

Materials and methods

1. Network Pharmacology Part

1.1 Identification of Candidate Components in Andrographis

All components of the Chinese medicinal herbs in Andrographis were retrieved from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (<http://tcmspw.com/>) (Ru et al., 2014).

1.2 Screening Strategy for Bioactive Components in Andrographis

All components were screened using the absorption, distribution, metabolism and excretion (ADME) model from the TCMSP database. In ADME processes, oral bioavailability (OB) (Tao et al., 2013; Xu et al., 2012) is a major pharmacokinetic parameter and represents the proportion of bioactive compounds that enter the blood circulation. Another parameter is the Druglikeness evaluation (DL) (Tao et al., 2013) which is used to assess the chemical suitability of a compound to act as a drug. The mean DL index of compounds in the DrugBank database is 0.18. Therefore, any compounds in Andrographis with $OB \geq 30\%$ and $DL \text{ index} \geq 0.18$ were regarded as active ingredients for the purposes of the present study.

1.3 Identification of drug targets and AF targets

The TCMSP database was used to predict protein targets of active substances present in Andrographis. GeneCards (<https://www.genecards.org>) was used to combine the relevant literature to generate a list of AF targets. DrugBank (<https://go.drugbank.com>), OMIM (<https://omim.org>) and KEGG databases (<https://www.kegg.jp/kegg>) were used to verify and replenish the targets. Targets were collated and entered into the STRING platform (<http://string-db.org/>) to obtain a PPI network and modified by Cytoscape 3.7.2 (Shannon et al., 2003). MCODE and the cytoHubba plug-in were applied to identify seed genes and various function clusters.

1.4 Gene Ontology and Pathway Enrichment Analysis for AF-Related Targets of Andrographis

The gene ontology (GO) and pathway enrichment analyses were conducted using the functional annotation tool of DAVID Bioinformatics Resources 6.8 (<http://david.ncifcrf.gov/>) (Huang da, Sherman, & Lempicki, 2009a, 2009b). Terms with thresholds of Count ≥ 2 and Expression Analysis Systematic Explorer (EASE) scores ≤ 0.05 were chosen as screening parameters for functional annotation clustering.

1.5 Molecular docking and molecular dynamics simulation

The 3D structure of the target Nrf2-Keap1 complex (PDB ID: 7K2M), was downloaded from the protein data bank (PDB) database (<https://www.rcsb.org/>) (Burley et al., 2017). The structures of Andr were downloaded from Pubchem (<https://pubchem.ncbi.nlm.nih.gov/>). Standard AutoDock protocol was used to convert ligand 2D MOL to 3D MOL, ligand 3D MOL to PDBQT, generate receptor structure, calculate docking energy, choosing the docking mode and mechanism of docking (Sulimov, Kutov, & Sulimov, 2019). The most favorable conformation was chosen based on the lowest binding energy on docking. The root-mean-square deviation (RMSD) value (in Å) between the best docking poses of compounds and the complex in co-crystal structures was selected as the evaluation standard for checking the accuracy of the docking program. The program was then applied to ligand-protein interactions of Nrf2-Keap1-compound complexes. RMSD values ≤ 2.0 Å were accepted as evidence of successful molecular docking (Sutherland, Nandigam, Erickson, & Vieth, 2007).

2. Experimental part

2.1 Animals

All institutional and national guidelines for the care and use of laboratory animals were followed. All animal protocols were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication No. 85-23, revised 1996). This study was conducted with approval by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xinjiang Medical University (Approval Number: IACUC-20170420-03). New Zealand white rabbit (male, healthy, 2.5–3.0 kg) were used for in vivo experiments. Animals were sacrificed at the end of this experiment by overdose anesthesia.

2.2 Rabbit models of acute AF and Andr treatment

Rabbits were randomly divided into 4 groups: the sham, control with AF surgery (AF), AF with Andr treatment (AF + Andr), and control with Andr treatment (Andr). Reagent-grade Andr (365645; Sigma-Aldrich, St. Louis, MO, USA) was tested $\geq 98\%$.

