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THE EFFECT OF LICOGLIFLOZIN (SGLT1/2 INHIBITOR) ON DIABETES AND CARDIAC COMPLICATIONS

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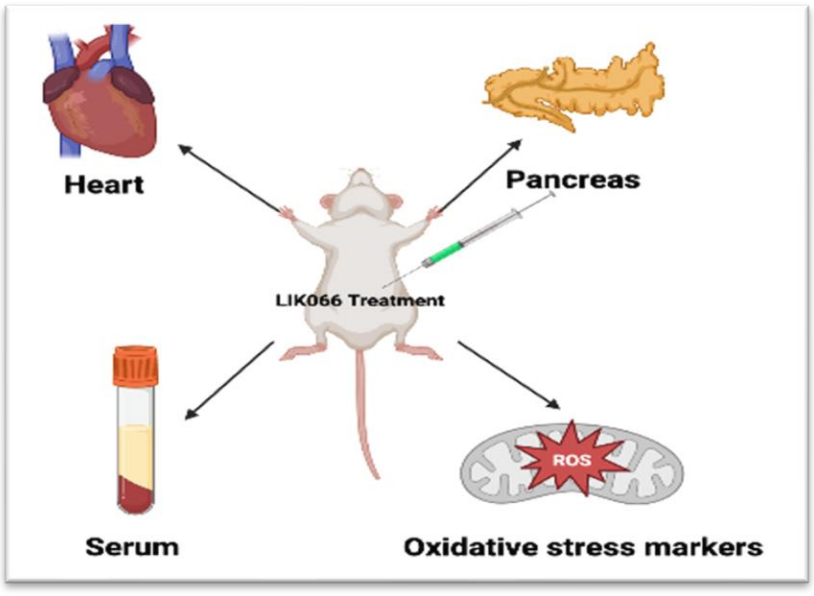

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Alanoud Gharib Alblooshi

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Biochemistry and Molecular Biology)

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Cover: The experimental diagram on the effect of LIK066 on rat's pancreas, heart, serum, and oxidative stress markers

(Photo: By Alanoud Gharib Alblooshi)

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Declaration of Original Work

I, Al Anoud Gharib Al Blooshi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The effect of licogliflozin (SGLT1/2 inhibitor) on diabetes and cardiac complications*", hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Ernest Adeghate, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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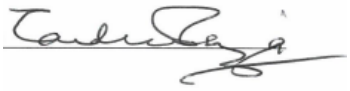
   Signature  Date 24th November 2022

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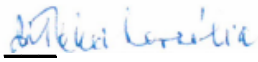
   Signature  Date 24th November 2022

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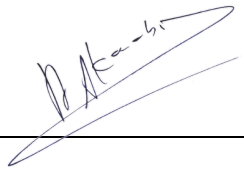
Signature 

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Abstract

Diabetes mellitus (DM) is a chronic endocrine disease affecting millions of people worldwide. In spite of the advances made in the management of DM, poor glycemic control and diabetes complications are still very common. There is a continuous search for new and more effective drugs to treat DM. One of the drugs currently in clinical trials for the treatment of DM is licogliflozin (LIK066), a dual SGLT1/2 inhibitor, which can be used to treat obesity and diabetes. LIK066 inhibits glucose reabsorption in the kidney and small intestine, thereby reducing hyperglycemia. This study aims to investigate the efficacy of licogliflozin on diabetes and cardiac complications in a rodent model of experimental diabetes. DM was induced by streptozotocin in male Wistar rats. This was followed by the administration of LIK066 at a dose of 0.588 mg/Kg given intraperitoneally for 4 weeks. The immunofluorescence technique was used to determine whether SGLT1/2 is found in the pancreas and to determine if it co-localizes with insulin in pancreatic islet cells. Markers of cardiomyopathy (Collage 3, TIMP4, Keap1, fibronectin) and oxidative stress (catalase, superoxide dismutase, glutathione reductase) were also examined using immunofluorescence and Masson staining and enzyme immunoabsorbent essay in heart homogenates and the serum. Moreover, oxidative stress markers were also studied in the pancreas using immunofluorescence techniques and in serum by colorimetric analysis. LIK066 causes slight weight reduction in diabetic rats. SGLT1 and SGLT2 expression were slightly higher after treatment with LIK066 in normal and diabetic rats. TIMP4 result shows a significant elevation in LIK066-treated diabetic rats compared to diabetic untreated causing improvement in diabetic cardiomyopathy. Oxidative stress is a hallmark of the pathogenesis of DM. In the pancreas, treatment DM rats with LIK066 caused a significant elevation of GSH compared to untreated DM rats. Heart muscle catalase shows a significant increase in diabetic rats treated with LIK066 compared to diabetic untreated rats. Also, GSH reductase was significantly increased in diabetics treated with LIK066. In conclusion, LIK066 may exert its beneficial cardiac effects by increasing the endogenous pool of antioxidants.

Keywords: Diabetes mellitus, Pancreas, Cardiovascular disease, Licogliflozin, Immunohistochemistry, Dual SGLT1/2 inhibitors, Oxidative stress.

Title and Abstract (in Arabic)

تأثير اللايكوجليفلوزين (مثبط SGLT1/2) على مرض السكري ومضاعفات القلب

الملخص

مرض السكري هو مرض مزمن يصيب الغدد الصماء يصيب ملايين الأشخاص في جملع أنحاء العالم. على الرغم من التقدم المحرز في إدارة مرض السكري، إلا أن لايزال شائع ضعف التحكم في نسبة السكر في الدم ومضاعفات مرض السكري. هناك بحث مستمر عن أدوية جديدة أكثر فعالية لعلاج مرض السكري. أحد الأدوية التي تخضع حاليًا للتجارب السريرية لعلاج مرض السكري هو لايكوجليفلوزين، يعتبر من مثبطات ناقلة مشتركة للصوديوم والجلوكوز ١ و ٢ والذي يمكن استخدامه لعلاج السمنة ومرض السكري. لايكوجليفلوزين يمنع امتصاص الجلوكوز في الكلى و الأمعاء الدقيقة، وبالتالي تقليل ارتفاع السكر في الدم. الهدف من هذه الدراسة هو التحقق من فعالية لايكوجليفلوزين على مرض السكري ومضاعفات القلب في نموذج القوارض لمرض السكري التجريبي. تم إحداث مرض السكري في ذكور فئران ويستار بواسطة الستربتوزوتوسين. تبع ذلك إعطاء ليكوجليفلوزين بجرعة 0.588 مجم / كجم داخل الصفاق لمدة 4 أسابيع. تم استخدام تقنية التوهج المناعي لتحديد ما إذا كانت مثبطات الصوديوم والجلوكوز ١ و٢ المشتركة توجد في البنكرياس ولتحديد ما إذا كانت تتشارك مع الأنسولين في خلايا (الكولاجين 3، تيمب 4، كيب ١، الفبرونكتين) والإجهاد التأكسدي (الكاتليز، سوبر اوكساید ديسميوتيز، الجلوتاثيون المختزل) تم فحصها أيضًا باستخدام التوهج المناعي وصبغ الماسون ومقال إنزيم الامتصاص المناعي في متجانسات القلب والمصل. بالإضافة إلى ذلك ، تم دراسة علامات الإجهاد التأكسدي في البنكرياس باستخدام تقنيات التوهج المناعي وفي المصل عن طريق التحليل اللوني. لايكوجليفلوزين يسبب انخفاضًا طفيفًا في وزن الفئران المصابة بداء السكري. مثبطات الصوديوم والجلوكوز ١ و ٢ يتم التعبير عنها في خلايا البنكرياس حيث يتم تواجدها مع الأنسولين في الجرذان العادية و المصابة بداء السكري. تيمب ٤ تظهر النتيجة ارتفاعًا ملحوظًا في الفئران المصابة بالسكري والمعالجة بـاللايكوجليفلوزين مقارنة بالفئران المصابة بالسكري الغير معالجة مما يؤدي إلى تحسن في اعتلال عضلة القلب السكري. الإجهاد التأكسدي هو السمة المميزة لمرض السكري. تسبب معالجة الفئران المصابين بالسكري بـاللايكوجليفلوزين في ارتفاع كبير في الجلوتاثيون المختزل بالفئران المصابة بالسكري الغير معالجة. يُظهر مستوى الكاتليز في عضلة القلب زيادة كبيرة في الفئران المصابة بداء السكري بعد علاجها بـاللايكوجليفلوزين مقارنة بالفئران المصابة بالسكري الغير معالجة. أيضاً الجلوتاثيون المختزل زاد بشكل ملحوظ في الفئران المصابة بالسكري والمعالجة بـاللايكوجليفلوزين. في الختام، لايكوجليفلوزين قد يمارس آثاره القلبية المفيدة عن طريق زيادة مجموعة مضادات الأكسدة الذاتية.

مفاهيم البحث الرئيسية: داء السكري، البنكرياس، أمراض القلب والأوعية الدموية، لايكوجليفلوزين، الكيمياء المناعية، مثبطات الصوديوم والجلوكوز ١ و ٢ المزدوجة، الإجهاد التأكسدي.

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Dedication

To my beloved parents and family

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List of Abbreviations

ACEIs	Angiotensin Converting Enzyme Inhibitors
AGEs	Advanced Glycation End Products
AKT	Protein Kinase B
AR	Aldose Reductase
BAT	Brown Adipose Tissue
CVD	Cardiovascular Disease
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DPP-4	Dipeptidyl Peptidase-4 Inhibitors
EM	Electron Microscopy
eNOS	Endothelial Nitric Oxide Synthase
FPG	Fasting Plasma Glucose
GDM	Gestational Diabetes Mellitus
GFR	Glomerular Filtration Rate
GLP-1	Glucagon-Like Peptide-1 Receptor Agonists
GLUT-2	Glucose Transporter-2
GSH	Glutathione
H2O2	Hydrogen Peroxide
HbA1c	Hemoglobin A1c
HF	Heart Failure
IDDM	Insulin-Dependent Diabetes Mellitus
IHC	Immunohistochemistry
IL-6	Interleukin-6
LIK066	Licogliflozin

MI	Myocardial Infarction
MODY	Maturity-onset diabetes of youth
NASH	Non-Alcoholic Steato-Hepatitis
NEFAs	Non-Esterified Fatty Acids
NGS	Normal Goat Serum
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NO	Nitric Oxide
IPGTT	Intraperitoneal Glucose Tolerance Test
PCT	Proximal Convoluted Tubules
PDR	Proliferative Diabetic Retinopathy
PKC	Protein Kinase C
PPAR-γ	Peroxisome Proliferator-Aactivated Receptor-Gamma
RAAS	Renin-Angiotensin-Aldosterone System
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SGLT1i	Sodium-Glucose Cotransporter 1 Inhibitors
SGLT2i	Sodium-Glucose Cotransporter 2 Inhibitors
SOD	Superoxide Dismutase
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a disorder related to insulin metabolism in the body, patients have either insufficient levels of insulin or insulin resistance as a result, their blood glucose level will increase, and this disease is mainly characterized by chronic hyperglycemia. The key hormone in the body regulating blood glucose is insulin secreted by the pancreas (Khan et al., 2019). Scientists have distinguished the complexity of the pancreas using histological techniques. The pancreas is an organ in the body that can have both exocrine and endocrine functions. The pancreas houses the islets of Langerhans which contain beta cells that are responsible for secreting insulin and alpha cells that produce glucagon. Human pancreatic islets are mostly made up of 54% beta cells, 35% alpha cells, and 11% delta cells. There are gamma and epsilon cells, but their numbers are very few. DM results in the abnormal metabolism of carbohydrates, lipids, and proteins (Marshall, 2020). The continued prevalence of DM is a major health problem worldwide. The various types of diabetes mellitus (Type 1, Type 2) share a common characterization like hyperglycemia due to insufficient or defective insulin function. This insulin insufficiency and defective insulin molecule may arise as a result of β-cell dysfunction. In 1997 the American Diabetes Association (ADA) classified DM as type 1 diabetes, type 2 diabetes, gestational diabetes mellitus (GDM), and other types. Type 1 diabetes mellitus (T1DM) results from the autoimmune destruction of beta cells causing an absolute absence of insulin, usually, it is common in children and adolescents, and its accounts for approximately 10% of all cases of DM (Kharroubi, 2015). Type 2 diabetes mellitus (T2DM) was formerly called adult-onset diabetes or silent killer, which reduces life expectancy by 20 years in patients. Type 2 DM accounts for almost 90% of all DM cases (Khan et al., 2019). Gestational diabetes mellitus (GDM) is a type of T2DM defined as hyperglycemia in pregnancy, which can develop before or during pregnancy. One of the risks coupled with GDM is macrosomia when the birth weight is equal to or greater than 4.5 kg. The other type of diabetes mellitus is monogenic diabetes, maturity-onset diabetes of youth (MODY) which is based on a genetic mutation of one gene in the pancreatic beta-cell. This mutation alters the function and decreases the number of pancreatic beta cells (Kharroubi, 2015).

1.2 Epidemiology of DM

Diabetes mellitus is the 5th leading cause of death worldwide and it has a positive association with the prevalence of hypertension and obesity. According to 2017 data from Imperial College London Diabetes Center, over 1 million individuals have diabetes in UAE. This number is expected to double by 2040 to 2.2 million. The UAE occupies 15th place worldwide with 17.3% of the UAE population aged between 20 and 79 having type 2 diabetes (Imperial College London Center, UAE Diabetes Trends). The worldwide diabetes trend in 2019 was approximately 463 million adults diagnosed with diabetes in people aged between 20 and 79. This trend is projected to increase worldwide, and it is expected to reach 700 million by the year 2045 (Imperial College London Center, Diabetes trends).

1.3 Classification and Diagnosis

1.3.1 Type 1 Diabetes Mellitus

Over the past 25 years, the world has recorded an increase in the number of T1DM patients. T1DM is a complex disease and the complexity came from the varying possibilities of the pathogenesis among individual cases such as environmental factors, genome, immune system, and metabolism (DiMeglio et al., 2019). T1DM is also known as juvenile diabetes or ketosis-prone diabetes. It is an autoimmune disease associated with the destruction of pancreatic beta cells through T-cell responses, inflammation, and humoral responses. T1DM is characterized by hyperglycemia due to insulin deficiency. Therefore, therapies for insulin replacement should be obtained. In case of any doubt regarding the diagnosis, there are other tests like the C-peptide test for the function of beta-cell to decide the right treatment. A low level of C-peptide will indicate insulin deficiency (Hoogwerf, 2020). Before diagnosing T1DM, autoantibodies will be detected in the patient's serum months or years earlier. These autoantibodies will be found in the serum of the patient and pancreatic islet cells and are considered indicators or biomarkers for T1DM. These antibodies include islet cell and insulin autoantibodies (IAA), protein tyrosine phosphatase (IA2 and IA2β), glutamic acid decarboxylase (GAD, GAD65) and zinc transporter protein (ZnT8A). All these antibodies can be measured and used for the clinical diagnosis of T1DM. More than 90% of people with T1DM carry

these antibodies. The etiology of T1DM has a strong association with some genes like HLA alleles combined with DR and DQ genes. Moreover, there are environmental factors related to the pathogenesis of T1DM. These environmental insults include vitamin D deficiency and viral factors including rotavirus and herpes virus. In addition, obesity can contribute to the early stages of childhood DM by increasing insulin resistance. T1DM symptoms include weight loss, tiredness, blurred vision polydipsia, and polyuria which means an increase in thirst and urination, respectively (Kharroubi, 2015). Ketoacidosis is a life-threatening condition related to T1DM but less common in T2DM. It is the process of building up ketone bodies in the blood at a higher level, due to the breakdown of fat, which is usually used as fuel instead of glucose (Bratton & Krane, 1992). The risks and stages of T1DM depend on the existence of multiple antibodies. Three stages are defined, first is the preclinical stage 1, which is associated with positive autoantibodies. This stage may take months or years to progress into stages 2 and 3 which is known as clinical diabetes, where metabolic abnormalities appear for example hyperglycemia (DiMeglio et al., 2019).

1.3.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) was formally called non-insulin-dependent chronic disease, adult-onset diabetes, or silent killer, and it reduces life expectancy by almost 20 years for patient's life. The most prevalent type of diabetes is T2DM, this hyperglycemic disease rises with two conditions deficiency in insulin secretion and β-cell improper function (Khan et al., 2019). The incidence of T2DM is now increasing among young people for many reasons, mostly as a result of lifestyle in general like living a sedentary life and consuming a bad diet. All of these will lead to obesity, a key factor for the development of insulin resistance, an important phenomenon in the pathogenesis of T2DM. T2DM is characterized by hyperglycemia, which is linked to many pathophysiological defects like inflammation, high secretion of hepatic glucose, and modified gut hormones that are responsible for regulating insulin and glucagon. The mechanism of insulin resistance is related to the defect of certain proteins which are responsible for the signaling pathways that regulate the cellular insulin response. These pathways contain molecules such as insulin-dependent receptor substrates IRS-1 and IRS-2. Protein kinase B (AKT) and protein kinase C (PKC). These are serine/threonine

kinases that phosphorylate Ser/Thr residues on IRS protein and control responses to insulin. IRS-1 wild-type protein has three tyrosine, but the mutant type will have alanine instead. In diabetes, AKT and PKC metabolism is disturbed leading to the development of many metabolic disorders related to diabetes mellitus such as hyperinsulinemia and dyslipidemia. Insulin resistance affects lipid and glucose metabolisms mainly in the liver and muscle tissues causing obesity. For example, the inability to transport, produce and store glucose in the liver (Kharroubi, 2015).

There are five clinical stages used to categorize a patient's condition in Type 2 diabetes mellitus. This classification allows the clinician to plan for the management of diabetic patients. Stage one is pre-diabetes, this stage is correlated to some other conditions the patients may suffer from like hypertension, obesity or overweight, and dyslipidemia which is an abnormality of lipid levels in the blood. Three tests can show whether diabetic patients are in the pre-diabetes stage or not. The usual test is fasting plasma glucose (FPG) pre-diabetes patients will have a level range between 100-125 mg/dL and if it exceeds 126 mg/dL they will consider diabetic. The second test is the oral glucose tolerance test (OGTT) patient will show levels between 140-199 mg/dL, and the last one is hemoglobin A1c (HbA1c) the percentage should be between 5.7-6.4%. This stage of T2DM may not be associated with complications and patients sometimes will not show insulin resistance. Furthermore, for a patient to diagnose with diabetes all the above three tests will show higher readings than the normal ranges mentioned above by a one-point increase or even more. Stage three is diabetes with mild complications, patients will start showing complications such as mild diabetic retinopathy and hyperglycemia. In stage 4 diabetic patients who have insulin deficiency with mild complications, the perfect treatment is therapies of insulin. And the last stage is the most severe, which is diabetes with serious complications of microvascular and macrovascular as well as hyperglycemia (Wu, 2015).

1.4 Pancreatic β-cell Dysfunction and Obesity

Despite their fragility, β-cells play a key role in controlling insulin production. The amount of insulin produced by β-cells fluctuates and varies depending on how much, and when the stimuli are delivered. Therefore, β-cells play a major role in maintaining the stability of blood glucose concentrations within a typical physiological

range in healthy subjects. The sensitivity to insulin and the regulation of β-cell activity declines in obesity. When β cells are stable, an adaptive response to insulin resistance occurs, resulting in the preservation of normal glucose levels. By comparison, in cases where there is pancreatic β-cell dysfunction, abnormal glucose tolerance or abnormal fasting glucose may occur, which may lead to T2DM (Al-Goblan et al., 2014).

One of the major causes of type 2 diabetes is a steady decrease in pancreatic β-cell activity. β-cell dysfunction leads to insufficient insulin secretion, fasting blood glucose and postprandial blood glucose levels will rise. Then the reduced efficiency of the absorption of hepatic and muscle glucose occurs with little or partial inhibition of the synthesis of liver glucose. Further blood glucose rise can lead to glucotoxicity effects on pancreatic β-cell and adverse effects on the absorption of insulin and the sensitivity of peripheral tissues. A second aspect that can lead to the ongoing loss of function of β-cells is an increase in plasma NEFA levels. Since NEFA plays a major role in insulin release, ongoing NEFA exposure is linked to severe malfunctions and decreased insulin biosynthesis in glucose-stimulated secretion pathways. Furthermore, the presence of insulin resistance *in vivo*, as well as a deficiency of the compensatory function of β-cells, will lead to an increase in NEFA levels made by the lipids (Al-Goblan et al., 2014).

Obesity has become an epidemic in developing countries and the major comorbidity of obesity is type 2 diabetes mellitus (T2DM) which has risen exponentially. In addition, it has other co-morbidities like chronic kidney disease, CVD, hypertension, and dyslipidemia. Diabesity is a term that links obesity with T2DM known since the 1970's (Pappachan et al., 2019). Obesity is a health problem represented by excess body fat resulting from the imbalance of nutrient intake (increase) and energy expenditure (decrease), in response to many hormones which are involved in this process. Body mass index is the tool to measure a healthy weight in which the BMI ranges between 18.5 to 24.9, and for overweight, the BMI is between 25 to 29.9 kg/m^2, and for obese people, their BMI will be equal to or above 30 kg/m^2 (Present, 2018). A hypothesis was conducted by Pories to explain the etiology of T2DM in obesity. They proposed that T2DM occurs when fasting hyperinsulinemia which has been induced by the gastrointestinal tract due to increased calorie intake so, after the meal the body does not respond to insulin (Busetto, 2015). Type 2 diabetes and obesity are both associated

with insulin resistance, however, not all obese patients suffering from insulin resistance will show hyperglycemia, because under normal conditions pancreatic β-cells will release insulin to overcome the low levels of insulin and keep normal glucose tolerance. Otherwise, if β-cells cannot compensate for the decrease in insulin levels, obesity and insulin resistance will cause T2DM. Some studies link insulin resistance and β-cell dysfunction in obese people to the fact culprit. The change in nutrient intakes such as high carbohydrates and high physical activity can make the level of insulin sensitivity fluctuate. Adipose tissue plays a major role in metabolism by controlling and secreting many hormones and substances, which will increase exponentially with obesity for example glycerol, leptin, adiponectin, cytokines, pro-inflammatory substances, and NEFAs which are observed at a higher level in people with T2DM (Al-Goblan et al., 2014).

1.5 Diabetes Mellitus Risk Factors

1.5.1 Lifestyle and Environmental Risk Factors

Two factors play an important role in the development of T2DM, genetic and environmental factors and they may interact to increase the risk of T2DM development in individuals. Environmental and lifestyle risk factors, show the importance of living a healthy lifestyle to protect people from certain diseases. Obesity is number one on the list which shows a strong correlation with T2DM, and various cross-sectional studies confirm that result. One is considered obese if the BMI is equal to or exceeds 30 kg/m^2, and the shows that 50% of patients with T2DM are obese, also 90% are overweight indicating a BMI over 25 kg/m^2. Studies have shown that decreasing body weight will lower the risk of T2DM. Recent studies have shown that waist circumference is a more efficient indicator for T2DM than BMI. There is a molecular aspect behind obesity and T2DM which is the increasing body level of some cytokines, such as namely interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFα). The high level of these components will cause adipose tissue to trigger a signaling pathway that stimulates inflammatory action in that tissue. To confirm the findings, a study was done by Barbarroja on the level of mRNA expression of IL-1β and IL-6. It was a high level in an

obese diabetic with insulin resistance compared to non-insulin resistance obese (Khan et al., 2019).

Another study was conducted on the health of nurses which shows obese that obese nurses are 49 times more at risk of developing T2DM than nurses with normal BMI. The second factor is a sedentary lifestyle, like watching TV, reading, writing, or even driving a car. The worst practice is watching TV; indeed, this was obtained because it shows the lowest metabolic rate. The statistics show that 112% of people with T2DM spend a long-time watching TV compared to the ones who watch TV for a shorter time. That has been recognized by a meta-analysis with 10 studies and a large (505,045) number of participants. A sedentary lifestyle such as watching TV could cause obesity and an increase in BMI which have a strong correlation to developing T2DM. Moreover, there is another reason related to acute muscle contraction which will facilitate the uptake of plasma glucose, and this will not be the case for physically inactive people. Aging increases the chance of having T2DM, this was confirmed according to the National Diabetes Statistics Report in the US, England, and a cohort study in Indonesia. The reason behind this is when the human body ages it will be less sensitive to insulin and also β-cells and may produce less insulin (US Department of Health and Human Services, 2020). Surprisingly there is a relationship between gender and sex with T2DM. The prevalence of T2DM is more in men than in women due to unknown reasons. This may be due to the attributes of the X or Y chromosomes, and the difference in brown fat (BAT) distribution between men and women. The BAT activity and the risk of diabetes are negatively associated, which means the higher activity of brown adipose tissue will decrease obesity and the risk of T2DM (Khan et al., 2019). Diabetes also is strongly associated with hypertension, so it is considered a risk factor as well, however, it is difficult to say which one is causing the other.

Smoking is also considered a risk factor for T2DM due to long-term studies on smokers and non-smokers. The number of cigarette consumption increases the risk of T2DM development. A study shows that if a smoker consumes 20 cigarettes per day, the risk will increase to 61% and if the number of cigarettes decreases to less than 20 cigarettes per day, the risk of T2DM will be 29%, mostly it decreases to half. Smoking cigarettes have an active chemical which is nicotine, which directly affects insulin

sensitivity due to decreasing the expression of a transcription factor called peroxisome proliferator-activated receptor gamma (PPAR-γ). The post-translator modification also plays an important role in smokers triggering modifications like increasing the phosphorylation of insulin receptor substrate IRS-1 at Serine 636, in response to this it will reduce the insulin signaling. Investigation and research show that pancreatic β-cells have nicotine receptors. Nicotine can directly cause apoptosis of pancreatic β-cells and reduce their numbers. The last risk factor is alcohol consumption and exceeding the threshold which is 63 g/day will cause a positive relation with T2DM (Khan et al., 2019).

1.5.2 Genetic Risk Factors

Genetics is considered a significant risk factor for T2DM Checked if genetics could play a role in the development of T2DM in patients. They used family-based linkage analysis, candidate gene approach, and genome-wide association studies (GWAS), to study genetics and T2DM. Studies found that some individuals from certain ethnic groups have a higher risk of developing T2DM than other ethnic groups. Also, a parent's history of having T2DM will increase the chance to 40% to have children with T2DM or 6 times more risk to develop it later in their life. There are more than 40 genes related to T2DM have been identified. Family-based linkage analysis is the analysis to check the chromosomes of the family members to find the genetic changes for T2DM. CAPN10 and ACRP30 are two genes that have been identified which can cause diabetes or are associated with the risk of this disease. However, there was no uniformity in the data of the studied population, so CAPN10 and ACRP30 genes are not proven to have a higher risk for T2DM. The Candidate Gene Approach is a study of genetic regions with certain and well-known biological functions. Other genes that are also related to T2DM are PPARG and KCNJ11. The KCNJ11 is a gene that increases the risk of T2DM and is regulated by the PPAR-γ. KCNJ11 codes for the potassium channel in the pancreas, especially for the subunit which forms the core of the potassium channel and uses ATP (Khan et al., 2019).

