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Cardio-protective effect of dapagliflozin against doxorubicin induced cardiomyopathy in rats

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Abstract. – OBJECTIVE: Cancer is the second most common non-communicable disease group in the world and its frequency is increasing. In parallel, side effects of drugs used in cancer treatment are frequently encountered. Doxorubicin (DOX) is one of the most effective multi-purpose anticancer drugs. However, its use is significantly limited due to the risk of cardiotoxicity. Sodium-glucose cotransporter-2 inhibitors are a group of antidiabetic drugs that have been shown to reduce cardiovascular events. Our aim is to examine the preventive effect of dapagliflozin on DOX-induced cardiac damage.

SUBJECTS AND METHODS: We used 30 albino rats. 20 of 30 rats were administered doxorubicin for cardiomyopathy model. The rats in the DOX arm were divided into two groups: those given penicillin and placebo. After the rats were terminated, tissues were prepared for histopathological and immunohistochemical examination. TNF-α, pro-BNP, troponin T and plasma FGF-21 levels were also measured in plasma.

RESULTS: The mean concentrations of cTnT and pro-BNP in the plasma of the DOX treated rats demonstrated a significantly higher value compared to the control group. Treatment with dapagliflozin caused a significant reduction in plasma cTnT, pro-BNP and TNF-α levels concentrations compared to the DOX control group ($p < 0.001$). The group of rats treated with dapagliflozin was effective in significantly decreasing the FGF-21 concentration and the percentage of fibronectin immunoexpression compared to the DOX control group ($p < 0.0001$).

CONCLUSIONS: This study revealed, for the first time, that dapagliflozin can improve DOX-induced cardiac dysfunction and pathological changes in non-diabetic rats. This result has shown that dapaglifozin, may be promising in terms of preventing cardiac damage that may develop in cancer treatment.

Key Words:
Dapagliflozin, Doxorubicin, Cardiomyopathy.

Introduction

After cardiovascular diseases, cancer is the second most common non-communicable disease worldwide, with 17.9 million cases. Together with respiratory system diseases and diabetes, these four groups of diseases account for 80% of early deaths from non-communicable diseases[1]. Since the increase in life expectancy means cancers are so frequently seen, avoiding the side effects of the drugs used in its treatment becomes more important. Doxorubicin (DOX) is one of the most effective multi-purpose anticancer drugs from the anthracycline family. However, its use is significantly limited due to the risk of cardiotoxicity, which can lead to cardiomyopathy and heart failure (HF)[2]. The severity of HF depends on the dose and time of use, and its prevalence increases further after 10-15 years of chronic exposure[3]. Arrhythmias, ischemia, systolic dysfunction, and heart failure are among the different manifestations of DOX-induced cardiotoxicity. Cardiac cell death and necrosis are the most common events leading to these complications. The mechanisms of DOX-induced cardiotoxicity may involve oxidative stress, inflammation, and apoptosis, which in turn lead to cardiac remodelling and dysfunction. Therefore, despite its high efficacy for various types of cancer, its long-term use in chemotherapy is limited because of the dose-dependent cardiotoxicity.

Sodium-glucose cotransporter-2 inhibitors (SGLT2i) are a new class of medications used to treat

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patients with type 2 diabetes mellitus (T2DM). SGLT2i class drugs lead to glucosuria, natriuresis, and diuresis by inhibiting the reabsorption of glucose in the proximal tubule of the nephron. Large randomized clinical trials[4,5] have demonstrated that SGLT2i drugs reduce major cardiovascular events and hospitalisations due to heart failure in patients with or without T2DM. The DAPA-HF trial revealed that the SGLT2i dapagliflozin, in addition to the standard HF therapy, had convincing clinical benefits in reducing hospitalization due to HF, cardiovascular mortality, and all-cause mortality, as well as improving quality of life[4]. After the groundbreaking results of the recently published DAPA-HF trial, the EMPEROR-Reduced study revealed that the SGLT2i empagliflozin, in addition to standard HF therapy, had major clinical benefits, whether or not patients had diabetes[5].

