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BIOCHEMICAL ANALYSIS AND MICRORNA PROFILING IN A HIGH GLUCOSE IN *VITRO* MODEL WITH RESVERATROL INTERVENTION

by

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A thesis submitted in fulfilment of requirements for the award of the Degree of Doctor of Philosophy (Ph.D.) in Biomedical Sciences

In the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology

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Bellville
November 2023

DECLARATION

I, Abegail Mukhethwa Tshivhase, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

27/11/2023

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ABSTRACT

Background: The prevalence of diabetes has reached an alarming level worldwide. Individuals with diabetes experience impaired glucose metabolism, which results in an augmented inflammatory response and heightened oxidative stress, contributing to the upregulation of inflammatory and pro-apoptotic genes. These effects ultimately exacerbate complications associated with diabetes, which significantly compromise patients' quality of life and life expectancy. Thus, there is an urgent need to identify safe and effective drugs that provide anti-diabetic benefits while protecting against complications of diabetes. Furthermore, alternative biomarkers are required to facilitate early identification of complications and risk management to improve the quality of life. Circulating miRNAs have emerged as potential contributors to disease etiology and progression, including diabetes; hence, they can be of significant use as novel markers with the potential for innovative diagnostic and therapeutic tools. Additionally, the aberrant expression of miRNAs may be implicated in various pathways, such as glucose metabolism, inflammation, oxidative stress, and apoptosis. The therapeutic effects of natural compounds have been widely recognized for centuries. This study aimed to investigate the effect of RES on oxidative stress, inflammation, apoptosis, and glucose metabolism under high glucose-induced conditions as well as investigate the effect of high glucose levels and evaluate the influence of RES on high glucose-induced miRNA dysregulation.

Methods: HepG2 liver cells were divided into six groups: control, High glucose (40 mM), Low resveratrol (LR) (25 μM), High resveratrol (HR) (50 μM), HG+LR, and HG+HR. The supernatant was collected after 48 and 72 hours of exposure; total RNA and miRNAs were extracted according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA and used for gene expression analyses. The extracted total miRNAs were used for miRNA expression analyses using quantitative Polymerase Chain Reaction (qPCR). The collected supernatant was utilized for ELISA, Bioplex, and lactate dehydrogenase (LDH) assays. All statistical analyses were performed using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California, USA). The Student's *t*-test and one-way analysis of variance (ANOVA) were used. All assays were performed in triplicate, and differences were considered statistically significant at $p<0.05$.

Results: A significant reduction was observed in the expression levels of miR-126-3p, miR-182-5p, and miR-30a-5p when HepG2 cells were exposed to high glucose conditions. Intriguingly, resveratrol treatment reversed the reduction of miR-126-3p, miR-182-5p, and miR-30a-5p caused by high glucose in HepG2 cells. Moreover, our research demonstrates that

high glucose resulted in an increase in Neuronal Differentiation 1 (*Neurod1*) expression in HepG2 cells. Conversely, the expression of *Neurod1* was found to be reduced in response to resveratrol. There was a significant increase in the mRNA expression of nuclear factor kappa B (*NF-kB*), IkB kinase α (*IKKα)*, and *IkB-α* when HepG2 cells were exposed to high glucose. Resveratrol treatment markedly reduced *NF-kB*, *IKKα*, and *IkB-α* expression levels. A notable increase in Sprouty-related EVH1 domain containing 1 (*SPRED1)* expression was observed in cells treated with high glucose, leading to augmented expression levels of tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), Cyclooxygenase 2 (COX2), and Interleukin-1 beta (IL-1β). Nevertheless, resveratrol treatments reduced the expression levels of *SPRED1*, TNF-α, IL-6, COX2, and IL-1β in HepG2 cells. In cells treated with high glucose, there was a significant increase in the expression of *FOXO1*. This increase subsequently led to an increase in the expression of genes associated with gluconeogenesis, namely phosphoenolpyruvate carboxykinase (*PEPCK*) and Glucose-6-phosphate (*G6P*). Simultaneously, there was a concurrent reduction in the expression of glucokinase (GCK). In contrast, resveratrol treatment reduced *FOXO1*, *PEPCK,* and *G6P* expression while increasing *GCK* expression. A significant reduction in nuclear factor erythroid 2–related factor 2 (*Nrf2*) expression, (p<0.0001) and antioxidant enzymes (*SOD*, Superoxide dismutase; *GPx1*, Glutathione peroxidase 1; *CAT* and *NQO1*, NAD(P)H quinone oxidoreductase 1) were observed when HepG2 cells were exposed to high glucose. Remarkably, resveratrol increased *Nrf2* expression, subsequently triggering an increase in genes associated with antioxidant enzymes (*SOD*, *CAT*, *GPx1*, and *NQO1)*. High glucose exposure notably decreased B-cell lymphoma 2 (*BcL-2*) gene expression, whereas resveratrol treatment significantly increased *BcL-2* expression. Prolonged exposure of HepG2 cells to high glucose (72 h) increased LDH release. Intriguingly, resveratrol treatment showed a noteworthy reduction in LDH release. High glucose exposure reduced Oxoguanine glycosylase-1 (*OGG1*) expression, while resveratrol significantly increased *OGG1* mRNA levels (p<0.0001).

