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Original Research Article

Network pharmacology study on mechanism involved in the use of YuShu Soup for the treatment of exertional heatstroke

Jing Dong[1]*, Zheng Yang[2], Chen Fan[3]
[1]Department of Emergency, First Affiliated Hospital of Ningbo University, Ningbo, Zhejiang Province 315000, [2]Department of Emergency, Ningbo Municipal Hospital of TCM, Affiliated Hospital of Zhejiang Chinese Medical University, Ningbo, Zhejiang Province 315010, [3]Intensive Care Unit, Ningbo First Hospital Longshan Hospital Medical and Health Group, Ningbo, Zhejiang Province 315311, China

For correspondence:* ***Email:* *jing_d0321@163.com;* ***Tel:*** *+86-057487086426*

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Abstract

Purpose: *To investigate the underlying mechanisms of action of YuShu Soup in exertional heatstroke (EHS) treatment.*
Methods: *This study utilized network pharmacology analysis, applied STRING database to collect compound targets, and visualized protein-protein interaction (PPI) network. Furthermore, EHS model was established on rats, and physiological indices and serum biochemical indicators were evaluated. Thereafter, morphological and histological assessments were carried out on renal tissues.*
Results: *The PPI network indicated that JUN, AKT1, MAPK3, MAPK1, STAT3, RXRA, CTNNB1, and RELA may be the vital proteins which YuShu Soup acts on. In terms of mechanism, YuShu Soup regulates the protein expression of HSP90 and Akt/Bcl-xL pathway significantly ($p < 0.01$).*
Conclusion: *YuShu Soup alleviates symptoms of EHS and apoptosis of renal cells in rats by regulating HSP90 and Akt/Bcl-xL pathway, thereby alleviating kidney damage. These findings shed light on the prevention and treatment of EHS with this traditional Chinese prescription.*

Keywords: *Exertional heatstroke, Heat shock protein 90, Network pharmacology, YuShu Soup*

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INTRODUCTION

Heat stroke is a severe medical condition defined as a rapid increase in core body temperature above 40 °C and abnormalities in central nervous system such as delirium, convulsions, or coma [1]. Heat stroke has two forms: classical heatstroke (CHS) and exertional heatstroke (EHS). Classical heatstroke occurs primarily in young or aged persons, whereas EHS is more common in athletes and military personnel who engage in strenuous outdoor exercises. Exertional heatstroke has a mortality rate of 5 - 50 % in humans [2]. Recently, treatment of heat stroke mainly includes cooling, intravenous rehydration and hemodiafiltration, but curative effect has been proven to be unsatisfactory [1,3].

It has been reported that acute stress reduces renal blood flow and causes ischemia, thereby

inducing acute kidney injury [4]. The anti-apoptosis and survival mechanism induced by heat shock protein 90 (HSP90) protects the kidney from stress injury. Transcription of HSP90 is upregulated to participate in restoration of protein homeostasis from heat stress [5]. It has been reported that HSP90 activates Akt phosphorylation and regulates Bcl-2 family protein Bcl-2/Bcl-xL, which functions in regulating mitochondrial function and apoptosis [6].

The traditional prescription, *Wang's Qingshu Yiqi* decoction is modified Yushu liquid, which is used in severe heat stroke, among which clearing heat, replenishing qi and nourishing yin have a higher status in treatment of severe heat stroke. Therefore, this study is aimed at investigating underlying mechanisms of *YuShu Soup* in exertional heatstroke (EHS) treatment using a network pharmacology model.

EXPERIMENTAL

Animals

Male SD rats were bought from SLAC Laboratory Animal Technology (Shanghai, China). This study was approved by Ethics Committee of the First Affiliated Hospital of Ningbo University (approval no. 2022012). All procedures for animal handling were in accordance with Guidelines for the Care and Use of Laboratory Animals [7]. The rats were raised in specific pathogen free (SPF) space with adequate food and drink and 12-h light/dark cycle. A total of twenty rats were randomly divided into 4 groups: Sham group, EHS group, EHS + *YuShu Soup* (YSS) (2 mL/100 g) group, and EHS + YSS (4 mL/100 g) group. The EHS + YSS groups were treated with *YuShu* by intragastric administration, with the indicated dose given three times per day. Sham and EHS groups were treated with saline only.

EHS experiment

A treadmill (Zhongshi Technology, Beijing, China) was set to provide a forced running system, and the rats were trained for one week to familiarize with the treadmill. The runway was previously designed with electrodes at one end, and the rats were forced to run on standard environment with 1 mAh electrical stimulation. Rats were free to run for 15 min on first day of training, and then they were forced to run at 10 m/min for 35 min. In the following days, daily exercise time was extended by 5 min, and speed was increased by 1 m/min. Finally, the speed was 15 m/min and the training time was 60 min on the sixth day. After a day rest (seventh day), EHS model was established. Two electromagnetic spectrum heaters (Changle Silicate, Chongqing, China) were arranged to preheat the treadmill, until the temperature rose to 37.5 ℃, while the indoor humidity was maintained. Rats were administered with *YuShu Soup* at doses of 2 mL/100 g, and 4 mL/100 g. The core temperature (Tco) of the rats was monitored using an electric thermometer. Subsequently, rats in EHS group and EHS + YSS groups were forced to run at a rate of 15 m/min with 1.0 mAh electrical stimulation. Core temperature (Tco) was monitored continuously until they refused to run for 5 sec (defined as fatigue), and 40.5 ℃ was considered onset of EHS disease. After the EHS procedure was completed, the rats were removed from the treadmill to a normal environment to recover.