To increase the use of DOX as a highly efficacious chemotherapeutic agent, studying potential drugs to reduce its significant cytotoxic effects is important. We aimed at evaluating the possible cardioprotective effects of dapagliflozin in the prevention of cardiac impairment after DOX administration.

Subjects and Methods

Animals

We used 30 mature male Sprague Dawley albino rats weighing 200-220 grams. All rats were fed *ad libitum* and kept in pairs in steel cages at a constant temperature (22±2°C) with 12-hour light-dark cycles. All experiments in this study were approved by the Animal Ethics Committee (20119-135/c). The Experimental Animal Laboratory of Science University provided the rats for the study. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, as confirmed by the US National Institutes of Health.

Experimental Protocol

Of the 30 male Sprague Dawley rats, 20 were administered DOX for the cardiomyopathy model. The remaining 10 rats were classified as the control group, and no treatment was applied. DOX was applied intraperitoneally (i.p.) at a dose of 2.5 mg/kg/day every second day for a total of 15 mg/kg six times for two weeks. The rats in the DOX arm were divided into two groups. Group 1 was the placebo group, in which the rats were given only 0.9% NaCl saline solution at a dose of 1 ml/kg/day; the group 2 rats were administered dapagliflozin (AstraZeneca, Trumpington, Cambridge, United Kingdom) at a dose of 1 mg/kg/day *via* oral gavage. After 15 days, blood samples were collected for biochemical analysis. After the rats were terminated, their hearts were removed for histopathological examination.

Histopathological Examination of Heart Tissue

The rats were anaesthetized with an i.p. dose of ketamine and xylazine and perfused with 200 mL of 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS) for histological and immunohistochemical studies. An Olympus BX51 microscope (Thermo Fisher Scientific, Waltham, MA, USA) with an Olympus C-5050 camera was used to take photos of all the sections. We used a computerized image analysis system to evaluate the morphological analysis. The cardiac tissue was examined by light microscopy for the degree of damage.

Fibronectin Immunoexpression

For the immunohistochemical examination, we incubated the sections with H_2O_2 (10%) for 30 minutes to eliminate endogenous peroxidase activity. After incubation, they were blocked with 10% normal goat serum for 1 hour at room temperature. Then they were incubated with primary antibodies for 24 hours at 4°C. We used the Histostain-Plus Bulk kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to measure the antibody against rabbit IgG and the 3.3 (DAB) to visualise the final product. An Olympus C-5050 digital camera mounted on an Olympus BX51 microscope was used to take photos. We used brown cytoplasmic staining in cardiomyocytes to determine CASPASE-3 positivity. The number of fibronectin (+) cells was assessed by systematically scoring at least 100 cardiomyocytes per field in 10 fields of tissue sections at ×10 magnification objective.

Measurement of Plasma TNF-α

We measured plasma TNF-α levels using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. The plasma samples were diluted 1:2, and TNF-α was determined in duplicate, according to the manufacturer's guide.

Measurement of Plasma Pro-BNP, Troponin T Levels

Plasma pro-BNP and cardiac Troponin T (cTnT) levels were detected using a commercially available ELISA kit.

Measurement of Plasma FGF-21

We used the rat ELISA kit to measure the plasma FGF-21 levels in the tissue supernatants. Measurements were made in duplicate for all samples from each animal according to the manufacturer's guidelines.

Statistical Analysis

Data were analysed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows. We used Student's *t*-test and analysis of variance (ANOVA) to compare the groups of parametric variables and the Mann-Whitney U test to compare groups of nonparametric variables. Results were presented as mean ± standard error of mean (SEM). A value of $p < 0.05$ was considered statistically significant, and $p < 0.001$ was considered statistically highly significant.

