Canagliflozin is a potential cardioprotective drug but exerts no significant effects on pirarubicin-induced cardiotoxicity in rats

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Abstract. Pirarubicin (THP), one of the anthracycline anti-cancer drugs, is widely used in the treatment of various types of cancer, but its cardiotoxicity cannot be ignored. Canagliflozin, the first sodium-glucose co-transporter-2 inhibitor approved by the USA FDA, has been shown to have a significant effect on cardiovascular damage caused by diabetes. However, it has not been reported whether it can resist THP-induced cardiotoxicity. The aim of the present study was to investigate the effect of canagliflozin on THP-induced cardiotoxicity and its mechanism. A rat model of cardiotoxicity induced by THP was established and canagliflozin treatment was performed at the same time. The changes of electrocardiography, cardiac coefficient and echocardiogram were observed. The levels of lactate dehydrogenase, brain natriuretic peptide, creatine kinase MB, cardiac troponin T, superoxide dismutase (SOD) and malondialdehyde were detected. The expression of SOD2, NADPH oxidase 2, pro/cleaved-caspase- and Bcl-2/Bax were evaluated by western blotting. The primary culture of cardiomyocytes was prepared to explore the effect *in vitro*. After eight weeks, a series of cardiotoxicity manifestations were observed in THP rats. However, canagliflozin treatment had no significant effect on the above adverse reactions. Similarly, further studies showed that canagliflozin had no significant effect on THP-induced cardiomyocyte injury *in vitro*. The present study showed that there was no significant protective effect of canagliflozin on THP-induced cardiotoxicity and cardiomyocyte injury.

Introduction

Pirarubicin (THP), an analogue of doxorubicin, can interfere with the synthesis of DNA and mRNA, block the cell into G1 phase in cell proliferation cycle, interfere with tumor cell division and inhibit tumor growth; thus it has strong anti-cancer activity (1,2). Its chemical structure is a tetrahydropyran group inserted into the OH group at the 4' position of the amino sugar part of doxorubicin, which greatly reduces the toxic and side effects of THP (3). However, its cardiotoxicity cannot be ignored (4). At present, there is no completely effective treatment for THP-induced cardiotoxicity and the approved dexrazoxane is expensive (5).

Canagliflozin, a sodium-glucose co-transporter-2 (SGLT2) inhibitor, can reduce blood glucose by decomposing glucose and excreting it through the kidney (6). In addition to blood glucose control, canagliflozin also has cardiovascular protective effects, including reducing cardiac preload, improving hemodynamics, reducing inflammation and oxidative stress and improving cardiac energy supply. Studies have shown that canagliflozin can alleviate the cardiovascular symptoms of diabetic patients with or without cardiovascular diseases and has prospects of broad application in the cardiovascular field (7-9).

Superoxide dismutase (SOD) is an important antioxidant enzyme, which is widely distributed in various organisms. It is often used to measure the antioxidant capacity of tissues or cells (10,11). NADPH oxidase (NOX) is the key enzyme of redox signal and the main source of reactive oxygen species (ROS) (12). NOX2 was mainly expressed in the heart and increased when oxidative stress increased (13).

The present study was only a preliminary study to explore the cardiotoxic effect of THP and to understand the corresponding protective effect of caglitazine. It aimed to provide a theoretical basis for clinical prevention and treatment of anthracycline cardiotoxicity and cardiovascular protective effect of canagliflozin.

Materials and methods

Materials. Pirarubicin, purity ≥98%, was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd.. Canagliflozin was obtained from Janssen-Cilag International NV. Brain natriuretic peptide (BNP; cat. no. MB-1608A), creatine kinase MB (CK-MB; cat. no. MB-6930A) and cardiac troponin T (cTnT; cat. no. MB-7278A) test kits were purchased from Shanghai Meixuan Biological Science and Technology Ltd. Malondialdehyde (MDA; cat. no. A003-1-2), superoxide dismutase (SOD; cat. no. A001-3-2) and lactate dehydrogenase (LDH; cat. no. A020-2-2) test kits were obtained from Nanjing Jiancheng Bioengineering Institute. SGLT2 inhibitor (SGLT2i) was purchased from MedChemExpress. The antibodies for SOD2 (1:3,000; cat. no. 13141T), pro/cleaved-caspase-(1:1,000; cat. no. 14220T/9664T), Bcl-2/Bax (1:1,000; cat. no. 4223T/2772T) were obtained from Cell Signaling Technology, Inc. The antibody for NOX2 (1:1,000; cat. no. 19013-1-AP) was obtained from ProteinTech Group, Inc.. All chemicals and reagents were analytical grade.

Animal model. The present study was performed according to the Guide for the Care and Use of Laboratory Animals (14) and was approved by the Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (CMU; approval no. 20195101). A total of 40 Male Sprague Dawley (SD) rats (180-200 g; age, 6 weeks) were obtained from the CMU experimental animal center. SD rats were housed at $23\pm2°C$ with humidity of 40-60% and a 12/12-h light/dark cycle. Rats were randomly divided equally into 4 groups (n=10 in each group): normal group (CON; normal-diet-fed rats), canagliflozin group (canagliflozin-diet-fed rats, 60 mg·kg⁻¹), THP group (normal-diet-fed rats; 3 mg·kg⁻¹ THP was injected via caudal vein once a week) and canagliflozin + THP group (canagliflozin-diet-fed rats, 60 mg·kg⁻¹; 3 mg·kg⁻¹ THP was injected via caudal vein once a week). The food consumption and body weight was measured twice a week.

