RESEARCH ARTICLE

Effects of SGLT2-inhibitor on The Expression of MicroRNA-21, Transforming Growth Factor-β1, and Matrix Metalloproteinase-2 in The Process of Cardiac Fibrosis in Hyperglycemic Model Rats

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Abstract

BACKGROUND: Sodium glucose co-transporter-2 inhibitor (SGLT2-i), a new oral antidiabetic drug, has been recommended for its morbidity and mortality benefits in patients with heart failure. This study aimed to determine the effect of acute SGLT2-i therapy on the relative ratio expression of microRNA-21 (miR-21), transforming growth factor-β1 (TGF-β1), and matrix metalloproteinase-2 (MMP-2) in the process of cardiac fibrosis in the hyperglycemia Wistar rat models compared to biguanide (metformin) therapy.

METHODS: We used Streptozotocin (STZ) to induce hyperglycemia in Wistar rats (n=31), randomly divided into four groups: negative control (NC, n=4), positive control (PC, n=10), hyperglycemia plus metformin (M, n=8), and hyperglycemia plus empagliflozin (E, n=9). After seven weeks, the rats were sacrificed and the heart tissue was taken for microRNA and messenger RNA (mRNA) extraction, followed by reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) examination. The data was analyzed using One-way ANOVA.

RESULTS: Results showed a decreasing trend in the gene expression relative ratio of miR-21 (1.0 *vs.* 1.9; p=0.079) and TGF-β1 (0.9 *vs.* 3.2; p=0.145), but a significant increase in MMP-2 gene expression (1.3 *vs.* 0.7; p=0.002) in the SGLT2-i (empagliflozin) *vs.* biguanide (metformin) groups.

CONCLUSION: Empagliflozin administration may play a significant role in preventing the occurrence of cardiac fibrosis in hyperglycemia.

KEYWORDS: sodium glucose co-transporter-2 inhibitor, microRNA-21, transforming growth factor-β1, matrix metalloproteinase-2, cardiac fibrosis

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Introduction

Diabetes mellitus (DM) is a major risk factor of cardiovascular disease. As reported by the International Diabetes Federation (IDF), DM affected an estimated 537 million people aged between 20 and 79 in 2021. (1) Hyperglycemia-induced diabetes will result in some consequences, the majority of which are linked to a higher risk of cardiovascular disease.(2) This predicament significantly amplifies the cardiovascular risk, including atherosclerosis, peripheral arterial disease, stroke, coronary artery disease, sudden cardiac death, heart failure (HF), and cardiac fibrosis.(3)

Sodium glucose co-transporter 2 inhibitor (SGLT2-i), a new oral antidiabetic therapy, has recently been used as monotherapy or effective combination therapy in type 2 DM (T2DM) and HF.(4) Empagliflozin is known as one of SGLT2-i. According to the Empagliflozin Cardiovascular Outcome Trial in T2DM Patients (EMPA-REG OUTCOME)

trial, using the drug has been associated with a decrease in cardiovascular events, including death and hospitalization rates in T2DM patients with HF.(5,6) The American Heart Association's (AHA) 2022 Heart Failure Guidelines and The European Society of Cardiology's (ESC) 2021 Heart Failure Guidelines both list SGLT2-i as one of the four required medication classes for patients with heart failure with reduced ejection fraction (HFrEF), reducing hospitalization and cardiovascular mortality rates, independent of the presence of T2DM.(7,8)

Various potential theories have elucidated the cardioprotective effects associated with SGLT2 inhibition. Proposed mechanisms encompass blood pressure reduction, erythropoiesis, diuresis/natriuresis, enhanced cardiac energy metabolism, sympathetic nervous system inhibition, inflammation reduction, prevention of ischemia/reperfusion injury, hyperuricemia reduction, Na^+/H^+-exchanger inhibition, promotion of autophagy and lysosomal degradation, SGLT1 inhibition, reduction of epicardial fat mass, augmentation of circulating pro-vascular progenitor cells, elevation of erythropoietin levels, enhancement of vascular function, reduction of oxidative stress, and prevention of adverse cardiac remodeling.(9,10) Cardiac remodeling, involving cardiac hypertrophy, inflammation, cardiomyocyte cell death, and cardiac fibrosis, is a crucial factor in heart failure.(9)

The pathogenesis of cardiac fibrosis is initiated by the activation of cardiac myofibroblasts, leading to increased collagen deposition in the extracellular matrix (ECM). Transforming growth factor-β1 (TGF-β1) plays a vital role in modulating collagen synthesis through activating procollagen type I genes (COL1A1/COL1A2) expression, and influencing collagen degradation through regulating matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) gene expression.(11) MMP-2 expression has been found in obese patients' adipose tissue and heart failure patients' heart tissue.(12-14) The degrading enzyme MMP will be expressed less in hyperglycemia, leading to ECM accumulation and diabetic cardiomyopathy. (15) In addition, other main variables involved in the process of cardiac fibrosis are inflammatory pathways and chemokines, reactive oxidative stress (ROS), Smad protein (16), and microRNA (17).

