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Original Article

Cardiac effects of dapagliflozin in diabetic rats with subacute exposure

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ABSTRACT

Background and Aims: Dapagliflozin (DAPA) is a sodium-glucose co-transporter 2 (SGLT2) inhibitor used for the treatment of type 2 diabetes mellitus (T2DM) as a monotherapy or combination therapy with other antidiabetic medicines. The Food and Drug Administration (FDA) recently approved DAPA to minimize the risk of hospitalization due to heart failure in patients with T2DM because of its antihypertensive and antihyperglycemic activities. However, further study of DAPA is necessary to ensure the safety of patients.
Methods: T2DM was induced by streptozotocin (STZ) injection (35 mg/kg b.w. i.p.) in male rats that were fed a high-fat diet for two weeks before STZ injection. The diabetic rats were exposed to 10 mg/kg DAPA by oral gavage during sub-acute treatment. Total cholesterol levels and oxidative stress parameters were evaluated. Heart tissues were histologically examined, and cardiac troponin T (cTnT) levels were measured.
Results: DAPA has the potential to inhibit diabetes-induced oxidative stress and morphologic damage to heart tissue, and increased cTnT levels of the heart, which is important for cardiac contractility.
Conclusion: DAPA might have a protective effect on the heart at a 10 mg/kg oral dose; however, further experimental and clinical studies are required to clarify the cardio-protective potential of DAPA.

Keywords: Dapagliflozin, SGLT2 inhibitor, Type 2 diabetes mellitus, Cardio-protection

INTRODUCTION

T2DM is one of the risk factors for the development of cardiovascular diseases (CVD) such as heart failure, atherosclerotic disease, and myocardial infarction. The risk is 2 to 4-fold higher in patients with diabetes (Fox et al., 2004; Ptaszynska, Hardy, Johnsson, Parikh, & List, 2013). It is well known that managing blood glucose levels is very important for preventing cardiovascular complications in diabetic patients (ESC, 2020). DAPA is one of the SGLT 2 inhibitors that inhibits glucose absorption from the kidney, and was approved in 2014 for the treatment of T2DM as a monotherapy or in combination with other antidiabetic medications (FDA, 2014; FDA, 2019). DAPA leads to natriuresis by decreasing sodium reabsorption concomitantly with glucose reabsorption from the kidney, thereby reducing systolic and diastolic blood pressure without affecting heart rate, which is different from standard antihypertensive agents (Reed, 2016).

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A large phase III multicenter clinical study (DECLARE-TIMI-58, NCT011730534) was designed to investigate the cardiovascular safety of 10 mg of DAPA daily, administered T2DM by health professionals in patients with or without T2DM. In that study, it was reported that DAPA lowered the rates of cardiovascular disease-related death and hospitalization for heart failure in patients who had or were at risk for atherosclerotic disease (Wiviott et al., 2019). Similarly, DAPA has been reported to reduce hospitalization for heart failure in patients with or without heart failure with reduced left ventricular ejection fraction, as well as the rate of cardiovascular disease-related death in patients with heart failure with reduced left ventricular ejection fraction (Kato et al., 2019). The FDA has also approved DAPA to reduce hospitalization for heart failure in patients who have CVD or CVD risk factors (Kelly, 2019).

The cardiovascular safety of DAPA has been evaluated by several studies in recent years (Ptaszynska et al., 2013; Wiviott et al., 2019; Kato et al., 2019). The effects of DAPA on the heart must be explicitly investigated due to the potential for DAPA usage rates to increase in cardiovascular patients following FDA approval. More research is needed to clarify the effects of DAPA on the heart with regard to patient safety. Therefore, we aimed to investigate the effects of DAPA on the heart after sub-acute DAPA exposure at 10 mg/kg b.w. dose. The effects of 10 mg/kg of DAPA on the levels of plasma cholesterol, glutathione (GSH), malondialdehyde (MDA), and the selective cardiotoxicity marker cTnT in the heart were evaluated.