Conclusion: Data obtained from this study showed that high glucose levels influence miR-126-3p, miR-182-5p, and miR-30a-5p in HepG2 liver cells. While resveratrol treatment reversed high glucose-induced downregulation of miR-126-3p, miR-182-5p, and miR-30a-5p in HepG2 cells. Thus, suggesting a promising role for resveratrol in regulating miRNA expression patterns implicated in diabetes. Our findings demonstrated that high glucose disrupts pathways (glucose metabolism, inflammation, oxidative stress, and apoptosis) related to diabetes. Moreover, our findings demonstrated that resveratrol may ameliorate the pathologic processes involved in DM complications by reducing inflammation and oxidative stress, increasing anti-apoptotic and DNA-repair genes, and regulating glucose metabolism.

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to each and every individual who played a part in my PhD journey:

- To my supervisor, Prof T.E Matsha, thank you so much for allowing me to grow, learn, and relearn under your supervision at the SAMRC/CPUT Cardiometabolic research unit. Thank you for your patience and support.
- To my supervisor, Dr. S Raghubeer, Thank you for your unwavering support. You corrected me when I was wrong, gave me a chance to develop, and allowed me to make mistakes and learn from them. Thank you for guiding me throughout the entirety of this Ph.D. Your insightful comments and suggestions are greatly appreciated. You were present during lab work and writing. Thank you for always proofreading my work. I am grateful.
- Special thank you to Dr. Davids and Ms. S. Chalklen for guidance, skills in the laboratory, and technical assistance.
- To my special doctors, Dr. Motshwari, Dr. Matshazi, Dr. Elvis, and Dr. Weale, I appreciate each of you. Thank you for all the talks, laughs, and guidance. You made this journey easy for me. Dr. Motshwari, thank you for being the big sister.
- My fellow students for all the assistance, support, and friendship
- To my husband, you've been nothing but the best. Your support has been invaluable throughout. You were there to bring smile on my face when I was feeling down.
- To my family, thank you for your support and love.
- I would also like to thank the National Research Foundation and the South African Medical Research Council for funding this project. I am grateful to the NRF for the scholarship and Department of Science and Innovation (DSI)-) Inter-bursary Support Programme-Council for Scientific and Industrial Research (CSIR) allowed me to take on PhD at the Cape Peninsula University of Technology.

DEDICATION

This thesis is dedicated wholeheartedly to my late grandfather (Mr R.M Tshivhase) and my beloved late Aunt (L.N Tshivhase). Your contribution to my life is unparalleled. I still hold onto the values you instilled in me. This one is for you.

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GLOSSARY

ABBREVIATIONS

%	Percentage
µL	Microlitre
µM	Micromolar
$2^{-\Delta Ct}$	Relative quantification of gene expression of each sample
$2^{-\Delta\Delta Ct}$	Relative quantification of gene expression detailing the difference between treated sample and control sample
cm	Centimetre
cm^3	Cubic centimetre
g	Grams
h	Hour (s)
mg/dL	Milligrams Per Decilitre
min	Minute (s)
mL	Millilitre
mM	Millimolar
mmol/L	millimoles per litre
nm	Nanometre
ºC	Degree Celsius
pH	Potential of Hydrogen
8-oxoG	8-oxo-7,8-dihydroguanine
ADA	American Diabetes Association
AGEs	Advanced glucose end-products
AGO2	Argonaute 2
AMPK	Adenosine monophosphate-activated kinase
AREs	Antioxidant response elements
AUSDRISK	Australian T2DM risk assessment tool
Bcl-2	B-cell lymphoma 2
BER	Base excision repair
C. Elegans	Caenorhabditis Elegans
CAT	Catalase
CCM	Complete culture medium
CO_2	Carbon dioxide
COX2	Cyclooxygenase-2
CSF	Cerebrospinal fluid

