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Effect of SGLT-2 Inhibitors on Zebrafish Model with Diabetic Heart Failure

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Abstract

Diabetes mellitus (DM) has the rapidly increasing pervasiveness worldwide that causes various cardiovascular complications mainly heart failure (HF). The number of people with DM worldwide is over 425 million and is expected to reach 700 million by 2045. Heart Failure (HF) is the heart's inability to provide sufficient blood to meet the body's needs. Heart failure (HF), a major public health problem with considerable morbidity and mortality, is often accompanied by various degrees of progressively pathological enlargement of the left ventricular and abnormal cardiac remodelling by 2030; almost 3% of the population is predicted to have HF. Most anti-diabetic drugs have the effect of increasing the metabolic risk of diabetes in heart failure, since they raise the risk of death and hospitalization for heart failure in patients with and without heart failure. Since lenient glycaemic control with newer therapeutic agents has shown to significantly reduce mortality, morbidity, and risk of developing heart failure in diabetic patients with proven cardiovascular disease, this effect may be related to a direct effect of the glucose-lowering molecules on the cardiovascular system and/or to a negative effect of excessive glucose lowering.

Keywords: Diabetes mellitus, Heart failure, zebrafish model, SGLT2 inhibitors

Introduction

Diabetes Mellitus and the Risk of Heart Failure

Individuals with diabetes mellitus are more than twice as likely to suffer heart failure (HF) than those without the disease. Even after controlling for additional risk variables such age, hypertension, hypercholesterolemia, and coronary artery disease, there is still a higher incidence of heart failure (HF) in people with diabetes. Therefore, when the term "diabetic cardiomyopathy" was first used more than 40 years ago, it was meant to refer to ventricular dysfunction in diabetic individuals who did not have hypertension or coronary artery disease. Potential pathways that could be targeted to Modulate specific pathophysiologic mechanism that contributes to diabetic associated heart failure-

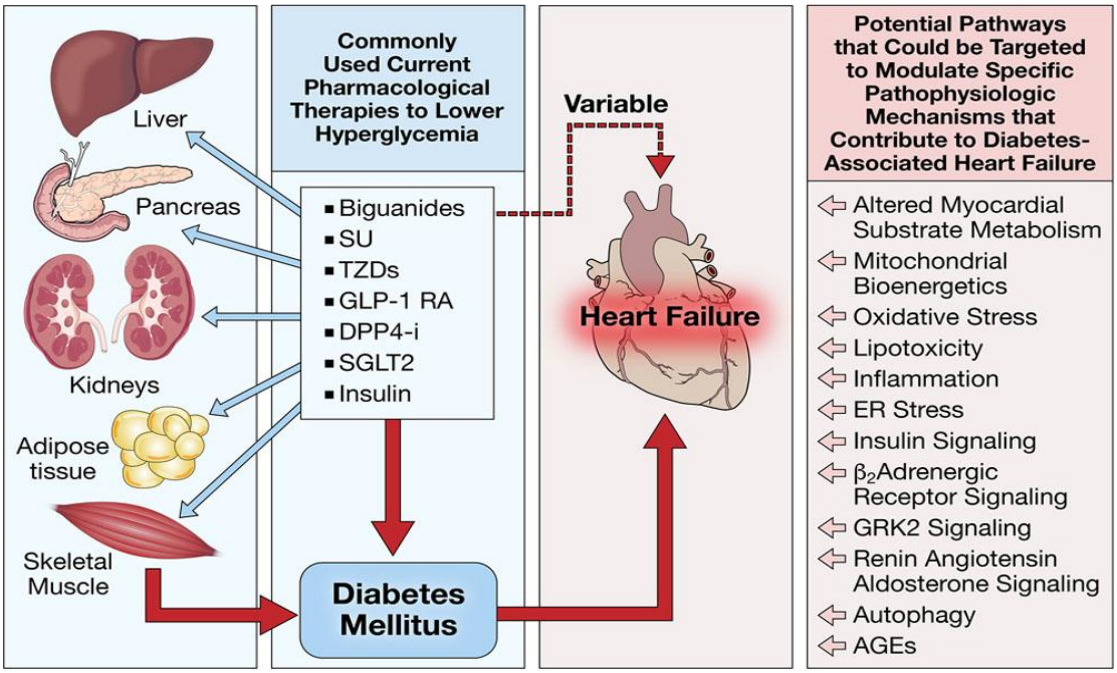


Fig 1. Present antihyperglycemic medications and prospective treatment targets that may influence heart failure linked to diabetes mellitus.

The multi-organ disease state known as diabetes mellitus is typified by hyperglycemia and dyslipidemia. Although the widely prescribed treatments of today may produce normoglycemia, their effects on the risk and course of heart failure are not all the same. The following is a summary of alternative targets that may be more susceptible to heart failure in people with diabetes mellitus and that may be treated with medication.

