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Comparative Cardioprotective Effects of SGLT2 Inhibitors in Preclinical Models of Diabetic Cardiomyopathy

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) often leads to severe cardiovascular complications such as diabetic cardiomyopathy (DCM) which is the main cause of significant morbidity and mortality. There is mounting evidence that sodium-glucose cotransporter 2 inhibitors (SGLT2i) offer cardioprotective effects that are not related to glucose-lowering mechanisms.

Objective: We conducted a systematic comparative study to assess the cardioprotective potential of SGLT2 inhibitors (dapagliflozin) versus glucagon-like peptide-1 (GLP-1) agonists (liraglutide) in type 2 diabetic rat models and also to figure out the molecular mechanisms behind this effect.

Methods: Thirty-two male Sprague Dawley rats were divided randomly into four groups: normal controls, untreated T2DM, T2DM + SGLT2i (1 mg/kg), and T2DM + GLP-1 (75 µg/kg) for four weeks. The evaluation was extensive and covered serum biochemistry, cardiac enzymes, myocardial oxidative stress markers (malondialdehyde, glutathione, catalase), inflammatory cytokine mRNA (TNF-α, TGF-β), apoptotic markers (caspase-3), sympathetic nervous system activity (norepinephrine, tyrosine hydroxylase), and histopathology.

Results: Treatment with SGLT2 inhibitor resulted in better glycemic control (95.9% blood glucose reduction) and stronger cardioprotection as compared to GLP-1 therapy. The SGLT2i group vigorously decreased myocardial oxidative stress (63.2% MDA reduction, 82.2% GSH restoration), inflammatory cytokines (69% TNF-α reduction, 73.3% TGF-β reduction), apoptosis (83.6% caspase-3 reduction), myocardial fibrosis (85% reduction), and sympathetic activity (63.3% norepinephrine reduction). The histopathological study showed almost normal myocardial structure with very little fibrosis in the hearts of the animals treated with SGLT2i as compared to the presence of fibrosis in those treated with GLP-1.

Conclusions: The results of this study show that SGLT2 inhibitors have greater cardioprotective efficacy against diabetic cardiomyopathy and that the mechanisms are glucose-independent and involve oxidative stress attenuation, anti-inflammatory effect, apoptosis inhibition, fibrosis suppression, and sympathetic nervous system modulation. These results position SGLT2 inhibitors as powerful cardioprotective drugs with a possible use in the clinic for the prevention of heart failure progression in type 2 diabetic populations.

KEYWORDS: SGLT2 Inhibitors, Diabetic Cardiomyopathy, Oxidative Stress, Fibrosis, Inflammation, Apoptosis, Sympathetic Nervous System, Type 2 Diabetes, Dapagliflozin, Liraglutide.

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INTRODUCTION

Diabetes mellitus remains a significant health problem that affects more than 415 million people worldwide, and the number is expected to reach over 642 million by 2040 (Ogurtsova et al., 2017). Among the complications of diabetes, cardiovascular diseases are the leading cause of about 65% of the deaths in diabetic patients, with diabetic cardiomyopathy (DCM) being recognized as the major contributor to this morbidity and mortality burden (Hussein et al., 2020). Diabetic cardiomyopathy is characterized clinically by the presence of left ventricular dysfunction and heart failure without coronary artery disease or hypertension. It is associated with both systolic and diastolic dysfunction and has an annual mortality rate of 15-20%.

The pathology of the DCM is based on a complicated network of molecular mechanisms. The chronic high blood sugar condition leads to the overproduction of reactive oxygen species (ROS) due to mitochondrial oxidative phosphorylation and NADPH oxidase activation, thereby exceeding the antioxidant defense of the heart. The increased oxidative stress results in apoptosis due to cytochrome C release from mitochondria and activation of caspase-3. At the same time, continuous hyperglycemia activates the renin-angiotensin-aldosterone system (RAAS), which leads to fibroblast-to-myofibroblast transition and excessive collagen deposition via the transforming growth factor-beta (TGF-β) signaling pathway. Moreover, a variety of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins act as the main orchestrators of myocardial inflammation and cardiac remodeling.

One of the significant breakthroughs in diabetic pharmacotherapy was the introduction of sodium-glucose cotransporter 2 inhibitors (SGLT2i) as a new class of drugs. These drugs have been found to exert beneficial effects on the heart that are independent of their glucose-lowering properties. Unlike conventional antidiabetic drugs, which primarily improve glucose homeostasis, SGLT2 inhibitors offer direct protection to the heart by changing the way cells handle sodium and calcium and energy.

Several clinical trials have shown that the use of SGLT2 inhibitors is associated with a decrease in hospitalizations due to heart failure and cardiovascular deaths. This effect seems to be mediated by mechanisms going beyond glucose control.

Despite the mounting clinical evidence, the specific mechanistic benefits of SGLT2 inhibitors over other cardioprotective drugs are still unclear. This exhaustive preclinical study provides a systematic comparison of the cardioprotective efficacy of SGLT2 inhibitors (dapagliflozin) versus glucagon-like peptide-1 (GLP-1) agonists (liraglutide) across multiple pathogenic pathways in the type 2 diabetic rat model. Our working hypothesis is that SGLT2 inhibitors would have a greater cardioprotective effect that is independent of glucose and that this effect would be related to the suppression of oxidative stress, anti-inflammatory effect, apoptosis inhibition, and sympathetic nervous system modulation.

LITERATURE REVIEW

2.1 DCM: Epidemiology and Pathophysiology

The development of DCM is mostly associated with molecular and cellular changes in the patient's body resulting from long-term hyperglycemia and insulin resistance.