1.6 Complications of Diabetes Mellitus

Brownlee & Cerami suggest that diabetes mellitus can be managed by insulin injection, oral medication, or even diet to avoid the development of other chronic complications affecting different parts of the body, including the kidneys, heart, nerves, and eyes (Brownlee & Cerami, 1981). Microvascular complications may cause retinal damage (diabetic retinopathy), nerve dysfunction (diabetic neuropathy), and kidney disease (diabetic nephropathy). On the other hand, macrovascular complications lead to cardiovascular diseases (CVD) like hypertension and atherosclerosis. Atherosclerosis may lead to heart complications like premature coronary artery disease (Kaur et al., 2018). This complication can affect the quality of a patient's life. Indeed, there are many ways to control DM depending on the patient's condition. If not, diabetes complications can lead to blindness or heart failure.

1.6.1 Diabetic Microvascular Complications

1.6.1.1 Diabetic Nephropathy

The term "microvascular" refers to small blood vessels in the body, and diabetes induces long-term damage due to hyperglycemia, which damages the microvascular network of some important organs like the kidney, eyes, and nerves. The kidney is an important organ in the body. It's a bean-shaped structure located on the sides of the spine. The kidney has an important role in keeping individuals healthy and active because it filters 180 liters of blood 60 times a day. Also, it keeps the homeostasis of the extracellular electrolyte by 99% filtration and reabsorption. The remaining 1%, will be excreted through urine. The functional unit in the kidney is the nephron, and it contains the proximal convoluted tubules (PCT), which have the most important function, and distal tubular segments. The proximal convoluted tubule reabsorbs filtered protein and solutes, as well as glucose, preventing it from being excreted in the urine (Kelsey C. Martin Mhatre V. Ho, 2012).

Diabetic nephropathy also known as diabetic kidney disease (DKD), is a disorder that causes a decline in renal function and is identified as the major cause of end-stage renal disease. The diagnosis and characterization of DKD have increased albuminuria and lowering in the glomerular filtration rate (GFR) (Cole & Florez, 2020). The data

shows a higher incidence of nephropathy in patients with T1DM than in patients with T2DM, comparing the numbers for IDDM in a range between 35-45% and T2DM in 4-20%. The factor diagnosed first in diabetic nephropathy patients with T1DM is microalbuminuria is range between 30–299 mg/day, in case there is extensive loss of protein in urine exceeding 300 mg/day this will be known as overt nephropathy. Indeed, the onset of diabetes in T1DM patients started with hyperglycemia, after five years microalbuminuria will be diagnosed with DKD. Albuminuria increases by 10-20 percent per year and can be upgraded in the next 5-10 years to overt proteinuria in the absence of medical treatment, also hypertension will arise. After 5-10 years next GFR will decline due to nephrotic syndrome and finally, it reaches end-stage renal disease which is the major cause of DKD, 23 years have been estimated for patients with IDDM to develop end-stage renal disease (Teitelbaum et al., 1993). There are some ways to prevent diabetic renal disease such as management of hyperglycemia, antihypertensive drugs, dietary protein restriction, and avoiding any nephrotoxic drugs (The et al., 1995).

1.6.1.2 Diabetic Retinopathy

Diabetic retinopathy is a disorder that affects the eyes, especially the retinal blood vessels, causing them to be dysfunctional. It happens in all types of diabetes, including T1DM and T2DM. This type of complication correlates well with the duration of diabetes. Furthermore, it is a major cause of blindness in patients diagnosed with diabetes for a long period, nearly over 15 years. Hyperglycemia and hypertension are considered risk factors due to their damage to retinal blood vessels, causing hemorrhage. There are two stages of proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (non-PDR) (Tripathi & Srivastava, 2006). In non-PDR, the biochemical change during this stage is started by a lesion in the blood vessel due to retinal hypoxia. In response, microaneurysms will form (Brownlee & Cerami, 1981). Dot and blot hemorrhages, emerge from microaneurysms due to erythrocyte escape. Microaneurysms, hemorrhage, and the last mark are hard exudates. They are yellowish intra-retinal lipids that appear due to the leakage of the serous fluid from the abnormal vessels. These three marks are known as background retinopathy or non-proliferative retinopathy. Furthermore, this happens often with diabetes. In proliferative retinopathy, new blood vessels start to form. This process is called neovascularization, in response to

occulted blood vessels, which causes ischemia. This stage is severe, and vision could be lost (Teitelbaum et al., 1993). On the other hand, IDDM patients have a 40% chance of developing proliferative retinopathy and a 20% chance of developing PDR NIDDM patients. There are some preventive measures to reduce the incidence of retinopathy. According to the Diabetes Control and Complications Trial (DCCT), glycemic control is one way to slow the progress of diabetic retinopathy and blood pressure control. In the early diagnosis of diabetic retinopathy, ophthalmological therapy and treatment should be used, like eye examination by an ophthalmologist, and severe cases treated with laser surgery and vitrectomy, as well as injection of anti-VEGF (The et al., 1995). The pathogenesis of diabetic retinopathy has been studied with different approaches. The investigation into different molecular pathways reaches a factor expressed in the retina of patients with PDR, found in a significant amount. It is a vascular endothelial growth factor (VEGF). It works to promote angiogenesis and hypertension, which will worsen the case of (Yamazaki et al., 2018).

1.6.1.3 Diabetic Neuropathy

Diabetic neuropathy is a typical complication associated with diabetes and causes nerve dysfunction, especially in peripheral nerves supplying the lower limbs in the form of loss of sensation in the toes and feet. The severity and abnormality of diabetic neuropathy are directly proportional to the duration of diabetes and hyperglycemia. Acute and chronic hyperglycemia has different side effects related to neuropathy. The impairment of nerve function is a result of acute hyperglycemia and chronic hyperglycemia is linked to more severe complications such as Wallerian degeneration (The et al., 1995). Wallerian degeneration develops after peripheral nerve damage and begins with axonal membrane and myelin sheath loss. Furthermore, the process of axonal regeneration will be followed by Schwann cells, and macrophages will facilitate axonal growth. The experiments show lower axon regeneration by 40% in diabetic rats, which is responsible for Wallerian degeneration. The polyol pathway is one of the causes of diabetic neuropathy, and it is due to the rise in glucose flow rate. The biochemistry behind this pathway is based on the increase of glucose flux, which will be reduced by the presence of NADPH sorbitol and NADP+. The enzyme that facilitates this reaction is aldose reductase (AR). Next is the oxidation of sorbitol to fructose, where NAD+ is

oxidized to NADH by sorbitol dehydrogenase. Sorbitol levels are inversely related to nerve conduction velocity (NCV). Increasing the level of sorbitol will decrease myoinositol, which leads to decreased Na/K ATPase activity and nerve conduction velocity. The cornerstone step is the consumption of NADPH by aldose reductase (AR), which also requires other pathways regulating oxidative stress like glutathione (GSH) reproduction by glutathione reductase and NADPH. The experiments show that using AR inhibitors as a treatment for nerve regeneration in diabetes is an effective (Chen et al., 2010).

1.6.2 Diabetic Macrovascular Complications

1.6.2.1 Cardiovascular Diseases (CVD)

Metabolic syndromes are increasing with the change in lifestyle and the unhealthy habits that people are practicing nowadays. These metabolic syndromes, such as dyslipidemia, obesity, hypertension, hyperglycemia, and insulin resistance, all together are risk factors for type 2 diabetes mellitus. The combination of metabolic syndromes with diabetes leads to damage to the vascular endothelium, increasing the risk of cardiovascular diseases (CVD) (Cade, 2008). Many studies show the comorbidity of diabetes with macrovascular complications, and it is considered a factor in sudden death (Beckman & Creager, 2016). When comparing the two types of diabetes and the risk of CVD. Type 2 diabetics are 15–400% more likely to experience a stroke. On the other hand, type 1 diabetics have a greater death rate due to ischemic heart disease. Patients with diabetes are more likely to have risk factors for CVD such as coronary artery disease, peripheral artery disease, stroke, myocardial infarction (MI), and heart failure (Khan et al., 2019).

Cardiovascular disease is the leading cause of death in 52% of T2DM patients and 44% of T1DM patients (Huang et al., 2017). The pathogenesis of DM coupled with CVD is hyperglycemia and insulin resistance, along with atherosclerosis. Atherosclerosis causes the narrowing of the blood vessels in the body, especially in the peripheral or coronary arterial wall. Atherosclerosis will cause endothelial injury and lipid accumulation from LDL oxidation. Furthermore, the process of atherosclerotic lesion formation is started by LDL oxidation facilitated by angiotensin II, followed by

monocyte invasion and differentiation in the arterial wall. It is differentiated to be macrophages which form foam cells from oxidized LDL. Macrophages will become T-cells and collagen will build up in the arteries to form an atherosclerotic lesion that is rich in lipids (Fowler, 2011). The response-to-injury hypothesis describes atherosclerosis as one pathogenesis of macrovascular diseases. Endothelial injury causes the accumulation of blood components in the subendothelial space, which forms an area called focal desquamation, which increases platelet circulation, and then platelets get in contact and adhere to the subendothelial. Some factors will be released in response to platelet adhesion, such as the platelet-derived growth factor, which is responsible for the migration and proliferation of smooth muscles. Moreover, plaque will form due to the formation of smooth muscles, lipids, and connective tissue. In vivo, experiments show hyperglycemia as a factor in facilitating the production of collagen in the arterial wall. Diabetes also causes lipid accumulation, decreases LDL degradation, and decreases the lysosomal enzyme in smooth muscle that aids in the hydrolysis of cholesterol esters (Brownlee & Cerami, 1981). Lowering the risk of cardiovascular death by changing and improving the lipid profile of diabetic patients with macrovascular complications is better than glycemic control. Controlling low-density lipoprotein and triglycerides is bad for health. Controlling these lipids can improve cardiovascular diseases. Also, intensive therapies can work. Diabetes alone will not increase LDL levels. However, LDL particles in T2DM can be glycated or oxidized (Tripathi & Srivastava, 2006).

1.7 Oxidative Stress

Oxygen is essential for life since it has a major role in energy production such as ATP through oxidative phosphorylation. On the other hand, it can turn toxic when it produces reactive oxygen species (ROS), which can target important biological molecules like protein, DNA, or lipids and cause damage or mutations (Burton & Jauniaux, 2011). ROS is one source of free radicals like superoxide anion, hydroxyl radical, and hydrogen peroxide which can damage the DNA, or cause lipid peroxidation. There is also reactive nitrogen species (RNS). The body must have a defense mechanism like antioxidants which remove free radicals. An example of antioxidant enzymes in the body is superoxide dismutase (SOD), glutathione reductase, and catalase (Buettner, 1993). Also, there are biological defenses which not include enzymes such as

glutathione, vitamin C, and vitamin E (Yang et al., 2011). The definition of oxidative stress is the imbalance between free radicals and the antioxidant (Sies et al., 2017).

1.7.1 Oxidative Stress and Diabetes Mellitus

Based on a hypothesis that started in 1991 up to date, the evidence shows oxidative stress is recognized as a key player in the development of diabetes mellitus (DM). The hypothesis concludes that higher ROS accumulation can trigger hyperglycemia which is responsible for many diabetic complications (West, 2000). In addition to being a major contributor to the development of diabetes, oxidative stress induced by hyperglycemia also directly causes cellular damage, which may occur in the period just before the beginning of many of the complications associated with the disease like nephropathy, retinopathy, neuropathy, vascular, and renal damage. Furthermore, oxidative stress could accelerate the development of DM by dysfunction β-pancreatic cells which are responsible for insulin production, and lead to a decrease in insulin sensitivity. In DM free fatty acids also cause the creation of ROS as a response it causes impairment of β-cells and mitochondrial DNA. Moreover, oxidative stress causes protein oxidation and inhibits many complexes responsible for mitochondrial respiration leading to blocking the process (Yang et al., 2011). Oxidative damage in diabetes will negatively affect the macromolecules such as lipids, DNA, and proteins and consider the change that occurs to these macromolecules as a biomarker for oxidative stress. Starting with DNA damage can be triggered by measuring 8-hydroxy-2′-deoxyguanosine in the blood which is used as an indicator for DNA damage. Lipid peroxidation is induced by free radicals, both isoprostanes and prostaglandin-like molecules formed by free radical-catalyzed peroxidation of arachidonic acid. Elevated amounts of the metabolite isoprostane have been found in the plasma and urine of diabetic individuals. Altering protein can lead to peptide fragmentation or cross-linking. The presence of both superoxide and nitric oxide (NO) can generate peroxynitrite which is a potent oxidant. Proteins attacked by peroxynitrite mostly result in the 3-position (ortho) nitration of tyrosine. Nitrotyrosine is suggested as a novel and intriguing marker of oxidative damage to proteins, in T2DM patients which shows an elevated nitrotyrosine in the plasma (Ceriello, 2006).

The two major processes causing diabetes are hyperglycemia and hyperlipidemia which affect the dysfunction of β-cell. Glucotoxicity and lipotoxicity increase oxidative stress and cause toxicity for β-cell. The evidence shows that long-term exposure to high glucose reduces insulin release and content as well as insulin mRNA levels in β-cells. An experiment was conducted using β-cell lines with two antioxidants N-acetylcysteine and aminoguanidine, and the result shows the effect of these antioxidants in protecting islet cells from the harmful consequences of being exposed to high glucose levels, by conserving both insulin mRNA expression and promoter activity. On the other hand, the effect of high fatty acids levels or glucolipotoxicity will disrupt insulin gene expression, inhibit insulin production stimulated by glucose, and promote cell death via apoptosis in β-cell with high oxidative stress (Pitocco et al., 2013).

1.7.2 Oxidative Stress in the Heart

The heart is a muscular pump located in the middle of the thorax between the lungs and slightly to the left. The heart's function is to collect deoxygenated blood from the body to the lung to exchange carbon dioxide with oxygen and then oxygenated blood will pump back to the body. The cardiac muscle is called the myocardium it contains four chambers. The upper two are receiving chambers called left and right atria. The lower two are pumping chambers with thicker walls called left and right ventricles which pump blood to the whole body. Between the chamber, there are valves to allow blood to flow in one direction. The normal cardiac muscle cells have single nuclei in the center, branched, and linked together at junctions called intercalated discs which hold the cells together. In cardiac muscle Ca^{2+} ions are responsible for the induction of contractions when it enters the cell. Since ions move freely between the network of cardiac cells this will transmit the electrical impulse, which triggers a coordinated contraction of all the muscle cells in response to the impulse (Iaizzo, 2005).

Mortality in diabetes mellites increases with the comorbidity of CVD. Hyperglycemia is characterized as the key factor of oxidative stress, and it is responsible for the excessive amount of superoxide produces by the mitochondrial electron-transport chain in response it will activate other pathways involved in diabetic complications through many mechanisms (Ceriello, 2006). Although the causes of diabetic cardiovascular problems are likely multifaceted and glyco-oxidative stress has been

proposed as the connecting element between the numerous molecular diseases seen in DM. In addition, inflammation can be a marker for cardiovascular events in DM such as atherosclerosis and coronary death due to an increase in the number of white blood cells, fibrinogen (Fbg), and C-reactive protein (CRP). The characteristic feature during an inflammatory response is the migration of phagocytic leukocytes to the injured tissue as a result of phagocytosis the production of oxidants will increase causing cardiovascular disease and vascular complications in diabetes. The polymorphonuclear (PMN) leukocytes are responsible for the production of hydrogen peroxide in diabetic patients. Unsaturated fatty acid-containing lipoproteins are especially susceptible to lipid peroxidation, which may trigger the formation of oxidants. Peroxynitrite is a potent oxidant of LDLs, and researchers have shown that oxidized low-density lipoprotein (LDL) is a reliable predictor of the progression of atherosclerosis events, via monocyte adhesion to the endothelium. These results provide a possible explanation for why antioxidants may suppress LDL oxidation in vivo but are not always effective in the atherosclerosis (Lipinski, 2001).

The pathogenesis of macrovascular complications is related to vascular endothelium damage and the limitation of vasodilation due to NO inhibition. The key enzyme for NO synthesis is endothelial nitric oxide synthase (eNOS). Hyperglycemia and insulin resistance both negatively affect the production of NO by inhibiting the eNOS enzyme and accumulating reactive oxygen species (ROS). The pathogenesis of macrovascular complications is related to vascular endothelium damage and the limitation of vasodilation due to NO inhibition. Furthermore, insulin resistance has another effect on adipose tissue, which can release free fatty acids. This can also increase ROS and decrease the activity of the eNOS enzyme (Cade, 2008). Thus, endothelial dysfunction is a major contributor to atherosclerosis and other vascular dysfunctions. Another study shows that significant cell death in myocytes and fibroblasts due to an increase in peroxynitrite formation will cause cardiovascular dysfunction in diabetic patients and experimental animals (Pitocco et al., 2013).

It has been hypothesized that prolonged oxidative stress due to hyperglycemia is the key to developing DM by speeding up the process of the formation of advanced glycation end products (AGEs) (Yang et al., 2011). AGEs are produced by the reaction

of both glucose and glycating compounds nonenzymatically in the heart's arterial endothelial cells. AGEs byproducts in the intracellular matrix can induce damage in the cell by many mechanisms. First, by modifying the function of proteins in the cell. The AGE precursors also will change the composition of the extracellular matrix by interacting with integrins receptors. In addition, AGEs precursors also affect plasma proteins and cause them to attach to the AGE receptor on cells like macrophages, vascular endothelial, and vascular smooth muscle cells. This process will result in the production of an excessive amount of ROS (Giacco & Brownlee, 2010).

The overproduction of ROS can activate the polyol pathway flux. The Aldo-keto reductase enzymes family will catalyze the reduction of carbonyl compounds by NADPH to form sugar alcohols (polyols). The enzyme aldose reductase is found in the vascular, glomerular, nerve, and retina cells. In these tissues, glucose is taken up by insulin-independent GLUTs which increase glucose concentrations in the cell due to hyperglycemic conditions, therefore, it will increase polyol pathway flux and cause damage to the cells. A study on diabetic mice that expressed a higher human aldose reductase showed atherosclerosis and a decrease in the expression of glutathione synthesis genes. The depletion of NADPH in the reaction of polyols formation promotes oxidative stress because NADPH is a cofactor necessary to produce reduced glutathione (GSH) which is a crucial antioxidant (Giacco & Brownlee, 2010).

The other mechanism contributing to hyperglycemia-induced diabetic vascular injury is the activation of protein kinase C (PKC) which works to phosphorylate other proteins. The activity of PKC depends on Ca^{2+} ion and phosphatidylserine, which is further boosted by diacylglycerol (DAG). The over-stimulating of PKC will cause tissue damage due to diabetes-induced reactive oxygen species. Also, the interaction between AGEs and their receptors can stimulate PKC activity. Indeed, the activation of PKC activity can lead to the buildup of some protein in the microvascular matrix via stimulating the expression of transforming growth factor (TGF)- β1, fibronectin, and type IV collagen in cells (Giacco & Brownlee, 2010). All three mechanisms are interconnected. They collectively induce ROS via hyperglycemia, which triggers the activation of proinflammatory NF-kB transcription factors and inflammatory gene expression. Therefore, increased polyol pathway flux and protein kinase C will be

activated and induce transforming growth factor (TGF)- β1 also AGEs formation will increase. After this cascade, the cell will get injured and apoptosis will take place (Yang et al., 2011).

1.8 Management of Diabetes Mellitus

The best way to control and manage diabetes is to maintain euglycemia, which is glycemic control. The maintenance of euglycemia will prevent both microvascular and macrovascular complications. According to the study by the United Kingdom Prospective Diabetes Study (UKPDS), it also reduces microvascular complications by 37% and myocardial infarction by 14%, which is considered a risk factor for cardiovascular diseases, and the numbers are considered significant (Hunter, 2015).

1.8.1 Lifestyle Modification

The cornerstone of managing diabetes is lifestyle modification, which includes diet control and increasing physical activity. Obesity and a sedentary lifestyle have a strong association with insulin resistance. Studies show that physical activity can increase insulin sensitivity. It works on muscle cells to facilitate the uptake of glucose and mitochondrial function, and improve cell function. On the other hand, being physically inactive will inactivate GLUT-4. This will block the entrance of glucose into the cell. Changing life habits can prevent pre-diabetic patients from becoming diabetics. Eating a healthy diet can decrease the risk of diabetes by 18%-40% (Khan et al., 2019).

The American Diabetes Association (ADA) recommends that diabetic patients consume a certain percentage of macronutrients. When a clinician recommends or prescribes a nutritional plan for any patient, it should be specific and unique depending on personal factors, medical status, and lifestyle that will perfectly suit that patient's situation, and this is known as medical nutrition therapy (MNT). There are three main sources of macronutrients: carbohydrates, fats, and proteins. The ADA recommends that diabetic patients have a higher total calorie intake from carbohydrates of about 45-65%, and clinicians should educate patients who are taking insulin as a treatment on carbohydrate counting for insulin injections to be adjusted according to the carbohydrate amount of the meal. Also, protein and fat percentages are very important in a balanced diet. It is recommended for diabetics to get 10-35% from proteins and 25-35% from fats

in a day. Obese patients have extra restrictions on both weight and calories so they can lose weight (Hunter, 2015).

1.8.2 Pharmacotherapy

Since 1995, there have been many drugs for treating diabetes that is considered antidiabetic agents. However, before that, the only drug found for T2DM in the US was sulfonylureas. More than half of diabetic patients were treated with sulfonylureas, 40% were treated with insulin, and very few were treated with a combination of both drugs. Recently, there have been many more antidiabetic drugs on the market, such as insulin and insulin analogs. These drugs mainly target hyperglycemia. The pharmacological effect of these drugs is to lower glycemia in both T1DM and T2DM. The key mechanism for the drugs is to control glucose in different ways, such as lowering glucose levels by eliminating insulin deficiency and inducing its production, reducing the amount of glucose produced by the liver, improving the use of muscle glucose, and slowing carbohydrate absorption (Skyler, 2004). Some examples of anti-diabetic drugs to treat different stages of DM with cardiovascular comorbidities include but are not limited to metformin, insulin, thiazolidinediones, α-Glucosidase inhibitors, and Glucagon-like peptide-1 (GLP-1) receptor agonists, Dipeptidyl Peptidase-4 (DPP-4) inhibitors, sulfonylureas, and sodium-glucose cotransporter (SGLT) inhibitors.

1.8.2.1 Insulin

Insulin is a hormone produced by pancreatic beta cells in an inactive form called proinsulin. Insulin has two polypeptide chains cross-linked by two disulfide bonds. The first chain (chain A) has 21 amino acids, and the second chain has 30 amino acids (chain B). The main function of insulin is to transport glucose from the bloodstream to target tissue like skeletal muscle and the liver, where the excess glucose is stored as glycogen. Since 1921, insulin was used to treat diabetic dogs, when it was first discovered. Exogenous insulin is the main treatment for T1DM to control glucose and glycogen levels in the body. T1DM patients use insulin injections repeatedly, usually more than once a day. These injections may contain different types of medications taken at different times of the day. Novorapid, Apidra, or Humalog are insulin analog injections used as immediate-acting insulin after meals (Wong et al., 2016). Lispro and Aspart are

considered short-acting insulin. Long-acting insulin is very critical because it can cause diabetic ketoacidosis, which can decrease electrolytes and water. An example of this is Glargine or Determir which is usually taken at bedtime or after dinner (Kahn et al., 2011). Other than subcutaneous injections, there are different routes for insulin administration, like nasal and pulmonary. However, the challenge is that only a limited amount of insulin can effectively enter the liver for the actual activity. The newest route is oral, but it currently faces several challenges (Wong et al., 2016).

1.8.2.2 Metformin

Metformin is one of the most effective treatments to control hyperglycemia by reducing glucose levels produced by the liver. Metformin is active only when insulin is around, which can be specifically prescribed for T2DM patients, not T1DM due to the complete absence of insulin. It also increases the rate at which glucose is transported into the muscular tissue (Modi, 2007). This drug is safely used for T2DM patients free from pulmonary, renal, or liver disease as well as heart failure; it should never be administered to such patients (Kahn et al., 2011). It has an advantage in weight loss in T2DM patients because it decreases HbA1c by 1.5% when it is combined with lifestyle treatments. Metformin protects patients with type 2 diabetes from developing cardiovascular problems due to its antioxidative and anti-inflammatory properties, which have the potential to enhance the microcirculation (Adeghate et al., 2021). On the other hand, gastrointestinal adverse effects impact up to 63% of patients when they begin therapy. To avoid the side effects, treatment with metformin should start with low doses like 500 mg and be increased to 850 mg after the first week if there are no other consequences. The highest dosage is 1000 mg two times a day (Pratley, 2013).

1.8.2.3 Glucagon-like peptide-1 (GLP-1) Receptor Agonists

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones secreted from the gastrointestinal tract (García-Ropero et al., 2019; Lotfy et al., 2014). The structure of GLP-1 has two forms: a peptide with 30 amino acids and a 31-amino acid, secreted specifically from the L-cells of the GI tract. In addition, GIP is secreted from K cells, which is a different part of the GI tract (Modi, 2007). Within a few minutes after ingesting a meal, GLP-1 will be secreted from

intestinal cells through the bloodstream, targeting the beta cells to stimulate the release of endogenous insulin and the alpha cells to suppress glucagon production. The GI tract plays a key role in secreting regulatory endocrine and exocrine hormones other than its main function in digestion. GLP-1 has a very short half-life before it is degraded by dipeptidyl peptidase-IV (DPP4), approximately 2 minutes. A new class of GLP-1 analogs has been created to take the role of GLP-1. Exenatide is an example of a synthetic incretin, which mimics naturally occurring exendin-4. Furthermore, exenatide is long-acting with a longer active half-life because it resists DPP4 action compared to GLP1 (García-Ropero et al., 2019; Lotfy et al., 2014). Since 2009, GPL-1 receptor agonists have been available on the market as an antidiabetic drug. It has been established that GLP-1 receptor agonists lower liver enzymes and affect reducing oxidative stress and weight loss, all of which help in the diabetes management (Sumida et al., 2020). Moreover, it helps to reduce cardiovascular risks like heart failure (HF) (Adeghate et al., 2021).

