Dapagliflozin Protects Doxorubicin-induced Cardiotoxicity in Breast Cancer Patients With Diabetes via Suppressing ER Stress

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Abstract

Background: Cancer patients with diabetes have an increasing risk of Dox-induced cardiotoxicity. Despite previous studies reporting benefits of dapagliflozin on the cardiovascular system, it remains unknown whether dapagliflozin has a cardioprotective effect in cancer patients with diabetes receiving Dox therapy. The purpose of this study was to investigate the potential of dapagliflozin for preventing doxorubicin (Dox)-induced cardiotoxicity.

Methods: Using Taiwan National Health Insurance Database, the incidence of heart failure of cancer patients with or without diabetes was investigated. Streptozotocin (STZ)-induced diabetic rats were pretreated with oral dapagliflozin (10 mg/kg/day) for 6 weeks followed by Dox (5 mg/kg/week) for 4 weeks via intraperitoneal injection. Sequential echocardiography was applied to assess cardiac function. For *in vitro* analysis, cardiomyocytes cultured in high glucose (30 mM) were treated with dapagliflozin at 10 μM and subsequently exposed to Dox at 1 μM. Apoptosis and endoplasmic reticulum (ER) stress-related protein expression were measured by immunohistochemistry and Western blotting.

Results: Among the studied patients, those with diabetes had a higher risk of major adverse cardiovascular events including the development of heart failure. In diabetic rats, dapagliflozin reduced cardiac fibrosis and significantly improved cardiac function. Dapagliflozin effectively inhibited Dox-induced apoptosis and reactive oxygen species in cardiomyocytes under high glucose. Mechanistically, we showed that dapagliflozin decreased the cardiac expression of Bax and cleaved caspase 3 but increased Bcl-2. Dapagliflozin also significantly reduced ER stress-associated proteins including GRP78, PERK, eIF-2α, ATF-4, and CHOP.

Conclusions: Our study revealed for the first time that dapagliflozin mitigated Dox-induced cardiomyocyte apoptosis in diabetes via inhibiting ER stress. These results indicate that dapagliflozin could be useful for preventing cardiotoxicity in diabetic cancer patients receiving Dox treatment.

Background

Doxorubicin (Dox), an efficient chemotherapeutic drug, is widely used to treat several tumors and increases survival of cancer patients. However, it induces cardiotoxicity and irreversibly results in degenerative cardiomyopathy and heart failure. These side effects have been reported in a wide variety of patients, especially cancer patients with diabetes [1, 2]. Several studies have demonstrated that patients with concomitant diabetes and cancer have a poorer prognosis than those without diabetes [3, 4]. It is reasonable to speculate that a cancer patient with diabetes is more susceptible to Dox-induced cardiotoxicity. Regarding the mechanisms of Dox-induced cardiotoxicity, reactive oxygen species (ROS) play a pivotal role. The Dox-exposed heart also lacks the anti-oxidant enzyme to detoxify ROS induced by oxidative stress. The accumulation of a large number of free radicals in the myocardium results in the destruction of the endoplasmic reticulum (ER), one of the critical organelles that control Ca^{2+} levels, membrane proteins, translocation, and apoptosis [5]. Recent evidence showed that activation of ER stress contributes to the

pathogenesis of cardiovascular diseases [6]. However, the detailed mechanisms of ER stress in Dox-induced cardiotoxicity have not been completely elucidated.

Dapagliflozin, a sodium glucose cotransporter 2 (SGLT2) inhibitor, is a novel class of glucose-lowering agent and is used to treat patients with type 2 diabetes. Beyond reducing glucose reabsorption and facilitating the elimination of blood glucose to the urine, dapagliflozin is also reported to have protective effects in cardiovascular diseases. The *DAPA*-HF study demonstrated that dapagliflozin reduced the primary composite outcomes including cardiovascular mortality and heart failure in patients with type 2 diabetes [7]. The cardioprotective effects of dapagliflozin have been demonstrated in models of diabetic cardiomyopathy, heart failure, and myocardial ischemia. Although the cardioprotective effects of dapagliflozin have been reported, the effects of dapagliflozin treatment on diabetic cancer patients receiving Dox therapy remain unclear. Herein, we studied the potential of dapagliflozin for preventing Dox-induced cardiotoxicity in diabetic cancer patients from both clinical and preclinical perspectives.

Materials And Methods

Data source

Taiwan's National Health Insurance (NHI) program, launched in 1995, is one of the largest and most complete population-based datasets worldwide that covers almost 99% of the Taiwanese population. In this study, we used the claims data from the *National Health* Insurance Research Database (NHIRD) that provides encrypted patient identification numbers, sex, date of birth, dates of admission and discharge, diagnoses, procedure codes from the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM), records of prescriptions, and covered and paid costs from NHI. The NHIRD has been described in detail in previous studies [8, 9]. The accuracy of diagnosis of major diseases in NHIRD has been validated [8, 9]. This study was approved by the Institutional Review Board of Chi-Mei Medical Center (CV code: 10406-E01).

Study design

This nationwide, population-based, retrospective cohort study was conducted to determine the association between diabetes and subsequent major adverse cardio- and cerebrovascular events (MACCEs) in patients with breast cancer receiving Dox therapy. The cohort initially comprised breast cancer patients receiving Dox therapy for the first time from January 2007 to December 2015. To specifically select patients at the time of initial enrollment, we excluded patients with breast cancer diagnosed < 18 years old, second cancer, history of MACCEs, or diagnoses date errors. Male patients were excluded given the very small population. After exclusion, the target group was divided according to patients with and without diabetes. Patients with diabetes were defined as receiving anti-diabetic medications for consecutive three months (shown in Supplement Figure 1). In addition to age and gender, chronic cardiovascular risk factors, including hypertension, diabetes, hyperlipidemia, coronary artery disease, stroke, heart failure, liver disease, chronic kidney disease (CKD), end-stage renal disease (ESRD), and chronic obstructive pulmonary disease (COPD) were analyzed and adjusted. The primary outcome was MACCEs, which included newly developed hypertension, acute coronary syndrome, heart failure, and stroke with hospitalization. We specifically

focused on the endpoint of new onset of heart failure. The second outcome was mortality, identified using the "in-hospital death" code at discharge. All of the patients were followed-up from the index date to either death, loss to follow-up, or December 31, 2015. ICD-9-CM codes were listed in Supplement Materials.

Animals

Adult male Sprague-Dawley rats weighing 300-350 g were purchased from the Animal Resource Center of Chi Mei Medical Center. The animal experiments were approved and conducted in accordance with the strict guidelines of the Subcommittee on Research Animal Care of Chi-Mei Medical Center (No. 106061521) and the standards met the Guide for the Care and Use of Laboratory Animals.

Dox-induced cardiotoxicity in diabetic rats

The Sprague-Dawley rats were randomly divided into four groups as follows. (1) Control group: rats received water p.o. (2) STZ group: rats received STZ at a single dose of 35 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) by intravenous injection (i.v.). The animals were considered diabetic if they had a plasma glucose concentration above 350 mg/dL (3) STZ+Dox group: STZ-induced diabetic rats receiving Dox at 5 mg/kg/week for 4 weeks via intraperitoneal injection (i.p.) (4) STZ+Dox+Dapa group: STZ-induced diabetic rats pretreated with oral dapagliflozin at 10 mg/kg/day for 6 weeks followed by Dox at 5 mg/kg/week for 4 weeks via i.p. injection. The rats' survival rate, cardiac function, and hemodynamic parameters were measured weekly.

