Evaluation of Pharmacological Interventions Targeted at Sodium Glucose Co-Transporter-1 (SGLT-1) in Diabetic Cardiomyopathy (DCM)

THESIS

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D. Deepika

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Under the supervision of

Prof. Arti Dhar

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CERTIFICATE

This is to certify that the thesis titled **"Evaluation of Pharmacological Interventions Targeted at Sodium Glucose Co-Transporter-1 (SGLT-1) in Diabetic Cardiomyopathy (DCM)"** submitted by **D. DEEPIKA** ID No. **2018PHXF0040H** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

Signature of the Supervisor:

Name in capital letters: **ARTI DHAR**

Designation: Professor

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ABSTRACT

Diabetes and associated cardiovascular (CV) complications are health problems of epidemic proportions worldwide. Diabetic individuals manifest a two to threefold greater risk of CV events compared with counterparts without diabetes. According to IDF (International Diabetes Federation) atlas, 425 million diabetes cases were recorded in 2017 and are expected to rise to 629 million by 2045. Moreover, India ranks second amongst the top 10 countries in the world with diabetes, where 1 in 12 adults has diabetes in India.

SGLT-1 and SGLT-2 are the two important Sodium-glucose co-transporters, where SGLT-1 is primarily responsible for glucose-galactose absorption in the small intestine and minute reabsorption of filtered glucose in renal tubule and SGLT-2 principally expressed in proximal convoluted tubule responsible for 90% glucose reabsorption in the kidney. SGLT2 inhibitors are the recently approved drugs for the treatment of diabetes mellitus. Among these, Canagliflozin and empagliflozin have shown approximately 35% reduction in heart failure hospitalization in large clinical trials of CANVAS (Canagliflozin Cardiovascular Assessment Study) and EMPA-REG outcome trials respectively. Intriguingly, 20-fold higher SGLT-1expression in the human heart than in the kidney, and perturbed expression of cardiac SGLT-1 in diseased myocardium added attention to explore the interventions targeted at cardiac SGLT-1.

The primary objective of the thesis is to investigate the expression pattern of SGLT-1 in rat cardiomyocytes (H9C2) and myocardial tissue under hyperglycaemic conditions. Further, to develop an invivo diabetic cardiomyopathy model (DCM) and to evaluate the pathogenic processes along with molecular mechanisms of SGLT-1 inhibition-induced cardiac benefits

with novel SGLT-1 inhibitors. To accomplish the objectives, rat cardiomyocytes were induced invitro DCM under glucolipotoxicity with 25 mM high glucose and 500 µM with or without, canagliflozin and dapagliflozin (10 µM). To demonstrate the molecular mechanisms confocal microscopy, Apoptosis, and ROS assay by flowcytometry, immunocytochemistry of SGLT-1 expression and RT-PCR were performed. Besides, a high-fat diet and low-dose streptozotocin-induced type-2 diabetes was induced in male Wistar rats and developed into diabetic cardiomyopathy. Furthermore, Canagliflozin was administered by oral gavage for 4 weeks and animals were sacrificed. The freshly harvested heart tissues were utilized to elucidate the molecular mechanisms of canagliflozin-mediated cardiac protection. OGTT, IPITT, Western blotting, RT-PCR, immunohistochemistry, and tissue histology were performed to demonstrate the disease induction and molecular mechanisms. Similarly in another experiment, KGA-2727 a novel SGLT-1 inhibitor was used in an invivo model of diabetic cardiomyopathy in male Wistar rats. Heart tissue was collected at the endpoint of the study and molecular mechanism was evaluated by various techniques like western blotting, RT-PCR, H&E stain, and Sirius red staining.

Taken together, the study findings suggest Cardiac SGLT-1 is upregulated in diabetic cardiomyopathy, which was blunted by canagliflozin, dapagliflozin, and KGA-2727. The pathogenesis of DCM progressed by increased apoptosis, hypertrophy, and fibrosis along with increased ROS. All these maladaptive changes were ameliorated by SGLT-1 inhibition proving to target cardiac SGLT-1 is a valid target.

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Abbreviations:

3P-MACE- 3Point-Major Adverse Cardiovascular Events

ACS- acyl-CoA synthase

AD- Anno Domini, Latin for “in the year of the Lord”

ADA- American Diabetes Association

AGE -glycation end products

AKI- Acute kidney injury

Akt- Ak strain transforming.

AMPK- AMP-activated protein kinase

anti-GAD- anti-glutamic acid decarboxylase

ApoE KO- *Apolipoprotein E knockout*

ATP- Adenosine triphosphate

BC- before Christ

Bcl-2- B-cell lymphoma

BETA2- Beta-2 adrenergic receptors

BNP- brain natriuretic peptide

BP- Blood pressure

Ca^{2+}- Calcium ions

CAD- coronary artery disease

cAMP- *Cyclic adenosine monophosphate*

CANVAS- CANagliflozin cardioVascular Assessment Study

CD36- Cluster of differentiation 36

CKD- Chronic kidney disease

COVT- Cardiovascular outcome trial

CREDENCE- Canagliflozin and Renal Events in Diabetes with Established Nephropathy *Clinical* Evaluation

CRP-C-reactive protein

CTGF- connective tissue growth factor

CVD- Cardiovascular disease

CVOTs- Cardiovascular outcome trials

Dapa- Dapagliflozin

DAPA-LVH- Dapagliflozin on left ventricular hypertrophy

db/db- *Diabetic*

DCM- Diabetic cardiomyopathy

DECLARE-TIMI 58- The Dapagliflozin Effect on Cardiovascular Events–Thrombolysis in Myocardial Infarction 58

DM- Diabetes mellitus

DPP-4- Dipeptidyl peptidase-4

EC- Endothelial cells

eGFR- estimated glomerular filtration rate

EMA- European Medicines Agency

Empa- *Empagliflozin*

EMPA-REG- EMPAgliflozin Removal of Excess Glucose Cardiovascular OUTCOME Trial

ERK1/2- Extracellular signal-regulated protein kinase

FA- Fatty acid

FABPpm- Fatty Acid binding protein plasma membrane

FADH2- flavin adenine dinucleotide

FATP- Fatty Acid transport protein

FATP1- Fatty acid transport protein 1

FDA- Food and Drug Administration

FGF-23- *Fibroblast growth factor-23*

FoxO1- Forkhead box-containing protein 1

GAPDH-glyceraldehyde phosphate dehydrogenase

GLP-1- Glucagon-like peptide 1

GLP-1Ras- GLP-1 receptor agonists

GLUT1- Glucose transporter 1

GLUT-4- Glucose transporter type 4

gr- Gram

HbA1c- Hemoglobin A1c

HDL-C High density lipoprotein- *cholesterol*

HF- heart failure

HFpEF- Heart failure with preserved ejection fraction

HFrEF- heart failure with reduced ejection fraction

HNF-1α- Hepatocyte nuclear factor-1 alpha

HNF-1β- Hepatocyte nuclear factor-1 beta

HNF-4α- Hepatocyte nuclear factor 4α

HTN- Hypertension

ICAM-1-intercellular adhesion molecule-1

IDDM- Insulin Dependent Diabetes mellitus

IL-1- Interleukin-1

IL-17- Interleukin-17

IL-18- Interleukin-18

IL-32- Interleukin-2

IL-6- Interleukin-6

IPF-1-Insulin Promoter Factor

IRS-1- Insulin receptor substrate 1

K^+- Potassium ions

Kcal- *kilocalories*

LADA-Latent autoimmune diabetes in adults

LDL-C- Low density lipoprotein- *cholesterol*

LPL- lipoprotein lipase

LVH- Left ventricular hypertrophy

m/s- Metre per second

MACE- Major Adverse Cardiac Events

mg- Milligram

mg/dl- milli gram per decilitre

MI- Myocardial infarction

miR-133a- muscle-enriched microRNA

miR-146a- *MicroRNA*-146a

miR-181a- *MicroRNA*-181a

miR-181b-5p- *MicroRNA*-181b-5p

miR-19b-3p- *MicroRNA*-19b-3p

miR-30c- *MicroRNA-30c*

miRNA- microRNA

mL- Millilitre

MMP2- Matrix metalloproteinase2

MnSOD- manganese superoxide dismutase

MODY 4- Maturity-onset diabetes of the young type 4

MODY2- Maturity-onset diabetes of the young type 2

MODY3- Maturity-onset diabetes of the young type 3

MODY-Maturity-onset diabetes of the young

Na^+- Sodium ions

NAD^+- Nicotinamide adenine dinucleotide

NADH- Nicotinamide adenine dinucleotide

NDDG- National Diabetes Data Group

NeuroD 1- Neurogenic differentiation 1

NF-kB- *Nuclear factor-κB*

NHE- *Sodium-hydrogen exchanger*

NIDDM- Non-Insulin Dependent Diabetes Mellitus

NO- Nitroc oxide

Nrf2- Nuclear factor erythroid 2–related factor 2

NRG-1- Neuregulin 1

PARP- peroxisome proliferator-activated receptor r

PECAM-1- platelet endothelial cell adhesion molecule

pH- Potential of Hydrogen

Pim1 gene- Proto-oncogene serine/threonine-protein kinase

PKC- protein kinase C

PKC- *Protein kinase C*

PKG- protein kinase G

PPAR-α- Peroxisome proliferator-activated receptor alpha

PPAR-β- Peroxisome proliferator-activated receptor beta

PPAR-γ- Peroxisome proliferator- activated receptor gamma

PTH- *parathyroid hormone*

Q10- Coenzyme *Q10*

RCTs- Randomized controlled trials

ROS- reactive oxygen species

SCAT- *subcutaneous adipose tissue*

SDF-1α- stromal cell derived factor-1α

SGLT-1- Sodium-glucose cotransporters 1

SGLT2- Sodium-glucose cotransporters 2

SGLT2i- Sodium-glucose cotransporter 2 inhibitors

SNS- Sympathetic nervous system

STZ- Streptozotocin

T1DM- Type-1 diabetes mellitus

T2DM- Type-2 diabetes mellitus

TECOS- Trial Evaluating Cardiovascular Outcomes with Sitagliptin

TFG-β1- Transforming growth factor beta 1

TG- *Triglycerides*

TNF-α- Tumour necrosis factor α

VAT- *Visceral adipose tissue*

VCAM-1-vascular cell adhesion molecule-1

VERTIS-CV- Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial

VIVIDD- Vildagliptin in Ventricular Dysfunction Diabetes

vs- *Vurses*

WHO- World health organization

α-SMA- α-smooth muscle actin

Chapter 1: Introduction

1. History of Diabetes

The word Diabetes was conceived by ancient Greek physician Apollonius of Memphis in about 230 BC which means ‘siphon’ in Greek, (dia – through, betes – to go) indicating “to pass through”(1).

However, the initial reference to diabetes was made in the early 1500BC in Egyptian literature, “Ebers papyrus” as a sugar disease with excessive formation of urine(2). At the same time, a great Indian physician and surgeon known as the father of surgery, Acharya Sushruta had mentioned diabetes as madhumeha (honey urine) in his work called “Samhita” and also described the clinical way to diagnose diabetes for the first time through testing of urine to attract ants and flies(3).

The first clinical definition of diabetes was made by the ancient Greek physician Aretaeus Of Cappadocia, in the 2nd century AD as a disease that melts flesh and limbs into the urine, where the patient never stops making urine with an unquenchable thirst and is short-lived (4). In the 5th century AD, Indian physicians Sushruta and Charaka differentiated diabetes into two types: the one which occurs in lean younger individuals (what is now type-1 diabetes) and the second being occur in obese adults (type-2 diabetes) (1). Later, in the 9th century AD Avicenna, an Arab physician, accurately documented the clinical characteristics and some manifestations of diabetes in his book “Kanon” or “Canon Avicennae”. Avicenna promoted the concept of the sweet taste of urine and made this idea known to European observers through his book(1,5).

Inventions made in Europe during the sixteenth and eighteenth centuries can be linked to the development of our current understanding of several elements of diabetes.

Paracelsus, a Swiss physician (1494–1541) noticed a white residue in urine of diabetic patients after allowing it to evaporate. He wrongly assumed that this residue was made up of salt and thought patients had excessive thirst and urination as salt buildup in the kidneys. Well ahead, Thomas Willis at Oxford observed the sugary taste of diabetic patients' urine in 1670(1,6). Investigates on the Urine of Diabetics by British scientist Matthew Dobson (1713–1794) was the first work to demonstrate that the sweet-tasting substance found in the urine of diabetic patients was sugar. He also observed that these people had sweet-tasting serum, which led him to the discovery of hyperglycaemia. Dobson proposed the hypothesis that diabetes was a systemic illness rather than a renal disease. In 1788, Thomas Cawley made the first mention of a connection between the pancreas and diabetes after noticing that those who had pancreatic injury also developed diabetes. In 1815, Eugene Chevreul demonstrated that the sugar in diabetics' urine was indeed glucose. In 1848, Von Fehling designed a quantitative test for urine glucose. Glucosuria thus became a recognized diagnostic criterion for diabetes in the nineteenth century(4). One of the most well-known and successful experimental physiologists of the nineteenth century, Claude Bernard (1813–1878), designed an experimental procedure of pancreatic duct ligation, which led to Pancreatic degeneration. Later studies looking for a pancreatic component that controlled glucose levels found this strategy to be extremely useful. Likewise Bernard revealed that the liver stores glycogen and produces sugary secretion into the blood. He believed that overproduction of this secretion causes diabetes. Bernard's hypothesis that excessive sugar secretion causes diabetes was widely accepted(5).

To distinguish diabetes from diabetes insipidus, John Rollo (1749–1809), the surgeon general of the British Army, added the adjective "Mellitus" to the word "diabetes"(7). Mellitus is derived from the Greek word for honey. For diabetic patients, Rollo created a high-protein, low-carbohydrate diet in 1797 that included blood pudding,

rancid meats, and a combination of milk and lime water(1). Leading American diabetologist Allen (1879–1964) thought that since people with diabetes could not utilize food well, decreasing the amount of food would help the condition. Dietary restriction therapy was stringent, and patients with type 1 diabetes receiving it frequently died from starvation(1,5). In 1889 Oscar Minkowski and Joseph von Mering noticed that the dogs with their pancreas removed suffered significant thirst, excessive urine, weight loss, and increased appetite. When Minkowski examined the urine of these dogs, he discovered glucose, confirming his suspicion that diabetes causes the symptoms(8). A French researcher Edouard Hedon (1863-1933) made a crucial discovery in 1893 by demonstrating that the entire pancreatectomy was required for the onset of diabetes. He transplanted a little portion of the pancreas under the skin after removing it. At this point, there was no indication that the experimental animals had diabetes. However, the moment the graft was removed, diabetic symptoms appeared(9). Besides, French scientist Gustave-Edouard Laguesse (1861–1927) hypothesized in 1893 that the material responsible for the regulation of blood glucose may originate from the minute islands of pancreatic tissue, later identified by Paul Langerhans in 1869. Langerhans described tiny clusters of pancreatic cells that were not emptied by pancreatic ducts in his doctoral thesis. The postulated substance secreted by the islets of Langerhans was given the name "insulin" in 1909 by the Belgian physician Jean de Mayer(5).

Many scientists sought to identify the active ingredient in internal pancreatic secretion. In 1902, John Rennie and Thomas Fraser extracted a chemical from the endocrine pancreas of a codfish (Gadus callurious), which has anatomically distinct endocrine and exocrine pancreata. They gave the dog the extract via injection, and it soon passed away, most likely from extreme hypoglycaemia. A German doctor Georg Ludwig Zuelzer in 1907 removed the dog's pancreas and then gave it an injection of pancreatic extract. His

research led to a decrease in glucosuria and an increase in blood pH. The extract was patented in the United States by Zuelzer under the name "acomatol"(8). In 1908, Zuelzer effectively used it to revive a diabetic patient who had fallen into a coma, but because the extract was probably contaminated with other materials, the procedure had significant side effects. However, he continued his research and created a new extract, but it developed a Convulsive behaviour most likely as a result of hypoglycaemia. In 1921, to extract a solution from the pancreas, Banting and Best extracted atrophied pancreatic glands from dogs, ground the tissue in a mortar, strained the mixture, and then injected the extract into the vein of a pancreatectomized (diabetic) dog. They continued the studies with other diabetic dogs after it was evident that the dog's condition had improved, with similarly spectacular outcomes. They also tried fresh pancreata, foetal calf pancreata, and various dosing methods (rectal, subcutaneous, and intravenous). At the end of 1921, James Collip joined the Banting and Best team and made a significant contribution to improving the extraction and purification of pancreatic extracts for successful animal trials(1).

Finally, Banting and Best's pancreatic extract was first given to 14-year-old diabetic patient Leonard Thompson in 1922. Thompson developed abscesses at the injection sites and fell sick after receiving 15 cm3 of a "thick brown" pancreatic extract. However, On January 23, a second injection was administered with better purity. This time, the patient's blood sugar dropped from 520 to 120 mg/dl in 24 hours, and the presence of urine ketones vanished. Thompson continued receiving treatment and lived for 13 years but died of pneumonia. This pancreatic extract was named ISLETIN, and later changed to INSULIN. In 1923 scientists were awarded the Nobel prize for the discovery of Insulin(8).

1.1. Diabetes –Types:

National Diabetes Data Group (NDDG) has categorized diabetes as Insulin Dependent Diabetes mellitus (IDDM), Non-Insulin Dependent Diabetes Mellitus (NIDDM) based on therapy in 1979 with WHO endorsement(10). Since numerous NIDDM patients also gradually develop insulin dependency, this classification was revised by American Diabetes Association (ADA) as Type-1 diabetes, Type -2 diabetes, gestational diabetes, and other types based on pathogenesis(11).

Type-1 diabetes is characterized by a complete lack of insulin. The condition is most prevalent in children and adolescents. Type-1 diabetes can be further classified as Type-1A and Type-1B. Type-1A is an autoimmune disorder, characterized by the presence of antibodies against islet cells, anti-glutamic acid decarboxylase (anti-GAD), IA-2, IA-2f3, and anti-insulin antibodies. Certain environmental factors such as viral infections and protein of cow's milk also trigger the immune reactions relatively faster as in the neonatal period or prolonged enough to get confused with Type-2 diabetes called LADA (Latent autoimmune diabetes in adults). Type-1B is mostly idiopathic without any characteristic islet cell antibodies(12,13).

Type-2 diabetes is the most common and accounts for 80-95% of diabetic cases worldwide. Type-2 diabetes is more complex in pathogenesis and characterized by an abnormality in insulin action and secretion. It is typically diagnosed in the middle-aged population, likely in the fourth decade of life. Insulin resistance in T2DM results in decreased glucose absorption in muscle and adipose tissue and increased glucose synthesis in the liver in a feedback loop. Additionally, the lower insulin release due to β-cell malfunction is insufficient to keep blood sugar levels within normal range. Taken together persistent hyperglycaemia causes deleterious effects at micro and microvasculature.

Important risk factors associated with the development of type-2 diabetes include a sedentary lifestyle, smoking, excessive alcohol consumption, and physical inactivity. T2DM is also attributed to several metabolic changes, including obesity, hypertension, dyslipidaemia, and an increased risk of atherosclerosis(14,15).

Gestational diabetes is detected primarily in pregnant women. However, the pathogenesis of gestational diabetes shows a similar pattern of genetic and physiological abnormalities seen in other diabetic patients(16). However gestational diabetes is majorly a result of insulin resistance due to elevated maternal adiposity and changes in placental hormones. Besides these, autoimmunity, insulin resistance, and monogenic diabetes are also associated with gestation. For instance, in most of the studies, women with gestational diabetes also expressed antibodies against pancreatic islets and beta cell antigens such as glutamic acid decarboxylase antibodies. Similarly, monogenic forms of gestational diabetes also found in some women, mostly with mutations of glucokinase (MODY2), hepatocyte nuclear factor1α (MODY3), and insulin promoter factor 1 (MODY 4). In addition, obesity is the known risk factor seen in women with gestational diabetes along with increased levels of circulating leptin, TNF-α, C-reactive protein, and decreased levels of adiponectin together contribute to insulin resistance. In conjunction with insulin resistance, the insulin signalling pathways also get disrupted in skeletal and adipose tissue, such that improper GLUT-4 translocation and reduced PPAR-γ play an important role in gestational diabetes (17).

Other types of diabetes include Maturity-onset diabetes of the young (MODY) usually seen in younger individuals and is triggered by genetic mutations in genes that regulate insulin secretion leading to hyperglycaemia. MODY can be further classified based on gene that is involved as 6 types, MODY 1 is attributed to mutations of HNF-4α (hepatocyte nuclear factor 4α), MODY 2 is involved with glucokinase gene mutations, MODY 3 has

mutated HNF-1α, MODY 4 has IPF-1 mutations (Insulin Promoter Factor), MODY 5 is characterized by HNF-1β mutation and MODY 6 has NeuroD 1 or BETA2 mutations(18).

Certain genetic defects of insulin receptors also cause severe insulin resistance associated with hyperglycaemia as seen in Donohue syndrome/ Leprechaunism and Rabson-Mendenhall syndrome. Patients with Acute pancreatitis also exhibit short-term hyperglycaemia. Similarly, pancreatic cancers and cystic fibrosis also cause diabetes. Diabetes is also caused secondary to chemical or drug intoxication as seen after clozapine, glucocorticoid, and nicotinic acid intake. Similarly, Thiazide diuretics induce diabetes due to hypokalaemia, which is a major adverse effect of thiazide diuretics. Beta-blockers induce hyperglycaemia by direct blocking of β-2 adrenergic receptors, which leads to inhibition of adrenergic-stimulated insulin release and glycogenolysis. Likewise, calcium channel blockers also induce hyperglycaemia by reducing intracellular calcium levels, which directly affects insulin release. Furthermore, Isoniazid, an antimycobacterial agent also induces hyperglycaemia by stimulating glucagon secretion and interfering in the krebs cycle. Some antiviral drugs are also associated with hyperglycaemia, as seen in ritonavir use, which inhibits GLUT-4 causing hyperglycaemia. Certain viral infections like coxsackie B and rubella viruses have been linked to autoimmune destruction of beta cells causing diabetes(19–22).

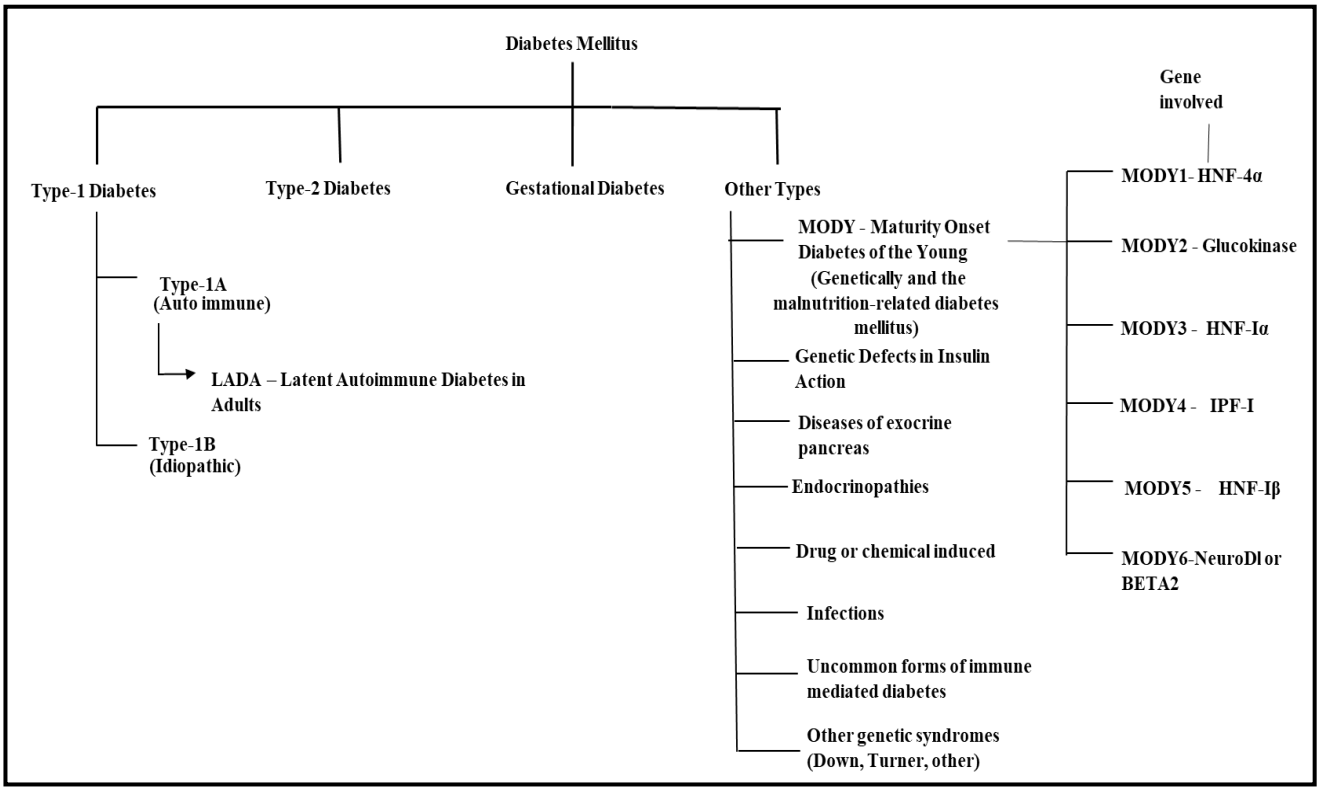

Figure 1.2 1 Classification of Diabetes

1.2. Diabetic Complications:

Over the past 40 years, diagnosed diabetes has become far more common both in the United States and around the world. Around 30 million individuals globally had diabetes in 1985; by 1995, this figure had risen to 135 million; In 2017 this number had projected to 425 million and is expected to rise to 629 million by 2045(23,24). It is concerning to learn that more than 47% of people worldwide still lack a diabetes diagnosis, and that number is only going to rise. Additionally, 20.9 million live births are impacted by some type of hyperglycaemia during pregnancy, of which 85.1% are caused by gestational diabetes, and 318 million people are projected to have impaired glucose tolerance. Type 2 diabetes is on the rise worldwide, although more than 80% of those affected live in low- and middle-income nations including India, Bangladesh, Bhutan, Pakistan, Sri Lanka, the Philippines,

and Indonesia. India ranks second amongst the top 10 countries in the world with diabetes. 1 in 12 adults has diabetes in India, collectively more than 74 million people, and 40 million cases of prediabetes, a condition that increases the risk of developing both diabetes and cardiovascular disease. The main causes of this rising incidence are changes in lifestyle, such as eating unhealthily and not exercising. Type 2 diabetes, which makes up more than 90% of cases of diabetes, is predicted to experience the majority of the estimated increase, whereas type 1 diabetes incidence is expected to remain steady. Even though T1DM and T2DM possess distinct aetiologies, both disorders are characterized by systemic metabolic abnormalities such as hyperglycaemia and dyslipidaemia(25).

Chronic hyperglycaemia is linked to long-term organ damage, dysfunction, and failure, particularly in the eyes, kidneys, nerves, heart, and blood vessels. These consequences are referred to as micro and macrovascular complications (26).

Long-term complications of diabetes mellitus include the progressive onset of retinopathy, which may result in blindness, nephropathy, which may result in renal failure, and/or neuropathy, which increases the risk of foot ulcers, amputation, charcot joints, and signs of autonomic dysfunction, such as erection problems. Cardiovascular, peripheral vascular disease, and cerebral vascular disease are all more common macrovascular complications(27).

Cardiovascular disorders are the leading cause of death among all macrovascular diseases. Cardiovascular disease (CVD) accounts for up to 65% of all fatalities and is the primary cause of death (70%) among T2DM patients(28). Insulin-mediated mitochondrial glucose oxidation is reduced in the heart of patients with T1DM and T2DM, due to either lack of insulin (T1DM) or resistance to insulin (T2DM)(29). Since insulin resistance in T2DM promotes increased free fatty-acid uptake through the fatty acid translocase CD36,

T2DM experiences more mitochondrial dysfunction and an accumulation of harmful lipid metabolites in the heart than in patients with T1DM(29–31). Ischemic heart disease is the most common CVD in DM. However, when considering proven heart failure risk factors such as hypertension or ischemic heart disease, population-based studies have found that the risk of heart failure in diabetic individuals (especially type 2) is much higher. This correlation was proven by Rubler and colleagues after evaluating post-mortem data from four diabetic patients who died from heart failure without signs of hypertension, myocardial ischemia, or congenital valvular heart disease. Similarly, a study by de Simone et al. found that patients with type 2 diabetes have an elevated risk of heart failure, regardless of whether they are also experiencing concomitant myocardial infarction or hypertension. In consequence, the term "diabetic cardiomyopathy" was introduced, which is defined as ventricular dysfunction in diabetics without pre-existing hypertension or coronary artery disease (CAD)(32–34).

1.2.1. Diabetic Cardiomyopathy:

Diabetic cardiomyopathy is a particular type of heart disease that is fueled by insulin resistance, compensatory hyperinsulinemia, and the progression of hyperglycemia, all of which happen independently of other cardiac risk factors like coronary artery disease (CAD) and high blood pressure(35). Four diabetics who experienced heart failure symptoms were the first to be diagnosed with diabetic cardiomyopathy in 1972. This was confirmed in a 1974 secondary analysis of the Framingham Heart Study, which discovered that after adjusting for other risk factors like age, hypertension, obesity, dyslipidemia, and coronary artery disease (CAD), the risk of heart failure was increased 2.4-fold in men and fivefold in women with diabetes compared to normal individuals(36,37). A 1977 study of 17 people with type 2 diabetes found conclusive indication of diabetic cardiomyopathy, which is characterized by elevated cardiac left ventricular end-diastolic pressure, decreased left

ventricular compliance, and reduced left ventricular ejection fraction with diffuse hypokinesis(38). These preliminary findings revealed that diabetes had a specific and direct effect on interstitial fibrosis, resulting in decreased left ventricular compliance and diastolic dysfunction. The clinical development of cardiac dysfunction in diabetes, progresses from subclinical cardiac abnormalities, such as left ventricular fibrosis and diastolic dysfunction, to severe diastolic heart failure with normal ejection fraction, and finally to systolic dysfunction coupled with heart failure with reduced ejection fraction(39,40).

Cardiomyopathy is a complex condition that has been linked to various etiologies. These include compromised autonomic and endothelial function, impaired fuel metabolism, abnormalities in several proteins that regulate ion flux, specifically intracellular calcium, as well as elevated stiffness of the left ventricular wall caused by the deposition of connective tissue and insoluble collagen. In diabetic heart utilization of glucose is hampered by insulin resistance(41,42). Together with an increase in FA supply, this modification shifts cardiac energy production to FA use. High FA absorption and metabolism enhance the generation of reactive oxygen species (ROS), which harms the heart, as well as accumulates FA intermediates and triglycerides(43).

1.2.1A. Metabolic Alterations in Diabetic Cardiomyopathy:

a. Alterations in Glucose absorption and metabolism:

Glucose is the important fuel for the heart under normal circumstances. Several processes control the metabolism of glucose, including glucose transport and glycolysis. The transmembrane glucose concentration and the number of sarcolemmal glucose transporters (GLUT1 and GLUT4) affect cardiac glucose uptake(44,45). GLUT1 is representative of basal cardiac uptake and has a more significant sarcolemmal location. In the adult heart, GLUT4 predominates over GLUT1 and, under basal circumstances, the majority of this

transporter is found intracellularly. Insulin is necessary for GLUT4 transit from the cytoplasm to the plasma membrane. Hence, T2DM heart shows reduced glucose uptake because of impaired insulin signalling and reduced GLUT4 translocation(45–47). However, recent studies in db/db mice suggested increased FA oxidation followed by decreased glucose metabolism(48). Remarkably, increased FA oxidation increases citrate levels which inhibit Phosphofructokinase, the rate-limiting enzyme in glycolysis. Similarly, high FA levels increase Phospho Diesterase Kinase4 which inhibits Pyruvate dehydrogenases after phosphorylation and also increases acetyl-CoA which inhibits pyruvate dehydrogenase(43). Glycogen accumulation is increased when the myocardial energy substrate is switched from carbohydrates to lipids. This is likely due to increased glycogen synthesis, decreased glycogenolysis, or a combination of these two processes. Interestingly, glycogen accumulation in skeletal muscle is reported to augment insulin resistance, however cardiac glycogen influence on insulin signalling is yet to be evaluated(49,50).

Hyperglycaemia leads to glucotoxicity of myocardium through the production of ROS and nitrogen species which in turn provoke cytochrome c –mediated caspase3 activation and apoptosis(51). Persistent hyperglycaemia activates chronic PARP [Poly(ADP-ribose)polymerase-1] activation, which depletes NAD+, an ATP-consuming process making myocardium energy deficit and apoptosis. Through the inhibition of glyceraldehyde phosphate dehydrogenase (GAPDH), PARP diverts glucose from glycolytic pathways into alternative fates, such as the formation of advanced glycation end products (AGE), hexosamine, polyol pathway flux, and protein kinase C (PKC), which is thought to be a mediator of the damage to cardiac tissue caused by hyperglycaemia(52,53). Accordingly, increased AGE formation causes several macromolecules, including collagen, to cross-link irreversibly, causing tissue fibrosis, inactivating SERCA2a and the ryanodine receptor calcium release channel, impairing cardiac relaxation and contractility, and increasing

ventricular stiffness. Finally, hyperglycaemia induce PKC-2 activation causes cardiomyopathy, which is characterised by left ventricular hypertrophy, cardiac myocyte necrosis, multifocal fibrosis, and reduced left ventricular performance(54–56).

b. Alterations in Fatty acid Uptake and metabolism:

In contrast to glucose uptake, FAs do not require any transporters to enter into cells owing to lipophilicity. However, FA uptake is hindered by proteases after reaching the saturation kinetics(43). Therefore, FA transporters are probably needed to facilitate this activity as well. Three FA transporters, CD36, FA transport protein (FATP), and FA binding protein plasma membrane (FABPpm), have been discovered in the heart. Increased cardiac CD36 and FABPpm expression in severe STZ-induced diabetes suggest that these transporters' transcription has been altered in diabetes, which significantly increases FA metabolism(57,58). It's worth noting that apart from transporters, cardiac lipoprotein lipase (LPL), a protein involved in FA transport, and cardiac peroxisome proliferator-activated receptor (PPAR), a protein involved in FA oxidation, Acyl-CoA synthase, AMPK also overexpressed in a cardiac phenotype that resembles diabetic cardiomyopathy(43).

LPL is an essential enzyme for hydrolyzing triglyceride-rich lipoproteins to release fatty acids, as the heart can synthesize and store only limited FA. Thus, LPL significantly regulates FA delivery to the heart, the upregulation of which accelerates the supply of FA to the heart. In contrast, cardiac knockout of LPL switches the substrate utilization from FA to glucose(59,60).

The first stage of FA metabolism is the esterification of FA to fatty acyl-CoA, which is catalyzed by acyl-CoA synthase (ACS)(61). Fatty acyl-CoA can be used to synthesize intracellular TG or transferred to the mitochondria for oxidation. Recent studies have shown that ACS is connected to CD36 or FATP on the cytosolic side of the sarcolemma, indicating

that ACS also affects FA uptake(62–64). Indeed, FA absorption and intracellular TG buildup are greatly increased when ACS is overexpressed in the heart or fibroblasts(61). Normally, 10–30% of the esterified FA that enters cardiomyocytes is converted to TG, whereas 70–90% is oxidized for ATP production(65). As lipolysis and esterification occur continuously, the TG pool is not static. The consistent intracellular TG levels in a healthy heart indicate that lipogenesis and lipolysis are in equilibrium. However, intracellular TG build-up is linked to lipotoxicity in conditions where the FA supply exceeds cellular oxidative capacity, as in obesity and diabetes. Although TG is not expected to directly cause apoptosis, increased lipolysis augments the levels of fatty acyl-CoA, a significant mediator of apoptosis(66,67).

