Cardioprotection of Sodium–Glucose Cotransporter 2 Inhibition in Rats With Isoproterenol-Induced Cardiomyopathy

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Research

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Abstract

**Background:** Sodium-glucose cotransporter 2 inhibitor (SGLT2i) has been reported to improve glycaemic control in patients with type 2 diabetes. The aim of this study was to investigate the effect of SGLT2i Dapagliflozin (Dapa) on cardiomyopathy induced by isoproterenol (ISO) and its potential mechanism.

**Methods:** Fifty male Sprague Dawley rats were randomly assigned to Control (n = 10) and ISO (2.5 mg/kg/day)-treated groups (n = 40). After 2 weeks, 28 survived rats with obvious left ventricular dysfunction in ISO group were randomized into three groups for medication including ARNI (angiotensin receptor neprilysin inhibitor, 68 mg/kg/day, n = 9), Dapa (3 mg/kg/day, n = 9) and ISO (saline, n = 10) for 4 weeks. After that, electrical programmed stimulation (EPS) was performed in all groups for the evaluation of the susceptibility of ventricular arrhythmias (VAs). Echocardiography was used to evaluate cardiac function.

**Results:** Echocardiography revealed significant left ventricular (LV) dysfunction in rats with ISO treatment for 2 weeks compared to the control group. Dapa administration for 4 weeks reduced the cumulative risk of death, myocardial fibrosis, plasma angiotensin II level and its functional receptor AT1R protein expression in the heart, and proinflammatory cytokines levels in the cardiac tissue of ISO-treated rats. It also improved cardiac function and inhibited oxidative stress when compared to the ISO group. These effects were similar to ARNI. However, Dapa showed a greater efficacy than ARNI in reducing left ventricular end-diastolic volume, lowering heart rate and VAs, and decreasing body weight and plasma glucose in ISO-treated rats.

**Conclusion:** Dapa effectively improved the myocardial remodelling and oxidative stress like ARNI in ISO-induced cardiomyopathy in rats, but Dapa may be more effectively in decreasing VAs, and improving cardiac function when compared to ARNI. The mechanisms by which Dapa exerts protective effects on cardiomyopathy may be related to its antioxidant capacity and hypoglycemic action.

Background

Sodium-glucose cotransporter 2 (SGLT2) transporter inhibitors (SGLT2i) such as Dapagliflozin (Dapa), as a new class of anti-diabetic drugs, are proven to have beneficial effects beyond the glucose-lowering effects, such as reducing visceral fat, inhibiting inflammation and oxidative stress, and having cardiac protective effects [1-4]. The Dapagliflozin And Prevention of Adverse-outcomes in Heart Failure (DAPA-HF) Trial outcomes showed that the SGLT2i markedly reduced mortality and improved heart failure remarkably, these benefits seemed to be similar in people with and without diabetes [5]. Angiotensin receptor neprilysin inhibitor (ARNI), beyond blocking angiotensin II signaling, augments natriuretic peptides by inhibiting their breakdown by neprilysin, and becomes class I drug recommended for the treatment of heart failure in the recent years [6]. At present, more attention was paid to the effects of SGLT2i on cardiovascular system [7-8]. A recent nationwide population-based longitudinal cohort study revealed that patients with type 2 diabetes prescribed with SGLT2i were associated with a lower risk of all-cause mortality and new-onset arrhythmias compared with those not taking SGLT2i in real-world practice [9].

Isoproterenol (ISO) as a synthetic nonselective β-adrenoceptor agonist is well accepted to induce myocardial damage in rats for evaluating cardiac dysfunctions [10]. The pathophysiological and morphological changes of ISO-induced myocardial changes are similar to those observed in human with myocardial infarction or heart
failure. Therefore, ISO-induced myocardial damage is a well-standardized animal model to study the protective effects of many drugs on cardiac dysfunctions. Persistent β-adrenergic stimulation with ISO results in cardiomyocytes injury, generation of reactive oxygen species (ROS), arrhythmias, ventricular hypertrophy and increased fibrosis, inflammation and collagen deposition [11]. Experimental and clinical studies have shown that SGLT2i therapy can prevent or ameliorate cardiac dysfunction through inhibition of oxidative stress, inflammation and so on [12].

