Research Article

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The in vitro cytotoxicity, genotoxicity and oxidative damage potential of dapagliflozin, on cultured human blood cells

 İnsan kan hücre kültürü üzerinde dapagliflozin’in in vitro sitotoksisite, genotoksisite ve oksidatif hasar potansiyeli

Abstract

Objectives: Dapagliflozin (DAPA), is a potent SGLT-2 inhibitor for the treatment of patients with type 2 diabetes. DAPA has a good clinical and biological tolerance profile. However little information is available on its potential effects on cultured human blood cells. The evaluation of the in vitro cytotoxicity, genotoxicity potential and antioxidant/oxidant activity of DAPA in primary human whole blood cell cultures was aimed in this study.

Materials and methods: Cell viability was measured by the MTT [3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and lactate dehydrogenase (LDH) leakage assays. The antioxidant/oxidant activity was determined by measuring the total antioxidant capacity (TAC) and total oxidative stress (TOS) levels. To assess the genotoxicity of DAPA, chromosomal aberration (CA) frequencies were determined.

Results: MTT and LDH release assay exhibited that exposure to different doses of DAPA did not changed significantly the proliferation of cells. The results of TAC and TOS assays were showed that TAC level was elevated while TOS level did not altered in DAPA-treated cells. Moreover, any increase in the frequency of CA did not found on cultures blood cells.

Conclusion: These data indicate that DAPA has not cytotoxic and genotoxic potential in cultured human blood cells, also, induces the increasing antioxidant activity.

Keywords: Dapagliflozin; MTT; LDH; Genotoxicity; Oxidant status.

Öz


Gereç ve yöntem: Hürce canlılığı, MTT [3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] ve laktat dehydrojenaz (LDH) sıçanması araştırıldı. Antioksidan/oksidan aktivitesi, total antioksidan kapasite (TAC) ve total oksidatif stres (TOS) seviyeleri ölçülenlerin birlikte. DAPA’nın genotoksisitesini değerlendirilmesi için kromozomal sapma (CA) frekansları belirlendi.

Sonuç: Bu veriler, DAPA’nın insan kan hücre kültürlerinde sitotoksik ve genotoksik potansiyele sahip olmadığını, ayrıca antioksidan aktivitenin artmasına neden olduğunu göstermektedir.

Anahtar kelimeler: Dapagliflozin; MTT; LDH; genotoksit; oksidan durum.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic multifactorial disease characterized by metabolic, hormonal, epigenetic and oxidative imbalances [1]. The incidence of T2DM continues to increase rapidly all over the world. According to National Diabetes Statistics Report data for 2017, approximately 30.3 million people in the USA (9.4% of the population) are affected by diabetes. The report stated that 23.1 million individuals had been diagnosed, while the others comprised an undiagnosed group [2].

As the pathophysiology and pathogenesis of T2DM have become better understood, new therapeutic options have been developed to target various key deficiencies in the disease [3]. Sodium glucose cotransporter-2 (SGLT-2) inhibitors have entered into use as novel and effective agents in the treatment of T2DM. SGLT-2 inhibitors exhibit blood glucose-lowering effects in inhibiting glucose re-uptake through an insulin-independent effect by inducing SGLT-2 inhibition in the renal proximal tubules. At the same time, they produce decreases in blood pressure, weight, and HbA1c values [4]. Glycosuria increases with SGLT-2 inhibition, while glucose toxicity decreases. It also exhibits positive effects, including improvement of beta (β) cell functions and increasing insulin sensitivity [5].

Dapagliflozin (Forxiga®; Bristol–Myers Squibb, New York, NY, USA; AstraZeneca, London, UK) was the first SGLT-2 inhibitor submitted to the US Food and Drug Administration (FDA) (December 2010) [6]. Dapagliflozin (Forxiga®) (DAPA) was approved for use in blood sugar control in addition to diet and exercise therapies in patients with adult T2DM by the European Medicines Agency (EMA) in 2012 and by the FDA in 2014 [7].