MATERIAL AND METHODS

Animals and study design

Male Sprague-Dawley (SD) rats aged 10-12 weeks were obtained from Acibadem University Laboratory Animal Application and Research Center. The rats were housed in polystyrene cages (up to five animals per room) at 21-23°C and humidity (55 ±5%). The rats were fed ad libitum throughout the experiments at Istanbul University Faculty of Pharmacy Animal Faculty Unit (EDEHAB). The study was approved by Istanbul University Local Ethics Committee of Experimental Animals (IUHADYEK; 2018/24). The rats were fed a high-fat diet for 2 weeks and then randomly divided into 3 groups as shown in Table 1. Diabetes was induced by a single i.p. injection of STZ (35 mg/kg in citrate buffer solution) in Groups II and III. Group I animals received a single i.p. injection of citrate buffer solution (Skovsø, 2014). One week after STZ treatment, the blood glucose levels were measured with a glucose analyzer device (Vivacheck, Biotech Inc., China) from the tail blood of the animals. The animals with blood glucose levels >270 mg/dL were included in the study (Furman, 2015).

No observed adverse effect level (NOAEL) of DAPA is 50 mg/kg/day in rats (EMA, 2012). One study showed a maximum glucose-lowering effect of DAPA at 10 mg/kg b.w dose in SD rats (Han et al., 2008). Additionally, another study reported that the area under the plasma drug concentration-time curve (AUC) at 10 mg/kg DAPA in rats is 130 times higher than in humans at the same dose, which means plasma concentration in SD rats is 130 fold that of human (Reilly et al., 2014). Therefore, we used a 10 mg/kg dose of DAPA, a relatively high dose compared to humans, suspended in 0.5% methyl cellulose, to evaluate the effects of DAPA in the present study. And, the rats in Group III were treated with 10 mg/kg b.w. DAPA (Table 1).

Table 1. Experimental groups in the study.

Groups	Sub-acute (28 days) treatment
Group I (n:5)	35 mg/kg citrate buffer injection (i.p.) 1 mL/kg water (p.o.)
Group II (n:8)	35 mg/kg STZ injection (i.p.) 1 mL/kg methyl cellulose (p.o.)
Group III (n:8)	35 mg/kg STZ injection (i.p.) 1 mL/kg DAPA (p.o.)

Plasma cholesterol level determination

The rats were euthanized by taking a large volume of blood under inhalation anesthesia on the 28th day of exposure. The blood samples were centrifugated at 3000 rpm at 4°C for 20 min (Hettich Universal 32R Germany); the supernatant was collected and used for cholesterol level determination.

The rats were weighed three times a week (Sartorius, Mettler H20, Germany) and observed for clinical signs. After sacrifice, the hearts were surgically dissected and weighed (Precisa XB220A, Switzerland), and the relative heart weights (%) were calculated (Liro, 1985). The plasma cholesterol levels were determined using a commercial kit according to the manufacturer's protocol (Sunred, 201-11-0785, China). The MDA and GSH levels in the tissue samples were evaluated using commercial kits followingthe manufacturer's guideline (Elabscience, E-EL-0060; E-EL-0026, USA). The tissues were homogenized in phosphate-buffered saline (PBS) (1:10, w/v) and kept at -80°C (Daihan-Scientific Wisecry, South Korea).

Hematoxylin and eosin (H&E) staining

For histological examination, the heart samples were fixed in 10% neutral buffered formalin and processed for routine paraffin embedding. The paraffin sections approximately 4 µm-thick were stained with Hematoxylin and Eosin (H&E). The sections were examined and photographed using a light microscope (BX51; Olympus Corp., Tokyo, Japan) attached to a digital camera (DP72; Olympus Corp., Tokyo, Japan). The cardiomyocyte and vascular morphology were evaluated in each section.

Determination of cTnT expression level

For immunohistochemistry, formalin-fixed paraffin tissue blocks were cut into 4-5 µm thick transversal sections using a Leica 1212 rotary-microtome (Germany). The paraffin sections were placed on poly-L-lysine coated microscope slides. Then, the sections were deparaffinized, rehydrated, and rinsed with distilled water. For antigen retrieval, the sections were heated in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven. The endogenous peroxidase activity was suppressed with 3% (v/v) H_2O_2 for 15 min. After the sections were blocked to avoid non-specific binding, the mouse monoclonal anti-cTnT antibody (1:100, Thermo, USA) was applied to the sections and stored overnight at +4°C. The peroxidase activity was visualized with 3-amino-9-ethyl carbazole (AEC). The sections were counterstained with Mayer's

Haematoxylin, mounted in glycerol vinyl alcohol (GVA), and observed with an Olympus BX53 microscope. For negative control staining, the sections were incubated with PBS.