The present study was designed to explore the cardiac effects of SGLT2i Dapa in rats with cardiomyopathy induced by ISO and compare the protective effects of ARNI on the heart.

Materials And Methods

Animals

Experimental animal care and use complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 8th edition, 2011). All experiment procedures were approved by the Nanjing Medical University. Fifty male Sprague-Dawley rats weighting 200-250 g were purchased from Nanjing Medical University Laboratory Animal Center. All rats were caged in a room with controlled temperature and humidity with a 12-h light/dark cycle and provided a standard chow and drinking water ad libitum. Rats were randomly assigned to Control group (n = 10) and isoproterenol induced cardiomyopathy group (ISO, n = 40). Rats in ISO group were intraperitoneally injected 2.5 mg/kg/d isoproterenol hydrochloride (Sigma, Switzerland) dissolved in normal saline, once a day for 2 weeks [13, 14]. Echocardiography was performed at the end of the 2nd week and the 6th week. After echocardiography measurement at the end of the 2nd week, 28 survival rats in ISO group were randomized into three groups including ARNI (angiotensin receptor neprilysin inhibitor, n = 9), Dapa (Dapagliozin, n = 9) and ISO (saline, n = 10) groups. ARNI (Novartis Pharma Schweiz AG, Chinese national medicine permission number J20190001) was administered intragastrically at a dose of 68 mg/kg, and Dapa (AstraZeneca Pharmaceuticals LP, Chinese national medicine permission number J20170040) was administered intragastrically at a dose of 3 mg/kg for 4 weeks, respectively. The medication method for ARNI and Dapa was adopted according to previously published literature [15, 16, 17].

Assay of Cardiac Function-related Parameters

Echocardiography was performed at the end of the 2nd week and the 6th week. The rats were anesthetized with ketamine, and then the cardiac function was evaluated using a Vevo 2100 (VisualSonics, Canada) system equipped with a MS-250, 16.0-21.0 MHz imaging transducer.

Electrical Programmed Stimulation

At the end of the 6th week, all rats underwent ventricular electrical programmed stimulation (EPS) for the evaluation of susceptibility of ventricular arrhythmias (VAs) before being sacrificed. They were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (50 mg/kg). Three needle electrodes were placed on the right upper limb and legs to perform electrocardiography. Then EPS was used to stimulate the left ventricular apex of the heart through a bipolar electrode and the incidence of ventricular arrhythmias (VA) was investigated. By a cycle length of 140 ms, the threshold potential for stable pacing was achieved. Pacing was
started with twice as much as the threshold and a cycle length of 140 ms, which was the interval of eight stimuli (S1). An extra stimulus (S2) was applied until it failed to induce ventricular depolarization, while the interval between S1 and S2 was progressively shortened by 10 ms.

**Samples and Histopathology**

Animals were sacrificed after EPS immediately. Blood was collected from the descending aorta. After being weighed and washed with ice-cold PBS, one part of the heart was cut and fast frozen by liquid nitrogen, then moved to − 80 °C for further detection. The other part fixed in 4% paraformaldehyde was used for staining. Masson's trichrome staining was performed to detect cardiac fibrosis. Five fields of each sample were randomly selected and collagen volume fraction (CVF) was assessed by Image-Pro Plus 6.0.

**Measurement of Plasma Angiotensin II (Ang II)**

Plasma level of Ang II was measured from the blood collected from the abdominal descending aorta. Blood was collected into tubes containing EDTA, and then centrifuged at 3000 rpm at 4 °C for 15 mins to separate the plasma. Plasma Ang II level was determined using enzyme linked immunosorbert assay (ELISA) kit. All steps were carried out in accordance with the manufacture's specifications (Abcam Inc, UK). The final solution was read by a microplate reader (ELX800, BioTek, Vermont, USA).