PPARs belong to the nuclear receptor superfamily and contain ligand-activated transcription factors which regulated FA metabolism. Three isoforms of PPARs exist: PPAR-α, PPAR-β (or -δ), and PPAR-γ(68,69). In tissues like the heart that have a high FA metabolism, PPAR-α is widely expressed. When intracellular FA levels rise, PPAR-α is activated, which promotes the expression of genes that regulate FA oxidation at various stages. These genes include genes that regulate, FA binding and uptake, like LPL, CD36, and FA binding protein, as well as those that regulate esterification, like ACS, and those that promote oxidation. Concurrently activated PPARα reduces the expression of genes involved in glucose metabolism(70,71).

According to several studies, severe FA overload causes lipotoxicity and aids in the onset and progression of cardiomyopathy. Furthermore, LPL, FATP1, PPAR-α and ACS overexpression also promote excess lipid uptake, and storage which induces lipotoxicity and contractile dysfunction attributed to diabetic cardiomyopathy(72,73). The mechanism behind FA metabolism-induced lipotoxicity is mainly oxidative stress. More fatty acid oxidation augments mitochondrial ROS generation which promotes cellular damage and

triggers apoptosis. Moreover increased FA also increases the generation of ceramide which in turn increase inducible nitric oxide synthase through activation of NF-kB and generates nitric oxide and peroxynitrite. As a highly reactive agent, peroxynitrite induces the opening of the mitochondrial permeability transition pore and the release of cytochrome c. On the other hand ceramide also directly reacts with cytochrome c, inducing its release from mitochondria. As a result, caspase is activated, starting the cell's apoptotic pathway(73,74).

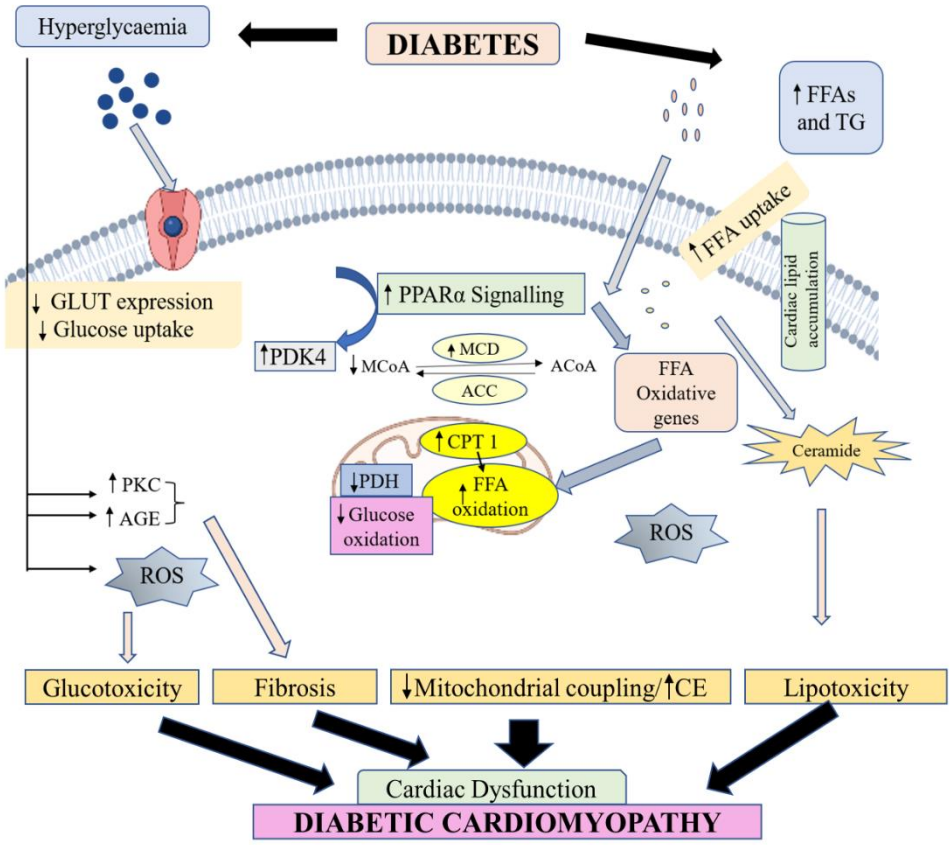

Figure 1.2 2 Potential contributors to Diabetic cardiomyopathy.

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c. Disrupted Calcium homeostasis:

Ion transporters including Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase preferentially rely on ATP produced during glycolysis. Thus, the elevated FA oxidation rates associated with obesity and diabetes may limit myocardial glycolysis, which may compromise intracellular Ca^{2+} homeostasis, a flaw that has been suggested to play a role in the development of cardiomyopathy(65,75,76).

d. Altered cardiac function and efficiency:

FA as a substrate produces 2.33 ATP per oxygen atom, whereas glucose produces 2.58 ATP/oxygen atom. Thus FA needs more oxygen compared to glucose to generate energy making it a low-efficient substrate. During ischemia or workload, this decreased efficiency makes the heart more prone to damage. Moreover, the diabetic heart utilizes 30% more oxygen, even though FA oxidation requires only 10% more oxygen compared to glucose making the diabetic heart less efficient(77–79).

e. Mitochondrial dysfunction:

The majority of the energy is generated by mitochondria in cells. The tricarboxylic acid cycle uses acetyl-CoA produced by FA oxidation or glycolysis to produce NADH and FADH2 in the body. The mitochondrial electron transport chain is where ATP is finally produced, and these electron carriers deliver electrons to it. Therefore, it is anticipated that cardiac mitochondrial dysfunction will have negative biological repercussions and eventually result in heart disease(80,81). Uncertainty exists regarding the processes through which mitochondrial malfunction leads to cardiomyopathy. ROS is one such target. Following excessive FA oxidation, increased ROS production causes oxidative stress and cell damage. It has been demonstrated that metallothionein, MnSOD, or catalase overexpression inhibits ROS, protecting against mitochondrial dysfunction and

cardiomyopathy(82,83). Ceramide, a sphingolipid that has been demonstrated to accumulate after FA excess, is an additional target. Ceramide is thought to cause the development of cardiomyopathy by either inhibiting the mitochondrial respiratory chain or causing the release of cytochrome c from the mitochondria and apoptosis(84,85).

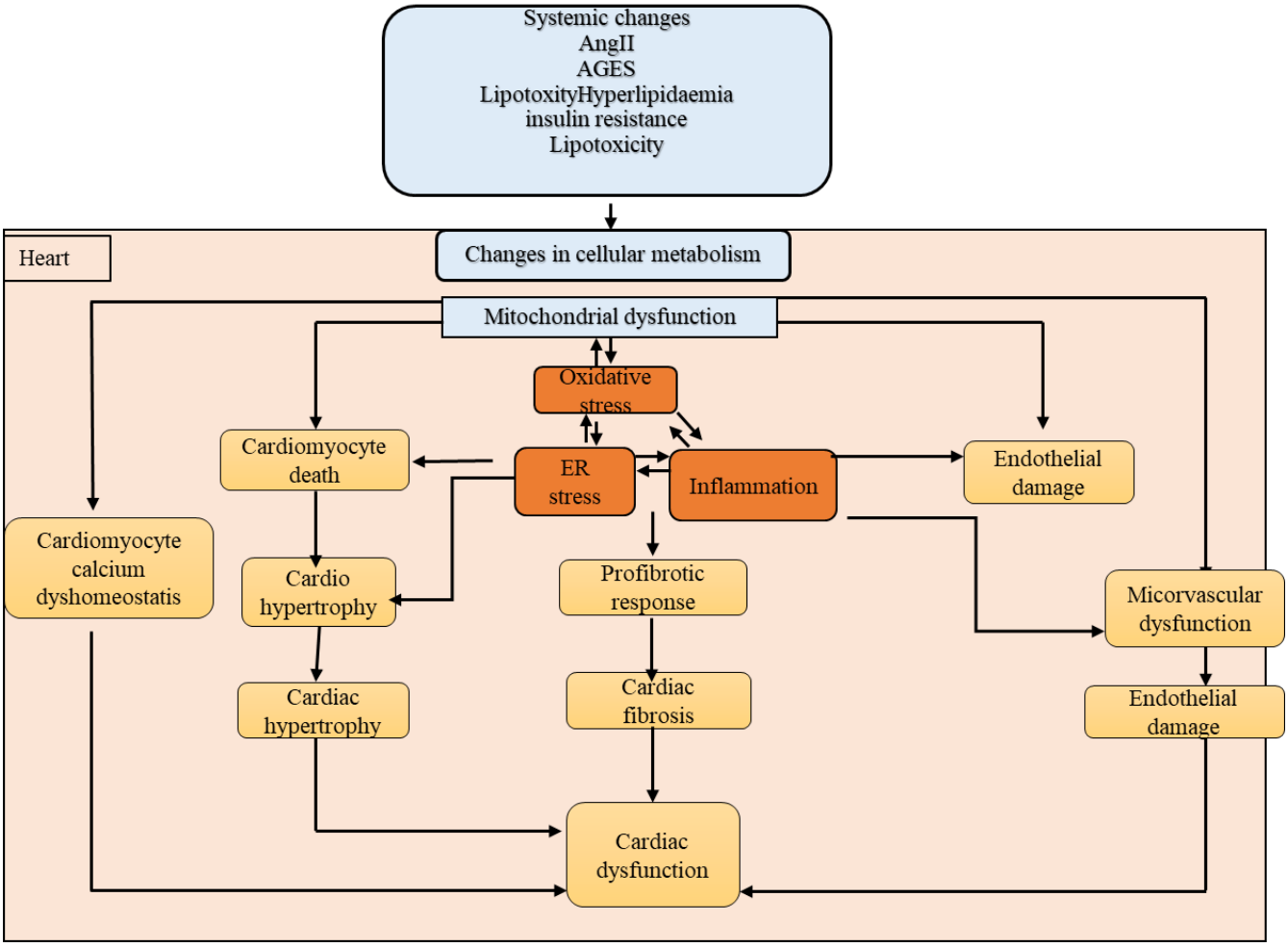


Figure 1.3 1 . Mechanism of Diabetic cardiomyopathy. Figure Adapted and reproduced from Reference (211)

1.2.1B. Therapeutic Strategies to Combat Diabetic Cardiomyopathy

a. Glucose Lowering Agents in Cardiovascular Safety

i. GLP-1 Receptor agonists:

Glucagon-like peptide 1(GLP-1) is an incretin released after food intake from L cells of the

ileum and colon (86). Upon activation, the GLP-1 receptor catalyzes the conversion of ATP into cAMP, this elevated cAMP in pancreatic-beta cells triggers insulin secretion. The effects of GLP-1 include delayed gastric emptying, suppression of glucagon secretion, reduced appetite, promotion of satiety, decrease in circulating lipoproteins, and reduction in blood pressure(87). Hence, GLP-1 agonists are the better choice of drugs to normalize hyperglycaemia without drug-induced hypoglycaemia. Moreover, the expression of GLP-1 receptor is reported in human atrial and ventricular tissue and has gained attention to develop novel GLP-1 receptor agonists (GLP-1RAs)(88–90). There are four GLP-1 analogues currently in use, exenatide, liraglutide, albiglutide, and dulaglutide (91).

ELIXA was the first cardiovascular safety trial on GLP-1 receptor agonist lixisenatide with 6068 diabetic patients with the acute coronary syndrome. This trial reported lixisenatide is safe in T2DM patients with CVD compared to placebo control(92). Similarly, another Cardiovascular outcome trial (COVT) LEADER on liraglutide with 9340 diabetic patients with established cardiovascular risks showed beneficial cardiovascular effects with a reduction in CV mortality, non-fatal MI, stroke, and all-cause mortality(93). Furthermore, trials like SUSTAIN-6 on semaglutide have shown positive CV outcomes, whereas EXSCEL trial on exenatide failed to demonstrate positive outcomes, but exhibited secondary positive outcomes. These inconsistent results are due to the variations in study population and inclusion criteria and follow-up periods among these studies(94,95).

ii. DPP-4 inhibitors:

Dipeptidyl peptidase-4 is an enzyme that shows exopeptidase activity on GLP-1 and other hormones involved in inflammation, and cell survival(96). B-type (brain) natriuretic peptide 1-32 (BNP 1-32) is an important peptide found in the ventricles of the heart, which regulates vascular tone, and fluid balance and also suppresses cardiac remodelling after

myocardial infarction. DPP-4 inactivates BNP by cleaving BNP (1-32) to BNP (3-32), which has reduced activity than native BNP. Similarly, DPP-4 also limits the activity of stromal cell derived factor-1α (SDF-1α) which promotes neovascularization by recruiting endothelial progenitor cells from bone marrow to the site of injury. Hence, inhibition of DPP-4 for cardioprotection is considerable. Several pre-clinical studies witnessed the cardioprotective effect of DPP-4 inhibition. For instance, in a rodent myocardial infarction model, DPP-4 inhibition has reduced the infarct size, and improved left ventricular ejection fraction in conjunction with neovascularization and overall survival. Similarly, in clinical studies, DPP4 inhibitor sitagliptin has shown cardioprotection by improving endothelial progenitor cell levels in diabetic patients, and also reduced blood pressure in hypertensive patients(91).

Upregulated plasma levels of DPP-4 correlate with heart failure outcomes in human and animal models(97). Previous studies revealed that DPP-4 inhibition protects the diabetic heart by improving ventricular remodelling and diastolic dysfunction through reducing fibrosis and oxidative stress(98). But in contrast, clinical outcomes trials showed slightly negative effects with saxagliptin, alogliptin, and sitagliptin, vildagliptin in SAVOR-TIMI, EXAMINE, TECOS, and VIVIDD trials respectively. However, the data has shown a large variation from no effect to a significant negative effect. Furthermore, recent in-vivo studies with prolonged DPP-4 inhibitor treatment demonstrated impaired cardiac function in a diabetic mouse model with transverse aortic constriction resulting from increased inflammation and fibrotic gene expression. Thus, augmented upregulation of inflammatory mediators could be a reason for increased negative outcomes of DPP-4 inhibitors(99–101).

iii. SGLT-2 inhibitors:

The EMPA-REG-OUTCOME trial was the first to document noticeably improved CV outcomes, including all-cause mortality in patients treated with Empagliflozin, a novel SGLT-2 inhibitor. In patients receiving empagliflozin, the researchers reported a substantial 14% decrease in the composite primary objective for CV mortality, non-fatal MI, and non-fatal stroke(102). Similarly, a CANVAS trial was performed with 10,142 T2DM patients at a high CV risk to investigate the overall CV safety and efficacy of canagliflozin (SGLT2i). Canagliflozin significantly decreased the rate of primary end events (comprised of CV death, non-fatal MI, and stroke) by 14% and HF hospitalization by 33%, in line with the findings from the EMPA-REG-OUTCOME study. Canagliflozin did not, however, significantly lower all-cause or CV mortality(103). Various possible mechanisms are hypothesized for CV benefit effects of SGLT2 inhibitors including weight loss through calorie loss, hemodynamic effects, natriuresis, glucosuria, blood pressure lowering osmotic diuresis, sodium hydrogen exchanger inhibition, and myocardial energy modulation(36).

b. Novel therapeutic strategies targeting Diabetic heart:

Cardiac tissue of T2DM animal models expressed upregulation of E3 ubiquitin ligase mitsugumin 3 which induces insulin resistance by the proteasomal degradation of both the insulin receptor and IRS-1. Additionally, transgenic mice showed an increase in fibrosis, indicating that inhibiting the E3 ubiquitin ligase mitsugumin 3 could be an all-encompassing possible therapeutic approach for the mitigation of diabetes cardiomyopathy(104).

FoxO1, a member of the Forkhead box-containing protein 1 (FoxO1) subfamily, also affects the IRS-1/Akt signalling pathway. Constant FoxO1 activation caused by metabolic stress leads to impaired Akt signalling and insulin resistance. In mice fed a high-fat diet, cardiac dysfunction was reversed and insulin responsiveness was preserved by

cardiomyocyte-specific deletion of FoxO1. FoxO1 could therefore be a viable target for future treatment strategies(105).

A characteristic attribute to HFpEF associated with T2DM is decreased chamber compliance, which is partially brought on by altered phosphorylation of the structural sarcomeric protein titin and an increase in the stiffness of cardiomyocytes as a result. A recent study reported that Neuregulin1 (NRG-1) boosts PKG and ERK1/2 activity and decreases PKC activity, which reversed the diabetes-related alterations in titin phosphorylation and was able to repair titin-based cardiomyocyte stiffness in diabetic mouse hearts(106).

Since, oxidative stress is the major contributor to DCM, novel antioxidant therapeutic strategies are always in need. A dietary isothiocyanate substrate called sulforaphane activates the transcription factor Nrf2, which controls the expression of several antioxidant proteins. Treatment with sulforaphane reduced the amount of ROS produced in the arterioles of diabetic mice and attenuated the remodelling and malfunction of the heart followed by a high-fat diet(107,108).

The effectiveness of coenzyme Q10 in lowering oxidative stress and pathological remodelling of the heart has been investigated in several studies undertaken over the last 40 years. Treatment with coenzyme Q10 reduces acting as a vasodilator, of systolic and diastolic blood pressure in diabetes patients. Additionally, coenzyme Q10 supplementation reduces heart fibrosis, hypertrophy, and inflammation in mouse models of T1DM and T2DM(109,110).

miRNA expression changes crucially in diabetic hearts, modulating many pathways. miRNAs are modulated in response to a variety of clinical stresses, such as inflammation, oxidative stress, hyperglycaemia, and high insulin levels. Unfortunately, glycaemic

management is unable to reverse hyperglycaemia-induced changes in miRNAs in the heart of streptozotocin-induced diabetic mice, suggesting that diabetic cardiomyopathy and the accompanying miRNA changes can persist even after blood glucose levels have returned to normal(111). miR-1, the most abundant miRNA in the heart, continues to rise with DCM progression. miR-1 primarily suppresses the expression of the anti-apoptotic and cardioprotective proteins Pim1 and Bcl-2(112). Surprisingly, anti-miR1 transfection in cardiomyocytes expresses prosurvival signals even after high glucose exposure. In contrast, other miRNAs, miR-133a, miR-30c, and miR-181a decrease in diabetes demonstrating upregulation in fibrosis, apoptosis, and hypertrophy. Interestingly, upregulation of these miRNAs attenuated diabetes-induced fibrosis, hypertrophy, and apoptosis(113). Similarly, expression of miR-146a is also diminished in hyperglycaemia, whereas cardio-specific higher expression abolishes cardiac remodelling and inflammation(114).

Moreover, miRNAs also can be considered biomarkers for cardiovascular disorders. Recent research found that miR-19b-3p and miR-181b-5p levels in the heart and blood were related to cardiac dysfunction during the development of diabetic cardiomyopathy in mice fed a high-fat diet, suggesting that these miRNAs could serve as biomarkers for the CV disease(115).

1.3. Development of SGLT Inhibitors:

In normal physiology, renal tubules reabsorb all filtered glucose, hence there will be no glucose in the urine.(116) Eighty to ninety percent of the filtered glucose is reabsorbed by SGLT2s in the early S1 segment of the proximal tubule, and ten to twenty percent of escaped glucose can be reabsorbed by SGLT1s in the S2/S3 segment(117). SGLT2s are also found in pancreatic α-cells and the cerebellum in addition to the renal proximal tubule, whereas

SGLT1s are primarily located in the intestine along with kidneys, colon, heart, lungs, and skeletal muscles(118).

The kidney can reabsorb blood glucose at 375 mg/dl. Meanwhile, the glomerulus filters glucose at a rate of 125 mg/min(119). Because of this, all filtered glucose is normally reabsorbed. However, filtered glucose is eliminated in the urine when plasma glucose concentration exceeds 180 mg/dL, called as renal “threshold for glycosuria". Glycosuria results from a lowering of the threshold for glycosuria, or a decrease in the capacity of the kidneys to reabsorb glucose(117). Even in patients with good glycaemic control, studies have revealed that adults with DM had raised levels of the renal threshold for glycosuria and greater renal glucose reabsorption capacity. When compared to individuals without diabetes, patients with DM have higher levels of SGLT2 expression in tubular epithelial cells, which is primarily responsible for this enhanced activity. This mechanism is the adaptability of the human body to stop energy loss, and it appears to play a role in the multifaceted aetiology of hyperglycaemia in people with diabetes(117,120).

The first naturally occurring SGLT inhibitor with high-affinity, selectivity, and competitive inhibitory activity for both SGLT1 and SGLT2 was phlorizin, which was extracted from the bark of apple trees in 1835. Phlorizin was initially used to treat fever, malaria, and other infectious disorders, however, it was soon found that phlorizin also causes glycosuria. Different analogues of phlorizin have been synthesized, each with varying potencies and selectivities against SGLT. Dapagliflozin, which was created in 2008, has a 1200 times greater affinity to inhibit SGLT2 than SGLT1. Another phlorizin derivative called Canagliflozin has 400-fold more potent anti-SGLT2 activity than anti-SGLT1 activity. Among the commercially available SGLT2i, Empagliflozin, the third agent in this class, displays the greatest selectivity for SGLT2 over SGLT1 (about 2700-fold). Ertugliflozin, the fourth phlorizin analogue synthesized, is 2200 times more selective for

SGLT2 than SGLT1. Dapa received FDA and EMA approval in 2012, Cana received FDA and EMA approval in 2013, and Empa received FDA and EMA approval in 2014. Ertu received FDA approval in 2017 and EMA approval in 2018.

1.3.1 SGLT-1/2 Inhibitors in Cardiovascular Outcome Trials:

After being used for many years as anti-diabetic medications, sodium-glucose cotransporter-2 (SGLT2) inhibitors achieved widespread recognition among cardiac therapeutics and were recently included in guidelines as a treatment of choice for heart failure with reduced ejection fraction (HFrEF)(121). Indeed, numerous trials on diabetic individuals on SGLT2 inhibitor treatment have consistently shown lower rates of unfavourable cardiovascular outcomes with these medications(103,122,123).

To determine the cardiovascular safety of these SGLT2 inhibitors, four specific double-blind, randomized, placebo-controlled trials were conducted. The first trial was the EMPA-REG OUTCOME (EMPAgliflozin Removal of Excess Glucose Cardiovascular OUTCOME Trial)(123), CANVAS (Canagliflozin Cardiovascular Assessment Study Program)(103), DECLARE-TIMI 58 (The Dapagliflozin Effect on Cardiovascular Events–Thrombolysis in Myocardial Infarction 58)(123), and VERTIS-CV (Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial)(124).

A benefit of empagliflozin on 3Point-Major Adverse Cardiovascular Events (3P-MACE) was apparent in EMPA-REG Outcomes including cardiovascular (CV) death, nonfatal myocardial infarction (MI), or nonfatal stroke. In this trial composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke was considered under primary outcome. The risk of hospitalization for unstable angina, death from cardiovascular causes, and the risk of myocardial infarction or stroke were under secondary outcomes. However, the primary outcome rate was substantially lower in the empagliflozin

group (10.5%) compared to the placebo group (12.1%). Whereas, 12.8% of patients receiving empagliflozin experienced the secondary outcome, compared to 14.3% of patients receiving a placebo. Additionally, empagliflozin markedly decreased all-cause mortality, cardiovascular mortality, hospitalization for heart failure, and hospitalization for heart failure or cardiovascular-related deaths (excluding fatal stroke)(102).

DECLARE-TIMI 58 was conducted with the largest sample size (n = 17,160), where 41% of participants had pre-existing CVDs, for the longest follow-up period of 4.2years. This trial is a secondary prevention trial because they already had a CVD at the time of recruitment. In these trials, 10 -24% of the participants already had heart failure. Interestingly, dapagliflozin was found non-inferior to placebo for the main safety outcome of MACE and resulted in a significantly lower rate of cardiovascular death or hospitalization for heart failure than placebo, with additional findings supporting a potentially lower rate of adverse renal outcomes. MACE, which stands for cardiovascular death, myocardial infarction, or ischemic stroke, was the primary safety outcome. MACE as well as a composite of cardiovascular death or hospitalization for heart failure were the two main efficacy outcomes. A new case of end-stage renal disease, death from renal or cardiovascular causes, or a persistent decline of 40% or more in estimated glomerular filtration rate (eGFR) was the first in the secondary outcomes , and Death from any cause was another secondary outcome(123).

10,142 people took part in the two CANVAS trials, 4330 in CANVAS and 5812 in CANVAS-R (R-Renal). 9630 out of a total of 9734 individuals completed the study. A similar significant 3P-MACE reduction was seen with CANVAS (canagliflozin). Additionally, heart failure hospitalizations were reduced with canagliflozin. In this trial composite of nonfatal myocardial infarction, nonfatal stroke, and death from cardiovascular causes made up the main primary outcome. Death from any cause, albuminuria progression,

and a composite of death from cardiovascular causes and hospitalization for heart failure were secondary outcomes planned for sequential conditional hypothesis testing. At the end point of the study, those randomized to canagliflozin experienced fewer adverse events than placebo (104.3 vs. 120.0). The difference in adverse events that led to cessation was not statistically significant (35.5 vs. 32.8 individuals per 1000 patients). With canagliflozin, there was an increased chance of lower extremity amputations (6.3 vs. 3.4 individuals with amputation per 1000 patients), with 71% of the afflicted people experiencing their highest amputation at the level of the toe or metatarsal. Patients with a history of amputation or peripheral vascular disease had the highest absolute risk of amputation, but the relative risk of amputation with canagliflozin as compared to placebo was similar across these subgroups(103).

The ertugliflozin component of VERTIS-CV failed to significantly improve 3P-MACE as compared to a placebo. Major adverse CV events (MACE), which included CV death, nonfatal myocardial infarction (MI), or nonfatal stroke, was the study's main endpoint. Secondary outcomes included the effect of ertugliflozin vs. placebo on time to (a) CV death or hospitalization for heart failure (HF); (b) CV death; and (c) a composite renal endpoint, which included renal death, end-stage kidney disease, or a twofold increase in blood creatinine. An equal number of individuals experienced the primary outcome in ertugliflozin (11.9%) vs placebo control (11.9%). Similar patterns were seen for all three MACE components, including CV mortality (1.8% with ertugliflozin vs. 1.9% with placebo), MI (1.7% with ertugliflozin vs. 1.6% with placebo), and stroke (0.8% with ertugliflozin vs. 0.8% with placebo). Although there was a trend toward slight improvement in the ertugliflozin group, the medication did not significantly lower the incidence of the renal composite compared to the placebo (3.2% vs 3.9%, respectively)(124,125).

Together, the CVOTs with SGLT2i showed not only cardiovascular safety,

and benefit in a few cases (empagliflozin, canagliflozin), but also showed a notable decrease in heart failure hospitalization with all four medications. A specialized trial with dapagliflozin was then carried out in individuals with heart failure with reduced ejection fraction. Regardless of the presence or absence of T2DM, Dapagliflozin showed its benefits in patients with congestive heart failure, leading to a notable decrease in heart failure hospitalizations and cardiovascular mortality. According to a meta-analysis of 46,969 individuals who participated in clinical outcome trials, there are consistent cardioprotective benefits throughout this SGLT-2i class of drugs. There was no discernible variability in the association between treatment with SGLT2 inhibitors and a lower incidence of MACE. Additionally, the risk decrease for heart failure hospitalization was consistent across trials, but there was a large amount of variation among trials for cardiovascular death(126).

1.3.2. Possible Cardiovascular Safety Mechanisms of SGLT-1/2 Inhibitors

a. Calorie loss / Reduction in Body Weight:

Obesity is a standalone risk factor for cardiovascular events. An increase in glucose elimination in the urine is caused by SGLT2 inhibition. Approximately, 400 mL per day of diuresis, eliminates 75 gr of glucose per day (or roughly 300 kcal per day) in the urine. According to clinical trial findings conducted thus far, these medications cause a total weight loss of 2- 3 kg (127). A meta-analysis of SGLT2i found that Cana 300 mg results in a larger weight loss (0.89 kg) when compared to Dapa 5 mg, however, Cana 100 mg doesn't appear to vary from other SGLT2i(128).

b. Reduction in blood pressure:

Reduction in arterial blood pressure and associated cardiac benefits in DM are well reported. Empagliflozin was successful in lowering both systolic and diastolic blood pressure in the EMPAREG-OUTCOME study without raising the heart rate. Various studies confirmed

these findings, and two meta-analyses proved that SGLT2i has a positive impact on blood pressure. More specifically, SGLT2i lower systolic and diastolic blood pressure by 2.46 mmHg and 1.46 mmHg, respectively. They also lower 24-hour ambulatory systolic and diastolic blood pressure by 3.76 mmHg and 1.83 mmHg, respectively. For this impact, several underlying pathophysiologic processes have been put forth. The first impact is a slight increase in natriuresis caused by the inhibition of SGLTs in the proximal tubule, while an additional osmotic diuretic effect is caused by increased glucose excretion per se. Second, it has been suggested that losing weight and reducing sympathetic nervous activity contributes to lower blood pressure. Even though all SGLT2i lower blood pressure, indirect results from a meta-analysis showed that Cana 300 mg caused a higher reduction in systolic blood pressure compared to other SGLT2i, while no differences were identified for diastolic blood pressure across other SGLT2i. However, SGLT2i has proven more successful and faster at reducing CV events, particularly HF than even BP-lowering medications(129,130).

c. Positive Renal outcomes:

Chronic kidney disease (CKD) raises CV risk in both DM patients and non-DM patients. The renal outcomes in each of the three CV trials for Empa, Cana, and Dapa were impressive. A sub-analysis of the EMPA-REG OUTCOME trial revealed that therapy with Empa prevented the onset or development of nephropathy in patients with existing CV disease(131). The evaluation of the effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants with Diabetic Nephropathy (CREDENCE) study revealed Cana administration reduced, the number of patients who died from renal or cardiovascular causes, and end-stage renal disease(132). These benefits are associated with SGLT2 inhibition which prevents sodium and glucose from being reabsorbed from the tubule, thus more sodium is supplied to the macula densa, which results in dilated afferent arterioles, lower intraglomerular pressure, and lessened hyperfiltration. Diuresis, which causes a

contraction in plasma volume, causes a loss of about 7% of plasma volume(133). Moreover, by utilizing ketones rather than free fatty acids, SGLT2i increases renal oxygen demand. The connection between SGLT2 and the sodium-hydrogen exchanger (NHE) or enhanced erythropoietin and haematocrit as a result of lower renal cortical oxygen tension has also been hypothesized as additional pathways(134). Therefore, the improvement in renal function may be a factor in the decline in CV events in DM patients.

d. Improved Lipid profile:

The frequent comorbidity of T2DM that raises CV morbidity and death is dyslipidaemia. LDL-cholesterol (LDL-C) has more tendency to stick to the inner wall of blood vessels, which leads to atheroma formation and stroke. Whereas, HDL-cholesterol (HDL-C) in contrast helps to clear the LDL-C by reverting it to the liver. However, recent research reports that there is no direct link between LDL-C and CV risk (135). Anyhow, the EMPA-REG OUTCOME experiment and the CANVAS program demonstrated that taking Empa or Cana raised both LDL-C and high-density lipoprotein cholesterol HDL-C in comparison to taking a placebo. These findings were supported by a meta-analysis of Empa studies, which showed a rise in LDL-C in the group receiving Empa. The treatment of SGLT2i (Dapa, Cana, Empa) enhanced HDL-C, LDL-C, and lowered serum triglycerides, according to a meta-analysis of 34 RCTs. The highest effects of serum lipids were linked to Cana. Similar findings from a recent meta-analysis were shown. Studies with SGLT2i have revealed alterations in lipoprotein sub-fractions aside from the quantitative impact of SGLT2i on blood lipids(136,137). According to a Japanese study, Dapa treatment reduced atherogenic small dense LDL-C while raising HDL-C, a healthy cardiometabolic marker(138). In conclusion, SGLT2i treatment is related to a modest increase in LDL-C and HDL-C levels and a modest decrease in triglyceride and small dense LDL levels.

e. Arterial Stiffness and Endothelial Function:

The endothelium is a thin layer of cells that covers the inner blood vessel wall. Nitric oxide (NO), which is produced by the endothelial cell (EC) enzyme calcium-calmodulin-dependent nitric oxide synthase is one of the main modulators of vascular tone. NO aids in maintaining vascular tone, preventing platelet aggregation and monocyte adherence to the endothelium, and inhibits excessive vascular smooth muscle cell proliferation. In contrast, endothelial dysfunction leads to reduced NO production, oxidative stress, and endothelial senescence. As a result, leukocyte adhesion and migration are increased, vasoconstrictor tone is elevated, and endothelium-dependent vasodilation is hindered. This endothelial dysfunction leads to the development of atherosclerosis and numerous macro and microvascular complications (139).

Arterial stiffness is a known risk factor for heart failure. Mechanistically, increased arterial stiffness worsens pump failure by placing a heavier load on the ventricles. Atherosclerotic arterial stiffness is decreased by cardio-protective drugs such as statins and angiotensin-converting enzyme inhibitors. Additionally, SGLT-2 inhibitor therapy significantly reduces arterial stiffness. Reduced total body sodium may be inferred, even if the underlying mechanisms for this finding are not well understood. Sodium overload contributes to endothelial glycocalyx destruction in heart failure (HF), which reduces the bioavailability of nitric oxide for the relaxation of vascular smooth muscle cells. It is evident from previous studies that dapagliflozin therapy lowers C-reactive protein expression in diabetic patients and provides positive effects of SGLT2 inhibitors on vascular health. Besides, SGLT-2 inhibition causes a decrease in plasma uric acid levels, which has also been linked to endothelial function in observational studies (140).

The "gold-standard" approach for measuring arterial stiffness and for independently

predicting CV disease morbidity and death is pulse wave velocity. In 16 individuals with T2DM, one study found that the pulse wave velocity decreased after 48 hours of Dapa treatment (10.1 1.6 to 8.9 1.6 m/s, p 0.05). A single study, however, cannot be used to draw any firm conclusions. Additionally, the same study by Solini et al. showed that Dapa administration had a positive impact on endothelial function as measured by the flow-mediated dilation method(141). Since established endothelial dysfunction-causing factors including hyperglycaemia and sympathetic stimulation were not found to alter endothelial function, researchers hypothesized that Dapa might directly affect the vascular endothelium. Further, a 6-month-long trial revealed that the treatment of Dapa enhanced the reactive hyperemia index, another indicator of endothelial function(142).

f. Modified Ventricular Preload and Afterload:

Preload is the pressure experienced by the ventricles as they fill with blood during diastole, whereas afterload is the pressure the heart must overcome to expel blood during systole. Fluctuations in preload and afterload directly impact the cardiac output as seen in heart failure. Low preload can be observed during hypotension, low fluid volume, and cardiac tamponade. Whereas, arrhythmias and heart failure induce elevation of preload. Similarly, afterload, which is essentially the vascular resistance found in the lungs and aorta, can fluctuate. During SNS stimulation, aortic stenosis, and HTN, afterload is often high. Vasodilators may be helpful in that situation. A low afterload can be seen in hypotension or septic shock.