Among the major factors that cause DCM is hyperglycemia-induced oxidative stress and this may eventually lead the heart to fail in different manners. The excess glucose changes the polyol pathway such that aldose reductase is activated and at the same time NADPH, which it needs from the cytosol, is being depleted. Simultaneously, glucose oxidation in mitochondria is increased making the electron transport chain the major source of ROS. AGEs are the final products when glucose binds non-enzymatically to proteins leading to the formation of RAGE that recognizes it, thus, inflammatory pathways are activated via NF-κB binding.

2.2 SGLT2 Inhibitors: Mechanisms and Cardioprotective Properties

It was a surprise that heart tissues also contain SGLT2, so direct cardiac effects can occur along with systemic glucose-lowering ones.

SGLT2 inhibitors employ one or more mechanisms to help the heart resist the damage, most of which are not related to glucose or metabolism. One of the ways the pathological calcium overload in the heart is reduced is by the disease-causing SGLT2 inhibition that results in the decrease of myocardial cytoplasmic sodium levels, thus the sodium-calcium exchanger gradient is restored and the pathological calcium overload is relieved (Baartscheer et al., 2017). Consequently, less ROS is generated because oxidative phosphorylation remains efficient through the facilitation of mitochondrial calcium uptake. Besides, SGLT2 inhibitors also relieve the cellular NADPH oxidase of its burden by eliminating sodium stress from the cell and, at the same time, they activate Nuclear factor erythroid 2-related factor 2 (Nrf2) thus leading to the enhancement of body's intrinsic antioxidant constituents.

2.3 GLP-1 Agonists: Alternative Cardioprotective Mechanisms

The principal cardioprotective pillars of GLP-1 agonists are entirely different from those of SGLT2 inhibitors. One of the outcomes is the induction of cardiomyocyte survival as the association of GLP-1 receptor on a cardiomyocyte with its ligand leads to the direct activation of cytoprotective signal transduction cascades comprising PI3K/Akt pathways. GLP-1 agonists intercept inflammatory cytokines by impeding their manufacture via the inhibition of receptor for advanced glycation end products (RAGE)-mediated NF-κB activation. The involvement of GLP-1 receptor in the brain diminishes the activity of the sympathetic nervous system, thus systemic hemodynamics are regulated, and myocardial workload gets reduced. Nevertheless, the question of comparative performance of GLP-1 agonists vs. SGLT2 inhibitors in an overall cardioprotective profile is still not convincingly resolved by the preclinical models.

2.4 Comparative Therapeutic Approaches

The authors of the previous studies in their separate works have mainly focused on both SGLT2 inhibitors and GLP-1 agonists as potential agents that could make the heart resistant to the adverse effects of diabetes in animal models. Specifically, SGLT2 inhibitorEmpagliflozin was instrumental in the reduction of the infarct zone and cardiomyocyte apoptosis in the heart through mitochondrial andSTAT3 dependent mechanisms (Andreadou et al., 2017). Dapagliflozin reversed diabetic cardiomyopathy symptoms in a type 2 diabetic mouse model through the blockade of the NLRP3 inflammasome and the relief of oxidative stress (Ye et al., 2017). In contrast, GLP-1 agonists like exendin-4 and liraglutide, demonstrated the ability to protect the myocardium from the ischemic insult as well as the subsequent remodeling through the anti-apoptotic and oxidative stress inhibitory pathways.

Besides, almost no preclinical study has dared to challenge that direct comparison of the relative cardioprotective efficacies of SGLT2 inhibitors and GLP-1 agonists across a wide range of molecular and histopathological endpoints. This work fills that void by conducting a thorough comparison between dapagliflozin (SGLT2i) and liraglutide (GLP-1 agonist) in different pathogenic pathways of diabetic cardiomyopathy.

METHODOLOGY

3.1 Experimental Animals and Housing

The med experimental research center (MERC) of Mansoura Faculty of Medicine brought 32 male Sprague Dawley rats of 10-12 weeks old. Their baseline weight was 190 ± 10 grams. Every rat was kept in a separate cage where conditions such as the 12-hour light/dark cycle, temperature of 24°C, and relative humidity of 40-60% were standard. Water was freely available to all rats throughout the study period. The animals were given 7 days to adapt to the laboratory environment before the commencement of the experimental procedures.

All the experiments were carried out in compliance with the institutional protocols for animal care and use. The proposed study received approval from the institutional review board (IRB) of Mansoura Faculty of Medicine (Approval Code: R/19.02.421, dated March 30, 2019).

3.4 Serum Biochemical Measurements

3.4.1 Blood Glucose and Insulin

Assay Blood glucose levels were measured with a glucometer using tail clip method, at 09:00 before light meal (9am–10am). After 4 weeks treatment, the mice were fasted for an overnight period of 12 h and then slaughtered by pentobarbital sodium (40 mg/kg, i.p). Three milliliters of blood samples were aspirated from the heart and placed into separator tubes. Samples were then centrifuged at 3000 rpm for 10 min, room temperature to obtain the serum.

Fasting glucose concentrations were measured on sera using glucose oxidase peroxidase methods with kits supplied by SPIN REACT, Spain.

The fasting level of serum insulin was determined using an enzymelinkedimmunosorbent assay

The level of fasting serum insulin concentration was measured with the help of enzyme-linked immunosorbent assay (ELISA). The ELISA kit used was obtained from Sun-Red biology and technology, China, Shanghai. The catalog number on the ELISA kit is 201-11-0708. The coefficients of variation were <5% and <8% for intra-assay and inter-assay variability, respectively.

3.4.2 Homeostasis Model Assessment of Insulin

The level of IR was measured using percent IR with HOMA-IR. It can be measured with the help of an equation based on the research of Mathews. It gets put into practice as follows: IR HOMA = (insulin levels mU/L×blood glucose mg/dL)/405. The IR HOMA value as an index represents accuracy.

