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Research Article

Effect of dapagliflozin on oxidative stress in heart embryonic H9c2 cardiomyocytes

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Abstract

Objectives: Dapagliflozin is a drug used to treat type 2 diabetes and is also used in certain heart failure and chronic kidney disease conditions. In this study, we investigated the effects of dapagliflozin (DAPA) on malondialdehyde (MDA), lipid hydroperoxide (LOOH), superoxide dismutase (SOD), total thiol (T-SH), and total antioxidant capacity (TAC) as oxidative stress parameters in heart embryonic H9c2 cardiomyocytes.

Methods: H9c2 cardiomyocyte cells were treated with methotrexate (MTX) (10-0.160 µM) and DAPA (10-0.150 µM). The cell viability and oxidative stress parameters were measured.

Results: MDA and LOOH levels were significantly lower in the control ($p<0.001$ for both) and DAPA groups ($p<0.001$; $p<0.05$, respectively) compared to the MTX groups, while SOD ($p<0.001$ for both), T-SH ($p<0.001$; $p<0.01$, respectively), and TAC ($p<0.01$; $p<0.05$, respectively) were significantly higher in the control and DAPA groups compared to the MTX groups. There was no significant difference between the control and DAPA groups in other parameters except for MDA. However, MDA levels were significantly higher in the DAPA group ($p<0.05$) compared to the control group. The decrease in MDA levels was significantly correlated with the increase in SOD activity (r: -0.814; p: 0.014) in the DAPA treatment group.

Conclusion: Cell viability increased, and the levels of MDA and LOOH decreased, while the SOD, T-SH, and TAC levels increased in H9c2 cardiomyocytes induced by oxidative stress. The findings obtained in this study suggest that DAPA may have beneficial effects in cardiomyopathy caused by oxidative stress.

Keywords: Dapagliflozin, H9c2 cardiomyocyte cells, malondialdehyde, methotrexate, oxidative stress, superoxide dismutase

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Dapagliflozin (DAPA), a sodium-glucose cotransporter 2 (SGLT-2) inhibitor, has been shown to have favorable effects on cardiac events such as cardiovascular death or hospitalization for heart failure (HF) in patients with type 2 diabetes mellitus (T2DM), along with a reduced risk of cardiovascular events [1, 2]. However, the cardiovascular safety of DAPA was studied in comparison to placebo and other drugs across a total of 21 studies, including 2 involving a large number of patients with a history of cardiovascular disease (CVD) [3]. The primary endpoints were the timing of cardiovascular death, hospitalization for myocardial infarction, stroke, or unstable angina, with secondary endpoints including unplanned coronary revascularization and hospitalization for HF in addition to the primary endpoints. When all the data were combined, it was concluded that cardiovascular risk would not increase with the use of DAPA [3]. DAPA may also attenuate cardiotoxicity by reducing oxidative stress, mitochondrial dysfunction, fibrosis, hypertrophy, and inflammation through PI3K/AKT/Nrf2 signaling [4, 5].

The mechanism of methotrexate (MTX)-induced cardiotoxicity is attributed to free radical formation, stimulation of lipid

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peroxidation, and subsequent alteration of cellular membrane integrity. This hypothesis is supported by the reported cytoprotective effect of antioxidants against MTX toxicity [6, 7].

Oxidative stress is a condition in which reactive oxygen-containing compounds are present in higher numbers than under normal conditions within a cell or group of cells [8]. Oxidative stress is a contributing factor in chronic CVD [8, 9] and is particularly significant in cardiovascular aging [10]. In recent years, many studies have focused on the role of various drugs in eliminating oxidative stress and reducing the damage caused by MTX [4, 5, 11, 12].

However, the exact mechanisms by which these favorable effects occur on MTX-induced cardiomyopathy are not fully understood. In our study, we aimed to determine the effects of DAPA treatment on MTX-induced oxidative stress by measuring (MDA), (LOOH), (SOD), (T-SH), and (TAC) as oxidative stress levels before and after DAPA treatment in H9c2 cardiomyocyte cells.