1.8.2.4 α-glucosidase Inhibitors

This class of drug was developed in the 1970s and authorized as a therapy for T2DM in the 1990s. As a result of its effects on the gastrointestinal tract, this medication helps to keep blood glucose levels more stable after meals. α-Glucosidase is an enzyme found in the small intestine, responsible for the breakdown of oligosaccharides into monosaccharides to improve their absorption in the intestinal mucosa. This causes a delay in the breakdown of carbohydrates. Therefore, the mechanism of action of α-glucosidase inhibitors acts as a competitive inhibitor. Hydrolysis is then carried out by the enzyme to cleave oligosaccharides, which are first bound to their binding site before they are hydrolytically broken down. Oligosaccharides and α-glucosidase inhibitors fight for the same active site on the enzyme. Acarbose, voglibose, and miglitol are examples of α-glucosidase inhibitors in the global pharmaceutical market. A meta-analysis proved that these agents reduce HbA1c, postprandial glucose, and body weight by 0.63 kg compared to the placebo (Kahn et al., 2011). Acarbose minimizes the risk of myocardial infarction, hypertension, and other cardiovascular events by 34-49% (E. A. Adeghate et al., 2021). Despite all the advantages, this class of drugs has many adverse effects. Many

participants withdraw from the study due to side effects like flatulence and diarrhea (Khan et al., 2019).

1.8.2.5 Sodium-Glucose Co-transporter (SGLTs) Inhibitors

Sodium-glucose cotransporters were identified in the 1930s and showed a significant association with DM. The molecular mechanisms underlying the beneficial effect of SGLT inhibitors in improving diabetes complications. In the 1930s, Chasis discovered SGLT compounds while examining phlorizin and its role in glucose excretion in the kidney. Phlorizin is found naturally in the root bark of apple trees and works by preventing the reabsorption of glucose in the kidney to reduce hyperglycemia in the blood. A study reported that lacking the SGLT gene in the kidney is incapable of reabsorbing glucose from the kidney exactly from proximal convoluted tubules (PCT). The studies support the association between SGLT and its role in regulating glucose and sodium in the GI tract and kidney. The SGLT gene was cloned in the 1980s by a group of scientists, confirming the existence of SGLT1 in the small intestine and SGLT2 function in the reabsorption of glucose from the PCT. The strategy of SGLT proteins is based on the belief that reducing hyperglycemia, a key contributor to oxidative stress can alleviate some of the symptoms and consequences associated with DM (E. Adeghate et al., 2019). The reduction of cardiovascular and renal complications is shown in several research studies (E. A. Adeghate et al., 2021).

The normal way glucose is filtered and reabsorbed is in the PCT where it is completely reabsorbed. Extra glucose is excreted in the urine when the glucose concentration exceeds the renal threshold for glucose reabsorption, which is 8.3 mmol/l. This condition is called glucosuria. In addition, glucose is transported into and out of cells by carrier proteins found in all cells, specifically in the cell membrane known as glucose transporters (GLUTs). GLUTs keep glucose levels stable across the membrane and are considered passive transporters, while SGLTs are active transporters that work against concentration gradients in transporting glucose and sodium (Brown et al., 2021).

1.8.2.5.1 Sodium-Glucose Cotransporter 1 Inhibitors (SGLT1i)

Sodium-glucose cotransporter 1 is a transmembrane protein with 664 amino acids and 14 domains. The gene encoded by SGLT1 is the SLC5A gene found on chromosome

22. SGLT1 plays an important role in glucose homeostasis in both the kidney and the small intestine. SGLT1 absorbs glucose and galactose in the small intestine. It also contributes to the reabsorption of glucose in the kidney since it is found in the S3 segment of proximal tubule renal epithelial cells (Abdul-Ghani et al., 2011). This induces hyperglycemia due to the increase in glucose absorption in the small intestine. In this type of protein transporter, glucose and sodium will cotransport at a ratio of 1:2 (Sano et al., 2020).

The location of SGLT1 in rats depends on the necessity for insulin to affect glucose delivery to myocytes like coronary arteries. Also, it can be found in the brain and the skeletal cells. The comorbidities of T2DM and obesity with end-stage cardiomyopathy in mice will show a higher SGLT1 expression (García-Ropero et al., 2019).

The SGLT1 inhibitors are designed to prevent the normal function of the SGLT1 transporter in the small intestine and the kidney and increase glucosuria. It inhibits the absorption of glucose in microvilli and the glucose reabsorption in PCT, and this is important in the carbohydrate metabolism (Adeghate et al., 2019). SGLT1-mediated renal glucose absorption is greatly enhanced when SGLT2 transporters are blocked. This group of inhibitors increases the delivery of glucose to the PCT and enhances the release of glucagon-like peptide-1 (GLP-1) from intestinal L-cells, which stimulate β-cells to secrete insulin and reduce glucose levels in the blood. In addition, GLP-1 reduces the amount of glucagon released, decreases appetite, and elevates β-cell mass. The beneficial effect of SGLT1 inhibitors appears to be in reducing reactive oxygen species produced by hyperglycemia. The influx of sodium with SGLT1 negatively affects myocardial cells and causes damage due to Ca^{2+} accumulation. Indeed, this can affect mitochondrial function. Also, SGLT1 function in glucose uptake promotes tumor growth. So, SGLT1 inhibitors can prevent these negative effects exerted by SGLT1 function. SGLT1 inhibitors have an adverse effect on T1DM patients because of the incidence of moderate diarrhea. Also, it causes polyuria and urogenital infections (Tsimihodimos et al., 2018).

1.8.2.5.2 Sodium-Glucose Cotransporter 2 Inhibitors (SGLT2i)

SGLT2 is a protein with 672 amino acids and is encoded by the SLC5A2 gene on chromosome 13. SGLT2 is responsible for the reabsorption of glucose in the renal PCT,

specifically localized in segments 1 and 2. In a euglycemic state, the reabsorption of glucose in the kidney by SGLT2 is highly significant, up to 90%, which is considered the main function. The glucose and sodium are co-transported in a ratio of 1:1(Sano et al., 2020).

SGLT2 inhibitors are a new class of drugs that have been developed as anti-diabetic agents known as gliflozins (Abdul-Ghani et al., 2011). SGLT2 inhibitors were used to prevent the reabsorption of glucose in PCT exactly in segment 1 (S1) (E. Adeghate et al., 2019). Indeed, this increases glucosuria, leading to a decrease in the glycemia (Chambergo-Michilot et al., 2021). SGLT2 inhibitors are recommended for T2DM patients and many studies show the effect of SGLT2i in improving cardiovascular outcomes (Täger et al., 2021). It is also prescribed for T1DM patients. This group of inhibitors increases the release of glucagon-like peptide-1 (GLP-1) from intestinal L-cells when it has been used with dipeptidyl peptidase IV (DPP-IV) inhibitors (Tsimihodimos et al., 2018). SGLT2i is proven to reduce the risk of heart failure and renal disease. Dapagliflozin, canagliflozin, and empagliflozin are examples of SGLT2i with a C-glucoside linkage, which prolongs the compound's half-life by protecting it from β-glucosidase hydrolysis. Recent clinical trials show the effectiveness in decreasing heart failure by 35% using canagliflozin and empagliflozin. However, dapagliflozin did not show any improvement in cardiovascular events. A recent study showed that canagliflozin raises GLP-1 levels in the bloodstream when taken before meals (Dominguez Rieg & Rieg, 2019).

The SGLT inhibitors have advantages in reducing glycosylated hemoglobin (HbA1c) by 0.5-1.0% (Herrington et al., 2021). It also ameliorates cardiovascular disease, for example, dapagliflozin helps in lowering low-density lipoprotein-cholesterol (LDL-C) and elevating high-density lipoprotein. Another study shows its effect in reducing weight from -1.0 kg to -0.3 kg. Empagliflozin reduces the absorption of cholesterol and enhances LDL-C excretion in feces. SGLT2i reduces systolic blood pressure and other vascular outcomes in T2DM. On the other hand, SGLT2i has adverse effects such as genitourinary infections due to glycosuria. This adverse effect affects about 10% to 15% of all patients taking SGLT2i, especially women. Also, ketoacidosis is one of the risk factors because it increases ketone body levels. It may cause acute renal

injury which has been reported in 100 individuals who had used SGLT2 inhibitor medications. Moreover, half of the participants reported experiencing symptoms within a month of using SGLT2i medication, and they show improvement after they stopped SGLT2i. The risk of bone fracture and amputations is also considered a side effect of SGLT2i (Pittampalli et al., 2018). As a result, hundreds of clinical studies were initiated, yielding valuable new information on the connection between type 2 diabetes and cardiovascular disease leading to the discovery of new medications controlling CVD and renal events in T2DM patients such as SGLT2i (Santos-Ferreira et al., 2020). A total of 15 studies combined with a meta-analysis were conducted to confirm the role of SGLT2i in improving cardiovascular events. Improved oxygen supply, cardiac fuel energetics, and mitochondrial activity can be achieved by SGLT2 inhibition in patients with diabetes as well as insulin resistance. Moreover, the advantages of SGLT2i in losing weight and protecting the renal system can help improve HF (Cardoso et al., 2021).

1.8.2.5.3 Dual SGLT1/2 inhibitors

The discovery of drugs that maintain glucose homeostasis sparked the development of new novel glucose-lowering medications. Several animal studies confirm the effect of dual SGLT1/2 inhibitors (SGLT1/2i) in glucose excretion throughout the kidney and glucose absorption in the small intestine which eventually ameliorates hyperglycemia. This inhibitor combined the mechanism of function of both SGLT1 and SGLT2 in reducing glucose uptake expressed in the intestine and kidney shown in figure 1. Also, SGLT1 is found in other body organs like the heart, liver, skeletal muscle, and lungs. Sotagliflozin is used for the treatment of T1DM and it is considered the first licensed dual SGLT inhibitor in Europe (Brown et al., 2021). Initially, sotagliflozin was designed to inhibit SGLT1 because it is expressed at the greatest levels and accounts for almost all glucose reabsorption in many organs. However, it exerts a similar effect to selective SGLT2i in excreting glucose in urine causing glucosuria. Dual SGLTi has cardiorenal benefits, sotagliflozin reduces the relative risk for heart failure by 33%. Also, it is very effective in reducing myocardial infarction and stroke. Insulin secretion and glucose absorption are both delayed when intestinal SGLT1 is blocked, which in turn increases the amount of GLP-1 released to enhance the glycemic control (Sayour et al., 2021). Like all pharmaceutical drugs on the

market dual SGLT1/2i have advantages and side effects. It helps in reducing the spike in blood sugar after eating, releases GLP-1 from the cecum, extends the effect of GLP-1 by inhibiting DPP4, and possible adjunct therapy for T1DM. On the other hand, the adverse effect includes diarrhea, diabetic ketoacidosis, and a higher risk of hypoglycemia (E. Adeghate et al., 2019).

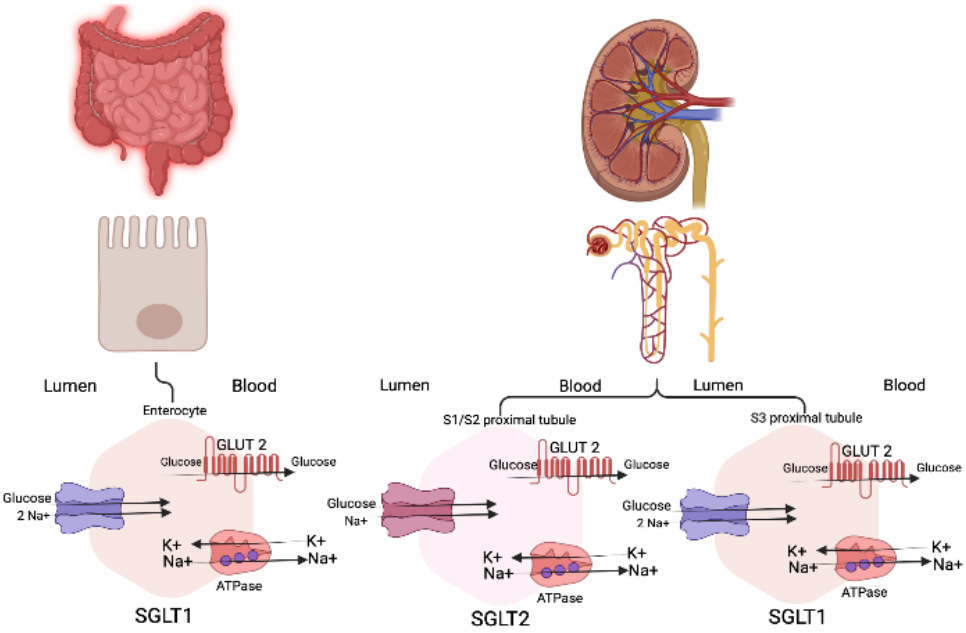


Figure 1: The mechanism of action of dual SGLT1/2 inhibitors in the enterocytes and the kidney. The image is created with BioRender.com

1.9 Licogliflozin

Licogliflozin (LIK066) is a dual SGLT1 and SGLT2 inhibitor (SGLT1/2i), with 30-fold SGLT2 selectivity. LIK066 is a safe, oral, and bioactive drug presumed to be effective for T2DM, obese, and heart failure patients (He et al., 2021). The mechanism of action is a combination of SGLT1i and SGLT2i. Regarding LIK066 SGLT1 represent IC50 of 20.6 nmol/L and SGLT1 0.58 nmol/L in human (IC50 is the amount of drug needed to achieve 50% inhibition). This result was obtained in healthy, T2DM, and obese patients. Oral glucose tolerance tests show improvement after taking LIK066, inhibiting insulin secretion and decreasing postprandial glucose. In addition, it promotes the excretion of glucose via the kidneys, resulting in weight reduction. The recommended dose for LIK066 to be safe can be a single or twice daily, 300 mg or 150

mg respectively, at larger dosages there was a significant rate of diarrhea (Wang-Lakshman et al., 2021). This study shows the significant effect of LIK066 on reducing body weight by 6% compared to the placebo (Bays et al., 2020).

There is a limited amount of research papers on LIK066. That was the reason behind this research and the investigations on the LIK066 effect on different metabolic parameters.

1.9.1 Structure of LIK066

LIK066 is a C-glycoside inhibitor that has C-C-linkage between the glucosyl group and the aromatic ring shown in figure 2. Therefore, improving the stability of enzymatic metabolic processes (A. M. Matos et al., 2021). The molecular weight of LIK066 is 416.5 and the molecular formula is $C_{23}H_{28}O_7$. The full IUPAC name of LIK066 is (2S,3R,4R,5S,6R)-2-[3-(2,3-dihydro-1,4-benzodioxin-6-ylmethyl)-4-ethylphenyl]-6-(hydroxymethyl) oxane-3,4,5-triol (Licogliflozin, 2022). This type of gliflozin is diarylmethane C-glucoside which has an aryl ring with a significantly wider structural variety, whereas glucosyl modifications are limited. The structure has a cyclic monosaccharide or anomeric structure with β configuration (A. M. Matos et al., 2021). The 2D structure of licogliflozin is shown in Figure (2) (Licogliflozin 2D Structure, 2011).

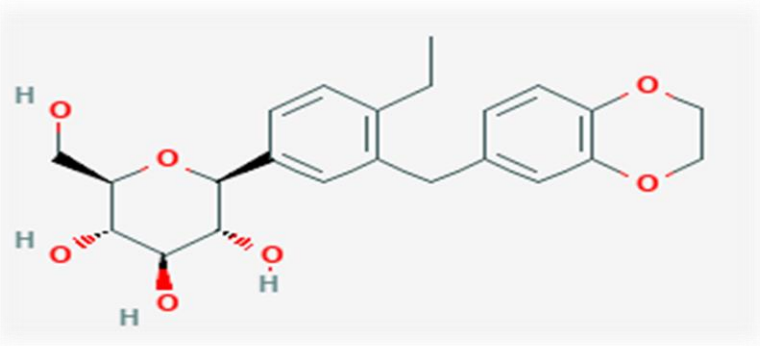


Figure 2: The 2D structure of Licogliflozin. Adapted from http://www.chemspider.com/Chemical-Structure.71045806.html

1.9.2 Licogliflozin's Mechanism of Action

LIK066 mechanism of action includes the inhibition of both SGLT1 and SGLT2. The SGLT1 inhibition happens in the small intestine which decreases glucose and galactose absorption, resulting in weight loss via multiple possible endocrine processes, including a decrease in insulin secretion after meals and reduce appetite due to GLP-1 and peptide YY secretion. Diarrhea and malfunction of the PCT can be a result of mutations in SGLT1 transporters, it can be controlled by cutting out the glucose and galactose from the diet completely (de Boer et al., 2020). Also, it causes loose stools due to the SGLT1 inhibition (E. Adeghate et al., 2019). On the other hand, SGLT2 action is exerted on the kidney by 90% reabsorption of filtered glucose in the PCT and 10% will be reabsorbed by SGLT1. The inhibition of SGLT2 results in weight loss, glucosuria, and a decrease in glucose levels in T2DM patients as well as the controls in many clinical trials (He et al., 2020). LIK066 is presently being researched as a therapeutic option for many diseases mainly for T2DM and its complications like heart failure, chronic kidney disease, obesity, and even non-alcoholic steatohepatitis (NASH).

1.9.3 The Clinical Benefits of Licogliflozin

1.9.3.1 Body Weight Reduction

Scientists came up with many anti-obesity drugs that operate mostly on the nervous system. LIK066, a dual SGLT1/2i, can also act as anti-diabetic at the same time while reducing body weight. LIK066 action cause calorie depletion in the intestine and kidney. The SGLT1 inhibition in intestinal L-cells enhances the production of weight-controlling incretin hormones like GLP-1 and PYY. Many studies conducted to investigate the effect of LIK066 on body weight and its effect on DM biomarkers like glucose, insulin, and incretin. A 12-week study of LIK066 treatment with a 150 mg dose on obese patients showed a weight reduction, especially with patients having dysglycemia by 6.4 kg from day 7 compared to placebo which reduce only 0.24 kg. Also, waist circumference was reduced significantly after the study. The maximal dose of LIK066 results in eliminated insulin secretion which supports the idea that LIK066 inhibits glucose transit in the gastrointestinal tract. In addition, LIK066 raised postprandial GLP-1 and PYY production and decreased GIP in individuals with type 2

diabetes. Eventually, LIK066 contributes to stimulating the release of GLP-1 which influences appetite, and this mechanism has an indirect effect on satiety thus it can reduce body weight. Glucagon has a great effect on the human body, it enhances energy expenditure and reduces fat tissue development. After 12 weeks of studying LIK066, the results significantly show glucagon inhibition which is also considered a factor of weight loss compared to placebo. Regarding the decrease in GIP levels in T2DM patients taking LIK066, the investigations have shown that GIP and GIP receptor gene alteration is linked to improved glucose management, weight loss, and improved insulin sensitivity. LIK066 also increases urinary glucose excretion by blocking its reabsorption. LIK066 is a safe drug in dosages of 150 x 2 mg or a single 300 mg on T2DM and obese individuals with adverse effects of a higher incidence of diarrhea and genitourinary infections. This might be a novel and effective way to control body weight in obese patients and patients with or without DM (He et al., 2019).

1.9.3.2 Cardiovascular Events in Diabetic Patients

According to recent research studies, the second most prevalent cardiovascular manifestation in people with type 2 diabetes is heart failure (HF) (Santos-Ferreira et al., 2020). Heart failure (HF) is becoming more common as people become older. Hospitalizations for heart failure are common in older persons, and 9.3% of all mortality from cardiovascular illness is attributed to HF which also shows a connection with atherosclerotic syndromes (Chambergo-Michilot et al., 2021). Diabetes mellitus can cause heart failure in numerous ways including ischemia from coronary artery disease, mitochondrial damage, malfunction of the microcirculation, hyperplasia of the myocyte, also an increase in sodium retention, and proinflammatory cytokines. Also usually, these abnormalities are combined with other co-morbidities like kidney disease, hypertension, or even obesity leading to HF with reduced ejection fraction (Cardoso et al., 2021). There are pharmaceutical drugs to manage HF with reduced ejection fraction, such as angiotensin-converting enzyme inhibitors (ACEis), or mineralocorticoid receptor antagonists (Chambergo-Michilot et al., 2021). For all commercially available medications that decrease blood glucose, the US Food and Drug Administration demanded proof of CV safety in 2008. The human heart uses energy from ATP and phosphocreatine. ATP is the major fuel molecule and a product of oxidative

phosphorylation in mitochondria, which produce 95% of ATP, and the rest 5% is generated from glycolysis. Normal heart metabolism needs a lot of energy, generated in the mitochondria in a process called the Krebs cycle starting with the Acetyl-CoA (García-Ropero et al., 2019). Sodium Na^+ homeostasis plays a major role in the function of cardiomyocytes, which regulated many ion channels and transporters that work together to keep Na^+ levels stable in cardiomyocytes. SGLT2i may be able to alter cellular processes to better handle Na^+ and hence slow the progression of HF. Sodium imbalance and oxidative stress are characteristics of HF Trump (Trum et al., 2021).

The SGLT1 protein expression was detected in the heart's blood vessels of both rats and humans. However, it was absent from small intestinal blood vessels. Heart muscle cells from humans and mice show SGLT1 in their plasma membranes. Cardiac SGLT1 is highly expressed in T2DM and diabetic cardiomyopathy. Its role is implicated in glucose transfer from the vessels to cardiomyocytes and there is no SGLT2 in the heart (Sano et al., 2020). A research study was conducted for 12 weeks to investigate the effect of dual SGLT1/2 inhibitor LIK066 on N-terminal pro-b-type natriuretic peptide [NT-proBNP] in individuals with T2DM and HF. The NT-proBNP is an HF severity and stress biomarker on the cardiac muscle wall. This study assigned patients randomly to different doses of LIK066 2.5, 10, or 50 mg daily. The one dose that shows a significant effect in reducing NT-proBNP which is 10 mg. This study concludes the benefit of LIK066 on cardiovascular events in diabetic and HF patients. There are other benefits like increasing urinary glucose excretion and decreasing HbA1c and obesity, which are considered risk factors for HF and CV mortality in T2DM patients. This study shows some adverse effects of LIK066 like mycotic infections, however only one patient gets affected and this is not significant, because of the small sample size limitation of this study. In addition, gastrointestinal side effects but no urinary tract infections or diarrhea was reported. Also, 4 patients significantly show hypoglycemia with the absence of ketoacidosis which is a good sign in T2DM patients. The study concludes that this dual SGLT1/2i (LIK066) has a great impact on patients with T2DM and HF by reducing NT-proBNP (de Boer et al., 2020). Other than the glycemic control and cardiovascular benefits, dual SGLT also reduces ROS production as heart failure exerts oxidative stress (Bode et al., 2021). In the case of LIK066, no information was provided for the

hospitalization for HF. Also, no data for LIK066 to avoid the progression of the renal function (Täger et al., 2021)

1.9.3.3 Renoprotection in Patients with T2D

A worldwide health concern, chronic kidney disease (CKD) affects around 10% of the world's population, with 1.2 million fatalities in 2015 due to renal failure. Although 50-70% of patients with severe CKD do not have diabetes, diabetic kidney diseases are common. LIK066, one of the most powerful and specific dual SGLT1/2i, enhances renal salt and glucose management by blocking both SGLT1 and 2 in the PCT. LIK066 has the potential to enhance or maintain glucose-lowering effects in individuals with varying degrees of CKD by affecting urinary glucose excretion within 24-h (UGE24). There is no effect of LIK066 on severe cases of kidney disease, however, it increases the UGE24 in CKD. One study that measured the effect of LIK066 on urinary excretion of electrolytes such as sodium, chloride, calcium, phosphate, and potassium showed that the sodium and chloride trend decreased after day 7 of a single LIK066 dose in healthy and moderate CKD subjects. Also, potassium excretion was reduced, but no exact trend for the calcium and phosphate excretion after LIK066 treatment. This study also showed the effect of LIK066 on the renin-angiotensin-aldosterone system (RAAS) (He et al., 2021). It is thought that aldosterone increases renal perfusion and causes hyperfiltration of the kidneys because it increases the sodium reabsorption, and synthesis in the adrenal cortex (Kobayashi et al., 2020). Also, potassium and protons are secreted by aldosterone in the collecting duct. RASS involve in slowing kidney disease progression. Angiotensinogen is generated in the liver and degraded by renin. Angiotensin I (AngI) is degraded with angiotensin-converting enzyme (ACE) activity and transforms into AngII, the active form. RAAS also functions in maintaining the blood pressure (Rüster & Wolf, 2006). After 7 days of LIK066 treatment renin and aldosterone levels increased. The increase in renin levels shows a decrease in blood pressure (BP). While the decreased BP may have resulted in an enhanced renin activity, higher aldosterone levels result in greater transport of sodium to the nephrons. This study concludes that LIK066 is safe for CKD patients.

1.9.3.4 Treatment of Nonalcoholic Steatohepatitis (NASH)

In Japan, 9.3% of T2DM death is associated with liver diseases. Nonalcoholic fatty liver disease (NAFLD) is the third leading cause of mortality in T2DM patients. Thus, NAFLD can progress to another severe form called nonalcoholic steatohepatitis (NASH) which causes liver carcinoma and hepatic failure. NASH is also known as "Diabetic Liver Disease" which is widely increased with metabolic syndromes like obesity and diabetes. The cornerstone of NASH therapy for nondiabetic patients is vitamin E and lifestyle interventions. However, this is not very effective for patients with NASH and T2DM. NASH pharmacotherapy can protect both liver and heart effects (Sumida et al., 2020). For the prevention of NASH and its associated consequences, pharmaceutical treatments are now being tested in clinical studies. One mechanism to treat NASH is targeting the efficiency in the use of energy and disposal approach which is the mechanism of SGLT1/2i without crossing the blood-brain barrier. Mainly renal glucose excretion by SGLT1/2i is considered an energy-wasting of 250-350 kcal of glucose per day. A study proved that LIK066 decreases liver fat (Neuschwander-Tetri, 2020). Another study shows that LIK066 reduces serum ALT, AST, and GGT which are liver biomarkers as well as liver fibrosis markers and liver fat. In addition, LIK066 lower HbA1c, weight, and insulin resistance (Negi et al., 2022). Furthermore, LIK066 shows an enhancement in transaminases and reduces steatosis in NASH patients, according to findings (Dewidar et al., 2020).

Chapter 2: Hypothesis, Aims, and Objectives

Sodium-Glucose Cotransporter Inhibitors (SGLTi) decrease glucose absorption from the kidney and the small intestine resulting in weight loss, euglycemia in diabetic patients, and improvement in cardiovascular diseases. We hypothesized that dual SGLT1/2i such as licogliflozin (LIK066) will alter the pattern of distribution concentration of insulin, markers of cardiac fibrosis, and oxidative stress.

Specific objectives of the study are:

- To study the effect of STZ-diabetes on fasting blood glucose level, intraperitoneal glucose tolerance test, and body weight.
- To determine whether SGLT1 and SGLT2 are present in the cells of pancreatic islets of Langerhans.
- To examine whether SGLT1 and SGLT2 co-localize with insulin in the endocrine pancreas.
- To examine whether licogliflozin influences the tissue concentration of selected markers of cardiac fibrosis.
- To determine whether licogliflozin alters the tissue concentration of selected markers of oxidative stress in the pancreas and myocardium.