CVD	Cardiovascular disease
DGCR8	DiGeorge Syndrome Critical Region 8
DKA	Diabetes ketoacidosis
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's minimum essential medium
ER	Estrogen reporter
ERK	Extracellular signal-regulated kinase.
ERK1/2	Extracellular signal-regulated protein kinase 1/2
EVs	Extracellular vesicles
FINRISK	Finnish Cardiovascular Risk Study
FOXO1	Forkhead box O1
FR	Free radicals
G6P	Glucose-6-phosphatase
G6P	Glucose-6-phosphatase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GLP1	Glucagon-like peptide-1 agonist
GLP1R	Glucagon-like peptide-1 receptor
GLUT4	Glucose transporter 4
GPx	Glutathione peroxidase
GR	Glutathione Reductase
GRB10	Growth Factor Receptor Bound Protein 10
GSH	Glutathione
GSK-3	Glycogen synthase kinase-3
H2O2	Hydrogen peroxide
HbA1c	Glycated haemoglobin A1c
HDL	High-density lipoprotein
HepG2	Human hepatoma G2
HG	High glucose
HHS	Hyperglycaemic hyperosmolar state
HIF1A	Hypoxia-inducible factor-1 Alpha
HMGB1	High mobility group box 1
HO-1	Heme oxygenase (decycling)-1
HPLC	High-Performance Liquid Chromatography
HR	High resveratrol

IDDM	Insulin-dependent Diabetes Mellitus
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IKK	IkappaB kinase
IL	Interleukin
IRS2	Insulin receptor substrate 2
JAK	Janus kinase
JNKs	c-Jun N-terminal kinases
KEAP1	Kelch-like ECH-associated protein 1
KLF7	Krupple-like factor 7
LA	Lactic acidosis
LDH	Lactate dehydrogenase
LR	Low resveratrol
MODY	Maturity Onset Diabetes of the Young
mRNAs	Messenger RNAs
mTOR	Mechanistic target of rapamycin
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NDM	Neonatal diabetes mellitus
NF-κB	Nuclear factor kappa B
NOD	Non-obese diabetic
NPM1	Nucleophosmin 1
NQO1	Quinine oxidoreductase-1
Nrf2	Nuclear factor erythroid 2-related factor 2
O_2^-	Superoxide anion radicals
OGTT	Oral glucose tolerance test
OH^-	Hydroxyl radicals
PBS	Phosphate-buffered saline
PEPCK	Phosphoenolpyruvate carboxylase
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PPAR-gamma	Peroxisome proliferator-activated receptor gamma
Pre-miRNA	Precursor-miRNA
Pri-miRNA	Primary miRNA transcripts
PSF	Penstrepfungizone
PTEN	Tensin homolog
qPCR	Quantitative Polymerase Chain Reaction

Ran-GTPase	Ras-related nuclear protein-guanosine-5' triphosphatase
RES	Resveratrol
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SGLT-2	Sodium-glucose transporter 2
SIRT	sirtuin
SOD	Superoxide dismutase
SP1	Specificity protein 1
SPRED1	Sprouty-related EVH1 domain containing 1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor necrosis factor-alpha
tRNA	Transfer Ribonucleic acid
UTR	Untranslated regions
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Introduction

Diabetes mellitus (DM) is a chronic metabolic, non-communicable disease characterized by elevated blood glucose levels due to impaired insulin secretion or action (Firdous *et al*., 2018). It is a major social and economic issue of the twenty-first century with serious public health challenges (Zimmet *et al*., 2016). The global prevalence of DM is rapidly increasing and has reached epidemic proportions (Firdous *et al*., 2018). The International Diabetes Federation (IDF) estimated that 536.6 million individuals (undiagnosed and diagnosed) worldwide had diabetes in 2021 (Sun *et al*., 2022). Projections indicate a potential surge to 783.2 million diabetes patients by 2045 if drastic prevention initiatives are not implemented (Sun *et al*., 2022). In Africa, it was estimated that 24 million individuals were living with diabetes in 2021, and this is projected to rise to 55 million by 2045 (Sun *et al*., 2022). In South Africa, the prevalence of diabetes increased from 4.5% in 2010 to 12.7% in 2019. Furthermore, in 2019, 4.58 million individuals aged between 20-79 years were estimated to have diabetes, of which 52.4% were undiagnosed (International Diabetes Federation, 2021). Globally, 87.5% of all undiagnosed diabetes cases occur in low and middle-income nations, with low-income countries having the highest proportion (50.5%). However, even in high-income countries, nearly one-third (28.8%) of people with diabetes have not been diagnosed (International Diabetes Federation, 2021). Alarmingly, diabetes resulted in the loss of approximately 4 million adult lives in 2017, equating to 1 death every 8 seconds (International Diabetes Federation, 2017). Given this escalating global prevalence, there is an urgent need to explore biomarkers that facilitate early detection of complications and effective management of diabetes.

Maintaining normal blood glucose levels relies on a delicate balance between insulin secretion and functioning within the body. The liver is a vital organ responsible for regulating glucose homeostasis by regulating various glucose metabolism pathways, such as glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis (Han *et al*., 2016). However, insulin resistance in the liver disrupts glucose metabolism, leading to elevated blood glucose levels in patients with diabetes (Demir *et al*., 2021). This metabolic dysfunction of glucose significantly impacts other cells, tissues, and processes, all contributing to the development of diabetes. Furthermore, inflammation arises from various pathological stimuli and tissue injuries associated with diabetes, playing a role in the induction of insulin resistance (Huang *et al*., 2020). Oxidative stress has also been shown as a key mechanism of insulin resistance (Hurrle & Hsu, 2017).