Results

Evaluation of Cardiac Biomarkers

The mean concentration of cTnT in the plasma of the DOX-treated rats demonstrated a significantly higher value compared to the control group. Treatment with dapagliflozin caused a significant reduction ($p < 0.001$) in plasma cTnT concentration compared to the DOX control group (Table I).

The DOX-treated rats had a significantly increased NT pro-BNP concentration as a specific marker in the left ventricular dysfunction compared to the control group. However, the dapagliflozin-treated rats showed a significantly lower ($p < 0.001$) value compared to the DOX-saline-treated rats (Table I).

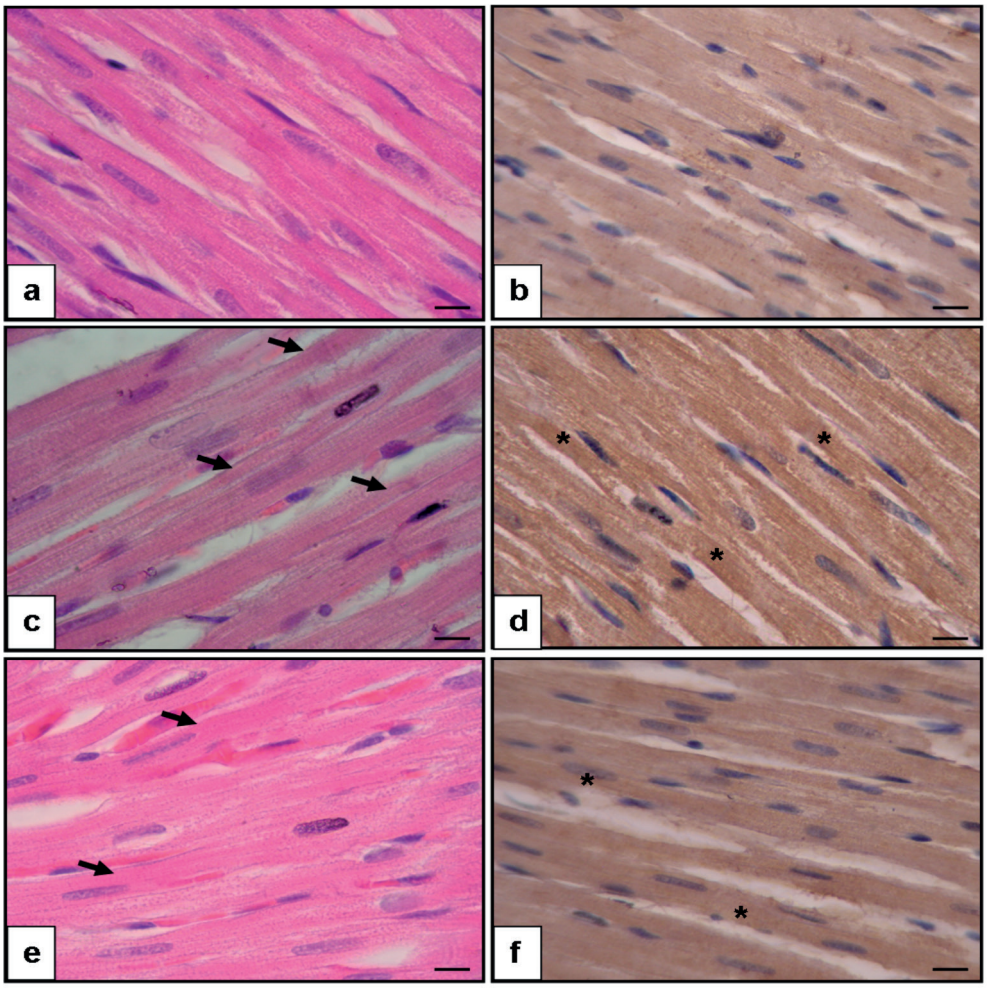

Figure 1. Cardiac tissue histopathology H&E and Fibronectin immunoexpression in cardiomyocytes (×100 magnification). **(a+b),** Normal cardiomyocytes, **(c+d),** Decreased thickness (arrow) and increased fibronectin expression of cardiomyocytes (*), **(e+f),** increased thickness (arrow) and Decreased fibronectin expression of cardiomyocytes (*).