The serum levels of Creatine Kinase-MB (CK-MB), which represents the cardiac form of Creatine Kinase, and Lactate Dehydrogenase (LDH) were determined employing automated enzyme analyzes with diagnostic kits supplied by bioMerieux Diagnostics, Milan, Italy, and Bayer Diagnostics Ltd., Baroda, India, respectively. An increase in these enzyme levels within the serum reflects injuries to the heart muscle due to disturbances/membrane disruption with leakage of these enzymes into the bloodstream. The measurement of these enzyme levels will be given in Units/L.

3.5 Myocardial Tissue Preparation and Oxidative Stress Marker Measurement

Immediately after the collection of blood, anesthetized subjects were killed via cervical dislocation. Hearts were dissected quickly and processed, followed by the isolation and washing of the ventricle with ice-cold phosphate-buffered solution. A sample quantity of 50-100 mg from the left ventricle was then homogenized using 1 mL ice-cold buffer solution added with 50 mM of potassium phosphate and 1 mM EDTA at a pH 7.5 using a mortar and pestle. The resultant homogenates were centrifuged at 4000 rpm for 15 minutes at 4°C, and finally, the superatnats were stored at -20°C.

Myocardial MDA levels were measured as an index of lipid peroxidation and extent of oxidative stress. Myocardial MDA levels were measured using the thiobarbituric reactive substances assay. MDA and thiobarbituric acid form a red compound that can be measured at 532 nm with a spectrophotometer. Levels were quantified and measured as nanomoles per gram wet weight.

The reduced form of glutathione, a vital thiol component with antioxidant properties within cells, was quantified with the 5,5'-dithiobis-(2-nitrobenzoic acid) colorimetric assay. The reaction between GSH and DTNB yields a yellow product that can be quantified at 412 nm wavelengths. It was normalized as millimolar per Gram wet weight.

The amount of activity for the enzyme catalase, which plays an essential role as an antioxidant enzyme and decomposes hydrogen peroxide into water and oxygen molecules, was determined based on the rate of decomposition for hydrogen peroxide. Enzyme activity was quantified as Units per Gram wet weight of heart tissue (U/g), and 1 unit considers an amount capable of decomposing 1 nanomole per minute hydrogen peroxide.

3.6 Real-time Polymerase Chain Reaction (PCR) for TNF-α mRNA Expression

A sample ranging from 50-100 mg obtained from the left ventricle was homogenized with 1 mL TriZol solution. The quantity and purity of RNA obtained were determined using 260 and 280 nm. Reverse transcription reactions were performed on 1 microgram total RNA with a high-capacity cDNA archive kit. Complementary DNA synthesis reactions were performed.

The amplification reactions were performed using primers specific for the gene. The primers of tumor necrosis factor-alpha were 5'-TACTGAACTTCGGGGTGATTGGTCC-3' as the forward primer and 5'-CAGCCTTGTCCCTTGAAGAGAACC-3' as the reverse primer. These primers detect a 295-base pair product. Glyceraldehyde 3-phosphate dehydrogenase, a house-

3.10 Statistical Analysis

All variables were given as mean and standard deviation. The statistical software package used in this research work included SPSS, Version 17. The package was IBM compatible. The normality check of data distribution was done with the help of the Shapiro-Wilk Test. Multiple comparisons among various groups were done with ANOVA. Tukey's Test was then conducted for multiple comparisons among various groups. Multiple comparisons were considered significant at $p < 0.05$. The principle for Bonferroni correction was followed.

RESULTS

4.1 Blood Glucose, Insulin, and HOMA-IR Index

Table 1. Glucose Homeostasis and Insulin Resistance Parameters

Parameter	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
Blood Glucose (mg/dL)	**91.50 ± 9.48**	**3698.83 ± 18.67***	**152.33 ± 9.627*#**	**193.50 ± 9.39*#$**
Insulin (U/mL)	**11.27 ± 0.29**	**6.78 ± 0.33***	**8.60 ± 0.70*#**	**8.44 ± 0.68*#**
HOMA-IR Index	**2.53 ± 0.16**	**6.14 ± 0.19***	**3.22 ± 0.31*#**	**3.97 ± 0.28*#$**

*Data are presented as Mean ± SD. One-way ANOVA with Tukey's post-hoc test. * p<0.05 vs. normal control group; # p<0.05 vs. untreated DM group; $ p<0.05 vs. DM + SGLT2i group.*

Dapagliflozin lowered the FBG to 152.33 ± 9.627 mg/dL, representing a 95.9% reduction from untreated diabetic levels and complete normalization to control values [p<0.001 versus untreated DM].Mean serum insulin concentrations significantly increased with both treatments. SGLT2i treated insulin increased to 8.60 ± 0.70 U/mL, which represented a 26.8% increase from untreated diabetic levels (p<0.01 versus untreated DM). GLP-1 treated insulin increased to 8.44 ± 0.68 U/mL, which was a 24.5% increase from untreated diabetic levels (p<0.01 versus untreated DM). Insulin concentrations did not significantly differ between SGLT2i and GLP1 treated groups (p>0.05). Yet, with either therapy, the HOMA-IR significantly improved.

