



RESEARCH ARTICLE

Dapagliflozin, a sodium glucose cotransporter 2 inhibitors, protects cardiovascular function in type-2 diabetic murine model

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Abstract. Diabetes mellitus and its complications are major international health problems in which there are many limitations to the orthodox approaches in the treatment. Sodium glucose cotransporter 2 (SGLT2) inhibitors are a new class of diabetic medications, with a different mechanism of action that may reduce risk of cardiovascular complications. To evaluate the effect of SGLT2 inhibitor monotherapy on cardiovascular complications in patients with type-2 diabetes and to compare its effect with the first-line therapy, metformin. Eighty rats divided into four groups were used: nondiabetic, diabetic nontreated, diabetic + met and diabetic + dapa. At the end, the arterial blood pressure and cardiac performance were assessed. Glycemic index, lipid profile, total antioxidant capacity, malondialdehyde, tumour necrosis factor α were measured. DNA changes were assessed from the hearts and aortae. Aortic tissue changes recorded using haematoxylin and eosin, Masson trichrome and iNOS immune stain. Glycemic index, lipid profile, oxidative stress and inflammatory parameters were significantly improved in both metformin and dapagliflozin treated groups with significant improvement in blood pressure and cardiac performance. Also, there were noticeable significant reduction in DNA fragmentation in aortic and cardiac tissues and reduction in collagen deposition and iNOS expression in aortic tissue. Dapagliflozin treatment results' significantly surpassed improvement of metformin treatment nearly in all parameters. Total genomic DNA extraction proved that SGL2 inhibitor (dapagliflozin) has superior glycemic control and cardiovascular protective effect over metformin especially in type-2 diabetes with high fat intake.

Keywords. type-2 diabetes; dapagliflozin; cardiovascular complication; cardiac fragmentation.

Introduction

Diabetes mellitus (DM) is a major international health problem characterized by relative deficiency in the production or the action of insulin, associated with hyperglycaemia that creates multiple bodily complications (ADA 2013). The most leading cause of death among them is cardiovascular disease (CVD) (ADA 2018) as the pathologic change caused by DM induces diabetic myocardial apoptosis (Wu *et al.* 2019). In type-2 diabetes, the more the intensive treatment of glycaemia the more the reduction of long-term CVD rates (Orchard *et al.* 2015).

Metformin as first-line therapy in type-2 diabetes, is generally acknowledged to restore the body's response to insulin, reduce gluconeogenesis in the liver and inhibit glucose uptake in the intestines (Zheng *et al.* 2015). Metformin has beneficial effects on the glycosylated haemoglobin (HbA1c) and cardiovascular mortality, however

most patients fail to maintain target glycated haemoglobin (A1C) levels with metformin monotherapy (Wexler 2019). The success of oral antidiabetics is limited by their mechanisms of action, which often address the symptoms rather than its underlying pathophysiology (Oguntibeju 2014). While there are many limitations to the orthodox approaches in the treatment of DM (Oguntibeju 2014), there is a very few systematic data available for other oral agents as initial therapy of type-2 diabetes (ADA 2019).

Sodium-glucose cotransporter 2 (SGLT2) inhibitor, also called gliflozins, is considered as a good therapeutic option for patients with type-2 diabetes owing to the fact that its comparatively better safety profile and their unique mechanism of action (Kalra *et al.* 2018), especially for people with established CVD, or chronic kidney disease, as it is shown to reduce risk of chronic kidney disease progression, cardiovascular events, or both (ADA 2019). The mechanism of action of this new class of drugs is to block the SGLT2

protein involved in 90% of glucose reabsorption in the proximal convoluted tubule, resulting in increased renal glucose excretion and lowered blood glucose levels (DeFronzo 2017). The aim of this research was to evaluate the effect of SGLT2 inhibitor monotherapy on cardiovascular complications in patients with type-2 diabetes using total cardiac and aortic DNA extraction and to compare its effect with the first-line therapy, metformin.

Materials and methods

Animals and experimental design

This study was carried out in accordance with the regulations of Animal Experimentation Ethics Committee of faculty of medicine Menoufia University. Eighty adults male Wistar albino rats weighing 120–150 g were used. The animals were housed at 20–24°C with 12-h light/ dark cycles and were provided with freely available standard rat chow and tap water.