Oxidative stress is the imbalance between oxidants and antioxidants (Francisqueti *et al*., 2017). The excessive generation of reactive oxygen species (ROS) has been associated with metabolic disorders, including diabetes (Shradha *et al*., 2010). Excessive ROS production results in oxidative damage to proteins, lipids, and genetic material, thereby disrupting

signalling and causing cellular malfunction, resulting in cell death (Costantini, 2019). ROS can also function as signalling molecules, facilitating cellular proliferation and apoptosis (Finkel, 1998). One example is oxidative stress-induced ROS production due to elevated glucose levels triggering apoptosis (Sun *et al*., 2012; Xu *et al*., 2012).

Diabetes increases the risk of developing life-threatening health complications, resulting in higher medical costs, reduced quality of life, and increased mortality (Baena-Díez *et al*., 2016). Diabetes mellitus is a leading cause of cardiovascular disease (CVD), including heart disease, stroke, renal failure, and blindness (due to diabetic retinopathy). Chronic hyperglycaemia leads to complications, such as neuropathy, nephropathy, and retinopathy, and an increased CVD risk (Danaei *et al*., 2011). Managing diabetes entails reducing blood glucose levels through lifestyle adjustments and anti-diabetic interventions. Unfortunately, current treatments may cause serious side effects, such as weight gain, hypoglycaemia, gastrointestinal discomfort, and contraindications that restrict their use (Öztürk *et al*., 2017; Su *et al*., 2022).

Recent advances in diabetes research highlight the crucial role of circulating miRNAs in the onset and progression of the disease, suggesting their therapeutic and diagnostic potential (Bartel, 2004). MiRNAs, intrinsic noncoding RNAs, regulate gene expression at the posttranscriptional stage, either by degradation of messenger RNAs (mRNAs) or translation inhibition (Bartel, 2004; Lewis *et al*., 2005). Due to their involvement in various biological processes, the aberrant expression of miRNAs may contribute to various pathophysiological conditions, making them promising targets for intervention/prevention of complications of DM (Mirra *et al*., 2018).

Resveratrol (RES; 3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin that has anti-inflammatory, anti-platelet aggregation, anti-carcinogenic, cartilage-protective, and anti-aging properties (Timmers *et al*., 2013; Zhu *et al*., 2017). Extensive research has demonstrated the role of RES in managing diabetes and its complications (Soufi *et al*., 2012; Turan *et al*., 2012; Singh *et al*., 2013; Vallianou *et al*., 2013) and researchers have demonstrated that RES can reduce blood glucose levels (Oyenihi *et al*., 2016; Brasnyó *et al*., 2011; Bhatt *et al*., 2012; Crandall *et al*., 2012; Szkudelski & Szkudelska, 2015). Previous research has demonstrated RES's efficacy in mitigating oxidative stress and apoptosis in various cell types (Liu *et al*., 2014; Kitada & Koya, 2013; Hoca *et al*., 2021; Do *et al*., 2012). Resveratrol has an antihyperglycemic effect that improves blood glucose parameters, inflammation, and insulin resistance (Imamura *et al*., 2017). Despite the potential benefits of RES in managing diabetes and its symptoms, including hyperglycemia, its effect on hyperglycemia-related miRNAs remains an under-researched area. Investigating how RES affects miRNA expression and regulates gene and protein expression may provide novel insights into disease onset and progression.

1.2 Problem statement / Rationale

The prevalence of diabetes has reached an alarming level worldwide. In 2021, it was estimated that 24 million people in Africa had diabetes, with 4.2 million originating from South Africa (Sun *et al*., 2022). The liver, a central regulator of glucose homeostasis, regulates critical glucose metabolic pathways, such as glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis (Han *et al*., 2016). However, insulin resistance in the liver leads to elevated blood glucose levels, disrupting glucose metabolism in individuals with diabetes (Demir *et al*., 2021). Diabetic liver damage is driven by a cascade of inflammatory response events and increasing oxidative stress, leading to upregulation of pro-apoptotic gene transcription and consequent hepatocyte impairment (Mohamed *et al*., 2016). The complications associated with diabetes significantly compromise patients' quality of life and life expectancy. Diabetes management relies on blood glucose reduction through lifestyle modifications and anti-diabetic medications. There is an urgent need to identify safe and effective drugs that provide anti-hyperglycemic benefits while protecting against complications of diabetes (Gupta *et al*., 2017). Furthermore, a biomarker is needed for the early identification of complications of DM and risk management to improve patients' quality of life. Circulating miRNAs have emerged as potential contributors to the etiology and progression of several diseases, including DM. Early identification of complications of DM and prediction of disease progression using biomarkers can offer profound insights into the intricate mechanisms underlying disease development. However, further research is needed to determine how miRNA expression and activity are disrupted in diabetes (McClelland & Kantharidis, 2014). Furthermore, the underlying mechanisms of diabetes development should be investigated to facilitate the design of therapeutic strategies. This knowledge will provide clinicians and scientists with advanced techniques for exploiting molecular targets as potential therapeutic interventions. The aberrant expression of miRNAs may be implicated in various pathways, such as glucose metabolism, inflammation, oxidative stress, and apoptosis. The therapeutic effects of natural products have been widely recognized for centuries. Hence, this study aimed to examine the influence of elevated glucose levels and RES (3,5,4-trihydroxy-trans-stilbene), a polyphenol phytoalexin compound, on key aspects of diabetes, including glucose metabolism, inflammation, oxidative stress, apoptosis, and miRNA regulation associated with diabetes.