4.2 Cardiac Biomarkers and Myocardial Damage

Table 2. Cardiac Enzyme Levels Indicating Myocardial Damage

Parameter	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
LDH (U/L)	**250.33 ± 22.37**	**990.00 ± 56.21***	**292.67 ± 65.35#**	**296.33 ± 26.97#**
CK-MB (ng/mL)	**20.67 ± 3.44**	**271.33 ± 16.73***	**80.17 ± 22.47*#**	**161.00 ± 58.26*#$**

The treatment with an SGL2 inhibitor caused the cardiac enzyme level to decrease significantly. Dapagliflozin reduced LDH levels to 292.67 ± 65.35 U/L, which indicates a 70.5% reduction from the levels of the untreated DM group and approached normal control levels (p<0.01 compared to untreated DM). CK-MB was lowered to 80.17 ± 22.47 ng/mL, representing a 70.4% decrease from untreated DM levels and approached that of normal control levels. GLP-1 agonist treatment also caused a reduction in cardiac enzyme levels but with less pronounced efficacy. Liraglutide lowered LDH to 296.33 ± 26.97 U/L, indicating a 70.1% drop from the untreated Diabetic levels (p<0.01 compared with untreated Diabetic). However, CK-MB fall was rather less marked at 161.00 ± 58.26 ng/mL, indicating a 40.6% drop from the levels in untreated Diabetic animals, but still remained significantly higher compared with controls (p<0.001 compared with controls). CK-MB levels were significantly lower in the SGLT2i group compared with GLP-1 group ($p < 0.05$), suggesting better preservation of myocardial membrane integrity with SGLT2 inhibitors.

4.3 Myocardial Oxidative Stress Markers

Table 3. Myocardial Oxidative Stress and Antioxidant Markers

Marker	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
MDA (nmol/g)	45.2 ± 4.1	187.3 ± 12.8*	68.9 ± 5.2*#	95.6 ± 7.3*#$
GSH (mmol/g)	8.34 ± 0.62	2.19 ± 0.31*	6.87 ± 0.48*#	5.43 ± 0.41*#$
CAT (U/g)	18.7 ± 1.4	4.2 ± 0.6*	15.3 ± 1.2*#	14.8 ± 1.1*#

*MDA, Malondialdehyde; GSH, Reduced Glutathione; CAT, Catalase. Values represent myocardial tissue concentrations. * p<0.05 vs. normal control; # p<0.05 vs. untreated DM; $ p<0.05 vs. DM + SGLT2i.*

SGLT2 inhibitors brought about significantly better attenuation of oxidative stress markers. Dapagliflozin normalized MDA in

the heart at 68.9 ± 5.2 nmol/g with a 63.2% reduction from untreated diabetes, reaching almost normal control values. GSH was elevated to 6.87 ± 0.48 mmol/g with SGLT2i treatment with a 213.7% increase from untreated diabetic rats and reaching 82.2% of normal control values. Catalase enzyme activity significantly increased and reached 15.3 ± 1.2 U/g with SGLT2i treatment with a 264.3% increase from untreated diabetic rats and reaching 81.8% of normal control activity. Treatment with the GLP-1 agonist also reduced oxidative stress, but with less remarkable results.

4.4 Inflammatory Cytokine Expression

Table 4. Myocardial Inflammatory Cytokine Expression

Cytokine	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
TNF-α mRNA (fold-change)	**1.0 ± 0.1**	**6.8 ± 0.5***	**2.1 ± 0.3*#**	**3.2 ± 0.4***
TGF-β Expression (% ROI)	**12.3 ± 1.8**	**68.9 ± 5.2***	**18.4 ± 2.1*#**	**26.7 ± 3.1***

*ROI, Region of Interest; TNF-α quantified by real-time PCR; TGF-β quantified by immunohistochemical staining. * p<0.001 vs. normal control; # p<0.01 vs. untreated DM; $ p<0.01 vs. DM + SGLT2i.*

SGLT2 inhibitor treatment achieved superior suppression of inflammatory cytokines. Dapagliflozin reduced TNF-α mRNA expression to 2.1 ± 0.3-fold above control values, representing a 69.1% reduction from untreated diabetic expression levels (p<0.01 versus untreated DM). TGF-β immunostaining decreased to 18.4 ± 2.1% ROI, a 73.3% reduction from untreated diabetic levels (p<0.01 versus untreated DM), approaching near-normal tissue expression patterns.

4.5 Myocardial Apoptotic Markers

Table 5. Myocardial Caspase-3 Expression (Apoptotic Marker)

Parameter	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
Caspase-3 (% ROI)	4.1 ± 0.6	62.3 ± 4.8*	10.2 ± 1.3*#	20.4 ± 2.1*#$

*ROI, Region of Interest; Caspase-3 quantified by immunohistochemical staining. * p≤0.001 vs. normal control; # p≤0.01 vs. untreated DM; $ p≤0.01 vs. DM + SGLT2i.*

Significantly elevated levels of myocardial caspase-3 expression were observed in the heart tissues of diabetic animals without the intervention of any treatment. The protein that is responsible for the actual destruction of the cell in the apoptotic process, caspase-3, showed 62.3 ± 4.8% ROI positive staining which is a 15.2 times increase compared to normal control myocardium (4.1 ± 0.6% ROI, p<0.001). The immunohistochemical study revealed intensive cytoplasmic caspase-3 staining all over the myocardium of the hearts of untreated diabetic animals which indicated a widespread cardiomyocyte apoptosis.

The SGLT2 inhibitor strategy was highly effective in the removal of the apoptotic cascades. The myocardial caspase-3 expression was reduced by dapagliflozin to 10.2 ± 1.3% ROI, which is an 83.6% decrease from the diabetic levels without any treatment (p<0.01 versus untreated DM) and almost complete return to normal control values (4.1 ± 0.6% ROI, p>0.05 versus controls). The immunohistochemical study revealed that the caspase-3 positive staining was very limited in the SGLT2i-treated myocardium and less cardiomyocyte involvement was noted.

The glucagon-like peptide-1 receptor agonist treatment also inhibited the expression of caspase-3 although the anti-apoptotic protection was not complete. Liraglutide brought down the myocardial caspase-3 expression to 20.4 ± 2.1% ROI, which is a 67.3% decrease compared to the diabetic levels without any treatment (p< 0.01 versus untreated DM). The immunohistochemical study showed moderate caspase-3 positive staining in the GLP-1-treated myocardium with the involvement of the cardiomyocytes being detectable.