Rats were randomly divided into four groups ($n = 20$ rats each) as follows; (i) Nondiabetic: injected intraperitoneally (IP) by single dose of citrate buffer (Adwic, Egypt). (ii) Diabetic nontreated: received fatty diet (HFD) for 4 weeks (Ozturk et al. 2015) and single IP injection of streptozotocin (STZ) (Sigma Chemical, USA) 35 mg/kg for induction of type-2 diabetes. Rats were considered diabetic when the fasting blood glucose level was above 113 mg/dL (Yazar et al. 2011). (iii) Diabetic + met: after being diabetic as in the previous group, rats were treated by oral metformin (Amoun, Egypt); (100 mg/kg by oral gavage once a day) (Chang et al. 2006). (iv) Diabetic + dapa: after being diabetic as in group 2, rats were treated by oral dapagliflozin (AstraZeneca, Sweden, UK) (0.1 mg/kg body weight once a day by oral gavage) (Han et al. 2008).

At the end of the experiment (after 4 weeks), 10 rats from each group were anesthetized for invasive measuring of blood pressure. Further, 3 mL of overnight fasting blood samples were collected from aortic cannula for biochemical analysis. After which the hearts and aortae were excised and preserved at -80 degree for DNA extraction and determination of rate of flow of extracted myocardial DNA. The other 10 rats from each group were decapitated and the chest wall of each rat was opened and the heart was excised and hooked from its apex. Cardiac contractility and heart rate were recorded. The thoracic aorta was fixed in 10% phosphate-buffered formalin solution and processed through paraffin embedding and prepared for histopathological studies.

Measurement of invasive blood pressure

Rats were anesthetized by using thiopental sodium (Epico, Egypt) (50 mg/kg (IP) (Zorniak et al. 2010). The aorta is

identified and cannulated using a cannula pre-filled with heparinized normal saline and the other end of the cannula was connected to a three-way stopcock saline filled syringe which was connected to a pressure transducer and the invasive blood pressure (SBP & DBP) was recorded using physiograph system (Narco Bio-Systems, UK). Further, mean arterial blood pressure (MABP) was calculated by using the formula:

$$\text{MABP} = \text{Diastolic blood pressure} + (\text{pulse pressure}/3) + 5 \text{ mmHg.}$$

Blood biochemical analysis

Three mL of aortic blood samples were collected as follows: 1 mL was collected in EDTA containing tube to be used for glycosylated haemoglobin (HbA1c) analysis (Riomidi, France) (Abraham et al. 1978); the remaining blood samples were collected in plain tube, left for clotting for 10 min and centrifuged at 4000 rpm for another 10 min to isolate the serum and maintained at -20°C for further analysis of serum glucose colorimetrically using a test reagent kit (EMAPOL, Poland) (Trinder 1969), serum insulin using ELISA commercial kits (DRG Instruments GmbH, Germany) (Judzewitsch et al. 1982), lipid profile; total cholesterol and high density lipoprotein (HDL) (mg/dL) levels were estimated using test reagent kits (Biodiagnostics, Egypt). Triglycerides (TGs) level (mg/dL) using reagent kit (EMAPOL, Poland) (Trinder 1969). Serum level of low density lipoprotein (LDL) was calculated using the formula: $\text{LDL} = \text{total cholesterol} - (\text{HDL} + \text{triglycerides}/5)$ as described by Friedwald et al. (1972), malondialdehyde (MDA) using test reagent kits (Biodiagnostics, Egypt), total antioxidant capacity (TAC) colorimetrically using a test reagent kit (Bio-diagnostic, Egypt) (Koracevic et al. 2001), and serum tumour necrosis factor alpha (TNF α) using solid phase (Elisa) kits (Assaypro, USA) (Taylor 2001). Further, homeostasis model assessment index (HOMA-IR) for insulin resistance was calculated: $\text{insulin} (\mu\text{U/mL}) \times \text{glucose} (\text{mg/dL}) \div 405$.