Since SGLT-2 inhibition causes natriuresis along with glycosuria leading to a decrease in preload volume, this osmotic diuresis improves the left ventricle's Franklin-Starling curve in T2DM patients. As mentioned, numerous studies have demonstrated that this new class of anti-hyperglycaemic medications reduces blood pressure, arterial stiffness,

and ventricular resistance(143). These effects may help patients with diabetes mellitus (DM) have better cardiac function by affecting the afterload, the second most vulnerable component of cardiac output.

g. Regulation of circulating Adipokine levels:

By endocrine and paracrine actions, adipokines secreted by perivascular and epicardial adipose tissue have been linked to the onset of HF. It has been shown that some adipokines, like leptin, encourage myocardial inflammation while others, like adiponectin, have anti-inflammatory and cardioprotective properties. It has been suggested that using SGLT2i will balance pro- and anti-inflammatory adipokines and end myocardial dysfunction. A recent study reported that Cana caused a 25% decrease in serum leptin levels and a 17% increase in serum adiponectin compared to an oral hypoglycaemic agent of sulphonylureas, glimepiride (144). Another study also demonstrated that Dapa decreases ectopic epicardial fat, which is essential for the development of HF(145). Recent studies revealed that phloretin, a breakdown product of phlorizin, directly and dose-dependently suppressed leptin release in adipocytes. However, the exact mechanism of SGLTi on adipokine levels needs further investigation (146).

h. Suppressed Cardiac fibrosis:

A crucial component of cardiac remodelling that results in HF is myocardial fibrosis. Extracellular matrix proteins are secreted in the heart by abnormally activated fibroblasts, which alter the ventricular function and cause contractile dysfunction(147). In post-myocardial infarction rat models, Lee et al. demonstrated that the injection of Dapa significantly reduces collagen synthesis by activating M2 macrophages and suppressing myofibroblast development. Additionally, it's reported that Empa inhibits the activation of fibroblasts caused by TFG-β1 and attenuates pro-fibrotic markers such as type I collagen,

α-smooth muscle actin, connective tissue growth factor, and matrix metalloproteinase2(148). From available data, it's evident that SGLT2i has positive effects on cardiac fibroblasts, one of the most important HF risk factors.

i. Modulating inflammatory cell infiltration:

The primary cell type involved in diabetic cardiovascular disease is the macrophage, and the cytokines that these cells secrete can alter the milieu where the disease develops. There is ample evidence that the IL-1 family, IL-6, IL-17, IL-18, IL-32, TNF-α, and C-reactive protein (CRP) are the main inflammatory cytokines involved in this process. Additionally, adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) are crucial in inflammation. It's well documented that SGLT-2 inhibitors are effective in neutralizing these inflammatory markers(149).

j. Effect on Adhesion molecules:

Adhesion molecules play an important role during inflammatory cell infiltration to the target tissue. ICAM-1 and VCAM-1 level in cardiomyocytes isolated from HFpEF human heart and obese rat models were both dramatically reduced by empagliflozin treatment(150). Additionally, VCAM-1-related vascular endothelial dysfunction caused by excessive hyperglycemia was similarly protected by empagliflozin(151). A similar finding from another study indicated that empagliflozin therapy can improve endothelial dysfunction in an obese rat model by lowering VCAM-1. Additionally, it is reported that Canagliflozin treatment for 12 weeks reduced atherosclerotic lesions in diabetic ApoE KO mice, while treatment for 8 weeks significantly reduced endothelial dysfunction, probably by blunting ICAM-1 and VCAM-1(152). Another crucial adhesion molecule in the development of diabetic cardiovascular disease is PECAM-1, which mediates Immunocyte

trafficking and is correlated with vascular integrity. It is reported that luseogliflozin can downregulate PECAM-1 and ICAM-1 in STZ-treated ApoE KO mice(153).

k. Effect on Cardiac hypertrophy:

Cardiac hypertrophy is a root cause of heart failure with preserved ejection fraction (HFpEF) as well as heart failure with reduced ejection fraction (HFrEF) in diabetic patients. Left ventricular hypertrophy is significant in HFpEF with a concentric left ventricular geometry, substantial diastolic dysfunction with elevated ventricular filling pressure, and dilatation of the left atrium. Interestingly, Dapagliflozin treatment for 12 months has reduced the left ventricular mass by -3.95± 4.85 gm vs. placebo group –1.13 ± 4.55 gm, resulting in a -2.82 gm absolute mean difference in a DAPA-LVH trial demonstrated by cardiac magnetic resonance imaging. Similarly, EMPA-HEART Cardiolink-6 trial also reported that empagliflozin treatment for 6 months has reduced the left ventricular mass in type-2 diabetic patients assessed by cardiac magnetic resonance imaging. These anti-hypertrophic effects of dapagliflozin and empagliflozin are attributed to their hemodynamic and blood pressure-reducing effects which inturn reduce ventricular filling pressure and after load. Besides, hemodynamic effects SGLT inhibitors also contribute to glucosuria and calorie loss which inturn reduces adipose tissue. Since, adipose tissue located in the viscera (VAT), subcutaneous (SCAT), and epicardial regions contribute to the release of pro-inflammatory and pro-oxidant substances, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) have been demonstrated to support transcriptional programs that induce LVH. It's interesting to note that in the DAPA-LVH trial, dapagliflozin treatment remarkably reduced both VAT and SCAT, with a substantial reduction in left ventricular mass (154,155).

l. Effect on Cardiac apoptosis:

Numerous cardiovascular disorders, such as atherosclerosis, myocardial infarction, and ischemic heart disease, have been linked to cardiac apoptosis. One of the key events leading to the onset of diabetic cardiomyopathy is accelerated cardiac apoptotic cell death. The diabetic heart is attributed to 85-fold increased myocyte apoptosis, and 4-fold increased necrosis compared to a normal heart. Hyperglycaemia is proven to be a major risk factor for cardiac apoptosis. Interestingly, SGLT-2 inhibitors have shown a significant reduction in cardiac apoptosis along with other hypoglycaemic treatments such as insulin, and metformin. Recent research has reported that specific SGLT-1 inhibition with mizagliflozin also significantly reduced apoptosis in a dose-dependent manner in diabetic cardiomyopathy(74,156,157).

1.3.3. Undesirable effects of SGLT inhibitors:

a. Keto acidosis:

Despite the benefits for metabolic health and HF hospitalization, some limitations are noticed in SGLT2 inhibitor safety. Across numerous trials, the risks of hypoglycaemia, fluid depletion, acute renal damage, thromboembolic events, and fractures were comparable in the SGLT2 inhibitor and placebo groups. Conversely, SGLT2 inhibitors induced little increase in the incidence of diabetic ketoacidosis compared to placebo which was evidenced by FDA Adverse Event Reporting System. With off-label usage in type 1 diabetes mellitus, the risk was significantly higher. Numerous factors, such as a reduction in insulin dosage due to concerns about hypoglycemia while taking SGLT2 inhibitors, an increase in ketone body production, a rise in renal reabsorption of ketone bodies from urine, and a reduction in insulin secretion by directly inhibiting alpha cells with SGLT2 all have the potential to raise the risk. The FDA has issued a warning regarding the use of SGLT2 inhibitors and instructions to take into account risk factors before beginning SGLT2 inhibitors, carefully

assess for ketoacidosis with euglycemia in the presence of ketoacidosis symptoms.

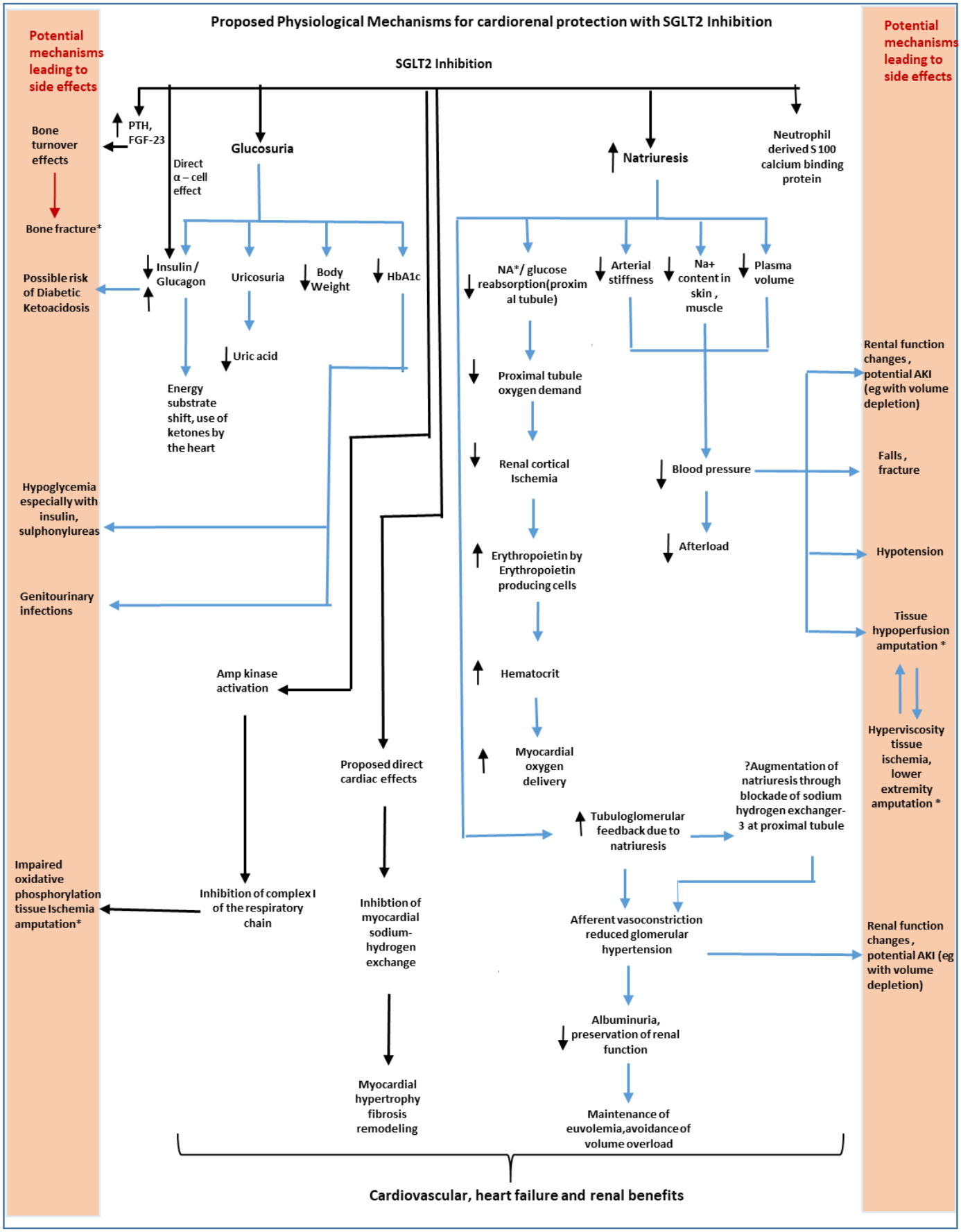
b. Urinary Tract infections:

SGLT2 inhibitor use is associated with an increased risk of genital infections, such as balanitis and phimosis, compared to placebo. However, the risk of severe infections necessitating drug withdrawal was minimal in the DECLARE TIMI-58 experiment. The elimination of glucose in urine creates an ideal environment for the growth and infection of bacteria and fungi. Comparing the empagliflozin group to the placebo group, urosepsis was more common. However, the rates of pyelonephritis, severe urinary tract infections, as well as urinary tract infections were comparable in both research groups(158).

c. Amputations:

The canagliflozin group experienced an increase in toe, foot, and limb amputations, according to the CANVAS program. Patients having an underlying diagnosis of peripheral vascular disease or a prior history of amputations had a greater risk of amputation than patients without these symptoms. Similar trends for low-trauma fractures were seen in the canagliflozin group, where fracture risks were greater than in the placebo group. In the EMPA-REG study, subgroup analysis for amputations did not reveal an elevated risk between the treatment and placebo groups. Moreover, there was no change in the rate of limb amputations between the therapy and placebo groups in the DECLARE-TIMI or DAPA-HF studies. It's unclear exactly how these negative events happen. Dehydration brought on by diarrhoea (perhaps due to SGLT1 suppression in the gut) may be a contributing factor. Canagliflozin use was found to be related with a greater risk of amputations than empagliflozin or dapagliflozin use in an analysis of amputations in SGLT2 inhibitor users from the FDA Adverse Event Reporting System(159). The FDA has issued a black box warning for increased amputation risk with canagliflozin use without any

particular prescription recommendation, even though a link between drug use and amputation cannot be proven(160).



Possible mechanisms for cardio-renal benefits and adverse effects of SGLT-2i(161)

Chapter 2: Research hypothesis and Objectives

- We hypothesize that SGLT-1 inhibition mitigates diabetic cardiomyopathy in in-vitro and in-vivo diabetic cardiomyopathy models.
- The principle objective of our study is to investigate the role of SGLT-1 in the structural and functional remodelling of myocardium in set of T2DM.

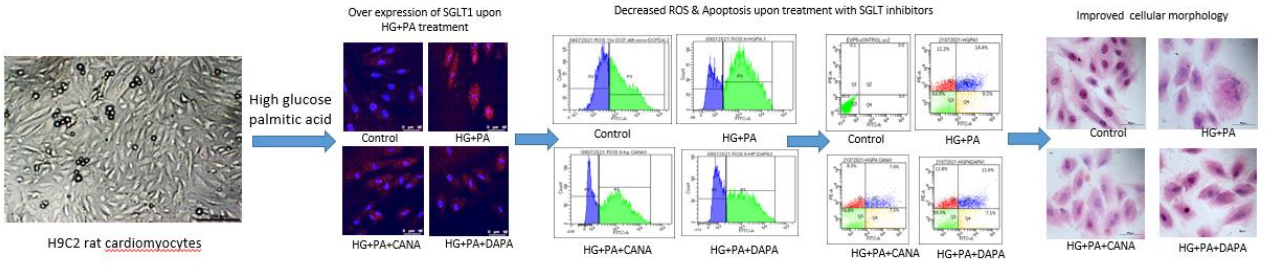
i. To investigate the concentration and time-dependent expression of SGLT1 in *in-vitro* cultured cardiomyocytes.

ii. To correlate the pathogenic process of diabetic cardiomyopathy *in-vitro* in cultured cardiomyocytes and *in-vivo* Wistar rats after exposure to high fat and high glucose.

iii. To investigate the mechanism, and role of SGLT1 in diabetic cardiomyopathy.

iv. To investigate the potential of novel SGLT1 inhibitors in diabetic cardiomyopathy.

Chapter 3: Canagliflozin and Dapagliflozin attenuate glucolipotoxicity-induced oxidative stress and apoptosis in cardiomyocytes via inhibition of Sodium-glucose Cotransporter-1



3.1. Introduction:

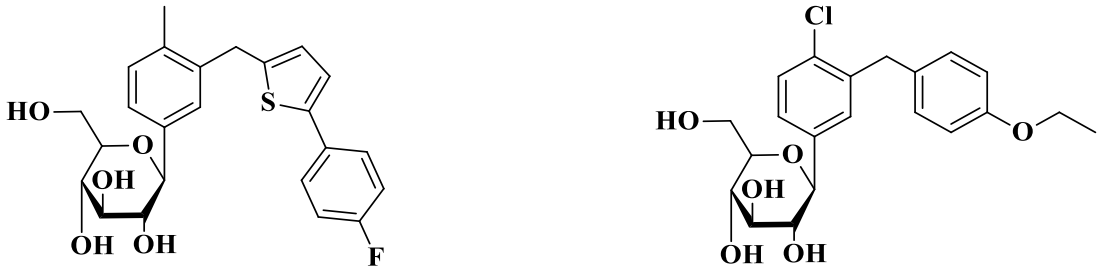
Diabetes and associated cardiovascular (CV) complications are health problems of epidemic proportions worldwide. Diabetic individuals manifest a two to threefold greater risk of CV events compared with counterparts without diabetes(162–165). In the majority of the cases of diabetes and heart diseases, the defects lie in glucose and energy homeostasis(166). Sodium-glucose co-transporter 1 (SGLT1) is a member of the sodium-dependent glucose co-transporter protein family, joined by SGLT-2, 3, 4, and 5 respectively (118,167). The two most well-known members of the SGLT family are SGLT1 and SGLT2, which are members of the SLC5A gene family(118,167). Lately, considerable attention has been focused on SGLT2, which is responsible for most of the glucose reabsorption in the kidney.

Likewise, SGLT1 expression has been reported in the heart of many species, and increased expression of SGLT1 is reported in diabetes mellitus and myocardial ischemia(168,169). SGLT1 transporter plays a key role in the translocation of sugar across epithelial cells in the small intestine and the renal proximal tubule (170,171). Apart from the intestine, a notable SGLT-1 expression has been detected in liver, lung tissues, and a lesser expression in the trachea and bronchi(170). Recent studies have reported expression of SGLT1 in the human heart mainly localized to the sarcolemma of the cardiac myocyte as well as heart capillaries and increased expression of SGLT1 is reported under diabetic conditions(172,173). It has also been reported recently that chronic pressure overload induces cardiac hypertrophy and fibrosis in mice via increased SGLT1 and interleukin-18 gene expression. Chronic pressure overload also increased the cardiac gene expression of atrial natriuretic peptide, B-type natriuretic peptide, and collagen type 1(174). Subsequently, overexpression of SGLT-2 has been observed in prostate, pancreas, and brain tumors, leaving SGLT-2 inhibitors as possible therapeutic agents to treat these cancers (175). SGLT2 inhibitors are the recently approved drugs, where canagliflozin (Invocana) being the first approved drug in 2013 for the treatment of diabetes mellitus. SGLT2 inhibitors function by increasing urinary excretion of glucose and have very less risk of induction of hypoglycaemia (176).

Some of the recent studies have reported the cardioprotective effect of SGLT2 inhibitors in preclinical as well as clinical studies (177,178). Canagliflozin (CANA) and Dapagliflozin (DAPA), two recently approved SGLT2 inhibitors for the treatment of type 2 diabetes have shown beneficial effects on the heart and reduction in the occurrence of CVD in patients in clinical trials, suggesting the cardiovascular-protective effect of SGLT2 inhibitors (161,179). Recently Lim et al had have reported Canagliflozin (CANA), attenuates myocardial infarction in the Zucker diabetic fatty rat and nondiabetic Zucker lean rat by upregulation of cardiac pro-survival pathway (180). Dapagliflozin (DAPA) is

reported to reduce intracellular calcium overload thereby reducing ROS production in the diabetic model of cardiomyopathy(181). However, to the best of our knowledge, the direct effect of CANA and DAPA in cultured cardiomyocytes which predominantly express SGLT1, and the comparison between the effects of two SGLT2 inhibitors have not been explored till now. The present study aimed to investigate the effect of CANA and DAPA in cultured cardiomyocytes under glucolipotoxic conditions and the underlying molecular mechanism.

Chemical structure of Canagliflozin (a) and Dapagliflozin (b)



(a) **(b)**

3.2. Materials and methods

3.2.1. Chemicals

Canagliflozin and Dapagliflozin were purchased from Sigma Aldrich (Sigma Aldrich, MO, USA). Primary antibody for SGLT1 was procured from Novus Biologicals, CO, USA while caspase-3 and B-actin and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DMEM (AL219A), BSA (TC348), trypsin (TCL007), MTT (TC191) were purchased from Himedia. FBS (10270-106) was purchased

from Gibco. Sodium Palmitate (P9767), 2′,7′-Dichlorofluorescin diacetate (D6883), 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Aldrich. TB Green® Premix Ex Taq™ II (RR820A), PrimeScript™ RT reagent (RR037A), RNAiso plus (9109) were purchased from Takara. 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (N13195) was purchased from (Invitrogen) Thermo Fischer Scientific, USA. TACS Annexin V-FITC Apoptosis Detection Kit (4830-250-K) was purchased from R&D Systems, NAC (N-acetyl cysteine) (A9165).

3.2.2. Cell culture

H9C2 cells (rat cardiomyocyte cell line) were obtained from NCCS, Pune, India, and were cultured on T25 flasks and coverslips using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin antibiotic solution. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. In vitro studies were done in rat H9C2 cardiomyocytes, glucolipotoxicity (GLP) was induced by exposing the cardiomyocytes with high glucose (25 mM) and palmitic acid (500 µM) in the presence or absence of Canagliflozin and Dapagliflozin (180). The concentrations of glucose and palmitic acid used were based on our previous studies with the same molecules (181–183).

3.2.3. Preparation of Sodium Palmitate solution by Saponification and Complexation with 2%BSA.

Palmitic acid (PA) was dissolved in a small volume of ethanol, then saponification was carried out to convert it into sodium salt of palmitate solution by addition of 10 mM NaOH. Then Sodium Palmitate solution was conjugated with 2% BSA in incomplete DMEM media in a ratio of 2:1 at 55º C in a shaking water bath for overnight incubation. Then Palmitate solution was stored at -20 C for further use (182).

3.2.4. Treatment

Cultured H9C2 cardiomyocytes were treated with Palmitate (500 µM) and High glucose (25 mM) and SGLT2 inhibitors, Canagliflozin (CANA), and Dapagliflozin (10 µM) for 0 h, 3 h, 6 h, 12 h, and 24 h respectively. The selected concentrations used are based on our previous studies with sample molecules and MTT assay (182,183).

3.2.5. MTT Assay:

H9C2 cells grown in complete DMEM were trypsinized and 1×10^6 cells were seeded in a 96-well plate. After 24 hrs, these cells were fasted with 1% FBS overnight and treated with high glucose (25 Mm), sodium palmitate (500 µM) with or without 10 µM canagliflozin, and Dapagliflozin for 24 hrs in 2% FBS media. After the treatment schedule, the supernatant was removed and cells were incubated with 100 µl of MTT (0.5 mg/ml) solution in PBS for 4hrs. The resultant formazan crystals were solubilized in DMSO and absorbance was read at 570 nM against blank (184).

3.2.6. Immunofluorescence and confocal microscopy

Briefly, H9C2 cardiomyocytes were grown in confocal dishes, after treatment cultured cells were fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.1% Triton X-100 for 5 min, then followed by blocking with 3% bovine serum albumin (BSA) for 1 hr. After that cells were incubated overnight with primary antibody 1:200 dilution in PBST (SGLT1) at 4^{o}c followed by incubation with Texas red conjugated secondary antibody 1:500 dilution in PBST for 1h at room temperature and counterstained with 6-diamidino-2-phenylindole (DAPI). Cells were observed under confocal laser-scanning microscopy (Leica DMi8 confocal microscope, Germany) (182).

3.2.7. Measurement of Reactive Oxygen Species (ROS)

Accumulation of intracellular peroxynitrite, hydrogen peroxide, and free radicals were determined by flow cytometry. Briefly, after treatment cells were incubated with non-fluorescent probe 2,7 '- dichlorofluorescein diacetate (CM-H2DCFDA) (5 µmol/l) and kept in the incubator for 30 minutes, maintained at 37 ° C. The cells were trypsinized, washed twice with PBS, and resuspended in PBS. The fluorescent intensity of DCF was measured using a flow cytometer ((BD FACSAria™ III) (182).

3.2.8. Measurement of mRNA expression of Catalase:

Briefly, H9C2 cells were cultured in a 6-well plate and after reaching sub-confluency, fasted for overnight, and treatments were given. After the treatment schedule RNA was isolated and converted to cDNA according to the manufacturer's protocol. A real-time polymerase chain reaction was carried out as mentioned previously(182). The forward and reverse primer sequences were. Catalase Forward: CATGGATCTGCTTAGGACTTCTG, Catalase Reverse: CCAGGCTGTGAGGTAACATAA.

3.2.9. Annexin V/fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining

Annexin V and PI double staining was carried out to differentiate the normal cells from apoptotic cells. Cultured H9C2 cells were incubated alone or in combination with different treatment groups for 24 hr, cells were trypsinized and harvested under cool conditions. Pellet was resuspended in 95µl of annexin binding buffer. 5 µl of FITC annexin V and 10 µl of the 100 µg/ml PI working solution were added to each 95 µl of cell suspension and incubated them at room temperature for 30 minutes. The final sample mixtures were immediately transferred onto the ice. Apoptotic cell percentage was analyzed by using BD imaging flow cytometer ((BD FACSAria™ III)(182).

3.2.10. Glucose uptake assay

Briefly, treated H9C2 cardiomyocytes were incubated with 100 µM 2-NBDG for 1hr and stimulated with or without 100 nm insulin for 10 min. Further cells were washed with PBS and the glucose uptake was measured by the relative fluorescent intensity of live cell images captured by fluorescent microscope (Leica DMi8 Germany) using 488nm laser(185).

3.2.11. H and E staining

After the treatment schedule, cells were fixed with ice-cold methanol for 10 min and stained with mayers hematoxylin for 5 min followed by a quick acid alcohol wash to remove excess stain and a PBS wash, as a bluing step, then stained with eosin for 30 sec. Later cells were washed with 2 changes of 95% alcohol and observed for changes in the shape of the nucleus and cytoplasm under a microscope (182,183).

3.2.12. Crystal violet staining

Treated cardiomyocytes were fixed with ice-cold methanol for 10 min and stained with 0.2% crystal violet solution (in 20-25% methanol) for 10 min. Further, cells were washed with 4-5 changes of double-distilled H_2O and dried. Images were captured using OLYMPUS IX53 microscope(186).

3.2.13. Statistical Analysis:

Data obtained from separate experiments are expressed as mean ± SEM. Statistical analysis was performed using ANOVA with post hoc Bonferroni's test. A *P* value of less than 0.05 was considered to be statistically significant.

3.3. Results:

Glucolipotoxicity associated with diabetes is one of the leading causes of CV complications. We have also reported previously high glucose as well as high lipid-mediated oxidative

stress and apoptosis under *in vitro* as well as *in vivo* conditions (182,183). SGLT1 is the main sodium-dependent glucose transporter expressed in the heart. Increased expression of SGLT1 has been reported in various CVDs (169,172,173). Although the therapeutic efficacy of SGLT2 inhibitors as antidiabetic agents is well established (187,188), very limited information is available about their cardioprotective effect and the possible molecular mechanism. In the present study, the effect of glucolipotoxicity *in vitro* in cultured H9C2 cardiomyocytes was investigated using two SGLT2 inhibitors, CANA and DAPA.

3.3.1. Concentration and time-dependent effect of CANA and DAPA on cell viability in cultured H9C2 cardiomyocytes

To investigate the effect of CANA and DAPA on the viability of cardiomyocytes, H9C2 cardiomyocytes were treated with different concentrations of CANA and DAPA. At a 10 µM concentration of CANA and DAPA, 80% of the cells were viable. However, at 30 µM, only 55% of the cells were viable with CANA and 75% of cells were viable with DAPA. Since 10 µM concentration was clinically relevant, as peak plasma concentration of canagliflozin and dapagliflozin is ≈ 1-10 µM after administration in patients as well as in healthy individuals(180). We chose, 10 µM concentrations of CANA and DAPA for all other experiments.

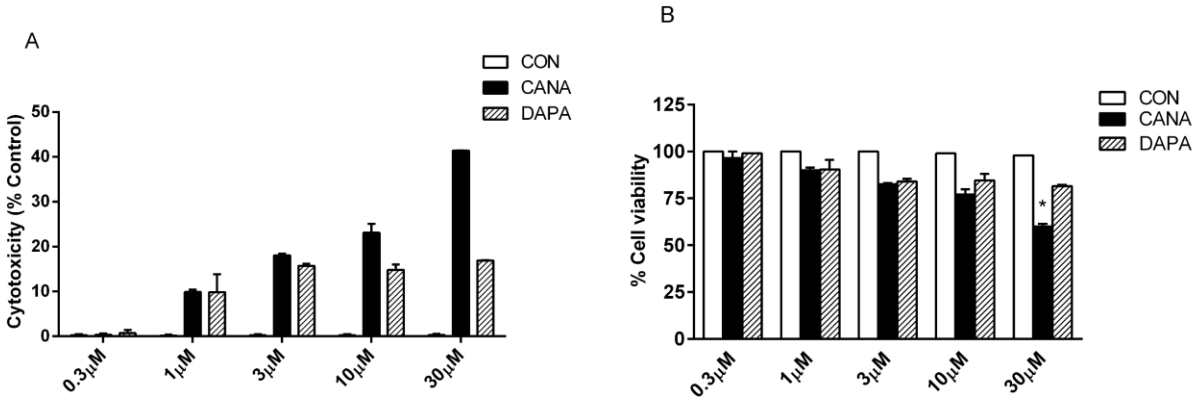


Figure 3. 1 : Concentration-dependent effect of CANA and DAPA on the viability of cultured cardiomyocytes

Fig.3.1. Cultured rat cardiomyocyte H9C2 cells were incubated with normal culture medium (control, Con) or medium containing high glucose (25 mM) for 24 h. CANA (0.3, 1, 3, 10, 30 µM) and DAPA (0.3, 1, 3, 10, 30 µM) were incubated alone or with HG for 24 h. Cytotoxicity (A) and cell viability (B) were measured by the MTT assay kit. n=5 for each treatment. *P<0.05 *vs*. respective control (Con) group.

3.3.2. Time-dependent effect of glucotoxicity on SGLT1 expression in H9C2 cultured cardiomyocytes

Glucolipotoxicity induces SGLT1 expression in cultured rat H9C2 cardiomyocytes: We checked whether glucolipotoxicity was able to activate SGLT1 in rat H9C2 cardiomyocytes. H9C2 cells were incubated with high glucose (25 mM) and palmitic acid (500 µM) for 3 h, 6 h, 12 h, and 24 h. The group which received high glucose and palmitic acid together showed a significant increase in SGLT1 expression compared to the control as well as high glucose and palmitic acid alone group (A, B). Moreover, when the cultured H9C2 cells were co-incubated with either CANA or DAPA in the presence of high glucose and palmitic acid, there was a significant decrease in SGLT1 expression (A, B). SGLT2 expression was quantified in cultured cardiomyocytes after incubation with high glucose (25 mM) and palmitic acid (500 µM) for different time points. However, no SGLT2 expression was detected in control in control as well as cells incubated with high glucose and palmitic acid

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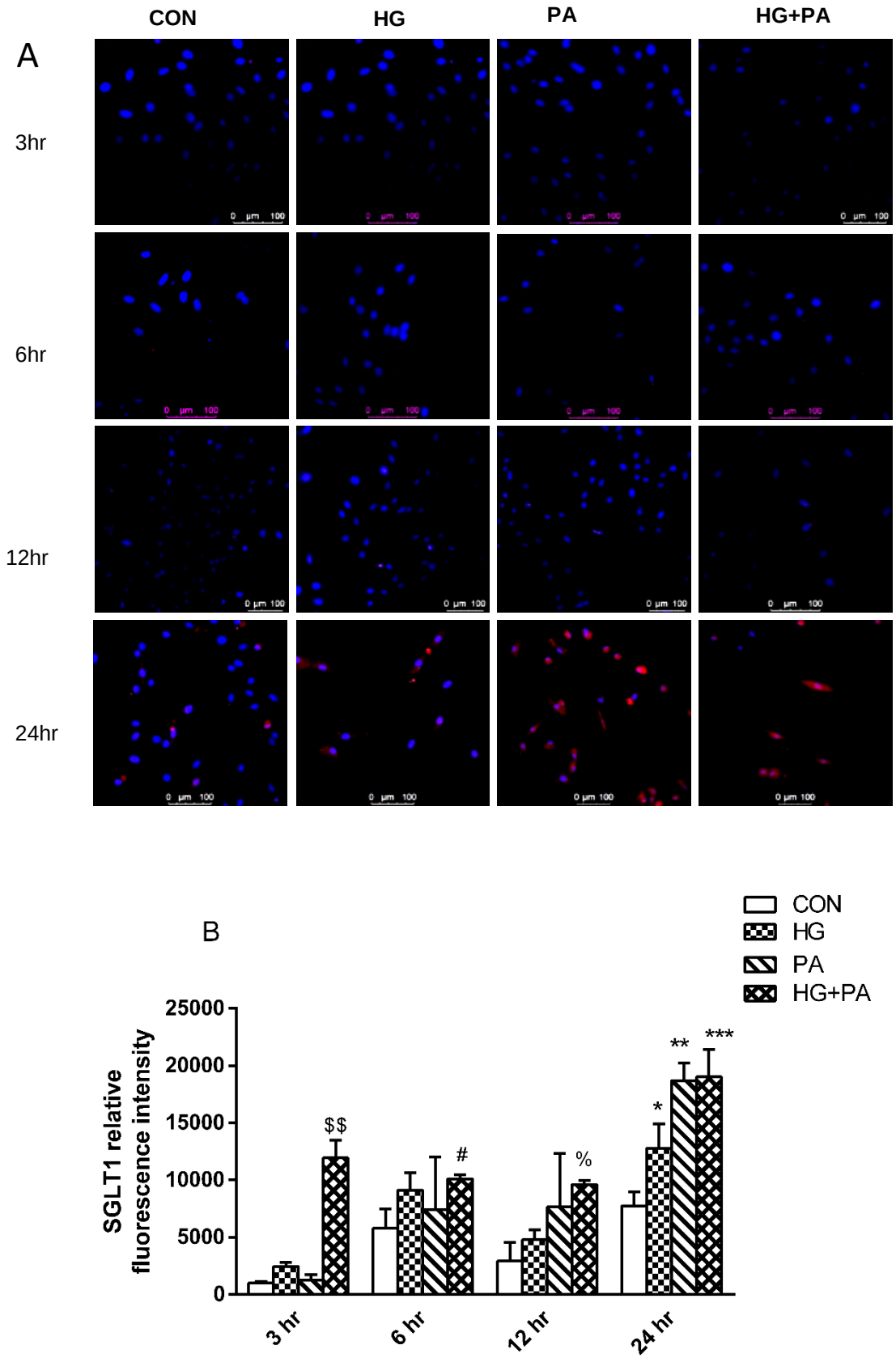


Figure 3. 2 Time-dependent expression of SGLT1 in cultured H9C2 cardiomyocytes

Fig 3.2: Time-dependent expression of SGLT1 in cultured H9C2 cardiomyocytes: Cultured rat H9C2 cardiomyocyte cells were incubated with normal culture medium (control, Con) or medium containing HG (25 mM) or Palmitic acid (500 µM) (A) for 3 h, 6 h, 12 h, and 24 h. SGLT1 expression was measured by immunofluorescence staining. n=5 for each treatment. (B) $^{\$\$}P<0.01$ *vs*. respective control (Con) at 3 h, $^{\#}P<0.05$ *vs*. respective control at 6 h, $^{\%}P<0.05$ *vs*. respective control at 12h, $*P<0.05$, $**P<0.01$, $***P<0.001$ *vs*. respective control at 24 h.