Electrocardiogram and Doppler echocardiography. The experiment ended at week 8. The rats were anesthetized with inhaled isoflurane (2%, maintenance dose was also 2%). Needle electrodes were inserted subcutaneously into the right upper limb, right lower limb and left lower limb respectively. The lead IV electrocardiography (ECG) was recorded by BL-420F biological function measurement system (Chengdu Taimeng Technology Company). The hair of the precordial region was removed and the Doppler echocardiography was measured by Vivid E95 ultrasonic diagnostic apparatus (General Electric Company).

Sample collection, preparation, section staining and biochemical indexes. At the end of the 8th week, the rats were weighed after fasting overnight and sacrificed by cervical dislocation

under anesthesia (inhalation of 2% isoflurane). Blood samples (1-2 ml per rat) were collected from abdominal aorta immediately after sacrifice and centrifuged at 314 x g, 4°C for 30 min within 6 h. The supernatant was frozen in a -80°C refrigerator and serum LDH, BNP, CK-MB, cTn-T, SOD and MDA contents were determined as soon as possible according to the operation procedure of the kit. Heart samples were excised and weighed. The left ventricular part of the heart was immersed in 10X its volume of 4% paraformaldehyde solution and stored for 4 h in a refrigerator. The rest of the left ventricular portion of the heart was stored in -80°C refrigerator for follow-up experiments. The next day, the heart tissue was dehydrated, dewaxed, embedded in paraffin and cut into 5 μm sections. Hematoxylin and eosin staining was performed according to the instructions of the kit (30°C, 30 min). TUNEL apoptosis detection kit (green fluorescence) was purchased from Beyotime Institute of Biotechnology. The paraffin section was dewaxed in xylene, dehydrated with absolute alcohol, washed with distilled water and then 20 μg/ml proteinase K without DNase added (37°C for 30 min), before washing with PBS for three times. TUNEL solution (50 μl) was added to the target area and incubated at 37°C for 60 min. DAPI staining solution (100%; Beyotime Institute of Biotechnology) was used to stain the nuclei (37°C, 3-5 min). After washing with PBS 3 times, an anti-fluorescence quenching sealing solution was used to seal the plates, which were observed under a fluorescence microscope (magnification, x200). A total of three fields of view were observed. Apoptosis level=apoptotic cells/total cells x100%.

Cell culture and treatment. A total of 20 neonatal SD rats (male, 1-3 days, CMU Experimental Animal Center) were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg) and disinfected with 75% ethanol. After the neonatal rats were sacrificed by cervical dislocation, the ventricles were quickly separated under aseptic conditions. The blood clots, blood vessels, fat and other tissues were washed 3 times in PBS buffer and then cut into sections with diameter <1 mm, digested by trypsin and II collagenase and then filtered, centrifuged, resuspended and seeded. Finally, primary rat cardiomyocytes were obtained by differential adhesion method [following 1.5 h culture in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (PAN-Biotech GmbH) and penicillin/streptomycin at 37.5°C with 5% CO_2, the culture supernatant containing cardiomyocytes was collected and re-seeded to obtain primary cardiomyocytes]. The primary cardiomyocytes were divided into four groups: Normal group (CON), canagliflozin group (canagliflozin, 60 μm, 14 h), THP group (THP, 10 μm, 12 h), THP and cana-gliflozin co culture group (canagliflozin, 60 μm, 14 h + THP, 10 μm, 12 h). In canagliflozin +THP group, the cells were pre incubated with canagliflozin (60 μm) for 2 h and then co cultured with THP (10 μm) for 12 h.