Cardiac contractility and heart rate

Rats were decapitated and the chest wall was opened, the heart was rapidly excised and immediately placed in a Petri dish containing ice-cold oxygenated modified Krebs–Henseleit solution; (EL-Nasr, Nasr, Egypt). The aorta was cannulated with an 18-gauge glass cannula, tied with a thread in a noncirculating Langendorff apparatus (Taegtmeier 1995). The isotonic contraction of the heart was recorded via tracing on a paper sheet of rotating drum of kymograph at a speed (5 mm/s) (Harvard, UK). Contractile function was assessed by measuring the heart rate and the height of cardiac contractility.

Aortic histopathology

Haematoxylin and eosin (H&E) staining: Several sections were cut from the paraffin-embedded blocks of thoracic aortae of 5 μ thickness and used for staining with H&E for evaluation of endothelium corrugation, endothelial vacuolization, smooth muscle vacuolization and thickness of aortic wall (Hassan 2013).

Masson trichrome technique: The aortic tissue was stained first with the acid dye, Biebrich scarlet, which binds with the acidophilic tissue components. Further when treated with the phospho acids, the less permeable components retain the red pigment, while the red was pulled out of the collagen. At the same time causing a link with the collagen to bind with the aniline blue (Bancroft and Stevens 1990).

Immuno histochemical technique using induced nitric oxide synthase (iNOS) antibody increased the intensity of staining which indicates increased iNOS enzyme activity (Capellini *et al.* 2010).

Total genomic DNA extraction and apoptosis detection in aortic and cardiac tissues

Nucleic acid extraction was done according to the modified extraction method of El-Garawani and Hassab El-Nabi (2016), in which the direct staining of DNA sample was done. Apoptotic bands of DNA fragmentation appeared and was located at 180 bp and its multiples 360, 540 and 720 bp against 100 bp plus DNA ladder (Thermo Scientific O'gene ruler) (Hassab El-Nabi and Elhassaneen 2008). The intensity of released DNA fragments was measured by image J software, as a mean of optical density values to assess total released left ventricular DNA fragmentation and total released aortic DNA fragmentation.

Statistical analysis

The data were analysed by SPSS Statistical Package software v. 20. Quantitative data were expressed as mean \pm

standard error of mean ($X \pm$ SEM). Data from control and test groups were compared using one-way analysis of variance (ANOVA) followed by Tukey post-Hoc test, values < 0.05 of probabilities were considered statistically significant ($P < 0.05$).

Results

Blood biochemical analysis

Fasting serum glucose, HbA1c%, serum insulin and HOMA-IR in diabetic nontreated group were significantly higher ($P < 0.05$) compared to their corresponding levels in nondiabetic group and were significantly lower ($P < 0.05$) compared to diabetic + met and diabetic + dapa groups, but still significantly higher in diabetic + met compared to nondiabetic. These parameters were significantly lower in diabetic + dapa compared to diabetic + met except for serum insulin (table 1).

Serum lipid profile (cholesterol, TGs and LDL) in diabetic nontreated group were significantly higher ($P < 0.05$) compared to nondiabetic group. In diabetic + met group, these parameters were significantly lower ($P < 0.05$) compared to diabetic nontreated group but still significantly higher compared to nondiabetic, except for triglycerides. These parameters were significantly lower in diabetic + dapa compared to diabetic nontreated and diabetic + met groups. HDL level in diabetic nontreated group was significantly lower ($P < 0.05$) compared to nondiabetic group, while it was significantly higher ($P < 0.05$) in diabetic + met group compared to their corresponding levels in diabetic nontreated group but still significantly lower compared to nondiabetic. This parameter was significantly higher in diabetic + dapa compared to diabetic nontreated and diabetic + met (table 1).

Serum TAC level in diabetic nontreated group was significantly lower ($P < 0.05$) compared to nondiabetic group, while it was significantly higher ($P < 0.05$) in

Table 1. Serum glucose, insulin, HbA1c%, HOMA-IR, lipid profile, MDA, TAC, and TNF levels in control, fructose and fructose plus melatonin treated groups.