1.3 Significance/Implications

Diabetes has placed a heavy burden on global healthcare systems due to its rising incidence and prevalence and is now an urgent public health threat. Treatment management and interventions required for diabetes are associated with high costs and adverse side effects. Given this scenario, researchers must urgently investigate cost-effective natural compounds

with anti-diabetic activity and fewer to no side effects. Several studies have demonstrated the beneficial effects of RES in managing diabetes (Soufi *et al*., 2012; Turan *et al*., 2012; Singh *et al*., 2013; Vallianou *et al*., 2013). In addition, RES possesses antioxidant, anti-inflammatory and antiapoptotic effects. Yet, it is still necessary to conduct additional research on the effects of RES on glucose metabolism, inflammation, oxidative stress, and apoptosis under hyperglycemic conditions. Additionally, comprehending the fundamental molecular pathways may aid in developing novel strategies to combat diabetes. Although RES may be beneficial for managing diabetes and its symptoms, including hyperglycemia, its effect on hyperglycemia-related miRNAs has yet to be thoroughly researched. The results of this study have the potential to establish a cornerstone for future research in ethnopharmacology, concentrating on the potential benefits of RES on miRNAs associated with diabetes.

1.4 Aims

1. To investigate the effect of RES on oxidative stress, inflammation, apoptosis, and glucose metabolism under high glucose-induced conditions.
2. To investigate the effect of high glucose levels and evaluate the influence of RES on high glucose-induced miRNA dysregulation.

1.5 Objectives

-To investigate the expression of inflammatory markers and glucose metabolism-related genes in HepG2 cells exposed to high glucose and RES for 48 and 72 hours.

-To assess high glucose-induced oxidative stress and apoptosis in HepG2 cells over 48 and 72 hours and evaluate the potential therapeutic effect of RES in this model.

-To identify upregulated or downregulated miRNAs in HepG2 cells using quantitative PCR (qPCR).

-To determine if RES could modulate the expression of miRNAs in HepG2 cells exposed to high glucose conditions.

1.6 Hypothesis

Exposing HepG2 cells to high glucose conditions with RES may alter their biochemical and miRNA profiles, thereby ameliorating adverse effects induced by high glucose conditions.

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CHAPTER 2: LITERATURE REVIEW

2.1 Diabetes mellitus (DM)

The classification of DM is based on its etiology and clinical presentation (Piero, 2015). Patients with diabetes have either type 1 or type 2 DM, and some have other types of diabetes, such as monogenic diabetes, Maturity Onset Diabetes of the Young (MODY), Neonatal diabetes mellitus (NDM), and gestational diabetes mellitus (GDM).

2.1.1 Type 1 diabetes mellitus (T1DM)

T1DM, also known as insulin-dependent DM (IDDM) or juvenile diabetes, is a form of diabetes caused by the autoimmune destruction of pancreatic beta cells, resulting in insulin deficiency (Mishra & Ndisang, 2014; American Diabetes Association, 2017). Anti-glutamic acid decarboxylase, islet cells, or insulin antibodies are thought to play a role in these autoimmune processes, causing beta-cell destruction and inhibiting insulin development and secretion. As a result, all T1DM patients must rely solely on insulin therapy to maintain normoglycemic conditions (Baynest, 2015). The combinations of genetic susceptibility and environmental factors, such as viral infection, toxins, or dietary factors, are triggers for autoimmunity (Goyal & Jialal, 2023). Type 1 diabetes accounts for 10-15% of all diabetes cases worldwide (Paschou *et al*., 2018), and is known to affect all age groups, but is commonly diagnosed in children and adolescents (American Diabetes Association, 2015). Furthermore, T1DM can be managed or treated using exogenous insulin (Grundlingh *et al*., 2022).