Western blotting. Heart tissue and primary rat cardio-myocytes was lysed in radioimmunoprecipitation (RIPA) lysis buffer. BCA kit was used to determine the protein concentration in the supernatant. Then ~50 μg heart tissue lysate or 20 μg of cell lysate was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) and
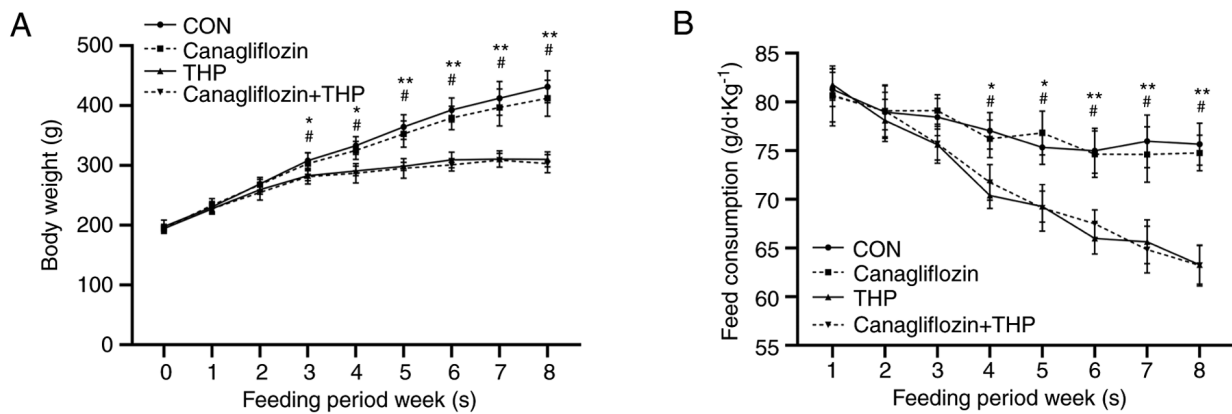
Figure 1. Effect of THP on body weight and food intake of rats and the therapeutic effect of canagliflozin. (A) From the third week, the body weight of rats injected with THP alone was significantly lower than that of normal rats (THP vs. CON; P<0.05). The weight loss of THP rats was further reduced after five weeks (P<0.01; THP vs. CON). The above changes were not significantly improved following canagliflozin (60 mg/kg) treatment. (B) From the fourth week, the food intake of rats injected with THP alone was significantly lower than that of normal rats (THP vs. CON; P<0.05). The food intake of THP rats decreased further after six weeks (P<0.01; THP vs. CON). The above changes were not significantly improved following canagliflozin (60 mg/kg) treatment. All values are the mean ± SD. *P<0.05 vs. CON; **P<0.01 vs. CON; #P>0.05 vs. THP. THP, pirarubicin; CON, normal group; NOX, NADPH oxidase; SOD, superoxide dismutase.

proteins were then transferred to an FL PVDF membrane (EMD Millipore) at 4°C for 1.5 h. After blocking with 5% blocking protein powder (room temperature), the first anti-body was incubated overnight at 4°C and the second antibody was incubated at room temperature for 1.5 h. The western blotting results were analyzed by BeyoECL Plus (Beyotime Institute of Biotechnology) in Image Lab (version: 5.2.1; Bio-Rad Laboratories, Inc.). The specific protein expression levels were normalized to GAPDH.

Statistical analysis. Data were presented as mean ± SD. The significance of differences between groups were analyzed statistically using one or two-way analysis of variance (ANOVA), followed by a Tukey's multiple-comparison post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

THP causes the decrease of body weight and food intake, but canagliflozin has no effect. The body weight (Fig. 1A, P<0.05 vs. CON) and food intake (Fig. 1B, P<0.05 vs. CON) of THP rats began to decrease in the third and fourth weeks, especially in the fifth and sixth week (P<0.01 vs. CON). However, there was no significant improvement in the above changes after treatment with canagliflozin (Fig. 1, P>0.05 vs. THP).

Canagliflozin does not improve the THP-induced changes of ECG and echocardiography in rats. At 8 weeks after THP injection, a series of ECG and echocardiographic (Fig. 2) changes occurred in SD rats, including: Ejection fraction (Fig. 2A) and fractional shortening Fig. 2B) decreased, left ventricular internal diameter end diastole (Fig. 2C) and left ventricular internal diameter end systole (Fig. 2D) increased; R wave (Fig. 2E) and T wave (Fig. 2F) increased; S wave (Fig. 2G) decreased; QT interval (Fig. 2H) was prolonged.
Following canagliflozin treatment, the above changes were not significantly improved (Fig. 2A-H; P>0.05 vs. THP).

Canagliflozin has no significant effect on THP-induced cardiac tissue changes and apoptosis in rats. As shown in Fig. 3, the arrangement of cardiomyocytes was disordered, the intercellular space was enlarged and the cardiomyocytes were focal vacuolization or steatosis in the rats injected with THP alone. Compared with THP group, the treatment of cana-gliflozin showed no significant improvement on cardiac tissue.
TUNEL staining (Fig. 3) showed that there was no cardio-myocyte apoptosis in CON and canagliflozin group, but there was regional cardiomyocyte apoptosis in THP injection group. The treatment of canagliflozin had no effect on THP-induced cardiomyocyte apoptosis. The quantitative results are shown in Fig. 3A.

The role of THP and canagliflozin in blood and heart tissue biochemical indexes. The SD rats were sacrificed after 8 weeks. Blood and heart tissue samples were collected and tested.
In blood, THP caused the decrease of SOD level (Fig. 4A) and the increase of MDA (Fig. 4B), LDH (Fig. 4C), CK-MB (Fig. 4D), cTnT (Fig. 4E) and BNP (Fig. 4F). However, the treatment of canagliflozin did not effectively improve the above changes (Fig. 4A-F; P>0.05 vs. THP).
The same was true of heart tissue, THP-induced the decrease of SOD level (Fig. 4G) and the increase of MDA (Fig. 4H), LDH (Fig. 4I), CK-MB (Fig. 4J), cTnT (Fig. 4K) and BNP (Fig. 4L) in rat heart. However, the treatment of canagliflozin does not effectively improve the above changes (Fig. 4G-L, P>0.05 vs. THP).