	Nondiabetic	Diabetic nontreated	Diabetic + met	Diabetic + dapa
Fasting serum glucose (mg/dL)	95.3 \pm 1.4	390.5 \pm 17.6*	150.6 \pm 3.7*#	110.9 \pm 2.5# ϕ
HbA1c% normal Hb	5.5 \pm 0.17	10.1 \pm 0.56*	6.9 \pm 0.15*#	5.9 \pm 0.14# ϕ
Serum insulin (μ IU/mL)	12.7 \pm 0.63	16.3 \pm 0.21*	14.1 \pm 0.25*#	12.92 \pm 0.16#
HOMA-IR index	3.05 \pm 0.13	7.16 \pm 1.27*	4.16 \pm 0.29*#	3.9 \pm 0.14# ϕ
Cholesterol (mg/dL)	100.5 \pm 1.7	230.75 \pm 14.68*	121.50 \pm 6.18#	110.35 \pm 2.1# ϕ
Triglycerides(mg/dL)	67.84 \pm 1.7	103.72 \pm 2.29*	81.70 \pm 2.94*#	70.95 \pm 2.75*# ϕ
LDL (mg/dL)	34.06 \pm 1.3	74.21 \pm 1.7*	44.68 \pm 1.9*#	38.28 \pm 2.26# ϕ
HDL (mg/dL)	58.60 \pm 3.3	30.92 \pm 3.00*	41.56 \pm 2.59*#	55.81 \pm 4.1# ϕ
Serum TAC (mM/L)	2.26 \pm 0.16	0.91 \pm 0.18*	1.93 \pm 0.11#	2.20 \pm 0.14# ϕ
Serum MDA (nM/mL)	4.76 \pm 0.18	14.80 \pm 0.27*	5.27 \pm 0.10*#	4.91 \pm 0.16#
Serum TNF α (pg/mL)	15.92 \pm 1.49	21.65 \pm 1.70*	19.23 \pm 1.81*#	16.34 \pm 2.31# ϕ

Data were expressed as mean \pm SE ($n = 20$). One-way ANOVA: * < 0.05 vs nondiabetic; # < 0.05 vs diabetic nontreated; $\phi < 0.05$ vs diabetic + met.

diabetic + met and diabetic + dapa groups compared to their corresponding levels in diabetic nontreated group. This parameter was significantly higher in diabetic + dapa compared to diabetic + met group. Serum MDA and TNF α in diabetic nontreated group were significantly higher ($P < 0.05$) compared to their corresponding levels in nondiabetic group, while these parameters in diabetic + met and diabetic + dapa groups were significantly lower ($P < 0.05$) compared to their corresponding levels in diabetic nontreated group but were still significantly higher ($P < 0.05$) in diabetic + met group compared to their corresponding levels in nondiabetic group. TNF α were significantly lower in diabetic + dapa compared to diabetic + met group (table 1).

Invasive blood pressure and cardiac performance

Systolic, diastolic and mean arterial blood pressure (measured by invasive technique) in diabetic rats showed significant increase ($P < 0.05$) compared to their corresponding levels in nondiabetic group, while these parameters were significantly lower ($P < 0.05$) in diabetic + met group compared to their corresponding levels in diabetic nontreated group but still significantly higher compared to nondiabetic. These parameters were significantly lower in diabetic + dapa compared to diabetic + met (figure 1a).

Cardiac contractility and heart rate in diabetic nontreated group were significantly lower ($P < 0.05$) compared to nondiabetic group, while they were significantly higher ($P < 0.05$) in diabetic + met compared to their corresponding levels in diabetic nontreated group but still significantly lower compared to nondiabetic, these parameters were significantly higher in diabetic + dapa compared to diabetic + met (figure 1b).

Aortic histopathological results

Histological results of aortae stained with H&E showed normal appearance and thickness of the aorta with normal endothelial corrugation in nondiabetic group, while diabetic nontreated group aorta showed loss of corrugation of the endothelium, endothelial vacuolization with intracellular lipid accumulation and smooth muscle vacuolization (foam cells). The thickness of the aortic wall media, extracellular lipid pools and adventitial lymphocytes significantly increased compared to nondiabetic. Diabetic + met showed significant reduction in thickness of wall of aorta with retrieved endothelial corrugation and small extracellular lipid pools. Diabetic + dapa showed significant reduced intimal endothelium damage, thickness of the aortic wall and adventitial infiltration compared to diabetic nontreated group (figure 2a).