**Measurement of Plasma Glucose and Cardiac MDA Levels**

Plasma level of glucose was detected by the glucose-oxidase method using a commercially available glucose assay kit from Jiancheng Bioengineering (Nanjing, Jiangsu, China). The level of MDA, in the heart tissue, was detected by using a lipid peroxidation (malondialdehyde; MDA) assay kit from Jiancheng Bioengineering (Nanjing, Jiangsu, China). Lipid peroxidation was determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present. The intensity of the color was measured spectrophotometrically at 505 nm for glucose and 532 nm for MDA.

**Measurement of Superoxide Anions**

The lucigenin-derived chemiluminescence method was used to examine superoxide anions level in the cardiac tissue. Superoxide anions can react with dark-adapted lucigenin (5 μM) resulting in photon emission which can be captured once every minute for 10 mins by a luminometer (20/20n, Turner, Sunnyvale, CA, USA). The superoxide anions levels were expressed as the mean light units (MLU) per minute per milligram of protein [18].

**Measurement of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Activity**

The enhanced lucigenin chemiluminescence method was used to detect NADPH oxidase activity. NADPH oxidase can react with NADPH substrate (100 μM) in the medium to generate superoxide anions which can react with lucigenin (5 μM) to produce light emission. A luminometer (20/20n, Turner, CA, USA) can capture the light emission once every minute for 10 mins. The NADPH oxidase activity could be expressed as the (MLU) per minute per milligram of protein [19].

**Western Blotting**
Protein expressions of angiotensin II type-1 receptor (AT1R, antibody from Endo Life Science Inc, USA), the superoxide (O$_2^-$)-generating NADPH oxidase isoforms (NOX2 and NOX4, antibodies from Abcam, Burlingame, CA, USA), and inflammatory markers including TNFα, IL-1β, IL-6 and IL-10 (antibodies from Proteintech, Chicago, IL, USA) in myocardial tissue were detected by Western blotting [20]. Simply, total cardiac proteins in the homogenate were extracted and measured. Antibodies AT1, NOX2, NOX4, TNFα, IL-1β, IL-6 and IL-10 were applied according to the manufacturer's instructions. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were used as secondary antibody. Protein expression level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, antibody from Proteintech, Chicago, IL, USA). The signals were quantified by using Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).

Kaplan-Meier Analysis

Survival over the 6-week experiment was analyzed according to the daily recording of deaths by the standard Kaplan-Meier analysis with the log rank test.

Statistics

Data are expressed as mean ± SEM and analyzed by GraphPad Prism v8.0.2 (GraphPad Software, CA). Comparisons between the two groups performed with two-tailed unpaired $t$ test. For multiple-group comparisons, data were performed using one-way ANOVA followed by Bonferroni's post-hoc test. A value of $P<0.05$ was considered statistically significant.

Results

Dapa Ameliorated ISO-induced Cardiac Dysfunction in ISO-treated Rats

At the end of the 2nd week, echocardiography showed significant increases in diastolic left atrial diameter and left ventricular interventricular diameter as shown by the representative tracings of echocardiography (Figure 1A), and a reduction in ejection fraction (EF) and fractional shortening (FS) in ISO group compared with the Control group ($P<0.05$, Table 1). The left ventricular end-diastolic volume (LVEDV) was increased in ISO group compared with the Control group ($P<0.05$, Table 1). These results showed that ISO-induced significant impairment of cardiac function. At the end of the 6th week, EF and FS were notably increased in Dapa and ARNI groups compared with ISO group ($P<0.05$, Table 1), while LAD and LVID increases were significantly improved in ARNI and Dapa groups compared with the ISO group (Figure 1B). But LVEDV and heart rate in Dapa group were lower than in ARNI group ($P<0.05$, Table 1).