Twenty-four hours prior to measuring or modeling, rabbits in AF + Andr group and Andr group were injected intraperitoneal with 10 mg/kg Andr. Acute AF was generated in New Zealand white rabbit. Briefly, rabbits were anesthetized with ketamine/xylazine (35 mg/5 mg/kg). Standard electrocardiograms (leads 1-aVF) were continuously monitored. After shaving the neck area and application of Betadine antiseptic, the right jugular vein was dissected and cannulated with a 4-French multi-electrode catheter. Under electrographic control, the catheter was passed into the right atrium to record atrial potentials in conjunction with the standard 6-lead ECG. RAP was delivered (stimulus voltage: 4V, S1-S1: 120 ms, 2 * threshold, 1 ms induration,) at the right atrial appendage for 6 hours.

2.3 HL-1 Cell Culture and Rapid Electronic Pacing Model Establishment

HL-1 atrial myocytes (SIGMA) were cultured in Claycomb medium supplemented with 10% FBS,

100 units/ml penicillin, 100 μ g/mL streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. HL-1 cells were plated in six-well plates, and transferred into 6-well C-Dish (IonOptix, Milton, MA) after cell adherence. Then RES was applied to cells for 24 hours (stimulus voltage: 10V, stimulation frequency: 5Hz, 10ms induration). One group of HL-1 seeded plates was treated with Andr 1 h before RES.

2.4 Apoptosis Assay

Apoptosis in atrial tissue was analyzed by TUNEL staining according to the manufacturer's instructions (Yeasen, China). Frozen sections were fixed with 4% PFA for 15 min, washed by PBS for 15 min, and costained with Proteinase K for 10 min. Sections were washed three times with PBS for 5 min each, costained with 1 \times Equilibration Buffer for 10 min, and then TdT for 60 min at 37 $^{\circ}$ C. Finally, sections were costained with PI and DAPI. TUNEL assay results were visualized by fluorescence microscopy (Olympus BX53, Japan), and TUNEL-positive nuclei were stained green in fluorescence images. Apoptosis in HL-1 cells was determined by flow cytometry with an Annexin V-FITC (AV)/propidium iodide (PI) apoptosis detection kit according to the manufacturer's instructions (40305ES20, Yeasen, China).

2.5 Immunofluorescence Analysis

Frozen sections were equilibrated to warm to room temperature, fixed with 4% PFA for 15 min, washed by PBS for 5 min 3 times, and blocked by QuickBlock™ Blocking Buffer for Immunol Staining (P0260, Beyotime, China) at 37 $^{\circ}$ C for 15 min. After blocking, primary antibodies were diluted in QuickBlock™ Primary Antibody Dilution Buffer for Immunol Staining (P0262, Beyotime, China) at 4 $^{\circ}$ C overnight. Slides were then washed by PBS for 5 min 3 times and incubated with the secondary antibody for 60 min at RT. After three washes with PBS for 5 min each, the slides were

sealed with anti-fluorescence quenching sealing tablets and observed using fluorescence microscopy (Olympus BX53, Japan). The primary antibodies were listed as follows: TNF- α (sc-12744, Santa, dilution 1/100), HO-1 (ab13248, abcam, dilution 1/100), cardiac Troponin T (15513-1-AP, Proteintech, dilution 1/200), Nrf2 (ab31163, abcam, dilution 1/200).

2.6 ROS Levels Measurement

ROS levels in the atrial tissue were measured by dihydroethidium (DHE) staining as previously reported (Su et al., 2020; Xie et al., 2020).

The frozen sections were washed in wash buffer for 10 min, PBS for 5 min and incubated with fluorescent dye (10 μ M) at 37 °C for 30 min according to the manufacturer's instructions (BestBio, China). Then, the frozen sections were washed three times and imaged by fluorescence microscopy (Olympus BX53, Japan). Fluorescence intensity was determined using ImageJ software.