Results of Masson trichrome staining the collagen of the aortae showed normal elastic lamellae and normal thickness of the aorta with normal endothelial corrugation in nondiabetic group, while diabetic nontreated showed significant increased collagenous tissue deposition between the elastic lamellae when compared to nondiabetic group. Diabetic + met showed significant reduction in collagen deposition when compared to diabetic group, while diabetic + dapa showed marked reduction in collagen deposition and marked reduction in the thickness of aortic wall that was significantly decreased when compared to diabetic and diabetic metformin treated groups (figure 2b).

Results of iNOS immune staining of aorta showed significant increase in the dark brown staining of the endothelial monolayer along the luminal surface of aorta in diabetic nontreated compared to nondiabetic group. Diabetic + met showed significant reduction compared to diabetic

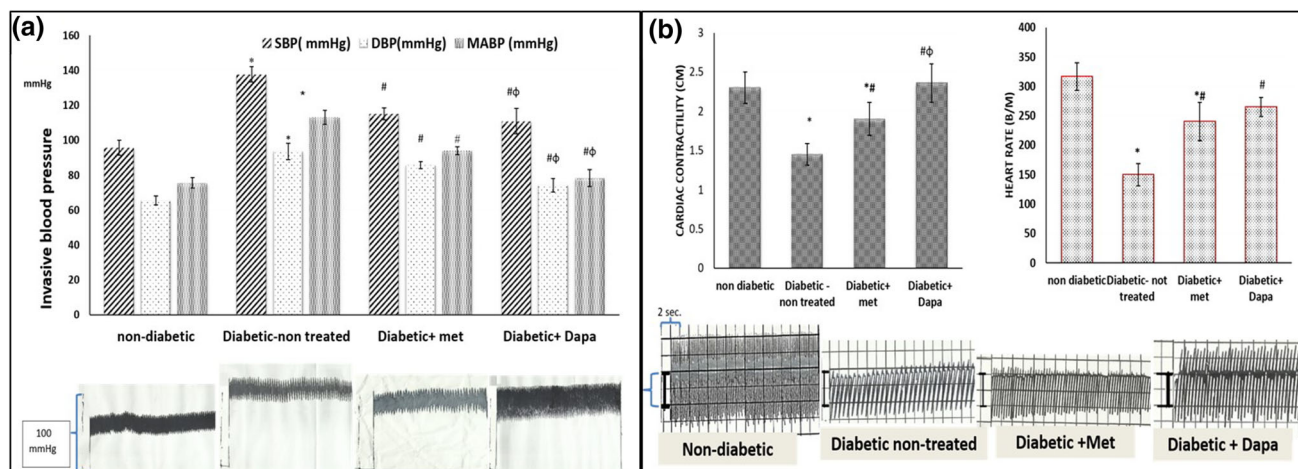


Figure 1. Invasive blood pressure and cardiac performance (hear rate and cardiac contractility) results among normal, diabetic, diabetic + met and diabetic + dapa groups. (a) Level of systolic, diastolic and mean arterial blood pressure (mmHg) among groups. (b) Heart rate (beat/minute) and magnitude of cardiac contractility (cm) among groups. Data were expressed as mean \pm SE ($n = 8-10$). One way ANOVA: * < 0.05 vs nondiabetic; # < 0.05 vs diabetic nontreated; φ < 0.05 vs diabetic + met.