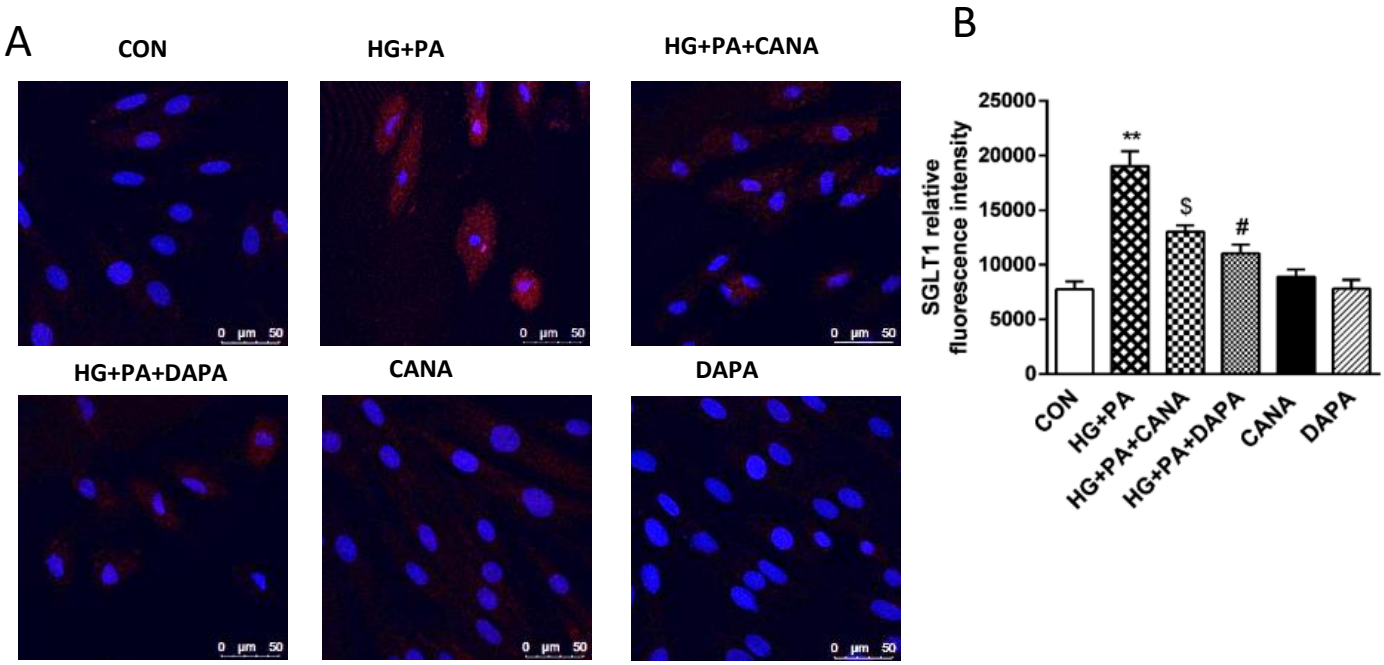


Figure 3. 3 Glucolipotoxicity induces SGLT1 expression in rat H9C2

Fig. 3.3: Glucolipotoxicity induces SGLT1 expression in rat H9C2: Cultured H9C2 cardiomyocytes were treated with high glucose (25 mM) and Palmitic acid (500 µM) (A) for 24 h. CANA and DAPA (10 µM) were incubated alone or with high glucose and palmitic acid for 24 h. SGLT1 expression was determined by immunofluorescence staining (A, B). n=5 for each treatment. **p<0.01 vs. respective control. $^{\$}p<0.05$, $^{\#}p<0.05$ vs. HG+PA group.

3.3.3. Effect of SGLT1 inhibition on reactive oxygen species production

We investigated the effect of glucolipotoxicity on ROS generation using FACS analysis. Treatment of cultured H9C2 cells with palmitic acid (500 µM) and high glucose (25 mM) for 24 h significantly increased reactive oxygen species generation which was significantly attenuated by CANA (10 µM) and DAPA (10 µM) and NAC (600µM) co-incubated with high glucose and palmitic acid (A&B). Additionally, we measured mRNA expression of catalase where CANA and DAPA treatments significantly increased the expression of antioxidant enzyme which alleviates the ROS. (5 C).

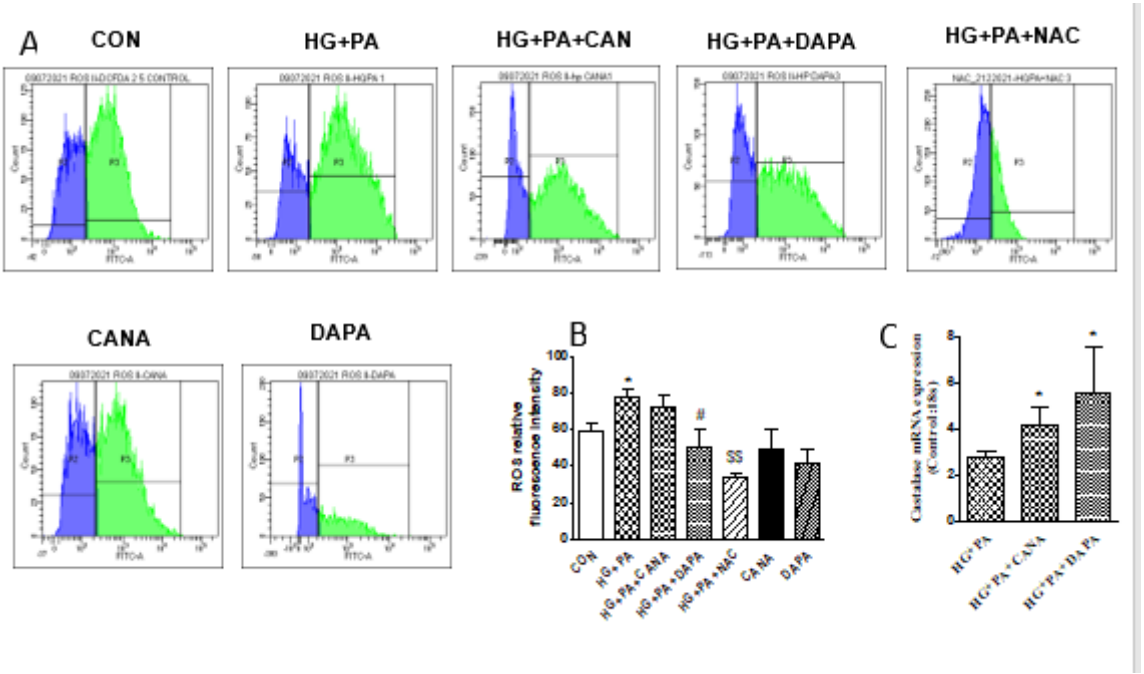


Figure 3. 4 Effect of SGLT1 inhibition on oxidative stress

Fig.3.4: Effect of SGLT1 inhibition on oxidative stress: Cultured H9C2 cardiomyocytes were treated with high glucose (25 mM) and palmitic acid (500 µM) (A, B) for 24 h. CANA, DAPA (10 µM), and NAC (600 µM) were incubated alone or with high glucose and palmitic acid for 3 h. ROS production was measured by FACS analysis using DCFDA (A, B), *$p<0.05$ vs. respective control. $^{\$\$}p<0.01$, $^{\#}p<0.05$ vs. HG+PA group. (C) mRNA expression

of catalase was measured in cultured cardiomyocytes incubated with HG+PA along with CANA, DAPA (10 µM) where *p<0.05 vs. respective HG+PA. Data is expressed as mean ± SD of at least three separate experiments.

3.3.4. SGLT1 inhibition protects the glucolipotoxicity induced apoptosis and morphological changes in rat H9C2 cardiomyocytes:

We examined whether the inhibition of SGLT1 protects the high glucose and palmitic acid-induced apoptosis by FACS analysis. In our findings, in the HG (25 mM) and PA (500 µM) group, the percentage of live cells were significantly decreased (40%), apoptotic and necrotic cells significantly increased compared to the control group, while the percentage of live cells were increased and apoptotic/necrotic cells significantly decreased in HG+PA treated cells co-incubated with CANA, DAPA (10 µM), and NAC (600 µM) (A, B). We also checked caspase-3 expression (C, D) using immunofluorescence staining, which was significantly increased in HG+PA treated group compared to the control whereas this decreased HG+PA cells co-incubated with CANA and DAPA. We also examined structural changes in H9C2 cardiomyocytes in HG+PA treated cells. Histology of H9C2 cardiomyocytes was observed by H and E and crystal violet staining (E, F). In the control group we observed that there was well-established morphology and well-defined nucleus whereas, in HG+PA, we observed the cells with morphological change and polymorphous nucleus. In HG+PA treated cells co-incubated with CANA and DAPA, morphology was preserved.

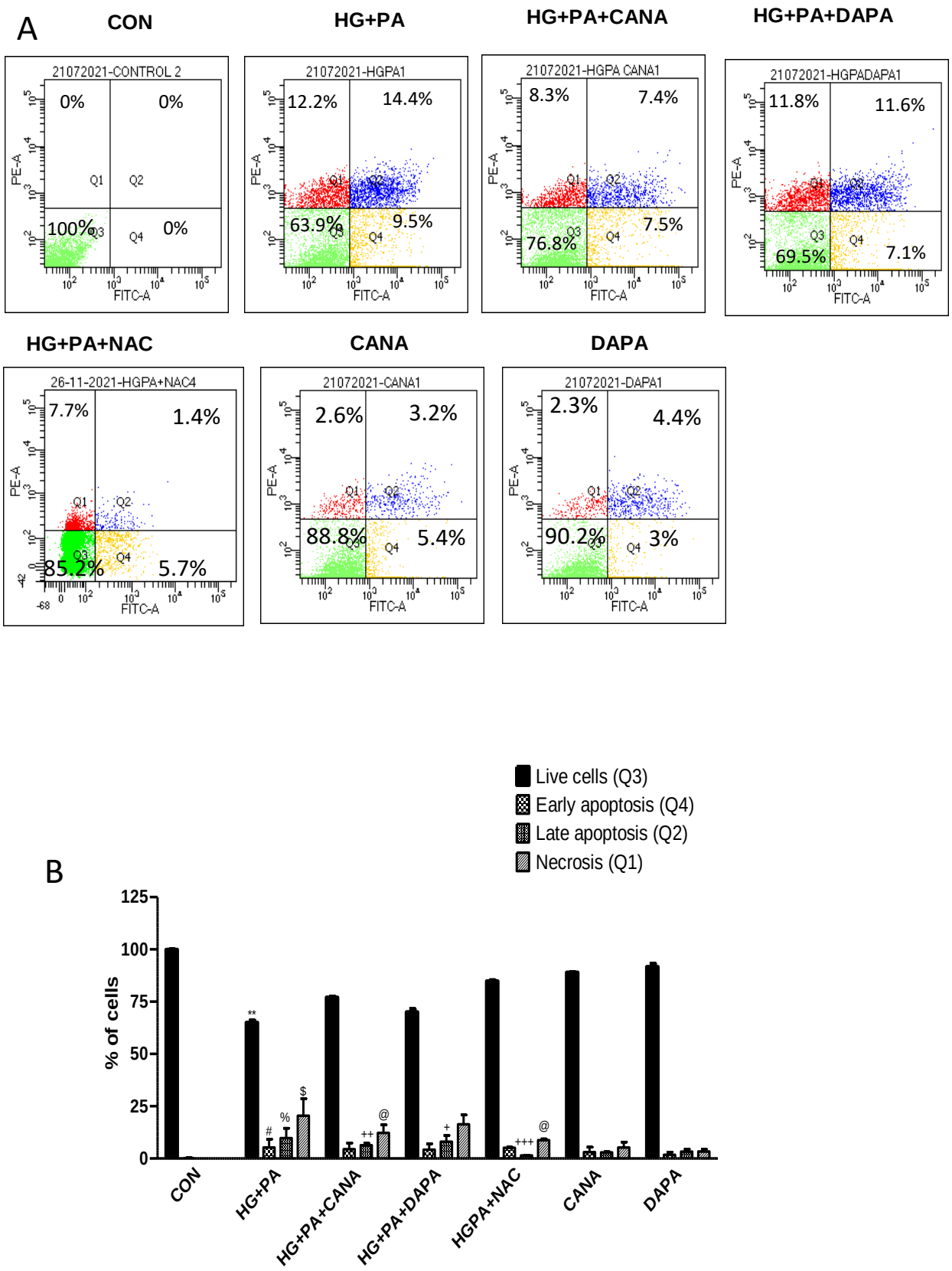


Figure 3. 5. SGLT1 inhibition protects glucolipotoxicity induced apoptosis in rat H9C2 cardiomyocytes.

Fig.3.5: SGLT1 inhibition protects glucolipotoxicity induced apoptosis in rat H9C2 cardiomyocytes: Cultured H9C2 cardiomyocytes were treated with high glucose (25 mM) and palmitic acid (500 µM) (A, B) for 24 h. CANA, DAPA (10 µM), and NAC (600 µM) were incubated alone or with high glucose and palmitic acid for 24 h. Apoptosis was measured by FACS analysis using Annexin-IV assay kit (A, B). n=4 for each group. **p<0.01, $^{\$}$p<0.05, $^{\#}$p<0.05, $^{\%}$p<0.05 vs. respective control (Con),$^{+++}$p<0.001, $^{++}$p<0.01, $^{+}$p<0.05, $^{@}$p<0.05 vs. HG+PA group.

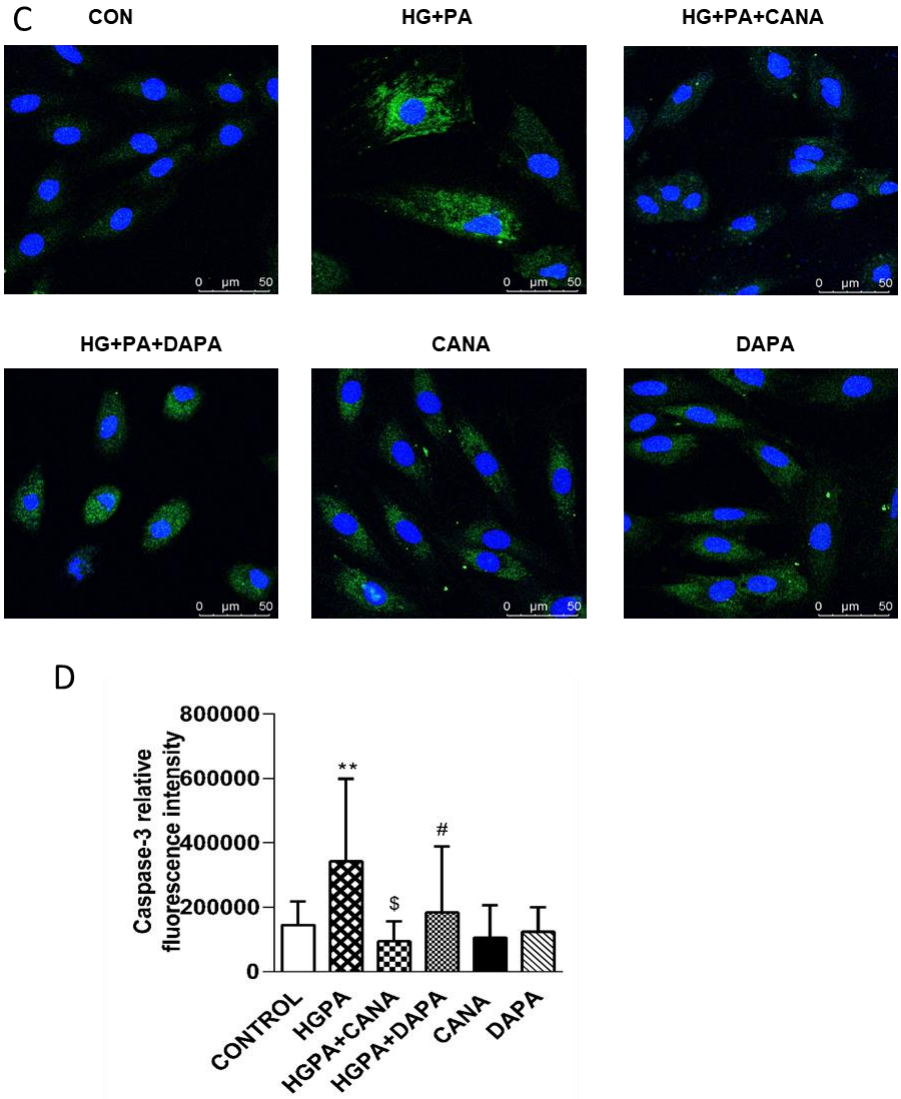
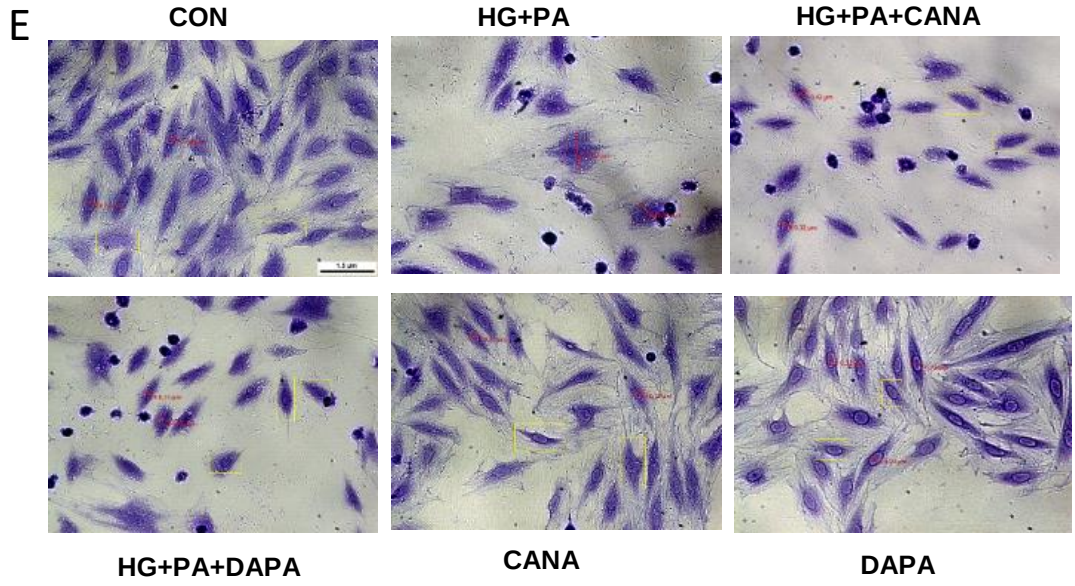
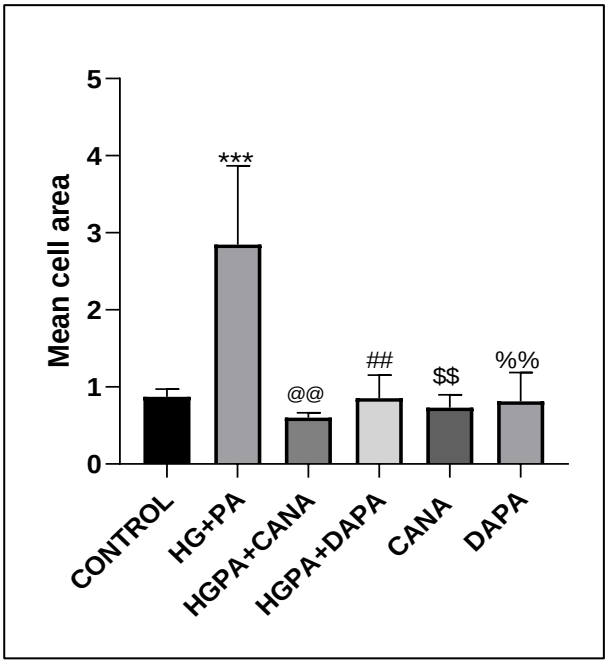


Figure 3. 6 SGLT1 inhibition protects glucolipotoxicity induced apoptosis in rat H9C2 cardiomyocytes.

Fig. 3.6: SGLT1 inhibition protects glucolipotoxicity induced apoptosis in rat H9C2 cardiomyocytes: Cultured H9C2 cardiomyocytes were treated with high glucose (25 mM) and palmitic acid (500 µM) (C, D) for 24 h. CANA and DAPA (10 µM) were incubated alone or with high glucose and palmitic acid for 24 h. Caspase-3 expression was determined by immunofluorescence staining (C, D). **p<0.01 vs. respective control, $^{\$}$p<0.05, $^{\#}$p<0.05 vs. HG+PA group. Data is expressed as mean ± SD of at least three separate experiments.





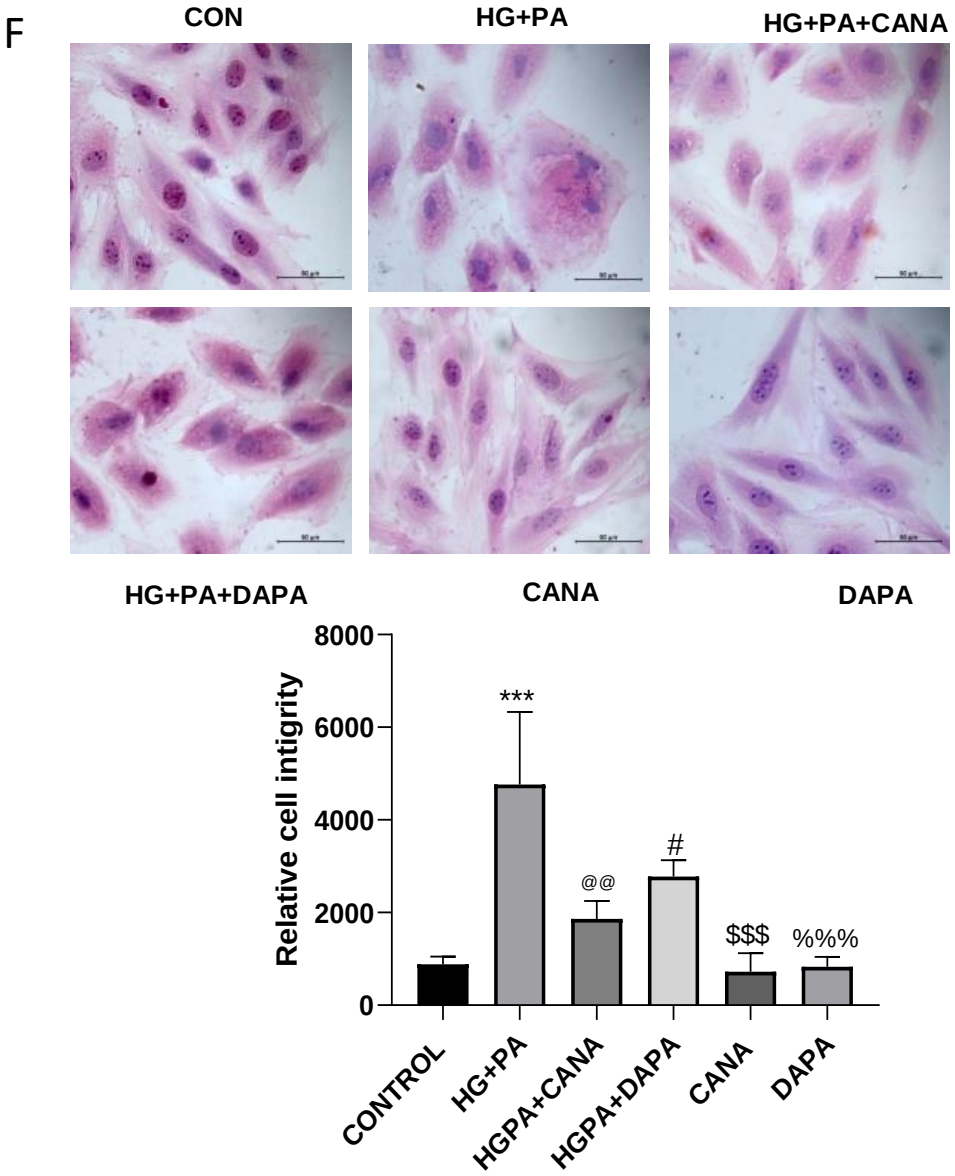


Figure 3. 7 SGLT1 inhibition protects glucolipotoxicity induced structural changes in rat H9C2 cardiomyocytes

Fig. 3. 7: SGLT1 inhibition protects glucolipotoxicity induced structural changes in rat H9C2 cardiomyocytes: Hematoxylin and eosin and Crystal violet staining was performed for assessment of cellular hypertrophy. Cellular hypertrophy/growth has been observed in cultured cardiomyocytes incubated with high glucose and palmitic acid for 24 h. Both CANA and DAPA (E, F) were able to reverse the structural changes induced by high glucose

and palmitic acid (E, F).***p<0.001 vs. respective control (Con) and, $^{@@@}$p<0.001, $^{@@}$p<0.01, $^{###}$p<0.001, $^{\#}$p<0.05 $^{\%\%\%}$p<0.001, $^{\$\$\$}$p<0.001, vs. HG+PA group.

3.3.5. Effect of SGLT1 inhibition on insulin-stimulated glucose uptake in H9C2 cells

Impaired glucose uptake was observed in high glucose and palmitic acid-treated H9C2 cultured cardiomyocytes. HG (25 mM) and PA (500 µM) treatment for 24 h caused a significant reduction in insulin-stimulated glucose uptake in H9C2 cultured cardiomyocyte cells (A, B). Both CANA and DAPA co-treatment with HG+PA caused a further reduction in insulin-stimulated glucose uptake.

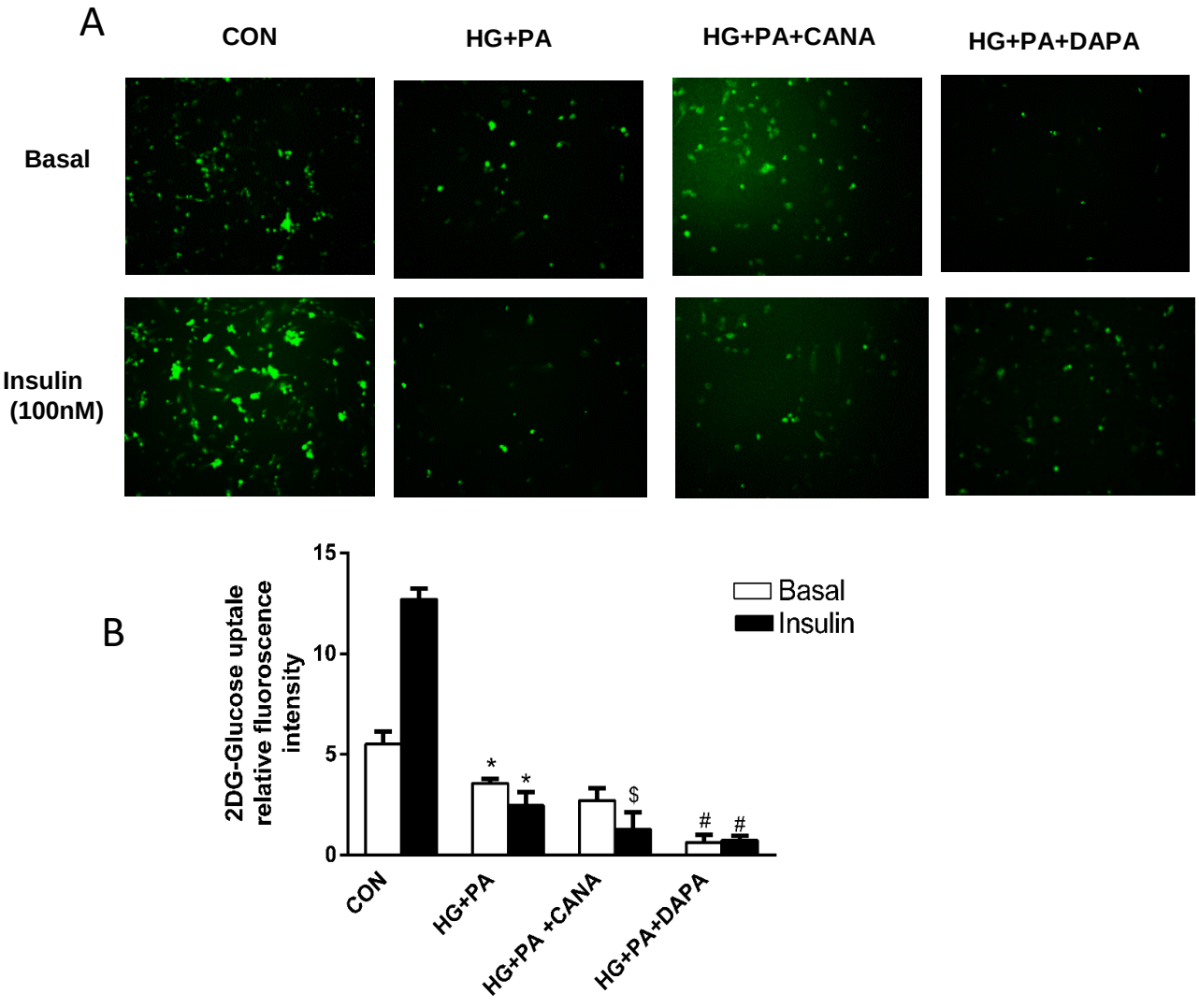


Figure 3. 8 Effect of SGLT1 inhibition on glucose uptake

Fig. 3.8: Effect of SGLT1 inhibition on glucose uptake: Cultured H9C2 cardiomyocytes were treated with high glucose (25 mM) and palmitic acid (500 µM) (A, B) for 24 h. Cells were stimulated with insulin (100 nM) for 10 minutes. CANA and DAPA (10 µM) were incubated alone or with high glucose and palmitic acid for 24 h. Glucose uptake was measured by immunofluorescence staining. (A, B) **$p<0.05$ vs. respective control. $^{\$}p<0.05$, $^{\#}p<0.05$ vs. HG+PA group. Data is expressed as mean ± SD of at least three separate experiments.

3.4. Discussion and Conclusion:

Diabetes-associated CVDs are major health problems globally. Understanding the fundamental cellular mechanisms may contribute to the discovery and development of novel drug molecules for the treatment of metabolic disorders. SGLT2 inhibitors are novel anti-hyperglycaemic agents approved for diabetes treatment (176,187–190). Their protective effect concerning CVD and the underlying molecular mechanism is still under investigation(191–193). The susceptibility of SGLT1- and SGLT2-expressing cells to glucose have consequences for many SGLT-expressing cell types, including cardiomyocytes, endothelial cells, and pancreatic β-cells (170,172,194). Although several studies are exploring the effect of high glucose and high lipid in cardiomyocytes (182,183), not much information is available under the conditions of glucolipotoxicity. In the present study, we have investigated the therapeutic efficacy of two SGLT2 inhibitors, CANA and DAPA in glucolipotoxicity-induced increased SGLT1 expression, oxidative damage, and apoptosis in cultured H9C2 cardiomyocytes. SGLT1 transporter plays a key role in the translocation of sugar across epithelial cells in the small intestine and the renal proximal tubule (170,172,173). Recent studies have reported expression of SGLT1 in the human heart mainly localized to the cardiac myocyte sarcolemma as well as heart capillaries and increased expression is observed under diabetic conditions(174,175). SGLT1 expression is

reported to be upregulated two to threefold in type 2 diabetes mellitus and myocardial ischemia (168). Secondly, knockdown of SGLT1 in the heart has been reported to prevent glycogen storage cardiomyopathy associated with mutation of gene gamma2 subunit of AMPK in a mouse model (169). Furthermore, epidermal growth factor (EGF) that is involved in cell growth, proliferation, and differentiation is reported to be implicated in blood pressure regulation, endothelial dysfunction, neo-intimal hyperplasia, atherogenesis, and cardiac remodelling as well as cardiac hypertrophy. EGF is reported to cause up-regulation of glucose absorption via increased SGLT-1 in rabbit jejunal brush-border membrane and differentiated Caco-2 cells (195). First, we determined the concentration-dependent effect of CANA and DAPA on cell proliferation in normal cardiomyocytes, and based on the cell viability at different concentrations of DAPA and CANA, 10 µM concentration was chosen for further experiments. In the present study, we report for increased SGLT1 expression in cultured cardiomyocytes after exposure to HG and PA in a time-dependent manner with a significant increase at 24 h. SGLT1 expression was attenuated by both CANA and DAPA with CANA showing slightly more inhibitory effect compared to DAPA.

In FACS analysis we found that inhibition of SGLT1 significantly reduced the increased generation of ROS in HG and PA treated cultured cardiomyocytes, which was comparable with the known anti-oxidant NAC. Similarly, the mRNA expression of antioxidant enzyme catalase also significantly increased, revealing that the protective effect of CANA and DAPA was greatly through the anti-oxidant effect. Numerous studies have shown high glucose and high fat as known inducers of oxidative stress. Oxidative stress is a contributing factor for numerous diabetes-associated CVD complications (196,197). In PA treated pancreatic beta-cells inhibition of ROS is associated with reduced ER stress and apoptosis. Oxidative stress can also induce inflammatory responses by activation of MAP

as well as of NF-kB signalling pathways(198,199), and increased inducible nitric oxide synthase expression has been reported in diabetic rat heart (200). Hence, by inhibiting ROS, CANA, and DAPA might be protecting the diabetic heart.

Previous studies including ours have shown saturated fatty acids and high glucose induce apoptosis in different cells and are associated with the development of various diseases, such as hypertension, diabetes, and atherosclerosis (201,202). Cardiomyocyte apoptosis and death are important factors that lead to patient morbidity and mortality, thus targeting the apoptotic signalling pathway can be one of the possible strategies which can be explored for therapeutic drug targeting (203,204). To know whether SGLT1 inhibition prevents high glucose and palmitic acid-induced apoptosis, we determined apoptosis by FACS analysis and caspase-3 expression by immunofluorescence, we observed that inhibition of SGLT1 prevents apoptosis. It has also been reported recently that SGLT1 inhibition can attenuate apoptosis by suppressing DCM development via the JNK and p38 pathway (157). We also observed significant necrosis in high glucose and palmitic acid-treated cells and this was attenuated by both CANA, DAPA, and NAC. By these findings, it is evident that the anti-apoptotic effect of CANA and DAPA is mainly through the anti-oxidant pathway Furthermore, we detected inhibition of SGLT1 preserves the morphology of cardiomyocytes. In the control group, we detected well-established cell morphology and well-defined nucleus whereas, in HG+PA, we observed the cells with structural change and polymorphous nucleus. In HG+PA treated cells co-incubated with CANA and DAPA, good morphology was observed. Several studies have reported insulin resistance is associated with pro-atherogenic changes in the vasculature as well as endothelial cells. Thus, cellular hypertrophy in cardiomyocytes could be related to insulin resistance and overexpression of IGF1 in response to HG and PA. Since increased glycogen deposition in the heart can lead

to cardiomyopathy due to increased cardiac glucose uptake, thus, chronic inhibition of SGLT1 would decrease this glycogen deposition associated with CVD (168).

To determine whether SGLT1 was associated with the change in cardiac glucose uptake and since SGLT1 is the main SGLT isoform localized in the heart and transports glucose by a secondary active transport mechanism which uses the sodium concentration gradient established by the Na^+/ K^+-ATPase pump, we wanted to observe the effect of CANA and DAPA on basal and insulin-stimulated glucose uptake in cardiomyocytes. We observed impaired glucose uptake in HG and PA treated cardiomyocytes under basal and insulin-stimulated conditions. Interestingly both CANA and DAPA reduced the basal and insulin-stimulated glucose uptake further in HG+PA treated cultured cardiomyocytes. There are no head-to-head comparisons of CANA and DAPA done so far, however, previous studies have reported both CANA and DAPA are equally effective in reducing uncontrolled hyperglycaemia, fasting blood glucose, postprandial glucose, HBIAc, body weight, and systolic blood pressure (205). In healthy volunteers, CANA provided greater 24h urinary glucose excretion, renal threshold for glucose excretion, and postprandial glucose excursion compared to DAPA (206). Recently clinical trials on DAPA-heart failure (assessing dapagliflozin) and EMPEROR-Reduced (assessing empagliflozin) showed SGLT2 inhibition reduced the combined risk of CV death or hospitalization for heart failure in patients with heart failure with reduced ejection fraction (HFrEF) with or without diabetes (207). In a meta-analysis, SGLT2 inhibitors were associated with a reduced risk of major adverse CV events. Also, the benefit across the class was for an associated reduction in risk for hospitalization due to heart failure and kidney outcomes (208).

