z) | RT (min) | Chinese Medicine | Ref.                        |
|-----|----------------------|-------------|-----------|---------------|--------------|-----------------------|------------|------------|----------------------|----------|------------------|-----------------------------|
| 34  | Gypenoside XVII      | 44584555    | 80321-69-3| C_{48}H_{82}O_{18} | ![Structure](image) | 946.5501              |            |            | [M–H]: 945.5458; [M+HCOO]−: 945.5458 | 8.36     | Panax ginseng C.A.Mey. | Xu et al. (2017)             |
| 35  | Acetyl ginsenoside Rd| 73818238    | 102805-32-3| C_{50}H_{84}O_{19} | ![Structure](image) | 988.5607              |            |            | [M–H]: 987.5566; [M+HCOO]−: 927.5345; [M–H–Ac]: 783.4946 | 8.72     | Panax ginseng C.A.Mey. | Yao et al. (2021)            |
| 36  | Ginsenoside Rg2      | 21599924    | 52286-74-5| C_{42}H_{72}O_{13} | ![Structure](image) | 784.4973              |            |            | [M–H]: 783.4946; [M+HCOO]−: 621.4363; [M–H–gulu]: 459.3836 | 9.39     | Panax ginseng C.A.Mey. | Chen et al. (2018)           |
| 37  | Ginsenoside Rg3      | 9918693     | 14197-60-5| C_{42}H_{72}O_{13} | ![Structure](image) | 784.4973              |            |            | [M–H]: 783.4946; [M+HCOO]−: 621.4363; [M–H–gulu]: 459.3836 | 9.43     | Panax ginseng C.A.Mey. | Chen et al. (2018)           |
Sprague-Dawley (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) and XBW group (MI/R + XBW, n = 6). In brief, SD rats were anaesthetized by intraperitoneal injection of 40 mg/kg 2% pentobarbital sodium. After anaesthesia, 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/RI model group (MI/R + XBW, n = 6) received 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 times with that of reference compounds or comparing their retention behaviours, empirical molecular formula and proposed fragmentation patterns with that in literature (Takayama et al. 1990; Boermans et al. 2006; Wu et al. 2012; Wang et al. 2014; Chen DX et al. 2015; Chen YJ et al. 2015; Xu et al. 2017; Chen et al. 2018; Cirlini et al. 2018; Du et al. 2018; Gong et al. 2019; Chen et al. 2019; Zhang et al. 2019; Wei et al. 2020; Yao et al. 2021), including ginsenoside Rgl-1, Rb1-3, Re, Ro, gynenoside XVII, aconine, mesaconine, isotalatizidine, scopoline, areno-bufagin, etc. The chemical components were derived from Fuzi, *Panax ginseng*, *Panax notoginseng*, *Datura metel* L., CCP and Chansu in XBW.

**H&E staining**

Hearts were fixed in the 4% PFA solution, dehydrated by gradient alcohol, and embedded with paraffin. Paraffin slices (5 μm) were rehydrated by gradient alcohol, and stained with haematoxylin and eosin (H&E). 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 webservice (http://targetnet.scbbd.com). In practice, protein targets with prediction score of >0.90 were selected. In addition, the targets of chemicals reported in literature were also collected.

**Collecting MI/RI-associated targets**

The targets related to MI/RI were selected from OMIM database (https://omim.org/) and the literature using ‘myocardial ischemia–reperfusion injury’ as keywords.

**Gene ontology (GO) and pathway enrichment of potential targets**

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

**Statistics analysis**

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

**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 behaviours, empirical molecular formula and proposed fragmentation patterns with that in literature (Takayama et al. 1990; Boermans et al. 2006; Wu et al. 2012; Wang et al. 2014; Chen DX et al. 2015; Chen YJ et al. 2015; Xu et al. 2017; Chen et al. 2018; Cirlini et al. 2018; Du et al. 2018; Gong et al. 2019; Chen et al. 2019; Zhang et al. 2019; Wei et al. 2020; Yao et al. 2021), including ginsenoside Rgl-1, Rb1-3, Re, Ro, gynenoside XVII, aconine, mesaconine, isotalatizidine, scopoline, areno-bufagin, etc. The chemical components were derived from Fuzi, *Panax ginseng*, *Panax notoginseng*, *Datura metel* L., CCP and Chansu in XBW.