Dapa Inhibited the Occurrence of Ventricular Arrhythmias in ISO-treated Rats

Electrical programmed stimulation (EPS) was performed in all groups in order to induce VAs. The original images of electrocardiography were shown in Figure 2A. The mean voltage level of all groups was similar. The incidence of pacing-induced VAs in the ARNI and Dapa groups was greatly reduced than that in ISO group (Figure 2B). It seemed that this effect was more effective in Dapa group than in ARNI group.

Dapa Improved Cardiac Remodeling in ISO-treated Rats
Cardiac fibrosis is well known to increase ventricular stiffness, leading to diastolic dysfunction. In this study, collagen volume fraction (CVF), a critical method to assess organic fibrosis, was evaluated in Masson's Trichrome Staining sections from hearts of rats. After 6 weeks of ISO treatment, the results showed that intraperitoneal injection of ISO resulted in increased myocardial fibrosis significantly. However, the myocardial interstitial fibrosis was obviously improved by ARNI and Dapa medication for 4 weeks (P<0.05, Figure 3).

**Dapa Reduced Body Weight, Cumulative Risk, and Plasma Glucose and Ang II Levels in ISO-treated Rats**

After 6 weeks of ISO treatment, the body weight and the plasma glucose level were significantly decreased (P<0.05, Figure 4A, 4C), but the plasma Ang II level (P<0.05, Figure 3D) were significantly increased in ISO-treated rats when compared with the control rats. Risk-Function was analyzed according to the daily recording of deaths for 6 weeks by Kaplan-Meier analysis. There were 3 of 10 animals dead in ISO-treated group, but there was only one of 9 animals dead in the Dapa or ARNI group. None of ten died in the Control group (P<0.05, Figure 4B). ARNI and Dapa both reduced the Ang II level and cumulative risks of rats induced by ISO (P<0.05, Figure 4B, 4D). However, Dapa further markedly reduced the body weight and the plasma glucose level when compared with the ISO group and ARNI group (P<0.05, Figure 4A, 4C).

**Dapa Inhibited Inflammation and AT1R Protein Expression in ISO-treated Rats**

Inflammation is the key mediator for myofibroblast formation and collagen deposition, which lead to cardiac fibrosis [21]. ISO action is partially mediated by inflammation through the activation of β1-adrenergic receptors in the heart [22]. In this study, the rats in ISO group revealed significant cardiac inflammation as shown by the increases in inflammatory factors including TNFα, IL-1β and IL-6 compared to the Control group. However, Dapa treatment significantly reduced myocardial TNFα, IL-1β and IL-6 protein levels (P<0.05, Figure 5A, 5B, 5C). ARNI treatment markedly decreased myocardial TNFα and IL-1β protein levels, but not IL-6 (Figure 5C). Dapa and ARNI both significantly upregulated the protein level of anti-inflammatory cytokine IL-10 (P<0.05, Figure 5D). AT1R serves as a major mediator of Ang II effects, including fibrogenic effect and increased ROS production [23]. In ISO-treated rats, cardiac AT1R protein level was higher than in the control rats. However, both Dapa and ARNI down-regulated the cardiac protein expression of AT1R (P<0.05, Figure 5E), and this may attenuated the pathogenic effects of Ang II via AT1R on the heart.