2.1.2 Type 2 diabetes mellitus (T2DM)

T2DM is a metabolic disorder caused by insulin secretion and/or action defects (Hurtado & Vella, 2019). T2DM is one of the most common chronic diseases that has become a growing global burden on modern society (Arora *et al*., 2021; Rosenberg *et al*., 2019; Shaw *et al*., 2010). T2DM is characterized by hyperglycemia resulting from inadequate insulin production and the body's reduced responsiveness to insulin, a condition called insulin resistance. During insulin resistance, the efficiency of insulin is compromised, leading to an initial compensatory response of increased insulin production to counteract elevated glucose levels. However, over time, this can progress to a state of relative inadequate insulin production (International Diabetes Federation, 2017) The first diabetes case was reported approximately three thousand years ago in Egypt (Leylabadlo *et al*., 2020). Epidemiological data in the year 2017 shows that approximately 425 million people (aged 20 to 79) have diabetes, with this number expected to rise to 629 million in the future (Wong & Sabanayagam, 2019). According to the IDF, it is estimated that the worldwide prevalence of T2DM among adults was 536.6% million (10.5%) in 2021, and this is projected to increase to 783.2 million (12.2%) by 2045 (Sun *et al*., 2022). The disorder is mainly caused by changes in the gene sequence (genetic factor) or an imbalance in lifestyle (environmental factor) (Azevedo & Alla, 2008), such as smoking and

alcohol consumption, unhealthy diet or a lack of physical activity (Liu *et al*., 2020). Obesity is a major contributor, accounting for approximately 55% of diabetes cases (Hu *et al*., 2001), particularly in teenagers and children, as obesity among the young is increasing alarmingly. Type 2 diabetes can be inherited, especially if first-degree relatives have the disease (Arora *et al*., 2021).

2.2 Global burden of diabetes

Diabetes represents a significant global public health concern, substantially burdening public health and socioeconomic development (Lin *et al*., 2020). Though some countries have observed a decline in the incidence rates, the prevalence of diabetes has notably increased in the past few decades across developed and developing countries (Patterson *et al*., 2019); (Wang *et al*., 2017; Dwyer-Lindgren *et al*., 2016). In 2019, diabetes ranked as the eighth most prevalent cause of mortality and morbidity globally (Vos *et al*., 2020). The escalating prevalence of diabetes worldwide is predictably accompanied by a corresponding rise in healthcare expenditure dedicated to treating or managing the disease (Sun *et al*., 2022). According to previous research, it was estimated that approximately 463 million individuals had diabetes worldwide in 2019 (Aschner *et al*., 2021; Teo *et al*., 2021; Williams *et al*., 2020). Furthermore, the IDF reported that the global prevalence of diabetes was estimated to be approximately 536.6 million in 2021. As mentioned, the figure is projected to increase to 643 million by 2023 and to 783.2 million by 2045 (Ogurtsova *et al*., 2022; Sun *et al*., 2022). Globally, it has been observed that approximately 50% of diabetes cases remain undiagnosed. This prevalence is particularly prominent in low-income and middle-income countries (Pheiffer *et al*., 2018).

2.3 Diabetes in South Africa

According to IDF, in 2021, 42 million individuals had diabetes in Africa, and this figure is projected to rise to 55 million by 2025. Furthermore, it was estimated that 54% of individuals with diabetes are undiagnosed. The IDF reported that diabetes was responsible for 416,000 deaths in Africa (International Diabetes Federation, 2021). The prevalence of diabetes in South Africa has demonstrated a significant surge, nearly tripling from 4.5% in 2010 to 12.7% in 2019. In 2019, the IDF approximated that out of the 4.48 million individuals with diabetes aged between 20 and 79 years, approximately 52.4% remained undiagnosed (International Diabetes Federation, 2020). The statistical data demonstrated that diabetes emerged as the second greatest underlying factor contributing to mortality in South Africa between 2016 and 2017 (Stats SA, 2017). According to IDF, South Africa exhibited the highest prevalence of diabetes, with a rate of 12.7% among all the African countries in 2019 (International Diabetes Federation, 2019). It was also reported that South Africa had the highest number of deaths associated with diabetes, with 89,900 fatalities (International Diabetes Federation, 2019). The health complications resulting from diabetes have significant implications for individuals and their

families, as they can hinder employment opportunities and limit financial resources. Additionally, the decrease in income adversely affects the national economy**.**

2.4 Complications of diabetes

Uncontrolled diabetes can lead to metabolic abnormalities, which can have serious consequences that necessitate immediate medical attention (Ahmed *et al*., 2020). Diabetes complications are classified as acute or chronic (Kaura Parbhakar *et al*., 2020). Diabetes ketoacidosis (DKA), hyperglycaemic hyperosmolar state (HHS), lactic acidosis (LA), and hypoglycemia are all examples of acute complications. These are severe complications of diabetes that can be fatal (Rewers, 2021). Patients with T1DM are more likely to develop DKA and severe hypoglycemia, whereas patients with T2DM are more likely to develop HHS without ketoacidosis (Negera *et al*., 2020; Rewers, 2021). Chronic complications are classified as macrovascular or microvascular. Retinopathy, nephropathy, and neuropathy are microvascular complications, while heart, peripheral vascular, and cerebrovascular diseases are examples of macrovascular complications (Table 2.1).