So far clinical studies have shown that CANA and DAPA have few risks and side effects, but not so severe as to discontinue the drug. The most common side effects are genital tract infections, lower limb amputations, bone fractures, electrolyte imbalance, and

risk of cancer. Anyhow, additional studies with a larger sample size are required to identify the long-term adverse effects (209).

In conclusion, our study reports glucolipotoxicity activates oxidative stress and apoptosis/necrosis via up-regulation of SGLT1. Thus, SGLT1 mediates glucolipotoxicity-induced cardiomyocyte damage. Thus inhibition of SGLT1 may be one of the possible strategies to inhibit cardiomyocyte damage, however further studies are needed to investigate its potential in different disease models.

Chapter 4: Canagliflozin protects the diabetic heart by mitigating fibrosis and preserving the myocardial integrity with improved mitochondrial function

4.1. Introduction

Diabetic patients are more likely to suffer from micro and macrovascular complications. Coronary artery disease, stroke, and peripheral vascular disease are major macrovascular complications. Diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy are the major deleterious effects of hyperglycaemia on microvasculature(26). Among different acute and chronic diabetic complications, cardiovascular complications contribute to more than 60% of mortality in diabetic patients compared to non-diabetic individuals due to poor prognosis(181,210).

Diabetic cardiomyopathy (DCM) is a myocardial disease with a characteristic structural and functional abnormality of the myocardium, seen in diabetic patients, without pre-existing cardiovascular complications such as hypertension, valvular and coronary artery disease(211,212). Although the pathogenesis of DCM is not clearly understood, persistent hyperglycemia, impaired glucose metabolism, insulin resistance, inflammation, oxidative stress, and cardiac fibroblast proliferation stands as the molecular basis for cardiac remodelling and diastolic dysfunction (211–213). Hyperglycaemia and poor glycaemic control are the greater risk factor for DCM and heart failure (HF), where a 1% increase in glycated haemoglobin (HbA_{1c}) tends to 30% increased risk of HF in Type 1 diabetes(T1D), and 8% in type 2 diabetes (T2D) patients(211).

Even though it is clear that there is a positive correlation between diabetes and negative cardiovascular outcomes, no cardiovascular outcome trials (COVT) were thoroughly performed to evaluate the cardiovascular safety of anti-diabetic drugs till 2008 until US food and drug administration (US-FDA) and the European medical agency (EMA) regulations request to assess the cardiovascular safety of all new anti-diabetic drugs(210). Thereafter, from 2008, several COVTs with different classes of drugs including Sodium-glucose co-transporter-2 (SGLT-2) inhibitors, DPP-4 inhibitors, and GLP-1 agonists named DECLARE-TIMI, DAPA-HF, CANVAS, DAPA-CKD, CREDENCE, EMPA-REG, EMPORER, SAVOR-TIMI, TECOS, ELIXA, EXAMINE, VERTIS-CV, LEADER, and SUSTAIN-6 have been initiated(210,214). Among all, Sodium-glucose co-transporter-2(SGLT-2) inhibitors have shown significant beneficial class effects of 11% reduction of major adverse cardiac events (MACE) and 23% reduction in heart failure hospitalization or cardiovascular death(214). Hence, further development of SGLT-2 and dual SGLT-1/2 inhibitors have gained attention in the recent past.

SGLT-1 and SGLT-2 are the two major classes of SGLTs responsible for active glucose transport, identified so far. SGLT-1 is highly expressed in the small intestine and also expressed in the heart, kidney, colon, testis, trachea, prostate, lung, and liver, Whereas SGLT-2 is exclusively expressed in the kidney, and a very little expression was observed in skeletal muscle, small intestine and testis(173). Intriguingly, 20-fold higher SGLT-1expression in human heart than the kidney, and perturbed expression of cardiac SGLT-1 in diseased myocardium added attention to explore the interventions targeted at SGLT-1/2(168).

The SGLT-2 inhibitors Canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin have recently been approved by US-FDA as a new class of anti-diabetic drugs. Predominantly, Canagliflozin and empagliflozin have shown 35% reduction in heart failure

hospitalization in the aforementioned large clinical trials CANVAS (Canagliflozin Cardiovascular Assessment Study) and EMPA-REG outcome trials respectively(215). To date, numerous studies have explored the role of SGLT-1 inhibition in cardiovascular protection *in-vitro* and *in-vivo*(216). From our previous study, we found that SGLT-1 inhibition can alleviate high glucose and high lipid-induced oxidative stress and apoptosis in in-vitro cultured cardiomyocytes(217). In the present study, we aim to explore the possible molecular mechanism behind the cardiovascular protection of canagliflozin in the most clinically relevant high-fat diet-induced type2 diabetic model in male Wistar rats.

4.2. Materials and Methods:

4.2.1. Chemicals:

Canagliflozin was purchased from Sigma Aldrich, Commercial kits for assessment of total cholesterol (TC), triglycerides (TG'S), high-density lipoproteins (HDL), and low-density lipoproteins (LDL), were purchased from tulip diagnostics (P) Ltd, Mumbai, India, and Arkray Healthcare Pvt. Ltd, Surat, India. Streptozotocin (STZ), hematoxylin, eosin, Sirius red, Poly-L-Lysine coating solution, and all primers were purchased from Sigma Aldrich,(St. Louis, Missouri, United States). Antibodies for a-SMA, TGF-b, and GAPDH were purchased from Santa Cruz Biotechnology (CA, USA). SGLT-1 antibody was purchased from Abcam (Milton, Cambridge, UK), Immun-Blot® PVDFMembrane, Trizol reagent, TMRE, MitoTracker Red (Invitrogen, Thermo fisher scientific), iScrip cDNA Synthesis Kit and iTaqUniversal SYBR® Green Supermix were purchased from Takara.

4.2.2. Animals and Experimental Design:

All the proposed animal experimental protocols were approved by Institutional Animal Ethical Committee (Protocol approval number: BITS/Hyd/IAEC/2020/14). 5-6 weeks old male Wistar rats were procured and maintained at our animal house facility with adlibitum

access to food and water. Animals were divided into two groups. One group was fed a high-fat diet (HFD)(218). And, the other group was fed with a normal pellet diet (NPD). After 4 weeks, an Oral glucose tolerance test (OGTT) and Intraperitoneal Insulin Test (IPITT) were performed to check the insulin resistance in the HFD group. Animals with insulin resistance were injected with a single dose (35 mg/kg) of streptozotocin and NPD animals were injected with vehicle citrate buffer (pH 4.4) intraperitoneally. After a week, blood glucose and serum biochemical parameters (HDL-high density lipoproteins, LDL-low density lipoprotein, TGs – Triglycerides, TC-total cholesterol, LDH-lactate dehydrogenase) were measured. Animals showing fasting blood glucose ≥250mg/dl were considered diabetic.

Diabetic animals were maintained for 4 more weeks on HFD. Serum biochemical parameters were measured to confirm the progression of the disease. Further, animals from HFD were randomly divided into diabetic, diabetic + CANA, likewise, the NPD group was divided into Control and CANA alone groups. According to the groups, appropriate treatments were given for 4 weeks, where 10 mg/kg canagliflozin was given orally in 5% HPMC to treatment groups, 5% HPMC was given orally control group. After completion of 12 weeks study period, end OGTT and IPITT were conducted and blood was collected by retro-orbital puncture. At the endpoint of the study, rats were euthanized and organs were collected after exsanguination.

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dl-Methionine	03
Yeast powder	01
Sodium chloride	01

Table 1. Composition of High-fat diet

4.2.3. OGTT:

In brief, animals were fasted overnight (16 hrs) with adlibitum access to water and then administered 2 gm/kg glucose by oral gavage. Further blood glucose levels were measured using the tail prick method by Accu-check glucometer at intervals of 0, 15, 30, 45, 60, 90, and 120 min(219).

4.2.4. IPITT:

Animals fasted for 6 hrs and were injected with 0.75 IU/KG insulin intraperitoneally and blood glucose levels were measured at 0,15,30,60 and 90 min intervals(219).

4.2.5. Histological analysis:

Freshly isolated tissue samples were processed in 4% paraformaldehyde followed by subsequent dehydration steps in gradient alcohols and xylene using an automated tissue processor. Dehydrated tissue sections were embedded in paraffin and 4-5 µm serial sections were taken using a microtome.

4.2.6. H&E Staining:

4-5 um thin paraffin-embedded tissue sections were deparaffinized and rehydrated with 2 changes of xylene and 100-70% alcohols for 2 min, then hydrated with dd H_2O. Following rehydration, sections were stained with hematoxylin for 30 sec, followed by washing and bluing. Now, sections were stained with eosin for 30 sec and dehydrated by 95%alcohol followed by 100% alcohol and xylene. Later sections were mounted with coverslips and images were taken using ZEISS AXIOLAB 5 Microscope(219).

4.2.7. Sirius red staining:

4-5 um thin tissue sections were rehydrated and stained with Sirius red stain for 1hour and washed with acidified H_2O to remove excess stain. Stained sections were dehydrated and mounted with coverslips(219).

4.2.8. Immunohistochemistry:

In brief, 4 µm thin OCT frozen heart tissue sections were fixed with 4% PFA for 15 min and permeabilized in 0.1% triton X100 for 10 min. Further tissue sections were blocked with 3% BSA for 1hr followed by overnight incubation with appropriate primary antibody at a concentration of 1:200 at 4˚C and 1:400 concentration of fluorescent-tagged secondary antibody for 1 hr at dark. DAPI was used to stain the nucleus for 10 min and the fluorescent images were captured using a confocal microscope (Leica DMi8, Leica Microsystems, Germany). Fluorescence was quantified using Image J(219).

4.2.9. Immunoblotting:

In brief heart tissue samples were homogenized in RIPA lysis buffer using bead homogenizer (Minilys Personal Homogenizer by Bertin Technologies) and quantified by Bicinconinic Acid assay, 40ug isolated protein was subsequently loaded and separated on

SDS-Poly Acrylamide Gel Electrophoresis at 70 Mv, further, the protein was blotted onto PVDF membrane (bio-rad) at 70 Mv for 90min, the membrane was blocked using 3% BSA followed by overnight incubation with appropriate primary antibody at 4˚C at a concentration of 1:1000 and HRP-conjugated secondary antibody for 1.30 hr at room temperature at a concentration of 1:2000. The target protein was detected and analysed by VILBER FUSION solo S western blot and chemi imaging system(219).

4.2.10. Real Time-PCR:

Total RNA was isolated using the TRIzol (Invitrogen) method, 1µg extracted RNA was reverse transcribed to cDNA, and real-time PCR was performed using SYBR green chemistry in an iCycler iQ apparatus. Primer sequences were designed using Rattus norvegicus gene sequences published on NCBI. Quantitative real-time-PCR was analyzed using Bio-rad CFX manager and data were quantified and represented as fold change using $2^{-\Delta\Delta CT}$ method(219).

Symbol	Forward primer	Reverse primer
NCX-1	AGTCTCCCACCCAATGTTTC	CTCCTGTTTCTGCCTCTGATC
NHE-1	GCCGTCTCAACTGTCTCTATG	ATCTCCTCCTCCTTGTCCTT
Na^+K^+ATPase	TCCCTACAGTCTCCTCATCTTC	TCAGTAGTACGTCTCCTTCTCC
SGLT-1	GTGTACGGATCAGGTCATTGT	CCATGAGGAACATAGGCAGTA
SGLT-2	GTAGAGGAAGGCTCTGAACTTG	ACCAATGACCAGCAGGA
TGF-β	CTTTAGGAAGGACCTGGGTTG	GTGTCCAGGCTCCAAATGTA
BNP	GCTCAGAGACAGCTCTCAAA	CCCAAAGCAGCTTGAACTATG

Table 2Primer Sequences

4.2.11. Cell culture:

H9C2 cell line (rat cardiomyocytes) was obtained from NCCS, Pune, India, and cultured on T25 flasks and coverslips using Roswell Park Memorial Institute (RPMI) 1640 medium

supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. High glucose (25 mM) and palmitic acid (150 µM) were used to mimic invitro diabetic cardiomyopathy with or without 10 µM Canagliflozin.

4.2.12. Measurement of mitochondrial membrane potential:

In brief, H9C2 cardiomyocytes were grown on coverslips in a 12-well plate, supplemented with 10% FBS in RPMI media. After the subconfluency, cells were treated with 150µM PA (palmitic acid) and 25 mM HG (High glucose) with or with canagliflozin. After 24hrs media was replaced with 200 nM TMRE in serum-free media and incubated for 15 min followed by a PBS wash. Live cell imaging was done using ZEISS AXIOLAB 5 Microscope, whereas for FACS analysis cells were trypsinized after treatments and resuspended in PBS for FACS analysis using PI filter(220).

4.2.13. Assessment of Mitochondrial morphology:

To assess the mitochondrial morphology, H9C2 cells were cultured in confocal dishes and treated with 150 µM PA, 25 mM HG with or without 10 µM canagliflozin for 24 hrs. After treatment, cells were incubated with 100nm mitoTracker red in serum-free media for 15min followed by a PBS wash. Live cell imaging was done using a confocal microscope (Leica DMi8, Leica Microsystems, Germany)(220).

4.2.14. Statistical analysis:

Data were analyzed using GraphPad prism8 software and presented as Mean ± SEM applying one-way or two-way ANOVA followed by tukey's or dunnets post analysis test, data were considered statistically significant at $P< 0.05$.

4.3. Results:

4.3.1. Canagliflozin treatment reverses insulin resistance in type-2 diabetic rats:

Obesity and insulin resistance are the clinical attributes of type-2 diabetes(221). In our study insulin resistance was evident in rats after 4 weeks of HFD with increased AUC measured by OGTT and IPITT (A and B). Blood glucose was significantly high after intraperitoneal injection of insulin, indicating insulin resistance with compromised glucose uptake by tissues in response to circulating insulin (B). Whereas 10 mg/kg canagliflozin treatment for 4 weeks improved the insulin sensitivity significantly compared to the DCM group (C and D)

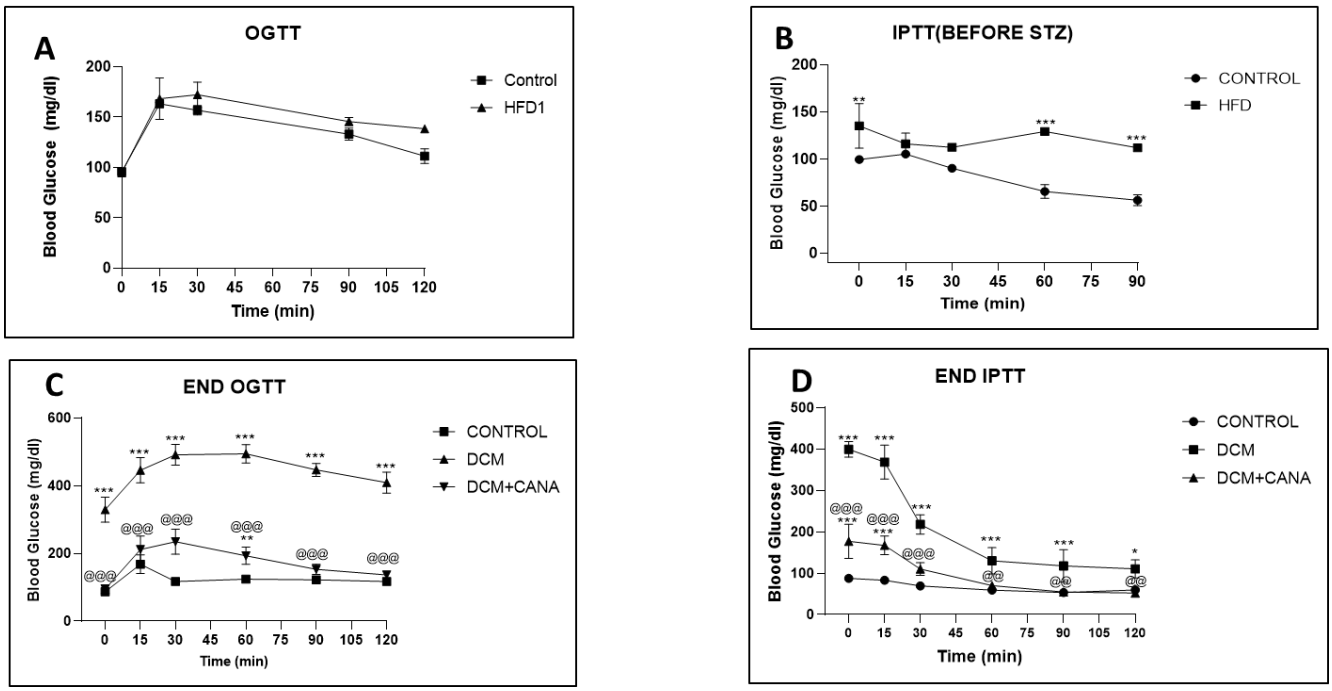


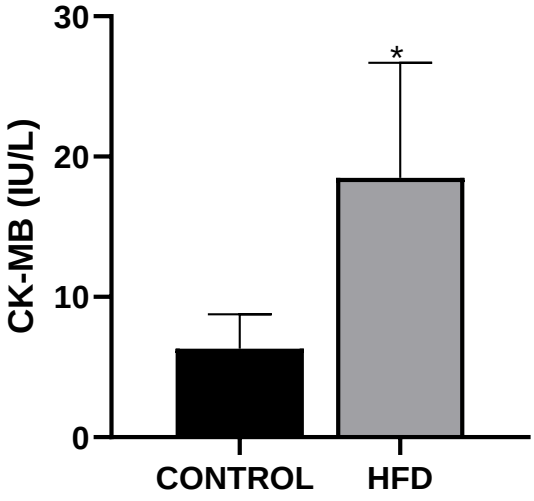
Figure 4. 1 Representative graphs of the Area under the curve (AUC) for OGTT and IPITT

Fig.4.1. Representative graphs of the Area under the curve (AUC) calculated for, oral glucose tolerance test and intraperitoneal insulin tolerance test, after 4 weeks of HFD (A, B), and 8 weeks of HFD along with 4 weeks of canagliflozin (C, D Data were expressed as

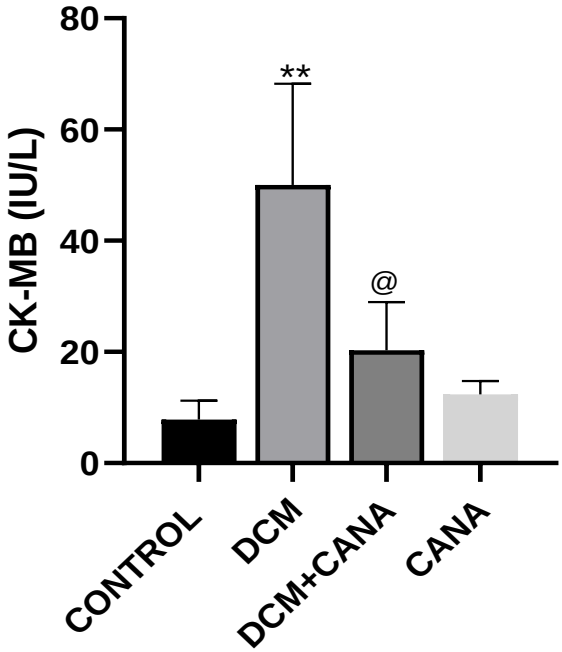
mean±SEM (where n≥6). Data were analyzed using ordinary two-way ANOVA followed by Tukey's multiple comparison test, where *P < 0.05,***P<0.001, ****P<0.0001 Vs respective control, $^{@}$P<0.05, $^{@@}$P<0.01, $^{@@@}$P<0.001, $^{@@@@}$P<0.0001 Vs DCM.

4.3.2. Systemic parameters in DCM rats:

Body weight was significantly decreased in DCM rats at the endpoint, whereas heart weight was increased. Likewise, serum triglycerides, LDL, and total cholesterol levels were significantly high and HDL levels were low in the DCM group compared to control and treatment groups accompanied by increased heart rate. In consistent with the above parameters, systolic and diastolic blood pressure was increased in the DCM group, where canagliflozin treatment normalized the blood pressure (Table.2)



CK-MB after 8 weeks of DM induction



CK-MB after canagliflozin treatment

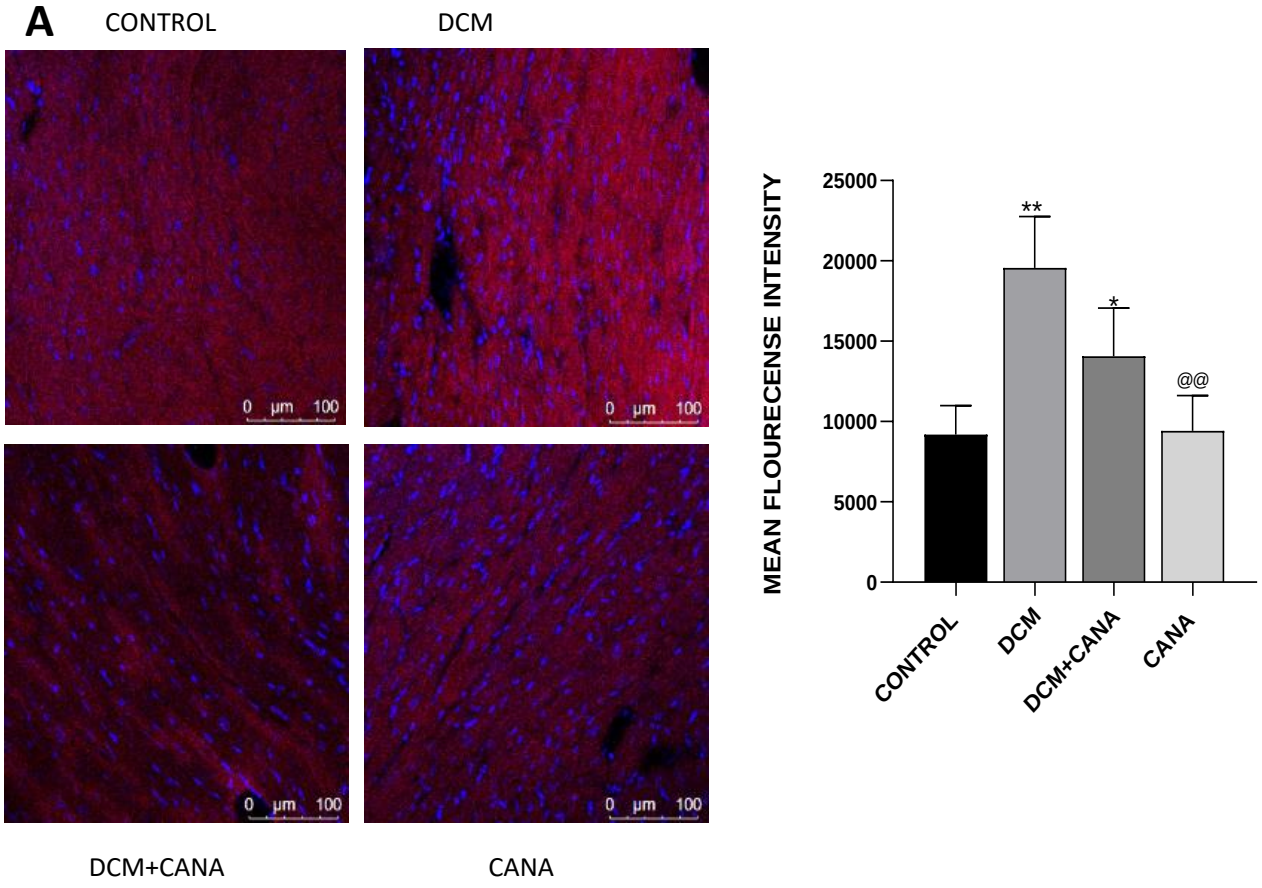
S.No	Parameter	Control	HFD + STZ	HFD + STZ +CANA	CANA
1	HDL(mg/dl)	36 ± 0.027	11 ± 3.010**	24 ± 2.100*	38 ± 0.012
2	LDL(mg/dl)	102 ± 0.52	164 ± 2.02***	112 ± 1.40*	98 ± 0.04
3	TG(mg/dl)	160 ± 0.09	404 ± 3.46***	200 ± 3.07**	180 ± 1.06
4	TC(mg/dl)	94 ± 0.200	116 ± 3.000*	105 ± 2.400	91 ± 0.601
5	HR(BPM)	313 ± 16	484 ± 25**	351 ± 15*	321 ± 15
6	SBP(mmhg)	129 ± 4	161 ± 11 **	129 ± 9	131 ± 9
7	DBP(mmhg)	82 ± 7	111 ± 12*	89 ± 8	81 ± 6
8	HW(gm)	0.926 ±0.05	1.305 ± 0.30**	0.955 ± 0.06*	0.869 ±0.02*
9	BW (gm)	339.68±8.14	292.46±13.63**	309.56 ± 9.69*	321.97 ±10.41

Table 3. Table representing serum biochemical parameters and CK-MB *P < 0.05, **P<0.01, ***P<0.001 Vs respective control, $^{@}$ P<0.01 Vs DCM

4.3.3. The diabetic heart shows upregulation of SGLT-1:

Upregulation of SGLT-1 in type-2 diabetic and ischemic hearts was reported in the previous study(168). Similar findings were observed in our present study, where immunofluorescent staining of DCM heart sections showed significantly higher expression of SGLT-1 compared to the control heart, whereas the canagliflozin treated group showed significantly low expression of SGLT-1 compared to the DCM group (A). Likewise, a similar trend was observed in mRNA expression of SGLT-1 in DCM heart with higher expression, which was

inhibited by canagliflozin treatment (B). However, mRNA expression of SGLT-2 showed negligible expression, where amplification started after 32 cycles, and in some samples, it was not amplified. Hence the resultant data showed more standard deviation (C).



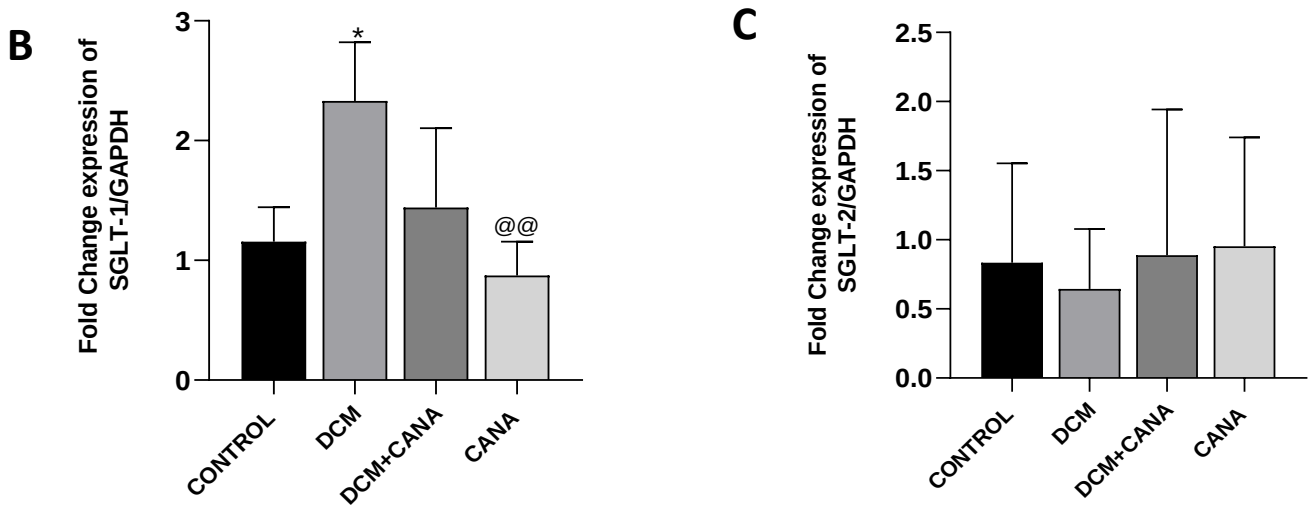
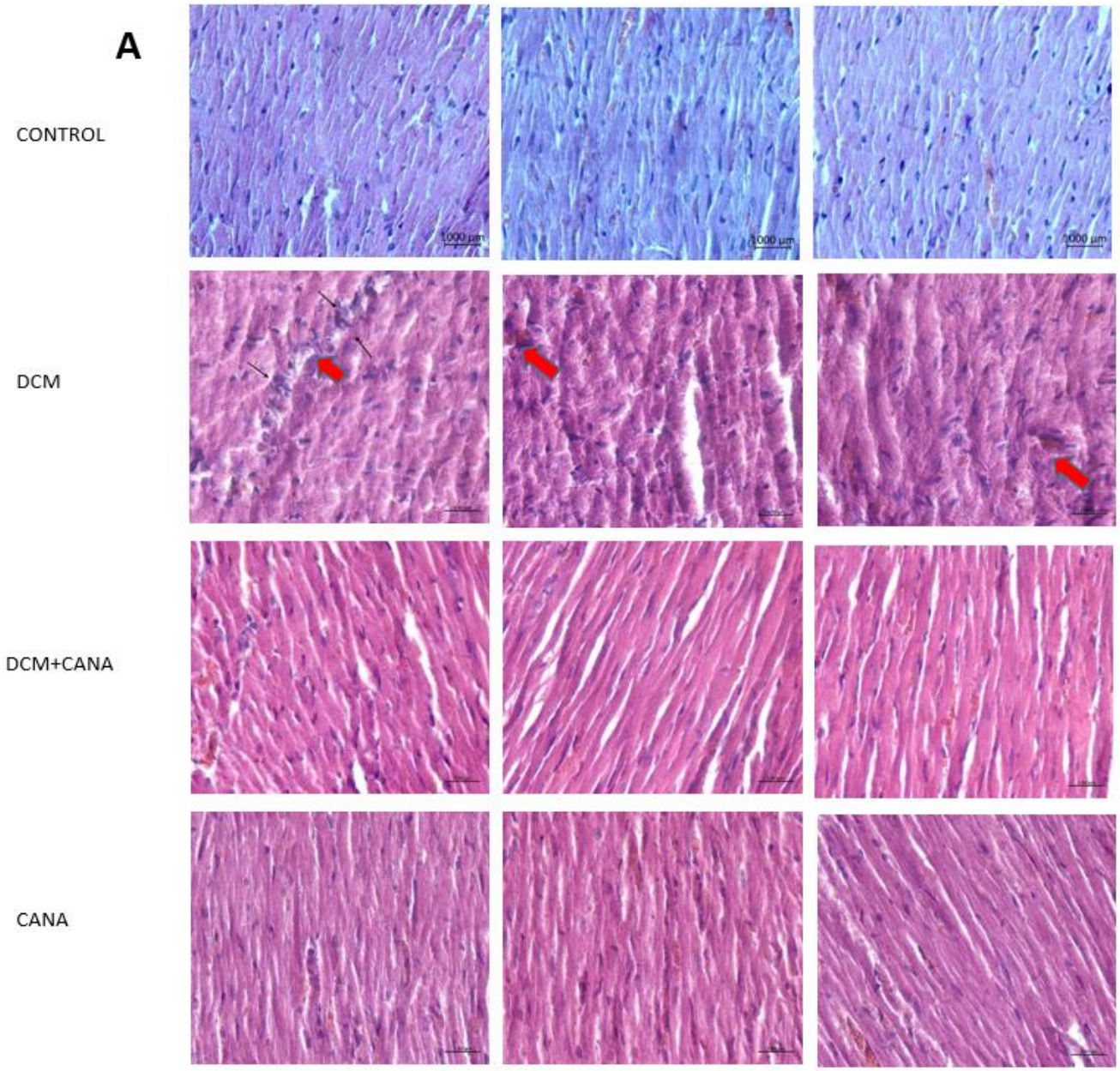


Figure 4. 2 .Representative images of SGLT-1 and SGLT-2 expression

Fig.4.2.Representative images of SGLT-1 expression in OCT sections of myocardium using immunoflorescence(A), mRNA expression of SGLT-1 and SGLT-2 in myocardial tissue (B and C). Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with single pooled variance, where *P < 0.05, **P<0.01, Vs respective control, $^{@@}$ P<0.01 Vs DCM.

4.3.4. Canagliflozin mitigates cardiac remodelling and hypertrophy:

Myocardial remodelling is a major hallmark of DCM progression and contractile dysfunction. In our present study, myocardial tissue morphology was examined using H&E staining and microscopical imaging followed by hypertrophy marker evaluation. DCM group showed significant tissue damage with disorganized myofibrils with pyknotic nuclei and inflammatory cell infiltration (A). DCM has damaged the intercalated discs and prominent myofibril hypertrophy was observed (B). Further, quantitative RT-PCR also revealed the elevated expression of BNP which was blunted by canagliflozin treatment (C).