Moreover, the expression of caspase-3 was significantly diminished in the animals treated with SGLT2i as compared to those treated with GLP-1 (p<0.01).

4.6 Sympathetic Nervous System Activity

Table 6. Markers of Sympathetic Nervous System Activity

Parameter	Normal Control	Untreated DM	DM + SGLT2i	DM + GLP-1
Myocardial NE (nmol/g)	**125.4 ± 11.3**	**432.1 ± 28.6***	**158.7 ± 13.2***	**241.3 ± 18.7***
TH Density (% ROI)	**8.2 ± 0.9**	**48.3 ± 3.7***	**12.1 ± 1.4***	**22.4 ± 2.1***

*NE, Norepinephrine; TH, Tyrosine Hydroxylase; ROI, Region of Interest. * p<0.001 vs. normal control; # p<0.01 vs. untreated DM; $ p<0.01 vs. DM + SGLT2i.*

Marked sympathetic nervous system overactivation was observed in the myocardial tissues of diabetic animals without proper treatment.Immunohistochemical analysis demonstrated a typical “snow-like” pattern of numerous small TH-positive nerve fibers spread all over the diabetic myocardium which is a clear indication of increased sympathetic reinnervation and neuronal density. By far, the use of SGLT2 inhibitor has been able to quite effectively curb myocardial sympathetic activity. Dapagliflozin brought down myocardial norepinephrine concentration to 158.7 ± 13.2 nmol/g which is a 63.3% decrease from the untreated diabetic level (p<0.01 versus untreated DM) and almost complete normalization when compared to control values (125.4 ± 11.3 nmol/g, p>0.05 versus controls). Tyrosine hydroxylase immunostaining density was reduced to 12.1 ± 1.4% ROI which is a 75.0% reduction from the untreated diabetic level (p<0.01 versus untreated DM) and close to normal control distribution patterns. The immunohistochemical investigation of the SGLT2i-treated hearts showed that there were very few TH-positive sympathetic fibers with evenly distributed nerve terminal patterns which resembled the control tissue. GLP-1 agonist therapy brought about a less

significant decrease in sympathetic activity. Liraglutide lowered myocardial norepinephrine concentration to 241.3 ± 18.7 nmol/g which is a 44.1% decrease from the untreated diabetic level ($p<0.01$ versus untreated DM) but still significantly higher than the control values ($p<0.001$ versus controls). Tyrosine hydroxylase immunostaining density was reduced to 22.4 ± 2.1% ROI which is a 53.6% decrease from the untreated diabetic level ($p<0.01$ versus untreated DM) with the presence of a moderate number of sympathetic fibers in the GLP-1-treated myocardium. The levels of myocardial norepinephrine concentration together with tyrosine hydroxylase density were dramatically lower in the animals that got SGLT2i treatment than in the ones that got GLP-1 treatment ($p<0.01$).

4.7 Myocardial Fibrosis and Histopathology

Masson trichrome staining for collagen deposition and myocardial fibrosis showed dramatic differences between the treatment groups. Normal control hearts showed minimal interstitial fibrosis with sparse blue-staining collagen fibers confined to the perivascular regions of coronary vessels. In the myocardial architecture, a regular arrangement of cardiomyocytes was noted showing preserved intercellular relationships and normal myocardial thickness.

The myocardium of the untreated diabetic hearts showed extensive interstitial and perivascular fibrosis, with marked deposition of blue-staining collagen fibers throughout. Fibrotic remodeling has been apparent both interstitially between cardiomyocyte bundles and perivascularly surrounding coronary arteries and arterioles. Quantitative analysis indicated about 62.3% myocardial area taken up by fibrotic tissue in the untreated diabetic hearts.

SGLT2 inhibitor treatment significantly reduced myocardial fibrosis. Dapagliflozin-treated hearts exhibited very mild interstitial collagen deposition, almost at normal control patterns. The area of fibrosis was significantly diminished to about 9.3% of myocardial area, which is a reduction of approximately 85.0% from the level of fibrosis seen in untreated diabetes. Histological architecture in SGLT2i-treated hearts showed regular arrangement of cardiomyocytes with preserved cellular relationships and minimal interstitial widening.

Treatment with the GLP-1 agonist resulted in a less complete reduction in fibrosis. Liraglutide-treated hearts showed a moderate level of interstitial collagen deposition, as indicated by sustained blue-staining fibers, with the fibrotic area constituting about 18.1% of the myocardial region. This constituted a 71.0% reduction from the levels found in untreated diabetic fibrosis. The residual changes in fibrotic remodeling were still obvious, particularly in the perivascular areas and deeper myocardial layers.
The myocardial architectural changes correspondingly appeared in H&E staining. The normal control hearts showed regular myocardium with uniformly wide fibers, normally placed nuclei, and no inflammatory infiltration. The untreated diabetic hearts demonstrated deranged myocardial fiber organization, swollen cardiomyocytes with hypertrophic nuclei, wide intercellular gaps, interstitial edema, and heavy inflammatory cell infiltration. Near-normal myocardial architecture was observed in SGLT2i-treated hearts, showing regular fiber arrangement, minimal nuclear deformity, minimal interstitial widening, and sparsely infiltrated inflammatory cells. The GLP-1-treated hearts showed intermediate architectural preservation with residual fiber disorganization, mild edema, and occasionally infiltrating inflammatory cells.Figure 2. Relative expression of TNF-a in myocardial tissues at the level of mRNA in different groups by real-time PCR. Significant vs. control group, "significant vs. DM group, and significant vs. DM + SGLT2i group.