A recent research found that 44% of patients hospitalized for heart failure had diabetes mellitus, compared to 10% to 15% of the general population who have the disease. The co-occurrence of comorbidities presents distinct clinical difficulties. The Framingham research, which indicated that men and women with DM had a two and four times, respectively, greater risk to develop HF compared with non-DM participants, gave rise to the idea that DM might cause or precipitate HF. Epidemiological studies have validated this association and shown that glycated hemoglobin levels, elevated blood glucose levels, and impaired glucose tolerance are linked to both the prevalence of diastolic dysfunction and the risk of systolic heart failure.

Clinical Manifestation

In the early stages, heart diseases usually don't have any symptoms but in the worsening cases symptoms will develop.

They may include:

- Shortness of breath
- Arrhythmia
- Fatigue
- Swollen feet and ankles
- Dizziness or fainting
- Chest pain

Pathophysiology

The alteration of cardiac function in diabetics occurs through several different mechanisms, such as decreased glucose transport and carbohydrate oxidation, increase in FFA utilisation, decrease in sarcolemmal calcium transport, and alterations in myofibrillar regulatory contractile proteins.

The switch of glucose to FFA oxidation that occurs in the diabetic heart has a significant negative effect on cardiac contractility and functioning thereby inducing left ventricular systolic and diastolic dysfunction even in the absence of coronary artery disease (CAD) or structured heart disease.

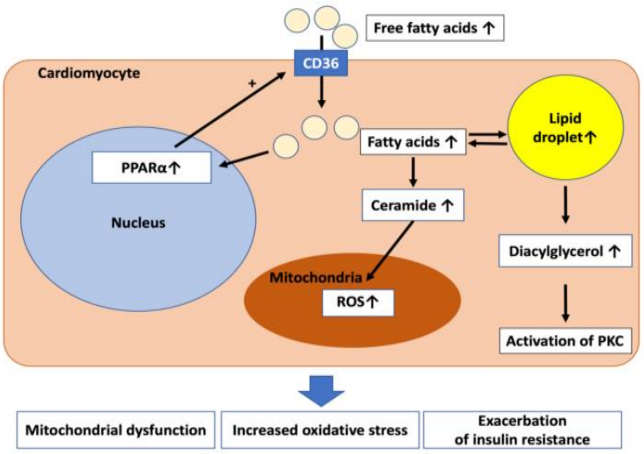


Fig 2.

Glycemic management and HF risk

Metformin, SGLT2i (sodium-glucose cotransporter 2 inhibitor), and certain GLP1RA (glucagon-like peptide 1 receptor agonist) are among the pharmaceutical drugs that may improve cardiovascular outcomes. Others, however, could worsen or raise the risk for HF. These include insulin, certain GLP1RAs, sulfonylureas (SUs), thiazolidinediones (TZDs), and some DPP4i (dipeptidyl peptidase 4 inhibitors). It is possible those factors other than glycaemia contribute to the increased risk of heart failure (HF) in people with diabetes mellitus, or that there are independent mechanisms that connect antihyperglycemic medications and remodelling of the left ventricle (LV). These observations suggest that lowering blood glucose may not be enough to prevent increased hospitalization and mortality from heart failure.

In the setting of diabetes mellitus, HF may be caused by a complex and linked pathophysiology that goes beyond the structural and functional alterations associated with diabetic cardiomyopathy. Some of this pathophysiology may be responsive to pharmaceutical treatment. Increased left ventricular mass and wall thickness, or cardiac hypertrophy, is an often observed condition in the diabetic myocardium. Studies on populations have shown a separate relationship between systolic dysfunction and heart hypertrophy and diabetes mellitus.

MATERIALS AND METHODS

5.1 Glucose colorimetric assay

Because zebrafish larvae are tiny, there are insufficient samples to determine blood glucose levels with standard techniques. Thus, we measured glucose in whole-body fluids using a different technique. In summary, the low-temperature shock approach was used to euthanize 10 larvae per sample after they

were moved to a 1.5 mL tube.After gently rinsing twice with phosphate-buffered saline (PBS), zebrafish larvae were homogenized using a homogenizer in 100 µL DDW. The homogenate was then centrifuged for 15 minutes at 4°C and 13,000 rpm to produce an eluate that contained free glucose. Following that, 50 µL of the eluted solution was put in a 96-well plate and left to react for 30 minutes at room temperature with 50 µL of the AmplexTM Red reagent/horseradish peroxidase/glucose oxidase mixed solution, which was provided in the AmplexTM Red Glucose/Glucose Oxidase Assay. The wavelength of excitation used to test the absorbance was between 530 and 560 nm.

5.2 Zebrafish maintenance

Artemia was fed twice a day to adult zebrafish (*Danio rerio*) kept in an automatic circulating tank system at 28 °C on a 14:10 h light-dark cycle. Experiments were conducted on hatched zebrafish embryos from 3 days post-fertilization (dpf) to 9.5 dpf.The embryos were kept at 28 °C in egg water containing 60 µg/ml ocean salts.