Echocardiography

Echocardiography was conducted using a GE Vivid S6 Dimension echocardiography platform with a 10 MHz linear array transducer (GE-Vingmed Ultrasound AS, Horten, Norway). The rats were anesthetized with 3% isoflurane mixed with oxygen to minimize the effects on the heart rate. Throughout the procedure, the heart rate was maintained above 200 beats/min and recorded at a frame rate of 300-350/s. Measurements included long- and short-axis views with ECG gating. Echocardiography was conducted in parasternal long- and short-axis views to measure the interventricular septum thickness in diastole (IVSd), left ventricular internal diameter in diastole (LVIDd), ejection fraction (EF), and fractional shortening (FS).

Pressure-volume (P-V) loop

The method of PV loop has been described previously [10]. In brief, the invasive hemodynamic assessments were conducted using a Millar pressure catheter (SPR-838; Millar Instruments, Houston, TX, USA) through the right carotid artery into the LV cavity. The left jugular vein was cannulated with hypertonic saline (10%) infusion to determine the conductance. The LV systolic function was evaluated using the end-systolic (Ves) and diastolic volumes (Ved), end-systolic (Pes) and diastolic pressures (Ped), maximal velocity of pressure rise (+dP/dt) and fall (-dP/dt), arterial elastance (Ea) and time constant of isovolumic pressure decay (tau). The end-systolic pressure-volume relationship (ESPVR) and end-diastolic pressure-volume relationship (EDPVR) were measured using inferior vena cava (IVC) temporal occlusion.

Assessment of LV fibrosis

At the end of the experiments, the rats were sacrificed and fresh heart tissues were immediately collected. The weight of the heart tissue was measured. For histopathological examination, the heart tissue was fixed in 4% paraformaldehyde and embedded in paraffin (Alfa Aesar, Lancashire, UK). Sections were stained with hematoxylin-eosin (HE) and Masson's trichrome stain. The rest of the heart tissue was frozen in liquid nitrogen and stored at -80°C for further biochemical assays.

Cell culture and treatment

H9C2 rat cardiac myoblast (H9C2) was obtained from the American Tissue Culture Collection (ATCC CRL1446, Manassas, VA, USA). The cell lines were maintained in DMEM medium (DMEM; GIBCO, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS, GE Laboratories Inc., Chicago, IL, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1 mM of non-essential amino acids (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in 5% CO_2. The H9C2 cells were randomly divided into four groups for treatment as follows: (1) control group; (2) high glucose group (HG) in which the cells were cultured in 30 mM of high glucose in DMEM for 24 h; (3) HG+Dox treatment group: the cells were cultured in high glucose and treated with 1 μM of Dox; and (4) HG+Dapa+Dox treatment group: the cells cultured in high glucose were pretreated with 20 μM of dapagliflozin before exposure to 1 μM of Dox.

Cell viability and ROS assay

Cell viability was determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-dimethyl-2H-tetrazolium bromide (MTT) assay kit (Bio-Rad, Hercules, CA, USA). The intracellular ROS levels were detected using a fluorescent 2',7'-dichlorofluorescindiacetate probe (H_2DCF-DA, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Measurement of cell apoptosis via flow cytometry

Apoptosis of the H9C2 cardiomyocytes was measured using the annexin V/propidium iodide (PI) double-staining method. After treatment, the cells were harvested and washed twice with ice-cold PBS. The cells were resuspended in binding buffer and then incubated with annexin V and PI working solution for 15 min in the dark at room temperature. Cellular fluorescence was measured via flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

TUNEL staining

A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was conducted using an in situ cell death detection kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol.

Western blotting

After the indicated treatments, the H9C2 cardiomyocytes were harvested and lysed with ice-cold RIPA buffer (Merck Millipore, Burlington, MA, USA). The total protein concentrations were determined using a BCA

Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The method has been addressed previously [4, 5]. Antibodies were listed in Supplement Materials.

Statistical analysis

The chi-squared test was used to compare differences in age and comorbidity frequencies between breast cancer patients with and without diabetes. After testing for normality, continuous variables were compared between diabetic and non-diabetic patients using the Mann-Whitney U test. The Kaplan-Meier method was used to plot MACCEs, and group differences were compared via the log-rank test. The hazard ratio (HR) of MACCEs between cancer patients with and without diabetes was estimated using the Cox proportional hazard regression model adjusted for the potential confounding factors age and comorbidities. A two-tailed *P* value < 0.05 was considered statistically significant for all of the tests. All of the analyses were conducted using SAS software version 9.4 (SAS Institute, Cary, NC, USA). Kaplan-Meier curves were plotted using STATA (version 12; Stata Corp., College Station, TX, USA).

Results

Diabetes increased the cardiovascular risks but not all cause mortality in patients with breast cancer

Compared with breast cancer patients naïve to anti-diabetic medications (N37, 962), those receiving more than three months of anti-diabetic medications (N = 762) were relatively older and had more comorbidities (Table 1). The breast cancer stages at diagnosis were similar between the two groups and only small amounts of patients received cardiovascular drugs. Notably, despite no significant difference in all-cause mortality between breast cancer with and without diabetes, significantly more of the diabetic cancer patients had subsequent MACCEs, especially hospitalization for heart failure. To reduce the potential bias, age and comorbidities were adjusted for the final study subjects. The adjusted hazard ratio showed that the diabetic cancer patients had higher risks of MACCEs (hazard ratio: 2.12; confidence interval: 1.75-2.57, P < 0.0001) and heart failure (hazard ratio: 2.17; confidence interval: 1.63-2.90, P < 0.0001) than those without diabetes (Table 2). Nevertheless, the risk of all-cause mortality was similar between the two groups. With the increases in age and clinical stages of breast cancer, the risks of MACCEs, heart failure, and all-cause mortality elevated incrementally. Even after adjusting for comorbidities, chronic kidney disease still contributed to the risks of all endpoints. Correspondingly, the *Kaplan-Meier* survival *plots* presented higher cumulative incidence rates of MACCEs and heart failure in the diabetic cancer patients than those free from diabetes. Interestingly, the probabilities of all-cause mortality were similar between the breast cancer patients with and without diabetes (Fig. 1).

Dapagliflozin improves cardiac function, survival and heart weight in Dox-treated STZ rats

To investigate whether dapagliflozin exerts beneficial actions on Dox-induced cardiotoxicity under high glucose, we established a Dox-induced cardiotoxicity model in the STZ rats. After the induction of diabetes using STZ, the rats were administered dapagliflozin for six weeks followed by another four weeks of Dox treatment (shown

in Supplement Figure 2). The Dox-treated STZ rats had significantly lower weight that the non-Dox treated rats. Pretreatment with dapagliflozin mitigated the reduction in body weight compared with the Dox-treated STZ rats (shown in Supplement Figure 3a, $P < 0.001$). Pretreatment with dapagliflozin decreased blood glucose but had no effects on heart rates compared with the Dox-treated STZ rats (shown in Supplement Figure 3b and c, $P < 0.001$). Cardiac function was measured by sequential echocardiography in the control, STZ, STZ+Dox, and STZ+Dox+Dapa groups. Although there was no difference in IVSd and LVIDd among the four groups, the STZ+Dox group presented a significant decline in left ventricular systolic function including EF and FS ($P < 0.05$) while pretreatment with dapagliflozin mitigated Dox-induced cardiotoxicity (Fig. 2a, $P < 0.001$). This result indicated the protective potential of dapagliflozin against Dox-induced cardiotoxicity in STZ rats.