Physiological indices and serum biochemical analyses

After measuring physiological indices such as weight, mean blood pressure (MBP), and Tco, the rats were anaesthetized using pentobarbital. Total blood was collected and centrifuged at 4000 rpm for 15 min. The serum was analyzed using an automated biochemical analyzer (c8000A-701, Roche Diagnostics, German) to quantify the serum creatinine (SCr), blood urea nitrogen (BUN), and creatine kinase-MB (CK-MB). The HSP90 concentration was measured using an HSP90 ELISA Kit (Elabscience Biotechnology Inc., Texas, USA) according to the manufacturer's protocols [8].

Morphological and histological analysis of renal tissues

The kidney tissues were fixed in 4 % paraformaldehyde and stored at -80 °C for histological assessment. The renal tissues were dehydrated, embedded into paraffin and sectioned (3 μm). Sections were stained with hematoxylin and eosin (H&E) for further morphological observation. Kidney tissue sections were deparaffinized in toluene and then stained using TUNEL fluorescent kit (Roche Diagnostics, German), according to manufacturer's instructions.

Protein extraction and western blot analysis

Kidney tissues were homogenized in lysis buffer (25 mM Tris-HCl pH 7.4, 250 mM NaCl, 1 mM EDTA, 0.1 % SDS, 0.5 % Sodium deoxycholate, and 1 mM Phenylmethanesulfonyl fluoride). Protein concentration was measured using bicinchoninic acid (BCA, Thermo Fisher, Rockford, IL, USA) quantification. An equal

amount of extracted proteins were denatured and then separated on sodium doceyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [9].

The samples were transferred to a nitrocellulose membrane, blocked with 5 % bovine serum albumin (BSA), incubated with primary antibodies against BAX (14796, Cell Signaling Technology, Danvers, Massachusetts, USA; 1:3000), cleaved caspase-3 (9661, Cell Signaling Technology; 1:4000), p-AKT (4060, Cell Signaling Technology; 1:2000), AKT (9272, Cell Signaling Technology; 1:1000), BCL-2 (PA5-27094, Thermo Fisher Scientific; 1:3000), Caspase 3 (PA5-77887, Thermo Fisher Scientific; 1:2500), HSP90 (13171-1-AP, ProteinTech, Rosemont, Illinois, USA; 1:3000) and β-actin (PA5-78715, Thermo Fisher Scientific; 1:5000) overnight at 4 ℃. Then, membranes were washed prior to incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (65-6120, Thermo Fisher Scientific; 1:5000). Immunoreactive protein bands were visualized using an enhanced chemiluminescence reagents (Bio-Rad, USA), and quantified using Image J software (National Institutes of Health, USA).

Screening of the active components and targets of *YuShu Soup*

The bioactive components and the corresponding targets of *Anemarrhenae rhizoma*, *Coptis chinensis*, *Phellodendron amurense*, *Reed rhizome*, *Lotus leaf*, *Adenophorae radix*, *Panax quinquefolius*, *Asparagus*, *Schisandra chinensis*, *Poria cocos*, *Rhizoma alismatis, Liquorice, Coix seed, Benincasae exocarpium*, *Lycii cortex*, and *Scrophulariae radix* were searched via the Traditional Chinese Medicine Systems Pharmacology database.

Collection of EHS-related targets

The disease targets of EHS were surveyed using five public databases: Online Mendelian Inheritance in Man (OMIM, https://omim.org/) database, GeneCards (https://www.genecards.org/) database, Archive Ensembl (https://www.ensembl.org/) database, Pharm GKB (https://www.pharmgkb.org/) database, Therapeutic Target Database (TTD, http://db.idrblab.net/ttd/), using the keyword ‘exertional heatstroke’.

Construction of drug regulatory network and protein interaction network

The potential targets of components in *YuShu Soup* and EHS-related targets were collected for further analysis using Cytoscape 3.8.2 software. The overlapping targets of the drugs and diseases in the intersection were analyzed using STRING database (https://string-db.org/) to establish protein interaction network which was then refined and visualized using Cytoscape 3.8.2.

Functional enrichment analysis

The gene ontology (GO) analysis and the Kyoto encyclopedia of genes and genomes (KEGG) analysis were performed to investigate biological function and signaling pathways involved in EHS. The KEGG and GO analysis were screened for $q < 0.05$.