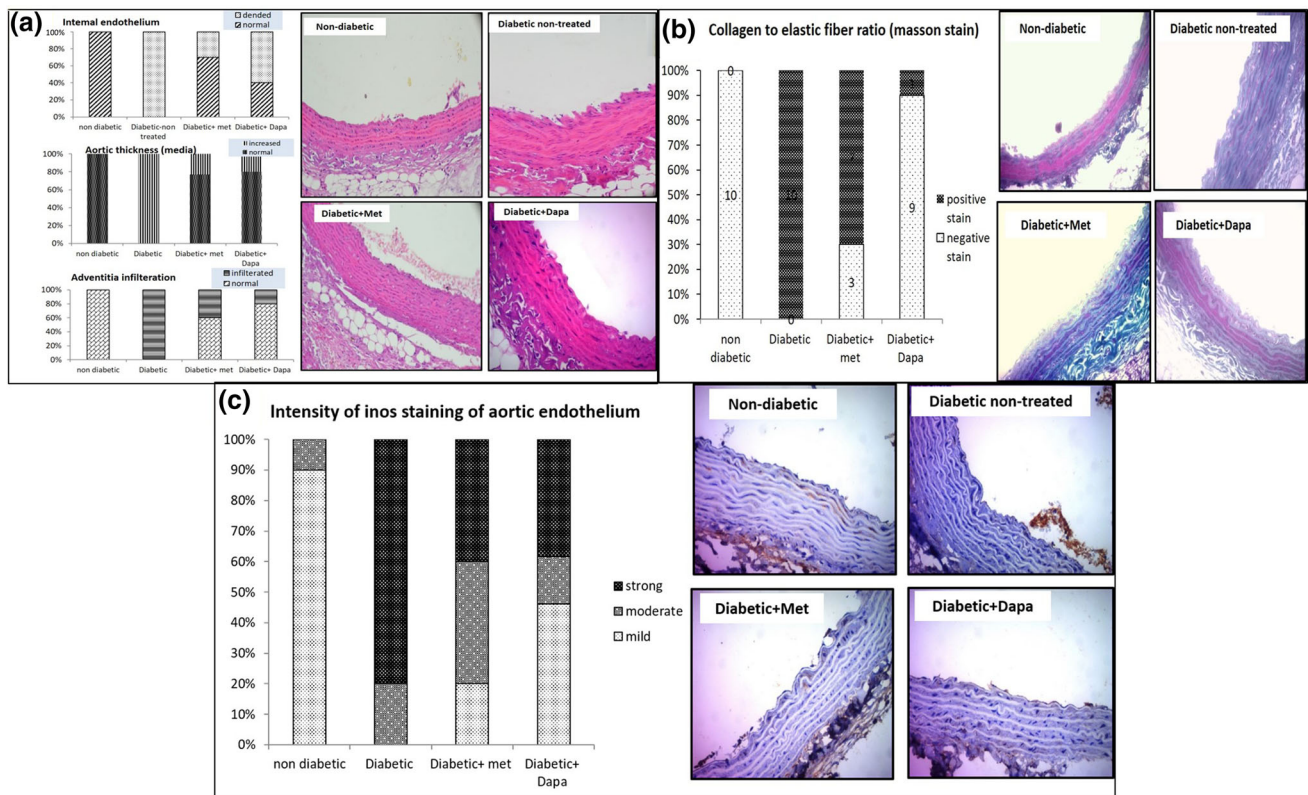


Figure 2. Aortic histopathology using H&E staining and masson trichrome stain results among normal, diabetic, diabetic + met and diabetic + dapa groups. (a) Histological results of aortae stained with H&E among groups. (b) Histological results of aortae stained with Masson trichrome for collagen detection among groups. (c) Histological results of aortae stained with iNOS immune stain among groups. Data were expressed as mean \pm SE ($n = 8-10$). One way ANOVA: * < 0.05 vs nondiabetic; # < 0.05 vs diabetic nontreated; $\phi < 0.05$ vs diabetic + met.

nontreated group. Diabetic + dapa showed marked reduction compared to diabetic and diabetic + met groups (figure 2c).

DNA electrophoresis results

The mean value of total released left ventricular DNA fragmentation (DNA optical density) of diabetic nontreated group was significantly higher, when compared to nondiabetic group. While in diabetic + met it was significantly lower when compared to diabetic nontreated group and still significantly higher when compared to nondiabetic group. In diabetic + dapa it was significantly lower when compared to diabetic group, diabetic + met and still significantly higher compared to nondiabetic groups (figure 3a).