Materials and Methods

All chemicals used in the study were provided by Sigma-Aldrich, Istanbul, Türkiye.

Cell culture and treatment

H9c2 (2–1) cardiomyocyte cells were purchased from The American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were subcultured with 0.25% trypsin-EDTA. The trypsin-EDTA-cell suspension was centrifuged at 120xg for 5 minutes. After centrifugation, the supernatant was discarded, fresh medium was added to the cell pellet, and cells were seeded into 96-well plates at 1×10^4/well. The effects on cell viability were analyzed by applying MTX and DAPA (Forziga®AstraZeneca, Türkiye) at different concentrations and times.

Cell viability assay

Cell viability was determined using the MTT reduction assay. Briefly, H9c2 cells were incubated with DMEM containing 10% FBS overnight in 96 well plates at a density of 1×10^4 cells/well. After reaching 80% confluence, the cells were washed twice with D-PBS and incubated with medium containing various concentrations of MTX (10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 µM) and DAPA (100, 50, 25, 12.5, 6.25, 3.125, and 1.562 µM) for 24, 48, and 72 hours at 37˚C in a humidified atmosphere containing 5% CO_2. The medium was removed, and 100 µL DMEM and 20 µL MTT (5 mg/mL) was added to each well for 3 hours. The formazan crystals formed in intact cells were dissolved in 100 µL DMSO. Absorbance was recorded at a wavelength of 490 nm and at a reference wavelength of 570 nm using a microplate reader (Multiskan GO-Thermo). Using optical density (OD) values, IC50 and EC50 values for MTX and DAPA were calculated as 2.2 µM and 10 µM for 48 hours respectively with the GraphPad Prism 9 program.

Experimental groups

Groups were formed to include control, 2.2 µM MTX, 10 µM DAPA, and 2.2 µM MTX+10 µM DAPA. Cells were seeded at $3–4\times10^5$ cells/well on 6-well plates for each group. 24 hours after cell seeding, the control group was replaced with normal medium, and the others with medium containing MTX and DAPA alone or in combination at the given concentrations. While the other groups were incubated for 48 hours, the MTX+DAPA group was treated with DAPA for 48 hours after 48 hours of MTX. At the end of the experiment, cell lysates were prepared for the measurement of oxidative stress parameters from all groups.

All measurements related to the cell culture model, cell viability assay, cell lysate preparation, and oxidative stress were done as clearly stated in our previous studies [11, 12]. Differently, the cells were incubated with MTX in complete culture medium for 48h prior to the addition of DAPA. H9c2 (2–1) cardiomyocyte cells were treated with DAPA (Forziga®AstraZeneca, Türkiye) (10–0.153 µM) for 24, 48, and 72h at 37˚C in a humidified atmosphere containing 5% CO_2. DAPA was dissolved in DMSO. 2.7 µM MTX and 2.5 µM DAPA were used for 48 hours. The cell viability was calculated by considering the control as 100%.

Cell lysate preparation

Using 1xRipa lysis buffer and a protease inhibitor cocktail set (Merck KGaA, Darmstadt, Germany), a cell lysate from all groups was created at the conclusion of the experiment. 300 µL of Ripa lysis buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) together with a protease inhibitor cocktail (1:200) were added after the cells had been washed twice with cold 1xPBS. The cells were lysed by pipetting on ice, and the cell suspension was incubated for 30 minutes at +4°C in a shaking water bath. It was then centrifuged at 14000×g for 30 minutes at +4°C. After centrifugation, the supernatants were transferred to fresh Eppendorf tubes, and the resulting cell lysates were stored in a deep freezer at –80°C until measurement.