Statistical analysis

The data are expressed as mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA) to compare means among groups, using SPSS 25.0 version [10]. To identify the core proteins of *YuShu Soup* intervention used for EHS, cytoscape software was applied to mine important targets, and then R project was used to perform statistical computing with data screening. $P < 0.05$ was considered statistically significant.

RESULTS

Potential targets of EHS and *YuShu Soup*

A total of 4656 targets of EHS disease were collected after de-duplication from five public databases: GeneCards, OMIM, PharmGkb, TTD, Archive Ensembl databases, with the keyword ‘exertional heatstroke (Figure 1 A). There were 113 overlapping recognitions obtained using intersectional targets between 148 *YuShu Soup*-related targets and EHS-related targets, which were designated as core targets for further study (Figure 1 B). Based on the peculiarity of multi-ingredients and multi-targets in traditional Chinese medicine prescriptions, the *YuShu Soup* component-target network was constructed (Figure 1 C). The blue rectangles in the middle are core targets, surrounded by bioactive ingredients of 16 traditional herbs in *YuShu Soup*. The 16 traditional herbs contain *Anemarrhenae rhizoma*, *Coptis chinensis*, *Phellodendron amurense*, Reed rhizome, Lotus leaf, *Adenophorae radix*, *Panax quinquefolius*, *Asparagus*, *Schisandra chinensis*, *Poria cocos*, *Rhizoma alismatis, Liquorice, Coix seed, Benincasae exocarpium*, *Lycii cortex*, and *Scrophulariae radix*.

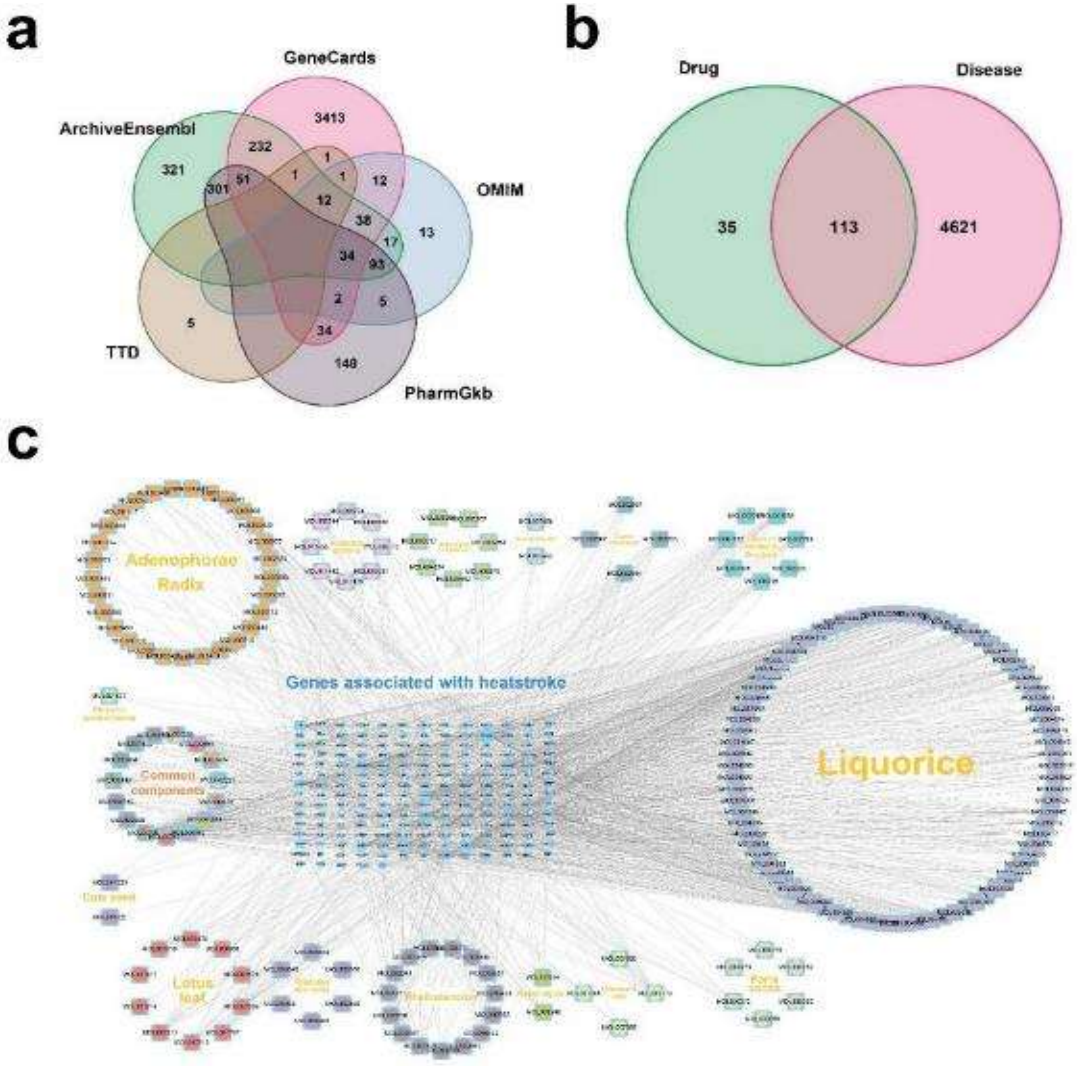

Figure 1: Targets of EHS and *YuShu Soup*. (a) Venn diagrams of EHS targets identified from five databases; (b) Venn diagram of the intersection targets in EHS and *YuShu Soup*; (c) herb-compound-target network of *YuShu Soup.* The ellipses represent the bioactive components of the indicated herbs from *YuShu Soup;* the blue squares represent the potential targets of EHS