Phase 3 trials and studies investigating the positive effects on treatment of DAPA monotherapy and two-and three-drug use with oral antidiabetic combinations and combinations with insulin have shown that in addition to producing a decrease in HbA1c, DAPA is safe and reliable, and has a positive impact on weight loss [8–10]. However, more studies are needed to allow a better assessment of the benefit-risk profiles of DAPA. For that reason, the current study was planned to evaluate the cytotoxic, genotoxic and antioxidant activities of DAPA in cultured human blood cells.

Materials and methods

Blood sampling

Whole blood was collected from 5 volunteer, healthy, male, nonsmoking human donors aged 25, 27, 29, 30 and 35, with no history of occupational exposure to any genotoxic agent obtained from the Erzurum Regional Training and Research Hospital (Erzurum, Turkey). Informed constant forms were signed. All blood samples used in this study were collected in June 2018.

Ethics

The study was approved by the local Ethics Committee (2019/03-30) and was in accordance with the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice. Written informed consent was obtained from all patients.

Cell isolation

PBMCs were obtained under sterile conditions by density gradient centrifugation of heparinized venous blood, over Ficoll-Paque (Sigma, St Louis, MO, USA). The collected cells were washed three times in PBS and suspended in RPMI-1640 (Gibco), supplemented with 1% penicillin/streptomycin (Sigma), and 10% fetal calf serum (FCS, Gibco). The PBMC were counted with a hemocytometer and their number was adjusted to $5 \times 10^6$/mL.

MTT assay

PBMCs were cultured in 96-well microplate containing the growth medium and various concentrations of DAPA (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 mg/L) for 48 h. Cell proliferation was measured using a MTT Cell Proliferation Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s manual. At the end of incubation, MTT solution was added to each well at 1:10 ratio. After 3 h, the microplates were centrifuged
at 800 g for 5 min and 150 μL DMSO was added to dissolve any formazan crystals. The absorbance was determined at a wavelength of 570 nm by using a microplate spectrophotometer (Synergy-HT; BioTek Winooski, VT, USA). Results were expressed as percentage of cell viability. Determined values are the mean of 4–6 replicates. Mitomycin-C (MMC; Sigma-Aldrich, USA) was used as a positive control.

**LDH release assay**

CytoSelect™ LDH Cytotoxicity Assay kit was used to determine membrane integrity and performed following the manufacturer’s instructions. PBMCs were cultured in 96-well microplate containing the growth medium and various concentrations of DAPA (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 mg/L). After 48 h of incubation, 90 μL of the supernatant was mixed with 10 μL of assay reagent in a new microplate and incubated at 37°C for 30 min. The optical density was measured at a wavelength of 450 nm by a microplate spectrophotometer (Synergy-HT; BioTek Winooski, VT, USA). Results were expressed as percentage of LDH activity. Determined values are the mean of 4–6 replicates. Mitomycin-C (MMC; Sigma-Aldrich, USA) was used as a positive control.

**Total antioxidant capacity (TAC) and total oxidant status (TOS) assays**

PBMCs were cultured in 96-well microplate containing the growth medium and various concentrations of DAPA (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 mg/L) for 48 h. At the end of incubation, TAC assay and total oxidant status (TOS) assay kits (Rel Assay Diagnostics®, Turkey) were used to determine antioxidative/oxidative potential of DAPA from plasma samples and performed according the providers manual.

**CA assay**

Human blood cells were cultured with DAPA at IC_{50} concentration at 37°C for 72 h. Two hours before the harvest of cells, 0.02 μg/mL of colchicine was added to the culture to arrest the dividing cells at metaphase. When the incubation period was completed, the cells were treated with hypotonic solution (0.075 M KCl) and fixed in methanol:acetic acid. Then the cells were collected by centrifugation and dropped on clean glass slides. Giemsa in phosphate buffer (pH 6.8) was used to stain the slides. Fifty well-spread metaphases were counted for chromosomal aberration (CA) according to the Environmental Health Criteria 46 for environmental monitoring of human populations (IPCS, 1985). Mitomycin-C (MMC; Sigma-Aldrich, USA) was used as a positive control in CA assay.