MicroRNA (miRNA) is a component of non-coding ribonucleic acid (RNA), which is about 18–24 nucleotides long.(18) MicroRNAs play an important role in protein synthesis, control of circulatory function, and other biological elements of the heart.(19,20) It has been reported that microRNA-21 (miR-21) plays some role in the development breast cancer (21) and also cardiac fibrosis (18). It is hypothesized that miR-21 influences the development of fibrosis through its effect on extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) in cardiac fibroblasts, as well as MMP-2, sprouty homolog-1 gene (Spry1), and phosphatase and tensin homolog (PTEN).(22,23)

However, the precise mechanism of SGLT2-i cardioprotective effect on cardiac fibrosis is not conclusive yet, especially regarding the role of miR-21. Thus, this study aimed to examine the effect of SGLT2-i on miR-21, TGF-β1, and MMP-2 gene expression in heart tissue compared to conventional DM therapy using biguanide to prevent cardiac fibrosis in hyperglycemic rats.

Methods

Experimental Animal

A total of 31 Wistar rats from the same strain, environment, and food were obtained from Biofarma (Bandung, Indonesia). Inclusion criteria included male, aged 8-10 weeks, body weight 180-220 grams, healthy, and had normal activities. On the other hand, the exclusion criteria were ill rats and those whose blood sugar did not increase after STZ injection. A regular diet for adult rats was given orally, and they were kept in a comfortable environment, using sawdust as a bedding medium, with room temperature set at 20-28°C. The humidity was set to be 50-10% with a 12-hour light/dark cycle. Animal laboratory facilities have been set up based on the Institutional Animal Care and Use Committee (IACUC) standard and supervised by the Indonesian Association for Laboratory Animal Science (IALAS). The experiment was approved by the Ethics Committee of Health Research, Faculty of Medicine, Universitas Syiah Kuala (Ethics approval No. 004/EA/FK-RSUDZA/2022) and performed in accordance with relevant guidelines and regulations.

Experimental Design

Animal care was carried out in accordance with the 3Rs principle (Replacement, Reduction and Refinement) to ensure animal welfare. After going through a preparation period, rats were randomly allocated into four groups: NC, negative control (healthy, no medication; n=4); PC, positive control (hyperglycemia, no medication; n=10); M, hyperglycemia plus metformin 150 mg/kg/day (n=8); and E, hyperglycemia plus empagliflozin 30 mg/kg/day (n=9). Randomization was carried out using online software after

coding process. The course of treatment lasted for seven weeks. We employed the medications biguanide metformin (Glucophage®, Merck KGaA, Darmstadt, Germany) and SGLT2-i empagliflozin (Jardiance®, Boehringer-Ingelheim, Ingelheim am Rhein, Germany) orally. Euthanasia was carried out using chemical techniques according to the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals by being exposed to high concentrations of carbon dioxide (30-70%) for 4-5 minutes in a carbon dioxide chamber, followed by exsanguination.(24) After being sacrificed, the heart tissue was removed and examined for the expression of miR-21, as well as the expression of TGF-β1 and MMP-2 messenger RNA (mRNA).

Hyperglycemia Induction

After two weeks of acclimatization and adaptation, the rats were given 60 mg/kgBW STZ intraperitoneally to induce hyperglycemia indicated by three consecutive blood glucose levels >250 mg/dL. An average of blood glucose levels obtained in hyperglycemic groups were: 301.2 mg/dL (PC group), 335.2 mg/dL (M group), and 275.2 mg/dL (E group). Blood sugar levels were measured using a Glucocard (Arkray, Kyoto, Japan), using blood taken from the tails of mice.