Zebrafish larvae were not utilized in the experiment if their survival rate was less than 80% at 24 hours post-fertilization (hpf). We employed transgenic zebrafish strains that expressed enhanced fluorescent protein (EGFP) in the myocardium22 and cardiac myosin light chain 7 (myl7). The IEAC approved all animal husbandry practices and experimentation protocols, and all studies were carried out in compliance with accepted GLP standards.

After being submerged in a 0.016% tricaine solution for five minutes, zebrafish larvae were rendered unconscious. As per the recommendations of the American Veterinary Medical Association (AVMA),24 zebrafish larvae were put to death using the hypothermic shock method, which involved exposing them to ice-cold water for a minimum of 20 minutes.

5.3 Production of the DM-HFrEF zebrafish model

First, a mixture of streptozotocin and D-glucose was used to create a DM-like state in zebrafish larvae. Zebrafish larvae were submerged in egg water with 40 mM GLU at 3 dpf. GLU was subjected to osmotic control using D-mannitol. 50 µg/ml STZ was introduced at 4 dpf, and the larvae were incubated for 2 hours in the dark. Terfenadine (TER), a potassium channel blocker that causes HF in zebrafish, was used to induce HF at 5 dpf. Analysis was done after more than 24 hours of incubation. Ertu and Cana were given at the appropriate concentrations at 5 dpf.

5.4 vFS Determination

After the zebrafish larvae anaesthesized, the beating heart was examined under an inverted fluorescent microscope. Every single zebrafish larva had its heart scanned and examined for thirty seconds. Cardiac contractility was measured as end-systole (VDs) and end-diastole (VDd) ventricular dimension (VD), which was then converted to ventricular fractional shortening (vFS). Below was the formula:

$$vFS = (VDd – VDs) / VDd \times 100.$$

5.5 Molecular docking analysis

Using the AlphaFold Protein Structure Database, created by DeepMind (London, UK) and EMBL-EBI (Cambridgeshire, UK), a protein structure prediction model for zebrafish NHE1 was created27. PubChem provided the 3D structures of GLU, EMPA, SOTA, and Amiloride (AMI) for molecular docking studies. Zebrafish NHE1 ligand binding sites and binding energy were examined using AutoDockVina.

5.6 Measurement of intracellular H+, Na+ and Ca2+ concentrations

Heart muscle cells grown in Dulbecco's modified Eagle's medium (DMEM) were used to quantify intracellular H+, Na+, and Ca2+ contents. Cultured cells were either treated with or without the addition of 40 mM D-glucose solution for 24 hours in order to apply high glucose (HG) stimulation on them. A fluorescence spectrophotometer was used to evaluate the fluorescence of

the grown cells at 560/580 nm, 340/500 nm, and 494/ 516 nm, respectively, after the cells had been treated with EMPA, SOTA, or AMI for two or twenty-four hours

RESULT

Production of the diabetic heart failure with reduced ejection fraction (DM-HFrEF) zebrafish model

Hyperglycemia and poor glucose homeostasis are hallmarks of diabetes mellitus (DM), which is caused by aberrant insulin expression or activity. Thus, in zebrafish treated with GLU/STZ, we verified the expression of insulin and phosphoenolpyruvatecarboxykinase (PEPCK), a crucial enzyme involved in gluconeogenesis. D-mannitol (MAN) at the same concentration served as the osmotic control for GLU.

Fig 3.

A. Larva before the glucose treatment

Fig 4.

B. Larva before the glucose treatment

Glucose Colorimetric Assay

When compared to the control, the whole-body glucose concentration increased significantly after receiving the GLU/STZ treatment. Although, not as much as GLU/STZ, the GLU-only group also saw a

rise in whole-body glucose concentrations. Furthermore, the whole-body glucose levels in the GLU/STZ group normalized after the high-glucose challenge more slowly than those in the control group and exhibited a tendency to fluctuate.

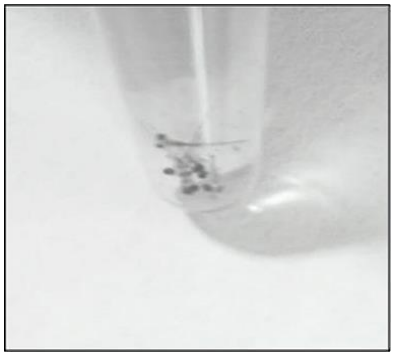


Fig 5.

c) Larvae for Glucose colorimetric assay

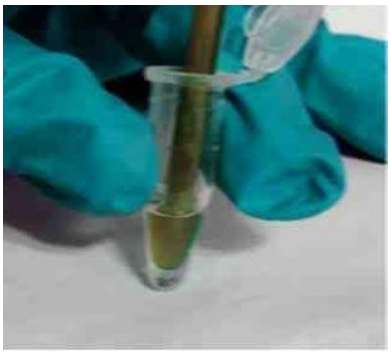


Fig 6.