**Dapa Inhibited Cardiac Oxidative Stress in ISO-treated Rats**

ISO-induced cardiotoxicity is assumed that it generates highly cytotoxic free radicals in myocytes, which causes oxidative stress involving in the structural and functional myocardial damage. Cytotoxic free radicals can be generated by an activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, increased levels of Ang II and proinflammatory cytokines and so on. In this study, the rats in ISO groups revealed significant alteration in cardiac oxidative stress including the increases in ROS level, NADPH oxidase activity, and the main isoform NOX2 of NADPH oxidase but not NOX4 protein expression in the heart when compared to the control group, which were significantly reduced by the treatment with Dapa and ARNI when compared to ISO-treated group (P<0.05, Figure 6A, C, D). Cytotoxic free radicals can cause lipid peroxidation (LPO) of intramembranous polyunsaturated fatty acids in the membrane. Malondialdehyde (MDA), as an important LPO by-product, had a significant increase in the cardiac tissue in the ISO-treated rats when compared to in the
control rats. The Dapa and ARNI treatment significantly decreased MDA level when compared to the ISO-treated group (P<0.05, Figure 6E).

**Discussion**

Dapa, a selective inhibitor of SGLT2, is widely used to treat with type 2 diabetes depending on the increase glucose excretion in urine. In addition to its special glycaemic effect, there are many other benefits of Dapa such as weight loss, slowdown of cardiovascular diseases progression and so on [1-4]. In this study, we evaluated the influence of the SGLT2 inhibitor Dapa on cardiac remodeling, function, VAs and oxidative stress in rats with cardiomyopathy induced by ISO. We observed that Dapa had obvious cardiovascular protective roles like ARNI in ISO-treated rats: 1) Dapa and ARNI both effectively improved the cardiac fibrosis and dysfunction, but the increase in left ventricular end-diastolic volume induced by ISO was improved by Dapa more markedly than ARNI; 2) Dapa reduced the incidence of pacing-induced VA, heart rate and body weight more effectively than ARNI; 3) Dapa and ARNI both decreased cumulative risk of death and ameliorated cardiac oxidative stress such as the decreases in ROS level and MDA content in the heart. Dapa may have stronger cardiac protective effects than ARNI in ISO-induced cardiomyopathy.

In recent years, many previous studies have focused on the effects of SGLT2i on the cardiomyopathy in animal models with type 2 diabetes [24]. However, the roles of Dapa on ISO-induced cardiomyopathy have not been explored. ISO, a synthetic nonselective β-adrenergic agonist, is commonly used to activate β1-adrenergic receptors that is associated with deleterious myocardial effects, including ventricular arrhythmia, left ventricular hypertrophy, increased ventricular collagen content and a reduced inotropic response [25]. Therefore, ISO-induced cardiotoxicity is one of the most widely studied model for chronic cardiac injury. Hung-Yi Chen et al [26] has reported that patients prescribed with SGLT2i were associated with a lower risk of new-onset arrhythmias compared with those not taking SGLT2 inhibitors in real-world practice. Therefore, we also studied the role of Dapa on VAs in ISO-treated rats. As well known, ARNI is commonly used in clinical treatment of heart failure. For instance, its effect on VAs prevention has been widely reported [27, 28, 29], but not SGLT2i. Therefore, we also used ARNI as a reference to compare the effects of Dapa in our present study. ISO-induced alterations including cardiac dysfunction, cardiac fibrosis and increase of VAs were found in our present study, and which were effectively improved by application of Dapa and ARNI. Moreover, Dapa was more effective in reducing LVEDV, VAs, heart rate and body weight than ARNI. In the present investigation, the reductions in LVEDV and BW from our results implied the reductions in cardiac preload and afterload, which suggested that Dapa may play an important role in mitigating ventricular loading. The reason for weight loss may be related to its hypoglycemic action. Moreover, the decreases in VAs and heart rate indicated that Dapa application may have greater potential to reduce the risk of ventricular arrhythmias. Therefore, the pharmacological intervention of Dapa to ameliorate ISO induced cardiac abnormalities may have the potential therapeutic value in preventing the initiation and progression of cardiomyopathy.