**Statistical analysis**

Statistical analysis was performed using SPSS software (version 18.0, SPSS, Chicago, IL, USA). The Duncan’s test was used for the statistical analysis of experimental values in the MTT, LDH, CA, TAC and TOS analysis. Statistical decisions were made with a significance level of 0.05.

**Results**

Cytotoxic activity of DAPA was assessed against human lymphocyte cells by MTT assay. It is evident from Figure 1, DAPA exhibited a low cytotoxicity against lymphocyte cells at higher concentrations (25–400 mg/L) while DAPA did not cause significant inhibition of cell growth at lower concentration than 25 mg/L. The lowest survival rate for lymphocyte cells belonged to mitomycin-C used as a positive control (4.21%). IC_{50} value of DAPA was calculated as 302.76 mg/L according to the MTT results.

In addition to MTT assay, LDH release assay was performed in order to determine anti-proliferative activities of DAPA on human lymphocyte cells. Positive control (mitomycin-c) application gave rise to the highest LDH level, while the control-group showed the lowest LDH release (6.12 μU/mL). The two highest concentration (200 and 400 mg/L) of DAPA caused the maximum LDH release (52.64 and 61.93 μU/mL, respectively) on cells (Figure 2).

In the present study, TAC and TOS levels in blood cells were assessed to determine the antioxidative protection and oxidative damage by treatments. Figure 3 depicts the alterations in the level of TAC on treatments with DAPA. The cells without any treatment had minimum TAC level (15.21 mmol Trolox equivalent/L) and the highest concentrations of DAPA did not change significantly (p > 0.05) TAC level as compared to the control group. Treatment with DAPA at the 6.25 and 3.125 concentrations had higher TAC levels (19.67 and 20.12 mmol Trolox equivalent/L, respectively). As shown in Figure 4, the cells without any treatment had the lowest TOS level (5.21 μmol H_{2}O_{2} equivalent/L)
while positive control, H2O2, exhibited the maximum TOS level (11.33 μmol H2O2 equivalent/L) on blood cells. There was no statistically (p > 0.05) significant alteration in oxidative stress levels of cells treated with all different concentrations when compared to the control cells.

Genotoxic potential of DAPA on healthy lymphocyte cells was measured by CA assay. Based on the results, the highest CA frequency was observed in the cells treated with Mitomycin C used as a positive control. IC50 concentration of DAPA did not significantly (p > 0.05) change the frequency of CA according to the control group (Figure 5).

Figure 1: Viability of human blood cells after 48 h of exposure to 0–400 mg/L DAPA. Each value is given as mean ± standard deviation (n = 3). Control (−): untreated cells; Control (+): Mitomycin-C treated cells.

Figure 2: LDH activities in cultured human blood cells treated with different concentrations (0–400 mg/L) of DAPA for 48 h. Each value is given as mean ± standard deviation (n = 3). Control (−): untreated cells; Control (+): Mitomycin-C treated cells.

Discussion

The objective in diabetes treatment is to achieve a close-to-normal blood glucose level and optimal glycosylated hemoglobin (HbA1c) by reducing glycemic variability to a minimum, and to reduce all micro- and macrovascular complications, particularly cardiovascular complications.

The drug molecules used to achieve the treatment objective having positive effects on lipid profiles, weight, blood pressure, and postprandial plasma glucose, independently of their blood glucose lowering effects, and
also the presence of an antioxidant and β cell reserve protective effect against oxidative stress developing secondary to hyperglycemia are an important component of the effectiveness of the treatment and in protection against complications.

According to data from phase 3 trials of therapeutic protocols involving DAPA monotherapy, and two- and three-drug oral antidiabetic and insulin combinations, in addition to producing an effective decrease in HbA1c, the drugs have been proved to be safe and reliable and to have positive effects on weight loss [8–10].