The mean value of total released aortic DNA fragmentation (DNA optical density) of diabetic nontreated and diabetic + met groups was significantly higher, when compared to nondiabetic group. While in diabetic + dapa it was significantly lower when compared to diabetic group and diabetic + met groups (figure 3b).

Discussion

The present investigation was assigned to study and compare the effects of SGL2 inhibitor (dapagliflozin) to the insulin sensitizer (metformin) on glycemic state, lipid profile, arterial blood pressure, cardiac performance, endothelial changes of thoracic aorta, cardiac and aortic nucleic acid changes in STZ-HFD induced diabetic rats.

In the present study, diabetes mellitus type-2 rat model was successfully established in fat-fed rats injected with low-dose streptozotocin (STZ-HFD) as evidenced by significant increase in glycemic index parameters and was associated with dyslipidaemia, oxidative stress and increased inflammatory marker, $\text{TNF}\alpha$. STZ-HFD resulted in development of insulin resistance and DM (Alfa *et al.* 2015); cardiovascular complications as elevation of blood pressure and decrease in cardiac performance (cardiac contractility and heart rate) with subsequent structural damage in cardiac and aortic tissues, left ventricular muscle and aortic tissue DNA fragmentation and apoptosis, which is one of the fundamental mechanisms leading to loss of contractile units, causing cardiac function impairments (Huynh *et al.* 2014).

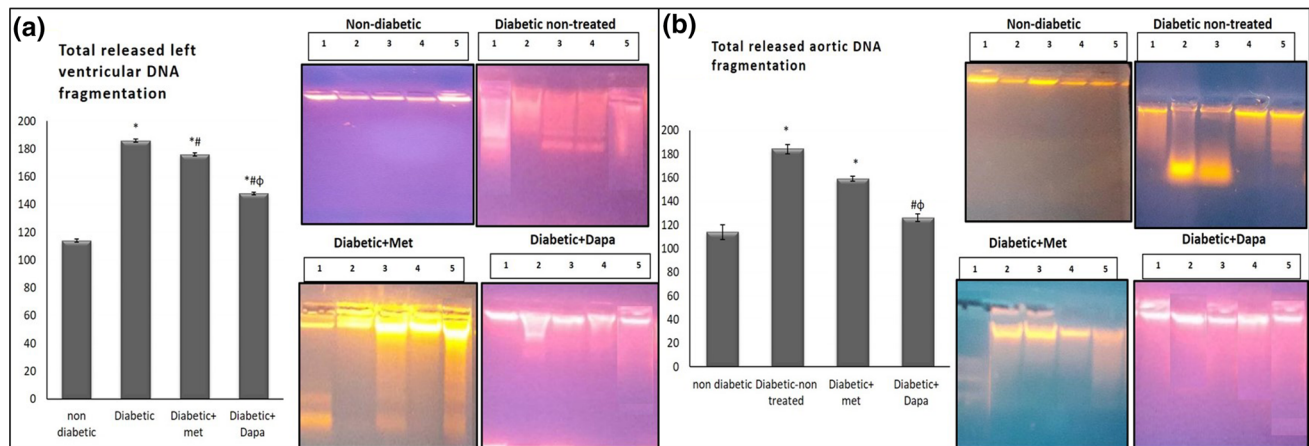


Figure 3. DNA electrophoresis results among normal, diabetic, diabetic + met and diabetic + dapa groups. (a) DNA electrophoresis results showing the mean value of total released left ventricular DNA fragmentation (DNA optical density) among groups. (b) DNA electrophoresis results showing the mean value of total released aortic DNA fragmentation (DNA optical density) among groups. Data were expressed as mean \pm SE ($n = 8-10$). One way ANOVA: * < 0.05 vs nondiabetic; # < 0.05 vs diabetic no-treated; $\phi < 0.05$ vs diabetic + met.