The mortality of the rats in the STZ+Dox+Dapa group was lower than that in the STZ+Dox group (Fig. 2b, $P = 0.16$). In the post-mortem study, we investigated the effect of dapagliflozin on cardiac structure and lung injury after Dox treatment. Dox-induced cardiac toxicities were observed through an increase in the ratio of heart to body weight (Fig. 2c, $P < 0.05$) as well as the weight to dry (D/W) lung weight ratio in the Dox-treated STZ rats while dapagliflozin significantly alleviated injuries (Fig. 2d, $P < 0.05$).

Dapagliflozin improves cardiac function and structure in Dox-treated STZ rats

Using the P-V loop analyses, we further studied the effect of dapagliflozin on hemodynamics in Dox-induced cardiac dysfunction in the STZ rats. Fig. 3a shows representative results of the P-V loop analyses with different preloads in the control, STZ, STZ+Dox, and STZ+Dox+Dapa groups. Compared with the controls, both Ves and Ved were higher in the STZ rats, especially those treated with Dox, and recovered by pretreatment with dapagliflozin (Fig. 3b, $P < 0.05$). Likewise, the maximal velocity of the pressure rise (+dP/dt) and fall (-dP/dt) was suppressed in the STZ rats treated with Dox and mitigated by dapagliflozin (Fig. 3c, $P < 0.05$). Despite no significant changes in arterial elastance (Ea), the decline in the exponential decay of the left ventricular pressure in isovolumic relaxation (tau) in the rats treated with STZ+Dox was also reversed by dapagliflozin (Fig. 3d, $P < 0.05$). Through temporal clamping the abdominal inferior vena cava, we found that although the EDPVR was not significantly different among the groups, the ESPVR was blunted in the rats treated with STZ+Dox, which was reversed by dapagliflozin (Fig. 3e, $P < 0.05$). Our findings implied that pretreatment with dapagliflozin mitigated the Dox-induced hemodynamic suppression in the STZ rats.

Dapagliflozin attenuates Dox-induced cardiac fibrosis and apoptosis through inhibiting ER stress in STZ rats

Compared with the STZ rats, cardiac fibrosis was significantly increased in the STZ rats treated with Dox for 28 days ($P < 0.001$) but significantly attenuated in the rats pretreated with dapagliflozin (Fig. 4a, $P < 0.001$). Using TUNEL and F-actin staining, we further evaluated the apoptotic cardiomyocytes in the four groups. Compared with the STZ group, Dox significantly increased the numbers of apoptotic cardiomyocytes ($P < 0.001$), which was alleviated in the rats pretreated with dapagliflozin (Fig. 4b, $P < 0.001$). The apoptosis-related proteins, including Bax, cleaved caspase 3, and Bcl-2, were measured in the cardiac tissue by Western blotting (Fig. 4c). The results showed that Dox treatment markedly upregulated the levels of pro-

apoptotic proteins, such as Bax and cleaved caspase 3 (P < 0.05), in the STZ rats, while protein expression was significantly suppressed in the rats pretreated with dapagliflozin compared with the control group (P < 0.001). Conversely, Bcl-2 expression, a key regulator of apoptosis, was markedly downregulated in the Dox-treated STZ rats (P < 0.05), whereas pretreatment with dapagliflozin preserved the expression of Bcl-2 (P < 0.05).

To study the effects of dapagliflozin on Dox-induced ER stress, we evaluated the cardiac expression of ER stress-associated proteins including GRP 78, p-PERK, eIF-2☐, ATF4, and CHOP among the four groups. Compared to the control group, Dox treatment significantly increased the expression of GRP 78, p-PERK, and eIF-2☐ (P < 0.01), while pretreatment with dapagliflozin suppressed the increase in p-PERK and eIF-2☐ expression (Fig. 4d, P < 0.01 and P < 0.05, respectively). For ER stress-induced apoptosis, CHOP is the downstream signal of the p-PERK-eIF2☐-ATF4 pathway in unfolded protein response. The results revealed that Dox treatment markedly induced the expression of ATF4 and CHOP in the cardiac tissue of the STZ rats (P < 0.05). Compared to the Dox+STZ group, pretreatment with dapagliflozin significantly inhibited the expression of ATF4 and CHOP (P < 0.05).

Dapagliflozin attenuates Dox-induced ROS generation and apoptosis mediated by ER stress in cardiomyocytes under high glucose

Using MTT assays, first we evaluated the effects of Dox and dapagliflozin on cell viabilities in the cardiomyocytes. The cells exposed to Dox at concentrations of 1, 10, and 100 ☐M for 24 h showed dose-dependent cytotoxicity (shown in Supplement Figure 4a; P < 0.01), while those exposed to dapagliflozin at concentrations of 0.1, 1, 10, and 20 ☐M presented no cytotoxic effects (shown in Supplement Figure 4b). To study whether dapagliflozin has beneficial actions on Dox-induced cardiotoxicity, cardiomyocytes were pretreated with 20 ☐M of dapagliflozin before exposure to 1 ☐M of Dox under high glucose (30 mM). Under high glucose, Dox enhanced the death of cardiomyocytes (P < 0.001) while dapagliflozin attenuated the death (Fig. 5a, P < 0.05). ROS is one of the main reasons for apoptotic death in cardiomyocytes. After exposure to Dox, the level of ROS in the cardiomyocytes increased (Fig. 5b, P < 0.05). Pretreatment with dapagliflozin for 1 h significantly suppressed the Dox-induced ROS generation (P < 0.01), which might lead cardiomyocytes to undergo apoptosis under high glucose. To confirm this hypothesis, we pretreated cardiomyocytes with or without dapagliflozin followed by administration of Dox under high glucose, and the percentages of apoptosis were determined by TUNEL assays and annexin V/propidium iodide staining (Fig. 5c and d, P < 0.001). Dapagliflozin significantly reduced Dox-induced apoptosis in the cardiomyocytes under high glucose (P < 0.001).

The apoptosis-associated proteins were detected by Western blotting. Dox administration significantly increased the expression of apoptosis-related proteins such as Bax and cleaved caspase 3 (P < 0.05 and P < 0.01, respectively). It also significantly decreased the expression of anti-apoptotic protein Bcl-2 in the cardiomyocytes under high glucose (Fig. 5e, P < 0.05). Furthermore, pretreatment with dapagliflozin effectively inhibited the expression of both Bax and cleaved caspase 3 (P < 0.01 and P < 0.05, respectively) but increased the expression of Bcl-2 (P < 0.05) in the cardiomyocytes after Dox exposure under high glucose. To investigate the anti-apoptosis mechanism of dapagliflozin on Dox-induced apoptosis under

high glucose, we further measured the expression of ER stress. Dox treatment significantly triggered the upregulation of GRP78 ($P < 0.001$), p-PERK ($P < 0.05$), eIF-2⬚ ($P < 0.05$), ATF4 ($P < 0.05$), and CHOP ($P < 0.01$) in the cardiomyocytes under high glucose (Fig. 5f). Pretreatment with dapagliflozin effectively inhibited ER stress-associated protein expression in the Dox-treated cardiomyocytes under high glucose. These results suggested that dapagliflozin could inhibit Dox-induced apoptosis via suppressing of ER stress under high glucose.