d) Homogenization of Larvae

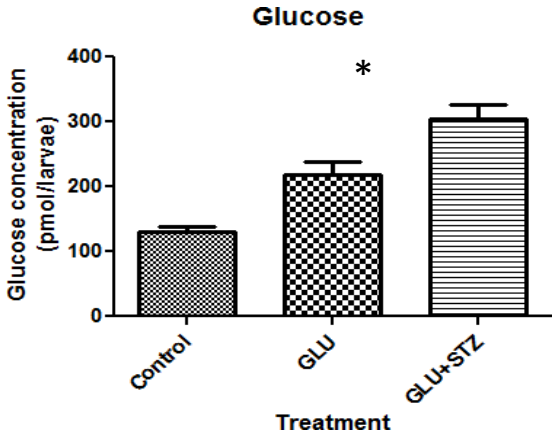


Fig 7

e) Glucose Concentration inLarvaeafter each treatment

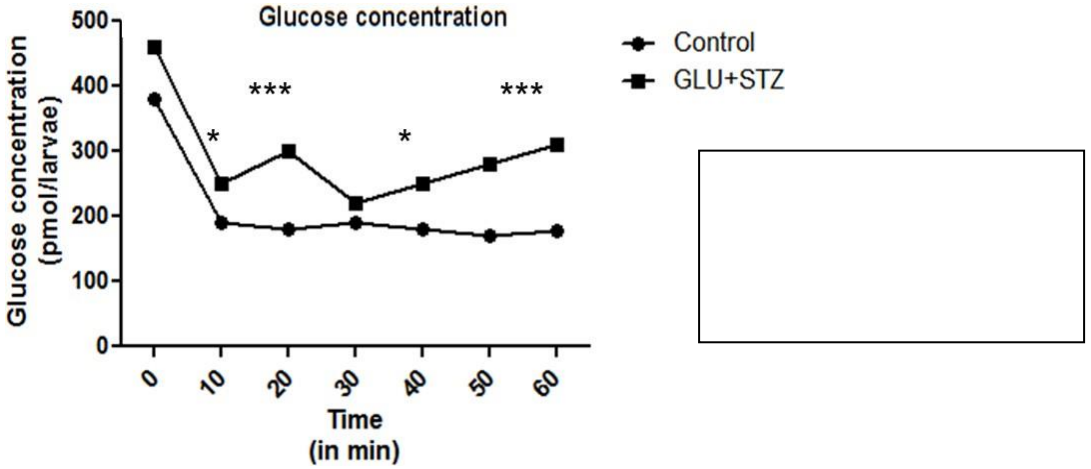


Fig 8 f) Glucose Concentration duringthe hour after treatment

Vs

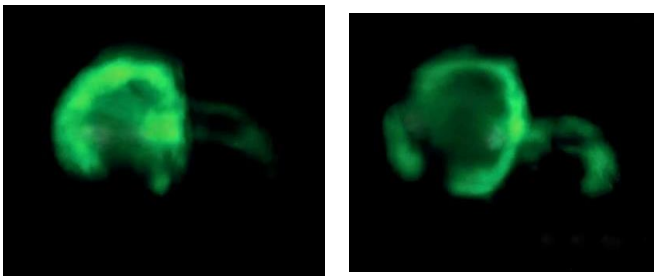
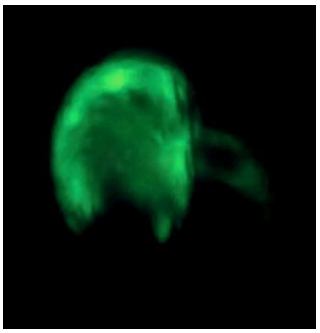
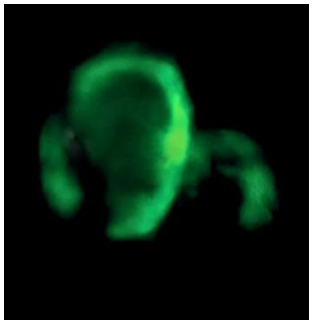


Fig 9 g),h) images of ventricular systole and diastole under Flouroscent microscope before inducing DM-HF

Vd



Control



DM-HF Induced

Fig 10 i),j) images of ventricular systole and diastole uner Fluoroscent microscope afterinducing DM-HF

Effect of Canagliflozin and Ertugliflozin on Relative fluorescence

Through the sodium-calcium exchanger (NCX), the activation of NHE1 exchanges intracellularH+ with external Na+. The influx of Na+ is then exchanged with Ca2+ once more, increasing intracellular Na+ and Ca2+31. It is examined whether EMPA or SOTA affected the concentrations of these ions in cardiomyocytes when they were exposed to high hyperglycemia (HG). Treatment with ERTU, CANA, or AMI inhibited the alterations in intracellular Na+ and Ca2+ concentrations brought on by HG. However, intracellular Na+ and Ca2+ were enhanced under HG conditions compared to low glucose (LG) settings.