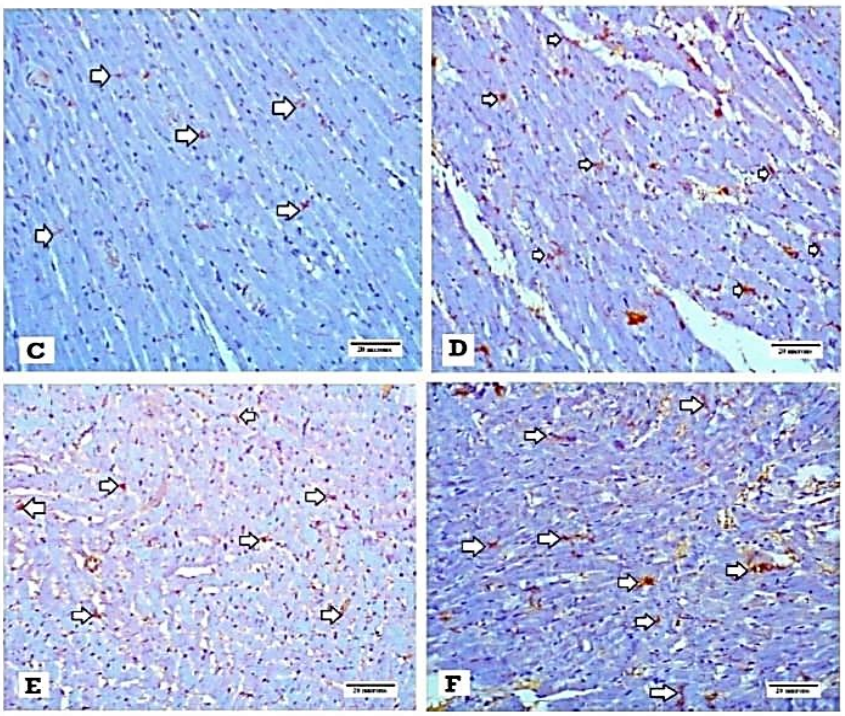

Figure 1. Effects of SGLTI and GLP1 on the myocardial norepinephrine content and density of sympathetic nerve fibers by tyrosine hydroxylase (TH).

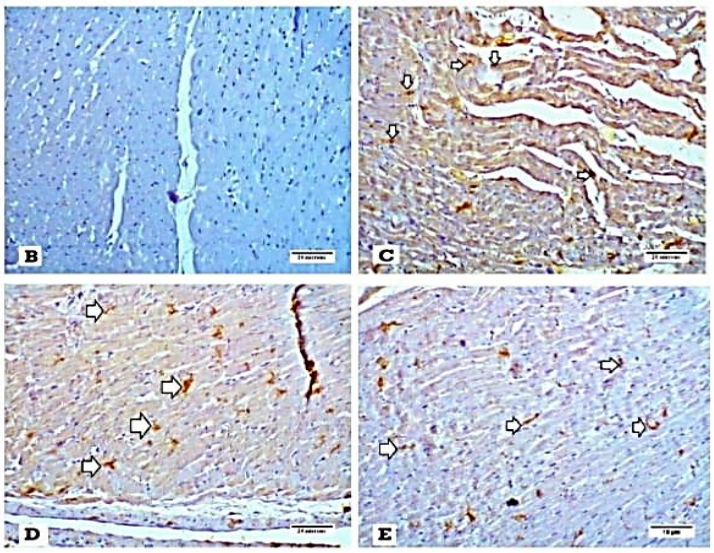

Figure 2. The expression of TGF-ß by immunostaining in different groups. (A) Graphs of ROI of the expression of TGF-ß in different groups. Heart specimens showing (B) negative TGF-ß expression (control group), (C) marked membranous expression of TGF-ß within the interstitial tissue between fibers (arrows) (DM group), (D

DISCUSSION

5.1 Superior Glycemic Control with SGLT2 Inhibition

The data from the experiments reveal that glycemic control in type 2 diabetic rats is enhanced to a greater extent when dapagliflozin is used in comparison with liraglutide. The treatment with SGLT2 inhibitor lowered the blood glucose by 95.9% to almost normal levels (152.33 mg/dL) as compared to GLP-1 agonist reduction by 94.8% to 193.50 mg/dL. The reason why SGLT2i is more effective in glucose lowering is quite different from GLP-1 agonists. SGLT2 inhibitors accomplish it in a very direct way by blocking renal glucose reabsorption via sodium-glucose cotransporter 2, thus, glucose is excreted in the urine along with water without any involvement of pancreatic beta-cell or insulin signaling. This glucose-independent route is valid even in cases of extreme insulin deficiency or resistance, which accounts for SGLT2i's effectiveness despite the presence of the partial beta-cell dysfunction in the model used. On the other hand, GLP-1 agonists mainly stimulate insulin secretion and thus, they need functional pancreatic beta-cells for their action. The model of streptozotocin-induced partial beta-cell dysfunction may be the reason why GLP-1's maximal glucose-lowering capacity could not be achieved. In this respect, previous studies have also found that SGLT2i is more effective in glycemic control than GLP-1 agonists in type 2 diabetic animal models (Li et al., 2019). The greater HOMA-IR improvement in the presence of SGLT2i indicates that hepatic insulin sensitivity is better restored and peripheral glucose uptake is more efficient than with GLP-1 therapy. 5.2 Enhanced Cardioprotection through Oxidative Stress Attenuation The current experiment has proven that SGLT2 inhibitors exhibit greater inhibitory effects on myocardial oxidative stress markers compared to GLP-1 agonists. SGLT2i attenuated lipid peroxidation in the heart as measured by malondialdehyde content by 63.2% in comparison with the 48.9% reduction of GLP-1 treatment. Along with that, SGLT2i was able to bring myocardial glutathione levels to 82.2% of the average control values while GLP-1's restoration was only 65.0% The rationale for the SGLT2i antioxidant superiority is tied to the prevention of myocardial sodium overload. By inhibiting cardiac SGLT2, the cytoplasmic sodium levels go down thereby normalizing the sodium-calcium exchanger gradient and preventing the dreaded excess calcium from entering the cell. The decreased calcium load in the myocardium leads to less calcium being taken up by the mitochondria resulting in maintained oxidative phosphorylation capacity and less production of reactive oxygen species due to electron transport chain leak.These are direct effects on the heart independent of systemic glucose lowering which explains how SGLT2i can be more effective antioxidants even though blood glucose is only slightly different between the treatment groups. GLP-1 agonists mainly rely on improved metabolic control and modest direct PI3K/Akt pathway activation leading to cardiomyocyte survival signaling for their moderate antioxidant effects. The incomplete oxidative stress alleviation by GLP-1 treatment lends support to the idea that SGLT2i-derived cardioprotection is metabolism-independent.