A
CONTROL
DCM
DCM+CANA
CANA

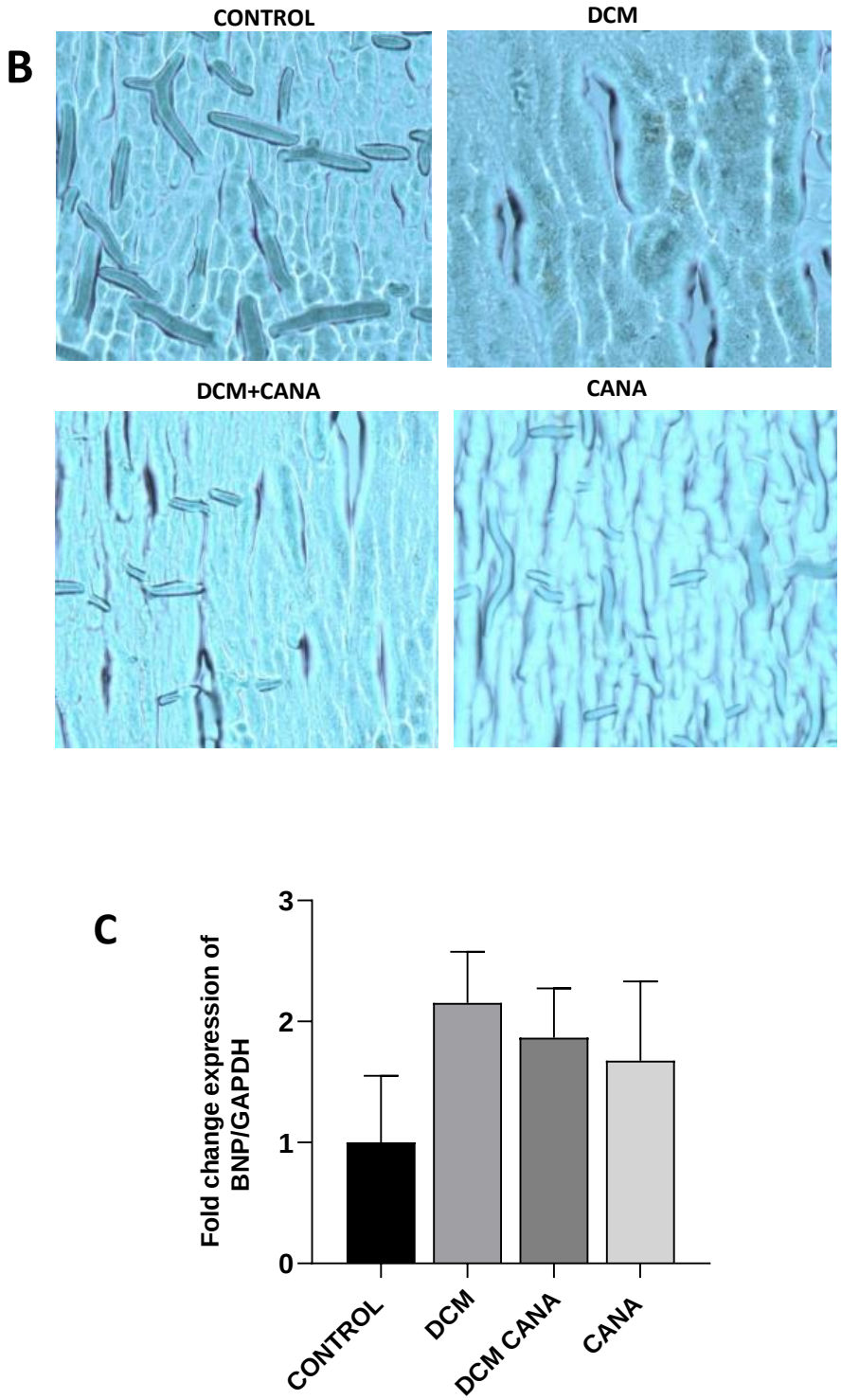


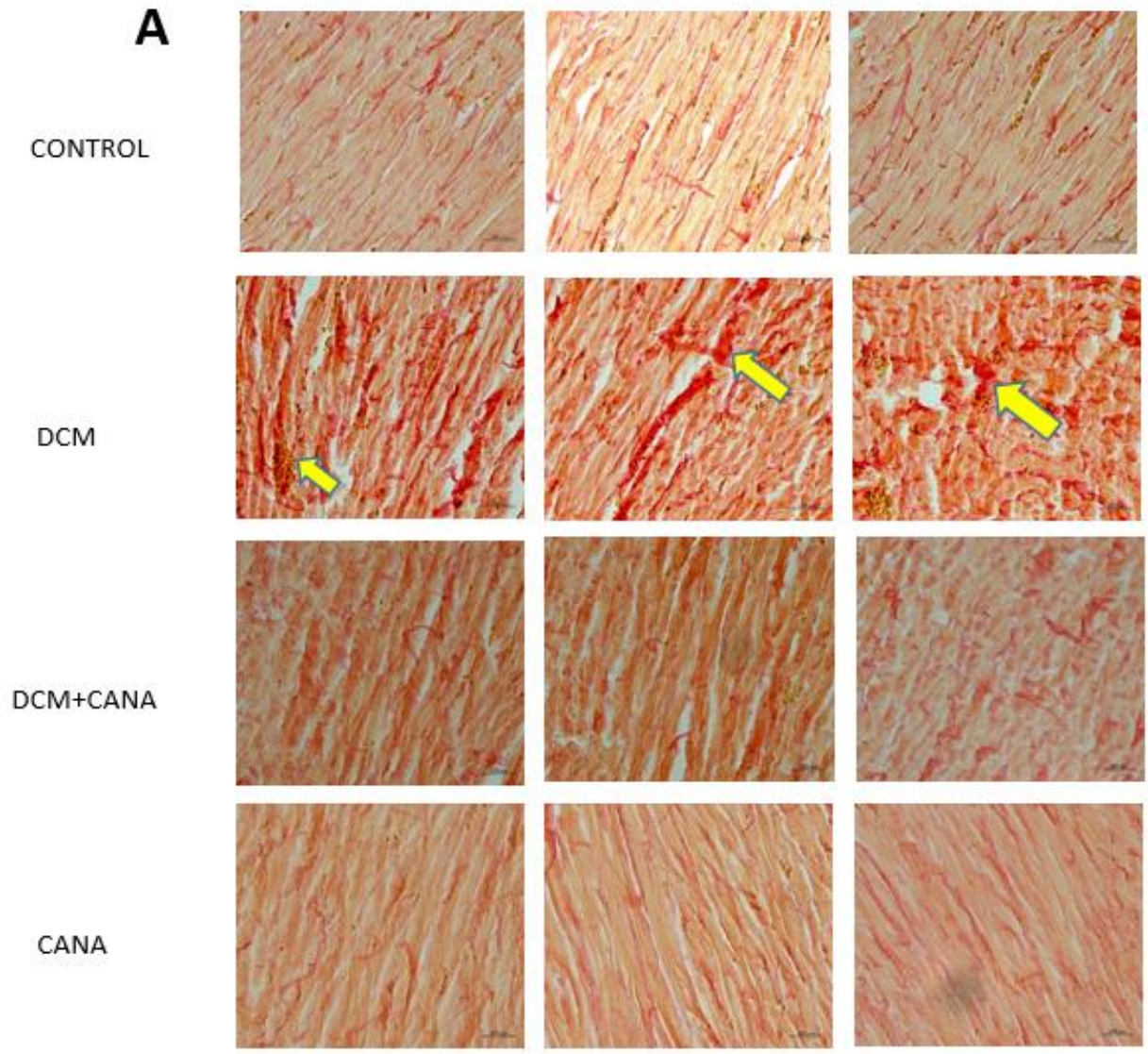
Figure 4. 3 Representative images of the effect of canagliflozin on cardiac hypertrophy

Fig4.3. Representative images of the effect of canagliflozin on the cardiac remodeling by histological assessment, using H&E stain. Red arrows represent inflammatory cell

infiltration in DCM group and disorganized myofibrils with pyknotic nuclei, interstitial edema, and hypertrophy. Whereas canagliflozin treatment improved the myocardial structural integrity (A). (B) Representative images of paraffin-embedded unstained heart tissue sections, where DCM heart showed severe myocardial tissue damage with lost intercalated discs and interstitial edema, whereas canagliflozin treatment preserved the structural integrity with well-defined intercalated discs. (C) Represents mRNA expression of BNP in myocardial tissue, data was represented as mean±SEM (where n=4) and analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with single pooled variance.

4.3.5. Canagliflozin ameliorates fibrosis in diabetic heart:

Persistent diabetes damaged the myocardium as demonstrated by H&E stain. This chronic damage led to interstitial collagen deposition, which was evident after the picrosirius red stain as shown in (A), whereas CANA treatment limited the collagen deposition (A). These findings were further accompanied by elevated protein levels of TGF-β, α-SMA demonstrating the elevated cardiac myofibroblasts (B,D &,C,E). Interestingly, CANA treatment attenuated these protein expressions. Furthermore, quantitative RT-PCR also demonstrated an elevated TGF-β expression (F), which was significantly high in DCM group compared to the control, whereas CANA treatment groups showed significantly low expression compared to DCM group.


A
CONTROL
DCM
DCM+CANA
CANA

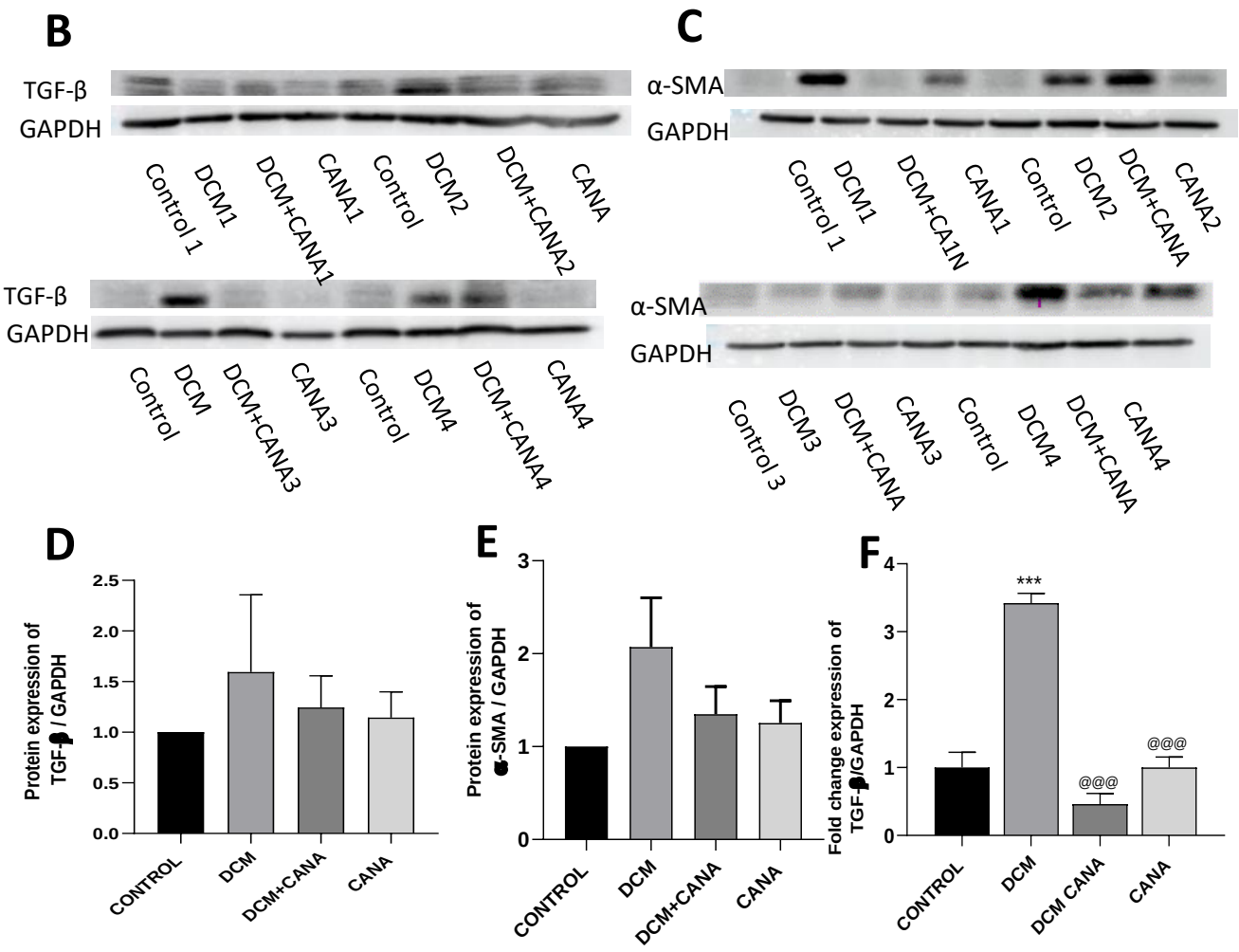
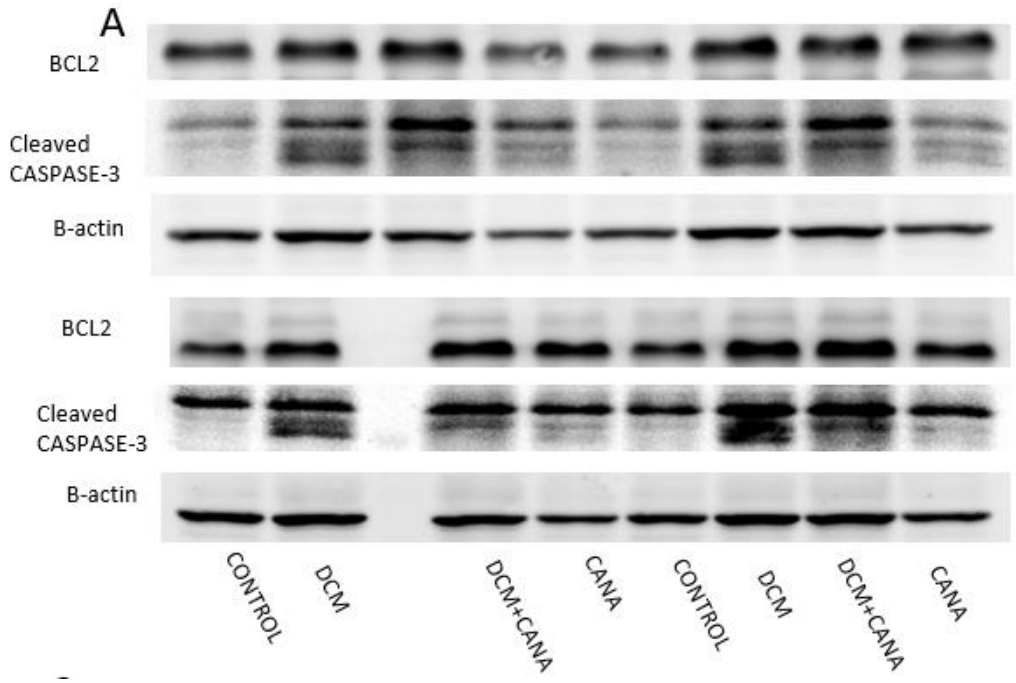


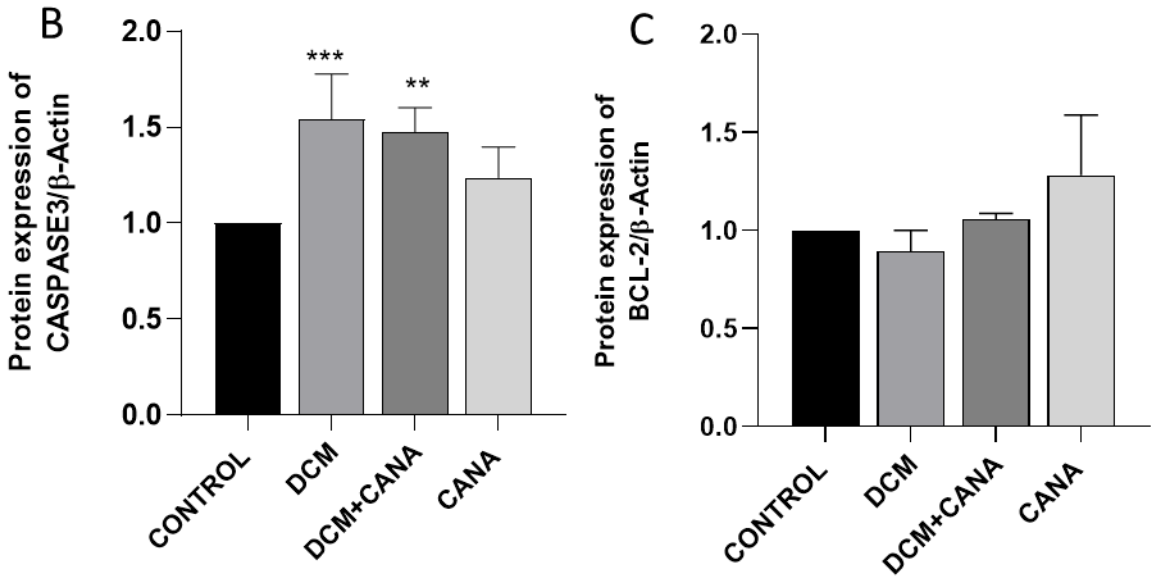
Figure 4. 4. Representative images of effect of Canagliflozin on fibrosis in diabetic heart

Fig 4.4. Representative images of the myocardial tissue sections stained with picro sirius red, where DCM group showed remarkable collagen deposition (yellow arrow), whereas canagliflozin treatment had ameliorated the Sirius red positive collagen deposition indicating decreased fibrosis (A). Myocardial Protein expression of fibrotic markers TGF-β (B, D) and α-SMA (C, E) using western blot. mRNA expression of TGF-β from myocardial tissue (F). Data were expressed as mean±SEM (where n=4) and analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance. , where ***P<0.001, Vs respective control, $^{@@@}$ P<0.001 Vs DCM.

4.3.6. Canagliflozin protects the diabetic heart by limiting apoptosis:

Apoptosis plays a vital role in the progression of DCM(217). Diabetes upregulated the cleaved caspase3 expression significantly, which is the executioner protein in the caspase cascade, anyhow Canagliflozin treatment showed the apparent downregulation of caspase3 (A, B). Furthermore, diabetes also tended to down-regulate the anti-apoptotic BCL2 expression demonstrated by immunoblotting, whereas Canagliflozin treatment improved the expression of BCL2 (A, C). Immunoblotting also revealed the apparent upregulation of P-JNK in DCM group, which was blunted by Canagliflozin treatment (D, E). In consistent with these results, quantitative RT-PCR also revealed a significant increase in caspase-3 expression in DCM group, which was limited by canagliflozin treatment (F).




B
Protein expression of CASPASE3/β-Actin
2.0
1.5
1.0
0.5
0.0
***
**
CONTROL
DCM
DCM+CANA
CANA
C
Protein expression of BCL-2/β-Actin
2.0
1.5
1.0
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CONTROL
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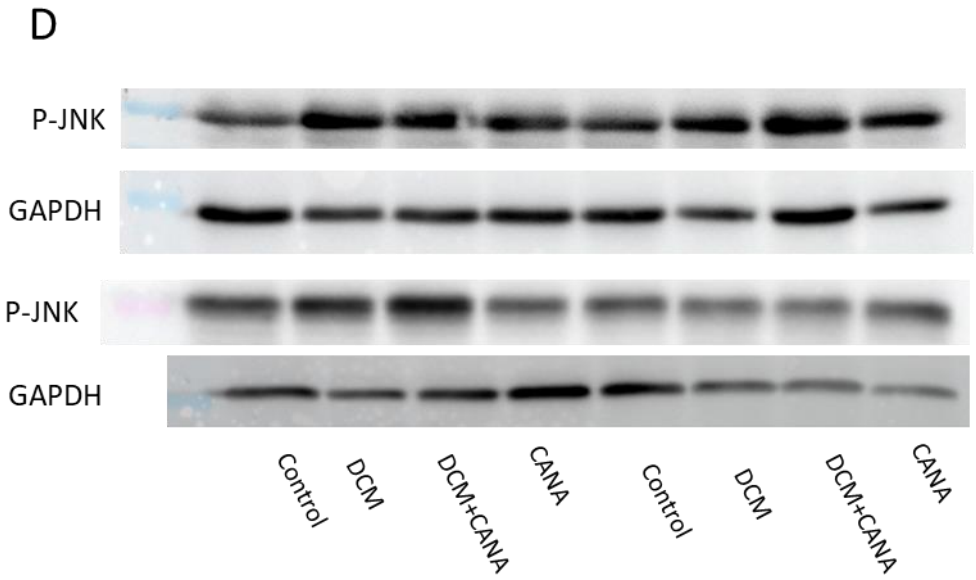
DCM+CANA
CANA


D
P-JNK
GAPDH
P-JNK
GAPDH
Control
DCM
DCM+CANA
CANA
Control
DCM
DCM+CANA
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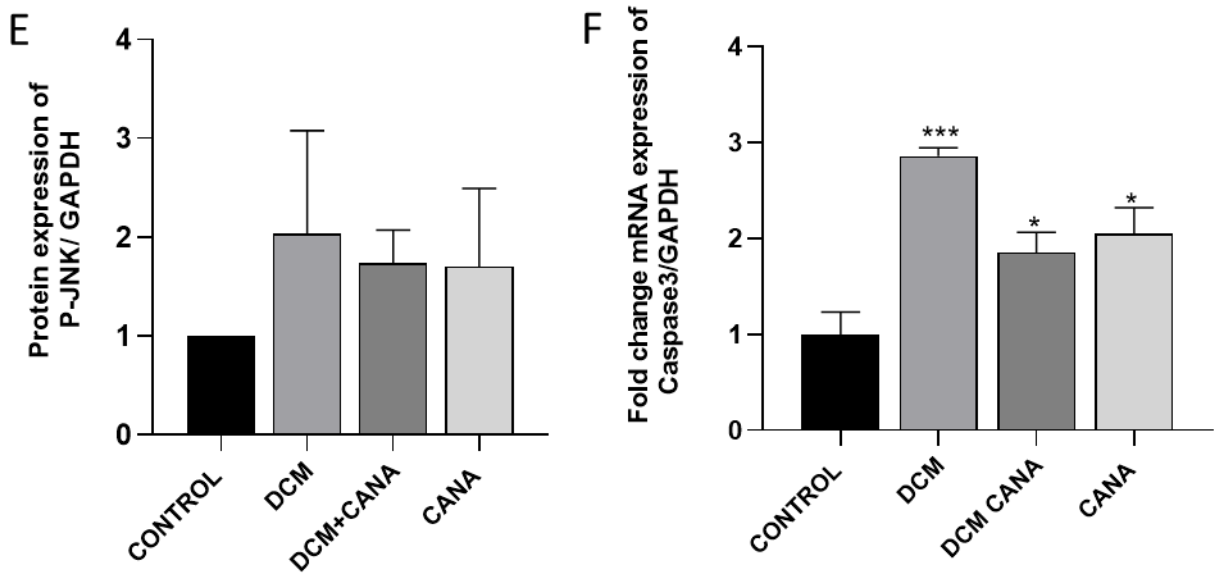


Figure 4. 5 Protein and mRNA expression of apoptotic markers

Fig 4.5. Protein expression of apoptotic marker Caspase-3, pro-survival BCL-2 (A, B, C), and P-JNK (D, E) using western blot,. mRNA expression of caspase3 (F). Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where *P<0.05, **P < 0.01, ***P<0.001, Vs respective control.

4.3.7. Canagliflozin improves mitochondrial energetics by modulating myocardial ion channels:

Since SGLT-1 inhibition also inhibits sodium transport, which may alter the different ion gradients inside the cell, we further investigated the different ion channel expressions in myocardial tissue to correlate the sodium gradient to myocardial protection of SGLT1 inhibitors. Our results revealed the upregulation of NCX-1, NHE-1 in DCM group which was blunted by canagliflozin treatment (4.7C and 4.7D), however, Na^+ K^+-ATPase expression tended to decrease in DCM, which was normalized with CANA treatment. It is evident that the ion gradient strongly affects mitochondrial biogenesis and also the diabetic

heart attributed by mitochondrial dysfunction, we further investigated the mitochondrial function by measuring mitochondrial membrane potential and mitochondrial mass in cardiomyocytes. TMRE-stained cardiomyocytes showed reduced mitochondrial membrane potential demonstrated by fluorescent microscopy (fig.4.6) and flow cytometry (fig.4.7A,B)

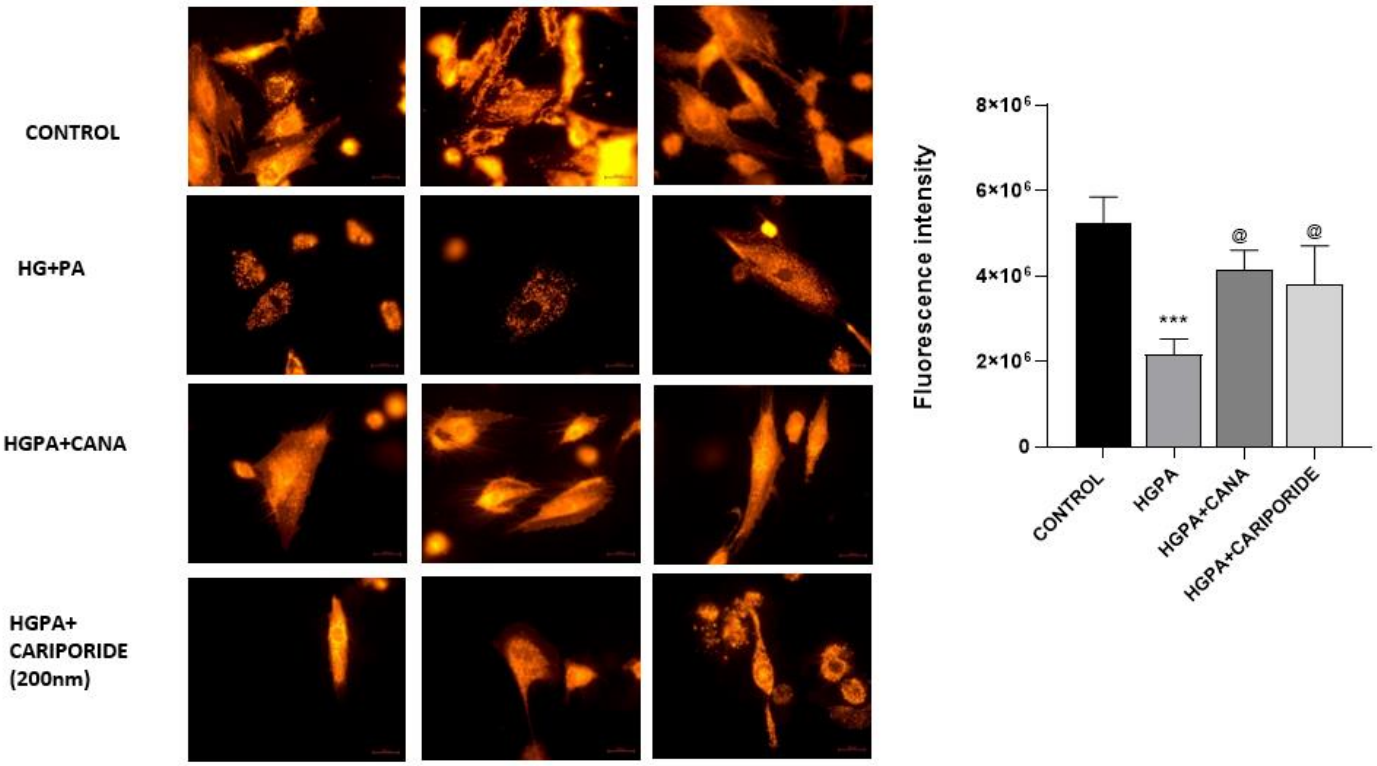


Figure 4. 6 Representative images of effect of canagliflozin on mitochondrial membrane potential.

Fig.4.6.Representative images of, the effect of canagliflozin on mitochondrial membrane potential. Fluorescent images of TMRE stained H9C2. Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where ***P<0.001 Vs respective control, $^{@}P < 0.05$ Vs HGPA.

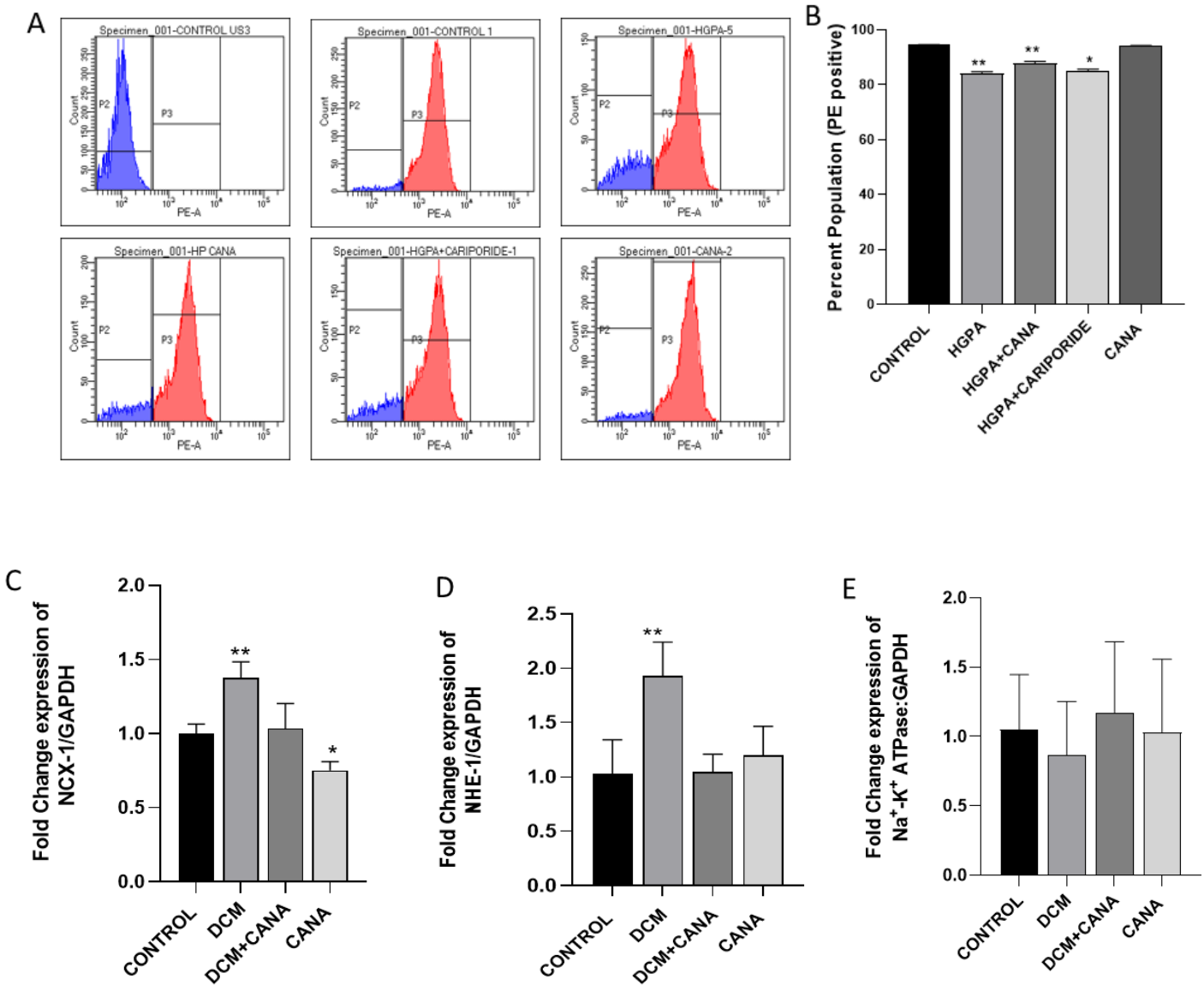


Figure 4. 8 Representative flowcytometry images of mitochondrial membrane potential and ion channel expression

Fig.4.7 Representative flowcytometry images of, the effect of canagliflozin on mitochondrial membrane potential using TMRE. (A, B). Representative mRNA expression of various ion channels in the myocardium (C) NCX-1 (Sodium calcium exchanger-1) ,(D) NHE-1 (Sodium hydrogen exchanger-1), (E) Na^+ K^+-ATPase (Sodium Potassium ATPase)

Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where *P < 0.05, **P<0.01, Vs respective.

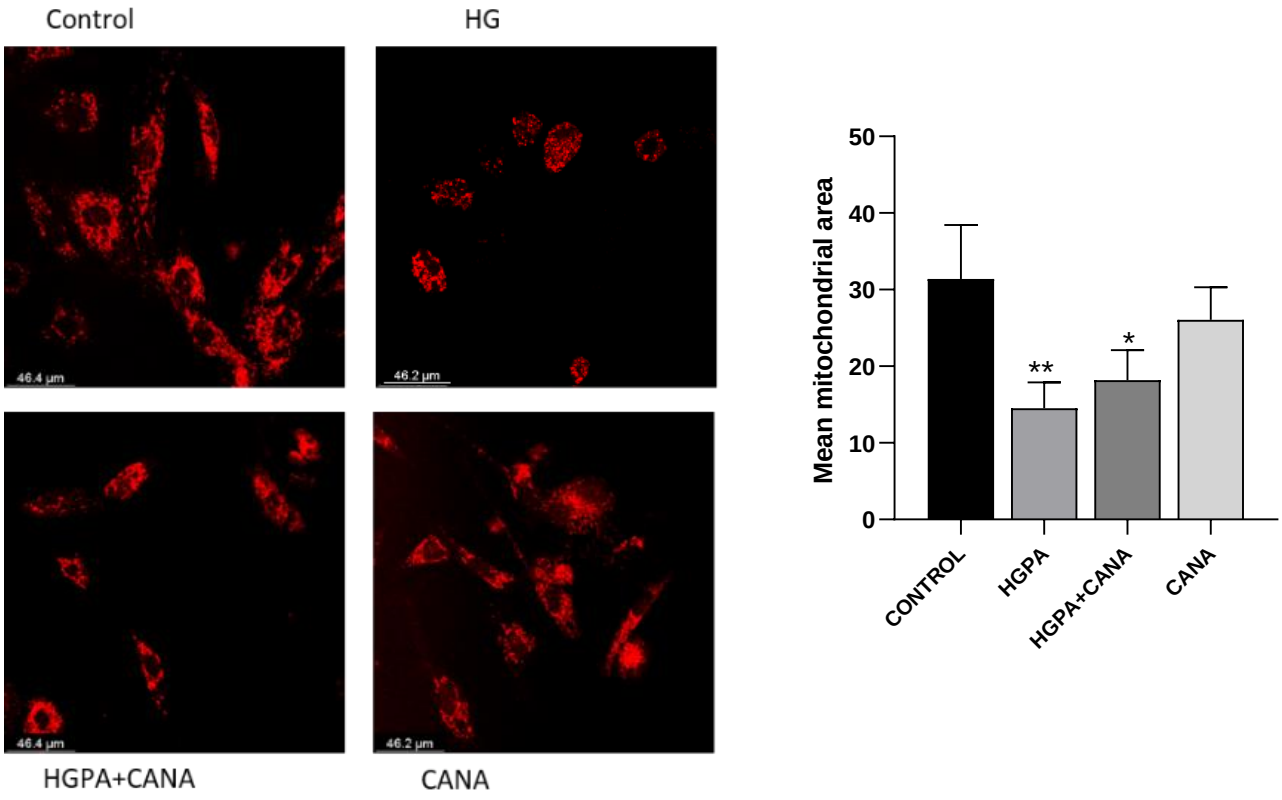


Figure 4. 9 Confocal images of H9C2 stained with MitoTracker® Red

Fig.4.8. Confocal images of H9C2 stained with MitoTracker® Red representing the mitochondrial morphology and the mean mitochondrial area. Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where *P<0.05, **P < 0.01, Vs respective control.

4.4. Discussion:

The principal objective of this study was to investigate the molecular mechanisms behind positive cardiovascular outcomes of canagliflozin in the diabetic heart. It is already evident that canagliflozin protects the diabetic heart by modulating hyperglycaemia, insulin resistance, body weight, visceral adiposity, blood pressure, lipid profile, and kidney function from the large clinical trial, CANagliflozin cardioVascular Assessment Study (CANVAS) Program(222). However, the underlying molecular mechanisms are yet to be investigated in conjunction with reversing the cardiac remodelling and improving cardiac function. In the present study, we have evaluated the cardiovascular benefits of canagliflozin in the most clinically relevant HFD and low STZ induced type-2 diabetic model of diabetic cardiomyopathy (DCM) in male Wistar rats. Our experimental results reveal that canagliflozin attenuates cardiac hypertrophy, and interstitial fibrosis secondary to hyperglycaemia and also re-establish mitochondrial biogenesis and preserves mitochondrial mass.

Insulin resistance is the preliminary characteristic of type-2 diabetes(221). We have successfully induced insulin resistance which was significantly reversed after canagliflozin treatment. Numerous clinical studies have already reported that canagliflozin improves insulin sensitivity by attenuating visceral adiposity, lipotoxicity-induced beta-cell damage, and also adipocyte-mediated inflammation in obese type-2 diabetic patients(223,224). However, canagliflozin moderately reduced the body weight of rats compared to the control group, whereas the body weight of diabetic rats was drastically reduced compared to the diabetic group treated with canagliflozin, which is a most likely case in type-2 diabetes(225,226). Furthermore, serum triglycerides, total cholesterol, and LDL levels were also attenuated with canagliflozin treatment in DCM rats, whereas HDL levels were re-established.