Additionally, there was no perceivable alter within the minor concentration move in intracellular H+ caused by HG that was decreased by treatment with ERTU, CANA, or AMI. These diminishes were seen two hours and twenty-four hours taking after ERTU, CANA, and AMI treatment. A concentration-dependent tendency was found within the inhibitory impact of ERTU and CANA organization on changes in intracellular Na+ and Ca2+, with factual importance being achieved at 5 μM. Particularly, there was a considerable variety within the intracellular Na+ substance of Cana-treated cells from 0.04 to 5 μM Cana. Intracellular Na+ and Ca2+ concentrations were surveyed in cardiomyocytes treated with ERTU and CANA consequent to AMI pre-treatment. As a result, critical contrasts in intracellular Na+ and Ca2+ were not watched between the AMI-only bunch and the ERTU post-treatment gather. Within the CANA post-treatment gather, no critical comes about were watched for intracellular Ca2+, but the intracellular Na+ concentration diminished altogether in that bunch compared to the other two bunches.

Time	HG	ERTU	CANA	AMI	Relative Fluorescence Intensity Range	Mean	SD
2hr	-				0.5 to ~1.5	1.0	±0.519
2hr	+				1.0 to ~2.0	1.5	±0.551
2hr	+	+			0.5 to ~1.5	1.0	±0.603
2hr	+		+		0.5 to ~1.0	0.8	±0.324
2hr	+			+	0.5 to ~1.0	0.8	±0.388
24hr	+				1.0 to ~2.0	1.5	±0.506
24hr	+	+			0.5 to ~1.5	1.0	±0.461
24hr	+		+		1.0 to ~2.0	1.5	±0.514
24hr	+			+	1.0 to ~1.5	1.2	±0.279

Table 1 Effect of drugs on Relative Fluorescence

(n = 6, Significant P < 0.05, values are expressed as mean ± SD)

All Values were expressed as mean ± SEM, (n=6), (*p <0.0001) compared to control, (*p<0.0001) (# = 0.0071) compared to HG group using One Way ANOVA followed by Dunnett's test.

Effect of ertugliflozin and canagliflozin on the survival of DM-HFrEF zebrafish

It was investigated whether ERTU and CANA treatment boosted the survival rate by looking at the viability of DM-HFrEF zebrafish treated at different concentrations. The groups treated with 0.2, 1, and 5 μM ERTU and CANA had considerably higher survival at 8 and 9 dpf, while the DM-HFrEF model had dramatically reduced survival at the same times. Notably, 25 μM ERTU was found to significantly improve survival, whereas 25 μM CANA did not.

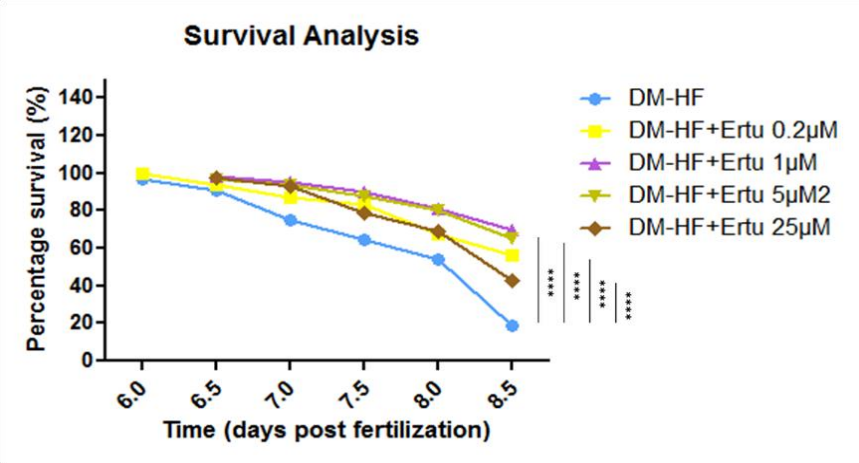
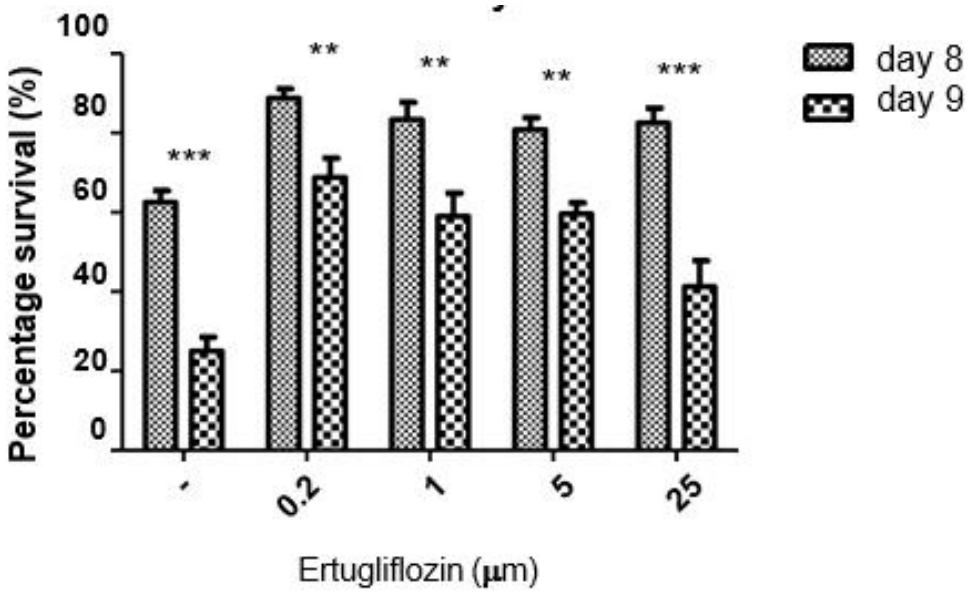