Evaluation of Plasma TNF-α Levels

When compared to the saline-treated group, the dapagliflozin-treated group revealed a significant reduction in plasma TNF-α levels (40.5±7.08 pg/mL *vs.* 51.5±6.5 pg/mL; p <0.0001) (Table I).

Evaluation of Plasma FGF-21 Levels

The concentration of FGF-21 in the plasma of the three groups of rats in the study is shown in Table I. The group of rats treated with DOX alone showed a significant increase ($p < 0.001$) in FGF-21 concentration compared to the control group, and treatment with dapagliflozin was effective in significantly decreasing ($p < 0.0001$) the FGF-21 concentration in plasma compared to the DOX control group.

Evaluation of Heart Tissues by Histopathological Findings

The percentage of fibronectin immunoexpression in heart specimens was significantly elevated in the DOX group when compared to the control group ($p < 0.001$; Table II, Figure 1). The percentage of fibronectin immunoexpression in heart specimens of the DOX group treated with dapagliflozin was significantly reduced when compared to the saline group ($p < 0.001$).

Discussion

This study revealed, for the first time, that dapagliflozin could improve DOX-induced cardiac dysfunction and pathological changes in non-diabetic rats. Studies[4,5] highlighting the cardiovascular benefits of SGLT2i drugs in HF have investigated the unclear, underlying mechanism of this protection. However, most of the *in vitro* studies have been limited to diabetic subjects. A recent study[6] with streptozotocin-induced diabetic rats revealed that dapagliflozin treatment effectively inhibited DOX-induced apoptosis by reducing endoplasmic reticulum stress-associated proteins including GRP78, PERK, eIF 2a, ATF-4 and CHOP in cardiomyocytes. This inhibition with dapagliflozin resulted in significantly improved cardiac function.

The potential mechanisms of cardiac protection with SGLT2i include renin-angiotensin system inhibition following decreased total body sodium content; reduced myocardial cytoplasmic sodium and calcium load; anti-apoptotic, anti-inflammatory and antioxidant effects; repair of diabetic myocardial microvascular injuries; reduced sympathetic overactivity; and inactivation of endoplasmic reticulum stress[7]. In a hypertensive heart failure rat model, empagliflozin was found to restore upregulated TNF-α and activated natriuretic peptides[8]. Increasing numbers of basic and clinical studies suggest that pro-inflammatory factors, including TNF-α and IL-6, participate in the pathogenesis of DOX-induced heart dysfunction. The synergistic effect of ATII and high glucose levels is well-attested[9]. Consistent with studies[10,11] showing the suppression of cardiac remodelling by ATII via TNF-α, we also concluded that TNF-α, which was increased by DOX, decreased more in the dapagliflozin group than in the control group. According to Maurea et al[12], dapagliflozin improves the DOX/trastuzumab-exposed cardiomyocyte viability by the reduction of intracellular Ca2+ overload, lipid peroxidation and pro-inflammatory cytokines, such as interleukin-1β, interleukin-8 and interleukin-6, which are related to cardiotoxicity. In a DOX-induced cardiac injury model, Wang et al[13] found that TNF-α expression was clearly elevated. In our experiment, treating with dapagliflozin reduced the plasma TNF-α values. Due to the important role of inflammation in the pathophysiology of left ventricular dysfunction and HF, this significant decrease in TNF-α is notable for the efficiency of dapagliflozin in DOX-induced cardiac dysfunction[14].

Fibroblast growth factor 21 (FGF21) is an essential metabolic factor in glucose and lipid metabolism. It is expressed in adipose tissue, liver and

Table I. Comparison of cardiac biomarker, TNF-α and FGF-21 levels of all three groups.