PPI network of EHS and *YuShu Soup*

A PPI network was created using STRING database, which was visually analyzed using Cytoscape 3.8.2. Edges indicate protein-protein and the nodes represent proteins (Figure 2 A). Matching components of *YuShu Soup* with EHS targets were lined while the acceptable core goals were highlighted in yellow (Figure 2 B). The results revealed that a new PPI network was acquired to uncover more critical proteins which were kept highlighted in yellow. The importance of components and targets was evaluated by degree of connectivity in the regulatory network (Figure 2 C). Ultimately, final PPI network was concentrated on the top 8 targets of *YuShu Soup* in treatment of EHS, and were sorted by degree values (Figure 2 D). Thus JUN (c-Jun), protein kinase B (AKT) 1, mitogen-activated protein kinase (MAPK) 3, MAPK1, signal transducer and activator of transcription 3 (STAT3), retinoid X receptor, alpha (RXRA), catenin beta 1 (CTNNB1) and RELA (NF-kappa B) were the top valued targets, and may be the vital proteins in EHS treatment with *YuShu Soup*.

Enrichment analysis of GO and KEGG

To investigate the potential signaling pathway linked to *YuShu Soup* in the treatment of EHS, GO and KEGG enrichment analysis were exerted based on potential targets. Response to xenobiotic stimulus pathways was identified as the most enriched biological progress by GO functional enrichment analysis (Figure 3 A). The PI3K/AKT and TNF signaling pathway were the main enriched signaling pathways screened via the KEGG pathway enrichment analysis (Figure 3 B).

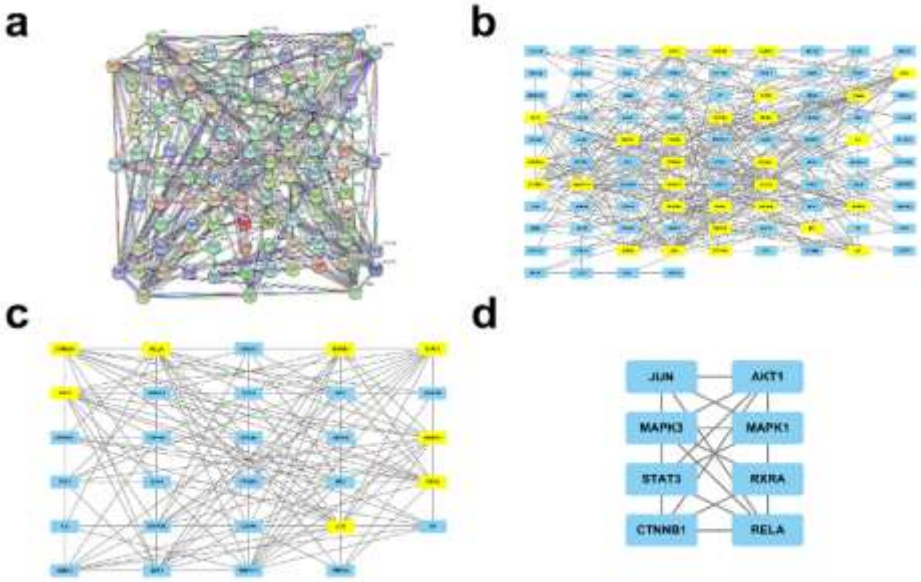

Figure 2: The PPI network and the enrichment analysis. (a) Interactive PPT network constructed from STRING database; (b) PPI network analyzed by R project; (c) PPI network of more critical proteins and (d) Core PPI network of top 8 proteins (Yellow represents the higher degree)