The purpose of the present study was to investigate the cytotoxic effect of DAPA in human blood lymphocyte cultures together with MTT and LDH release measurements.
MTT analysis is widely used for the in vitro measurement of the metabolic activity of cell cultures exposed to different culture conditions [11]. The LDH release test is a marker of cell cytotoxicity based on the principle of cell membrane integrity [12]. In the current study, DAPA showed no major effect on cell viability. In accordance with current results, it was reported that administration of DAPA at >5000 times the maximum recommended human dose (MRHD: 10 mg) did not exhibit any toxic effect and to be well tolerated in rat and dog studies [13]. In addition, safety of DAPA was investigated in pooled analyses of phase IIb/III studies and it was found that DAPA has a favorable and predictable tolerability profile [14].

Increased oxidative stress is widely recognized as a factor in the development and progression of diabetes and diabetes-related complications [15]. Hyperglycemia-related oxidative stress leads to endothelial dysfunction (ED), which plays a central role in the pathogenesis of micro- and macrovascular diseases [16, 17]. ED is an early marker of diabetic vascular diseases and an independent predictor for cardiovascular diseases [18]. Oxidative stress also plays an important role in the neogenesis, proliferation and survival of β cells under both physiological and pathophysiological conditions. Pancreatic β cells are susceptible to oxidative stress due to their endogenous production of reactive oxygen species and low antioxidant enzyme synthesis [19].

Based on our TAC and TOS analysis, DAPA increased antioxidant capacity, but not TOS. We therefore conclude from this finding that DAPA may have beneficial impacts on the prevention of progression of diabetes and mortality-morbidity due to its positive effects on diabetic vascular complications and β cells reserve in the clinical setting. Considering DAPA’s glucose-lowering effect, it is also possible that both positive effects will be more effectively reflected in the clinical setting. Consistent with our results, many previous studies have reported that lowering blood glucose levels with DAPA monotherapy or two- and three-way drug therapies including DAPA as a component have an improving effect on β cell functions by reducing the toxic effect of glucose on these cells [20–22]. Studies have also investigated the healing effects of DAPA and other SGLT-2 inhibitors on endothelial cells and have reported improved cell functions due to positive effects on oxidative stress. Solini et al. reported better vascular and endothelial functions in T2DM patients treated with DAPA, and attributed this to a decrease in oxidative stress [23]. Shigiyama et al. monitored T2DM patients using metformin by adding DAPA to their treatment and reported lower oxidative stress markers and better endothelial functions in the DAPA group [24].

Type 2 diabetes is a pathological condition that increases the risk of cancer. This is because T2DM is a disease characterized by hyperinsulinemia, and hyperinsulinism exhibits mitogenic and anti-apoptotic effects on cancer cells [25]. It is therefore important that drugs to be used for therapeutic purposes should have no additional genotoxic effect. The in vitro genotoxic effects of DAPA were also investigated in this study. The CA assay results revealed that DAPA had no genotoxic potential. Similar to our findings, Reilly et al. reported that exposure to DAPA at >100 times the human clinical dose over a period of 2 years caused no difference in the incidence of tumors and proliferative/paraneoplastic lesions in the urinary bladder in CD-1 mice and Sprague Dawley rats, and that DAPA and its metabolites were not genotoxic. The same study also reported no increase in tumors or paraneoplastic lesions in dogs exposed to a >3000-fold DAPA dosage for more than a year or in SGLT2−/− mice observed over 15 months [26]. Although an increase has been reported in the incidence of bladder and breast cancer with DAPA use in some publications [27, 28], data from the current literature show no evidence of a causal relation between DAPA and bladder cancer [29, 30].

As a conclusion, it was revealed that DAPA has a low cytotoxic effect on healthy lymphocyte cells. In addition, TAC level is increased by DAPA at certain doses. Furthermore, DAPA did not show any significant genotoxic activity against blood cells. Overall, these results exhibited that DAPA is a well-tolerated drug and has a positive clinical benefit/risk balance in blood cells.

**Conflict of interest:** There is no conflict interest of any author.

**References**