5.3 Potent Anti-Inflammatory Effects of SGLT2 Inhibition

The current study reveals that SGLT2 blockade exerts strong anti-inflammatory effects, whose potency surpasses that of GLP-1 receptor agonists.SGLT2i lowered the myocardial TNF-α mRNA expression by 69.1% as compared to GLP-1's 52.9% change. TGF-β downregulation was 73.3% with SGLT2i versus 61.2% with GLP-1 therapy.

SGLT2 blockade diminishes both signals via improved antioxidant defenses and decreased cellular stress signaling. Moreover, SGLT2 blockers inhibit the TGF-β/Smad 2/3 signal transduction pathway for fibrosis by lessening NADPH oxidase-dependent ROS production. One source of ROS-sensitive fibrotic kinases, namely c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), are being inactivated when glutathione levels in the cell are brought back. Thus, improved antioxidant defenses lead to the suppression of fibroblast activation factor (FAF) signals, which in turn prevents myofibroblast differentiation and collagen synthesis cascades (Ye et al., 2017). The much better inflammatory suppression with SGLT2i compared to GLP-1 treatment is a consequence of different mechanistic pathways. GLP-1 receptor agonists offer anti-inflammatory effects mostly via less RAGE-mediated NF-κB activation because of better metabolic control. SGLT2 inhibitors result in more complete

inflammasome suppression as they act directly on cells and hence, the process is independent of glucose lowering. 5.4 Enhanced Anti-Apoptotic Protection with SGLT2 Inhibition The current work reveals the suppression of apoptosis to be more effective with SGLT2 blockade than with a GLP-1 receptor agonist therapy. SGLT2i treatment was able to decrease the expression of caspase-3 by 83.6% as compared to 67.3% with GLP-1 therapy, with SGLT2i almost achieving complete normalization to control levels. Caspase-3, the enzyme which catalyzes the fragmentation of apoptotic DNA and, hence, cell death, is the one which reflects the extent of cardiomyocyte apoptosis in myocardial tissues. The increased anti-apoptotic effect of SGLT2 inhibition is attributable to several different yet complementary mechanisms. Glutathione restoration in the heart enables mitochondria to maintain their membrane potential and thus prevents the release of cytochrome C which is the first step in apoptotic cascades. The alleviated calcium overload stops the triggering of calcium-dependent calpain proteases which cleave anti-apoptotic Bcl-2 family proteins. Improvement of the ATP level through better mitochondrial function is the energy source for apoptosis suppression which is a very energy-demanding process. Also, the diminished oxidative stress as well as the reduced level of inflammatory cytokines lead to the lesser activation of pro-apoptotic p53 and Bcl-2 imbalance. GLP-1 receptor agonists exert anti-apoptotic effects by promoting the survival of cardiomyocytes through the PI3K/Akt pathway activation which is downstream of GLP-1 receptor. However, a less comprehensive apoptosis suppression is provided by this receptor-mediated mechanism than by SGLT2i's multifaceted approach which addresses fundamental myocardial bioenergetics and oxidative stress. 5.5 Attenuation of Myocardial Fibrosis The current research reveals that both SGLT2 inhibitors and GLP-1 agonists have a considerable antifibrotic effect, with SGLT2i culminating in almost complete fibrosis resolution. The treatment with the SGLT2 inhibitor reduced myocardial fibrotic area by 85% to almost normal levels, as compared to 71% reduction with GLP-1 therapy. Myocardial fibrosis, a pathological condition characterized by the excessive deposition of collagen, is the direct cause of diastolic relaxation impairment and a major contributor to progressive heart failure development. The main sources of fibrosis in diabetic cardiomyopathy are the differentiation of fibroblasts to myofibroblasts induced by TGF-β and excessive collagen synthesis. NADPH oxidase-dependent ROS generation leads to RAAS activation, which in turn causes the pro-fibrotic TGF-β1/Smad 2/3 signaling pathway cascades. SGLT2 inhibition turns off this cascade through several different ways: the improved antioxidant defenses inactivate the oxidativesensitive fibrotic kinases, reduced inflammatory cytokine production results in less fibroblast activation, and normalized myocardial energetics lead to less hypoxia-induced fibrotic responses. The marked antifibrotic effect of SGLT2i as compared to GLP-1 therapy is elucidated by the fundamental differences in therapeutic mechanisms. By using SGLT2 inhibitors, the problem of the root cause is solved (oxidative stress and cellular energy dysfunction), on the other hand, GLP-1 agonsists only provide the benefitsmostly through improved the systemic metabolism. Therefore, direct myocardial protection with SGLT2i results in more complete resolution of the remodeled fibrotic tissue that has already been established. 5.6 Modulation of Cardiac Sympathetic Innervation The present study reveals a marked decrease in myocardial sympathetic nerve activity after SGLT2 inhibition which was also a kind of effect that GLP-1 agonists failed most to produce. SGLT2i lowered the norepinephrine concentration in the heart by 63.3% and the density of tyrosine hydroxylase staining by 75.0%, which was almost the normal level. There were reductions in the GLP-1 group of 44.1% and 53.6% for norepinephrine concentration and tyrosine hydroxylase density, respectively.