Oxidative stress parameters

Each experimental group was repeated at least three times. Lipoperoxidation was ascertained by the formation of malondialdehyde (MDA), which was estimated using the modified thiobarbituric acid (TBA) method [13]. LOOH levels were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange version 2 (FOX2) [14]. Cu, Zn-SOD activity was determined using the method of Sun et al. [15] by inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine/xanthine oxidase used as a superoxide

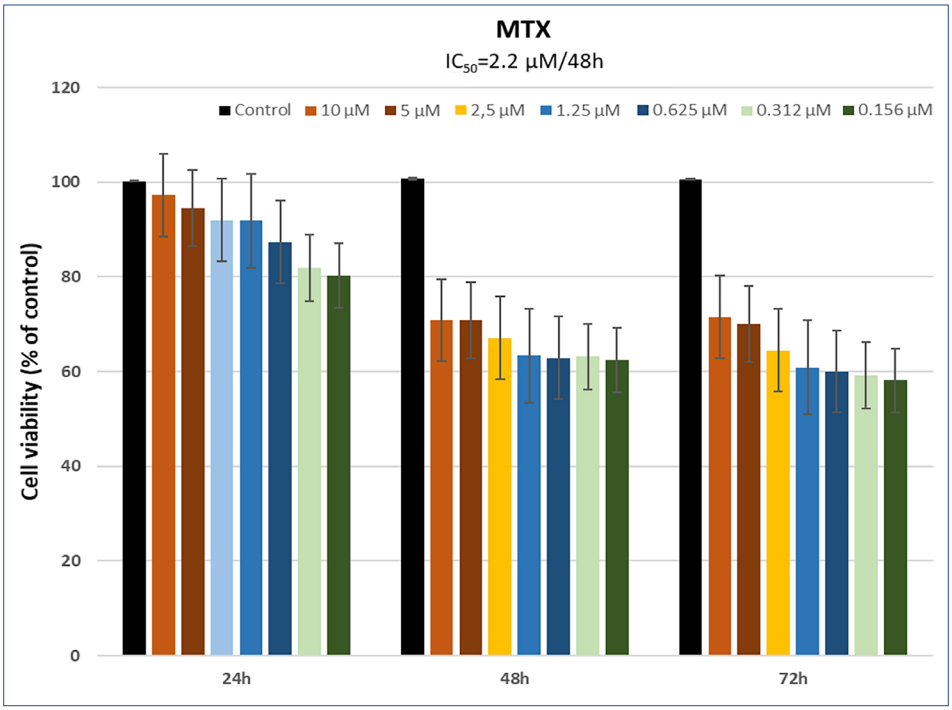

Figure 1. Time- and dose-dependent effects of MTX and DAPA in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8). MTX: Methotrexate; DATA: Dapagliflozin.

generator. T-SH levels were determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) as introduced by Hu [16]. The non-enzymatic TAC levels were measured with the ferric reducing antioxidant power assay and were performed according to the protocol of Benzie and Strain [17].

Statistical methods

All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) v. 22.0 (IBM, Armonk, NY, USA) package program. The distribution of all analyzed parameters was confirmed using the Shapiro-Wilk test. All parameters were normally distributed and expressed as mean± standard deviation. One-way ANOVA and the Tukey test as *post-hoc* were used in the comparison of groups. Correlation analysis was performed using Spearman's correlation analysis. Correlation/scatter graphs were plotted with Jamovi 2.3.18. A p-value below 0.05 was considered significant.

Results

The results of an MTT assay demonstrated that the number of viable cells decreased in response to increased concentration and times (24, 48, and 72 hours) of MTX treatment (Fig. 1). Conversely, the number of viable cells increased in response to increased concentration and times of DAPA treatment (Figs. 2, 3).

MDA ($p<0.001$; $p<0.05$, respectively) and LOOH ($p<0.001$ for both) levels were significantly lower in the control and DAPA groups compared to the MTX groups, while SOD ($p<0.001$ for both), T-SH ($p<0.001$; $p<0.01$, respectively), and TAC ($p<0.01$; $p<0.05$, respectively) were significantly higher in the control and DAPA groups compared to the MTX groups. There was no significant difference between the control and DAPA groups in other parameters except for MDA. However, MDA levels were significantly higher in the DAPA group ($p<0.05$) compared to the control group (Table 1).