Effects of THP and canagliflozin on the expression of related proteins in vivo. As shown in Fig. 5, THP injection for 8 weeks led to the decrease of the protein expression of SOD2, pro-caspase- and Bcl-2/Bax and the increase of the protein expression of NOX2 and cleaved-caspase-, which suggested that THP caused oxidative stress and increased apoptosis in rat heart. However, treatment with canagliflozin does not effectively improve the above changes (Fig. 5; P>0.05 vs. THP). Further evidence was provided by quantitative analysis (Fig. 5).
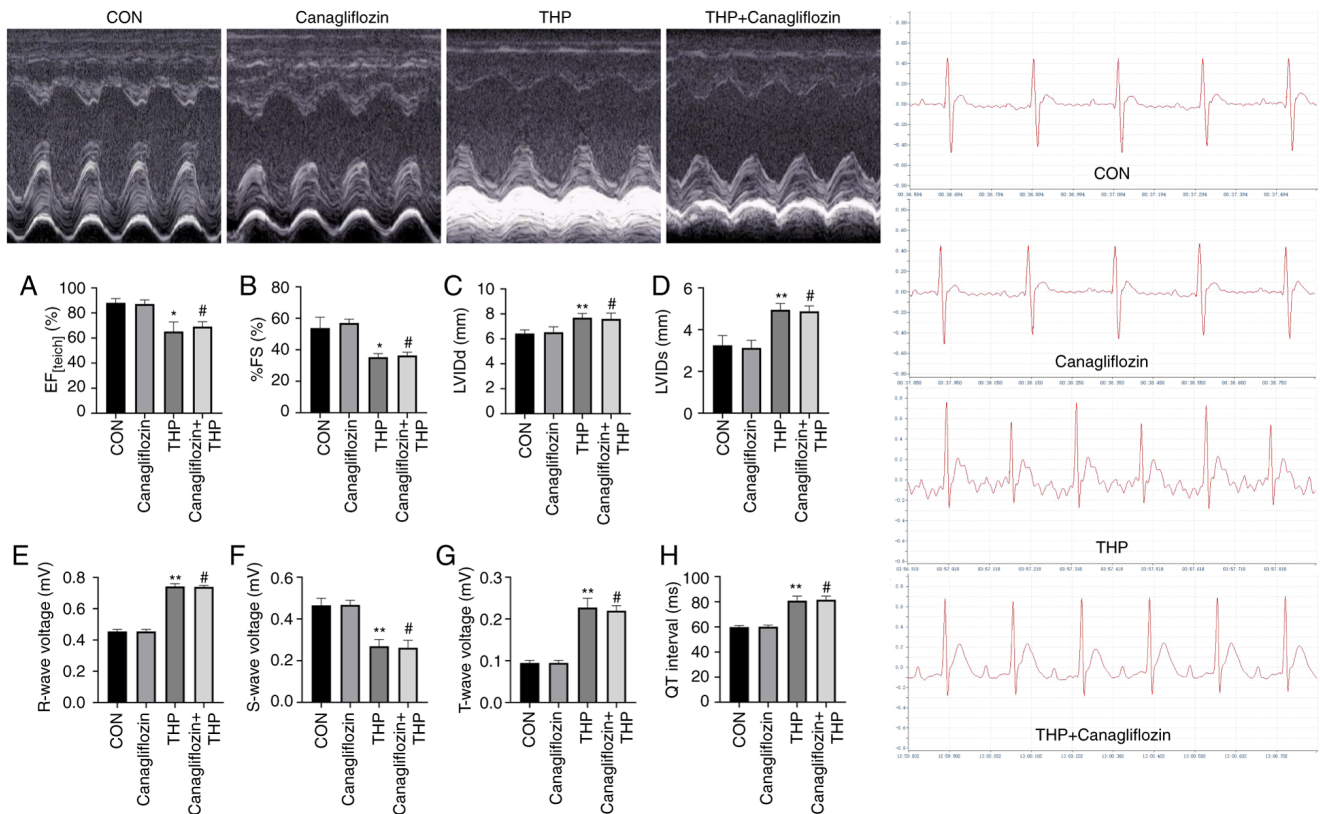
Figure 2. Canagliflozin does not improve the THP-induced changes of ECG and echocardiography in rats. THP caused the changes of ECG