	Normal Group Rats	DOX+saline	DOX+DAPA
Plasma TNF-alpha (pg/ml)	15.8 ± 1.02	51.5 ± 6.5 **	40.5 ± 7.08 ±
Plasma Pro-BNP (pg/ml)	3.86 ± 1.1	27.6 ± 5.2 **	10.9 ± 3.3 ±±
Plasma cTroponin T (pg/ml)	1.1 ± 0.06	3.03 ± 0.9 *	2.8 ± 0.5 ±
Plasma FGF-21 (pg/ml)	95.6 ± 17.2	165.8 ± 21.5 **	245.9 ± 14.01 ±±

**p<0.001, DOX+Saline group compared with Normal group. *p<0.05, DOX +Saline group compared with Normal group. ±±p<0.0001, DOX+DAPA group compared with DOX + Saline group. ±p<0.001, DOX+DAPA group compared with DOX + Saline group.

Table II. Comparison of histopathological findings of all three groups.

	Normal Group Rats	DOX + saline	DOX + DAPA
Cardiomyocyte's thickness (% of control)	100	75.7 ± 18.4 *	86.9 ± 15.1
Fibronectin immunoexpression (percent)	6.5 ± 0.9	54.2 ± 8.33 $^{+}$	11.04 ± 4.9^{++}

*$p < 0.05$, DOX +Saline group compared with Normal group. $^{+}p < 0.001$, DOX + Saline group compared with Normal group. $^{++}p < 0.001$, DOX+DAPA group compared with DOX + Saline group.

the myocardium and works as an anti-inflammatory and antioxidative agent. Recent *in vivo* studies have demonstrated FGF21-mediated protection against cardiac ischemia and reperfusion injury[15]. In a rat model, FGF21 was found to regulate genes involved in antioxidant pathways in an autocrine manner and prevent reactive oxygen species production in cardiac cells[16]. Plasma FGF21 levels were found to be elevated in HF patients with reduced ejection fraction, and these high levels were associated with cardiac cachexia[17]. In another study, the role of circulating FGF21 in diastolic HF was prospectively investigated in 95 patients[18]. Circulating FGF21 levels were correlated with echocardiographic and invasive parameters of diastolic function and associated with 1-year cardiac events. In our DOX-induced cardiomyopathy model, initially elevated plasma FGF21 levels decreased following dapagliflozin treatment. This finding supports dapagliflozin's anti-inflammatory and antioxidant effects on doxorubicin-induced cardiomyopathy. Demonstrating this relationship between FGF21 and dapagliflozin is important to help elucidating the mechanism of the cardioprotective effects of SGLT2i drugs, which is still not completely understood.

Many studies[6-11] attempting to explain the cardioprotective mechanism of action of SGLT2i drugs have focused on their anti-inflammatory properties and their effects on the Na^{+} and Ca^{++} mechanisms. This makes our results more important because they demonstrate the direct effect of the significant decline in the percentage of fibronectin immunoexpression in heart specimens of the DOX group treated with dapagliflozin. Similarly, another study[19] in the literature showed the direct cardiac effects of SGLT2i. In that study, the researchers revealed direct reversible effects on endothelial activation and nitric oxide synthase deficit.

Conclusions

cTnT and pro-BNP levels are among the most sensitive and commonly used biomarkers, and their release from cardiomyocytes is proportional to the size and extent of cardiac tissue injury because of doxorubicin toxicity. In this study, the DOX control group showed a marked increase in troponin and pro-BNP concentrations, indicating extensive damage to the cardiac tissues in the presence of DOX. However, the treatment with dapagliflozin showed a marked reduction of the cTnT and pro-BNP values, suggesting that dapagliflozin may reduce cardiac cell injury caused by DOX, even in non-diabetic subjects.

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All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, as confirmed by National Institute of Health (USA).

Ethical Approval
All experiments in this study were approved by Animal Ethics Committee (20119-135/c).

Availability of Data and Material
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest
The authors declare no conflicts of interest.

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Authors' Contributions
All authors contributed to the conception and design of the study, data collection, analysis or interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published.

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