**Effect of XBW against myocardial ischaemia–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 3(A,B), XBW administration significantly reduced infarct size from 40.64% to 8.79%. Creatine kinase MB (CK-MB) is one of the biomarkers in serum for MI/RI, and XBW administration decreased the level of CK-MB induced by MI/RI from 2.83 to 1.63 U/L in plasma (Figure 3(C)). Meanwhile, the levels of cTnI and cTnT in plasma were also decreased from 155.8 ± 5.880 to 133.9 ± 2.047 pg/mL (p < 0.01), from 311.5 ± 7.663 to 266.± 15.81 pg/mL (p < 0.05), respectively (Figure 3(D,E)). Figure 3(F, G) also shows that XBW could decrease the level of cTnI and cTnT in the heart tissue from 1.047 ± 0.061 to 0.808 ± 0.078 ng/g (p < 0.05) and from 2.166 ± 0.111 to 1.868 ± 0.092 ng/g (p < 0.05). Echocardiography in Figure 3(H) exhibited that XBW improved cardiac function. H&E staining showed that XBW administration attenuated inflammatory cell infiltration, and disordered myocardial fibre induced by MI/RI (Figure 3(I)). In in vitro study, XBW protected H9c2 cell against OGD/R injury from 40.08% to 58.8%, 77.9%, 80.1% at 60, 240 and 720 μg/mL, respectively (Figure 4(A,B)). These results suggested that XBW ameliorated MI/RI.

**Target identification and network analysis**

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

After GO enrichment analysis of the targets by the String database, the top 15 enrichment results listed in BPs, MFs and CCs are shown in Figure 6(A), 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 is performed in Figure 6(B), which exhibited the top 20 related signalling pathways excluding the specific cancer related pathways, HIF-1 signalling pathway, PI3K-Akt signalling pathway, autophagy, FoxO signalling pathway, apoptosis, etc. Based on the protein–protein interactions (PPI) analysis (Figure 6(C)), CASP3, MTOR (rapamycin), SIRT1, HIF1A, ATF4, GRP78 (BIP, glucose...
and ATG5 expressions were detected (Figure 8(A–E)). The expressions of Beclin-1 and LC3II in cardiac tissue were markedly increased in MI/RI group, while significantly decreased in XBW administration group from 1.668 ± 0.143 to 1.067 ± 0.161-fold \((p < 0.05)\) and from 2.053 ± 0.137 to 1.060 ± 0.100-fold \((p < 0.001)\). Meanwhile, the expression of p62 was reduced in the MI/RI group, while increased in XBW group from 0.653 ± 0.044 to 0.899 ± 0.100-fold \((p < 0.05)\). However, ATG5 was no obvious changes between three groups. In *in vitro* study, XBW could also decrease Beclin-1 expression induced by OGD/R in H9c2 cells (Figure 8(H,G)). 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 8(H,I), 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 ischaemia–reperfusion injury is a difficult clinical problem in myocardial infarction therapy; however, current medications for treating MIRI are not ideal (Thind et al. 2015). TCM exhibits unique advantages in the treatment of cardiovascular diseases, based on multiple components and multiple targets (Hao M et al. 2017; Hao P et al. 2017). XBW is a patented traditional Chinese herbal formula, which has been listed in China for more than 30 years. It is used for treating ischaemic heart disease and chronic heart failure (He et al. 2020). However, there is a lack of evidence 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 treat coronary heart disease and chronic heart failure (Li et al. 2018). 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 left ventricular (LV) function. Moreover, the *in vitro* results revealed that XBW could also reduce OGD/R-induced cell injury. XBW is composed of nine Chinese medicines. 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* and Fuzi, which are monarch drugs in XBW. For example, *Panax ginseng* exhibits unique advantages in the treatment of cardiovascular diseases, based on multiple components and multiple targets (Hao M et al. 2017; Hao P et al. 2017). XBW is a patented traditional Chinese herbal formula, which has been listed in China for more than 30 years. It is used for treating ischaemic heart disease and chronic heart failure (He et al. 2020). However, there is a lack of evidence 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.
that ginsenoside Rg3, Rg1, Rb3, arenobufagin and notoginsenoside R1 had high degrees, which may be the active compounds of XBW. Importantly, it is reported that notoginsenoside R1 attenuated MIRI by inhibiting oxidative stress- and ER stress-related signalling pathways (Yu LM et al. 2016; Yu YL et al. 2016).