in rats, but there was no improvement after canagliflozin (60 mg/kg) treatment. So is echocardiography (A) EF and (B) FS decreased; (C) LVIDd and (D) LVIDs increased; (E) R wave and (F) T wave increased; (G) S wave decreased and (H) QT interval was prolonged. Following canagliflozin treatment, the above changes were not significantly improved (Fig. 2A-H; P>0.05). All values are the mean ± SD. *P<0.05 vs. CON; **P<0.01 vs. CON; #P>0.05 vs. THP. THP, pirarubicin; ECG, electrocardiography; EF, ejection fraction; FS, fractional shortening; LVIDd, left ventricular internal diameter end diastole; LVIDs, left ventricular internal diameter end systole; CON, normal group.
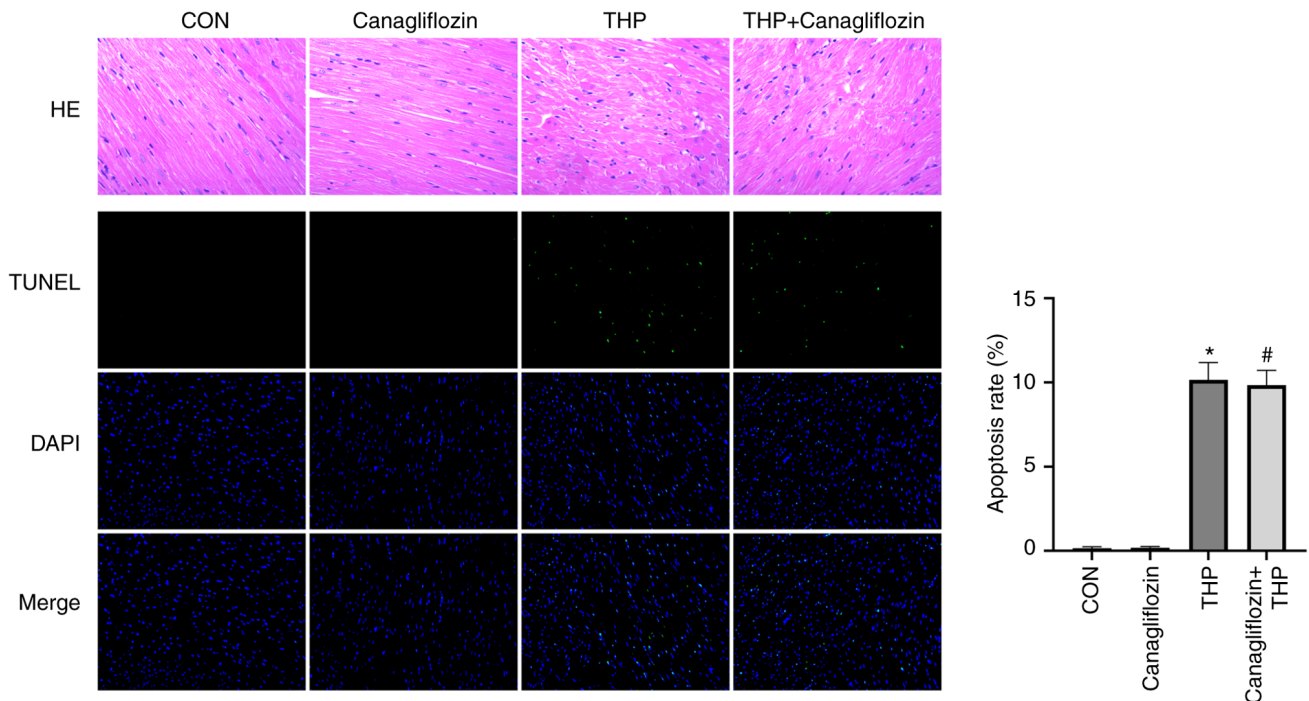