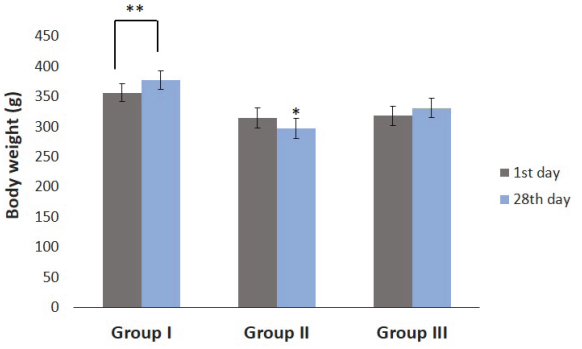
The statistical differences between the experimental groups were analysed by one-way analysis of variances (ANOVA) using SPSS v.20 (Chicago, IL). Significant differences were determined by Tukey post-hoc test. The results are given as values ± standard error of the means (SEM). All biochemical experiments were performed in triplicate for each animal. H-score of cTnT immune-reactivity was calculated using the formula: [1 x (weak cells %) + 2 x (moderate cells %) + (dense cells %)]. The intensity of staining was graded as 1, weak; 2, moderate; or 3, dense; plus, the percentage of positive cells.

RESULTS

Changes in body weights and relative heart weights

In diabetic groups (Group II and III), an increase in water consumption, diuresis, and fatigue were observed. At the end of the 28-day exposure period, the body weight was significantly lower in Group II compared with Group I, which observed a significant increase in the body weight, whereas the body weight of Group II decreased insignificantly ($p>0.05$) in comparison with the beginning of treatment (Figure 1).

The relative heart weights did not show significant changes in diabetic groups (Figure 2).



**p<0.05 vs Group I, **p<0.05 vs 1st day of the treatment.*

Figure 1. Effects of DAPA on the body weights. Data were shown as mean value ±SEM.

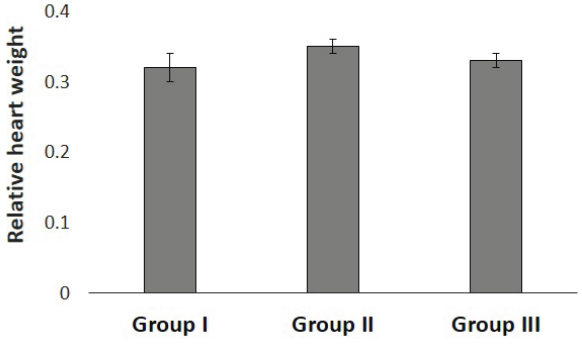


Figure 2. Effects of DAPA on relative heart weights. Data were shown as mean ±SEM.

Effects of DAPA on the plasma cholesterol level

As shown in Figure 3, the plasma cholesterol levels increased slightly in Group II, which was not found statistically significant ($p>0.05$).

Changes in MDA and GSH levels in the heart tissue

In Group II, the GSH levels in heart tissues decreased significantly, whereas the MDA levels increased significantly compared with Group I. DAPA treatment distinctively ameliorated reduced GSH levels in Group II (Figure 4).

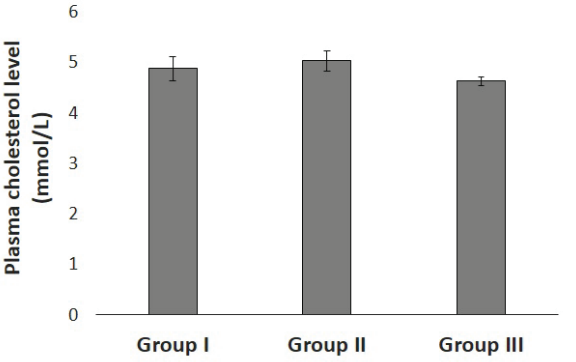
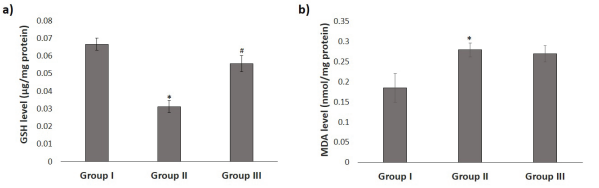


Figure 3. Effects of DAPA on plasma cholesterol levels. Data were shown as mean ±SEM.