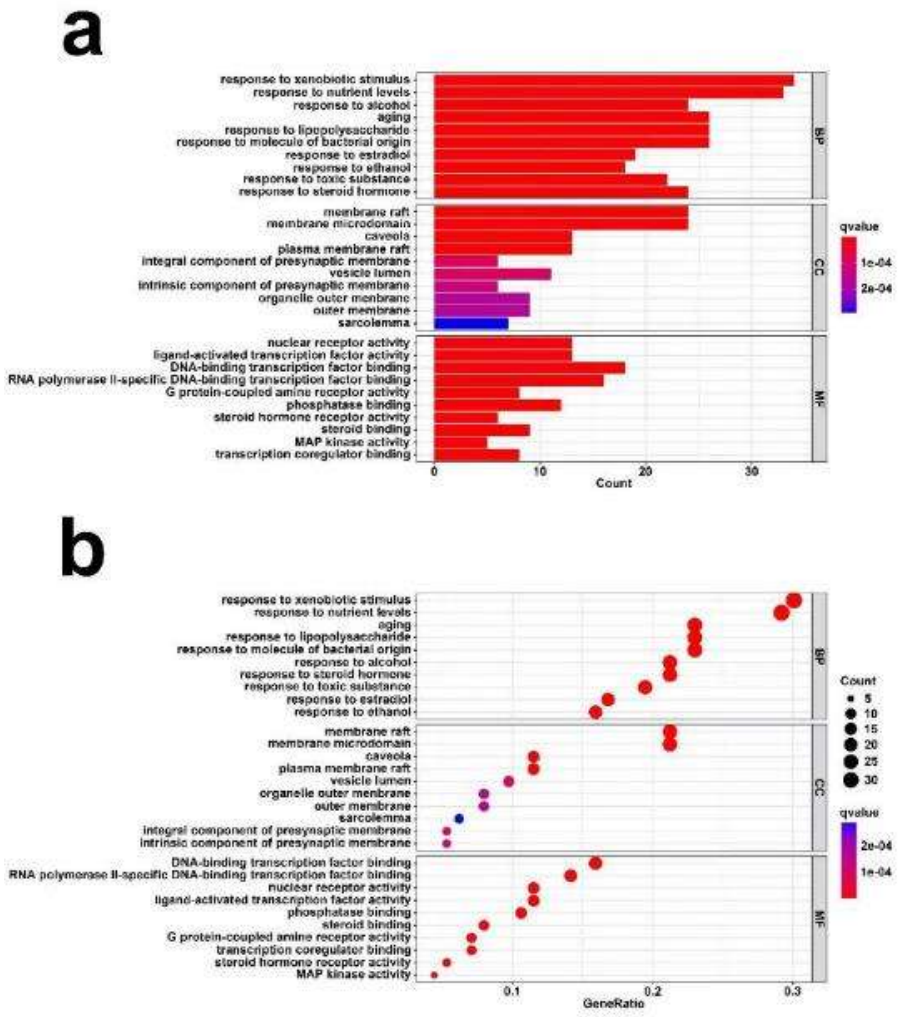

Figure 3: (A) Bar plot diagram of GO analysis for the main biological pathways and (B) KEGG enrichment analysis of the mainly enriched genes

Effects of *YuShu Soup* on EHS-induced physiology and kidney damage in rats

The Tco of rats which experienced EHS were significantly increased, whereas rats from *YuShu Soup* treated groups showed a lower Tco (Figure 4 A). The MBP of rats in EHS group was significantly reduced, while the reduction was neutralized by *YuShu Soup* treatment (Figure 4 B). Serological examination showed that expression of BUN and CK-MB increased in EHS group when compared to sham group, but no significant difference in SCr level among groups. Moreover, *YuShu Soup* significantly reduced production of BUN and CK-MB compared to EHS group (Figure 4 C). Severe tubular damage accompanied with glomerular injury was observed in EHS rats, while *YuShu Soup* at a dose of 4 mL/100 g significantly ameliorated tubular and glomerular damage induce by EHS. There was no significant improvement of tubular damage at low dose *YuShu Soup* (2 mL/100 g) (Figure 4 D). Therefore, *YuShu Soup* inhibited EHS-induced physiology and kidney damage at a dose-dependent manner.

***YuShu Soup* alleviated renal apoptosis induced by EHS**

The TUNEL staining assay revealed that EHS significantly aggravated apoptosis in renal tissue of rats. However, apoptosis was attenuated among rats in EHS + YSS (2 mL/100 g) and EHS + YSS (4 mL/100 g) groups (Figure 5 A). Expressions of apoptosis-related proteins (cleaved-caspase 3, Bax and Bcl-2) were estimated using western blotting assay. The results suggested that cleaved-caspase 3 and Bax levels in EHS model rats were significantly increased, while Bcl-2 expression was significantly reduced compared to sham group. More importantly, significant suppression was observed in EHS + YSS groups in a dose-dependent manner (Figure 5 B).

***YuShu Soup* enhanced HSP90 and AKT/Bcl-xL pathway**

Western blotting revealed that HSP90 expression in renal samples was significantly enhanced after EHS establishment, and further increased in *YuShu Soup*-treated rats (Figure 6 A) as well as serum samples (Figure 6 B). Furthermore, phosphorylation level of AKT was increased in renal cells of EHS model rats, and in EHS + YSS rats as well (Figure 6 C). In addition, protein expression level of Bcl-xL was reduced in kidney of rats and was activated by *YuShu Soup* therapy (Figure 6 D).