Figure 3. Effects of canagliflozin in THP-induced histopathology changes and apoptosis in cardiac tissue. The rats in CON group showed normal structure of heart tissue. There was no significant change in canagliflozin group. In THP group, the arrangement of myocardial cells was disordered, the intercellular space was enlarged and the myocardial cells exhibted focal vacuolization or steatosis. Compared with the THP group, there was no significant improvement in cardiac tissue in the canagliflozin + THP group. Magnification, x200. All values are the mean ± SD. *P<0.05 vs. CON; #P>0.05 vs. THP. THP, pirarubicin; CON, normal group; HE, hematoxylin and eosin stain.
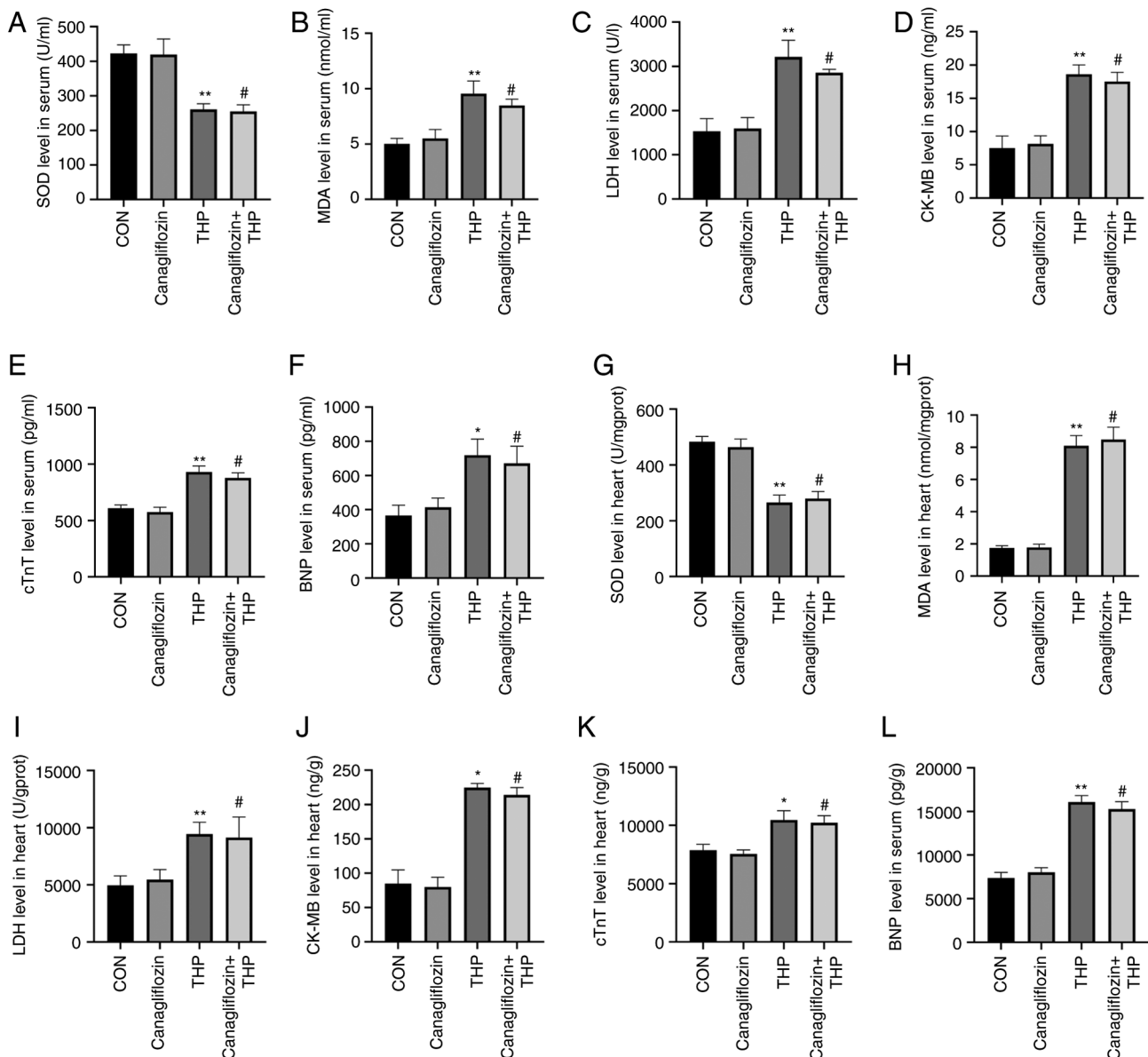
Figure 4. Canagliflozin cannot effectively improve the level of serum and heart tissue related biochemical markers of THP-induced heart injury. (A) SOD, (B) MDA, (C) LDH, (D) CK-MB, (E) cTnT and (F) BNP levels in serum. (G) SOD, (H) MDA, (I) LDH, (J) CK-MB, (K) cTnT and (L) BNP levels in heart. All values are the mean ± SD. *$P<0.05$ vs. CON; **$P<0.01$ vs. CON; #$P>0.05$ vs. THP. THP, pirarubicin; SOD, superoxide dismutase; MDA, malondialdehyde; LDH, lactate dehydrogenase; BNP, brain natriuretic peptide; CK-MB, creatine kinase MB; cTnT, cardiac troponin T; CON, normal group.