**p<0.05 vs Group I, and #p<0.05 vs Group II, GSH: Reduced glutathione, MDA: Malondialdehyde*

Figure 4. Effects of DAPA on (a) GSH levels and (b) MDA levels in heart tissue. Data were shown as mean ±SEM.

Histological changes in the heart tissue

According to the histological examination, cardiomyocytes with regular morphology and typical vascular morphology were seen in Group I. Severe vascular congestion and disorganized myofibrils in cardiomyocytes were detected in Group II. Moderate vascular congestion and a decrease in myofibril disorganization in cardiomyocytes were observed in Group III (Figure 5).

Changes in cTnT expression levels

As shown in Figure 6, cTnT levels in heart tissue were significantly lower (20%, $p\leq0.01$) in Group II than in Group I. DAPA treatment ameliorated 17% of this decrease in Group III as compared with the diabetic groups.

DISCUSSION

DAPA is a novel therapeutic option for the treatment of T2DM. It is known that DAPA reduces blood pressure without affecting heart rate (Inzucchi et al., 2015), and this can be a major advantage over standard antihypertensive drugs used for

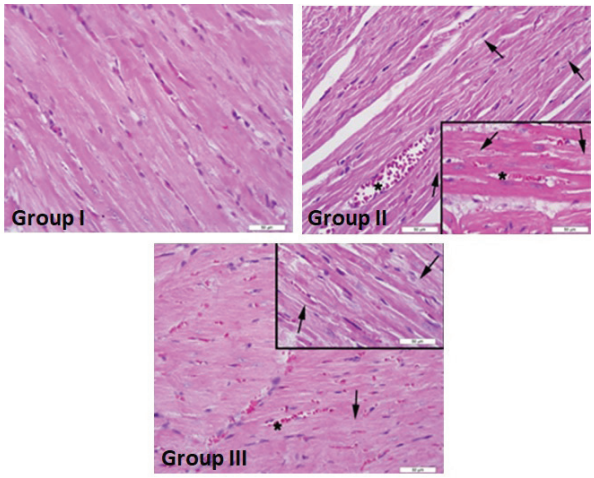
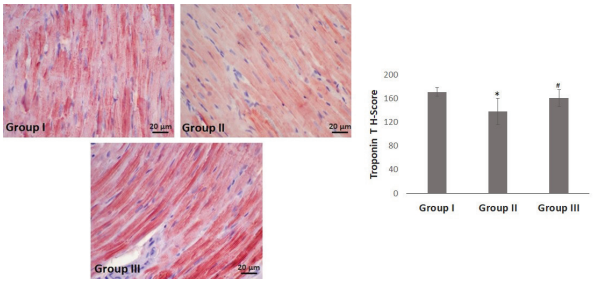


Figure 5. Representative photomicrographs of heart tissue in the experimental groups. Regular cardiomyocyte and vascular morphology are seen in Group I. Vascular congestion (asterisk) and disorganization of myofibrils (arrow) of cardiomyocytes are seen in Group II. Mild vascular congestion (asterisk) and decreased disorganized myofibrils in cardiomyocyte (arrow) are seen in Group III. H&E staining scale bars: 50 µm.



**p<0.05 vs Group I, and #p<0.05 vs Group II.*

Figure 6. Changes in cTnT expression in the heart in different experimental groups. Histological sections representing cTnT immunopositivity in the heart of different experimental groups. Graph representing H-score of cTnT immunoreactivity in the heart. Data were expressed as mean ±SEM.

diabetic patients with hypertension. Following FDA approval, DAPA may increase in usage for patients with a risk of heart failure, making it necessary to identify its cardiotoxic potential. For this reason, we investigated the biochemical and histological effects of DAPA on heart tissue at a 10 mg/kg dose.