There was a negative correlation between MDA and SOD (r: –0.814; p: 0.014) in the DAPA treatment group (Fig. 4). No correlation was observed between other oxidative parameters.

Discussion

In the present study, we found that in MTX-induced H9c2 cells, MDA and LOOH levels significantly increased, while Cu/Zn-SOD, T-SH, and TAC levels significantly decreased. These results indicate that the activation of systemic reactive oxygen species (ROS) triggers a sequence of events leading to cardiomyopathy. To the best of our knowledge, this is the first study to show that DAPA administration was able to improve oxidative stress induced by MTX. DAPA achieved this goal by reducing oxidative stress, increasing antioxidant status, and preventing cellular injury.

Free radicals, which occur during normal metabolism or pathologically, cause many damages in cells and tissues. Since oxidative damage caused by ROS affects biomolecules such as proteins, lipids, and nucleic acids, tests for oxidative products of these biomolecules have been used for many

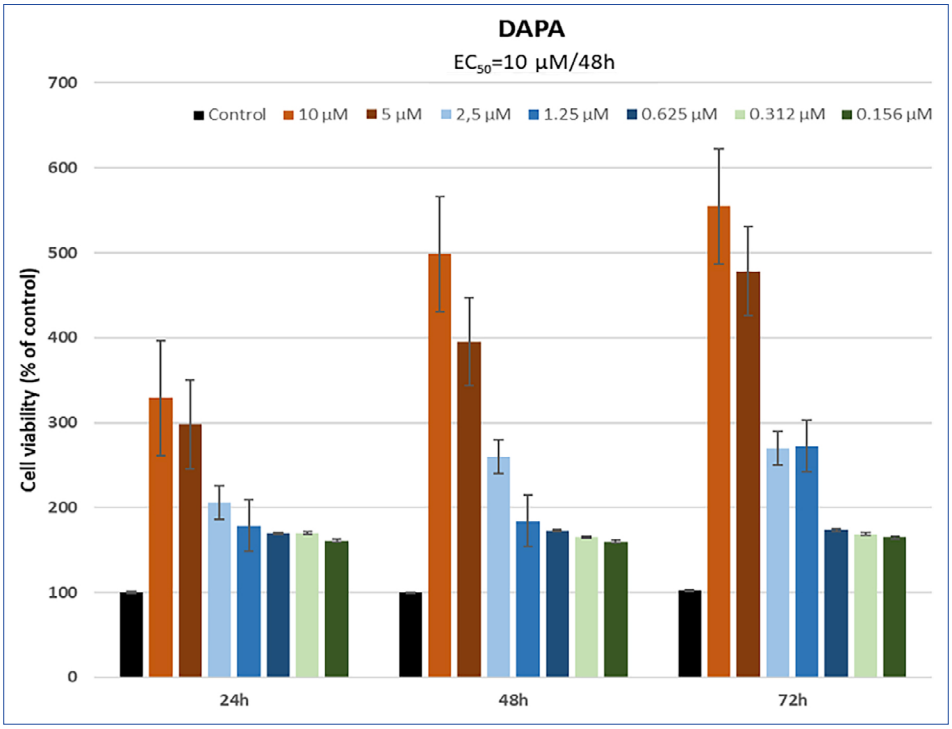

Figure 2. Protective effects of DAPA against MTX-induced decrease in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8).

years in demonstrating oxidative stress [18]. Recent studies suggested that oxidative stress [19] may be held responsible for the development of coronary artery disease (CAD). The heart is one of the most vulnerable organs to oxidative stress due to its specific structure and function [20]. It is clear that the production of ROS in cardiac dysfunction is a major factor contributing to heart diseases including cardiomyocytes, endothelial cells, and neutrophils [21].