Chapter 3: Methods

3.1 Animal Model

This study used three-month male Wistar rats weighing approximately 250 g. The Research Animal Facility in the College of Medicine and Health Sciences at UAE university provides this study with all animals. The animals were kept in a pathogen-free environment at 22-25°C with 12 hours light/dark cycle. Polycarbonate cages with wood chips bedding were utilized to house three animals in each cage. The cages were changed twice a week for non-diabetic animals and every two days for diabetic animals. They were fed a conventional rat chow diet and given free access to tap water. The study was authorized by the Institutional Animal Ethics Committee of the United Arab Emirates University (Approval #: ERA-2022-8481).

3.2 Establishing the Experimental Diabetes Model

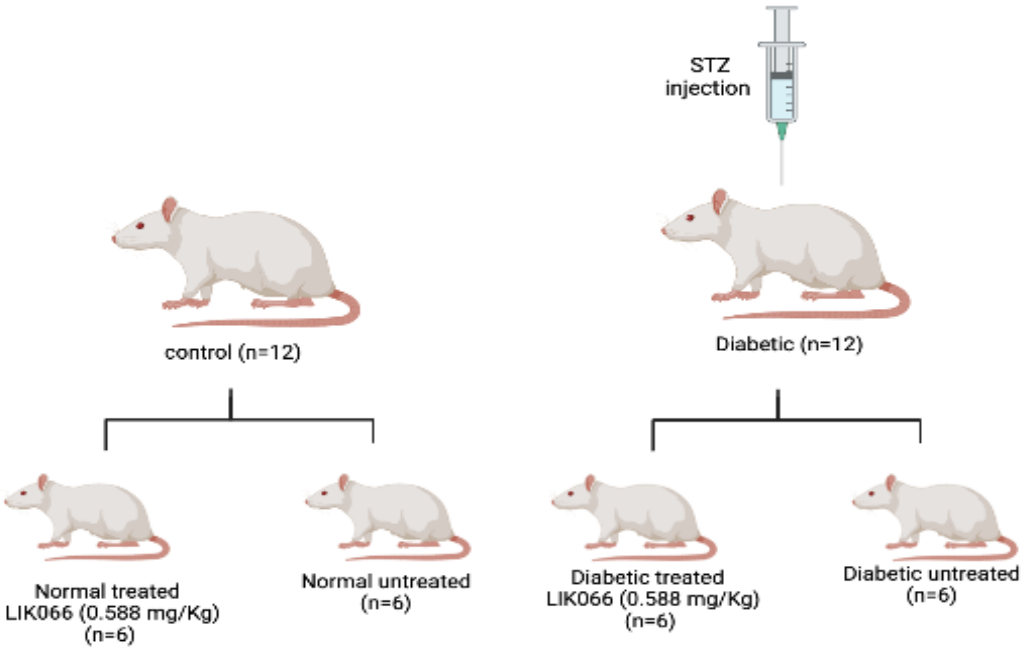
The diabetic animal model was established with a single dose of streptozotocin (STZ). STZ was administered intraperitoneally to Wistar rats at a dosage of 60 mg/kg body weight. Injectable streptozotocin was newly prepared by dissolving it in citrate buffer (pH 4.5, 0.5 M) and stored on ice. Fasting blood glucose (FPG) levels were checked after one month and the animals with FPG levels of more than 250 mg/dl were declared diabetic and exclude animals with readings below the level. It has been reported in the literature that STZ induces diabetes in rodents by generating ROS to destroy beta islet cells of the pancreas (West, 2000).

3.3 Experimental Design

Starting by sorting the animals into two groups, control, and diabetic. Then each group was subdivided into treated and untreated which is shown in the schematic diagram in figure 3. The treated was injected with 0.588 mg/Kg of LIK066 five days/week for a period of 4 weeks and the untreated was injected with the vehicle (phosphate buffered saline).

During the 4 weeks of treatment, the body weight was checked once a week, as well as blood glucose levels, which were measured from the tail vein using an

OneTouch® Ultra® glucometer (LifeScan, Inc., Milpitas, CA, USA). At the end of the 4 weeks, an intraperitoneal glucose tolerance test (IPGTT) was administered for all groups after 12 hours of fasting. The process was as follows: intraperitoneal injections of glucose which differ between each rat depending on the rat's weight are given according to the following equation (Glucose dosage = 0.002 X rat's weight) (mg/Kg). Blood glucose was then measured from the tail vein sample. The process was repeated at



different time intervals between 0 and 120 minutes (0, 30, 60, 90, and 20 minutes).

Figure 3: Schematic diagram of the animal category in each group included in this study. (Created by biorender.com)

3.4 Blood/Tissue Collection and Tissue Processing

Before tissue collection, IPGTT was done for all the groups. After 4 weeks of treatment, animals were sacrificed, first anesthetized using a ketamine and xylazine cocktail, and then tissue was collected. To operate, an incision was created in the rat's abdominal wall to collect blood and certain organs such as the heart and pancreas. Using a 5-ml syringe, blood was drawn from the inferior vena cava which is the largest vein in the body, located at the posterior abdominal wall and carries deoxygenated blood back to the heart (S. J. Li et al., 2021). The samples of the heart and pancreas were isolated and separated for immunohistochemistry (IHC), and electron microscopy (EM) investigation.

After tissue collection, the samples were prepared for fixation. All the samples were rinsed with PBS before fixation take place and then samples for immunohistochemistry analysis were embedded in Zamboni fixative, whereas samples for electron microscopy (EM) analysis were embedded in McDowell fixative.

Tissues were preserved and fixed for a week before being processed for further study. Tissues from samples fixed in Zamboni for IHC were dehydrated using a succession of ethanol concentrations ranging from 70% to 95%, followed by three cycles in 100% ethanol for one hour each, the following process is to produce paraffin blocks. After ethanol three rounds of xylene for 30 minutes, and the final step is three rounds of dipping in paraffin wax at 55°C. After that, the tissues were placed in paraffin blocks, cooled, and then stored in the freezer until the paraffin had solidified. Tissue blocks were removed, trimmed, and sectioned by the microtome into 5 μm sections. Before being deposited onto gelatin-coated slides, sections were heated in a water bath at 48°C so they can spread out properly. We let the slides sit out overnight so the parts could dry and adhere to the slides correctly.

The tissue samples kept in McDowell fixative for electron microscopy were cut into small pieces, removing fat and connective tissues. Followed by three washes with PBS each for 10 minutes, then spin down the cells at 3000rpm for 5 minutes. Following a 60-minute incubation in 1% osmium tetroxide, the tissues underwent a dehydration stage by being submerged in ethanol solutions of progressively higher concentrations (50%, 70%, and twice at 95%) for 20 minutes each. The next step is infiltration and embedding, starting by incubating the tissue at a ratio of 1:1 of LR White resin and 95% ethanol for one hour on a rotator at room temperature. The tissues were later infiltrated with pure LR White resin overnight at 4°C. The next morning, 100% fresh LR White has been incubated with the tissues for two hours on a rotator at room temperature, and specimens were embedded in pure resin in gelatin capsules. The last step is polymerization. Gelatin capsules were placed in a holder una der a UV lamp to irradiate with the wavelength of 360-365 nm in a chamber for 24-36 hours at room temperature to polymerize the tissue. The tissue was sectioned after resin blocks were extracted from the gelatin capsules and cut to reveal the surface. Using a diamond knife, ultrathin portions were cut, then deposited on nickel grids and dried on filter paper.

3.5 Immunofluorescence Staining of Paraffin Sections

IHC, or immunohistochemistry, is the process of using antibodies to identify antigens in histological sections of a tissue sample such as the pancreas and heart (Ramos-Vara, 2017). Fluorescent dyes, colloidal gold, or even an enzyme-substrate colored reaction are all possible markers for detecting this specific interaction between antigen and antibody which is further analyzed by light microscopy. One of the most important applications of IHC is the investigation of the treatment indicators and prognosis markers for certain diseases (L. L. de Matos et al., 2010). Once tissue section slides are generated, they can be processed for immunohistochemical analysis. The IHC process starts with dewaxing the slides using the oven and then start submerging the slides in a double wash of xylene for 5 minutes. Slides were then rehydrated by being incubated for 3 minutes at a time in a declining sequence of ethanol concentrations (two changes of 100%, 95%, 75%, and 50%). To retrieve antigens from the slides, they were first immersed in distilled water for 5 minutes before being transferred to a citrate buffer solution for pancreatic tissue, however, 1X tris EDTA was used for heart tissues and then heated in the microwave at high power for 2 minutes and low for 20 minutes. The purpose of antigen retrieval is to recover the antigens and exposes the hidden epitopes that were masked by the fixation's chemical cross-linking (Zhang et al., 2017). The slides were left at room temperature for 20 minutes, after which they were washed three times for 5 minutes each in PBS. The slide section will have the tissue of interest, using a PAP pen which is a hydrophobic marker used to make a circle around the tissue which is important for adding the antibodies. Next is the permeabilization step by adding 0.1% of triton for 5 minutes. Then the blocking of the tissue sections by adding blocking buffer and incubating for 45 minutes at room temperature to prevent any nonspecific binding. Followed by immunolabelling by adding the primary antibodies shown in table 1, to the sections overnight at 4°C. The primary antibodies for pancreatic tissue are insulin anti-guinea pig, SGLT1 anti-rabbit, SGLT2 anti-mouse, glucagon anti-rabbit, superoxide dismutase anti-rabbit, catalase anti-rabbit, and glutathione reductase anti-mouse. On the other hand, heart tissue was single-labeled with only primary antibodies such as Collagen III anti-mouse, Fibronectin anti-rabbit, Keap1 anti-mouse, and Tipm4 anti-rabbit shown in table 2. The next day, the slides were out of the fridge for one hour at

room temperature, and then the slides were washed with PBS 3 times each for 5 minutes, and then double labeling will take place for pancreatic tissue by adding the secondary antibody and incubating for 1 hour at room temperature. The secondary antibodies for pancreatic tissue were FITC anti-guinea pig, FITC anti-rabbit, RRX anti-rabbit, and RRX anti-mouse. The last step is washing the slides three times with PBS for 3 minutes, and 5 minutes in distilled water. The sections were infiltrated with a mounting medium (DakoCytomation Fluorescent mounting medium). The results were interpreted using a fluorescent microscope, a digital camera equipped with an AxioCam HRc, and a computer running AxioVision 3.0 software (Carl Zeiss, Oberkochen, Germany). Images were captured and edited in image J 1.47V.

Table 1: Primary and secondary antibodies used in IHC for pancreatic tissues and their dilution

Primary antibody	**Dilution**	**Source**	**Secondary antibody**	**Dilution**	**Source**
Insulin anti-guinea pig	1:2000	DAKO	FITC anti-guinea pig	1:100	Jackson Immuno
Glucagon anti-rabbit	1:500	Abcam	FITC anti-rabbit	1:100	Jackson Immuno
Superoxide dismutase anti-rabbit	1:200	Rockland	RRX anti-rabbit	1:100	Jackson Immuno
Catalase anti-rabbit	1:500	Abcam	RRX anti-rabbit	1:100	Jackson Immuno
Glutathione reductase anti-mouse	1:200	Abcam	RRX anti-mouse	1:100	Jackson Immuno
SGLT1 anti-rabbit	1:100	ThermoFisher	RRX anti-rabbit	1:100	Jackson Immuno
SGLT2 anti-mouse	1:500	Santa Cruz	RRX anti-mouse	1:100	Jackson Immuno

Table 2: Primary antibodies used in IHC for heart tissues and their dilutions

Primary antibody for heart	Dilution	Source
Collagen III anti-mouse	1:100	Abcam
Fibronectin anti-rabbit	1:200	Abcam
Keap1 anti-mouse	1:1000	Abcam
Tipm4 anti-rabbit	1:100	Abcam
Catalase anti-rabbit	1:100	Abcam
GSH Reductase anti-mouse	1:100	Abcam
SOD anti-rabbit	1:100	Rockland

3.6 Immunoelectron Microscopy

The immunoelectron microscopy experiment started with washing the grids till we remove all sorts of contamination. In trying to eliminate any contamination, we filter each solution before using 0.2 µm filters and wearing gloves and a face mask. On day 1, the pancreatic sections on the grids were jet washed with deionized water. The grids were then subjected to a 10-minute incubation in a solution of 10% hydrogen peroxide (H2O2) in water. Then wash the grids using 10 ml of deionized water one time. The grid was immersed in 0.5 M NH4Cl in 0.01 M PBS (pH 7.7) for 20 minutes. After this, follows the step of washing all the grids with 10 ml of washing buffer in PBS (pH 7.3) containing 1% BSA and 0.1% tween-20. Blocking the tissue is the next step by blocking a buffer containing 20% normal goat serum (NGS) diluted in washing buffer for 20 minutes. Ultrathin sections were incubated overnight at 4°C in primary antibody which is (anti-rabbit insulin) diluted 1:100 in PBS, PH7.3 1% BSA, 0.1% tween-20and 5% NGS (antibody buffer) shown in table 3. On the next day, the grids were brought to room temperature for one hour. Then washed with two full 10 ml syringes of washing buffer and incubated with blocking buffer for 20 minutes at room temperature. We added the secondary antibody (goat anti-rabbit IgG coated with 6 nm colloidal gold particles) diluted 1:20 in antibody buffer and incubated for 2 hours at room temperature. Later we washed two times with full 10 ml syringes of washing buffer. Then fixed in glutaraldehyde (2.5% aqueous) for 5 minutes. The sections were washed two times with

full 10 ml syringes of distilled water. Put the grids on filter paper to dry overnight. On the next day, the grids were contrasted with uranyl acetate for 20 minutes and centrifuged at 3000 RPM for 1 hour before using it, so the sediment particle will be in the bottom by this, we eliminate any contaminations. This was followed by washing two times with full 10 ml syringes of distilled water. The same is for the lead citrate, centrifuge for 1 hour and add it to the grids for 10 minutes. Washing was performed two times with full 10 ml syringes of distilled water. Drying was allowed overnight on filter paper. On the following day, we viewed the grids under Transmission Electron Microscope (Tecnai BiotwinG2 Spirit FEI Company Netherlands).

Table 3: Primary and secondary antibodies used in TEM for pancreatic tissues and their dilution

Primary antibody	Dilution	Source	Secondary antibody	Dilution	Source
Insulin anti-rabbit	1:100	Abcam	6 nm gold particles anti-rabbit	1:20	Jackson Immuno

3.7 Markers of Oxidative Stress in the Heart and Serum

Two techniques were used to analyze oxidative stress markers. The first technique is immunofluorescent double labeling for the pancreas and single labeling for heart tissue. The second one is the colorimetric analysis of the serum and heart homogenate. The oxidative stress markers superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GHR) were the focus of all the oxidative stress experiments.

Before the colorimetric analysis of the heart, samples were homogenized. A piece of heart tissue was placed in a tube and 1 ml of the mixture of RIPA buffer with protease and phosphatase inhibitors was added before homogenization. The samples were kept on ice for 15 minutes and then centrifuged at 14000 rpm for 15 minutes at 4°C. After each use of the homogenizer, the head of the homogenizer was washed with 70% of ethanol and other wash with distilled water to prevent contamination with other samples. The heart tissues homogenate is created by using the rotator-stator homogenizer (Omni International homogenizer, GLH 850). Also, protein quantification was applied for the antioxidant assay from heart tissue extract.

3.7.1 Measurement of Catalase

Catalase (CAT) is a protein encoded by the CAT gene in human chromosome 11 and is highly expressed in the liver, kidney, and erythrocytes (Nandi et al., 2019). CAT activity was measured in rat serum samples using the catalase assay kit from Cayman Chemical, which includes catalase assay buffer (10X), catalase sample buffer (10X), catalase formaldehyde standard, catalase (control), catalase potassium hydroxide, catalase hydrogen peroxide, catalase purpled (Chromogen), catalase potassium periodate, high-binding 96-well solid plate, and 96-well cover sheet. Other than the catalytic activity of catalase it also has a peroxidatic activity characterized by this reaction "H2O2 + AH2 → A + 2H2O" using this reaction catalase activity has been measured. This kit used chromogen to measure the oxidation changes from colorless to purple color, which shows catalase activity. Some reagents should be diluted to 1:5 as catalase assay buffer, catalase sample buffer, catalase, and catalase hydrogen peroxide, otherwise, the rest is ready to use. The first step included the preparation of serum samples followed by a plate set up to record the content of each well so each sample would be in duplicate. To prepare the standard first a stock solution of 4.25 mM formaldehyde should be obtained by diluting 10 µL of catalase formaldehyde standard and adding 9.99 ml of diluted sample buffer. Seven tubes were labeled from A-G with each tube having different concentrations from (0-75). Each tube contained formaldehyde and sample buffer, respectively. The content of the tubes are as follows: A tube (0 µL, 1000 µL), B (10 µL, 990 µL), C (30 µL, 970 µL), D (60 µL, 940 µL), E (90 µL, 910 µL), F (120 µL, 880 µL), and G (150 µL, 850 µL). The next step performing the assay in the wells which were formaldehyde standard wells and sample wells. We added 100 µL of diluted assay buffer in the formaldehyde standard wells and also 30 µL of methanol, and 20 µL of standard which were prepared in tubes A-G. In addition, the same will be added to positive control wells: added 100 µL of diluted assay buffer and 30 µL of methanol but the difference will be 20 µL of diluted catalase.

For the serum sample wells, we added 100 µL of diluted assay buffer and 30 µL of methanol, and 20 µL of serum sample in duplicates. Then we added into each well, 20 µL of diluted hydrogen peroxide to initiate the reaction and incubate for 20 minutes at room temperature on the shaker and cover the plate. For the termination of the reaction,

we added 30 µL of potassium hydroxide to all the wells as well as add 30 µL of the chromogen catalase Purpald and cover the plate. The plate was placed on the shaker for 10 minutes at room temperature. For the final step, we added 10 µL of catalase potassium periodate to all the wells and incubated it on the shaker for 10 minutes at room temperature. Using Emax plus microplate reader, we took the reading of absorbance at 540 nm on SoftMax Pro 7.0.2 for the analysis. To determine the rate of the reaction, we calculated the average absorbance for all standards and samples, using the average absorbance of standard A subtracted from the absorbances of standards and samples. The software created the formaldehyde standard curve, which plots formaldehyde concentration (µM) on the X-axis and absorbance of the standards on the Y-axis. We substituted the absorbance values of each sample in the linear equation from the standard curve to calculate the formaldehyde concentration of the samples and finally calculated the catalase activity of the samples (Cayman Chemical, 2017).

3.7.2 Measurement of Glutathione

Glutathione is a tripeptide with a relatively simple composition of cysteine, glycine, and glutamic acid. The quantity of glutathione in most cells is remarkably high, at about 5 millimolar because it is endogenously synthesized in the cells in a reduced form (Rossi, 1989). Glutathione has a key role in antioxidant defense, and 20% of glutathione is found in mitochondria due to the high production of ROS by the electron transport chain. GSH scavenges hydroxyl radicals, superoxide anion, and nitric oxide. Glutathione is widely believed to reduce the oxidative stress-induced lipid peroxidation of cellular membranes (Kerksick & Willoughby, 2005). In a cell, glutathione can be in either its reduced (GSH) or oxidized (GSSG) form, mainly in the reduced form inside the cell. The ratio between the oxidized and reduced form is a key indicator of cellular redox status, indeed if the GSH/GSSG ratio is more than 100 the cell is considered healthy, but if the ratio decreases between 1-10 it means the cell is subjected to oxidative stress (Rossi, 1989).

A glutathione assay kit made in-house was used to measure reduced glutathione in serum and heart homogenates, 50 µL of the samples were taken and 50 µL of 5% sulphosalicylic acid was added (1:1) ratio for the deproteinization process then vortex and incubated on ice for 10 minutes. Next, the samples were centrifuged at 10000 rpm

for 10 minutes at 4°C to remove the precipitate protein as a pallet, and the supernatant was transferred into a 96-well plate for GSH assay for colorimetric analysis. The kit contains DNTB and NADPH stock solutions, 5% sulphosalicylic acid (SSA), and a glutathione standard stock solution. Next, was preparing the working solutions: first was the assay buffer by adding 100 mM potassium phosphate and 1 mM EDTA. The assay buffer was diluted by adding 2.4 ml to 9.6 ml of distilled water. The second was enzyme solution diluting: 3.8 µL of glutathione reductase was added to 250 µL of dilute assay buffer. Then, onto the NADPH solution, we added 10 µL of NADPH stock solution to 2.5 ml dilute assay buffer. This was followed by the preparation of the working mixture that has 8 ml of dilute assay buffer, 228 µL of dilute enzyme solution, and 228 µL of DNTB stock solution. 150 µL of the working mixture was added to all the wells and incubated for 5 minutes on the shaker at room temperature followed by the addition of 50 µL of diluted NADPH. Using a plate reader BioTek ELX800 measures the absorbance at 412 and takes 5 readings every minute by the software Gen5 Elisa to show GSH activity which is a kinetic assay. This was performed in the same for both serum and heart tissue homogenate samples.

3.7.3 Superoxide Dismutase

Superoxide dismutase (SOD) activity was measured in rat serum samples and heart homogenate samples using the SOD assay kit from Cayman Chemical which includes assay buffer (10X), sample buffer (10X), SOD standard, catalase (Control), radical detector, Xanthine oxidase, 96-well solid plate, and 96-well cover sheet. The SOD activity is one of the antioxidant defense mechanisms in the cell. It is a scavenger for oxygen-free radicals. SOD is a metalloenzyme, it contains copper/zinc, manganese, or iron. SOD activity has been measured using an assay kit that uses a tetrazolium salt for the detection of superoxide radicals produced by xanthine oxidase and hypoxanthine. As determined by the rate of change in absorbance per minute at 25°C, one unit of SOD is the quantity of enzyme required to dismutation the superoxide radical by 50%. This assay is a quick, easy, and reliable way to measure SOD activity in blood serum and heart tissue homogenates. All reagents were diluted. For serum, the dilution was, 1:5 with heart homogenate, and 1:100. The first step was: the preparation of serum and heart tissue homogenates followed by a plate set up to record the content of each well so each

sample should be in duplicate also SOD standards. [To prepare the standard first 20 µL of SOD standard should be diluted with 1.98 ml of diluted sample buffer to have SOD stock solution then label seven tubes from A-G each tube will have different concentrations. Each tube will contain SOD stock solution and diluted sample buffer respectively, A tube (0 µL, 1000 µL), B (20 µL, 980 µL), C (40 µL, 960 µL), D (80 µL, 920 µL), E (120 µL, 880 µL), F (160 µL, 840 µL), and G (200 µL, 800 µL)]. The next step: perform the assay in the wells which are SOD standard wells and sample wells (Add 200 µL of the diluted radical detector and 10 µL of standard in the wells (tubes A-G). In addition, the same was added to positive control wells adding 100 µL of diluted assay buffer and 30 µL of methanol but the difference will be 20 µL of diluted catalase. For the sample, wells add 200 µL of the diluted radical detector and 230 µL of the samples in duplicates. Then add to each well 20 µL of diluted Xanthine oxidase to initiate the reaction and incubate for 30 minutes at room temperature on the shaker and cover the plate. Using Emax plus microplate reader take the reading of absorbance at 450 nm on SoftMax Pro 7.0.2 for the analysis. To determine the rate of the reaction, calculate the average absorbance for all standards and samples. The software will create the SOD standard rate curve which plots SOD concentration (µM) on the X-axis and absorbance of the standards on the Y-axis. Calculate the SOD activity of the samples using the linear regression graph created for the standard (Cayman Chemical, 2008).

3.7.4 Nitric Oxide

This assay was used to measure nitric oxide in serum and heart homogenate, 50 µL of the samples were taken and 50 µL of 5% sulphosalicylic acid was added (1:1) ratio for the deproteinization process then vortexed and incubated on ice for 10 minutes. Next, centrifuging at 10000 rpm for 10 minutes at 4°C to clear the turbidity of the serum. Then the supernatant was transferred into a 96-well plate for colorimetric analysis of NO. Incubate 50 µL of the deproteinized sample with 50 µL of reagent A (H3PO4, Sulfanilamide, Naphthyl ethylenediamine dihydrochloride) for 5 minutes and read at an absorbance of 540 nm.

3.8 Masson Staining for Heart Tissue

Mason's Trichrome stain can be used to stain connective tissue fibers in tissue sections such as the heart section, which will stain it in a range of pink to red colors. The heart tissue sections were deparaffinized and hydrated in distilled water before immersion in hot Bouin's Fluid for 60 minutes. The sections were later allowed to cool for 10 minutes before washing with tap water until the section is completely clear. This was followed by rinsing in distilled water. The sections were then stained with Weigert's Iron Hematoxylin for 5 minutes. They were later rinsed before staining with Biebrich Scarlet /Acid Fuchsin Solution to slide for 15 minutes. The sections were rinsed again in distilled water before differentiation in phosphomolybdic/phosphotungstic acid solution for 15 minutes or until the collagen is not red, followed by staining with aniline blue solution for 10 minutes. The slides were treated with an acetic acid solution (1%) to slide for 5 minutes, and dehydrated very quickly in two changes of 95% alcohol, followed by two changes of absolute alcohol. The sections were cleared in xylene and mounted in synthetic resin.

3.9 Statistical Analysis

Statistics and data were reported as mean ± standard error of the mean. Prism GraphPad software's two-way ANOVA test (grouped - ordinary - two data sets) was used to analyze the data, and the Tukey Karmer *post hoc* test was used to calculate the significance of the p-value when comparing the samples which are considered significant if $p < 0.05$. Moreover, a one-way ANOVA test was used to analyze oxidative stress markers in the serum and the heart samples including Catalase, SOD, Glutathione, and NO (GraphPad Software, San Diego, CA, USA).

Chapter 4: Results and Discussions

4.1 Metabolic Parameters

The effect of LIK066 on metabolic parameters such as body weight, blood glucose, and glucose tolerance measured by IPGTT is shown in figures 4, 5, and 6. The different experimental groups including normal (treated, untreated) and diabetic (treated, untreated) were compared.

4.1.1 Body Weight

Figure 4 shows the effect of LIK066 treatment on weight changes in both normal and diabetic animals over the 4 weeks of the study. The weight range of all the animals before we induced diabetes was between 200-250g. From week 1 to the end of the study, STZ-induced diabetic treated, and untreated rats showed a significant decrease in body weight in comparison with the normal treated and untreated groups, which shows weight gain during the experimental period. Neither normal nor diabetic rats had any significant weight change after LIK066 treatment. It is worth noting that, STZ-induced diabetic rats treated with LIK066 showed the lowest body weight compared to all animals included in the study after 4 weeks of treatment.