Our results showed that body weight decreased significantly in Group II and III compared with Group I as a result of STZ injection-induced diabetes. Diabetes has been shown to reduce body weight in many experimental studies (Thomson et al., 2012; Rebolledo-Solleiro & Fernández-Guasti, 2018). At the end of the experiment, the body weight of Group III, which was treated with 10 mg/kg b.w. DAPA slightly increased compared with the beginning. This could be associated with an ameliorative effect of DAPA on diabetes by reducing blood glucose levels and inducing hyperphagia (Devenny, Godonis, Harvey, Rooney, Cullen & Pelleymounter, 2012). It is known that relative heart weight increased in diabetic cardiomyopathy, and this may result from cardiac hypertrophy (Zhang et al., 2018). In our study, relative heart weights did not show significant change among the groups because exposure time may not be enough to increase the relative heart index. Similarly, Singh et al. did not show a difference in the relative heart index one week after diabetes induction (Singh, Le, Khode, Baker, & Kumar, 2008).

It has been reported that DAPA leads to an increase in cholesterol levels in patients with T2DM (Gallwitz, 2018). However, plasma cholesterol levels did not show a significant change after sub-acute DAPA exposure in the present study. This might be associated with the duration of exposure, which was not sufficient to detect changes in plasma cholesterol levels.

Oxidative stress is one of the underlying mechanisms of cardiac dysfunction and diabetic complications. Oxidative stress may lead to lipid peroxidation and membrane damage (Dhalla, Temsah & Netticadan, 2000; Liu, Wang & Cai, 2014). MDA, an oxidized lipid, indicates lipid peroxidation and oxidative stress (Gawel, Wardas, Niedworok & Wardas, 2004). Reduced GSH levels are very important for antioxidant defense and protect cells from reactive species (Forman, Zhang & Rinna, 2009). An increase in MDA and depletion of GSH levels have been seen in the plasma or some tissues of diabetic patients (Wei, Liu, Tan, Liu, Li & Cai, 2009; Tiwari, Pandey, Abidi & Rizvi, 2013). DAPA has been demonstrated to elevate GSH levels and reduce MDA levels in heart tissue at 1 mg/kg concentration after 4 weeks of exposure (Hussein, Eid, Taha, Elshazli, Bedir & Lashin, 2020). Similarly, we observed that GSH levels of heart tissue decreased in diabetic Group II, and this decrease was significantly ameliorated by DAPA treatment in Group III. The MDA levels in heart tissue were elevated in Group II, and DAPA treatment slightly inhibited the elevation of MDA levels in Group III. Some studies also showed that DAPA reduced oxidative stress markers in rats at 1 mg/kg and 10 mg/kg doses (Tanajak et al., 2018; Kıngır et al., 2019). Our histological results parallel the increase in oxidant damage parameters in Groups II. DAPA might have ameliorated morphological damage via the regulating of the oxidant/antioxidant balance in STZ-induced cardiotoxicity.

Troponin is important for cardiac contractility, and it plays a structural and modulator role in the heart (Gomes, Barnes, Harada & Potter, 2004). cTnT, one of the cardiac-specific troponin isoforms and a sensitive marker for cardiotoxicity, is widely used in the clinic (Lorenzo-Almoros, Tuñón, Orejas, Cortés, Egido & Lorenzo, 2017). An increase in serum/plasma cTnT levels indicates myocardial damage (Wallace et al., 2004). However, decreased cTnT levels in heart tissue have been observed in cardiac damage in experimental models (Sehnert et al., 2002; Jankowski et al., 2010). Similarly, our study showed that DAPA treatment significantly raised cTnT levels in heart tissue as compared with diabetic groups.

In conclusion, DAPA showed a protective effect on the heart by reducing oxidative stress and inflammation at a 10 mg/kg dose by sub-acute exposure. Still, further studies are needed to confirm our results by identifying the long-term effects of DAPA on the heart.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The study was approved by Istanbul University Local Ethics Committee of Experimental Animals (IUHADYEK; 2018/24).

Author Contributions: Conception/Design of Study- T.B., B.U.K., G.Ö.; Data Acquisition- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.; Data Analysis/Interpretation- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.; Drafting Manuscript- T.B., B.U.K., A.K.K.; Critical Revision of Manuscript- E.K., F.E., G.Ö.; Final Approval and Accountability- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.

Conflict of Interest: The authors have no conflict of interest to declare.

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