Gene Expression Analyses

Total RNA miRNA was extracted using the Absolutely RNA miRNA kit according to the manufacturer's instructions. A Tecan infinite M200 multimode reader with A260/A280 at a ratio of 1.8-2.0 was used to measure the amount of RNA. The miRNA 1st strand complementary deoxyribonucleic acid (cDNA) synthesis kit reacted with a 30 ng RNA template to create cDNA. The bioanalyzer 2100 was used to analyze miRNA from RNA and cDNA. The 100 ng of cDNA were reacted with miRNA qPCR Master Mix. The primers used were miR-21 and micro-RNA 92 (miR-92) (as controls). The ariaMX real-time polymerase chain reaction (PCR) engine was used for the amplification process.(25) Specific primers for the microRNA used could be seen in Table 1.

The steps taken to analyze the mRNA for TGF-1 and MMP-2 are as follows (26): Total cellular RNA was extracted using GENEzol reagent according to the manufacturer's instructions. RNA levels were quantified using a Tecan Infinite M200 multimode reader with A260/A280 at a ratio of 1.8-2.0. The 100 ng RNA template was reacted with the master mix sensiFAST SYBR® No-ROX one-step kit. The primers used were TGF-β1, MMP-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with the last used as a control. Specific primers for mRNA can be seen in Table 2. The amplification process was done using the ariaMX real-time PCR machine (26,27).

There were three main phases of real-time PCR: denaturation, annealing, and extension.(28) The delta-delta Ct (ΔΔCt) technique was used to analyze one step reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) findings.(29-31)

Results

The use of metformin elevated miR-21 expression 1.89 times more than NC and PC groups (p=0.045 and p=0.045, respectively). MiR-21 expression was not substantially increased by empagliflozin treatment when compared to the NC and PC groups. Compared to the metformin group, miR-21 expression in the empagliflozin group was lower but not significantly different (mean: 1.9 *vs.* 1.0; p=0.079) (Table 3 and Figure 1).

Metformin administration resulted in an over threefold increase in TGF-β1 mRNA expression compared to the NC (mean: 3.2 *vs.* 0.5) and PC (mean: 3.2 *vs.* 0.4) groups. Conversely, the administration of empagliflozin did not significantly increase TGF-β1 mRNA expression compared to the NC (mean: 0.9 *vs.* 0.5) and PC (mean: 0.9 *vs.* 0.4) groups. Additionally, while TGF-β1 mRNA expression in the empagliflozin group was notably lower than in the metformin group (mean: 0.9 *vs.* 3.2; p=0.145), this difference was not statistically significant (Table 4 and Figure 2).

Metformin administration did not significantly change the level of MMP-2 mRNA expression when compared to the NC (mean: 0.7 *vs.* 0.8) and PC (mean: 0.7 *vs.* 1.0) groups. In contrast, empagliflozin administration significantly increased MMP-2 mRNA expression compared to the

Table 1. Specific primers for microRNA.

Targeted microRNA (rat)	Primer Sequences
miR-21	GGA CGG TAG CAA GCA AAG AGT GTG GAC AGC CCA TC
miR-92	GGA CGG TAG CAA GCA AAG AGT GTG CAG GCC GGG AC

Table 2. Specific primers for mRNA.

Targeted mRNA (rat)	Forward Primer Sequences (5' – 3')	Reverse Primer Sequences (5' – 3')
TGF-β1	CCCCTGGAAAGGGCTCAACAC	TCCAACCCAGGTCCTTCCTAAAGTC
MMP-2	AGACCGCCATGTCCACTGTT	TGGTCGCACACCACATCTTT
GAPDH	GTT ACC AGG GCT GCC TTC TC	GAT GGT GAT GGG TTT CCC GT

metformin group (mean: 1.3 *vs.* 0.7; p=0.002). However, this increase was not statistically significant compared to the NC and PC groups (Table 5 and Figure 3).

Tabel 3. Results of the relative ratio of miR-21 gene expression which has been normalized with the comparison gene (miR-92) and the average value of each group.

Sample Code	$2^{-\Delta\Delta Ct}$ miR-21	Average	SEM
NC1	10.000	10.007	0.05
NC2	10.210		
NC3	11.173		
NC4	0.8645		
PC1	0.7579	10.014	0.07
PC2	0.8950		
PC3	0.8888		
PC4	0.8293		
PC5	12.058		
PC6	12.658		
PC7	14.540		
PC8	0.9593		
PC9	0.8123		
PC10	0.9461		
M1	14.044	19.000	0.25
M2	26.759		
M3	11.487		
M4	11.173		
M5	25.140		
M6	28.879		
M7	13.947		
M8	17.777		
E1	0.9013	10.861	0.12
E2	11.487		
E3	11.975		
E4	18.150		
E5	0.5510		
E6	0.9461		
E7	12.226		
E8	0.7955		
E9	11.975		

SEM: Standard error mean; NC: Non-hyperglycemia (negative control); PC: Hyperglycemia (positive control); M: Hyperglycemia + Metformin; E: Hyperglycemia + Empaglifozin.