Table 2.1: The major microvascular and macrovascular complications linked with DM (Ahmed *et al*., 2020).

Microvascular	Macrovascular
Eye: Hyperglycemia and hypertension can cause damage to blood vessels, resulting in retinopathy, cataracts, and glaucoma.	**Brain:** Increased risk of stroke and cerebrovascular disease, including transient ischemic attacks and cognitive impairment.
Kidney: High blood pressure destroys small blood vessels, and high blood glucose overloads the kidneys, causing nephropathy.	**Heart:** Hypertension and insulin resistance both increase the risk of coronary heart disease.
Neuropathy: Hyperglycemia affects peripheral nerves. This can cause discomfort or numbness. Infected foot wounds may result in gangrene.	**Extremities:** Peripheral vascular disease is caused by blood vessel narrowing, which increases the risk of reduced or absent blood flow in the legs. Foot wounds are likely to take a long time to heal, which could lead to gangrene.

2.5 Management of diabetes

It was previously believed that once patients develop diabetes, they will remain diabetic for life; however, DM can go into remission (Panunzi *et al*., 2016). The primary goals of diabetes treatment are to maintain normal blood sugar levels and to avoid diabetic complications. DM can be managed by altering one's diet, engaging in physical activity, maintaining healthy body weight, monitoring lipid profiles, and using appropriate medications when necessary. Diet modification is an effective method for managing diabetes (Alam *et al*., 2021). Blood sugar control can be achieved through the consumption of low glycaemic foods, as well as a high content of protein and polyunsaturated fatty acids. Moderate physical activity has been found to contribute to reducing blood glucose levels by facilitating insulin-independent glucose transport into muscle. Gestational diabetes mellitus is a significant risk factor for postpartum T2DM development. Breastfeeding for three months reduces the risk of postpartum T2DM by 40% and improves early postpartum glucose tolerance (PGT) (Ziegler *et al*., 2012). Patients with T1DM and 25–30% of those with T2DM require insulin (Martin *et al*., 1992; Tisch & McDevitt, 1996). Inadequate insulin dosing can occasionally result in hypoglycemia, a more dangerous condition than hyperglycemia. To combat this phenomenon, patients with diabetes who are prescribed insulin therapy are frequently advised to keep some sugar or chocolate on hand (Alam *et al*., 2021). Certain medications are frequently prescribed to treat T2DM. The most frequently used medications include a class of biguanides, thiazolidinediones, α-glucosidase inhibitors, and a glucagon-like peptide-1 agonist. Proton pump inhibitors are believed to have some effects on diabetes management (Takebayashi, 2015). The various classes of drugs exhibit a distinct mechanism of action, yet collectively they play a role in regulating blood glucose levels within the physiological range. Nonetheless, several of these medications are associated with metabolic side effects. For example, thiazolidinediones (TZDs) have a well-defined set of adverse effects associated with their action in adipocytes as a peroxisome proliferator-activated receptor gamma (PPAR-gamma) agonist. As a result, proper use of these prescription medications will benefit diabetes control (Lai *et al*., 2012). Hence, it is imperative to research natural therapeutic agents that do not exhibit any detrimental side effects.

2.6 Diagnosis of diabetes

Since untreated diabetes can result in significant complications, early screening for diabetes or pre-diabetes enables earlier intervention and may help prevent serious complications. The primary symptoms of diabetes are high blood glucose levels over an extended period, frequent urination, increased thirst, and increased hunger, among other things (Alam *et al*., 2021) In the current guidelines, four diabetes diagnostic tests are recommended: measurement of fasting plasma glucose; 2-hour post-load plasma glucose after performing a 75 g oral glucose tolerance test (OGTT); glycated haemoglobin A1c (HbA1c); and a random blood glucose test in the presence of diabetes-related signs and symptoms (World Health Organization, 2019).

Additionally, numerous questionnaires have been created to screen for the risk of undiagnosed diabetes, including the Finnish Cardiovascular Risk Study (FINRISK), the Australian T2DM risk assessment tool (AUSDRISK), and The Indian Diabetes Risk Score (IDRS) (Chen *et al*., 2010; Lindström & Tuomilehto, 2003; Mohan *et al*., 2007). Individuals with fasting plasma glucose levels of ≥ 7.0 mmol/L (126 mg/dL), 2-hour post-load plasma glucose levels of ≥ 11.1 mmol/L (200 mg/dL), HbA1c levels of ≥ 6.5 % (48 mmol/mol), or random blood glucose levels of ≥ 11.1 mmol/L (200 mg/dL) in the presence of signs and symptoms are classified as having diabetes (World Health Organization, 2011; International Diabetes Federation, 2017) (Figure 2.1). If elevated values are found in asymptomatic people, it is recommended that they be tested again as soon as possible on a subsequent day, preferably with the same test to confirm the diagnosis (World Health Organization, 2011). Despite their advantages, a constraint inherent in existing methodologies is their dependence on a singular measurement, which may not adequately capture an individual's comprehensive glucose regulation. Moreover, it should be noted that these techniques may lack the necessary sensitivity to accurately identify the initial phase of diabetes or pre-diabetes. Hence it is imperative to explore novel methodologies that can offer enhanced precision and comprehensive assessment, thereby facilitating improved disease management. A diabetes diagnosis has significant ramifications for individuals, not just their health but also their work, health, life insurance, driving status, social prospects, and other cultural, ethical, and human rights issues (World Health Organization, 2019). Recent advancements in diabetes research have demonstrated the role of miRNAs circulating in the blood as crucial in its onset and progression. Hence, they possess immense therapeutic and diagnostic potential.