Discussion

In this study, we found that first, among cancer patients receiving Dox therapy, those with diabetes had higher risks of cardiovascular events and especially heart failure, but not all-cause mortalities. This emphasized the importance of treating diabetes optimally in cancer patients preparing for Dox therapy, which is another stress on the heart. Second, by suppressing ER stress-associated proteins, dapagliflozin significantly reduced myocardial fibrosis and restored cardiac function in the Dox-treated rats. Also, in the cardiomyocytes, pretreatment with dapagliflozin effectively inhibited Dox-induced apoptosis and reactive oxygen species. Based on these findings, dapagliflozin may mitigate chemotherapy-induced cardiotoxicity in patients with concomitant diabetes by regulating ER stress (Fig. 6).

Dox is one of the most common chemotherapeutic drugs and is typically used to treat patients with breast cancer [11]. However, Dox-induced myocardial dysfunction remains a major challenge in clinical practice. The mechanisms involved in Dox-induced cardiotoxicities have been reported including oxidative stress by ROS [12], mitochondria dysregulation and topoisomerase II b inhibition[13]. This suggests that Dox-induced cardiotoxicity is an indicator of multiplex biological processes. Some strategies have been reported to possibly prevent Dox-induced cardiotoxicity such as dexrazoxane, *an iron chelator.* It has been found to bind free iron and remove iron from its complex with Dox [14]. Despite being approved as a cardioprotective agent to prevent Dox-induced cardiotoxicity, dexrazoxane has side effects [15]. It may increase risks of infection, cause secondary malignant neoplasms, reduce the efficacy of Dox, and is expensive. Thus, many ongoing studies that attempt to discover new agents against Dox-induced cardiotoxicity focus on interfering with oxidative stress, inflammation, and apoptosis [5]. Nevertheless, to date an optimal regimen for preventing and managing Dox-induced cardiotoxicity remains lacking.

With the increasing prevalence of diabetes in cancer patients, it remains unclear how Dox affects the diabetic heart. Dox-induced irreversible cardiotoxicity may happen in a variety of patients, especially cancer patients with diabetes [1]. Reports have demonstrated that ROS-mediated apoptosis in cardiomyocytes is a major mechanism of Dox-induced cardiotoxicity [16]. Likewise, increased ROS has been regarded as a central mechanism of cardiac dysfunction in patients with diabetes [17] ROS generation may contribute to a double hit of stress in diabetic cancer patients receiving Dox treatment. Therefore, inhibiting the generation of ROS could be a therapeutic target to reduce Dox-induced cardiotoxicity in diabetic cancer patients.

The goal of diabetes treatment is not only to control blood glucose but also maintain myocardial function. The EMPA-REG, CANVAS, and DAPA-HF trials have shown that SGLT2 inhibitors have cardioprotective effects in reducing cardiovascular adverse events including heart failure hospitalizations and

cardiovascular mortality [18]. Therefore, SGLT2 inhibitors could be a priority choice in diabetic patients who are vulnerable to cardiovascular stress such as Dox therapy. In diabetic cardiomyopathy animal models, dapagliflozin has been found to improve cardiac morphologic and function including cardiac hypertrophy, fibrosis, and heart failure as well as both systolic and diastolic left ventricle function [19]. From another perspective, while some studies indicated that dapagliflozin could possibly decrease Dox-induced cardiotoxicity, the effect of dapagliflozin treatment on diabetic cancer patients receiving Dox therapy remains largely unknown. In this study, we recruited breast cancer patients with diabetes and found that diabetes increased their cardiovascular risks. Further, echoing our clinical findings, using a diabetic cardiotoxicity animal model, we illustrated that Dox-induced cardiac dysfunction, fibrosis, and activated ER stress could be significantly reserved by pretreatment with dapagliflozin.

Previous studies indicated that ER stress plays a significant role in mediating Dox damage in hearts [20]. Accumulating studies demonstrated that Dox-induced oxidative stress and ER stress subsequently cause myocardial cell death through the apoptosis pathway [5, 21]. Collectively, these studies indicated that the heart is more susceptible to Dox-induced oxidative stress given its high mitochondrial density. Further, the heart also lacks the anti-oxidant enzyme to detoxify oxidative stress-induced ROS. Hence, a large number of free radicals accumulating in the myocardium results in the destruction of the mitochondrial membranes and ER [22]. In this study, we hypothesized that the diabetic heart would be more susceptible to Dox-induced cardiotoxicity. Our results demonstrated that high glucose exacerbated Dox-induced apoptosis in cardiomyocytes by upregulating ER stress.

Conclusion

Dapagliflozin mitigated Dox-induced cardiomyocyte apoptosis in diabetes via inhibiting ER stress. Our results suggest that dapagliflozin could be useful for preventing cardiotoxicity in diabetic cancer patients receiving Dox treatment.

Abbreviations

Dox: doxorubicin; Dapa: dapagliflozin; STZ: Streptozotocin; ER: endoplasmic reticulum; ROS: reactive oxygen species; SGLT2: sodium glucose cotransporter; NHI: National Health Insurance; NHIRD: National Health Insurance Research Database; MACCEs: major adverse cardio- and cerebrovascular events; CKD: chronic kidney disease; ESRD: end-stage renal disease; COPD: chronic obstructive pulmonary disease; IVSd: interventricular septum thickness in diastole; LVIDd:left ventricular internal diameter; in diastole; EF: ejection fraction; FS: fractional shortening; P-V: Pressure-volume; HE: hematoxylin-eosin; Ves: end-systolic; Ved: diastolic volumes; Pes: end-systolic; Ped: diastolic pressures; +dP/dt and -dP/dt: maximal velocity of pressure rise and fall; Ea: arterial elastance; tau: time constant of isovolumic pressure decay; ESPVR: end-systolic pressure-volume relationship; EDPVR: end-diastolic pressure-volume relationship; IVC: inferior vena cava; MTT:3-(4,5-dimethyl-2-thiazolyl)-2,5-dimethyl-2H-tetrazolium bromide; H2DCF-DA: fluorescent 2',7'-dichlorofluorescindiacetate; PI: propidium iodide; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Declarations

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Tables

Table 1. Baseline characteristics and outcomes of breast cancer patients with and without diabetes (DM)