Fig 11. Survival Analysis After ERTU treatment

Survival Analysis



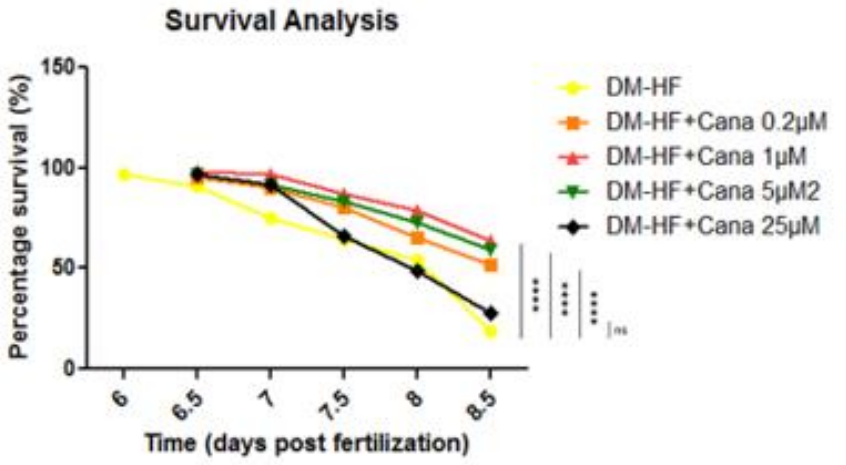
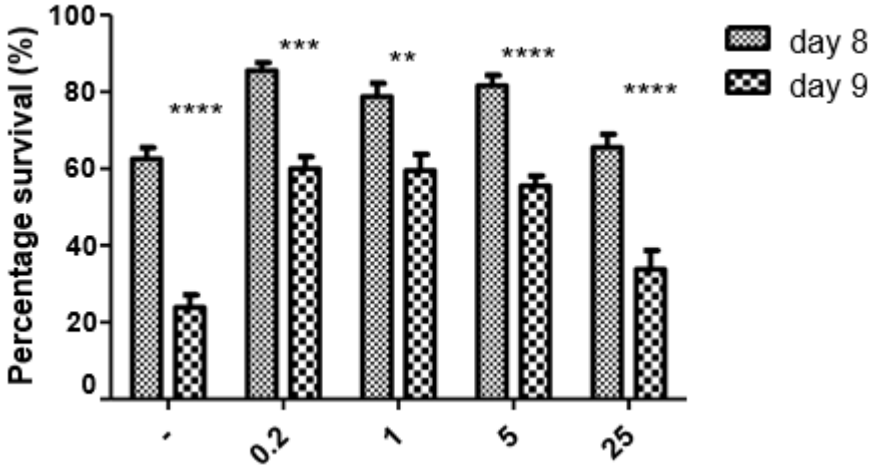


Fig 12. Survival Analysis after CANA treatment

Survival Analysis



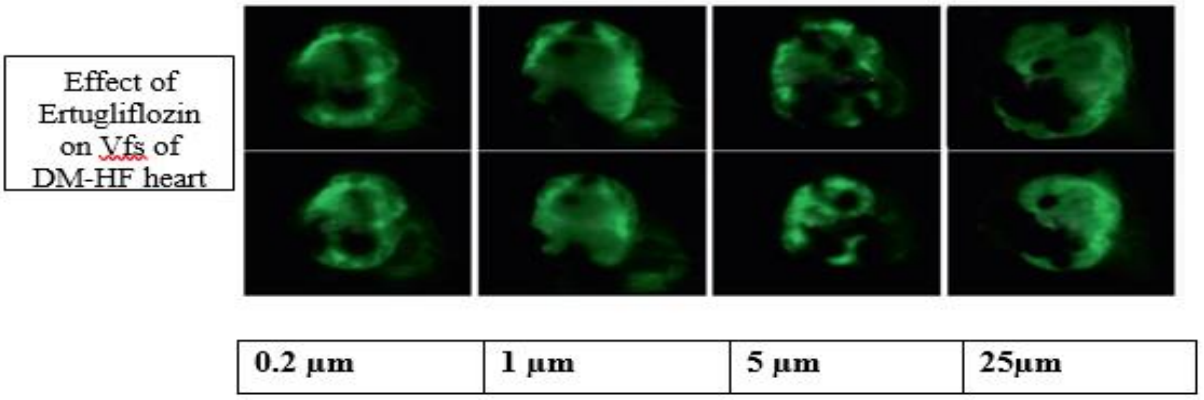
Canagliflozin (□m)

ERTU, CANA treatments significantly increased the survival. There was significant increase in percent survival by treatment of Ertugliflozin (#p < 0.0001) and Sotagliflozin (*p<0.0001) as compared to the inducing treatment group, when compared to control (*p<0.0001) using Two Way ANOVA.