Banerjee et al reported that diabetic hearts show two-three fold upregulation of SGLT-1 in humans and mice(168). Indeed, our findings also revealed a significant upregulation of SGLT-1 in the DCM group demonstrated by immunofluorescence and quantitative RT-PCR results. Recent studies reported that specific SGLT-1 inhibition by mizagliflozin ameliorates cardiomyocyte apoptosis and improves the diastolic function in DCM(157). Similarly, novel SGLT-1 inhibitor KGA-2727 also mitigated ischemic cardiomyopathy by attenuating cardiac remodelling demonstrated by left anterior descending coronary artery ligation (LAD) in mice through inhibiting cardiac hypertrophy and fibrosis(227). Furthermore, Sun Z et al, reported that SGLT-1 knockdown in diabetic mice attenuates the inflammation-induced myocardial damage and pyroptosis in cardiomyocytes attributed to glycaemic variability of type-2 diabetes(228). Despite these findings, Bode D et al, revealed that dual SGLT1/2 inhibition by sotagliflozin ameliorates the left atrial cardiomyopathy in a leptin receptor mutated ZSF-1 obese rat model well known for Heart failure with preserved ejection fraction HFpEF along with improved calcium handling and mitochondrial function(229). Hence, in the current study, we explored the cardioprotective effect of canagliflozin in correspondence with SGLT-1 inhibition as canagliflozin has higher selectivity towards SGLT-1 amongst glifozins (empaglifozin: 2680-fold, dapaglifozin: 1242-fold, canaglifozin: 155-fold selective to SGLT-2 over SGLT-1)(229).

Left ventricular hypertrophy is a clinical attribute of DCM. Hyperglycaemia, oxidative stress, inflammation, activated RAAS, AGEs, and hyperinsulinemia mediate cardiac tissue damage and remodelling through PKC, which mediates the activation of fibroblast and extracellular matrix deposition. Nevertheless, the above-mentioned clinical attributes also upregulate ANP, and BNP which tend to cardiac remodelling(230). To demonstrate, the degree of myocardial damage and hypertrophy in DCM, we stained the myocardial sections with H&E stain. The results revealed that DCM induced severe

myocardial damage with hypertrophic myofibrils associated with the upregulation of BNP demonstrated by quantitative RT-PCR results. Similarly, unstained tissue sections also showed tissue damage with no intercalated discs in the DCM group. These results are consistent with our previous results, where canagliflozin ameliorated high glucose and palmitate-induced hypertrophy in cultured rat cardiomyocytes(217). Deposition of extracellular matrix proteins, predominantly collagen makes the myocardium more vulnerable to DCM progression in setting to hyperglycemia(231). Sun- Pengbo et al demonstrated canagliflozin abolished hypertrophy and fibrosis in HFD/STZ induced type-2 diabetic mice(232). Consistent with these results, our findings also revealed that canagliflozin attenuates fibrosis in DCM group demonstrated by the picrosirius red stain. Similarly, canagliflozin treatment abolished TGF-β and α-SMA expression in the DCM induced group.

Hyperglycaemia plays a critical role in inducing myocardial apoptosis followed by cardiac remodelling attributed to diabetic cardiomyopathy(233). Therefore, in the current study, we also explored apoptotic markers in conjunction with DCM progression. Our findings revealed the upregulation of caspase3, P-JNK, and reduced expression of pro-survival marker Bcl-2 in DCM-induced rats. Intriguingly, canagliflozin inhibited apoptosis by attenuating the expression of these genes. A similar trend was observed in our previous studies, where high glucose and palmitate-induced cardiomyocyte apoptosis, mediated through caspase-3 which was blunted by canagliflozin(217). Lu Cai et al had reported previously: hyperglycaemia induces apoptosis in mouse myocardium by caspase-3 activation(74). Recent studies also suggest that SGLT-1 inhibition protects the diabetic heart via modulating P-JNK, Bcl-2, and Caspase3 expression(157).

It is well-reported that metabolic disorders adversely affect mitochondrial function and biogenesis. Type-2 diabetes also notably impairs mitochondrial function through

hyperglycaemia-induced ROS, which readily damages the cell by reacting with DNA and cellular proteins(221). To understand the cardioprotective effect of canagliflozin, we investigated the mitochondrial membrane potential in rat cardiomyocytes treated with high glucose and palmitate using TMRE dye. Indeed, HG+PA-treated cells showed decreased mitochondrial membrane potential which was re-established with canagliflozin treatment. Consistent with this, Wei Dan et al, reported canagliflozin also improves mitochondrial function via PPARα in adipocytes(234). Alike, Mitochondrial quality is also an attribute to mitochondrial function(221), we have investigated the mitochondrial integrity, area using mitotracker red. Interestingly, HG+PA reduced the mitochondrial area, whereas canagliflozin limited glucolipotoxicity on mitochondria. More recently it has become debatable that SGLT-2 inhibitors act via inhibiting membrane NHE-1 associated with decreased Na^+ input, consequently decreased cytosolic Ca^{+2} and increased mitochondrial Ca^{+2}, which ultimately improves mitochondrial energetics(235–237). To investigate whether NHE-1 inhibition contributes to mitochondrial health, we further treated cells with specific NHE-1 inhibitor cariporide. Intriguingly, the HG+PA+Cariporide group showed a relatively similar effect to the HG+PA+CANA group. Thus, we speculate that canagliflozin not only protects the myocardium via inhibiting glucose transport but also via modulating other ion transporters of the myocardium. Therefore, here we have measured the expression of different ion channels in myocardial tissue. Interestingly, the expression of NHE-1, NCX-1 was upregulated and Na^+-K^+ATPase expression was decreased in DCM, whilst canagliflozin treatment inhibited NHE-1 and also normalized NCX-1 and Na^+-K^+ATPase expression. Numerous studies revealed that SGLT-2 inhibitors could benefit the myocardium by blocking NHE-1 receptor in a glucose-independent manner. Ye Y et al, reported dapagliflozin inhibits NHE-1 in cardiofibroblasts modulated by LPS-induced pro-inflammatory state(238). Another study revealed empagliflozin also abolish Ang-II induced

hypertrophy in cardiomyoblasts by inhibiting NHE-1(239). However, these studies do not demonstrate the effect of NHE-1 inhibition under hyperglycaemia.

4.5. Conclusion:

From our findings, it is evident that SGLT-1 is upregulated in the diabetic heart that plays a significant role in disease progression and cardiac remodelling attributed by cardiac hypertrophy, fibrosis, and mitochondrial dysfunction. Our study proves that Canagliflozin benefits diabetic hearts by attenuating deleterious effects of hyperglycaemia and also partly by modulating ion channel expression. Therefore, we suggest specific SGLT-1 inhibition in the heart could be a novel therapeutic target for diabetes-related cardiovascular abnormalities.

Chapter 5: Specific SGLT-1 inhibitor KGA-2727 ameliorates Diabetic cardiomyopathy by mitigating cardiac hypertrophy and fibrosis

5.1. Introduction:

Hyperglycemia is the major attribute of diabetes mellitus, which ultimately causes vascular and tissue damage (240). Hence treatments targeting excess glucose reabsorption have grown as a promising strategy. Sodium-glucose co-transporters (SGLTs) are the membrane proteins involved in the transport of various substrates like sugars, some ions, vitamins, inositols, Lactate, Choline, urea, proline, and amino acids majorly across the apical and basolateral membrane of the lumen in the kidney and small intestine. SGLT-1 and SGLT-2 are the major isoforms among 12 different isoforms of SGLTs which actively transports sugars across cell membranes in conjunction with sodium transport. SGLT-1 discovered in 1987 by expression cloning is primarily responsible for glucose-galactose absorption in small intestine and minute reabsorption of filtered glucose in renal tubule, whereas SGLT-2 was discovered in 1994 and is principally expressed in proximal convoluted tubule responsible for 90% glucose reabsorption in the kidney. Considering these physiological roles of SGLT-1 and SGLT-2, drug discovery research focusing on transporters meets its rationale.

Phlorizin was the first dual inhibitor of SGLT-1 and SGLT-2 developed in 1987, shown to treat experimental diabetes in rats with partial pancreatotomies. In later years, Phlorizin was used as a lead molecule in the development of SGLT inhibitors, leading to the synthesis of SGLT-2 inhibitors, which were successfully introduced to the market in 2012 dapagliflozin being the first drug. It has been demonstrated that SGLT-2 inhibitors

lower the incidence of cardiovascular mortality, infarction, and stroke in patients with established atherosclerotic cardiovascular disease. However, treatment with SGLT-2 inhibitors lowered the likelihood of hospitalization for heart failure and the development of renal disease, regardless of the presence of atherosclerotic cardiovascular disease or heart failure. The fundamental goal of this technique is to lessen the body's burden of glucose by preventing dietary glucose from being absorbed in the intestine (through SGLT-1) and eliminating filtered glucose through the kidneys (by SGLT-2 and SGLT-1). In addition, there is convincing evidence that SGLT-2 inhibitors can regulate body weight, blood pressure, lipid profiles, endothelial functions, and cardiac output efficiency in addition to lowering blood glucose and glycated haemoglobin (HbA1c) levels. These actions have significant renal- and cardioprotective benefits that can lower the frequency of severe cardiovascular problems that are frequently linked to DM. conversely, SGLT-2 inhibitor's side effects, such as an increased risk of genitourinary infections, ketoacidosis, and bone fractures, have raised concerns. However, these oral diabetes medications were welcomed for their novel insulin-independent mechanism of action and attracted increasing attention, which led to the development of new derivatives. Later, it was shown that inhibiting the SGLT-1 cotransporter, which is primarily expressed in the intestine, can also play a significant role in glycaemic control by diminishing intestinal glucose absorption, as well as the increasing release of incretins by enteroendocrine cells, improving cellular response to insulin signalling through a variety of mechanisms. . This would also prevent the glycosuria-related side effects of SGLT-2 inhibitors, especially genital tract infections. These results served as additional inspiration for the development and testing of new SGLT-1 inhibitors to find novel antidiabetic options. Moreover, postprandial hyperglycaemia is a risk factor for diabetes-associated cardiovascular mortality and microangiopathy. Since SGLT-1 is primarily responsible for mediating glucose uptake from the small intestine,

treating postprandial hyperglycemia with an SGLT-1 inhibitor would unquestionably be an effective treatment. KGA-2727, a selective n SGLT-1 inhibitor, alleviated postprandial hyperglycemia in diabetic rats after a single dosage, and its chronic treatment decreased hemoglobin A1c levels, indicating that SGLT-1 inhibition could sustain effective glycemic control over the long run. Plasma insulin levels and plasma glucose levels were decreased in an oral glucose tolerance test with KGA-2727, and beneficial effects on the pancreas are also anticipated. Considering these facts, we have further evaluated cardiovascular benefits of KGA-2727 in a high-fat diet and streptozotocin-induced type-2 diabetes-associated diabetic cardiomyopathy in male Wistar rats.

5.2. Methodology:

5.2.1. Chemicals:

KGA-2727 (HY-123797, 99.04% pure) was purchased from Life technologies india Pvt. Ltd, Commercial kits for assessment of total cholesterol (TC), triglycerides (TG'S), high-density lipoproteins (HDL), and low-density lipoproteins (LDL), were purchased from tulip diagnostics (P) Ltd, Mumbai, India, and Arkray Healthcare Pvt. Ltd, Surat, India. Streptozotocin (STZ), hematoxylin, eosin, Sirius red, Poly-L-Lysine coating solution, and all primers were purchased from Sigma Aldrich,(St. Louis, Missouri, United States). Antibodies for a-SMA, BNP, and GAPDH were purchased from Santa Cruz Biotechnology (CA, USA). SGLT-1 antibody was purchased from Abcam (Milton, Cambridge, UK), Immun-Blot® PVDFMembrane, Trizol reagent, iScrip cDNA Synthesis Kit and iTaqUniversal SYBR® Green Supermix were purchased from Takara.

5.2.2. Animals and Experimental Design:

All the proposed animal experimental protocols were approved by Institutional Animal Ethical Committee (Protocol approval number: BITS/Hyd/IAEC/2021/30). Male Wistar rats

of 5–6 weeks old were obtained and kept at our animal house facility with ad lib access to food and water. Animals were split into two groups. One group had a diet high in fat (HFD) and the other group received a regular pellet diet (NPD). To examine the insulin resistance in the HFD group, an oral glucose tolerance test (OGTT) and an intraperitoneal insulin test (IPITT) were carried out after 4 weeks. Streptozotocin was given intraperitoneally to rats with insulin resistance in a single dosage (35 mg/kg), while animals with NPD received a vehicle injection of citrate buffer (pH 4.4). Blood sugar levels and serum biochemical markers, including HDL (high density lipoprotein), LDL (low density lipoprotein), TGs (triglycerides), TC (total cholesterol), and LDH (lactate dehydrogenase), were assessed after a week. Animals showing presenting fasting blood glucose ≥250 mg/dl were considered diabetic.

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dl-Methionine	03
Yeast powder	01
Sodium chloride	01

Table 4: Composition of high fat diet

Animals with diabetes were kept on HFD for an additional six weeks. To validate the disease's course, serum biochemical markers were assessed. Additionally, animals from the HFD group

were divided into groups of diabetic, diabetic + KGA-2727 animals from the NPD group as Control. All groups were given respective treatments for four weeks. KGA-2727 was dissolved in a solvent mixture as 10% DMSO >> 40%PEG300 >> 5% Tween-80 >> 45% saline and given at a dose of 0.1 mg/kg which was selected from previous study (241). After the 12-week experimental period, the final OGTT and IPITT tests were conducted, and blood was drawn through the retro-orbital puncture. Rats were exsanguinated at the study's conclusion, following which their organs were harvested.

5.2.3. OGTT:

In summary, animals were given 2 gm/kg of glucose via oral gavage after 16 hours of fasting with adlibitum access to water. The Accu-check glucometer was used to take further blood glucose readings at intervals of 0, 15, 30, 45, 60, 90, and 120 minutes and area under curve was plotted using graphpad prism.

5.2.4. IPITT:

After a 6-hour fast, rats were injected an intraperitoneal injection of 0.75 IU/KG insulin, and blood sugar levels were checked at intervals of 0, 15, 30, 60, and 90 minutes and area under curve was plotted.

5.2.5. Histological analysis:

Freshly extracted tissue samples stored in 4% paraformaldehyde for overnight were dehydrated in gradient alcohols and xylene in an automated tissue processor. Further tissue was infiltrated in paraffin for 2hrs and embedded in paraffin. 4-5 µM serial sections were cut using a microtome from paraffin blocks.

5.2.6. H&E Staining:

4-5 µM thin paraffin-embedded tissue sections were deparaffinized and rehydrated with 2 changes of xylene and 100-70% alcohols for 2 min, then hydrated with dd H_2O. Following rehydration, sections were stained with hematoxylin for 30 sec, followed by washing and bluing. Now, sections were stained with eosin for 30 sec and dehydrated by 95%alcohol followed by 100% alcohol and xylene. Later sections were mounted with coverslips and images were taken using ZEISS AXIOLAB 5 Microscope.

5.2.7. SIRIUS RED staining:

Rehydrated tissue sections with a thickness of 4-5 µM were stained for 1 hour with Sirius red stain, then the excess stain was washed away with acidified water. Sections that had been stained were dehydrated and mounted on coverslips.

5.2.8. Immunoblotting:

Briefly, heart tissue samples were homogenized in RIPA lysis buffer using a bead homogenizer (Minilys Personal Homogenizer by Bertin Technologies), and their concentration was estimated by the Bicinconinic Acid assay. 40 µg of isolated protein was then loaded and separated on SDS-Poly Acrylamide Gel Electrophoresis at 70Mv, and the protein was then blotted onto PVDF membrane (bio-rad) at 70Mv for 90 min. The VILBER FUSION solo S western blot and chemi imaging system was used to identify and analyze the target protein.

5.2.9. Real Time-PCR:

The TRIzol (Invitrogen) technique was used to isolate total RNA. One gram of extracted RNA was reverse transcribed into cDNA, and real-time PCR was carried out using SYBR green chemistry in an iCycler iQ device. Rattus norvegicus gene sequences that have been published on NCBI were used to design primer sequences. Using Bio-rad CFX manager,

quantitative real-time PCR was analyzed. Data were quantified and expressed as fold change using the $2^{-\Delta\Delta CT}$ method.

Symbol	Forward primer	Reverse primer
NCX-1	AGTCTCCCACCCAATGTTTC	CTCCTGTTTCTGCCTCTGATC
NHE-1	GCCGTCTCAACTGTCTCTATG	ATCTCCTCCTCCTTGTCCTT
Na^+K^+ATPase	TCCCTACAGTCTCCTCATCTTC	TCAGTAGTACGTCTCCTTCTCC
SGLT-1	GTGTACGGATCAGGTCATTGT	CCATGAGGAACATAGGCAGTAG
TGF-β	CTTTAGGAAGGACCTGGGTTG	GTGTCCAGGCTCCAAATGTA

Table 5. Primer sequences

5.2.10. Statistical analysis:

Data were analyzed using GraphPad prism8 software and presented as Mean ± SEM applying one-way or two-way ANOVA followed by tukey's or dunnets post analysis test, data were considered statistically significant at $P< 0.05$.

5.3. Results:

5.3.1. Treatment with KGA-2727 reduces insulin resistance in type-2 diabetic Rats:

The clinical characteristics of type-2 diabetes include obesity and insulin resistance. In our work, higher AUC determined by OGTT and IPITT after 4 weeks of HFD in rats indicated insulin resistance (A and B). After intraperitoneal insulin injection, blood sugar spiked considerably, showing insulin resistance and impaired tissue glucose uptake in response to circulating insulin. When compared to the DCM group a 4-week therapy with 0.1 mg/kg of KGA-2727 dramatically increased insulin sensitivity (C and D).

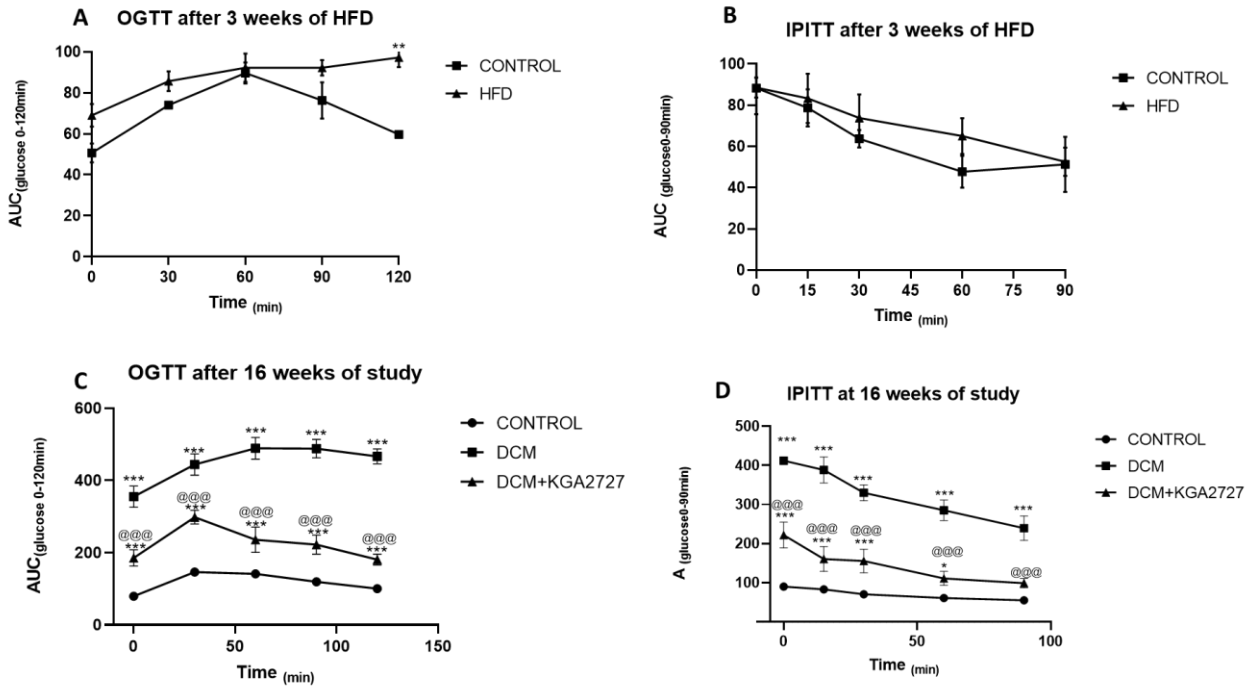


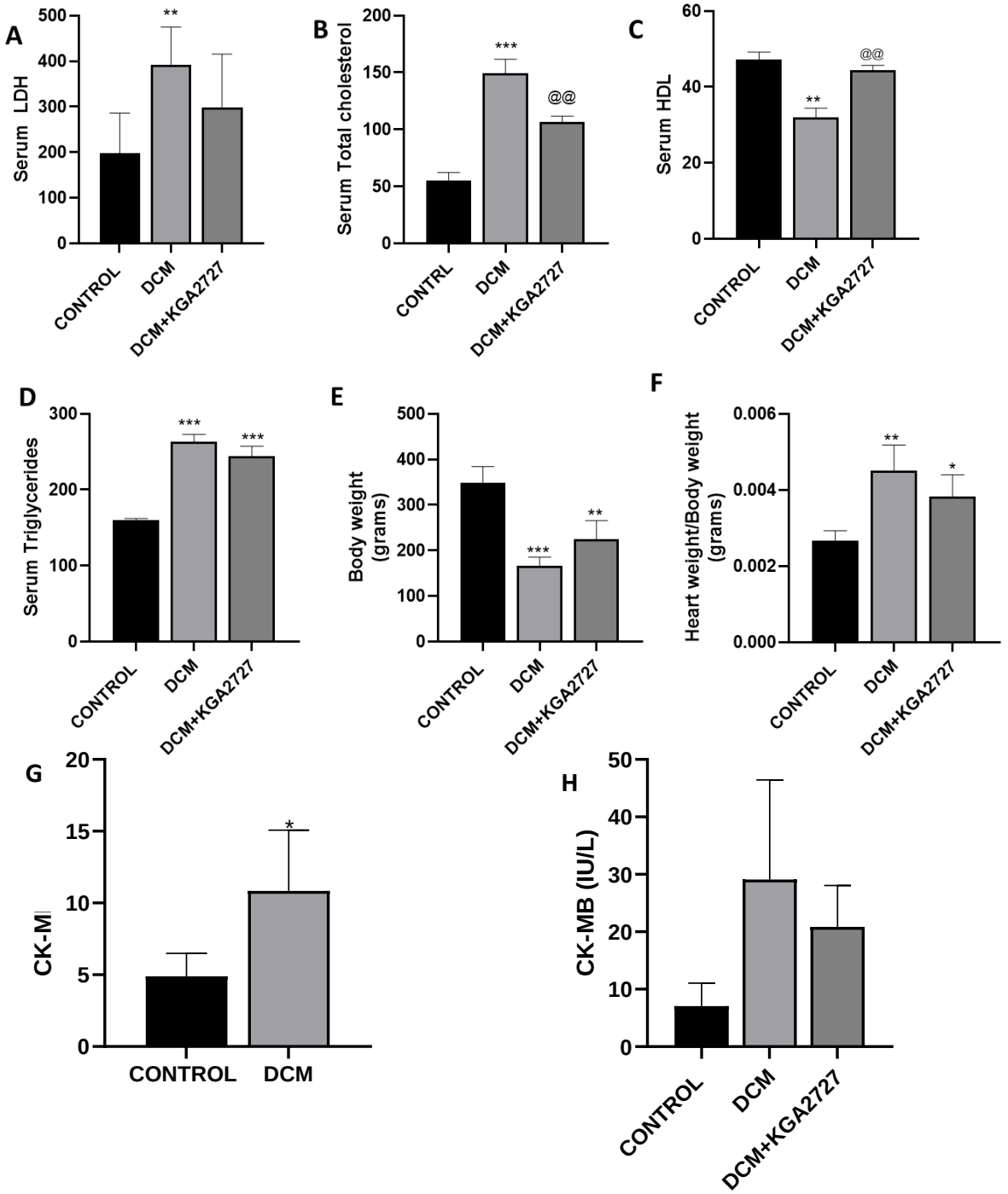
Figure 5.1 Representative Area under the curve (AUC) graphs for OGTT and IPITT

Fig 5.1. Area under the curve (AUC) graphs depicting the findings of the oral glucose tolerance test and intraperitoneal insulin tolerance test after 4 weeks on the HFD (A,B) and 8 weeks on the HFD plus 4 weeks of KGA2727 are (C,D) respectively. Data were presented as mean±SEM (where n = 6). Following a standard two-way ANOVA, Tukey's multiple comparison test was used to examine the data. Where, *P < 0.05,***P<0.001, Vs respective control, $^{@}$ P<0.05, $^{@@}$ P<0.01, $^{@@@}$ P<0.001, Vs DCM.

5.3.2. Systemic characteristics in DCM rats:

At the endpoint, DCM rats' body weight had drastically dropped while their cardiac weight had grown. The DCM group also had elevated serum LDH, triglycerides, total cholesterol, and lower HDL levels compared to the control. KGA-2727 treatment considerably normalized the serum parameters. In contrast to the aforementioned criteria, the DCM group's systolic, diastolic, and mean arterial blood pressure dropped significantly along with

heart rate; however, KGA-2727 treatment re-established systolic blood pressure and heart rate but diastolic blood pressure was further dropped in treatment groups.



CK-MB after 8 weeks of DM induction

CK-MB after treatment with KGA-2727

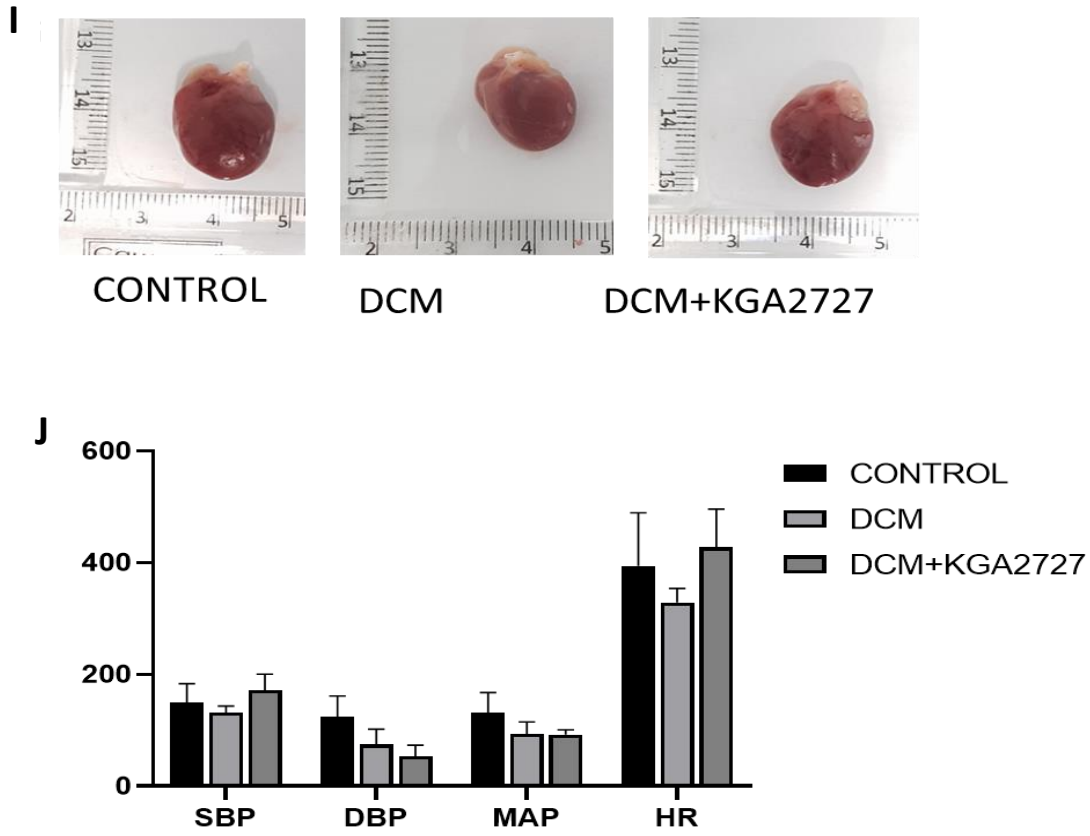


Figure 5.2 Representative images of Systemic characteristics in DCM rats

Fig.5.2.Representative bar diagrams of serum lipid profile illustrating LDH (A), Total cholesterol, (B), HDL (C), Triglycerides (D), Body weight (E), Heart weight to body weight ratio(F). CK-MB (G, H). And heart images representing increase in size (I), cumulative bar diagram of SBP, DBP, MAP heart rate (J). Data were expressed as mean±SEM (where n=4). Data were analysed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with single pooled variance, where, *P < 0.05, **P<0.01, ***P<0.001 Vs respective control, $^{@@}$ P<0.01 Vs DCM.

5.3.2. The SGLT-1 gene is upregulated in the diabetic heart:

The earlier study indicated that type-2 diabetes and ischemic hearts had upregulated SGLT-1. Similar results were seen in the current investigation, where immunoblot of DCM heart revealed significantly higher SGLT-1 expression compared to the control heart, while

the KGA-2727 treated group expressed significantly lower SGLT-1 expression compared to the DCM group (A). Similarly, increased mRNA expression of SGLT-1 in the DCM heart was seen, and this expression was blunted by KGA-2727 treatment (B).

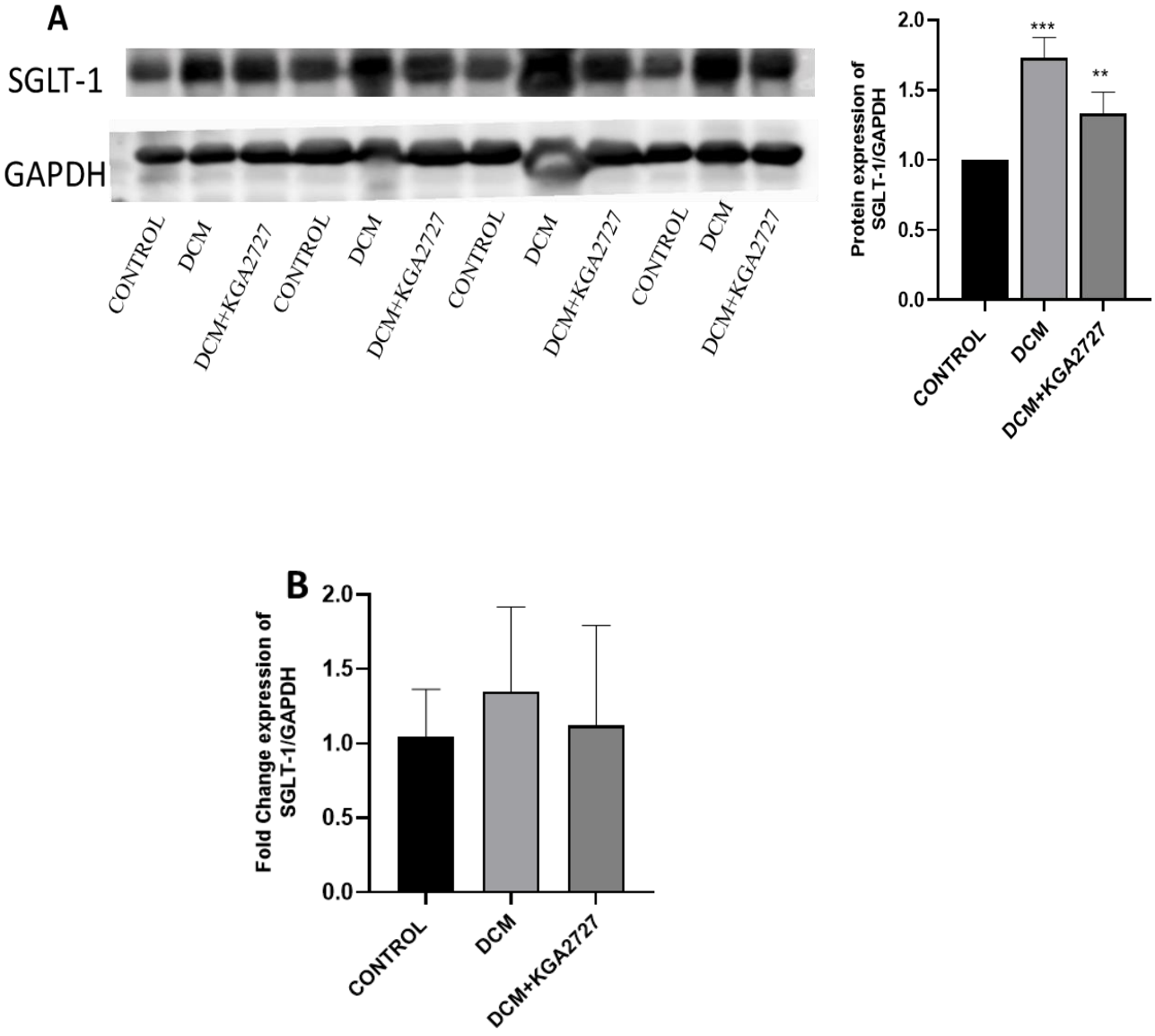
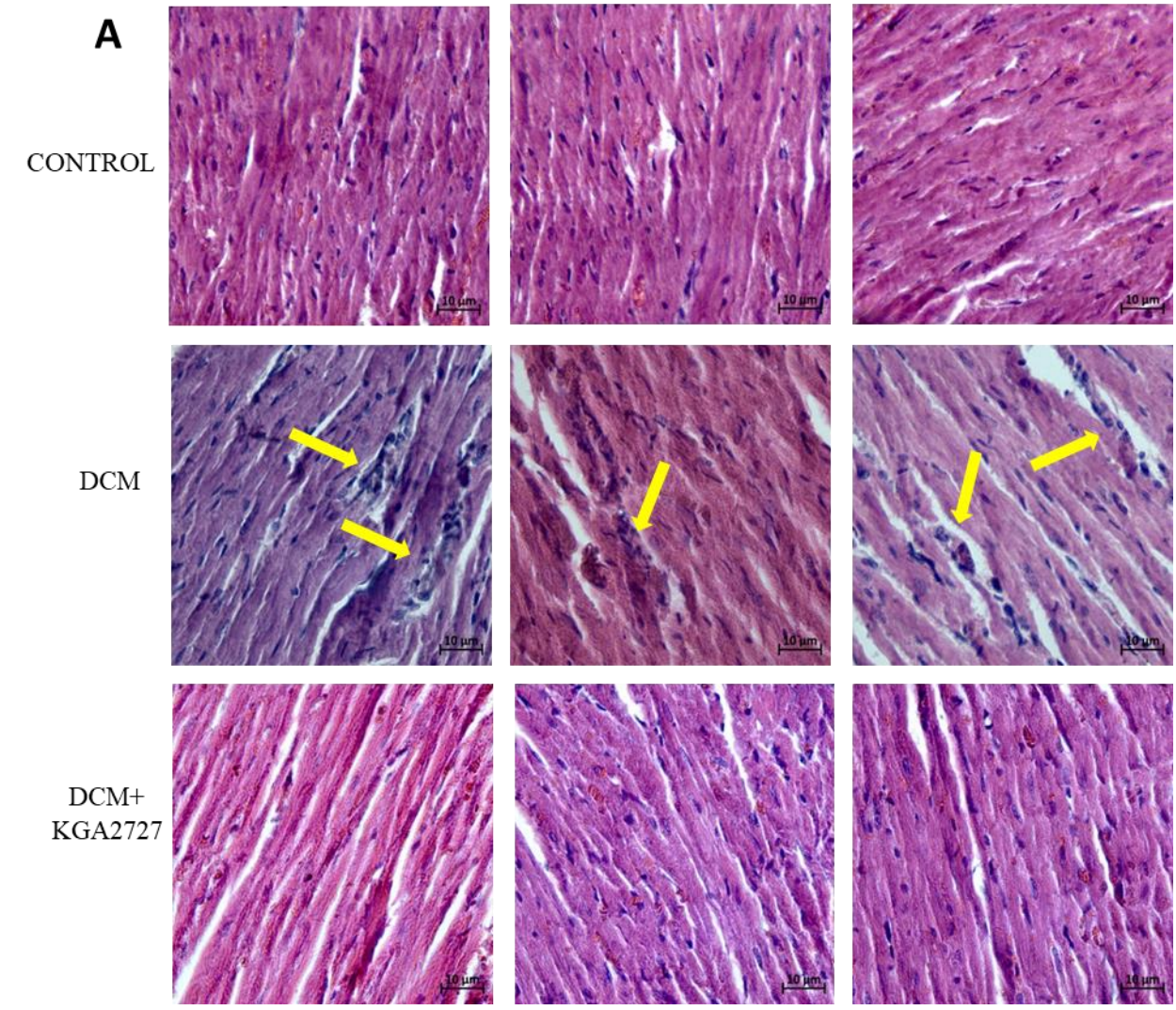


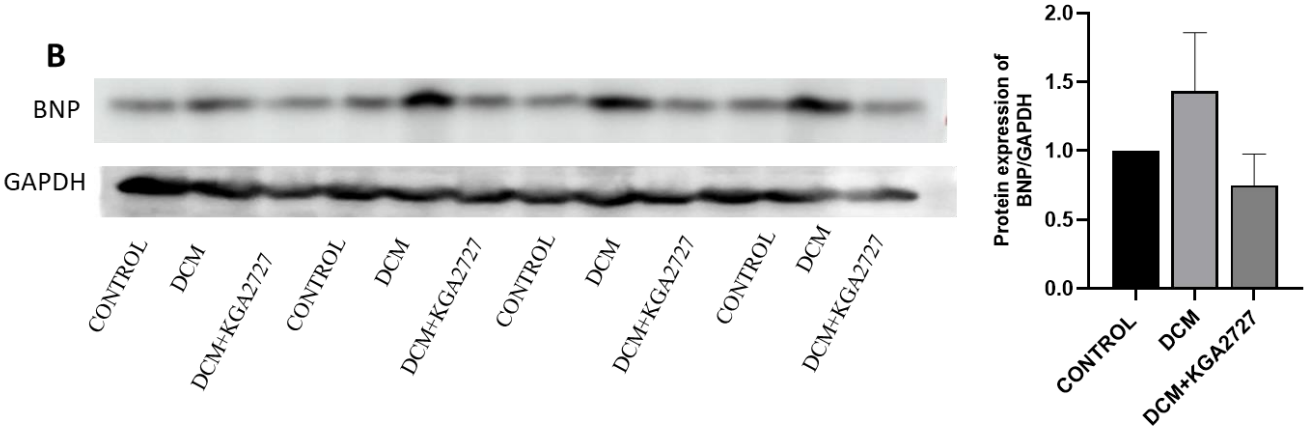
Figure 5.3 Representative images of SGLT-1 expression in myocardium tissue

Fig.5.3.Representative immunoblot of SGLT-1 expression in myocardium tissue using western blot (A), mRNA expression of SGLT-1 in myocardial tissue (B). Data were expressed as mean±SEM (where n=4). Data were analysed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with single pooled variance, where *P < 0.05, Vs respective control.