The relationship between oxidative stress and atherosclerosis has been investigated by various research groups both in humans and experimental animals [22]. In many studies where MDA was used as a marker, high levels of MDA were observed to play an important role in the development of atherosclerosis in rabbit aorta, and a positive correlation was found between atheromatous plaques and MDA levels [23]. On the other hand, blood samples were generally used in human studies and lipid peroxidation indicators were mostly analyzed. Many studies have shown that MDA, diene conjugates, or LOOHs increase in serum in atherosclerotic CVD [24–26]. In our previous studies [11, 12], MDA and LOOH levels were found to be increased in H9c2 rat cardiomyocyte cells by MTT assay. Su et al. [27] detected increased MDA activity and decreased SOD activity in H9c2 cells after H_2O_2 stimulation. Zilinyi et al. [28] showed that injection of 6×3 mg/kg doxorubicin (DOX) was associated with considerably elevated MDA levels compared to the control group. In line with the literature, our results also show increased oxidative stress evidenced by elevated MDA and LOOH levels in MTX-treated H9c2 rat cardiomyocyte cells.

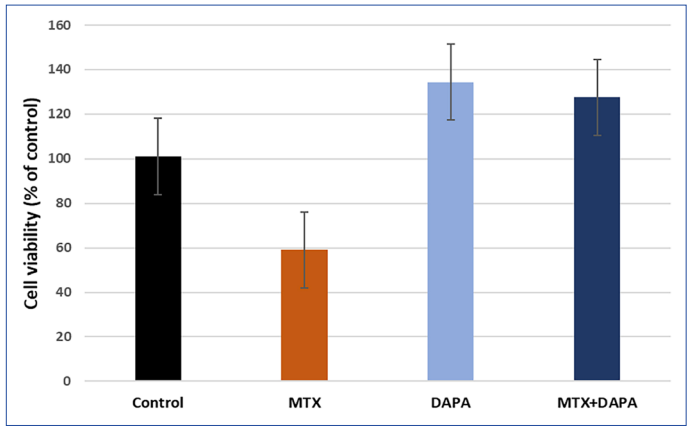

Figure 3. Protective effects of DAPA against MTX-induced decrease in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8).

Studies have investigated the healing and antioxidant effects of DAPA on endothelial cells. DAPA has been reported to improve cell functions due to positive effects on oxidative stress [29, 30]. According to TAC and total oxidant status (TOS) analysis, DAPA increased TAC, but not TOS, in cultured human blood cells [31]. An animal study showed that DAPA administration attenuated macrophage polarization in infarcted rat hearts by regulating macrophage polarization via the STAT3-signaling pathway [32]. Solini et al. [33] reported significant reductions in blood pressure (BP) and oxidative stress due to the acute effects of DAPA on systemic and renal vascular function.

Table 1. Oxidative stress markers in H9c2 cardiomyocyte control cells, cells exposed to MTX without any treatment, and cells treated with DAPA

	Control (n=8)	MTX (n=8)	DAPA (n=8)
	Mean±SD	Mean±SD	Mean±SD
MDA (nmol/mL)	0.63± 0.06	0.78±0.07^{a***}	0.69±0.06^{a*,b*}
LOOH (nmol/mL)	13.67±1.81	21.36±2.31^{a***}	13.68±2.32^{b***}
SOD (U/mL)	3.94±0.39	3.06±0.28^{a***}	3.79±0.22^{b***}
T-SH (mM)	1.52±0.15	0.94±0.24^{a***}	1.38±0.26^{b**}
TAC (µg ascorbic acid equivalent/mL)	19.46±1.41	16.87±2.24^{a**}	19.16±2.35^{b*}

*: p<0.05; **: p<0.01; ***: p<0.001. ^{a}: versus control; ^{b}: versus MTX. MTX: Methotrexate; DAPA: Dapagliflozin; MDA: Malondialdehyde; LOOH: Lipid hydroperoxide; SOD: Superoxide dismutase; T-SH: Total thiol; TAC: Total antioxidant capacity.