Effects of THP and canagliflozin on the expression of related proteins in vitro. The same applied *in vitro*. As shown in Fig. 6: THP treatment of cardiomyocytes led to the decrease of the protein expression of SOD2, pro-caspase- and Bcl-2/Bax and the increase of the protein expression of NOX2 and cleaved-caspase-, which suggested that THP caused oxidative stress and increased apoptosis in rat cardiomyocytes. However, the treatment of SGLT2i does not effectively improve the above changes (Fig. 6; $P>0.05$ vs. THP). Further evidence was provided by quantitative analysis (Fig. 6).

Discussion

In accordance with parts of the hypothesis of the present study, the body weight and food intake of rats were significantly decreased after intravenous injection of 10 mg·kg⁻¹/day

THP for 8 weeks. A series of cardiotoxic manifestations were observed, including changes in echocardiography and electrocardiogram outputs, increased LDH, CK-MB, cTnT and BNP levels in serum and heart. Additionally, THP effectively induced oxidative stress and apoptosis in the heart, reduced SOD activity and increased MDA levels in serum and heart, leading to significant changes in protein expression in the heart. However, against parts of the hypothesis of the present study, adding canagliflozin (60 mg·kg⁻¹/week) to rat diet did not improve these THP-mediated conditions. In brief, the *in vitro* studies failed. Western blotting data showed that THP still induced oxidative stress and apoptosis in cardiomyocytes, but canagliflozin could not improve this state and similarly no significant differences was observed when compared with the THP group. These results suggested that the cardioprotective effect of canagliflozin may not
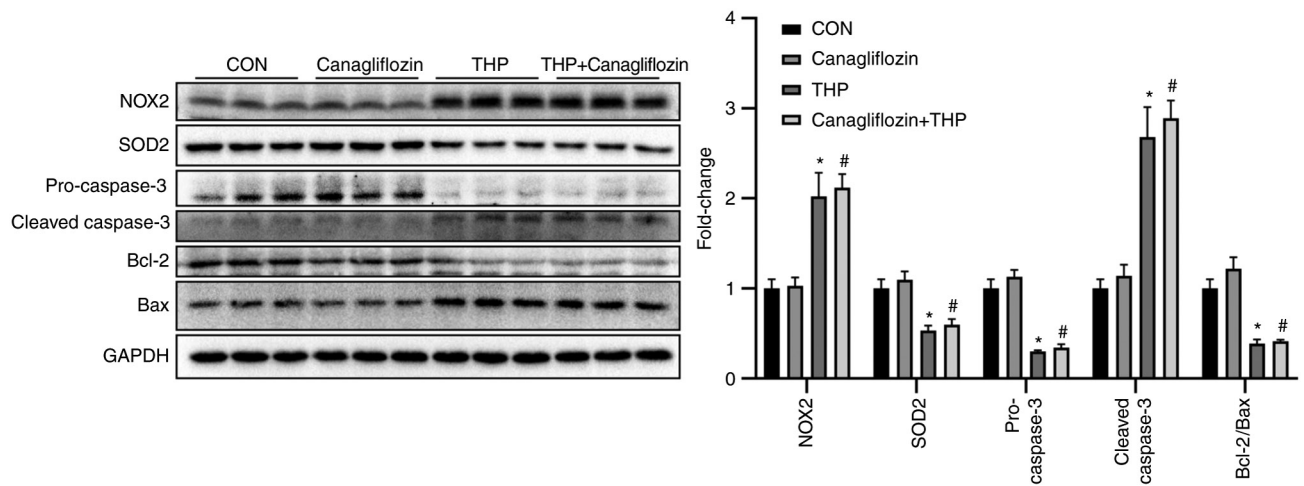
Figure 5. Effects of THP and canagliflozin on the expression of related proteins *in vivo*. THP injection for 8 weeks led to the decrease of the protein expression of SOD2, pro-caspase- and Bcl-2/Bax and the increase of the protein expression of NOX2 and cleaved-caspase-3 in rat heart. However, the treatment of canagliflozin did not effectively improve the above changes. All values are the mean ± SD. *P<0.05 vs. CON; #P>0.05 vs. THP. THP, pirarubicin; SOD, superoxide dismutase; NOX, NADPH oxidase.
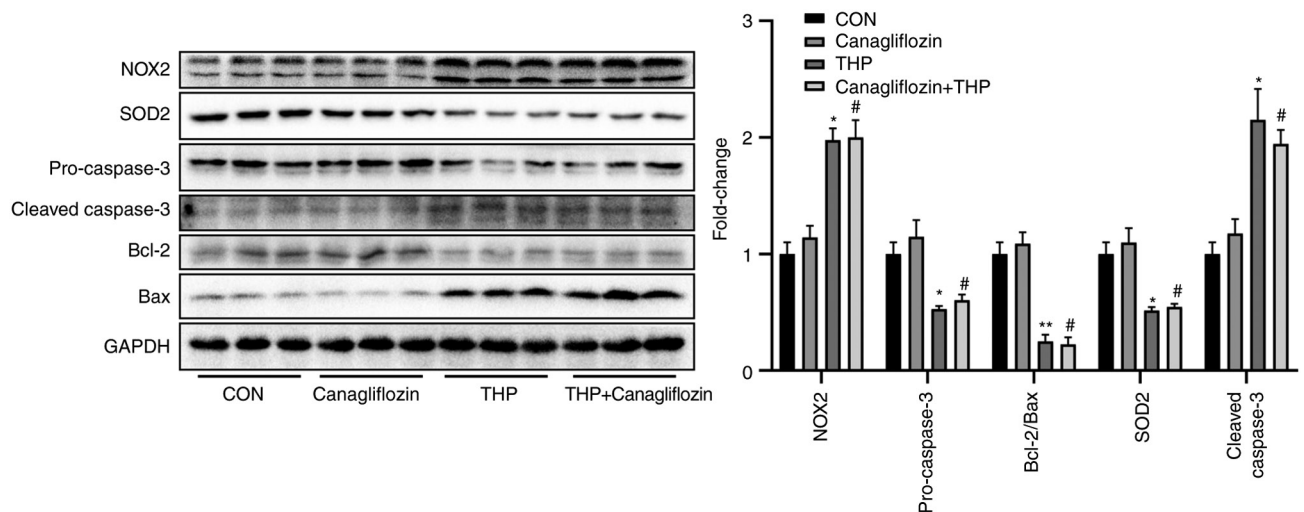

Figure 6. Effects of THP and canagliflozin on the expression of related proteins *in vitro*. THP injection for 8 weeks led to the decrease of the protein expression of SOD2, pro-caspase- and Bcl-2/Bax and the increase of the protein expression of NOX2 and cleaved-caspase- in rat cardiomyocytes. However, the treatment of SGLT2i did not effectively improve the above changes. All values are the mean ± SD. *P<0.05 vs. CON; **P<0.01 vs. CON; #P>0.05 vs. THP. THP, pirarubicin; SOD, superoxide dismutase; NOX, NADPH oxidase; SGLT2, sodium-glucose co-transporter-2.

function during THP-induced cardiotoxicity and myocardial cell injury.