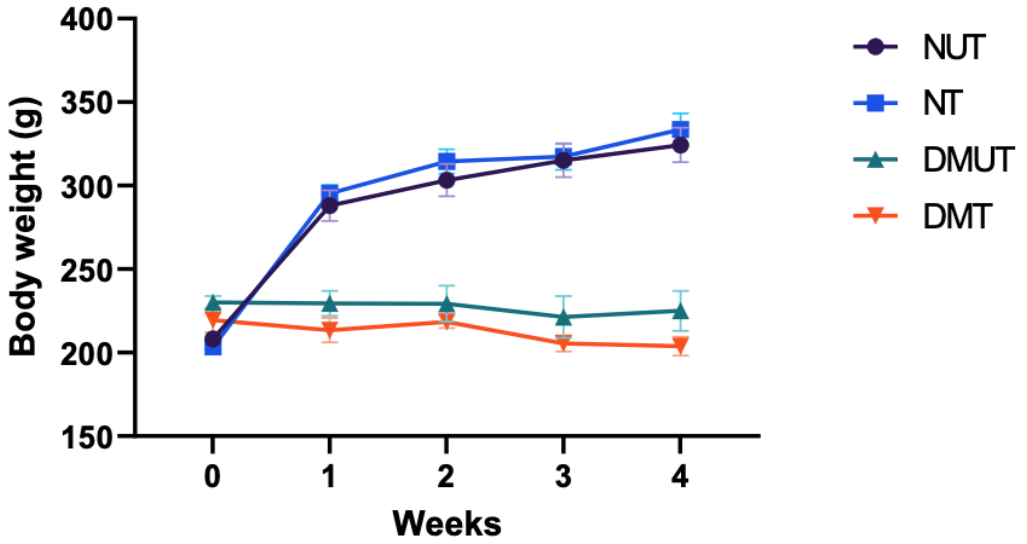


Figure 4: Effects of LIK066 treatment on weight gain in normal and diabetic animals. LIK066 treatment did not cause significant differences in body weight in normal and diabetic animals. Values are represented as mean ± SEM, n=6

The result showed that diabetic rats treated with LIK066 lost a small but not significant weight gradually up to the end of the study compared to diabetic untreated. In the literature, one of the effects of LIK066 in patients is weight reduction. One study examined the dose-response relationship in a 12-week regimen on individuals with obesity, and whether or not they have diabetes. The result shows a significant weight reduction higher than 5.7% from baseline in patients with dysglycemia seen with an optimal dose of 150 mg of LIK066 compared to placebo-controlled individuals (Kalra et al., 2020). The mechanism of LIK066 in reducing weight is the inhibition in intestinal L-cells that enhances the production of weight-controlling incretin hormones like GLP-1 and peptide YY which influence appetite and this mechanism have an indirect effect on satiety thus it can reduce body weight (He et al., 2019). On the other hand, in the LIK066-treated normal group, the result shows weight gain which is the opposite of the evidence of the LIK066 effect. This may be associated with the duration of the studies (longer in human studies) or the doses of LIK066 (higher in the reported human studies). The higher doses were coupled with adverse effects like diarrhea (Wang-Lakshman et al., 2021).

4.1.2 Blood Glucose Levels

The effect of LIK066 on blood glucose levels in both normal and diabetic animals over the 4 weeks of the study is shown in Figure 5. The blood glucose readings were taken once a week during the study. The STZ-induced diabetic rats had significantly elevated blood glucose levels compared with normal rats. The treatment with LIK066 had no significant result on the blood glucose of diabetic rats compared with non-treated diabetic rats.

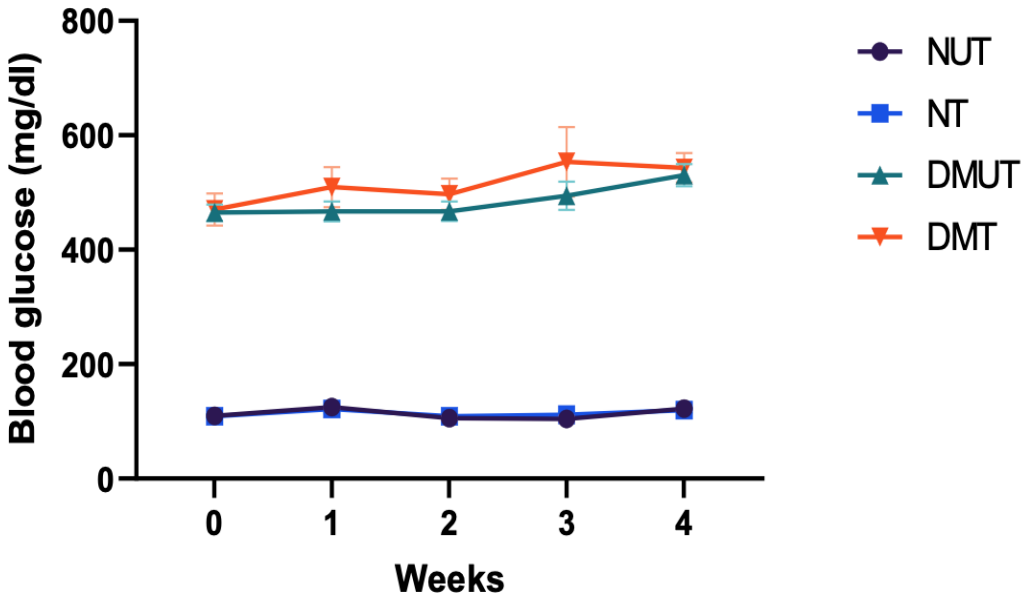


Figure 5: Effects of LIK066 on blood glucose levels in normal and diabetic animals. Time course graphs representing the effect of LIK066 treatment on blood glucose levels in normal untreated, normal LIK066-treated, diabetic untreated, and diabetic LIK066-treated rats. Values represented as mean± SEM, n=6

Figure 5 shows that plasma glucose levels were not significantly reduced by LIK066 treatment in the diabetic groups, even though they were higher after the 4 weeks of study. However, it has been reported that LIK066 has an anti-diabetic effect exerted by SGLT1 and SGLT2 inhibition which reduces intestinal and renal glucose reabsorption resulting in low glucose levels. A study was conducted to prove the effect of LIK066 on FPG. The result shows the significant effect of LIK066 in reducing FPG in 10 mg, 25 mg, and 50 mg doses by −1.03, −1.07, and −1.30 mmol/L, respectively. However, a dose of 2.5 mg did not induce a significant weight (Yokote et al., 2020). This clinical study was done on a human population, so the result may differ from that of animal models. Note that the dose and duration of treatment in this research are quite low when compared to these clinical studies. Also, the rats in this study were not fasting before the glucose reading was taken which would be another factor influencing the result.

4.1.3 IPGTT

In the glucose tolerance test, the rate at which the body eliminates a glucose load after the injection is monitored for all the animals at 0, 30, 60, 90, and 120 minutes. It's a diagnostic tool for diabetes risk assessment by identifying deviations in glucose

metabolism. The effect of LIK066 on blood glucose in IPGTT for both normal and diabetic animals at the end of the 4 weeks study is shown in figure 6. Before receiving glucose injections, diabetic animals' FPG was considerably higher than those of normal rats. After the second glucose injection at 30 minutes, LIK066-treated diabetic rats reached the highest peak in blood glucose level and continue to plateau, almost similar to the glucose value after 30 minutes same as diabetic untreated. However, diabetic untreated shows lower FPG levels of glucose challenge compared to diabetic treated but the difference was not significant. The treatment with LIK066 had a slight improvement in FPG of normal-treated rats and no improvements in responding to a glucose load.

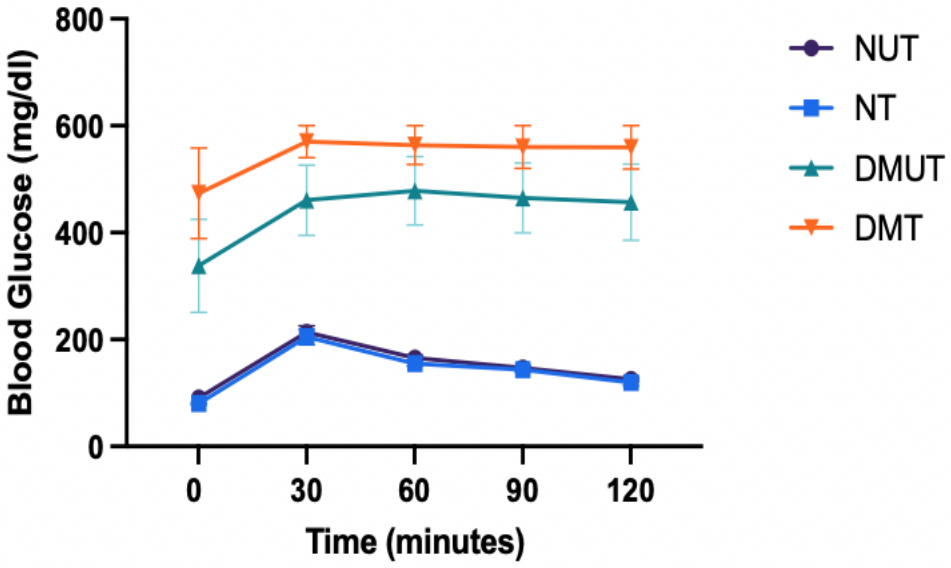


Figure 6: Effects of LIK066 on IPGTT 4 weeks after treatment of normal and diabetic animals. Values represented as mean± SEM, n=6

There are limited publications on the effect of LIK066 on IPGTT levels. On other hand, LIK066 shows improvement in oral glucose tolerance tests to inhibit insulin secretion and decrease the postprandial glucose (Wang-Lakshman et al., 2021). A study was performed to examine the improvements in glycemic control in mice deficient in SGLT1 and SGLT2, mimicking the mechanism of LIK066 in inhibiting SGLT1 and SGLT2. The result of IPGTT shows a decline in the fasting blood glucose levels and a further reduction on 30 and 60 min after the glucose challenge, also enhancing glucose tolerance compared to normal animals (Powell et al., 2013).

4.2 Immunohistochemical Analysis

4.2.1 Immunohistochemical Study in the Pancreas

4.2.1.1 Co-localization Analysis of SGLT1 with Insulin in β-cells

In a bid to determine whether SGLT1 is present in pancreatic endocrine cells, co-localization of insulin with SGLT1 was investigated by the immunofluorescence staining technique. The double-labeling of pancreatic islets of Langerhans of normal and diabetic Wistar rats using antibodies against insulin and SGLT1 shows the distribution of insulin and SGLT1 and insulin-positive cells as shown in Figure 7. LIK066 treatment shows a slight increase in SGLT1 and insulin in diabetic rat pancreas. The merged pictures demonstrate that SGLT1 is co-localized with insulin in the pancreatic β-cells of islets of Langerhans. The colocalization between insulin and SGLT1 is elevated slightly in diabetic treated compared to diabetic untreated also normal treated shows a slight increase compared to normal untreated animals.

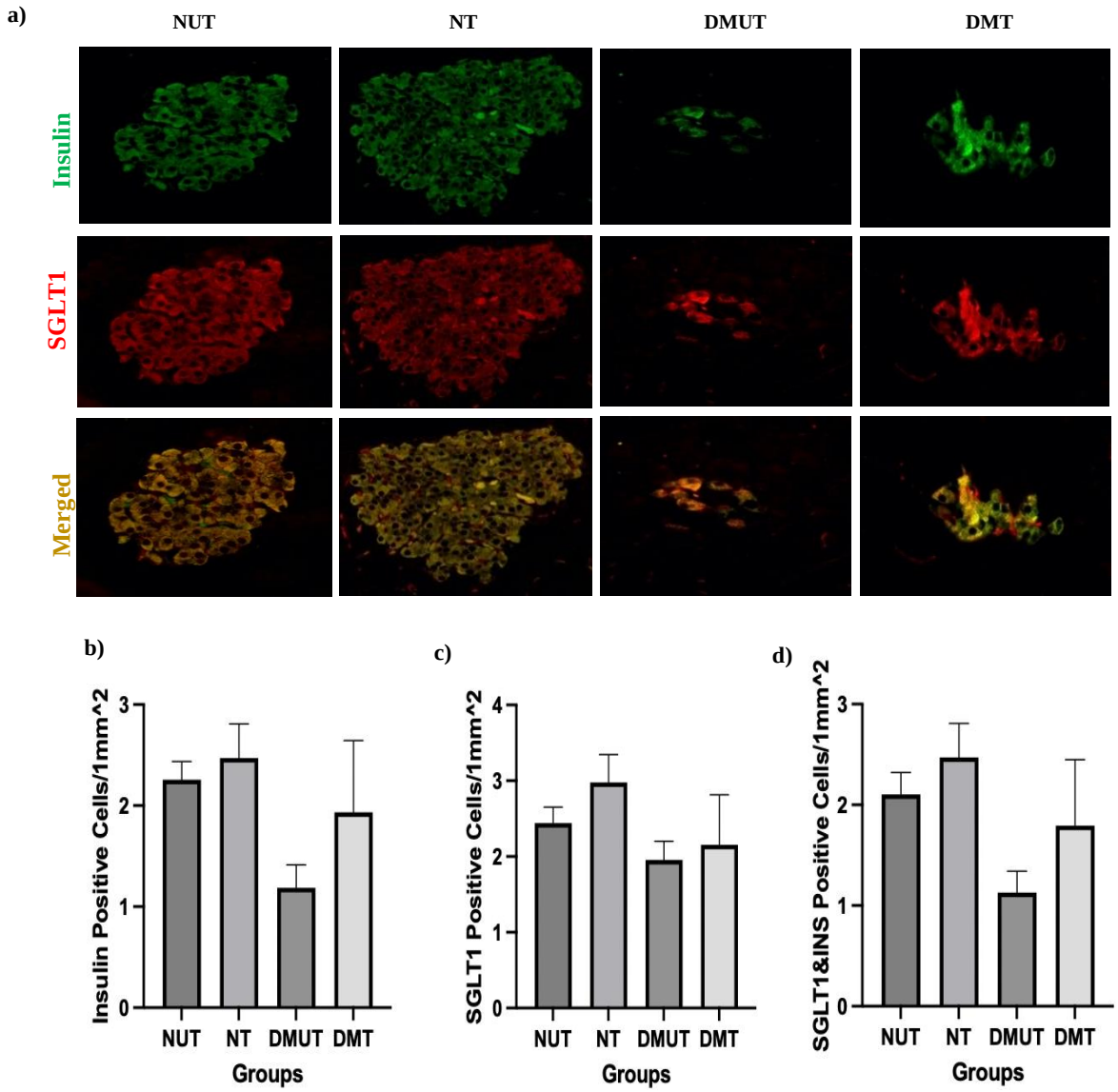


Figure 7: Co-localization of SGLT1 with insulin in pancreatic islet cells of normal and diabetic rats. (a) Insulin-positive cells are shown by green fluorescence (FITC) while SGLT1-positive cells are shown in red (RRX). The yellow color demonstrates the merging and co-localization of both SGLT1 and insulin. The presented pictures are taken on a scale of X40. The morphometric analysis of (b) insulin-positive cells/1mm^2, (c) SGLT2-positive cells/1mm^2, and (d) SGLT2 & insulin-positive cells/1mm^2 of NUT, NT, DMUT, and DMT group. Values represented as mean± SEM, n=6

In a diabetic rat model induced by STZ, SGLT1 will be highly expressed in the intestine and kidney (Sano et al., 2020). However, our study shows SGLT1 is expressed in pancreatic β-cells of diabetic and normal rats. Treatment of rats with LIK066 caused a slight but not significant increase in the number of insulin and SGLT1-positive cells.

4.2.1.2 Co-localization Analysis of SGLT2 with Insulin in β-cells

The colocalization of insulin with SGLT2 using antibodies against insulin and SGLT2 was investigated by the immunofluorescence staining technique. The distribution of insulin and SGLT2-positive cells is shown in Figure 8. LIK066 treatment caused a slight but not significant increase in the number of SGLT2-positive cells in diabetic and normal animals. The merged pictures demonstrate that SGLT2 is co-localized with insulin in the pancreatic β-cells of islets of Langerhans. The data were obtained from six different animals.

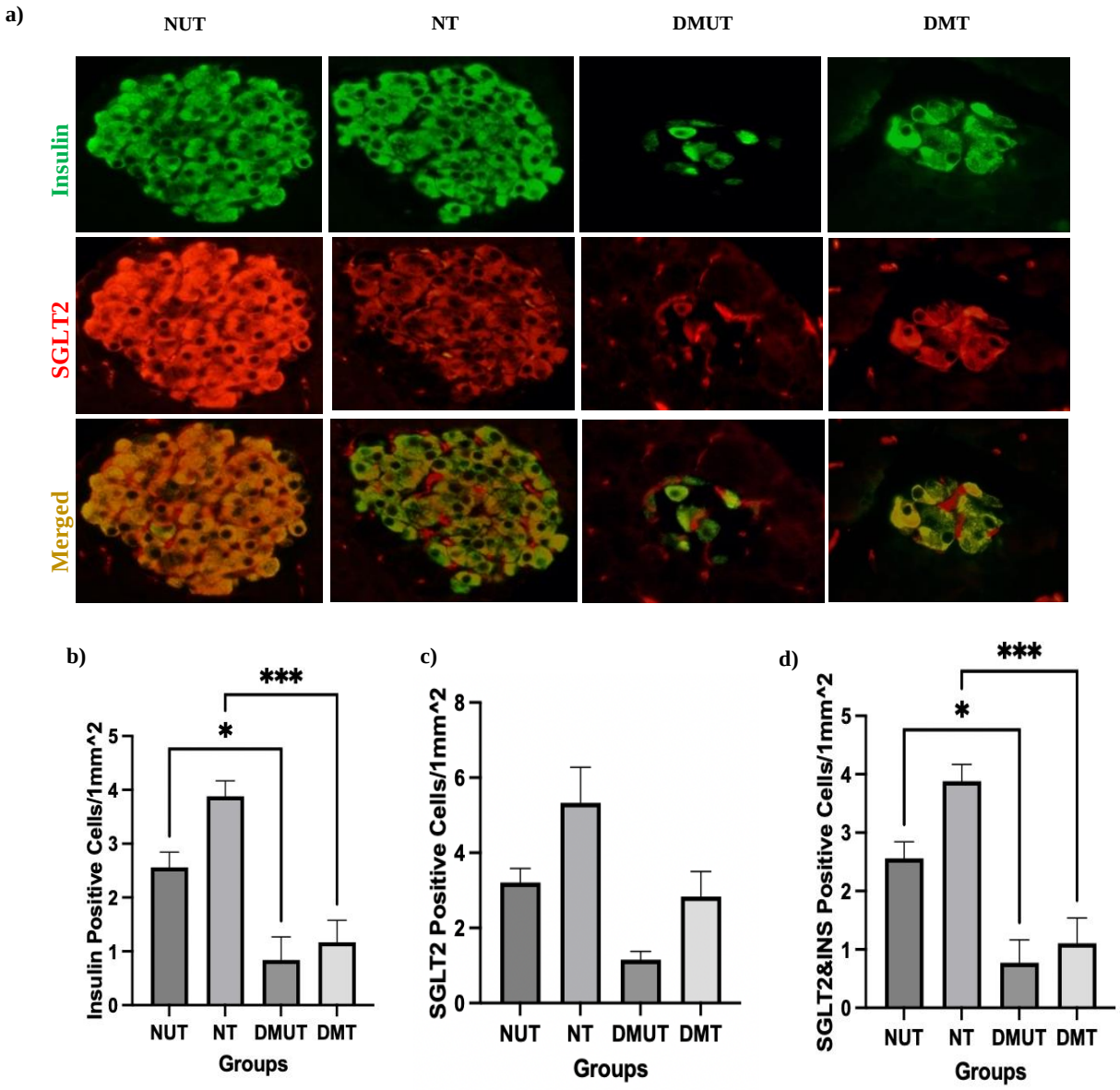


Figure 8: Co-localization of SGLT2 with insulin in pancreatic islet cells of normal and diabetic rats. (a) Insulin-positive cells are shown by green fluorescence (FITC) while SGLT2-positive cells are shown in red (RRX). The yellow color demonstrates the co-localization of both SGLT2 and insulin. They presented pictures on a scale of X40. The morphometric analysis of (b) insulin-positive cells/1mm^2, (c) SGLT2-positive cells/1mm^2, and (d) SGLT2 and insulin-positive cells/1$mm^2$of NUT, NT, DMUT, and DMT group. Values represented as mean± SEM, n=6

SGLT2 expression is highly increased in PCT in the case of diabetes, which enhances the reabsorption of glucose in the kidney (Sano et al., 2020). In our study, the number of SGLT2 immunoreactive cells is slightly elevated in the pancreas of diabetic treated and normally treated compared to of diabetic untreated and unnormal treated. The

double-labeling of insulin and SGLT2 confirms the significant co-localization of insulin and SGLT2 in islets of Langerhans of normal compared to diabetic animals.

4.2.2 Immunohistochemical Study of the Remodeling of the Myocardium Heart

4.2.2.1 Collagen III

The effect of LIK066 on collagen III was examined in the myocardium using immunofluorescence analysis. Figure 9 shows the percentage distribution of collagen III-positive cells in the myocardium of diabetic and normal Wistar rats. A significant reduction in the number of collagen III positive cells in STZ-induced diabetic untreated rats compared to normal untreated. There is a slight increase in % of cells of diabetic treated than diabetic untreated, but the difference is not significant.

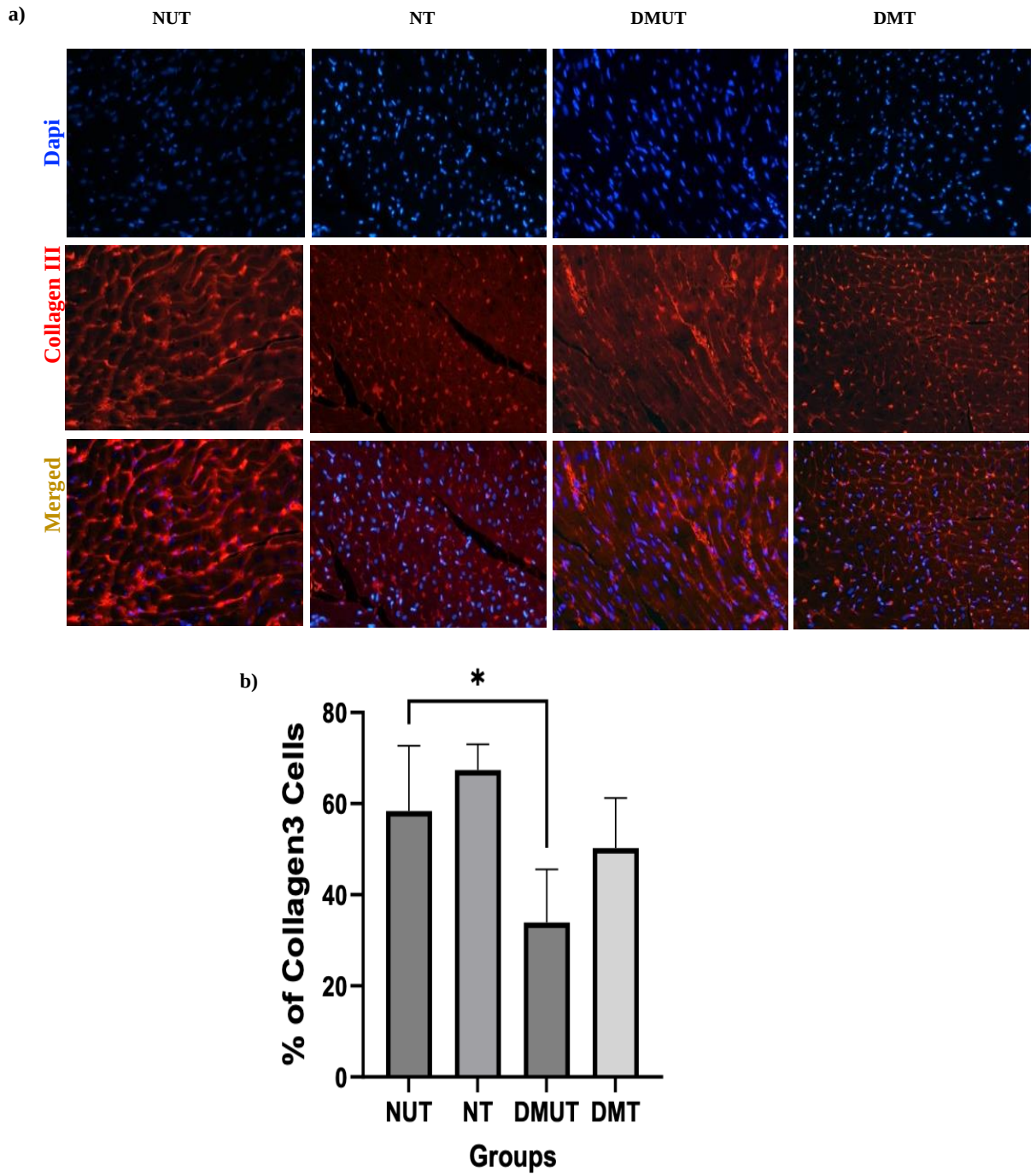


Figure 9: The effect of LIK066 on collagen III remodeling in the heart muscle of normal and diabetic rats. (a) single staining of IHC shows collagen III-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number will be counted. All pictures are taken on a scale of X40 (b) The % distribution of collagen III cells in diabetic untreated is significant compared to normal untreated. Values represented as mean± SEM, n=6

The intensity of collagen III staining was reduced after treatment with LIK066. To the best of our knowledge, there are no reported data on the effect of LIK066 on collagen III in the myocardium.

It has been reported that DM is associated with a higher risk of cardiovascular complications. DM is mainly characterized by hyperglycemia and oxidative stress which alter the structure and function of cardiac muscle cells because cardiac fibroblasts induce an overproduction and accumulation of collagen III in the extracellular matrix (Guo et al., 2018). Collagen is a structural protein found in the extracellular matrix it has an important role in sustaining wall thickness and myocardial form. A study on an interstitium of the heart in DM patients shows a significant increase in collagen III by cardiac fibroblasts found in DM patients and the mechanism was unknown (Shimizu et al., 1993). Thus, this increase can change cardiac function and make the myocardium stiffer (Guo et al., 2018).

4.2.2.2 Fibronectin

The effect of LIK066 on fibronectin was examined in the myocardium using immunofluorescence analysis. Figure 10 shows how the percentage distribution of fibronectin-positive cells in the myocardium of normal and diabetic Wistar rats. There is no significant difference in the percentage distribution of fibronectin-positive cells between the groups.