In the current study, SGLT2 inhibitors (dapagliflozin) produced profound cardiovascular protection in STZ-HFD rat model as verified by amelioration of the elevated blood pressure and the reduced cardiac performance. This was in agreement with several other studies (Dziuba *et al.* 2014; Joubert *et al.* 2017; Arow *et al.* 2020). SGLT2 inhibitors is a new strategy for the management of diabetes controlling hyperglycaemia with reduction of HbA_{1c}, body weight and vascular complication with insulin-independent mode of action (Briasoulis *et al.* 2018). It may decrease preload through promoting osmotic diuresis which reduces volume overload in heart failure patients improving myocardial stretching mechanisms and contractility (Cherney *et al.* 2014). Arow *et al.* (2020) attributed cardiovascular protection with SGLT2 inhibitors to multiple pleiotropic effects beyond hypoglycaemia.

Dapa improved dyslipidaemia, which is the cause of chronic low-grade inflammation on vascular endothelium, that leads to fibrosis, cell death, cardiac remodelling (Arow *et al.* 2020), hypertension and impaired cardiac function (Panth *et al.* 2016).

Dapa improved oxidative stress, which accelerate pathogenesis of the cardiovascular diseases by the associating hypertension and increased serum cholesterol level (Cheng *et al.* 2017). The former is supported by the link between reactive oxygen species (ROS) and the development of atherosclerosis (Yung *et al.* 2006), cell apoptosis and necrosis (Feidantsis *et al.* 2018).

The DNA laddering pattern which is the diagnostic pattern of apoptosis found in diabetic nontreated group of this study indicated left ventricular muscle and aortic tissue DNA fragmentation was improved after dapa treatment. The improvement in aortic and cardiac DNA fragmentation may be implicated to reduction of iNOS which is a major mediator of inflammation and cellular apoptosis (Nakazawa

et al. 2017); improvement of diabetic-induced oxidative damage responsible for activation of the death pathways implicated in cell apoptosis and necrosis (Feidantsis *et al.* 2018); and reduction in inflammatory mediator TNF α . Dapa also succeeded to alleviate endothelial dysfunction and atherosclerotic changes evidenced in histopathological findings of aortic samples stained with H&E, Masson trichrome and iNOS immune staining.

Treatment with metformin indicated the importance of glycemic control and blood lipid control in the treatment of type-2 diabetes induced hypertension and cardiomyopathy. However, the limited results of metformin in this HFD/low-dose STZ diabetic rat model evidenced by significant disturbed parameters compared to nondiabetic and dapa treated groups was in agreement with Wexler (2019) who reported that the majority of patients fail to maintain target glycated haemoglobin (A1C) levels with metformin monotherapy. Wessels *et al.* (2014) proved that treatment with metformin compromises *in vivo* and *ex vivo* oxidative capacity in a dose-dependent manner, in both healthy and diabetic rats. Reduced mitochondrial oxidative capacity may contribute to cardiac dysfunction especially with obesity (Boudina *et al.* 2005). Combination therapy between metformin with other hypoglycaemic drugs produced better results than metformin alone. For example, the results of Lunder *et al.* (2018) showed that metformin alongside empagliflozin, a new SGLT2 inhibitor, has significantly improved arterial stiffness compared to metformin alone.

The National Institute for Health and Care Excellence (NICE) and the Scottish Intercollegiate Guideline Network (SIGN) recommend considering SGLT2 inhibitors treatment in patients with type-2 diabetes alongside other glucose-lowering medicines as metformin if unable to achieve glycemic control, or as a first-line treatment in cases of metformin intolerance (NICE 2017, <http://nice.org.uk/guidance>;

SIGN 2017). According to Liu *et al.* (2019), SGLT2 inhibitors should be a part of the therapeutic regimen for all eligible type-2 diabetic patients with established cardiovascular disease and those with high risk. This approach is further supported by the recently published results of the Wiviott *et al.* (2019) in which there was cardiovascular and renal outcome benefits with dapagliflozin in patients with established disease and even extend to lower-risk patients, compared to standard of care.

In conclusion, high fat diet and low-dose streptozotocin injection in rats induced type-2 diabetes associated with disturbed glycemic index, serum lipid, inflammation, oxidative stress parameters and was complicated with cardiovascular structural and functional damage. Dapagliflozin has superior glycemic control and cardiovascular protective effect over metformin especially in type-2 diabetes with established CVD or at susceptible risk, this is proved by laboratory results and other functional investigation and by the results of total genomic DNA extraction.

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