Characteristic	Total	Breast cancer patients without DM	Breast cancer patients with DM	p
	(n=38724)	(n=37962)	(n=762)	
*Age	n(%)*				
18-49	22546(58.22)	22394(58.99)	152(19.95)	<.0001
50-64	14448(37.31)	13965(36.79)	483(63.39)	
65-74	1456(3.76)	1342(3.54)	114(7.83)	
≥75	274(0.71)	261(0.69)	13(1.71)	
*Comorbidities	n(%)*				
Liver diseases	553(1.43)	490(1.29)	63(8.27)	<.0001
COPD	638(1.65)	616(1.62)	22(2.89)	0.0066
Hyperlipidemia	1202(3.10)	804(2.12)	398(52.23)	<.0001
Chronic kidney disease	81(0.21)	72(0.19)	9(1.18)	<.0001
Clinical stage				
0	5452(14.08)	5358(1411)	94(12.34)	0.2591
1	12006(31.00)	11750(30.95)	256(33.60)	
2	15424(39.83)	15117(39.82)	307(40.29)	
3	3298(8.52)	3244(8.55)	54(7.09)	
4	2544(6.57)	2493(6.57)	51(6.69)	
Cardiovascular drugs				
Anti-coagulants	14(0.04)	14(0.04)	0(0.00)	0.5960
ACEIs/ARBs	119(0.31)	78(0.21)	41(5.38)	<.0001
β-blockers	1366(3.53)	1319(3.47)	47(6.17)	<.0001
Statins	937(2.42)	611(1.61)	326(42.78)	<.0001
Digoxin	11(0.03)	11(0.03)	0(0.00)	0.6384
MRAs	128(0.33)	120(0.32)	8(1.05)	0.0005
*Outcome	n(%)*				
death	4020(10.38)	3931(10.36)	89(11.68)	0.2352
MACCEs	2876(7.43)	2736(7.21)	140(18.37)	<.0001

Heart Failure	1198(3.09)	1135(2.99)	63(8.27)	<.0001

DM= diabetes mellitus; COPD= chronic obstruction pulmonary disease; ACEIs/ARBs = Angiotensin converting enzyme inhibitors/Angiotensin II receptor blockers; MRAs = Mineralocorticoid receptor antagonist; MACCEs= major adverse *cardiac* and cerebrovascular events

Table 2. The risk of mortality, MACCEs and heart failure among breast cancer patients with DM compared those without DM (n=38724)

	All-cause mortality			MACC			Heart Failure		
	AHR*	(95% CI)	p	AHR*	(95% CI)	p	AHR*	(95% CI)	p
Overall									
patients without DM	1.00	Ref.		1.00	Ref.		1.00	Ref.	
patients with DM	1.09	(0.87-1.37)	0.4491	2.12	(1.75-2.57)	<.0001	2.17	(1.63-2.90)	<.0001
Age (years)									
18-49	1.00	Ref.		1.00	Ref.		1.00	Ref.	
50-64	1.23	(1.15-1.32)	<.0001	1.70	(1.57-1.84)	<.0001	1.81	(1.60-2.05)	<.0001
65-74	1.65	(1.45-1.89)	<.0001	3.44	(3.01-3.94)	<.0001	4.23	(3.47-5.17)	<.0001
≥75	2.31	(1.86-2.86)	<.0001	6.22	(4.94-7.82)	<.0001	8.20	(5.90-11.39)	<.0001
Comorbidities									
Chronic kidney disease	1.94	(1.00-3.73)	0.0485	1.89	(1.10-3.28)	0.0223	3.15	(1.63-6.10)	0.0007
Liver diseases	1.96	(1.57-2.45)	<.0001	1.16	(0.89-1.52)	0.2707	1.20	(0.8-01.80)	0.3789
Hyperlipidemia	0.87	(0.69-1.10)	0.2393	0.98	(0.80-1.19)	0.8241	0.92	(0.68-1.25)	0.6107
COPD	1.13	(0.89-1.43)	0.3195	1.16	(0.89-1.50)	0.2741	1.50	(1.05-2.13)	0.0244
Clinical stage									
0	1.00	Ref.		1.00	Ref.		1.00	Ref.	
1	2.64	(1.94-3.59)	<.0001	1.16	(1.00-1.33)	0.0436	1.04	(0.84-1.28)	0.7385
2	8.73	(6.52-11.68)	<.0001	1.54	(1.35-1.76)	<.0001	1.41	(1.15-1.72)	0.0008
3	32.20	(24.00-43.20)	<.0001	2.59	(2.22-3.03)	<.0001	2.25	(1.77-2.85)	<.0001
4	129.69	(96.99-173.42)	<.0001	3.69	(3.13-4.36)	<.0001	2.91	(2.22-3.80)	<.0001

*Adjusted hazard ratio | adjusted for the patients' age and comorbidities. Abbreviations as Table 1
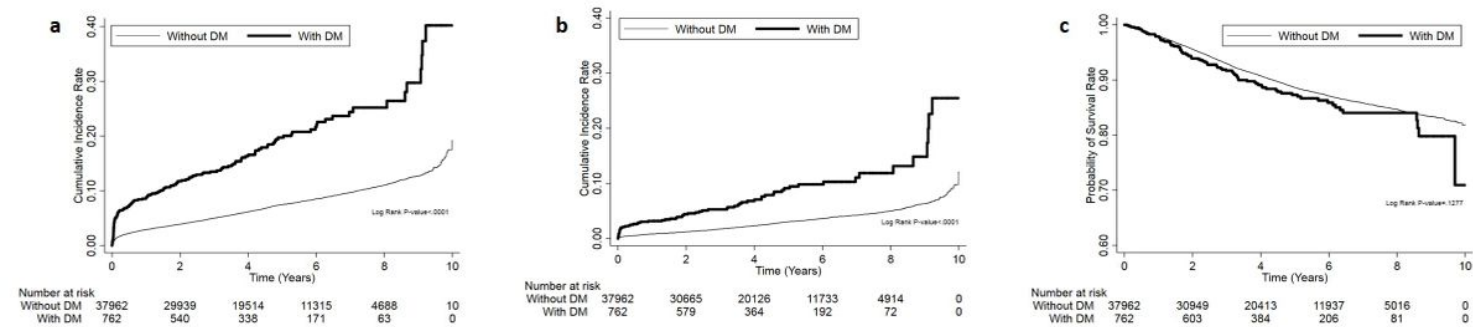
Figures