Effect of ertugliflozin and canagliflozin on the ventricular fractionalshortening (vFS)

In a DM-HFrEF zebrafish model, treatment with ERTU and CANA enhanced heart function. The vFS parameter is used to estimate ventricular contractility. DM HFrEF zebrafish showed a considerable drop in vFS when compared to DM-only zebrafish, but there was no difference in vFS between non-DM and DM-only zebrafish. The vFS of DM-HFrEF zebrafish treated with varying dosages of ERTU or CANA was dramatically increased. vFS was markedly improved by treatment with 0.2–5 μM of both medications. ERTU's vFS preservation effect peaked at 5 μM, while CANA's peaked at 1 μM.

Ertugliflozin, Canagliflozin on Vfs of DM-HF heart of zebrafish larvae



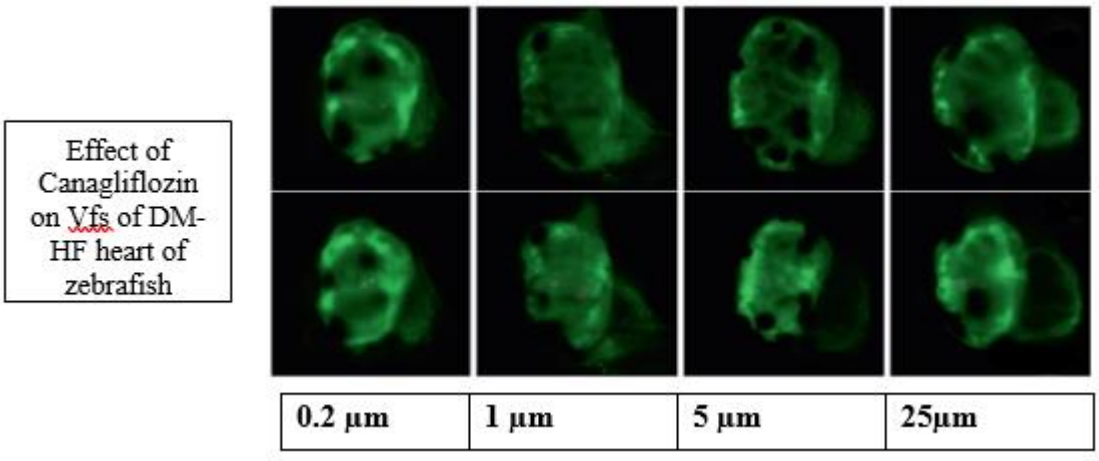


Fig 13. Effect of Ertugliflozin, Canagliflozin on Vfs of DM-HF heart of zebrafish larvae

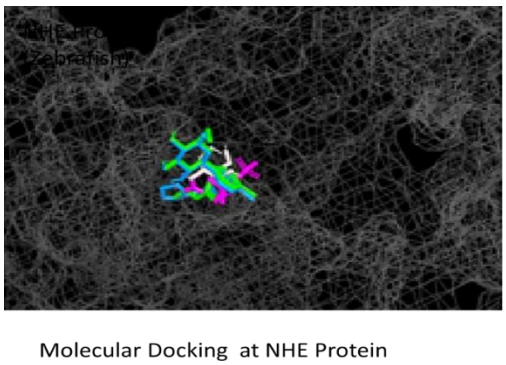
Group	Treatment	vFS
I	Control	30.00±6.6500
II	D-glucose + Streptozotocin	32.500±8.0312*
III	D-glucose + Streptozotocin + Terfenadine	14.700±5.4579*
IV	D-glucose + Streptozotocin + Terfenadine + ERTU	28.300±9.4169*
V	D-glucose + Streptozotocin + Terfenadine +CANA	26.600±6.9634

Table 2 Effect of drugs on vFS

All Values were expressed as mean ± SEM, (n=6), (*p<0.0001) compared to control, (*p <0.0001) compared to treatment group using One Way ANOVA followed by Tuckey'sComparison test.

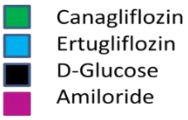
Molecular Docking

The morphology of the hearts did not significantly differ between the groups either. Zebrafish NHE1 was structurally bound by both ERTU and CANA, which both reduced the function of the protein. In the docking analysis, ERTU, CANA, and the selective NHE1inhibitor AMI were all coupled to the same location in zebrafish NHE1.