Discussion

As seen in Figure 1, empagliflozin administration for seven weeks showed a decreasing trend in miR-21 expression levels when compared to metformin, indicating potential benefit of empagliflozin compared to metformin in preventing cardiac fibrosis. In line with this study, a previous study reported a greater reduction in miR-21 expression after three months of treatment with SGLT2-i empagliflozin 10 mg compared to metformin in the blood of HFpEF patients with diabetes and frailty.(31)

Cardiac fibrosis (23) and hypertrophy (23) are linked to the increased of miR-21 circulation. Studies have demonstrated that miR-21 influences cardiac hypertrophy by inhibiting the Spry2 gene. Moreover, it has been shown to impact cardiac fibrosis by inhibiting not only the Spry1 gene but also PTEN (32), ERK/MAPK, and MMP-2 pathways (22,23). This finding enriches information regarding the role of SGLT2-i on miR-21 expression in cardiac fibrosis process and suggests further research on this issue.

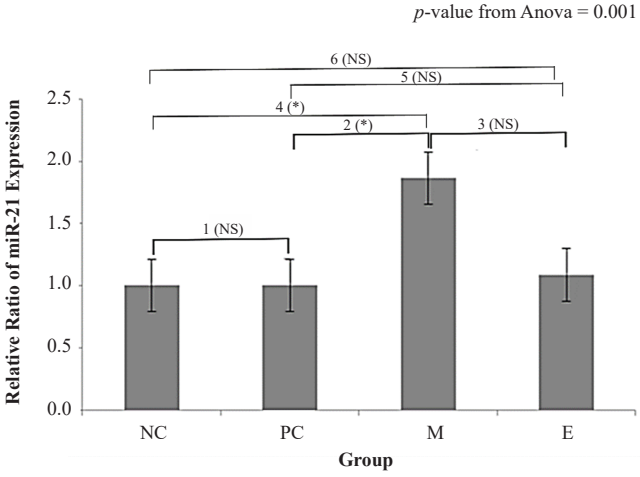

Figure 1. Relative ratio of miR-21 expression among groups. $2^{-\Delta\Delta Ct}$ was presented as the mean±SEM, using One-way ANOVA analysis. Games-Howell's post-hoc analysis between groups is indicated by numbers: 1 indicates NC *vs.* PC (p=1.000); 2 indicates PC *vs.* M (p=0.045*); 3 indicates M *vs.* E (p=0.079); 4 indicates NC *vs.* M (p=0.045*); 5 indicates PC *vs.* E (p=0.927); and 6 indicates NC *vs.* E (p=0.909). *p<0.05 is considered significant. NS: not significant.

Table 4. Results of the relative ratio of TGF-β1 gene expression which has been normalized with the comparison gene (GAPDH) and the average value of each group.

Sample code	$2^{-\Delta\Delta Ct}$ TGF-β1	Average	SEM
NC1	0.1843	0.5324	0.22
NC2	0.8066		
NC3	0.1387		
NC4	10.000		
PC1	0.8706	0.4939	0.13
PC2	#		
PC3	0.4730		
PC4	0.2132		
PC5	0.1103		
PC6	0.5322		
PC7	0.2717		
PC8	#		
PC9	#		
PC10	0.9862		
M1	0.1948	32.639	0.89
M2	37.321		
M3	65.887		
M4	66.346		
M5	42.871		
M6	20.562		
M7	23.457		
M8	0.2717		
E1	0.1961	0.913	0.42
E2	24.116		
E3	#		
E4	#		
E5	0.4633		
E6	#		
E7	12.658		
E8	0.2269		
E9	#		

SEM: Standard error mean; NC: Non-hyperglycemia (negative control); PC: Hyperglycemia (positive control); M: Hyperglycemia + Metformin; E: Hyperglycemia + Empaglifozin. # indicates values that are excluded because they are extreme and inconsistent.