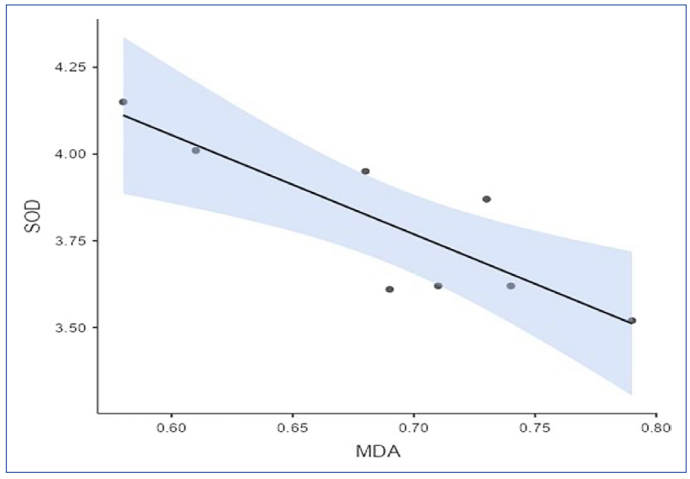

Figure 4. Correlation between MDA and SOD activity.
MDA: Malondialdehyde; SOD: Superoxide dismutase.

Shigiyama et al. [34] monitored T2DM patients using metformin by adding DAPA to their treatment and reported that urine 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative stress, was significantly lower and endothelial functions were better in the DAPA group than in the metformin group at 16 weeks. Buyukaydin et al. [35] demonstrated that patients using SGLT2 inhibitors (empagliflozin or dapagliflozin) had statistically higher total antioxidant status (TAS) levels in T2DM patients. While increased TAS may appear to be related to lower glucose values, there was no statistically significant difference in HbA1c between patients using SGLT2 inhibitors or not. DAPA has been reported to reduce mitochondrial ROS formation in aortic tissues and also to prevent atherosclerosis formation and suppress macrophage infiltration [36]. DAPA also suppressed high-glucose-induced oxidative stress in cultured mProx24 cells [37]. In addition to modulating inflammation, endothelial activation, and oxidative damage, DAPA regulated tubular ion channel expression and the non-classic renin-angiotensin-aldosterone system (RAAS) [38].

Limitations of the study

Our study has some limitations. The most significant shortcoming is the lack of transmission electron microscopic data. Another limitation is the absence of in vivo (animal and human studies) experiments in our study. Additionally, including another drug with a proven antioxidant effect that reduces oxidative stress could have enhanced the study.

In our study, MDA and LOOH values, which are lipid peroxidation products, were analyzed as markers of oxidative stress. SOD, T-SH, and TAC levels were analyzed as antioxidant indicators. MDA and LOOH levels were significantly lower in DAPA groups, while SOD, T-SH, and TAC were significantly higher in DAPA groups compared to the MTX groups. Additionally, there was a negative correlation between MDA and SOD activity in the DAPA treatment group. It was shown that oxidative stress markers increased in case of injury and then decreased significantly with DAPA treatment.

Conclusion

The results of the study demonstrate that DAPA reduces oxidative stress by decreasing the production of ROS and increasing antioxidant levels, in line with the literature. DAPA exhibited an antioxidant effect by reducing oxidative stress markers in heart embryonic H9c2 cardiomyocytes. However, the mechanism behind the positive effect of SGLT2 inhibitors (DAPA and EMPA) on cardiac function is not yet fully understood. Further studies are needed to explore how and why these changes occur in humans and through experimental research.

Conflict of Interest: The authors declare that there is no conflict of interest.

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Authorship Contributions: Concept – Z.D., H.U.; Design – Z.D., H.U.; Supervision – Z.D., H.U.; Funding – Z.D., H.U.; Materials – Z.D., H.U.; Data collection &/or processing – Z.D., H.U.; Analysis and/or interpretation – Z.D., H.U.; Literature search – Z.D., H.U.; Writing – Z.D., H.U.; Critical review – Z.D., H.U.

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