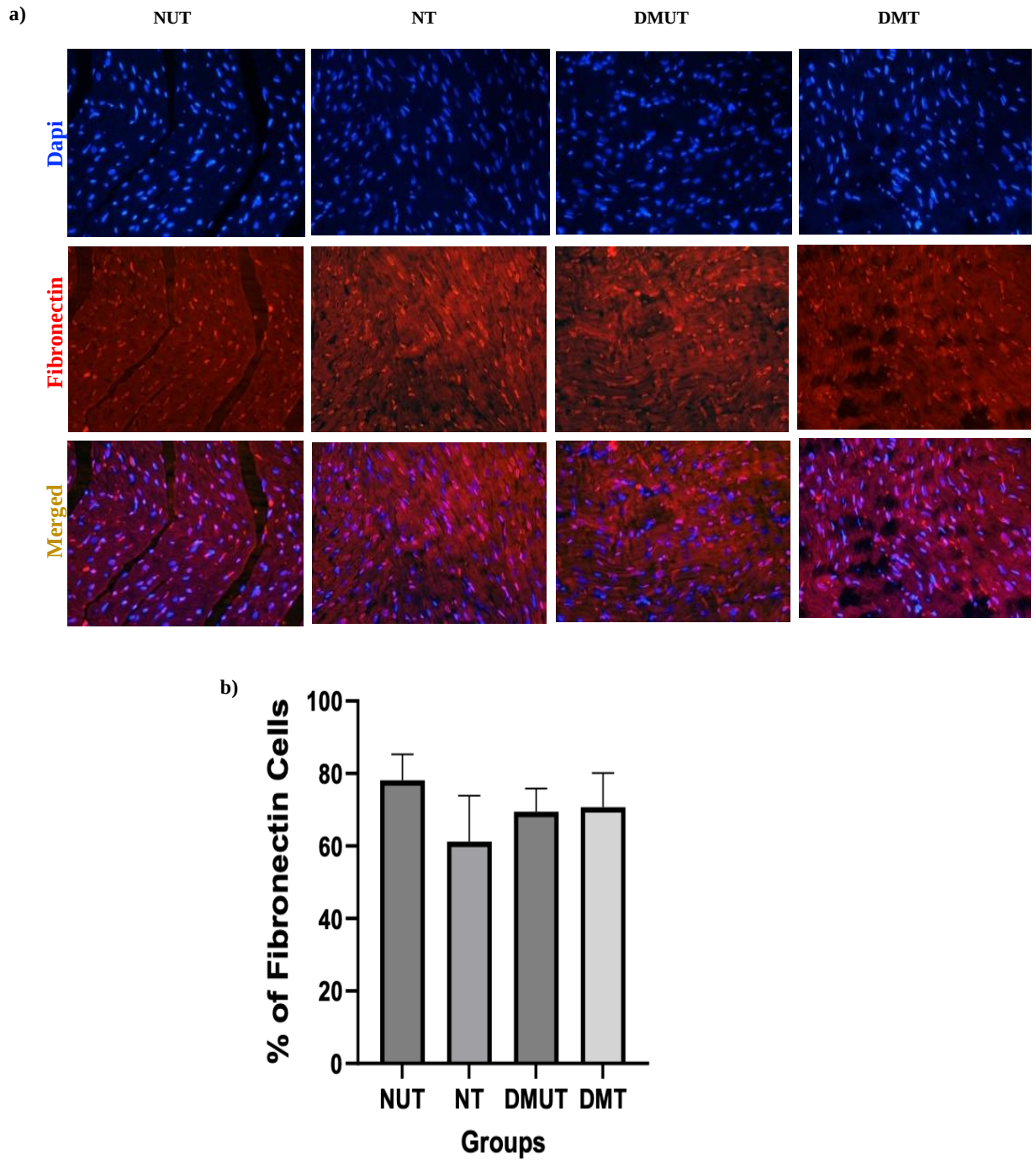


Figure 10: The effect of LIK066 on fibronectin distribution in the heart of normal and diabetic rats. (a) single staining of IHC shows fibronectin-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number was counted. All pictures are taken on a scale of X40 (b) The % distribution of fibronectin cells is not significant in all the groups. Values represented as mean± SEM, n=6

The results show no significant effect of LIK066 in the myocardium of normal and diabetic rats irrespective of LIK066 treatment. No data have been reported on the effect of LIK066 on fibronectin in the heart of experimental animals.

Diabetes modifies the gene expression of some proteins such as fibronectin. Intracellular hyperglycemia can cause overexpression of fibronectin mRNA levels in the hearts of diabetic rats, especially in vascular endothelial cells. The conclusion they reached in this study even when diabetic rats have been treated with insulin the effect of cardiac fibronectin upregulation cannot be reversed (Roy et al., 1990).

4.2.2.3 Tissue Inhibitor of Matrix Metalloprotease 4 (TIMP4)

The effect of LIK066 on TIMP4 expression was examined in the myocardium of normal and diabetic rats treated with LIK066 using immunofluorescence analysis. Figure 11 shows the percentage distribution of TIMP4-positive cells in diabetic and normal Wistar rats, with and without LIK066 treatment. A significant elevation was observed in the number of TIMP4-positive cells in diabetic rats treated with LIK066 compared to diabetic untreated rats. This increase shows that LIK066 has a protective effect on the heart by stimulating TIMP4.

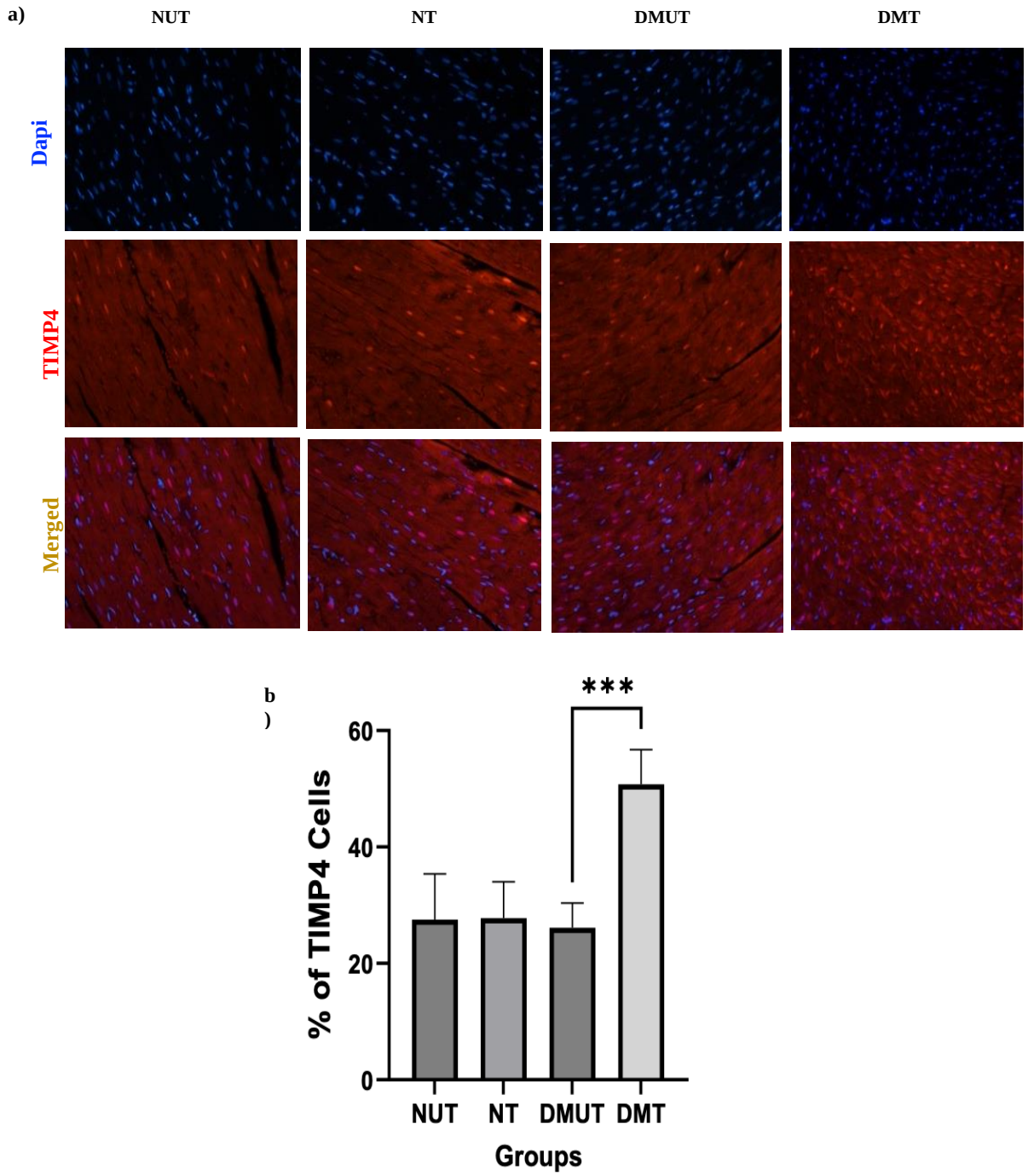


Figure 11: The effect of LIK066 on TIMP4 distribution in the heart muscle of normal and diabetic rats. (a) single staining of IHC shows TIMP4-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number was counted. All pictures are taken on a scale of X40 (b) The % distribution of TIMP4 cells is significant in STZ-induced diabetics-treated rats. Values represented as mean± SEM, n=6

LIK066 improves the CVD events in diabetes as our result shows the upregulation of TIMP4 and by this MMPs will be lower due to the inhibition. No data were reported on the effect of LIK066 on TIMP4.

The tissue inhibitor of metalloproteases (TIMP4) is an inhibitor for matrix metalloproteinases (MMPs). The MMPs remodeling is one of the mechanisms the extracellular matrix (ECM) is altered through the degradation of the ECM by proteases. In diabetes, hyperglycemia and ROS can induce MMP production and one example of MMPs related to a higher incidence of the macrovascular disease is the increase in MMP-2 which is associated with endothelial dysfunction and also increase fibrosis, thrombus formation, and lower elastin content (Peeters et al., 2017). Moreover, myocardial infarction is associated with an increase in MMP-9, and the overexpression of TIMP4 can inhibit MMP-9 and lower ROS production (Chaturvedi & Tyagi, 2016).

4.3 Electron Microscopy

The effect of LIK066 on the pattern of insulin distribution in the secretion granules of pancreatic β-cells was studied using electron microscopy. Figures 12, 13, and 14 show electron micrographs of pancreatic β-cells of normal and diabetic rats. The number of number insulin-labeled particles in the secretory granules of diabetic rats was significantly lower compared to normal. Figure 15 shows no insulin-labeled gold particles were observed in the exocrine cells which indicate good staining.

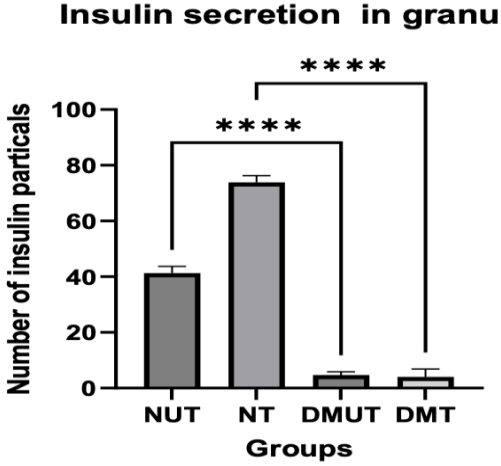


Figure 12: The effect of LIK066 on insulin level in the secretory granules of pancreatic β-cells of normal and diabetic animals. The electron micrographs are typical for 6 different animals in each group. Values represented as mean± SEM, n=6

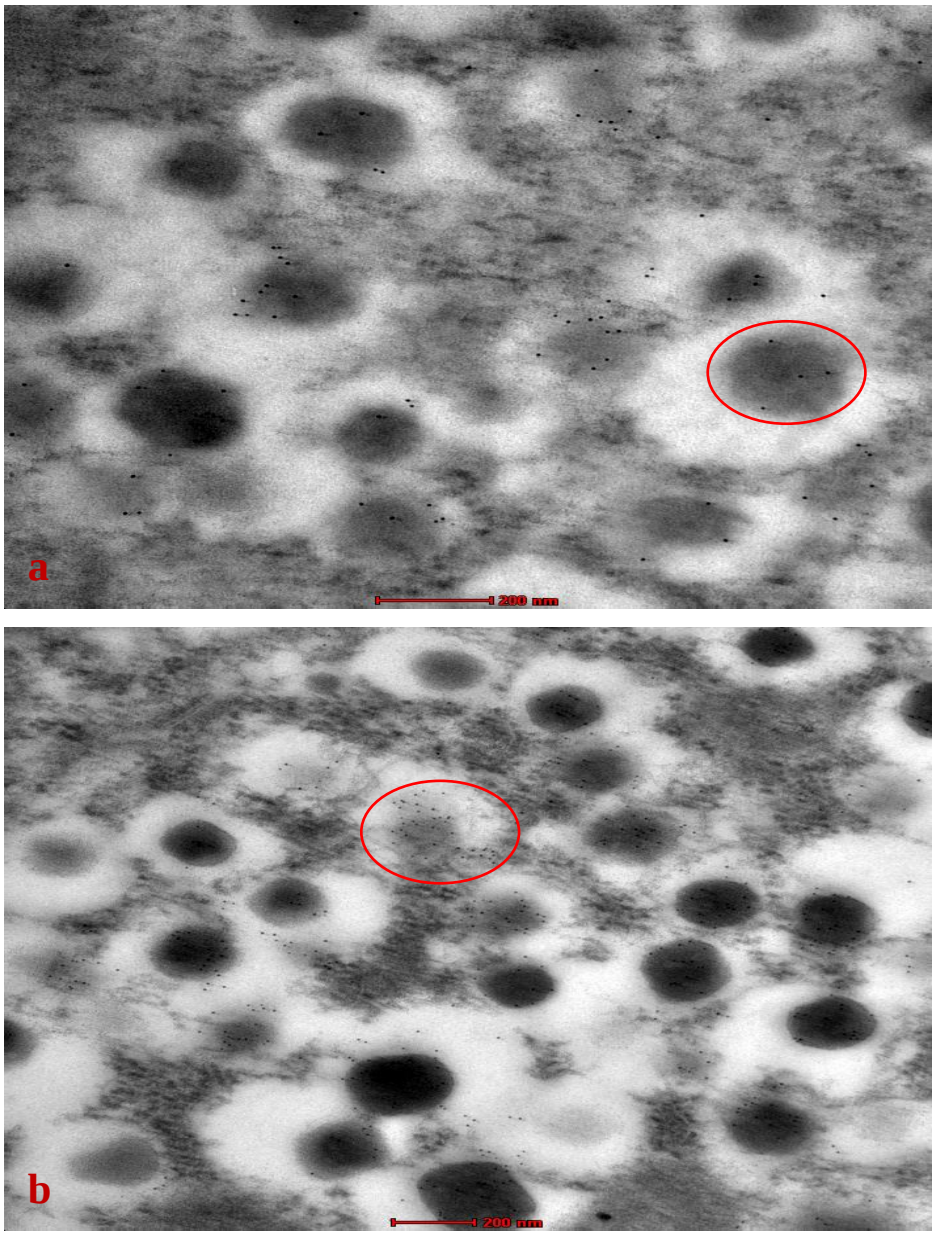


Figure 13: Electron micrograph showing (a) β-cell of normal rats untreated had a few insulin particles per secretory granules compared to normal treated in (b) β-cell of normal rats treated with LIK066 showed more insulin particles. Circled areas show insulin particles. The electron micrographs are typical for 6 different animals in each group and the pictures were taken at a 200 nm scale

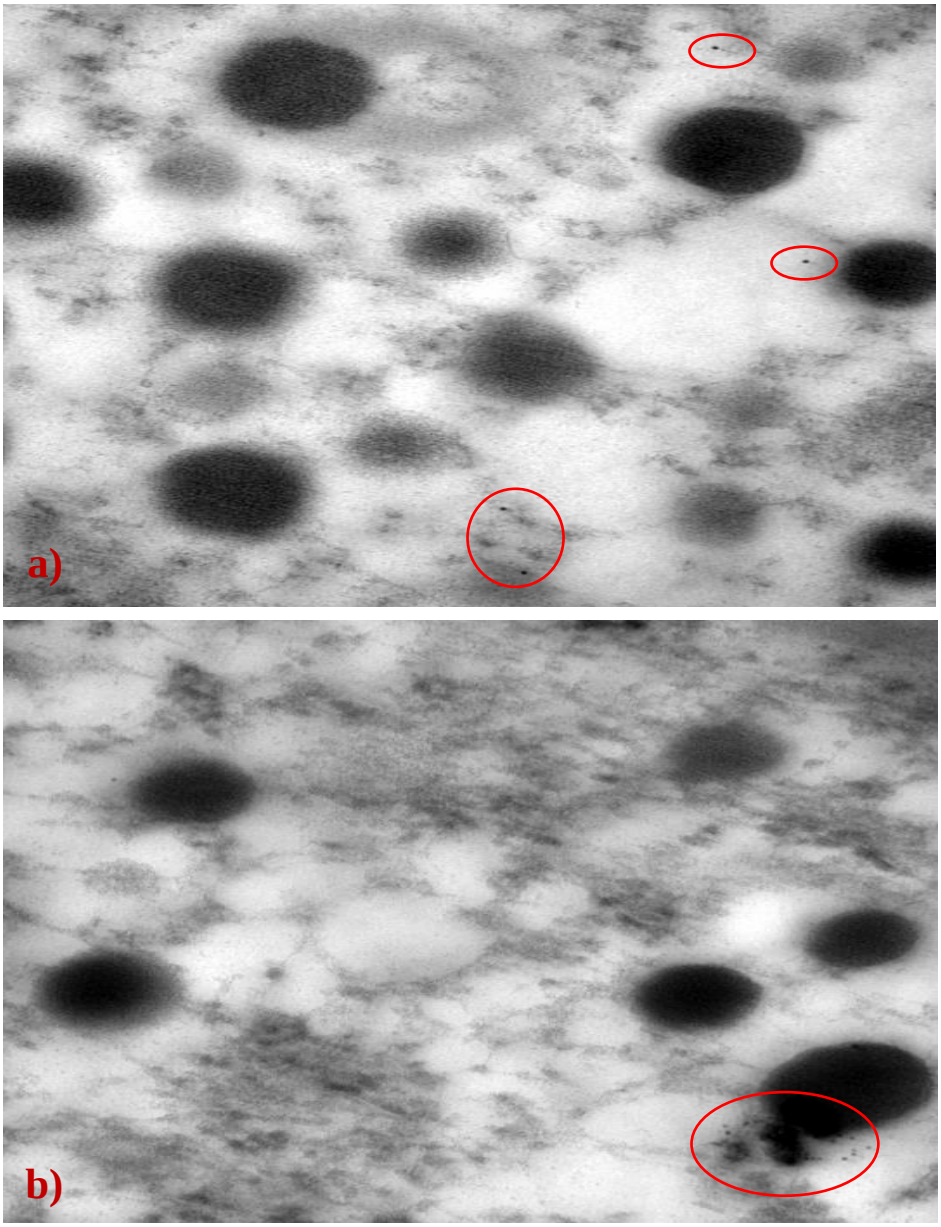


Figure 14: Electron micrograph showing (a) β-cell of diabetic rats untreated with LIK066 had very few insulin particles in the secretory granules and (b) β-cell of diabetic rats treated with LIK066 showed more insulin particles. Circled areas show insulin particles. The electron micrographs are typical for 6 different animals in each group and the pictures were taken at a 200 nm scale

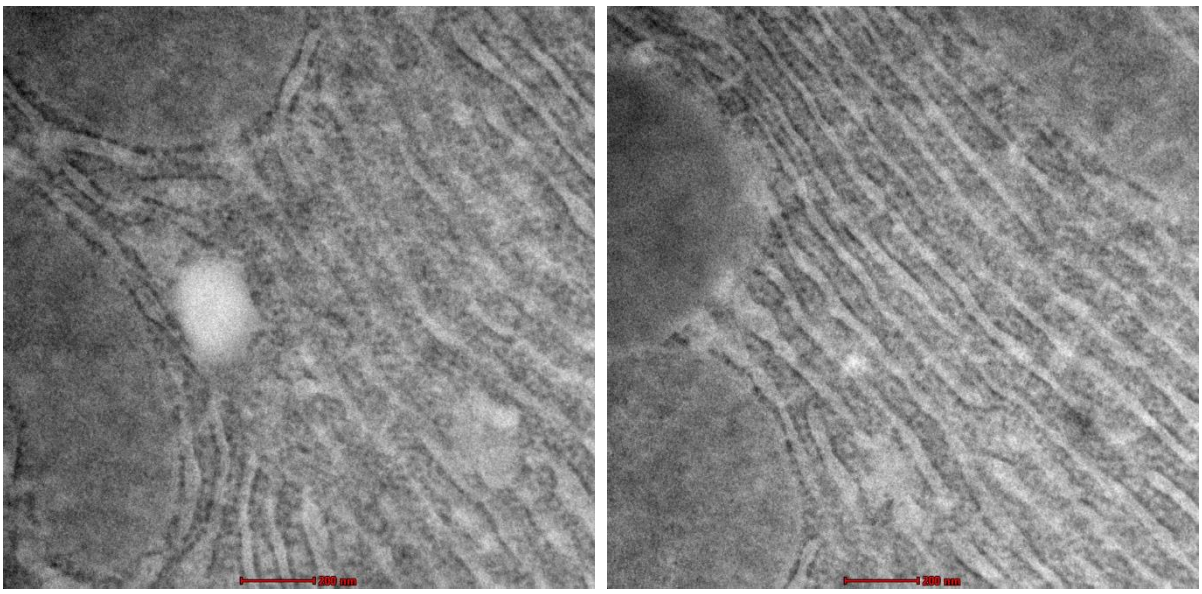


Figure 15: Electron micrograph of normal untreated rat's exocrine pancreatic cells. Note that no insulin-labeled gold particles were observed in the exocrine cells. The pictures were taken at a 200 nm scale

4.4 Markers of Oxidative Stress

The antioxidant activity of LIK066 was tested in the pancreatic tissue, heart tissue, and serum using both immunofluorescence analysis and colorimetric analysis.

4.4.1 Oxidative Stress Markers in the Pancreas

4.4.1.1 Catalase

Figure 16 shows the pattern of distribution of catalase in pancreatic islet cells of normal and diabetic rats after treatment with or without LIK066. The number of catalase-positive cells was significantly lower after the onset of DM. Treatment with LIK066 did not significantly alter the number of catalase-positive cells in either normal or diabetic rats. Catalase colocalized with insulin (Figures 16b and 16d).

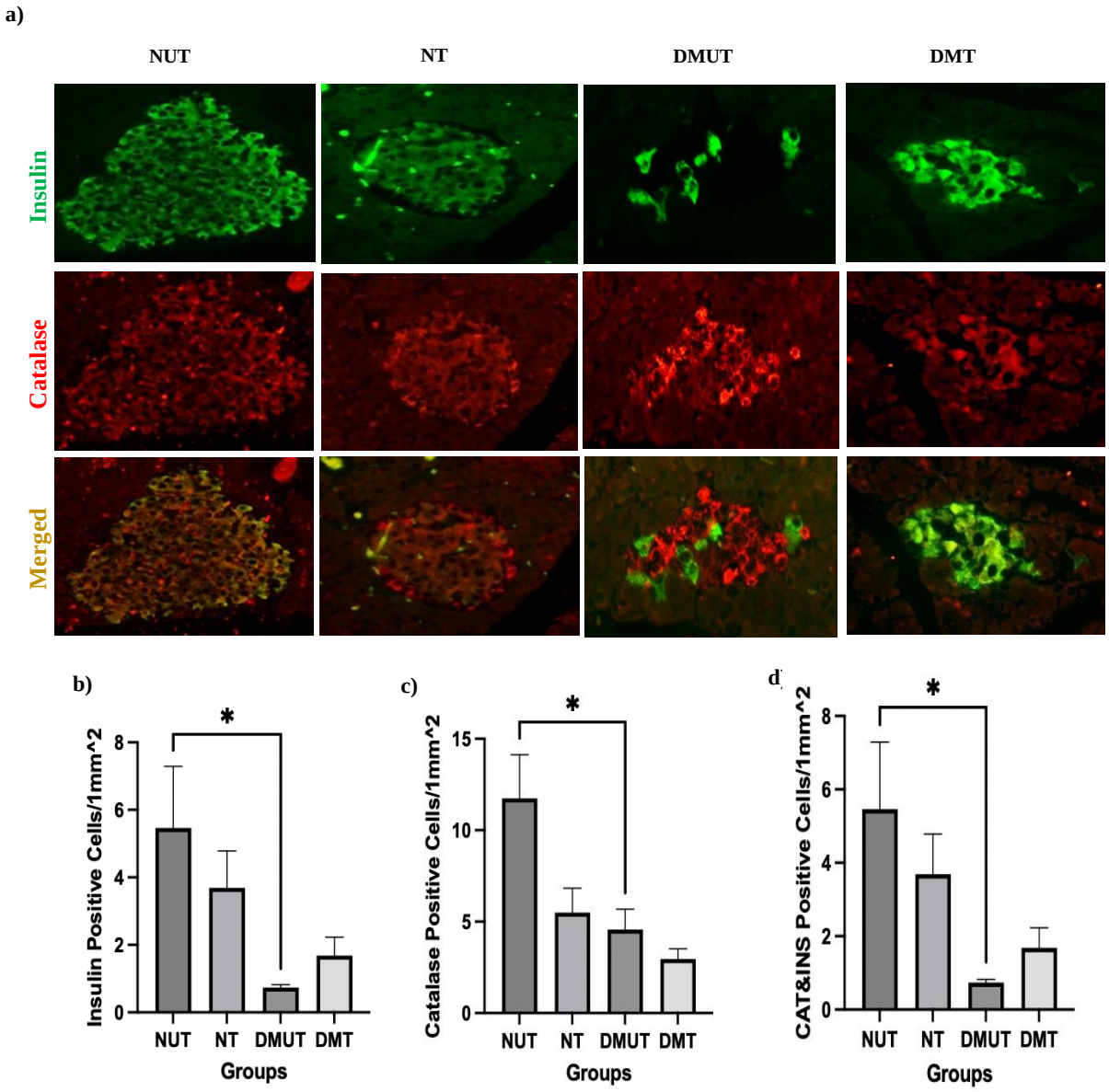


Figure 16: Shows images of insulin and catalase-positive cells in the pancreatic islet cells of normal and diabetic rats after LIK066 treatment. (a) insulin-positive cells are shown by green fluorescence (FITC) while catalase-positive cells are shown in red (RRX). All pictures are taken on a scale of X20. The morphometric analysis of (b) insulin-positive cells/1mm^2, (c) catalase-positive cells/1mm^2, and (d) catalase & insulin-positive cells/1$mm^2$of NUT, NT, DMUT, and DMT group. Values represented as mean± SEM, n=6

Oxidative stress is a hallmark of the pathogenesis of T1DM and T2DM. Free radicals create serious damage in diabetes by glucose oxidation, lipid peroxidation, and glycation of proteins. Antioxidant enzymes like catalase is a scavenger against free

radicals specifically it regulates hydrogen peroxide due to their effect on damaging lipids and DNA if it builds up to toxic levels. A significant decrease in catalase-positive cells in diabetic untreated compared to normal untreated animals. In diabetes, catalase deficiency causes pancreatic β-cells dysfunction through the overproduction of ROS in their mitochondria. Increased H_2O_2 generation and decreased CAT gene expression were both associated with the hyperglycemia (Asmat et al., 2016).