Molecular Docking at NHE Protein

Ligand	Affinity (kcal/mol)
D-Glucose	-4.7
Ertugliflozin	-7.4
Canagliflozin	-7.2
Amiloride	-6.75



DISCUSSION

The protective benefits of specific SGLT2 inhibitors like CANA and ERTU against DM-HFrEF are further illuminated by this study. Initially, both ERTU and CANA had comparable impacts on survival at the same molarity; generally, CANA performed somewhat better than ERTU. Furthermore, in the DM-HFrEFzebrafish model, the anticipated substantial additive cardioprotective impact of ERTU was not seen. Second, the morphological anomaly and dramatic drop in survival rate shown in the group given with a high dose of CANA suggest that CANA may have unintended effects on zebrafish larvae. Third, the primary mechanism behind the cardioprotective effect of ERTU and CANA may be the structural and functional inhibition of NHE1. Novel diabetic medications, such as SGLT2 inhibitors, effectively lower blood glucose levels11, 14. These inhibitors are used as diabetes medications, but they also have excellent cardioprotective properties.

Both medications significantly decreased HF hospitalization as well as overall mortality in DM patients, according to the CANVAS and VERTIS trials. Furthermore, therapy with ERTU and CANA prevented myocardial fibrosis, hypertrophy, and inflammation in studies employing an animal model of diabetes mellitus32, 33. In any case, considers comparing the cardioprotective impacts of ERTU and CANA are still deficiently. The comes about of this consider utilizing the DM HFrEFzebrafish demonstrate appear that treatment with each of these drugs incorporates a surprising and comparable cardioprotective impact and survival-promoting impact. In any case, in this ponder, the two inhibitors conferred comparable cardioprotective impacts and survival rate changes at different molarities (0.2–5 μM). Each drug's maximal cardiac effects were comparable. One of the measures employed here to assess heart function, vFS, showed a modest variation between the two medications. Although the difference between the concentrations at which ERTU and CANA had their maximal effects was not statistically significant, the two medications' similarly demonstrated cardioprotective effects point to NHE1 inhibition as the mechanism behind these benefits in the DM-HFrEFzebrafish model. In any case, advance considers are required to analyze the commitment of NHE1 and SGLT2 restraint to cardiac work security. In clinical hone, the measurements of these drugs are 5 mg ERTU and100 mg CANA based on clinical trials for DM patients.Furthermore, when compared to ERTU at the same molarity, the survival rate was considerably lower with high-molarity CANA. These findings

imply that zebrafish may experience adverse consequences from treatment with a high molarity of CANA. Because cardiomyocytes do not express SGLT2, NHE1 has been identified as an off- target ligand of SGLT2 inhibitors in a number of investigations investigating the mechanism underlying the cardioprotective action of these drugs15,16. Stimuli associated to diabetes mellitus promote the induction of NHE1 expression and activation. NHE1 is shown to be upregulated in the ventricular tissue of patients with HF18 as well as in DM patients. Furthermore, in an experimental HF model, specific inhibition of NHE1 prevented ventricular hypertrophy and fibrosis, improving cardiac function.

According to these results, NHE1 is a molecule that is important to the pathophysiology of DM-HFrEF and may be a novel target for DM-HFrEF therapeutic approaches. Nevertheless, this mechanism is still up for debate. It was demonstrated that ERTU and CANA bind to NHE1 structurally and prevent its functions in vitro as well as in silico. The potential for inhibition was demonstrated through the measurement of intracellular Na+ and Ca2+ variations. In example, it was evident from inhibitor competition assays of AMI, CANA, and ERTU that CANA and ERTU inhibit NHE1. There are various restrictions on this study.

To begin with, in spite of the fact that the center was on the restraint of NHE1, to explain the precise

atomic component of the cardioprotective impact, it is fundamental to ponder the intuitive more accurately through loss-of-function and gain-of-function tests on NHE1, and SGLT2. Moment, in spite of the fact that the utilize of zebrafish as an creature demonstrate in this ponder has different preferences, it may be troublesome to apply the comes about to people since zebrafish are nonmammalian. Third, the inhibitory impact of SGLT inhibitors on NHE1 was affirmed as it were in cardiomyocytes; it is still essential to affirm the impacts of SGLT inhibitors in different cells other cells constituting the heart, such as endothelial cells and safe cells. At long last, the cause of the diminished survival and morphological anomalies watched within the high-dose CANA-treated gather has not however been explained. In conclusion, this think about appeared that ERTU and CANA, specific SGLT2 inhibitors, give comparative cardioprotective impacts in a zebrafish show of DM-HFrEF. In any case, both inhibitors appeared a tall authoritative partiality for NHE1. Hence, proposition propose that NHE1 hindrance is an basic instrument for the cardioprotective impacts of SGLT2 inhibitors.

CONCLUSION

In conclusion, it is verified that EMPA and SOTA effectively suppress NHE1 in vitro. Through the sodium-calcium exchanger (NCX), the activation of NHE1 exchanges intracellular H+ with external Na+. The influx of Na+ is then exchanged with Ca2+ once more, increasing intracellular Na+ and Ca2+31. It is examined whether EMPA or SOTA affected the concentrations of these ions in cardiomyocytes when they were exposed to high hyperglycemia (HG). Treatment with ERTU, CANA, or AMI inhibited the alterations in intracellular Na+ and Ca2+ concentrations brought on by HG. However, intracellular Na+ and Ca2+ were enhanced under HG conditions compared to low glucose (LG) settings.

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