ISO-induced cardiotoxicityis highly associated cytotoxic free radicals in myocytes, which causes oxidative stress leading to inflammation and structural and functional myocardial damage [30]. Oxidative stress is generated due to reactive oxygen species (ROS) and imbalanced antioxidant defence mechanisms. ROS can be generated by an activated NADPH oxidase, increased Ang II and proinflammatory cytokines and so on [31-32]. These major factors for promoting ROS generation were investigated in our present study. Indeed, the
application of ISO produced the obvious increases in ROS level, NADPH oxidase activity and the proinflammatory cytokines production. Moreover, Ang II level in plasma and its functional receptor AT1R protein expression in the heart, and oxidative stress-caused lipid peroxidation (LPO) product malondialdehyde (MDA) content in cardiac tissue were evidently higher than those in the control group. However, these adverse alterations were effectively reduced by the administration of Dapa and ARNI. These results indicated that treatment with Dapa and ARNI both significantly attenuated oxidative stress in cardiac tissue as shown by the decreases in ROS level and MDA content. It may be through reducing NADPH activity, AT1R protein levels and the production of proinflammatory cytokines in the heart. These results also revealed that Dapa had the antioxidant property by protecting cardiac muscle from ISO-mediated oxidative damage.

In conclusion, the protective effects of Dapa observed in this study may be due to its potent antioxidant properties, which protected cardiac tissue from the oxidative damage and helped in maintaining the myocardial cell membrane integrity and function. Our experimental results also provide an effective basis for the further clinical application of Dapa in the prevention and treatment of structural and functional myocardial damage. However, the potent protective mechanisms of Dapa in ISO-induced cardiotoxicity need to be further explored.

**Declarations**

**Authors’ Contributions**

All authors participated in interpretation of the studies and review of the manuscript. ZZL and YBZ designed the study. FZW, WBW, HYW and PQ conducted the experiments. XL, JYH and WYJ performed the data analysis. ZZL and YBZ wrote the manuscript, and YBZ revised.

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**Declaration of Conflicting Interests**

The Authors declare that there is no conflict of interest.

**Availability of data and materials**

The datasets used and/or analyzed in this study will be made available by the authors on reasonable request.

**Consent for publication**

This study consists of animal data and is devoid of any human data.

**Ethics approval and consent to participate**
This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Experimental Animal Care and Use Committee of Nanjing Medical University, and conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011).

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**Tables**

**Table 1.** Echocardiography parameters and heart rate at the end of the 2nd and 6th week.
| Week | Group  | EF%          | FS%       | LAD (mm)   | LVID (mm) | LVEDV (uL) | HR (bpm) |
|------|--------|--------------|-----------|------------|-----------|------------|----------|
| 2    | Control | 70.17±1.66  | 41.04±1.67| 4.01±0.07  | 7.86±0.08 | 341.8±11.03| 333±6   |
|      | ISO    | 43.34±1.71* | 28.77±1.03*| 4.78±0.06* | 8.33±0.1* | 394.38±4.78*| 355±9* |
| 6    | Control | 69.37±1.37  | 40.01±1.97| 4.22±0.08  | 8.17±0.19 | 369.7±9.31 | 339±7   |
|      | ISO    | 40.07±1.87* | 26.21±1.68*| 4.97±0.08* | 8.81±0.12*| 422.6±7.37*| 377±6*  |
|      | Dapa   | 60.56±1.73# | 36.04±1.72#| 4.28±0.04# | 8.29±0.12#| 341.83±10.37#$| 356±6# |
|      | ARNI   | 63.58±0.97# | 38.42±1.33#| 4.24±0.06# | 8.39±0.18#| 382.0±6.4# | 358±10  |

ISO: isoproterenol; ARNI: angiotensin receptor neprilysin inhibitors; Dapa: Dapagliflozin; EF: ejection fraction; FS: fractional shortening; LAD: left atrial diameter; LVID: left ventricular internal diameter; LVEDV: left ventricular end-diastolic volume; HR: heart rate.*P<0.05 vs. Control, #P<0.05 vs. ISO, $P<0.05 vs. ARNI.