An important study outcome was that THP induced cardiotoxicity in rats, which may have been caused by oxidative stress and increased cardiomyocyte apoptosis. Currently, it is generally accepted that anthracycline induced cardiotoxicity is cumulative and dose-dependent (15). Reactive oxygen species (ROS), oxidative stress induced by lipid peroxidation and cardiomyocyte apoptosis all have dominant roles in anthracycline induced cardiotoxicity (16). SOD is one such important antioxidant enzymes in organisms (17), with the SOD2 protein expressed in mitochondria (18). Previous studies have shown that excessive consumption of mitochondrial SOD2 causes mitochondrial damage and apoptosis (18,19). NADPH oxidase consumes oxygen and produces superoxide which is also the main source of

ROS in cardiovascular system (20). NOX2 is a classical representative structural model of NADPH oxidase and is also the main form expressed in cardiomyocytes (20). NOX2, via its quinone structure, generates high ROS levels during metabolism, leading to cardiomyocyte apoptosis and necrosis (3,21,22). In addition, THP also chelates iron ions and triggers oxygen free radicals, resulting in lipid peroxidation of myocardial cell membranes and mitochondrial DNA damage (23). Paglia and Radcliffe (24) reported that increased iron ion levels enhances the sensitivity of cardiomyocytes to DOX, thereby increasing ROS free radical production, leading to oxidative stress and damage to myocardial tissue ultrastructures and cardiomyocytes. THP also induced cardiomyocyte apoptosis, which was putatively related to decreased Bcl-2/Bax ratios and caspase family activation (25,26). The Bcl-2/Bax ratio is typically reflective

of the degree of apoptosis (27). When this ratio decreases, permeability of the mitochondrial outer membrane changes, releasing cytochrome c and apoptosis-inducing factors to the cytoplasm, caspase cascade reaction and caspase-independent pathways are involved in the occurrence of apoptosis (28-30).

Another unexpected outcome of the present study was that canagliflozin, which is believed to have strong cardiovascular protection potential (7,8,31), did not exhibit corresponding cardiovascular protection in a THP-induced cardiotoxicity model. A similar phenomenon was also apparent in the *in vitro* studies. As previously mentioned, the cardiotoxicity induced by THP is mainly due to the THP accumulation in cardiomyocytes, concomitant with excessive ROS production and eventual apoptosis (29,32). Canagliflozin inhibits SGLT2, with studies showing that SGLT2 is mainly distributed in the renal cortex and specifically binds to the SGLT2 receptor at this location (32). In addition to blood glucose control, the cardiovascular protective effect of canagliflozin are attractive qualities with a broad application base (7,33). Canagliflozin increases urinary sodium excretion, reduces water and sodium retention, alleviates pre- and post-cardiac loads and exerts cardiovascular protection (34). THP-induced cardiotoxicity also causes hemodynamic changes to a certain extent, but the condition is not caused by sodium and water retention, but by direct damage to the heart (35). The present study hypothesized that this is one of the main reasons why canagliflozin cannot exert its effect. In addition, previous studies have shown that canagliflozin reduces inflammation and oxidative stress in patients with T2DM and atherosclerosis (36,37). The present study hypothesized that this beneficial protective effect is closely related to weight loss and hypoglycemic effect, but THP does not lead to abnormal increase in blood glucose and blood lipid levels in rats, which may be another important reason for the ineffectiveness of canagliflozin. Increasing myocardial energy metabolism efficiency, inhibiting Na^+-H^+ exchange protein activity, reducing cytoplasmic Na^+ and Ca^{2+} concentration and increasing mitochondrial Ca^{2+} concentration may be another way for canagliflozin to exert myocardial protective effect, which has practical significance for THP-induced cardiotoxicity (38,39). The present study hypothesized that this effect may not be the main pharmacological action of canagliflozin in protecting heart, but its effects on improving THP cardiotoxicity are limited.

The present study showed THP-induced cardiomyocyte injury *in vivo* and *in vitro*, possibly caused by increased oxidative stress and apoptosis. It was only a preliminary study and there are a number of deficiencies, including the lack of positive control drugs. However, the authors of the present study suggested that the cardiac toxicity model based on THP is a mature model, which does not affect the experimental conclusion: In the present study, it appeared that caglitazine did not improve the cardiac toxicity induced by THP. Future studies are required to analyze the potential cardioprotective effects of canagliflozin.

Acknowledgements

Funding

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to patent application but are available from the corresponding author on reasonable request.

Authors' contributions

HS, QZ, PP and RF conceptualized the study and analyzed and interpreted data. YW, HY and HT analyzed and interpreted data and revised the manuscript critically for important intellectual content. DW designed the study and analyzed the data. PP and RF drafted the manuscript. All authors confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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