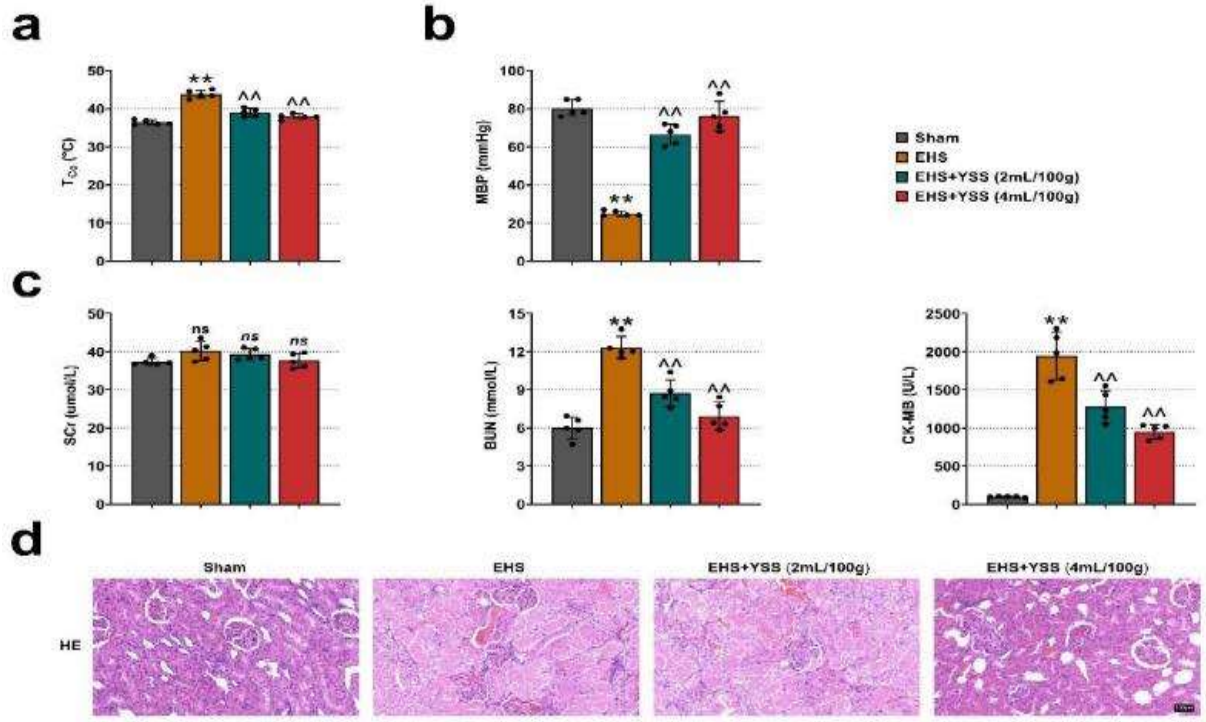

Figure 4: Effects of *YuShu Soup* on EHS-induced physiology and kidney damage in rats. The results of Tco (A) and MBP (B) in different group; (C) Serum concentrations of SCr, BUN and CK-MB in different groups; (D) Representative renal histopathological images of rats from indicated groups. Scale bar: 100 nm. Data are presented as mean ± SD (n = 5). ***P* < 0.01 compared to sham group. ^^*P* < 0.01 compared to EHS group