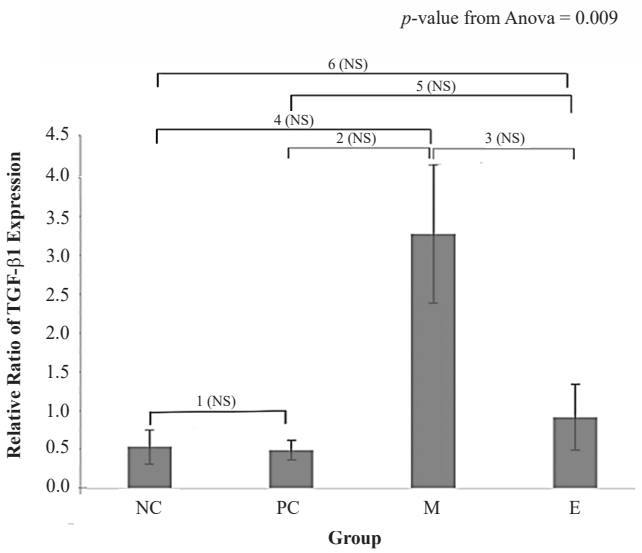

Figure 2. Relative ratio of TGF-β1 mRNA expression among groups. $2^{-\Delta\Delta Ct}$ was presented as the mean±SEM, using One-way ANOVA analysis. Games-Howell's post-hoc analysis between groups is indicated by numbers: 1 indicates NC *vs.* PC (p=0.999); 2 indicates PC *vs.* M (p=0.065); 3 indicates M *vs.* E (p=0.145); 4 indicates NC *vs.* M (p=0.070); 5 indicates PC *vs.* E (p=0.781), and 6 indicates NC *vs.* E; (p=0.852). NS: not significant.

Figure 2 revealed that treatment with empagliflozin resulted in a lower expression trend of TGF-β1 than the metformin group. Similarly, a study reported that SGLT2-i empagliflozin had a favorable effect in rats at a dose of 30 mg/kg/day for four weeks in lowering the production of TGF-β1 and Smad3, which was correlated with a reduction in the development of cardiac fibrosis.(33) These results are consistent with a study in 2019 which demonstrated that oral administration of 10 mg/kg/day empagliflozin for eight weeks to 30 diabetic rats could reduce cardiac fibrosis and improve myocardial structure and function by reducing oxidative stress and inhibiting the expression of via the TGF-β/Smad pathway.(34) Likewise, a study discovered an increase in miR-21 in cardiac fibroblast cells at specific TGF-β1 exposure level, suggesting a function for TGF-β1 in miR-21 production.(35)

As shown in Figure 3, our research revealed that the empagliflozin group significantly increased the expression of MMP-2 in comparison to the metformin group. This increase in MMP-2 may promote more collagen degradation, which can be protective against cardiac fibrosis.

However, few prior studies reported differently regarding this issue. An *in vitro* study argued that treatment with empagliflozin for 72 hours reduced the size of human atrial myofibroblast cells, and the quantity and expression of MMP-2, a marker of collagen formation in the extracellular matrix, without exploring hyperglicemic factor.(36) In addition, employing empagliflozin to T2DM and coronary artery disease (CAD) patients for six months reduced left ventricle extracellular volume (ECV), indexed extracellular compartment volume (iECV), and mass index measured with cardiac magnetic resonance (CMR), but did not change MMP-2 expression.(37) In addition, another *in vitro* study unveiled that MMP-2 levels remained unaffected when exposed to SGLT2-i dapagliflozin at dosages of 10 µM

Table 5. Results of the relative ratio of MMP-2 gene expression which has been normalized with the comparison gene (GAPDH) and the average of each group.

Sample code	$2^{-\Delta\Delta Ct}$ MMP-2	Average	SEM
NC1	10.000	0.87	0.13
NC2	10.943		
NC3	0.5000		
NC4	0.8888		
PC1	10.140	1.01	0.09
PC2	0.6974		
PC3	0.6690		
PC4	0.7846		
PC5	10.281		
PC6	12.397		
PC7	13.755		
PC8	0.7955		
PC9	15.583		
PC10	0.9330		
M1	0.6643	0.70	0.11
M2	0.4665		
M3	0.3392		
M4	0.7526		
M5	0.6373		
M6	0.4698		
M7	0.9266		
M8	13.566		
E1	0.9075	1.31	0.10
E2	0.9461		
E3	15.052		
E4	13.104		
E5	12.397		
E6	12.658		
E7	19.725		
E8	13.013		
E9	13.379		

SEM: Standard error mean; NC: Non-hyperglycemia (negative control); PC: Hyperglycemia (positive control); M: Hyperglycemia + Metformin; E: Hyperglycemia + Empaglifozin.