Figure 1

Kaplan-Meier survival plots of the cumulative incidence rates of (a) major adverse cardio- and cerebrovascular events (MACCEs), (b) heart failure, and the probabilities of free from (c) all-cause mortality in breast cancer patients with diabetes
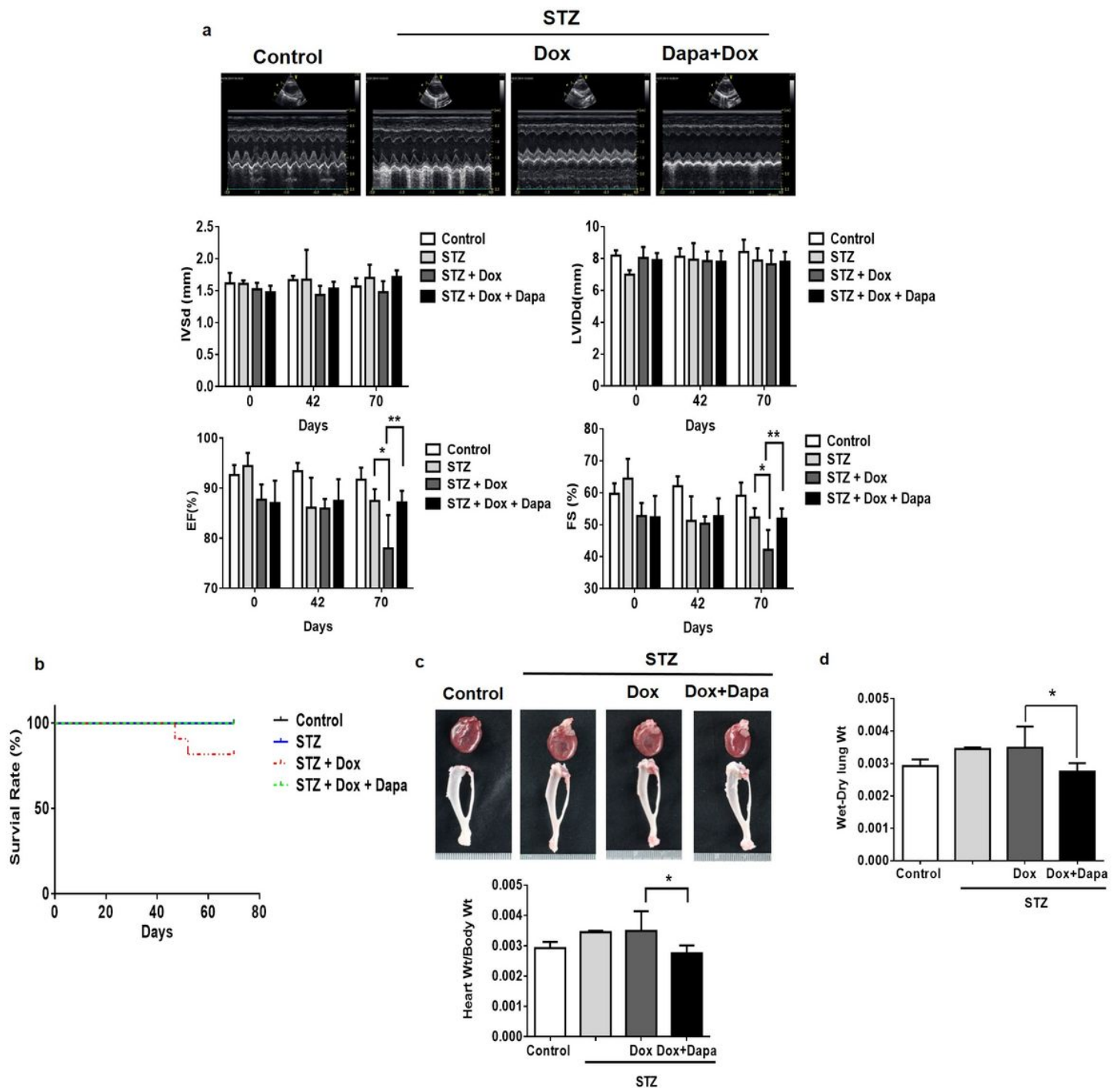
Figure 2

Effects of dapagliflozin (Dapa) on cardiac function, survival, and heart weight in the doxorubicin (Dox)-treated STZ rats. (a) Sequential measurements of echocardiography in the control, STZ, STZ+Dox, and STZ+Dox+Dapa rats. Echocardiographic measurements of interventricular septal thickness at end-diastole (IVSd), left ventricular internal dimension at end-diastole (LVIDd), ejection fraction (EF), and fractional shortening (FS). (b) The survival rate and (c) quantitative analysis of heart weight/body weight, (d) the wet to dry lung weight ratio in the control, STZ, STZ+Dox, and STZ+Dox+Dapa groups on day 70 after the initial injection. *P < 0.05 and **P < 0.01 compared with the indicated groups (N = 3-6).
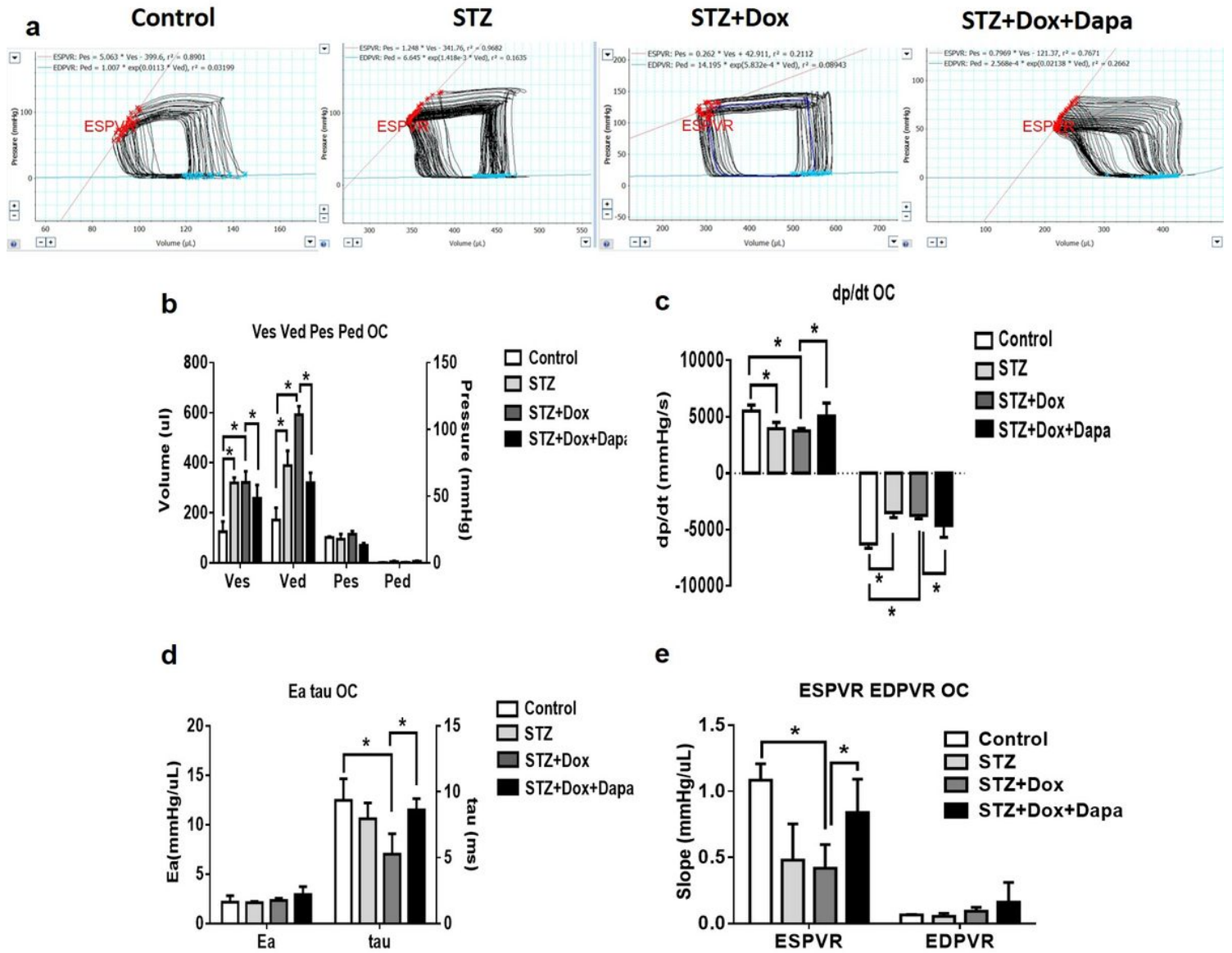
Figure 3