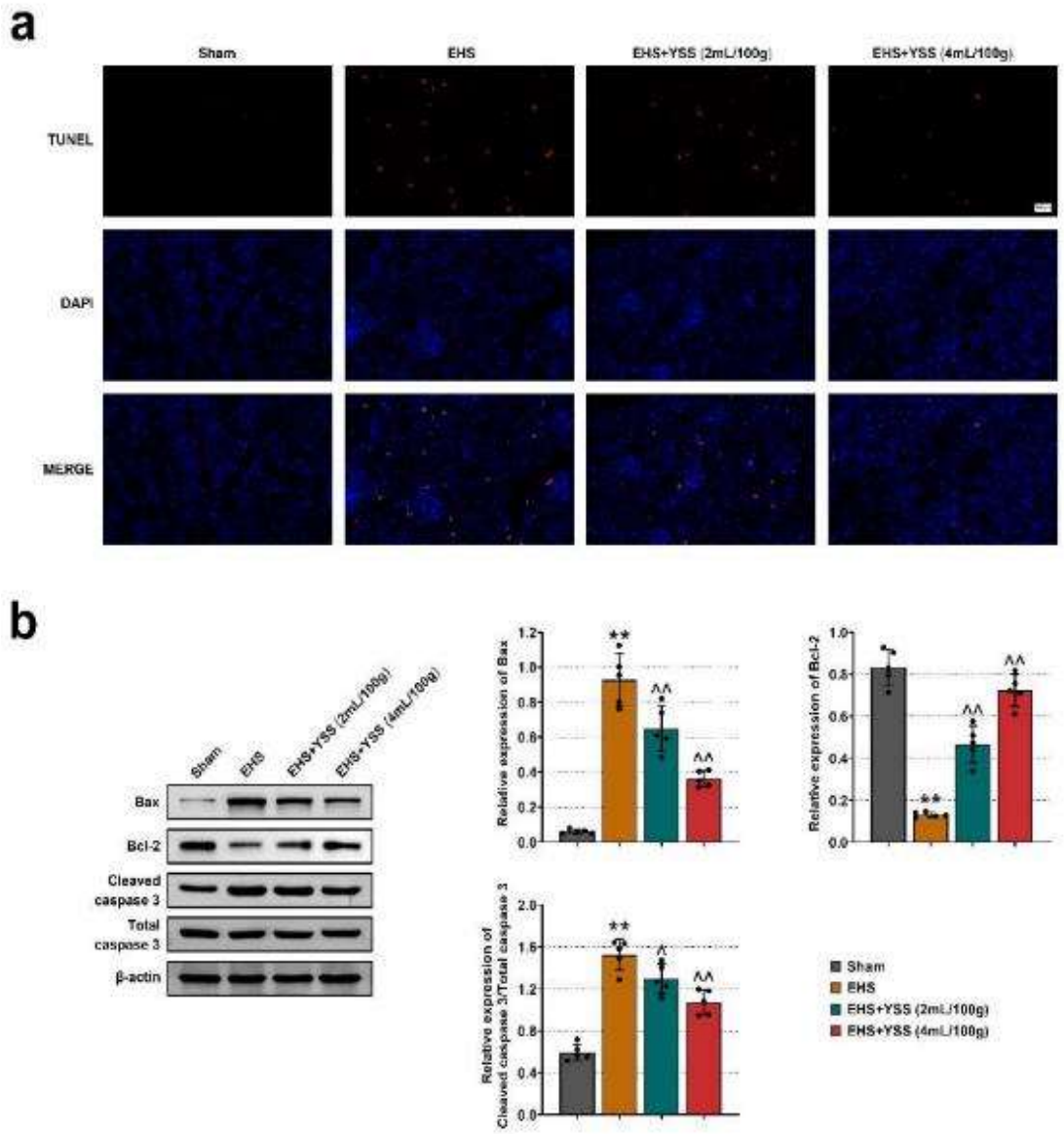

Figure 5: *YuShu Soup* alleviated renal apoptosis induced by EHS. (A) Representative TUNEL staining images of kidney tissues from rats of the indicated groups; (B) Protein expressions (left) and quantifications (right) of cleaved-caspase 3, total caspase 3, Bax and Bcl-2 in renal tissues of the rats treated as indicated. β-actin served as the loading control (n = 3). Data are presented as mean ± SD. ** $P < 0.01$ compared to sham group, ^$p < 0.05$, ^^$p < 0.01$ compared to EHS group

DISCUSSION

Exertional heatstroke (EHS) has a mortality rate of 5-50 % in humans, but all treatments for EHS have proved unsatisfactory. While traditional prescription *YuShu Soup* has been widely used for severe heat stroke, the mechanism underlying its use in EHS treatment remains unclarified. In this research, a network pharmacology was conducted to analyze the mechanism of *YuShu Soup* in EHS treatment. A total of 4656 targets of EHS disease were acquired from five public databases and the results revealed that *YuShu Soup* alleviated symptoms of EHS, apoptosis of renal cells in rats by regulating HSP90 and Akt/Bcl-xL pathway, thereby alleviating kidney damage. Elucidating these mechanisms would encourage clinical use of *YuShu Soup* to treat EHS.

Network pharmacology analysis is a novel approach which combines high-throughput histology, bioinformatics and systems biology, which were applied in this study on therapeutic effects of *YuShu Soup* in EHS treatment [11,12]. The traditional prescription of *YuShu Soup* is composed of 16 bioactive traditional herbs, and it has been widely utilized in heat stroke treatment in ancient times and until now.