Multiple evidence has shown that ginsenoside Rg1 protected heart against MIRI partially by activating PI3K/Akt/mTOR and inhibiting autophagy (Zhang et al. 2012; Qin et al. 2018). Ginsenoside Rb3 and Rg3 also improved cardiac functions and protected MIRI via suppressing apoptosis and inflammation (Liu et al. 2014; Zhang et al. 2016). The results of the studies were consistent to our network pharmacological analysis. Although
Some active components have been reported in MI/RI, it still has several compounds without pharmacological verification, thus, 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 activation is a biochemical hallmark of apoptosis (Choudhary et al. 2015); B cell lymphoma-2 (BCL2) plays an important role in the negative regulation of apoptosis during MI/IR (Huang et al. 1997; Wang G et al. 2016); eukaryotic initiation factor 2 alpha (eIF2α) and activating transcription factor-4 (ATF4) can mediate myocardial ER stress (Yu LM et al. 2016; Yu YL et al. 2016). mTOR and Beclin-1 are two key autophagy-related proteins in MI/R injury (Shi et al. 2019). The results illustrated that XBW might regulate above proteins to show cardioprotective effect. GO enrichment analysis showed that XBW can treat MI/RI by regulation of cell death, apoptosis process and response to stress. KEGG enrichment analysis demonstrated that apoptosis, autophagy, HIF-1 signalling pathway, PI3K/Akt signalling pathway and FoxO signalling 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 signalling pathways helps prevent myocardial injury (Jennings 2013; Zhu et al. 2015). 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

**Figure 6.** The GO and KEGG analysis. (A) The GO CC-MF-BP analysis diagram. (B) The KEGG analysis diagram. (C) PPI network graph of 50 hub Nodes based on their interactions.
has the potential effects for attenuating myocardial apoptosis for the patients with MI/RI.

Emerging evidence has indicated that ER stress is involved in the development and pathogenesis of MI/RI (Huang et al. 1997; Wang J et al. 2016). During MI/RI, the balance of the homeostasis for the ER is broken, subsequently unfolded or misfolded proteins are accumulating in myocardial cells, and eventually triggering ER stress (Wu et al. 2016). 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 signalling pathway activation (Li et al. 2019). GRP78 (BIP) is a calcium ion-binding molecular chaperone in the ER. When undergoing ER stress, GRP78 and ER cross to activate the downstream CHOP-associated apoptotic signalling pathways (Wu LX et al. 2018). 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 (Nishida et al. 2009). Several studies showed that reduction of autophagy clearance in myocardial cells during MI/RI threatens cell survival (Ma et al. 2012; Hao M et al. 2017; Hao P et al. 2017). Promoting autophagy moderately may protect cell and mitochondrial injury in MI/RI (Tannous et al. 2008; Wu SY et al. 2018). However, at the late stage of MI/RI, it induces excessive activation of autophagy, resulting in cytotoxic cell death (Zhu et al. 2007; Kroemer and Levine 2008; Huang et al. 2017). Thus, prevention of excessive autophagy activated during MI/RI may be benefit to reduce cardiomyocyte

**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 ± SEM. n = 5–6, *p < 0.05, **p < 0.01, Sham vs. MI/R group; #p < 0.05, ##p < 0.01, MI/R + XBW vs. MI/R group.
death and improve cardiac function. In the present study, XBW treatment inhibited MI/RI-induced beclin-1 and LC3II expression 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 be solved in the future work. By using UPLC-Q-TOF-MS/MS, 37 major compounds from six medicinal materials were identified, but the volatile constituents of three others, including Cinnamomum cassia Presl, moschus and Borneolum syntheticum had not been detected, which needs QC-MS analysis in our later work to enrich 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.

Conclusions

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. Thirty-seven chemical constituents in XBW were identified, 50 potential MI/RI targets and five 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 (Figure 9).

Author 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 partially analysed the data. All authors reviewed and revised the manuscript.

Disclosure statement

All authors have no conflict of interest to disclose.

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