Dapagliflozin (Dapa) mitigated the doxorubicin (Dox)-induced hemodynamic declines in diabetic rats. (a) Representative pressure-volume loops at different preloads in the control, STZ, STZ+Dox, and STZ+Dox+Dapa rats. Hemodynamic measurements of (b) the mean end-systolic volume (Ves), end-diastolic volume (Ved), end-systolic pressure (Pes), and end-diastolic pressure (Ped). (c) The maximal velocity of pressure rise (+dP/dt) and fall (-dP/dt), (d) mean arterial elastance (Ea), time constant of isovolumic pressure decay (tau), and (e) mean slopes of the ESPVR and EDPVR are shown for the four rat models. *P < 0.05 compared with the indicated groups (N = 3-6).
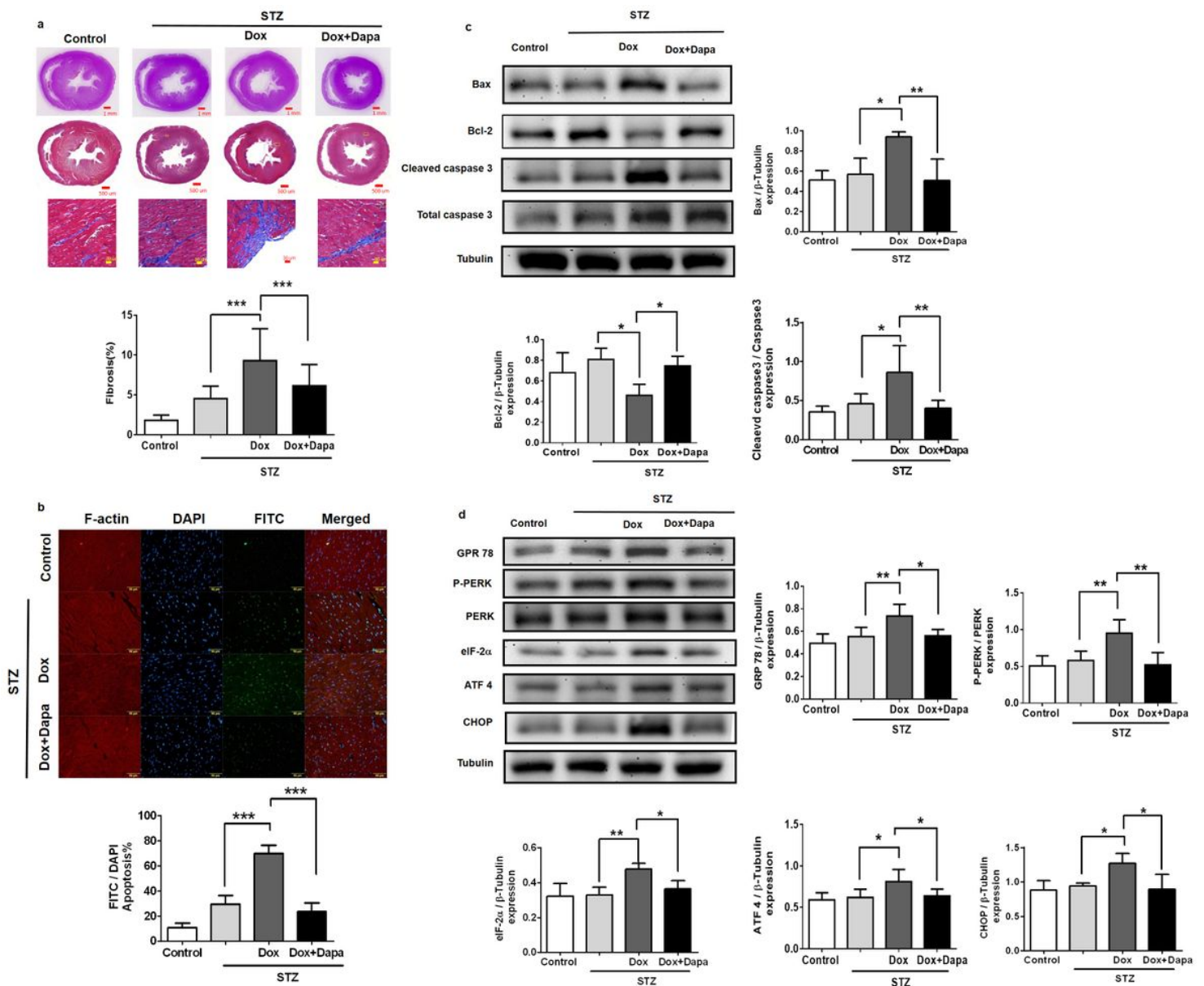
Figure 4

Dapagliflozin (Dapa) prevented doxorubicin (Dox)-induced cardiac fibrosis and apoptosis in the STZ rats. (a) Representative sections of hearts stained with Masson's trichrome for fibrosis detection (blue); scale bars, 30 μm (top panel). Quantification of cardiac fibrosis in the indicated groups of rats (bottom panel). (b) Representative sections of hearts stained with TUNEL assay for apoptosis detection (green); scale bars, 50 μm (top panel). Quantification of cardiac apoptosis in the indicated groups of rats (bottom panel). (c) Expression of apoptosis-associated protein, including Bax, Bcl-2, and caspase 3, in the rat hearts were measured by Western blotting. (d) Expression of ER stress-associated protein, including GRP78, p-PERK, eIF-2α, ATF4, and CHOP, in the rat hearts were measured by Western blotting. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the indicated groups (N = 3-6).
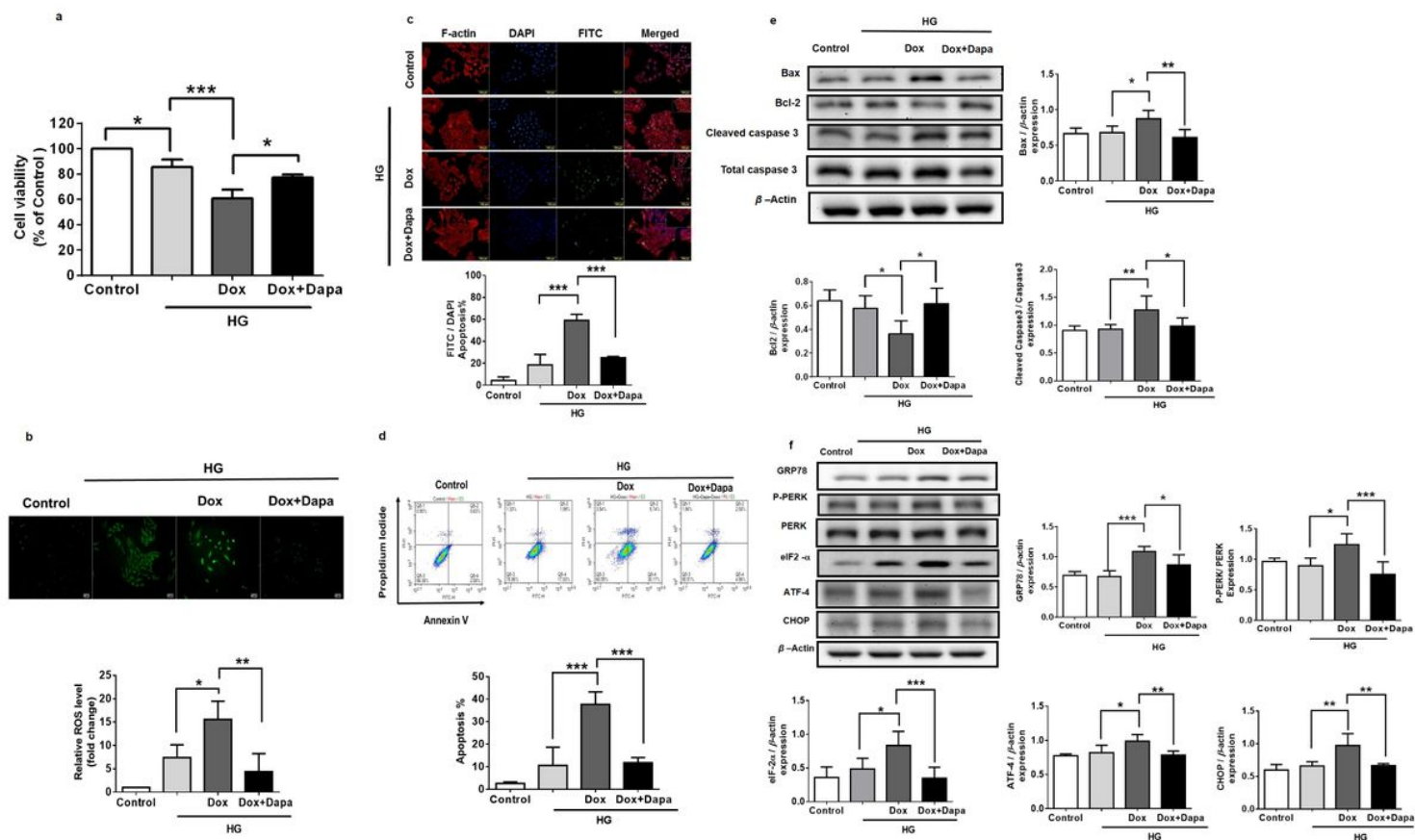
Figure 5