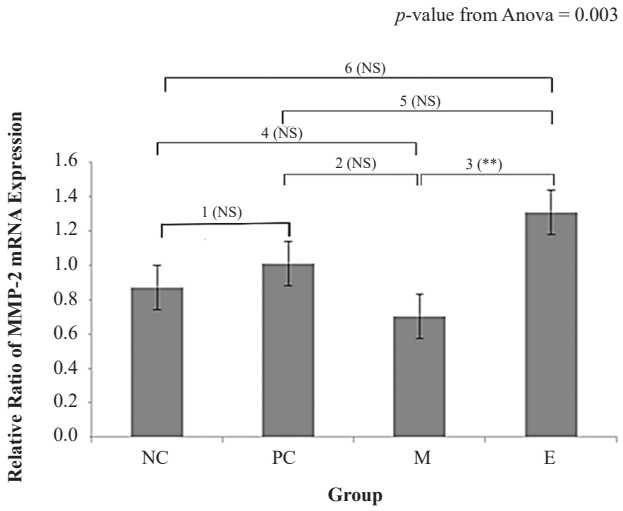

Figure 3. Relative ratio of MMP-2 mRNA expression among groups. 2–ΔΔCt was presented as the mean±SEM, using One-way ANOVA analysis. Tukey's post-hoc analysis between groups is indicated by numbers: 1 indicates NC *vs.* PC (p=0.868); 2 indicates PC *vs.* M (p=0.170); 3 indicates M *vs.* E (p=0.002**); 4 indicates NC *vs*. M (p=0.802); 5 indicates PC *vs.* E (p=0.166); and 6 indicates NC *vs.* E (p=0.103). *p<0.005 is considered very significant. NS: not significant.

and 100 µM, even when combined with glucose in human cardiac fibroblast cells.(38) This shows that the role of SGLT2-i on MMP-2 in the process of collagen degradation and synthesis requires a more comprehensive explanation. In humans, the collagen degradation process associated with fibrosis repair can be influenced by factors beyond MMP-2. Moreover, the MMP-2 levels analyzed in this study were derived from rat heart tissue, which contains a diverse array of cells. Consequently, additional variables may exert an impact, distinguishing it from the context of the three preceding human studies.

Several studies claim that metformin does not have a long-term heart-healthy preventive impact. A study involving 4,030 T2DM patients with myocardial infarction concluded that administration of metformin at the first attack of myocardial infarction increased the risk of cardiovascular disease and death in T2DM patients compared to other anti-diabetic drugs used in the study. (39) Concerning major adverse cardiac events, such as cardiovascular death, recurrent myocardial infarction, or target lesion revascularization, a study of metformin usage in post-percutaneous coronary intervention ST-elevation myocardial infarction (STEMI) patients without T2DM found no significant long-term difference or effect between the use of metformin and placebo.(40)

This study's limitation arises from administering orally given medication via probe, exclusive to the interventional groups (metformin and empagliflozin groups). This approach may introduce varying stress levels and potentially serve as a source of bias among the groups. Furthermore, the study faces constraints due to the relatively brief duration of empagliflozin exposure (only seven weeks), posing challenges in attaining optimal results.

Further studies are suggested to add the hyperglycemia model rat group with sham treatment (placebo) for a more

equal comparison between the intervention and control groups and to lengthen the period of exposure. The application of inhibitors targeting miR-21 and TGF-β1 has garnered significant interest among researchers searching for novel potential treatments for cardiac fibrosis and remodeling.

Conclusion

This study concluded that empagliflozin administration may potentially prevent the development of cardiac fibrosis in hyperglycemia. This conclusion is substantiated by the observed declining trends in relative ratios of miR-21 and TGF-β1 mRNA expression. Furthermore, it is noteworthy that there was a statistically significant increase in MMP-2 mRNA expression compared to the metformin-treated group.

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Authors Contribution

MR conducted this research and prepared the manuscript. HD was responsible for conception, funding and review. MS initiated ideas, drafting, and revision. RL edited, reviewed manuscripts and assisted in funding acquisition. LM prepared initial study and was responsible for animal treatment. All authors have read and approved the manuscript.

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