5.3.3. KGA-2727 mitigates cardiac remodelling and hypertrophy:

Myocardial remodelling, a key indicator of the evolution of DCM and contractile dysfunction, is mitigated by KGA-2727. In the current investigation, H&E staining, and hypertrophy marker analysis were used to assess the morphology of the myocardial tissue. The DCM group displayed severe tissue damage, including inflammatory cell infiltration, myofibrils with disordered, pyknotic nuclei and pronounced myofibril hypertrophy has been seen (A). Additionally, immunoblot and quantitative RT-PCR demonstrated the increased expression of BNP and caspase-3 expression which was reduced after KGA-2727 treatment (B, C)


A
CONTROL
DCM
DCM+
KGA2727
10 μm


B
BNP
GAPDH
CONTROL
DCM
DCM+KGA2727
CONTROL
DCM
DCM+KGA2727
CONTROL
DCM
DCM+KGA2727
CONTROL
DCM
DCM+KGA2727
Protein expression of BNP/GAPDH
2.0
1.5
1.0
0.5
0.0
CONTROL
DCM
DCM+KGA2727

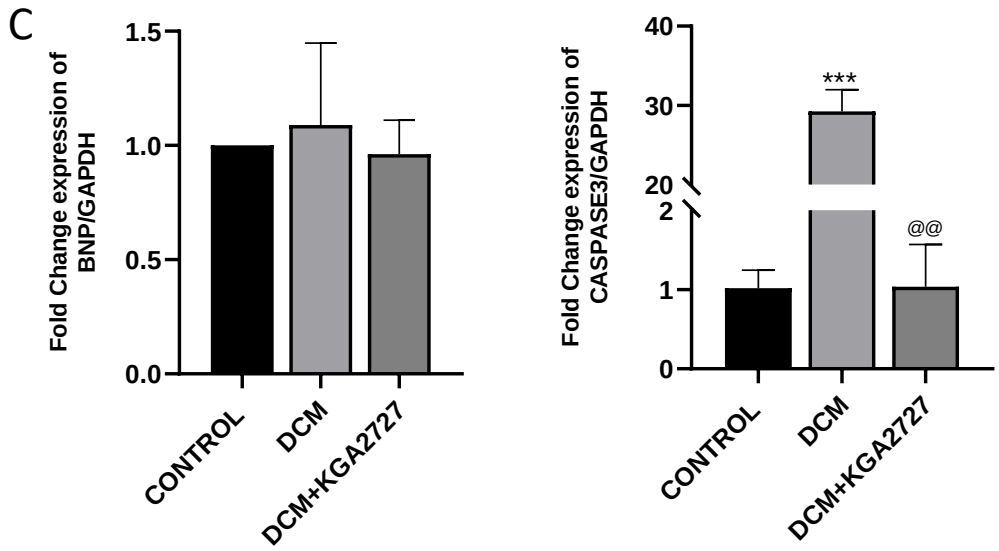


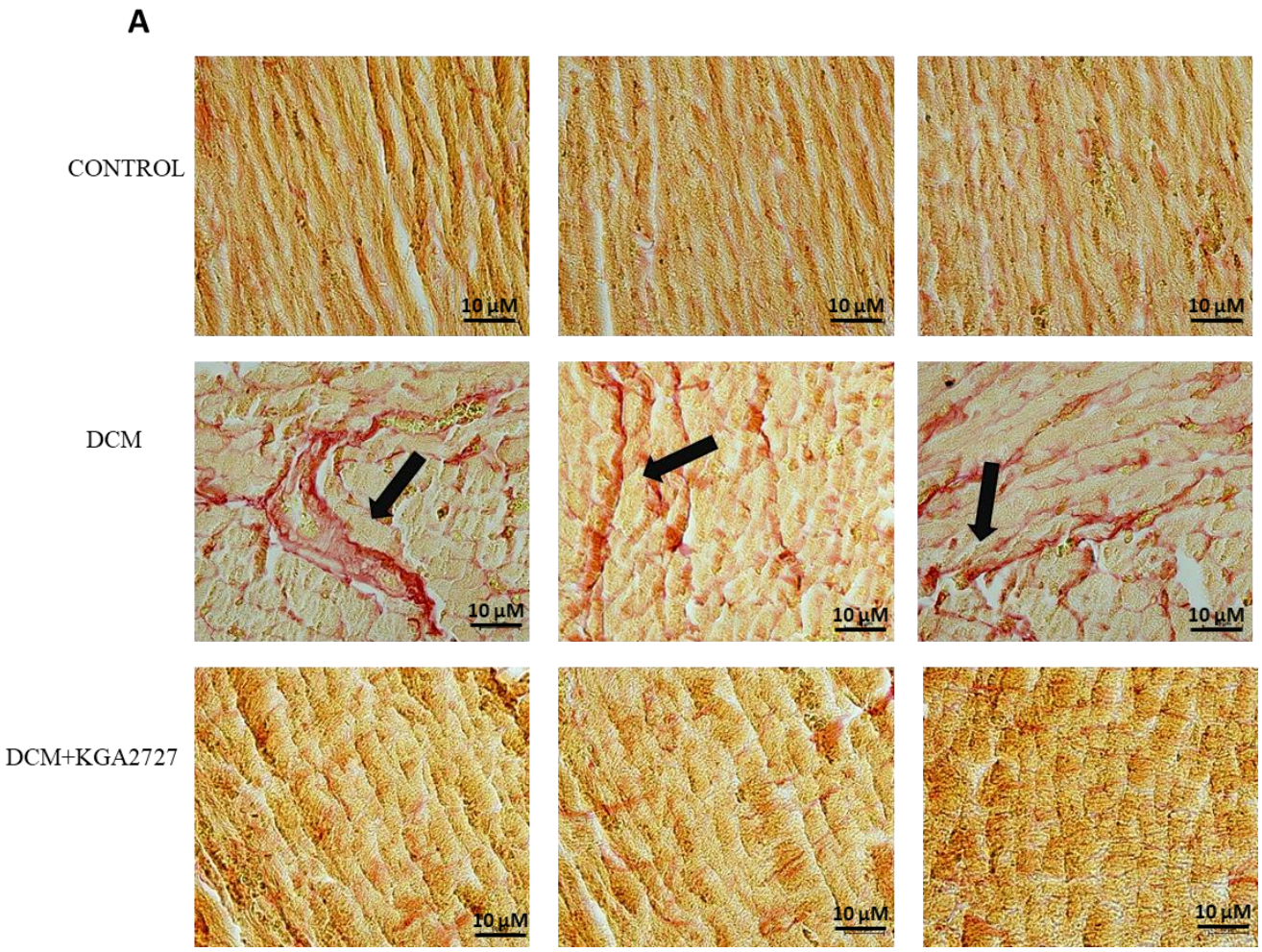
Figure 5.4 Illustrations of KGA2727's effects on cardiac remodelling and hypertrophy

Fig 5.4. Illustrations of KGA-2727's effects on cardiac remodelling as determined by histological analysis and H&E stain. Inflammatory cell infiltration in the DCM group, disordered myofibrils with pyknotic nuclei, interstitial edema, and hypertrophy are all represented by yellow arrows. Whereas KGA-2727 treatment enhanced the structural integrity of the myocardium (A). Immunoblot of BNP (B) expression along with qPCR has shown elevated BNP and caspase3 mRNA expression in cardiac tissue shown in (C). Data was expressed as mean ±SEM (where n=4) and evaluated using a standard one-way ANOVA before Dunnet's multiple comparison test utilizing a single pooled variance. , where ***P<0.001, Vs respective control, $^{@@}$ P<0.01 Vs DCM.

5.3.4. KGA-2727 ameliorates fibrosis in the diabetic heart:

H&E staining revealed myocardial damage from ongoing diabetes. As seen in Fig. 4A after the picrosirius red stain, this persistent injury caused interstitial collagen deposition, whereas KGA-2727 treatment prevented further collagen deposition (A). These results were further supported by increased α-SMA-protein, TGF-β mRNA and collagen mRNA levels,

which showed increased cardiac myofibroblasts (B, C). Curiously, KGA-2727 treatment reduced the expression of these proteins.



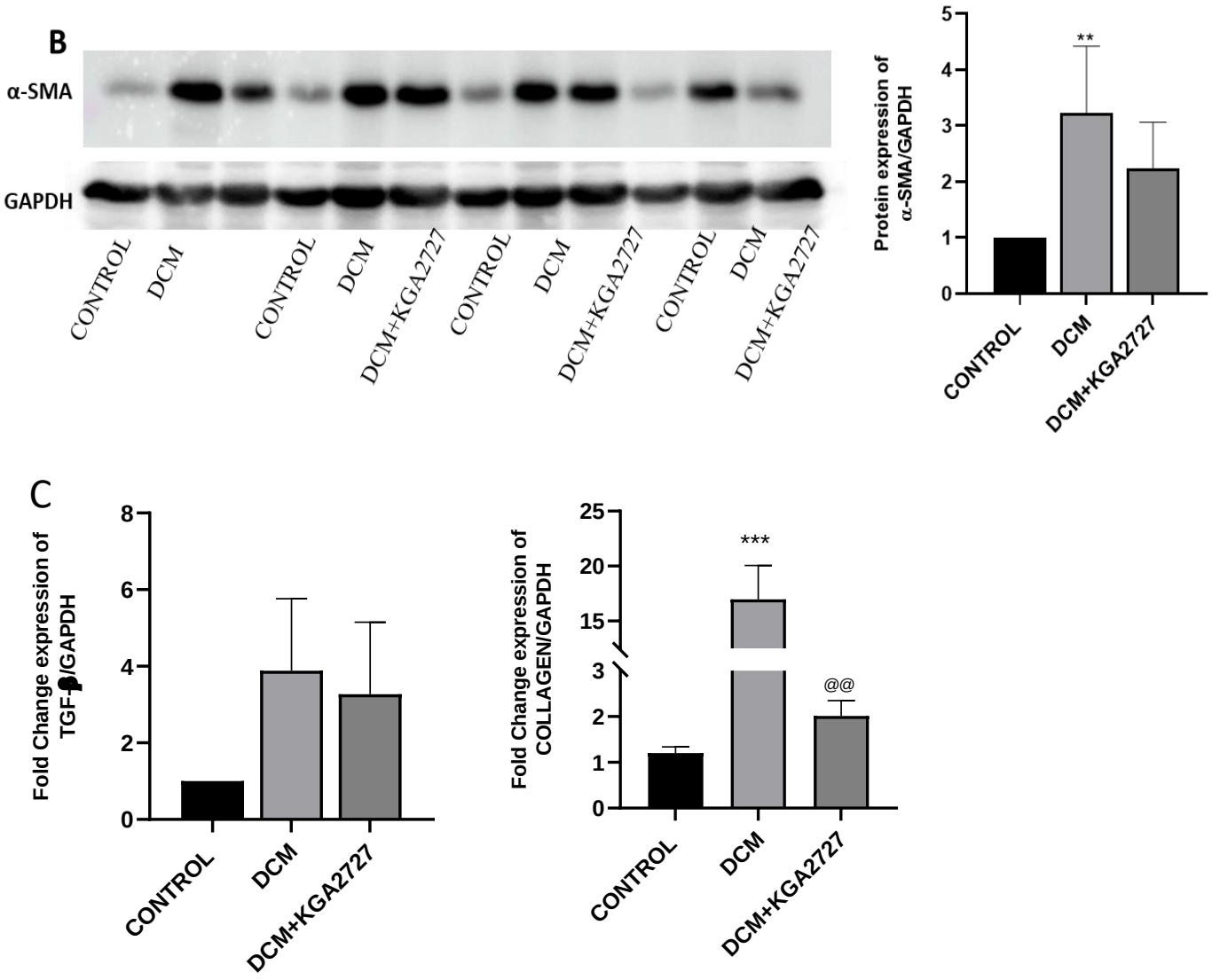


Figure 5.5 Effect of KGA-2727 on fibrotic markers

Fig 5.5. The DCM group displayed notable collagen deposition (black arrow) in representative images of the cardiac tissue sections stained with picrosirius red; however, KGA-2727 treatment had reduced the Sirius red positive collagen deposition, indicating decreased fibrosis (A). TGF-β and collagen mRNA expression from cardiac tissue and myocardial protein expression of fibrotic markers α-SMA were demonstrated by qPCR and western blot (B, C). Using a standard one-way ANOVA and Dunnett's multiple comparison test with a single pooled variance, the data were reported as mean±SEM (where n=4). Data

were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where **P < 0.01, ***P < 0.001 Vs respective control and $^{@@}$ P<0.01 Vs DCM.

5.3.5. KGA-2727 inhibits myocardial NHE-1 in diabetic cardiomyopathy:

The upregulated NHE-1 in diabetic cardiomyopathy leads to increased sodium concentrations in the myocardium, this increased sodium in the myocardium stimulates calcium release from mitochondria and interferes with mitochondrial energy production. KGA-2727 inhibited NHE-1 to abnormally lower levels (A), whereas NCX-1 was not altered much (B). Furthermore, Na^+ K^+-ATPase was normalized to normal levels after KGA-2727 treatment in DCM.

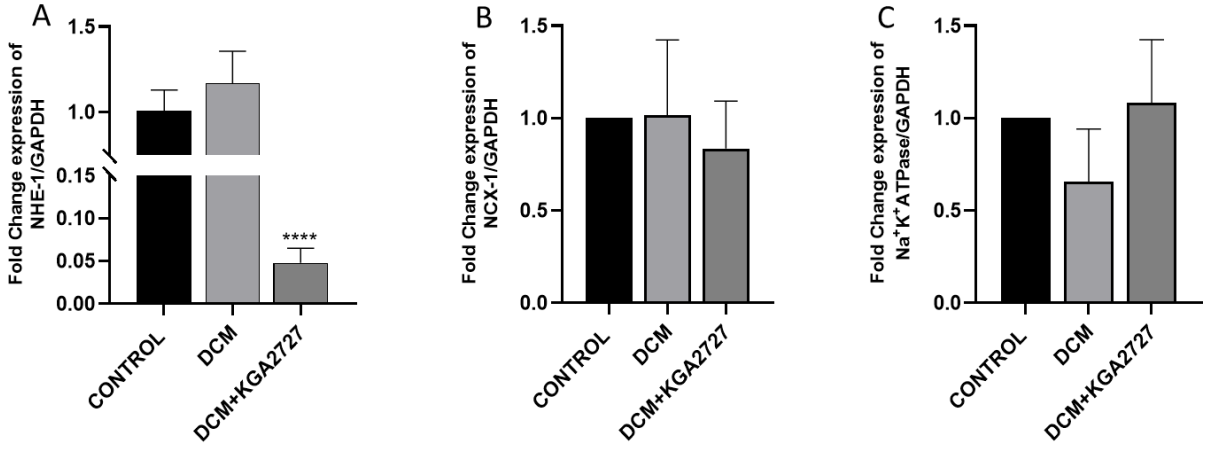


Figure 5.6 KGA-2727 inhibits myocardial NHE-1

Fig 5.6 Representative mRNA expression of various ion channels in the myocardium (A) NHE-1 (Sodium hydrogen exchanger-1),(B) NCX-1 (Sodium calcium exchanger-1), (D) Na^+ K^+-ATPase (Sodium Potassium ATPase) Data were expressed as mean±SEM (where n=4). Data were analyzed using ordinary one-way ANOVA followed by Dunnet's multiple comparison test with a single pooled variance, where ****P < 0.0001 Vs respective control.

5.4. Discussion:

Under normal physiological conditions, SGLT-1 is mainly responsible for dietary glucose absorption from the brush border membrane of small intestine and approximately 3% of filtered glucose reabsorption from the late proximal convoluted tubule of the kidney. However, in diabetic conditions, SGLT-1 role is considered detrimental along with SGLT-2, since there is overexpression of SGLT-1 in vital organs like the heart and kidney which mainly contributes to target organ remodelling as seen in diabetic cardiomyopathy and chronic kidney disease respectively(242,243). In the current study, we aimed to inhibit specific SGLT-1 in a high-fat diet and low-dose streptozotocin-induced type-2 diabetes-associated diabetic cardiomyopathy. Our findings revealed that SGLT-1 is upregulated in cardiac tissue along with significant myocardial tissue remodelling attributed to ventricular hypertrophy and fibrosis. Conversely, specific SGLT-1 inhibition by KGA-2727 has reduced pathological remodelling by attenuating myocardial apoptosis, hypertrophy, and fibrosis. These findings indicate pharmacological interventions targeted at specific SGLT-1 have a profound beneficial effect on diabetic cardiomyopathy.

Type-2 diabetes' initial defining feature is insulin resistance. We were able to successfully induce insulin resistance demonstrated by an oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT). Moreover, Addressing insulin resistance or hyperinsulinemia might be a valid therapeutic strategy to blunt the development of diabetic cardiomyopathy because both conditions independently promote diabetic cardiomyopathy. Insulin resistance shifts the cardiac substrate utilization from glucose to fatty acids, where excessive fatty acid β-oxidation ultimately increases oxidative stress and excessive buildup of fatty acids in the myocardium causes lipotoxicity and limits the normal physiological autophagy, which alters the morphology and structure of the heart tissue and compromises myocardial function(244,245). Intriguingly, KGA-2727 treatment

has significantly lowered the AUC of OGTT and IPITT performed at the end point of treatment resulting in reduced insulin resistance.

In conjunction with insulin resistance, Patients with T2DM have elevated free fatty acids and triglycerides due to increased lipogenesis in hepatocytes and elevated lipolysis in adipocytes. consequently, lipotoxicity can accelerate cardiomyocyte death and directly impair myocyte metabolism and contractility through increased ROS production and endoplasmic reticulum stress(162,246). Hence, the serum lipid profile has a characteristic impact on diabetic cardiomyopathy. In our study, we observed elevated serum TGs, TC, and reduced HDL in the diabetic group, whereas the KGA-2727 treatment normalized the total cholesterol, triglycerides, and HDL levels. These findings were consistent with a previous study, where dual SGLT-1/2 inhibitor phlorizin attenuated diabetic cardiomyopathy by modulating fasting blood glucose, serum TGs, TC, and improved cardiac energy metabolism(247). Furthermore, systolic blood pressure (SBP), diastolic blood pressure (DBP), Mean arterial pressure (MAP), and heart rate (HR) were considerably reduced in diabetic animals, whereas KGA-2727 treatment has improved SBP and HR but not the DBP and MAP. These findings are contradictory to previous studies on diabetic cardiomyopathy, where blood pressure and HR were significantly elevated(247). In this scenario, we speculate that diabetic autonomic neuropathy could be a possible reason for bradycardia and conduction abnormalities(247).

In both humans and animals, diabetic hearts exhibit a two- to three-fold increase in SGLT-1 expression. In fact, the DCM group showed a considerable overexpression of SGLT-1, which was shown by the results of immunoblotting and quantitative RT-PCR. Recent investigations have shown that mizagliflozin's targeted SGLT-1 inhibition reduces cardiomyocyte death and enhances diastolic function in DCM. Moreover, KGA-2727 also reduced the severity of ischemic cardiomyopathy by reducing cardiac remodelling as seen

in mice after left anterior descending coronary artery (LAD) ligation by preventing ventricular hypertrophy and fibrosis. In addition, Sun Z et colleagues found that in diabetic mice, SGLT-1 knockdown attenuates inflammation-induced cardiac damage and pyroptosis in cardiomyocytes attributable to the glycaemic fluctuation of type-2 diabetes. Despite these results, Bode D et al. found that dual SGLT-1/2 inhibition by sotagliflozin improves calcium handling and mitochondrial function while alleviating left atrial cardiomyopathy in a leptin receptor mutated ZSF-1 obese rat model, which is known for heart failure with preserved ejection fraction (HFpEF).

In clinical examinations of early-stage diabetes, LV hypertrophy and associated impairment in ventricular relaxation and filling were the most often found cardiac abnormalities. In addition, it is well-documented that SGLT-1 overexpression also contributes to cardiac remodelling in mice, including hypertrophy and an increase in interstitial fibrosis(169,248). Our findings revealed that the DCM group has a significant increase in heart-to-body weight ratio, ventricular hypertrophy demonstrated by H&E staining, and upregulation of BNP demonstrated by immunoblotting and qPCR, which was blunted after KGA-272 treatment. Similar findings were reported by Rajaratnam et. al. where transgenic overexpression of cardiac SGLT-1 induces pathologic myocardial hypertrophy which was partially abolished by SGLT-1, probably due to the involvement of multiple factors(169).

An unfavourable remodelling of the heart can result from cardiac fibrosis, a pathological process that forms and deposits a fibrotic extracellular matrix (ECM). For instance, Heart failure, myocardial infarction, and hypertension are also attributed to cardiac fibrosis as a significant pathogenic feature. Studies have also discovered a connection between cardiac fibrosis and the emergence of ventricular hypertrophy and diabetic cardiomyopathy(249,250). Our observations in the study are also indeed consistent with

these discoveries. Heart tissue showed significant collagen deposition evidenced by picrosirius red stain, we also observed the changes in fibrotic markers like α-SMA and TGF-β, collagen demonstrated by immunoblot and qPCR respectively. According to numerous research, cardiomyocyte apoptosis is the major hallmark of DCM. Diabetes-induced cardiomyocyte apoptosis rose 85-fold in diabetic patients compared to non-diabetic patients, indicating that cardiomyocytes are susceptible to apoptosis(156,251). Hence, we have evaluated the Caspase3 gene expression using qPCR, which was upregulated in DCM and considerably abolished after KGA-2727 treatment. Consistent with our findings Lin N et al also stated that SGLT-1 inhibition can mitigate apoptosis to prevent the onset of DCM through the JNK and p38 pathway, making it a valid target in DCM. In his findings, specific SGLT-1 inhibitor mizagliflozin has attenuated caspase3 expression in hyperglycaemic conditions(157).

Numerous studies suggest a prominent role of NHE-1 in mediating cardiac hypertrophy and promoting DCM. NHE-1 upregulation also induces hyperglycaemia in DCM by PKC-dependent processes. Additionally, Apoptosis, fibrosis, and compromised myocardial function were also observed, along with an increase in the ratio of heart weight to body weight(252,253). Hence, we tried to correlate how specific SGLT-1 inhibition modulates ion channel expression in DCM cardiac tissue demonstrated by qPCR. The expression pattern was in line with previous observations. Cardiac NHE-1 was upregulated in DCM myocardial tissue, whereas KGA-2727 treatment has abolished NHE-1. Similarly, cardiac NCX-1 was also upregulated and in contrast, NKA was downregulated in DCM. Intriguingly, KGA-2727 has normalized the ion channel expression.

5.6. Conclusion:

The results reported here confirm that the novel SGLT-1 inhibitor KGA-2727 improves insulin sensitivity in T2DM animals. Additionally, KGA-2727 proved to increase HDL levels and reduce the cardiac remodelling demonstrated by reduced collagen deposition. Herein, KGA-2727 was found to be effective in inhibiting cardiac SGLT-1 and thus inhibits deleterious effects of hyperglycemia-induced cardiac remodelling. Furthermore, KGA-2727 showed efficacy in inhibiting cardiac hypertrophy and apoptosis in diabetic cardiomyopathy. The overall conclusion of the study is specific inhibition of cardiac SGLT-1 is effective aginst T2DM induced diabetic cardiomyopathy.

Chapter 6: Conclusion and future perspectives

Type-2 diabetes and cardiovascular diseases are growing hand in hand devastatingly around the world. Individuals with type-2 diabetes have 2 to 4-fold increased risk of CV morbidity compared to non-diabetic individuals. Hence, it's essential to develop and screen therapeutic interventions which can be beneficial to curb diabetes to develop severe CVDs. The primary goal of this thesis is to evaluate the molecular mechanism of cardiovascular benefits associated with novel SGLT-1/2 inhibitors.

Initially, Canagliflozin and dapagliflozin were screened against glucolipotoxicity induced in-vitro diabetic cardiomyopathy model in rat cardiomyocytes (H9C2). The study findings revealed canagliflozin and dapagliflozin inhibit cardiac SGLT-1 and protect the myocardium by mitigating glucolipotoxicity-induced ROS and apoptosis. Additionally, cana and dapa alone were proven to be safe on H9C2 at their therapeutic levels.

In conjunction with the invitro study, Canagliflozin was best selected to be evaluated in the invivo diabetic cardiomyopathy model in male Wistar rats. The study findings were appreciable to inhibit the myocardial remodelling associated with type-2 diabetes. Moreover, canagliflozin ameliorated diabetic cardiomyopathy by blunting the over-expression of myocardial SGLT-1 and NHE-1 in the diabetic heart. Furthermore, canagliflozin reduced cardiac hypertrophy, fibrosis, and apoptosis with preserving structural integrity and mitochondrial biogenesis.

Later, KGA-2727 a specific novel SGLT-1 inhibitor was evaluated in an invivo diabetic cardiomyopathy model, which was found to be relatively safe and effective. The study

observations revealed that KGA-2727 is effective in maintaining blood glucose levels and protects the myocardium mainly by inhibiting SGLT-1 and diabetes-associated cardiac hypertrophy, and fibrosis.

Therefore, our overall study concludes inhibition of cardiac SGLT-1 is a valid target for reducing diabetic cardiomyopathy.

Future perspectives:

Inhibiting SGLT-1 is proven to be beneficial to ameliorate diabetic cardiomyopathy. However, the side effects associated the canagliflozin include genitourinary tract infections and limb amputation mainly due to its SGLT-2 inhibiting effect at reno-vasculature. Similarly, specific SGLT-1 inhibition at the small intestine cause diarrhoea and gastric discomfort. To minimize these side effects and to increase the benefits, further research needs to be done to design and evaluate myocardial targeted drug delivery of SGLT-1/2 inhibitors, since. invitro experiments on isolated cardiomyocytes also showed cardiovascular benefits. Besides, further research needs to evaluate the fate of various ion exchanger expression patterns in diabetic cardiomyopathy and how SGLT-1 inhibitors affect these ion exchangers that contribute to cardiac contractility.

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Appendix

List of publications (Thesis work)

- **Dasari D**, Bhat A, Mangali S, Ghatage T, Lahane GP, Sriram D, Dhar A. Canagliflozin and Dapagliflozin Attenuate Glucolipotoxicity-Induced Oxidative Stress and Apoptosis in Cardiomyocytes via Inhibition of Sodium-Glucose Cotransporter-1. ACS Pharmacology & Translational Science. 2022 Mar 9.
- **Dasari D**, Goyal SG, Penmetsa A, Sriram D, Dhar A. Canagliflozin protects diabetic cardiomyopathy by mitigating fibrosis and preserving the myocardial integrity with improved mitochondrial function. European Journal of Pharmacology. 2023 Apr 11:175720

PUBLICATIONS UNDER REVISION:

- **Dasari D**, Dhar A. Selective inhibition of SGLT-1 by KGA-2727 improves Diabetic cardiomyopathy in Wistar rats.

List of publications (Others)

- Mangali S, Bhat A, **Dasari D**, Sriram D, Dhar A. Inhibition of double stranded RNA dependent protein kinase (PKR) abrogates isoproterenol induced myocardial ischemia in vitro in cultured cardiomyocytes and in vivo in wistar rats. European Journal of Pharmacology. 2021 Jun 1:174223.
- Udumula MP, Mangali S, Kalra J, **Dasari D**, Goyal S, Krishna V, Bollareddy SR, Sriram D, Dhar A, Bhat A. High fructose and streptozotocin induced diabetic impairments are mitigated by Indirubin-3-hydrazone via downregulation of PKR pathway in Wistar rats. Scientific Reports. 2021 Jun 21;11(1):1-1.

- Li L, Mangali S, Kour N, **Dasari D**, Ghatage T, Sharma V, Dhar A, Bhat A. Syzygium cumini (jamun) fruit-extracted phytochemicals exert anti-proliferative effect on ovarian cancer cells. Journal of Cancer Research and Therapeutics. 2021 Oct 1;17(6):1547.
- Kalra J, **Dasari D**, Bhat A, Mangali S, Goyal SG, Jadhav KB, Dhar A. PKR inhibitor imoxin prevents hypertension, endothelial dysfunction and cardiac and vascular remodelling in L-NAME-treated rats. Life Sciences. 2020 Dec 1;262:118436..
- Kalra J, Mangali SB, **Dasari D**, Bhat A, Goyal S, Dhar I, Sriram D, Dhar A. SGLT1 inhibition boon or bane for diabetes-associated cardiomyopathy. Fundamental & clinical pharmacology. 2020 Apr;34(2):173-88.
- KALRA J, **Dasari D**, Bhat A, Dhar A. SUN-172 SELECTIVE INHIBITION OF PKR AMELIORATES HYPERTENSIVE NEPHROPATHY AND AORTIC REMODELLING IN L-NAME TREATED WISTAR RATS. Kidney International Reports. 2020 Mar 1;5(3):S271.
- Bollareddy SR, Krishna V, Roy G, Dasari D, Dhar A, Venuganti VV. Transfersome Hydrogel Containing 5-Fluorouracil and Etodolac Combination for Synergistic Oral Cancer Treatment. AAPS PharmSciTech. 2022 Feb;23(2):1-1.

Poster presentations

- **Deepika Dasari** , Lahane GP, Dhar A, Canagliflozin Ameliorates SODIUM DEPENDENT GLUCOSE TRANSPORTER-2 INHIBITORS ATTENUATE GLUCOLIPOTOXICITY-INDUCED OXIDATIVE STRESS AND APOPTOSIS IN CARDIOMYOCYTES VIA INHIBITION OF SODIUM-GLUCOSE COTRANSPORTER-1**.** Poster Presented at NIPER PHARMACON-2022 International Conference on Research, SAS Nagar, Punjab, INDIA. November 10-12, 2022.

- **Deepika Dasari** , Srashti Goel, Dhar A, Canagliflozin Ameliorates In-Vitro And In-Vivo Diabetic Cardiomyopathy By Targeting Cardiac SGLT-1. Poster Presented at NIPER PHARMACON-2022 International Conference on Research, SAS Nagar, Punjab, INDIA. November 10-12, 2022.

Deepika Dasari

Biography

Mrs. Deepika completed her Bachelor of Pharmacy from Smt. Sarojini Ramulamma College of Pharmacy, Osmania University, Telangana in the year 2013. She pursued Master of Pharmacy with specialization in Pharmacology from University college of Pharmaceutical sciences, Kakatiya University, Telangana in the year 2016. She worked as Assistant Professor Guru Nanak Institute of Pharmacy, Ibrahimpatnam, Hyderabad, Telangana. Later, Mrs. Deepika joined Prof. Arti Dhar's lab at Birla Institute of Technology and Science, Pilani, Hyderabad Campus for her doctoral studies. Her doctoral research work involved evaluation of pharmacological interventions targeted at sodium glucose co-transporter-1 (SGLT-1) in diabetic cardiomyopathy. Mrs. Deepika Dasari has published 2 research publication from her thesis and co-authored 8 scientific peer review publications in well-renowned international journals.

Prof. Arti Dhar

Biography

Dr Arti Dhar is currently working as an Associate Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Dr. Dhar received her PhD from College of Medicine, University of Saskatchewan, Canada in the year 2010. During her PhD she received scholarships from Heart and Stroke Foundation of Canada (HSFC) and Arthur Smith Memorial Scholarship from University of Saskatchewan, Canada. Dr. Dhar also won numerous travel awards from Canadian Physiological Society and Canadian Hypertension Society. Her PhD thesis was nominated for Governor General's Gold medal and her thesis work was presented on CBC channel Canada in March 2011. She did her postdoctoral trainings from Lakehead University, Ontario, Canada and University of Saskatchewan, Canada from the year 2010 to 2013. After joining BITS Pilani, Hyderabad Campus in 2014, she has received research funding from DST-SERB, CSIR, ICMR and from BITS under additional competitive grant. She has published more than 42 research publications in peer-reviewed international journals. She has guided three doctoral students, six master's students and six undergraduate students in fulfilment of their dissertation work. Currently 5 students are pursuing PhD under her supervision. Her main research interests are centred on novel therapeutic targets for cardiovascular, metabolic disorders and cancer. She has won numerous awards at national and International level.