Dapagliflozin (Dapa) attenuated doxorubicin (Dox)-induced ROS generation, apoptosis, and ER stress in the H9C2 cardiomyocytes under high glucose. H9C2 cardiomyocytes were pretreated with Dapa for 1 h in the absence or presence of Dox for 6 h. (a) Effects of Dapa on the MTT test measured the cell viability, (b) intracellular expression of reactive oxygen species (ROS) measured by H2DCF-DA, (c) TUNEL assays in the H9C2 cardiomyocytes pretreated with or without Dapa followed by treatment with Dox and (d) apoptosis detected by annexin V expression in flow cytometry. (e) Expression of apoptosis-associated proteins, including Bax, Bcl-2, and caspase 3, in the cardiomyocytes. (f) Expression of ER stress-associated protein, including GRP78, p-PERK, eIF-2⍺, ATF4, and CHOP, in the cardiomyocytes. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the indicated groups (N = 3-5).
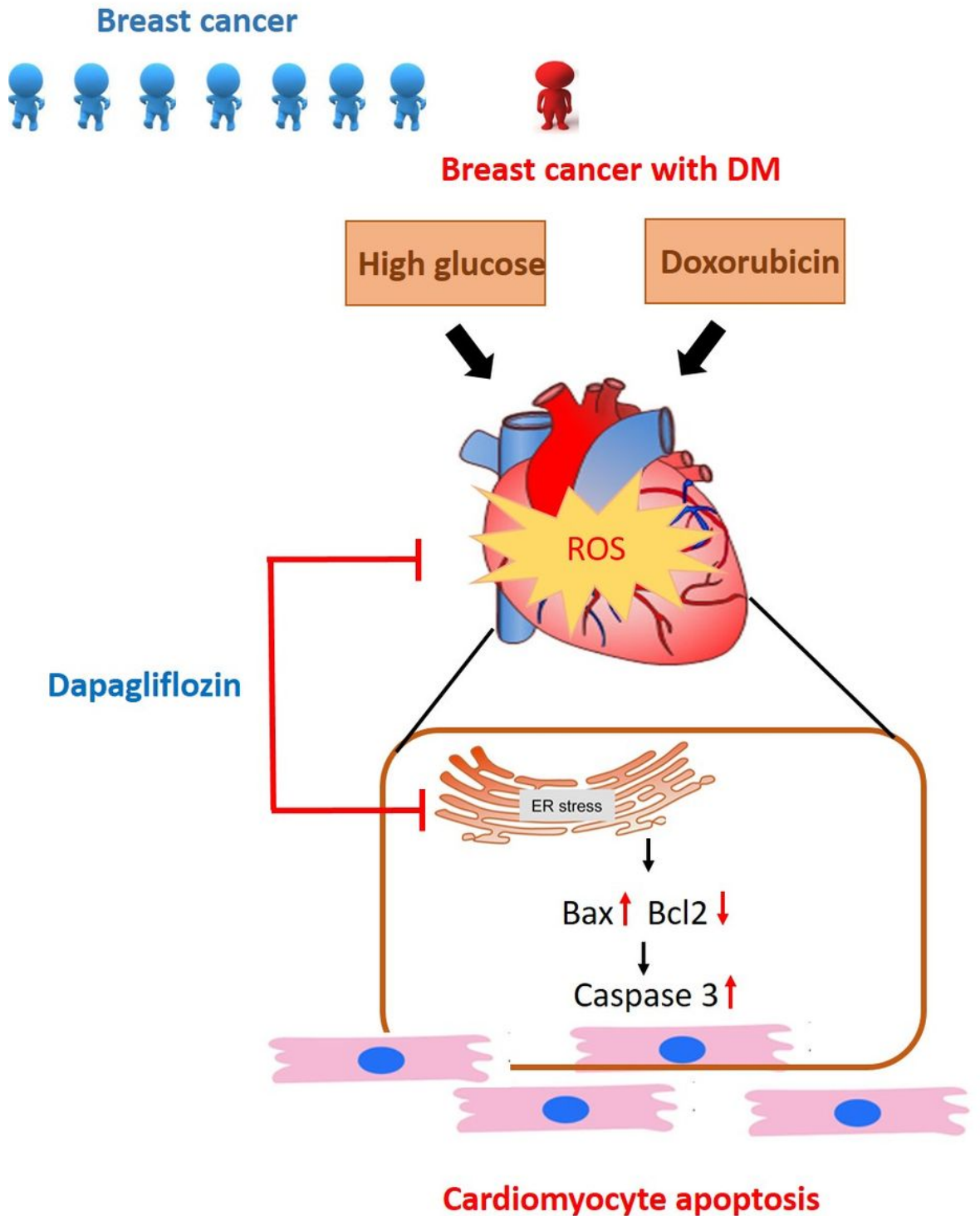
Figure 6

Summary of the double-hit stresses of diabetes and doxorubicin therapy in patients with cancer through ROS, apoptosis, and ER stress that could be prevented by treatment with dapagliflozin.

Supplementary Files

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