4.4.1.2 Superoxide Dismutase

Treatment with LIK066 does not affect SOD levels in pancreatic β-cells of normal and diabetic animals shown in figure 17. However, a small but not significant decrease in the number of SOD-positive cells was observed after the treatment of diabetic rats with LIK066.

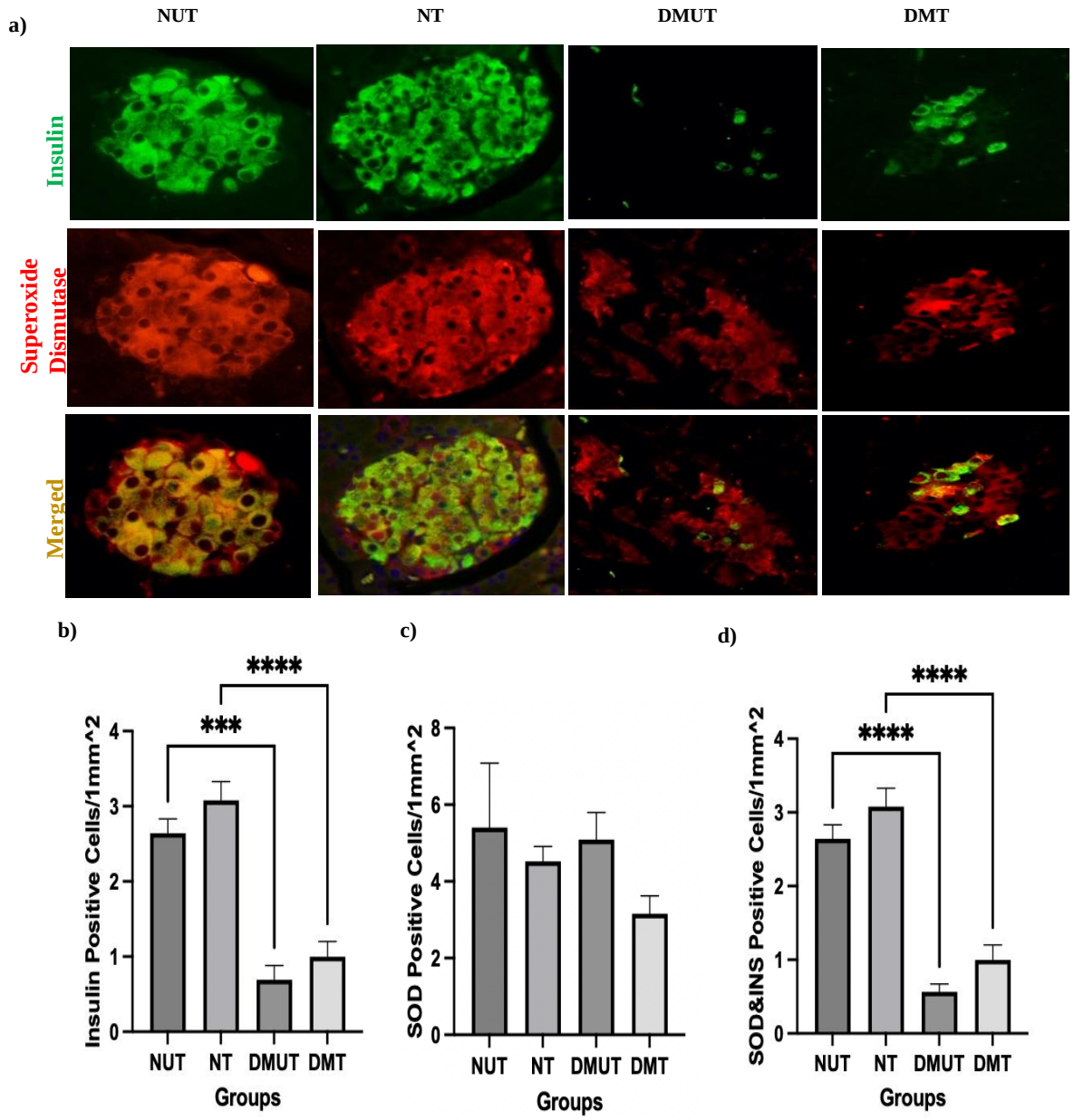


Figure 17: Shows images of insulin and SOD-positive cells in the pancreatic islet cells of normal and diabetic rats after LIK066 treatment. (a) insulin-positive cells are shown by green fluorescence (FITC) while SOD-positive cells are shown in red (RRX). All pictures are taken on a scale of X40. The morphometric analysis of (b) insulin-positive cells/1mm^2, (c) SOD-positive cells/1mm^2, and (d) SOD & insulin-positive cells/1$mm^2$of NUT, NT, DMUT, and DMT group. Values represented as mean± SEM, n=6

ROS production in diabetes can destroy islet cells. In hyperglycemia, the dismutation of the free radical by dismutase is low in T1DM and T2DM. Hyperglycemia decreases SOD activity by glycation which means the binding of glucose to proteins.

(West, 2000). During diabetes, there is an increased level of oxidative stress. This increase in oxidative stress triggers increases SOD production. Treatment with LIK066 reduces the level of oxidative stress.

4.4.1.3 Glutathione Reductase

There is a significant increase in GSH reductase-positive cells in normal treated compared to normal untreated animals in figure 18. Moreover, there is a significant increase in the number of GSH reductase-positive cells in islets of Langerhans of LIK066 treated diabetic rats compared to untreated diabetic rats.

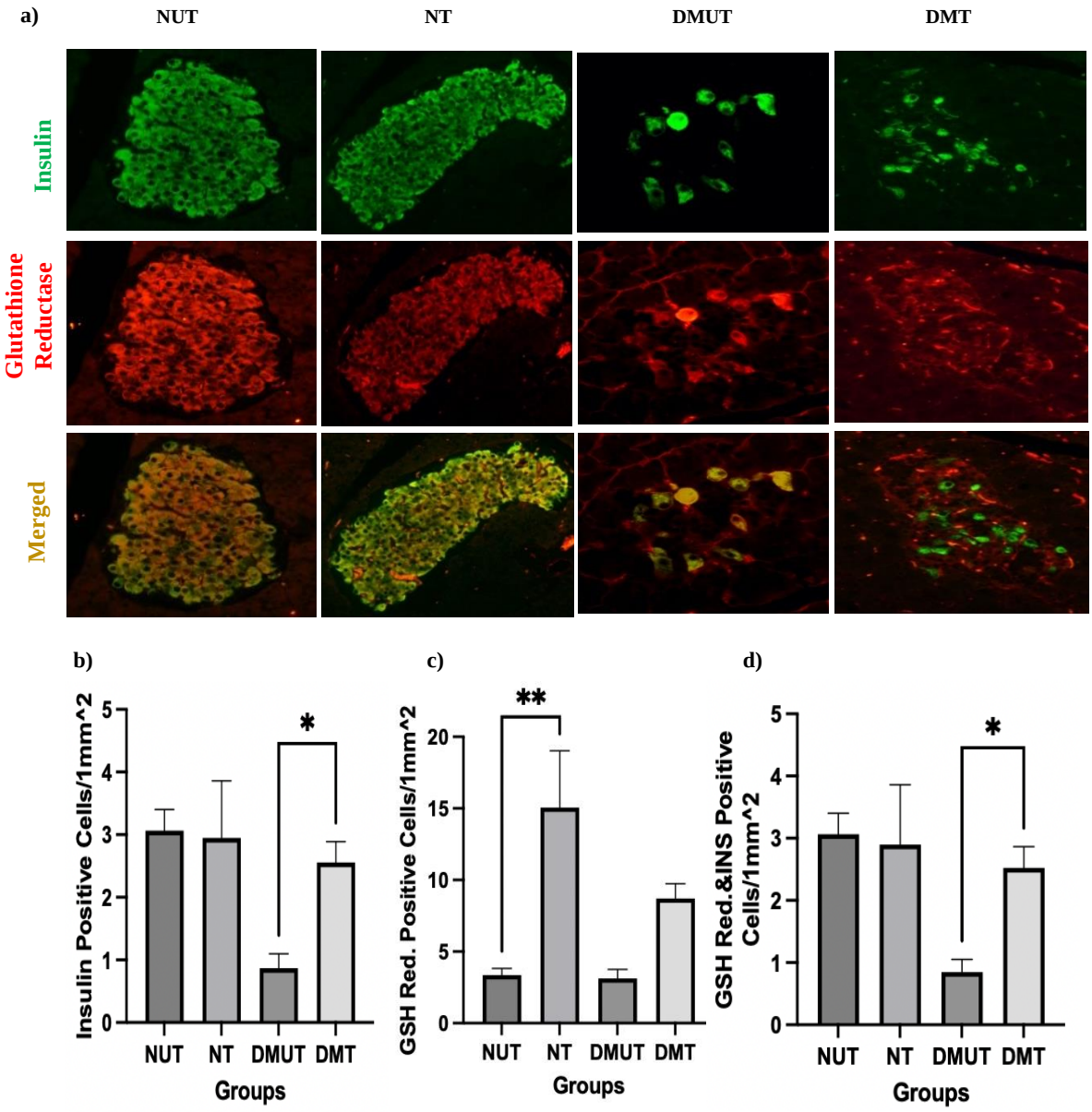


Figure 18: Shows images of insulin and glutathione reductase positive cells in the pancreatic islet cells of normal and diabetic rats after LIK066 treatment. (a) insulin-positive cells are shown by green fluorescence (FITC) while GSH reductase-positive cells are shown in red (RRX). All pictures were taken on a scale of X40. The morphometric analysis of (b) insulin-positive cells/1mm^2, (c) SOD-positive cells/1mm^2, and (d) SOD & insulin-positive cells/1$mm^2$of NUT, NT, DMUT, and DMT group. Values represented as mean± SEM, n=6

Chronic hyperglycemia is associated with oxidative stress and causes long-term damage. Glutathione reductase is involved in the mechanism of free radical detoxification using reduced and NADPH as a cofactor. NADPH is necessary for converting oxidized glutathione to its reduced form and it's stimulated by insulin. In

diabetes, NADPH production is very low which decreases the activity of glutathione reductase thus reduced glutathione will be lower too. Glutathione reductase levels decrease in T2DM (Likidlilid et al., 2007). Our result support what is in the literature that a decrease in GSH reductase is observed in untreated diabetic subjects. Here we showed that treatment with LIK066 increases GSH reductase levels in pancreatic β-cells of diabetic-treated animals.

4.4.2 Effects of LIK066 on Antioxidant Enzymes Activities in the Heart

4.4.2.1 Catalase

Figure 19 shows the percentage distribution of catalase-positive cells in the heart tissue of diabetic and normal animals using immunofluorescence. A highly significant increase in the percentage distribution of catalase-positive cells was observed in the heart of diabetic rats treated with LIK066. This shows that LIK066 indeed has an antioxidative effect on the heart.

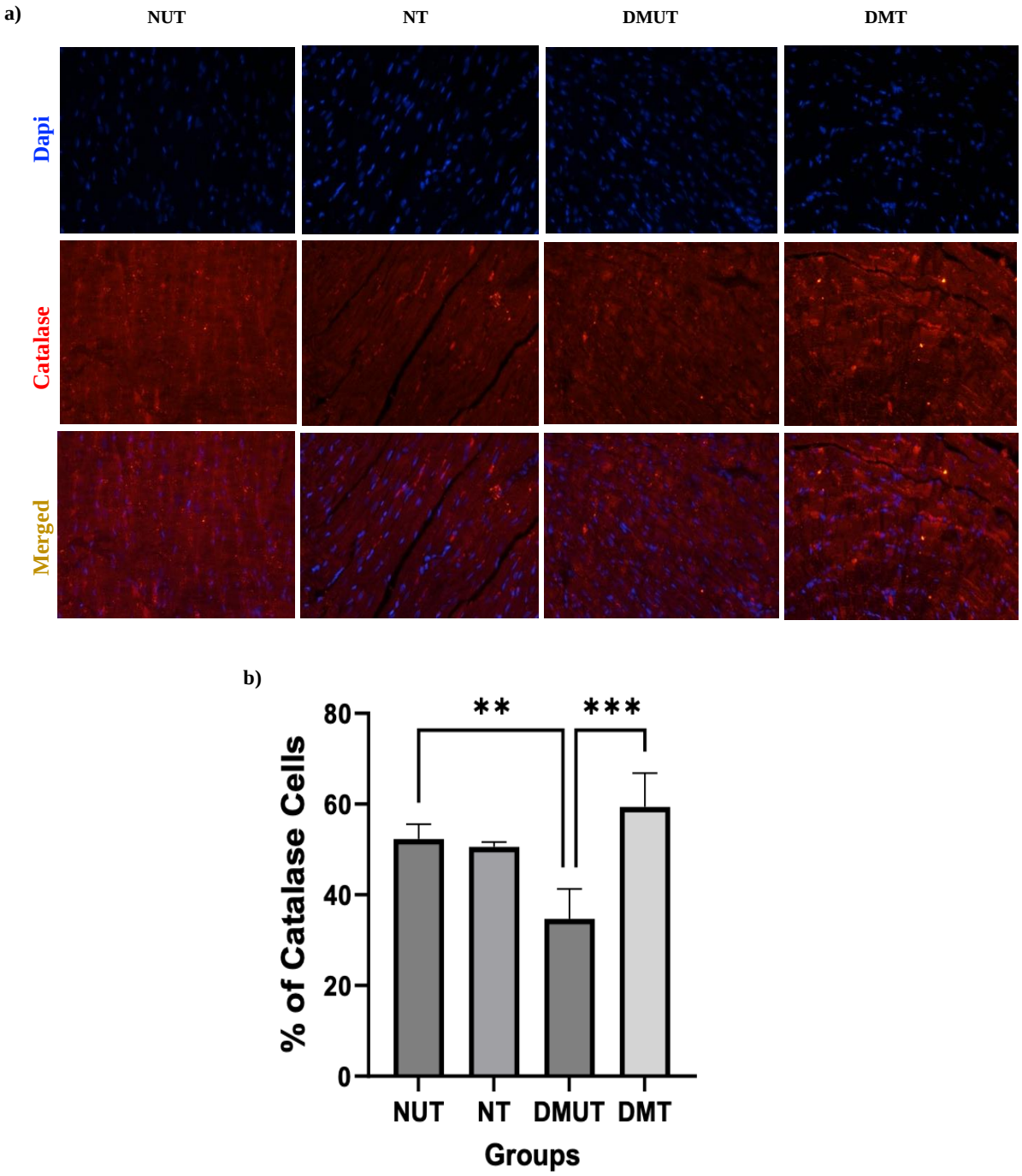


Figure 19: The antioxidant effect of LIK066 on heart muscle of normal and diabetic rats. (a) single staining of IHC shows catalase-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number will be counted. All pictures are taken on a scale of X40 (b) The % of catalase-positive cells in the four groups show significant result comparing diabetic treated and untreated. The graphs are representative of six animals.

The results show that catalase is present in the heart of diabetic and normal rats. LIK066 treatment *in vivo* causes a significant increase in the heart of diabetic rats compared to diabetic untreated rats. To the best of our knowledge, there are no reported data available on the effect of LIK066 on catalase in the heart.

Catalase has an important role as an antioxidant enzyme that significantly reduces oxidative stress by breaking down hydrogen peroxide into water and oxygen. Catalase has been shown to have a role in mutagenesis, and inflammation which are highly associated with oxidative stress. Diseases like diabetes and cardiovascular diseases are linked to the dysfunction of catalase or its deficiency (Nandi et al., 2019). The overproduction of ROS may lead to diabetic complications like diabetic cardiomyopathy because the cell's ability to detoxify ROS will be reduced (Ye et al., 2005). Catalase is founded in the peroxisomes which are very few in myocytes in humans and rodents. Unfortunately, cardiac cells are particularly very vulnerable to the damaging effects of oxidative stress. In the T1DM model, catalase plays a major role in preventing cardiomyocyte dysfunction. It was shown that suppressing the heart's NF-κB signaling pathway by overexpressing catalase decrease the buildup of reactive oxygen species (ROS). This result of catalase can affect cardiovascular complications in the diabetes (Cong et al., 2015).

4.4.2.2 Superoxide Dismutase

Figure 20 shows the percentage distribution of SOD-positive cells in the heart of diabetic and normal animals using the immunofluorescence method. There is no significant difference in the percentage distribution of SOD-positive cells in diabetic rats compared to normal. However, in diabetics, there is a slight increase in SOD-positive cells when compared to normal. The treatment with LIK066 had no significant effect on the number of SOD-positive cells in the heart.

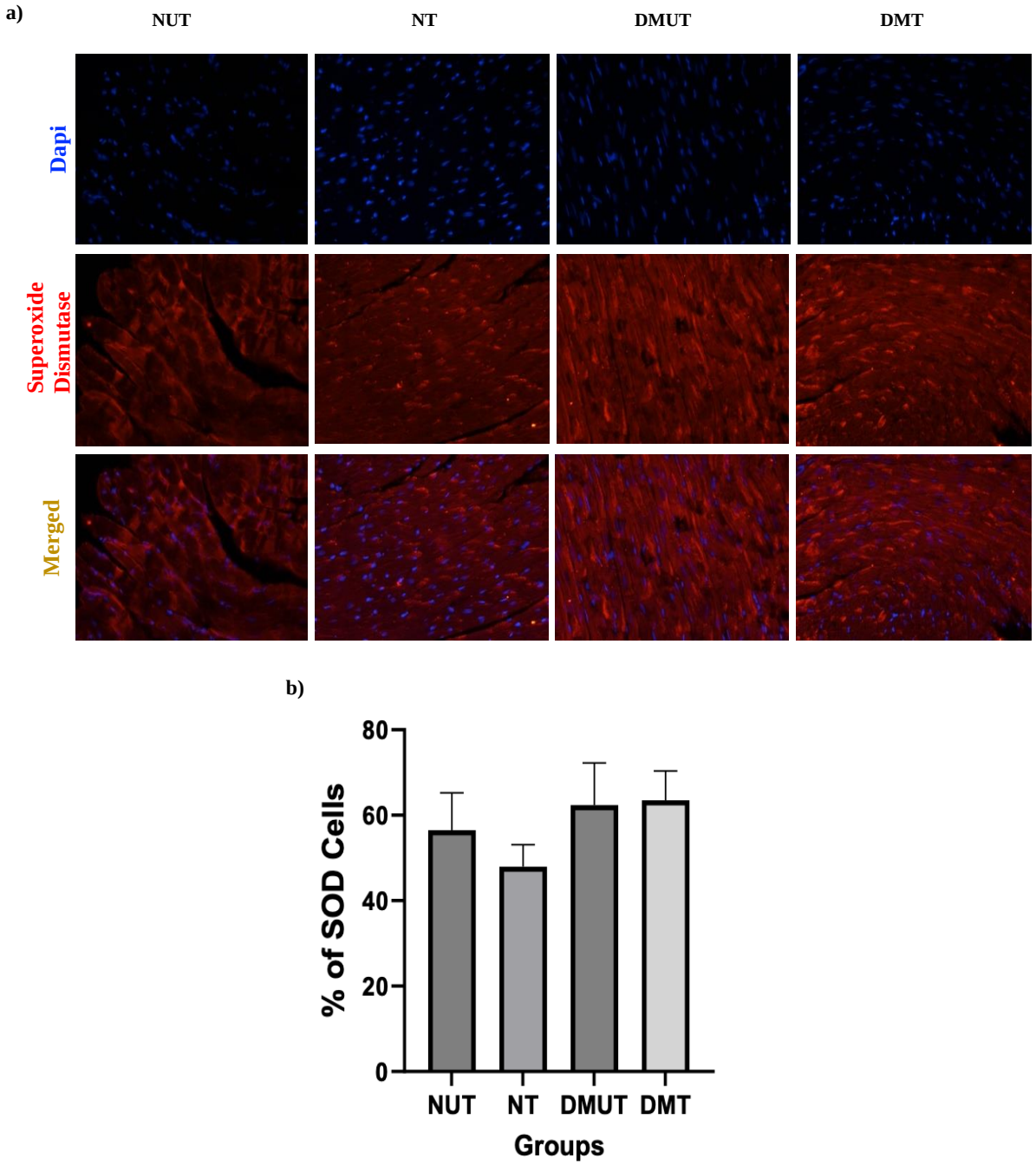


Figure 20: Immunofluorescence images of SOD in the heart of normal and diabetic rats treated with and without LIK066. (a) single staining of IHC shows SOD-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number will be counted. All pictures are taken on a scale of X40 (b) The % distribution of SOD-positive cells in NUT, NT, DMUT, and DMT show no significant result when comparing all the groups. Values represented as mean± SEM, n=6

SOD levels show no difference between diabetic treated and untreated rats. Showing no improvement in cardiac heart muscle after LIK066 treatment. Superoxide

dismutase catalyzes the reaction of superoxide into oxygen and hydrogen peroxide. SOD is one of the most important antioxidant enzymes found outside of cells and is called extracellular superoxide dismutase (EC-SOD or SOD3). The studies show that EC-SOD is capable to protect CVS from oxidative stress due to its high expression in the arterial walls. However, ROS overproduction has a negative effect on endothelial function and the vasodilatory capacity of the endothelium due to the inhibition of NO leading to atherosclerosis (Mohammedi et al., 2015).

4.4.2.3 Glutathione Reductase

Figure 21 shows the percentage distribution of GSH reductase-positive cells in the heart tissue of diabetic and normal animals using the immunofluorescence method. A significant elevation in the number of GSH reductase-positive cells was observed in the heart of diabetic rats 4 weeks after being treated with LIK066.

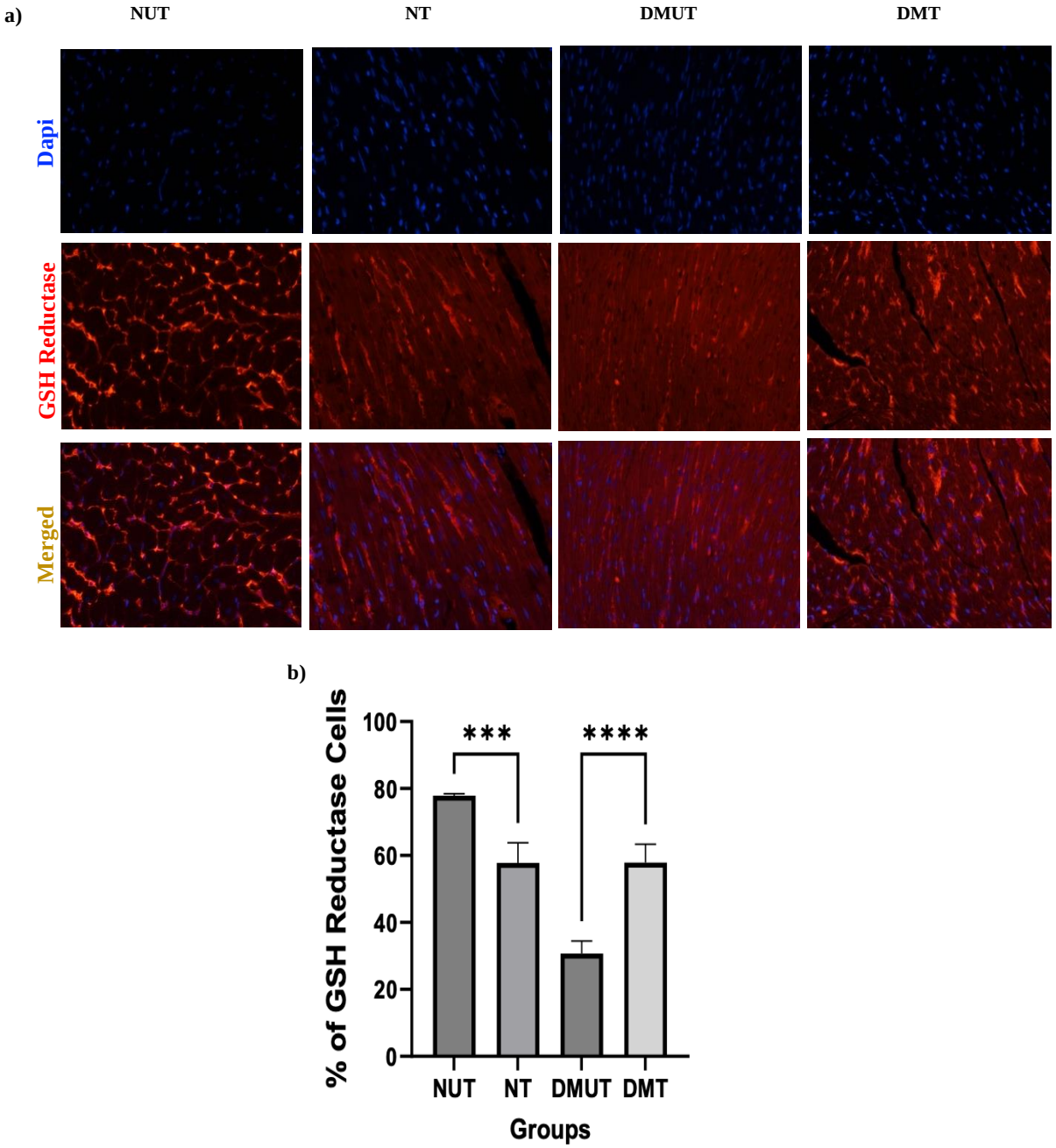


Figure 21: Immunofluorescence images of GSH reductase in the heart of normal and diabetic rats treated with and without LIK066. (a) single staining of IHC shows GSH reductase-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number will be counted. All pictures are taken on a scale of X40 (b) The percentage distribution of GSH reductase-positive cells in NUT, NT, DMUT, and DMT show significant results comparing diabetic treated and untreated. Values represented as mean± SEM, n=6

The treatment with LIK066 showed increases in the tissue level of the GSH reductase enzyme and thus can induce the reaction of reducing GSSH to GSH in the

presence of NADPH as a cofactor (S. Li et al., 2007). GSH detoxifies ROS, however, in diabetes, the overproduction of ROS due to hyperglycemia can reduce the GSH levels as shown in Figure 19, where untreated diabetic rat hearts showed significant depletion in GSH reductase levels.

4.4.2.4 Kelch-like ECH-associated Protein 1 (Keap1)

The effect of LIK066 on keap1 expression has been studied in the heart using the immunofluorescence method. Figure 22 shows the % of keap1 cell distribution in diabetic and normal Wistar rats. There was no significant difference in the number of keap1-positive cells while comparing all the groups. The STZ-induced diabetics treated show a slight but not significant decrease in the number of Keap1 cells compared with the three other groups (NUT, NT, DMUT).