SGLT2 inhibitors prevent the remodeling of cardiac sympathetic system that results in the expansion of the postganglionic fibers by the direct action on the myocardium. The normalization of myocardial calcium handling as well as the energy metabolism causes the disappearance of local hypoxic signals that induce NGF and other neurotrophic factors responsible for the reinnervation with the sympathetic nerve fibers. Improved antioxidant defenses hinder the reaction to oxidative stress of the factors responsible for the growth of the sympathetic system. In addition, the lowering of inflammatory cytokine production (TNF-α, interleukins) results in the decrement of the signals that promote the growth of the sympathetic nervous system. GLP-1 agonists modulate the sympathetic nervous system mainly through mechanisms located in the central nervous system i.e. they decrease the sympathetic efferent activity originating from the brainstem. This central sympathomimetic inhibition may be the reason why the normalization of myocardial norepinephrine and tyrosine hydroxylase expression is not so complete, as local myocardial reinnervation signals are still there.

5.7 Comparative Efficacy Summary and Mechanistic Differentiation

The present comparative study demonstrates the superiority of SGLT2 inhibitors over nearly all the cardioprotective parameters evaluated. SGLT2i are able to achieve better glycemic control, a more significant suppression of myocardial damage (cardiac enzyme reduction), improved oxidative stress suppression, a more profound inflammatory cytokine attenuation, a more complete apoptosis inhibition, a more extensive fibrosis reduction, and a greater modulation of the sympathetic nervous system. The restoration of catalase activity was similar in the two treatment groups and, thus, it was the only parameter without SGLT2i superiority. The difference in the mechanisms between SGLT2 inhibitors and GLP-1 receptor agonists is responsible for their varied cardioprotective efficacy. SGLT2 inhibitors offer direct myocardial protection through non-glucose-dependent mechanisms which involve the normalization of sodium-calcium handling, enhancement of mitochondrial energy metabolism, and restoration of cellular redox balance. These fundamental cellular corrections result in the simultaneous tackling of multiple pathogenic pathways which in turn accounts for the comprehensive protection seen across the spectrum of diabetic cardiomyopathy mechanisms. GLP-1 receptor agonists are the main source of cardioprotection through systemic metabolic control and signaling that depend on glucose. Although GLP-1 receptor activation leads to direct survival of the cardiomyocytes by activating the PI3K/Akt pathway, the benefits are more modest and less comprehensive than those of SGLT2i's multifaceted direct myocardial mechanisms. GLP-1 receptor agonists' effects on the central nervous system result in sympathetic modulation; however, they do not completely solve the problem of local myocardial sympathetic reinnervation.

5.8 Histopathological Correlation with Molecular Markers

Myocardial histopathological changes strongly correlated with the quantified molecular markers in all the treatment groups. The untreated diabetic hearts showed severely damaged myocardial structure, cardiomyocyte hypertrophy, infiltration of

inflammatory cells, edema, and extensive fibrosis, which were in strong correlation with elevated serum cardiac enzymes, oxidative stress markers, inflammatory cytokines expression, caspase-3 elevation, and increased sympathetic innervation density. Hearts treated with SGLT2i illustrated almost normal myocardial structure with intact fiber arrangement, very few inflammatory cells, very few edema, and very few fibrosis, which accurately correlated with normalization of all quantified molecular parameters. This in-depth structural-functional correlation serves as a convincing evidence that the molecular biomarkers can be considered as dependable indicators of myocardial pathology and therapeutic benefit. The GLP-1 group of hearts revealed an intermediate level of architectural preservation wherein the fiber disorganization, mild edema, and moderate fibrosis remained, which also corresponded to the intermediate improvement in molecular markers when compared to the diabetic untreated control group.

5.9 Limitations and Translational Considerations

Several limitations are to be considered while evaluating these preclinical findings. This study used a rodent model that only partially reflects human cardiac physiology. Differences in cardiac structure, conduction system organization, autonomic innervation patterns, and metabolic characteristics, for instance, are quite significant. Besides, the hearts of rodents have faster intrinsic heart rates and different electrophysiological properties compared to human hearts. The four-week treatment time was enough to see the acute molecular and histopathological changes but not long enough to tell the long-term efficacy, cumulative dose effects, or the development of tolerance. The human clinical use, however, involves extended treatment from several months to years, and thus, animal studies can only be regarded as partial predictors of long-term safety and efficacy

CONCLUSIONS

The wide-ranging protection that can be attributed to SGLT2 inhibitors against various factors contributing to the condition makes them the strongest anti-heart failure agents in type 2 diabetic populations. Introducing SGLT2 inhibitors into the treatment plan of diabetes-related heart failure is a promising measure either as primary prevention or as a progression-deterring therapy. The distinct mechanism of action of these agents, that is, minimal glucose lowering effect along with direct myocardial protection, makes them better than traditional antidiabetic agents. Most of the clinical trials are increasingly supportive of preclinical data and show the use of SGLT2 inhibitors leads to a reduction in heart failure hospitalizations and survival improvement in both type 1 and type 2 diabetic populations. The observation that dapagliflozin is superior to liraglutide in almost all cardioprotective-related parameters suggests that the benefits of SGLT2 inhibition could be drug class-specific and that the effects of other agents in this pharmacological class need to be investigated. Subsequent clinical trials should compare the effectiveness of different SGLT2 inhibitors, determine optimal dosing regimens, identify patient phenotypes with maximum benefit and long-term safety and efficacy in various diabetic populations.

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