ROS levels in the HL-1 cells were measured by DHE staining. Cells were incubated with DHE for 30 min at 37 °C in the dark, washed with Hanks' balanced salt solutions 3 times to remove background fluorescence and imaged by fluorescence microscopy.

Superoxide dismutase (SOD) activity was determined using a SOD assay kit (S0103, Beyotime, China) based on the WST-8 method. ATP contents was determined using an ATP assay kit (S0026, Beyotime, China).

2.7 ELISA

Blood samples clotted overnight at 4 °C and were centrifuged for 15 min at 12,000 rpm 4 °C. Serum was collected and frozen at -80 °C until use. ELISA kits for Rabbit TNF- α were purchased from CLOUD-CLONE CORP. (CCC, USA). ELISA was performed according to manufacturer instructions.

2.8 Real-Time Quantitative PCR

Total RNA was extracted from the atrial tissues and HL-1 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the instruction manual. Total RNA was qualified and quantified using NanoDrop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA) and reverse-transcribed into cDNAs with PrimeScript RT (Takara, Japan) in accordance with the manufacturer's instructions. Real-time PCR was performed using PCR reaction mixture (11203ES08, Yeasen, China) on a LightCycler 480II system (Roche). The sequences of primers were as follows:

GAPDH (forward, 5'-CGGGAAGCTTGTGATCAATGG-3', reverse, 5'-

GGCAGTGATGGCATGGACTG-3'),

TNF- α (forward, 5'-CTCTTCTCTTTCTGCTCGTG-3', reverse, 5'-

GCCACAGGGTTGACTAGATG-3'),

IL-1 β (forward, 5'-TACAACAAGAGCTTCCGGCA-3', reverse, 5'-

GGCCACAGGTATCTTGTCGT-3'),

NQO1 (forward, 5'-CATACAGCATCGGGCACAC-3', reverse, 5'-

TTTTCTGCTCATCTTGACCT-3'),

Nrf2 (forward, 5'-TTAGTGCTTTTGAGGATTCTTTCCG-3', reverse, 5'-

AATTCTGTGCTTTCAGGGTGGTTCT-3'),

SOD1 (forward, 5'-GACGCATAACAGGACTGACCG-3', reverse, 5'-

AACACATCAGCGACACCATTG-3'),

SOD2 (forward, 5'-TGACGGCTGTGTCTGTTGGT-3', reverse, 5'-

GCAGGTAGTAAGCGTGTTC-3').

2.9 Western Blot

Right atrial tissues and HL-1 cells were lysed in RIPA lysis buffer (Fdbio science, Hangzhou, China) with PMSF for 10 min. Cell debris was pelleted by centrifugation for 10 min at 12,000 rpm at 4 °C. The supernatant containing protein was collected and protein concentration was determined. Samples were denatured at 100 °C for 10 min prior to separation by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to PVDF membranes (Millipore, Sigma-Aldrich, Ireland). After blocking in 5% non-fat milk, the following primary antibodies were incubated overnight: β -tubulin (66240-1-Ig, Proteintech, at 1/5000 dilution in 5% BSA), HO-1 (ab13248, abcam, at 1/1000 dilution in 5% BSA), cardiac Troponin I (21652-1-AP, Proteintech, at 1/1000 dilution in 5% BSA), Total OXPHOS Rodent WB Antibody Cocktail (ab110413, abcam, at 1/1000 dilution in 5% BSA). After washing membranes the following day, the blots were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1h. Signals were detected using enhanced chemiluminescence reagents (Fdbio science, Hangzhou, China) and quantification was performed with Image Lab software for the ChemiDoc XRS system (Bio-Rad, Hercules, CA).

2.10 Statistical analysis

Data were presented as mean \pm SD and were statistically analyzed using GraphPad Prism 8 software. Differences between two groups were compared using two-tailed unpaired Student's t-test. For more than two groups, statistical analyses were performed using two-way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine the differences within and between groups. No samples/results were excluded from the analyses. The significance between samples was denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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