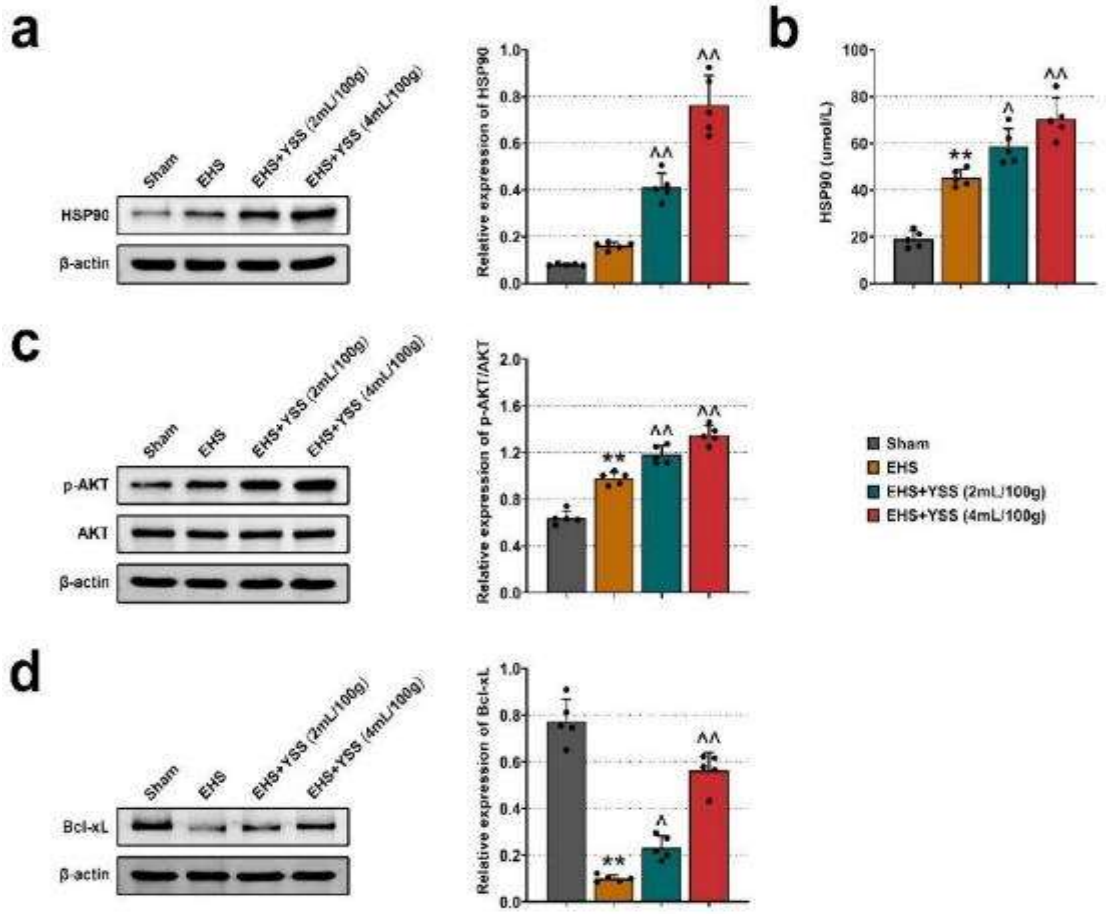

Figure 6: *YuShu Soup* promotes HSP90 and AKT/Bcl-xL pathway. (A) Protein expression (left) and quantification (right) of HSP90 in renal tissues in rat groups; (B) Serum HSP90 expression in rats groups; (C) AKT and p-AKT protein expression (left) and quantification (right) in kidneys of rat groups; (D) Protein expressions (left) and quantification (right) of Bcl-xL in kidneys of indicated rats (n = 3 for each group). β-Actin was the loading control. Data are presented as mean ± SD. ***P* < 0.01 compared with sham group, ^*p* < 0.05, ^^*p* < 0.01 compared with EHS group

Currently, it is known that traditional Chinese prescription has been characterized by multi-components and multi-targets, which is incomparable to Western drugs with single chemical components [13]. From the massive 4656 targets of EHS disease, a new subject of PPI network was acquired for a comprehensive understanding of components and potential pathways of *YuShu Soup* [14]. With heat wave around the world and increasing risk of EHS morbidity, EHS has attracted increasing attentions and become a new popular research [1]. Regrettably, the clinical therapies of EHS such as cooling, intravenous hydration, and hemodialysis filtration have proved to be unsatisfactory. In addition, there are few measures that aid the prevention of EHS [3,15]. This study has shown that *YuShu Soup* treatment contributed to significant improvements in physiological impairment, kidney injury and renal cell apoptosis induced by EHS.

Previous studies have reported that HSP90 expression significantly increased during heat stress, and they were closely associated with acute kidney injury and cell apoptosis [16]. However, underlying mechanism of HSP90 on renal impairment caused by EHS remains unclear, especially in treatment with *YuShu Soup*. Recent studies on myocardial cells suggested that interaction of AKT with HSP90 was required for AKT activation to perform its function of inhibiting cellular apoptosis through downstream signal proteins [17,18]. Similarly, this study revealed that protein expression of HSP90 in kidney, AKT phosphorylation as well as Bcl-xL expression were activated by *YuShu Soup*.

CONCLUSION

This study demonstrates that JUN, AKT1, MAPK3, MAPK1, STAT3, RXRA, CTNNB1 and RELA are the vital proteins in EHS treatment

using *YuShu Soup*. The data from rat experiments suggests that *YuShu Soup* alleviates renal apoptosis and kidney damage induced by EHS by regulating HSP90 expression and AKT/Bcl-xL pathway. This finding sheds light on prevention and treatment of EHS with the traditional Chinese prescription, *YuShu Soup*. Data derived from this research was only obtained from male rats; further investigation is necessary to determine whether female rats experience similar beneficial effect with *YuShu Soup* administration.

DECLARATIONS

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Ethical approval

This work was approved by Ethics Committee of the First Affiliated Hospital of Ningbo University (approval no. 2022012).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jing Dong designed the study and carried them out; Jing Dong, Zheng Yang and Chen Fan supervised the data collection, analyzed the data, interpreted the data; Jing Dong prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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