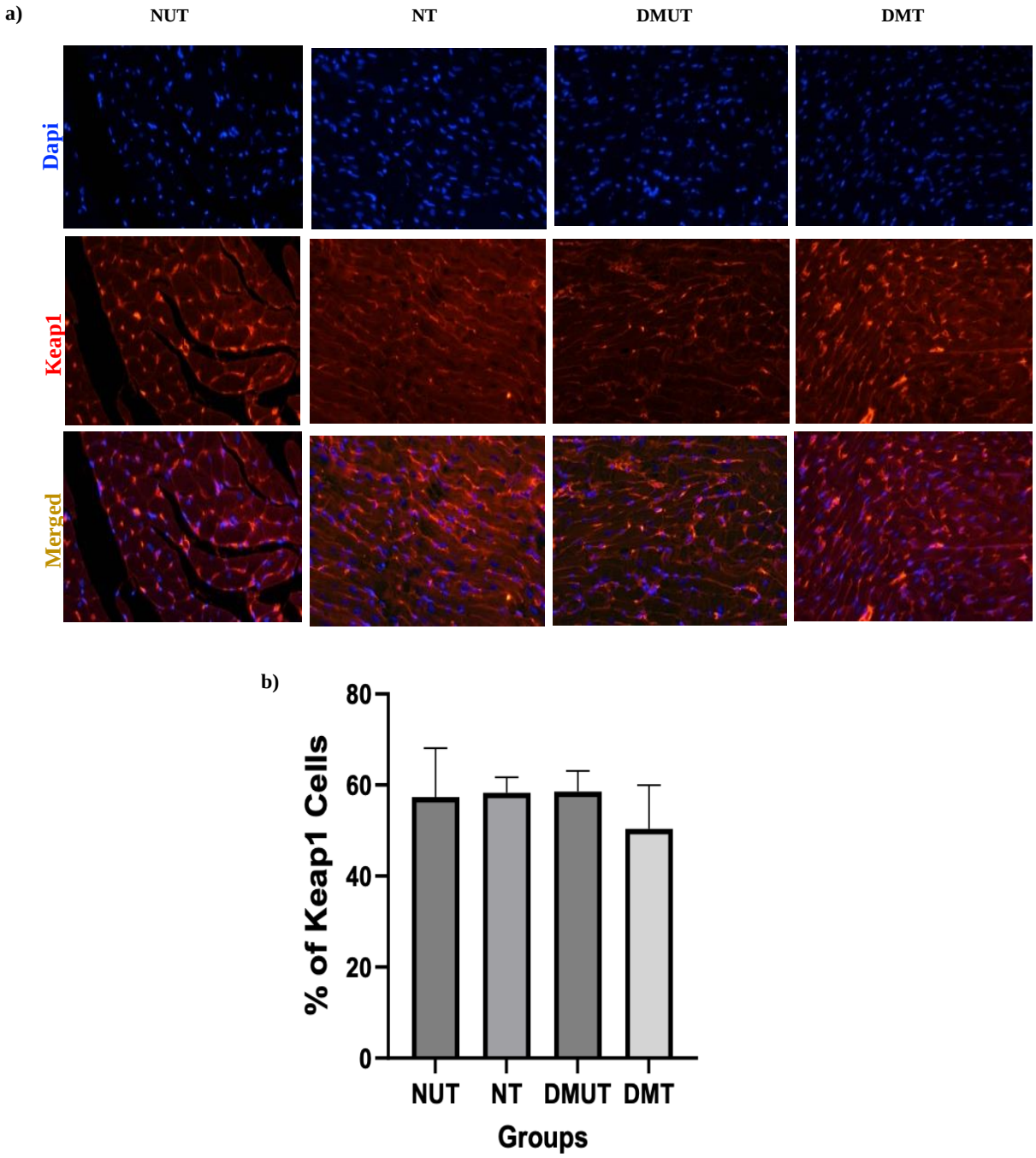


Figure 22: Immunofluorescence images of Keap1 in the heart of normal and diabetic rats treated with and without LIK066. (a) single staining of IHC shows keap1-positive cells in red fluorescence (RRX) and the blue is (DAPI) stain, after merging both RRX and DAPI total cell number will be counted. All pictures are taken on a scale of X40 (b) The % distribution of keap1 cells is not significant in all the groups. Values represented as mean± SEM, n=6

Keap1positive cells decreased slightly but not significantly after treatment with LIK066 in diabetic rats compared to diabetic untreated rats.

DM is associated with long-term complications like diabetic cardiomyopathy which is characterized by oxidative stress leading to cardiac abnormality and myocardial fibrosis. The activation of the Keap1/Nrf2/ARE signaling pathway regulates the expression of most antioxidant genes like NADPH. A study on the heart of diabetic mice shows that hyperglycemia is downregulating Nrf2 and upregulated Keap1. Then The dissociation of the Keap1/Nrf2 complex can have a positive impact on diabetic cardiomyopathy by decreasing oxidative stress due to a decrease in keap1 levels and an increase in Nrf2 (Ying et al., 2018). This is the crucial transcription factor binding to the cis-regulatory antioxidant response element (ARE) sequence. Furthermore, transcriptional upregulation of several antioxidant enzymes is triggered by Nrf2. In a non-stress environment, Nrf2 is suppressed by Keap1, this pathway is regulating oxidative stress in disease conditions. In diabetes, the level of Nrf2 will be depleted which induces apoptosis in β-cell within pancreatic islets. Also, Nrf2 has a great impact on cardiovascular health by preventing the dysfunction of endothelial cells and protecting the heart muscle (David et al., 2017).

4.4.3 Effects of LIK066 on Antioxidant Enzymes Activities in Serum and Heart Homogenate

4.4.3.1 Catalase Activity in Serum and Heart Tissue

The antioxidant activity of LIK066 was tested in the serum and the heart tissue homogenate using colorimetric analysis. Figure 23a shows serum catalase activity was slightly increased in untreated diabetic rats compared to all the groups. In heart tissue, catalase activity is not significant in all the groups shown in Figure 23b.

a) b)

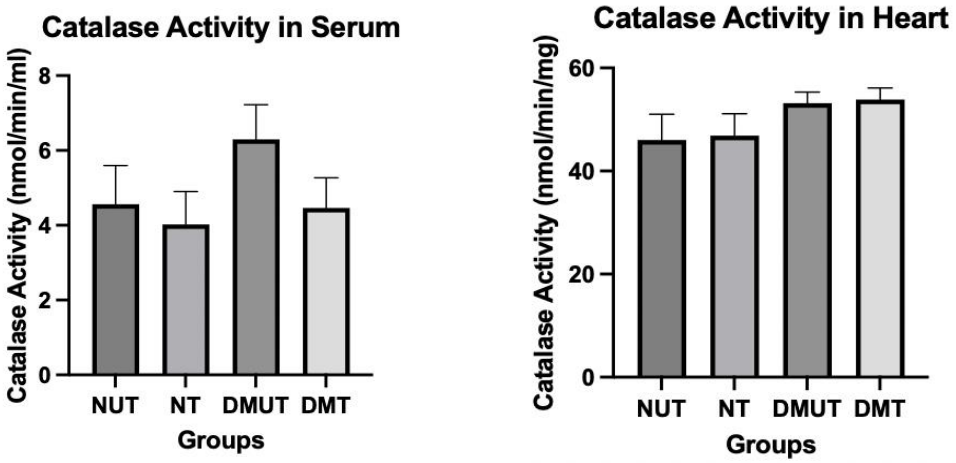


Figure 23: Catalase (CAT) activity in the serum and heart of normal and diabetic rats. (a) represents the colorimetric analysis of catalase activity in NUT, NT, DMUT, and DMT. (b) CAT activity in the heart in NUT, NT, DMUT, and DMT. No significant changes were observed between the groups. Values represented as mean± SEM, n=6

The mechanism by which diabetes induces free radicals is by glycosylation of proteins, glucose oxidation by itself, or elevated glucose metabolism through the sorbitol-polyol pathway. Diabetes and its complications like hypertension cause serum antioxidant enzyme activity depletion (Sözmen et al., 1999). LIK066 treatment has no effect on catalase activity in the heart. Some studies have shown that serum catalase activity increased slightly in the diabetes (Singh et al., 2013).

4.4.3.2 Superoxide dismutase (SOD) Activity in Serum and Heart

The antioxidant activity of LIK066 on SOD was tested in the serum and heart tissue homogenate using colorimetric analysis. SOD activity in the heart was significantly higher in the heart of diabetic rats compared to untreated diabetics shown in Figure 24b. However, in Figure 24a, analysis of serum shows no significant changes in SOD activity in the serum between the different groups of all the groups. Treatment with LIK066 thus shows that this dual SGLTi increases SOD enzyme activity in the heart, thus contributing to antioxidant protection for the heart.

a) b)

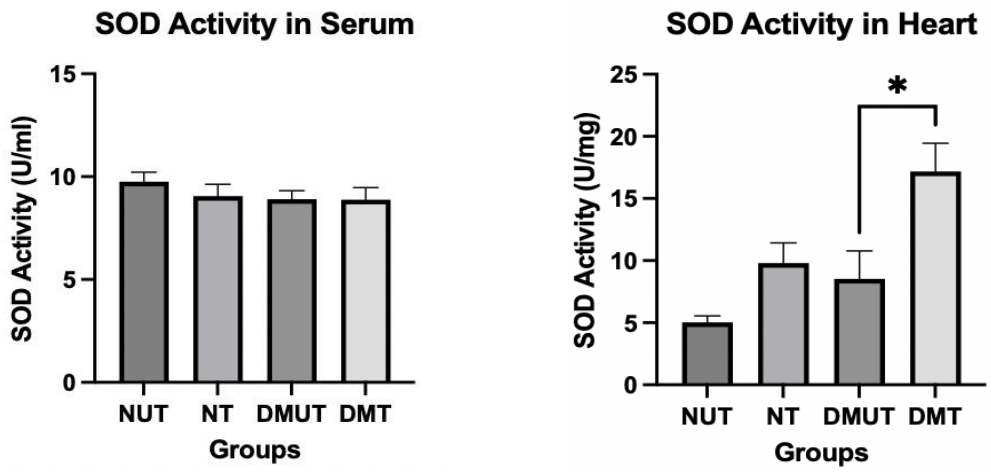


Figure 24: Superoxide dismutase (SOD) activity in the serum and heart of normal and diabetic rats. (a) represents the colorimetric analysis of SOD activity in NUT, NT, DMUT, and DMT. (b) SOD activity shows no significant difference in the serum of all groups. Values represented as mean± SEM, n=6

The activity of SOD in serum and heart can be an indicator of diabetes and its complication. In the serum, there was no effect of LIK066 on SOD levels in either diabetic or normal rats. However, studies reported in the literature showed a lower serum SOD activity is associated with diabetes and atherosclerosis (Fei et al., 2021). In the case of heart tissue, as shown in Figure 24b, LIK066 increases SOD activity significantly in the heart of diabetic rats when compared to untreated diabetics. The expression of SOD in arterial walls is considered a defense mechanism against oxidative stress in the diabetes (Mohammedi et al., 2015).

4.4.3.3 Glutathione Activity in Serum and Heart

Glutathione (GSH) activity in the heart homogenate of rats treated with LIK066 was significantly higher when compared to their respective controls in Figure 25. The GSH activities measured in the serum and heart homogenate of normal and diabetic groups appeared to be higher than that of normal rats. The reason for this observation may be due to a compensatory mechanism, where this endogenous antioxidant is trying to compensate for the increased oxidative stress in diabetes.

a) b)

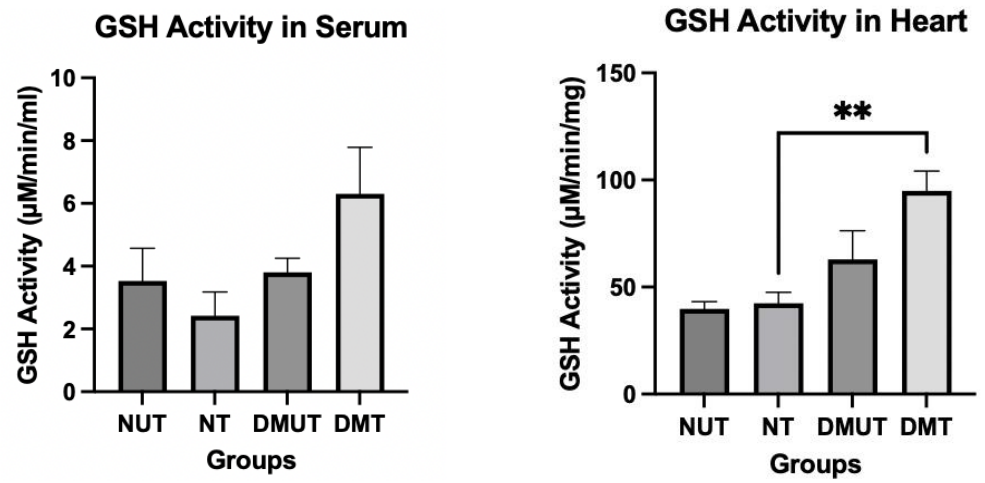


Figure 25: Glutathione (GSH) activity in the serum and heart of normal and diabetic rats. (a) represent the colorimetric analysis of GSH activity in NUT, NT, DMUT, and DMT. (b) GSH in heart homogenate. Values represented as mean± SEM, n=6

Both serum (figure 25a) and heart tissue homogenate (figure 25b) show a slight increase in GSH activity in diabetic treated compared to diabetic untreated animals after LIK066 treatment. However, the tissues of control animals produce fewer free radicals so the GSH production will be low. A study conducted on STZ-induced diabetic rats showed that oral GSH treatment can elevate myocardial GSH levels and protect the heart from oxidative stress (Yur et al., 2013).

4.4.3.4 NO Activity in Serum and Heart

Figure 26 shows the activity of NO in the serum and heart tissue homogenate using colorimetric analysis. Nitric Oxide (NO) activity in the heart homogenate was significantly higher in the LIK066-treated group compared to the control in Figure 26b. However, Figure 26a shows non-significance changes in the level of NO activity in the serum of diabetic and normal animals. The treatment with LIK066 shows a significant increase of NO activity in the heart homogenate, but no effect on serum.

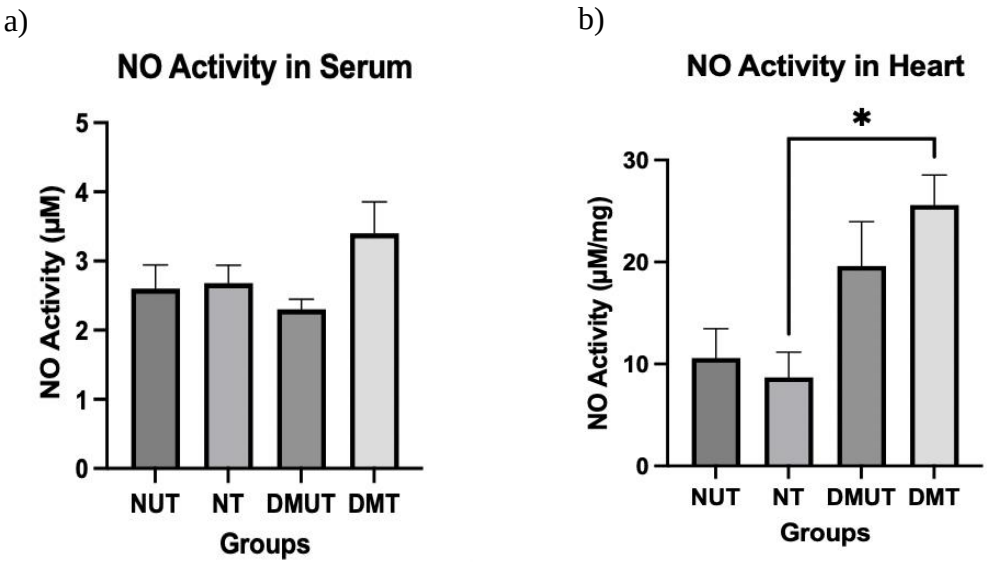


Figure 26: The activity of NO in serum and heart tissue homogenate of normal and diabetic rats. Both (a) and (b) represent the colorimetric analysis of NO activity in NUT, NT, DMUT, and DMT groups which show significance in heart homogenate while comparing diabetic-treated and control-treated and no significance in the serum comparing all the groups. Values represented as mean± SEM, n=6

In diabetes, the hallmark of vascular complications is the elevation of ROS and endothelial dysfunction due to a decrease in NO levels which play a role in the endothelium relaxing and control of muscle contraction in the cardiovascular system (J. C. Li et al., 2021). One study reported that STZ-induced diabetic rats show higher NO levels and NO metabolites excreted in urine which is related to the glomerular hyperfiltration rather than arteriolar vasodilatation (Maree et al., 1996). We found that STZ-induced diabetic treated rats show a significant increase in NO activity in the heart compared to normal treated animals. A study conducted shows an increase in endogenous DMA in diabetic rats which is considered an inhibitor of NO synthase in serum thus it causes vasorelaxation (Xiong et al, 1997).

4.5 Heart Morphology

4.5.1 Masson Staining of heart tissue fibrosis

Figures 27 and 28 show Masson trichrome staining for connective fibers in the myocardium was performed to investigate whether the pattern of connective tissue distribution has been altered. Impaired distribution of connective tissue in the heart may

point to cardiac fibrosis and a process of heart remodeling. In diabetic untreated rat hearts, the arrangement of cardiomyocytes is random with a lot of spaces, however, in diabetic treated the arrangement of cardiomyocytes is more regular.

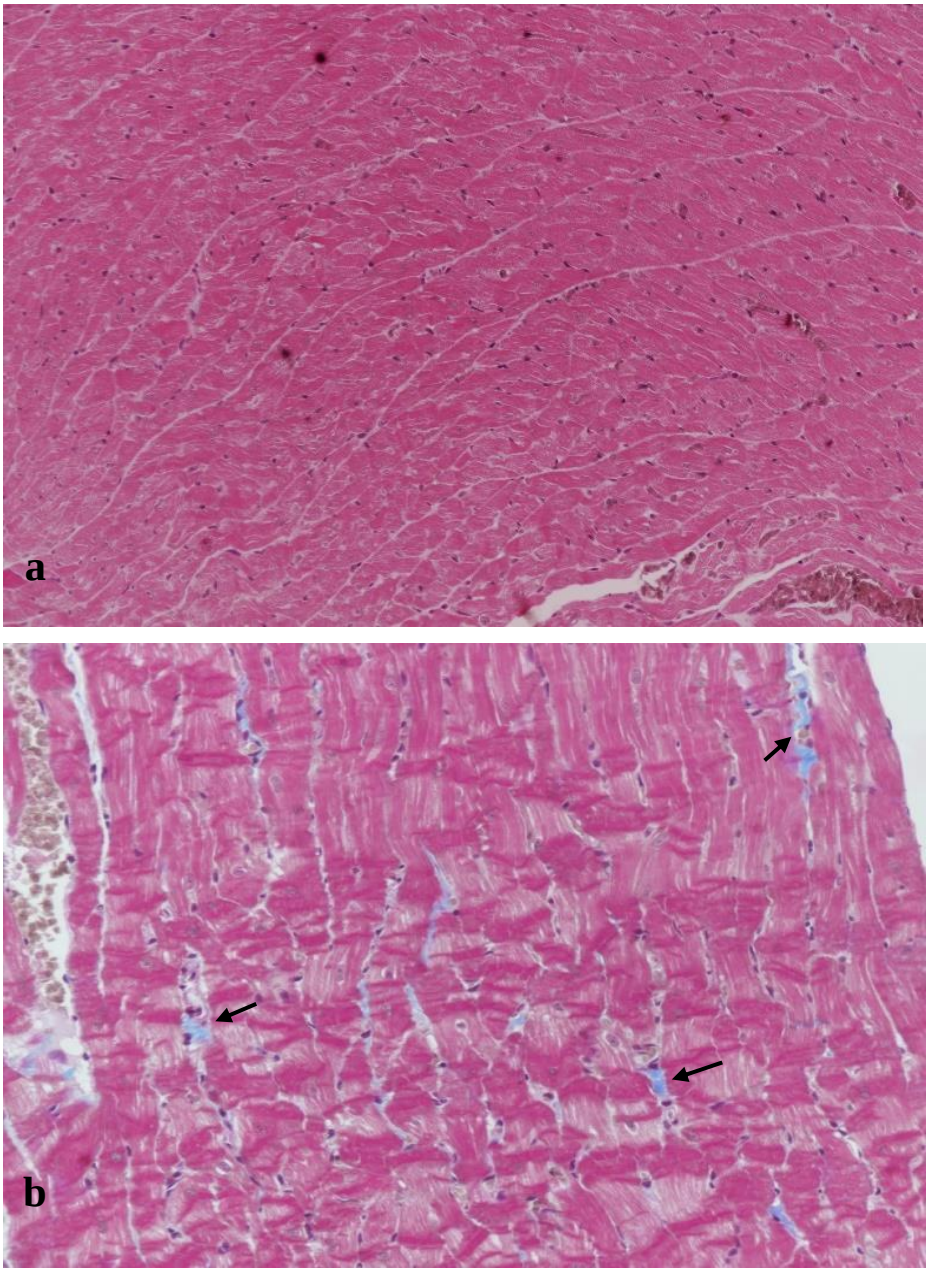


Figure 27: Masson trichrome staining for collagen fiber in the myocardium (a) normal untreated rats (b) normal treated rats.

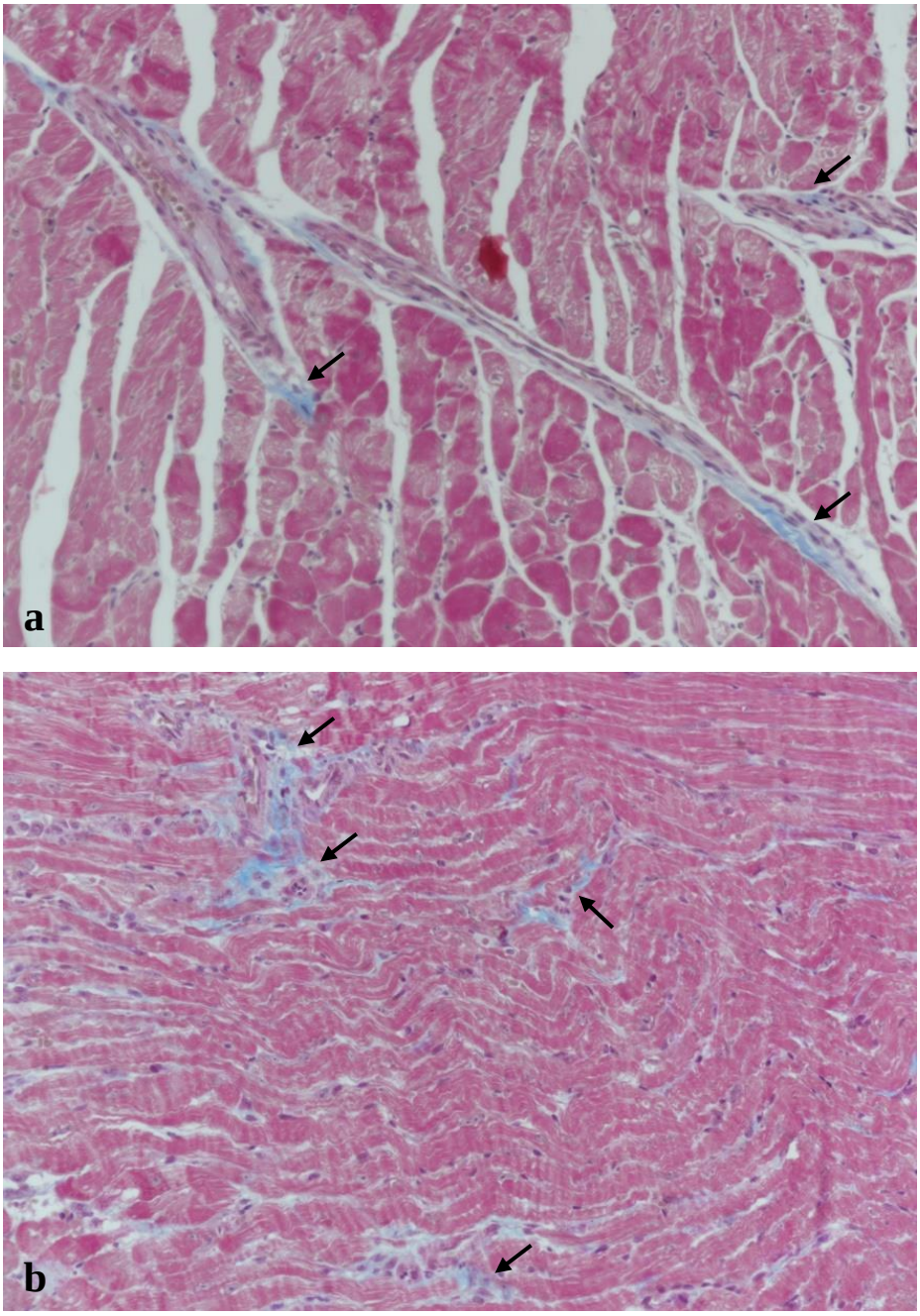


Figure 28: Masson trichrome staining for collagen fiber in the myocardium (a) diabetic untreated rats (b) diabetic treated rats.

Chapter 5: Conclusions

This study provided evidence that SGLT1 and SGLT2 co-localized with insulin in the pancreatic β-cells of normal and diabetic rats. The administration of LIK066 increased the number of insulin-positive cells in the islets of Langerhans. We also examined the effects of in vivo LIK066 treatment on the pattern of distribution of collagen III, fibronectin, and TIMP4 in the heart of normal and diabetic rats, to determine whether it will ameliorate the severity of cardiac fibrosis. The result shows that TIMP4 tissue level increased significantly in the heart of rats treated with LIK066 compared to the control. Furthermore, LIK066 showed significant antioxidant activity by increasing CAT and GSH reductase levels in the heart of diabetic rats compared to untreated controls. Finally, regarding the effect of LIK066 on antioxidant enzyme activities in serum and in heart homogenate some enzymes show an increase in their activity as SOD, GSH, and NO in heart homogenate. Also, LIK066 showed a significant antioxidant activity by increasing GSH reductase in normal treated compared to normal untreated. In conclusion, LIK066 may project its cardioprotective effects by inducing the release of endogenous antioxidants.

Limitations of the study

When considering the limitations of this study, it is worth noting that the experimental model used in this study is the streptozotocin (STZ)-induced DM model. Although we know that STZ is a toxin that has a very specific effect on pancreatic β-cells, its potential effects on other organs remain unclear. In addition, the dosage used and treatment duration in this study was less than those described in the literature for LIK066's efficacy in weight loss and glycemic control.

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This study provided evidence that SGLT1 and SGLT2 co-localized with insulin in the pancreatic β-cells of normal and diabetic rats. The administration of LIK066 increased the number of insulin-positive cells in the islets of Langerhans. We also examined the effects of in vivo LIK066 treatment on the pattern of distribution of collagen III, fibronectin, and TIMP4 in the heart of normal and diabetic rats, to determine whether it will ameliorate the severity of cardiac fibrosis.

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