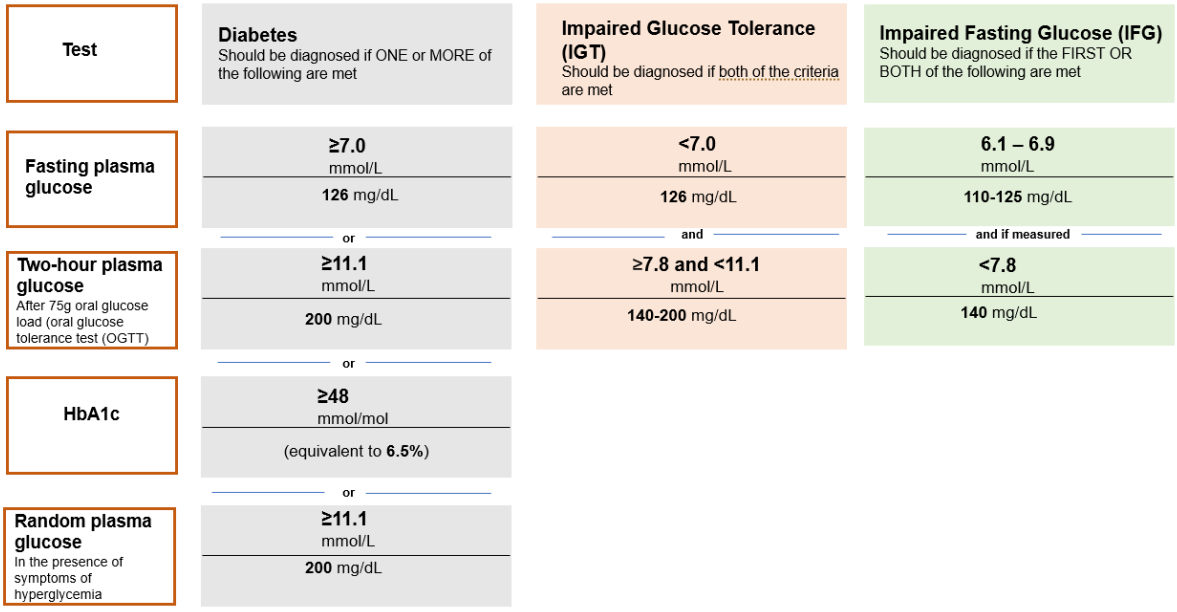

Figure 2.1: Diagnostic criteria for diabetes (International Diabetes Federation, 2017; International Diabetes Federation, 2021).

2.7 MicroRNAs (miRNAs)

2.7.1 History

Lin-4 was the first miRNA to be discovered in 1993 by collaboration between the laboratories of Ambros and Ruvkun (Lee *et al*., 1993; Wightman *et al*., 1993). Years before, lin-4 was characterized by Horvitz's lab as one of the genes that regulate the temporal development of *Caenorhabditis Elegans* (*C. Elegans*) larvae (Horvitz & Sulston, 1980; Chalfie, 1981). Seven years later, after identifying the first miRNA (in *C. Elegans*), the first miRNA was identified in humans (let-7) (Reinhart *et al*., 2000). Since then, miRNAs have been discovered in vertebrates, plants, and some viruses; some are highly conserved across species (Davis & Hata, 2010; Li *et al*., 2010; Friedländer *et al*., 2014). It is estimated that thousands of miRNAs have been identified in humans and other animals, and these can be found in online sequence repositories for miRNAs (Griffiths-Jones *et al*., 2007; Griffiths-Jones *et al*., 2006). More than 2,500 miRNAs are cited in the global miRNA database, miRbase (http://www.mirbase.org/). New miRNAs are often discovered, and their importance in gene regulation is well understood (de Rie *et al*., 2017).

2.7.2 Overview

MiRNAs are a group of small non-coding ribonucleic acid (RNA) molecules with a length ranging from 19 to 24 nucleotides (Krol *et al*., 2010); (Chandrasekaran *et al*., 2012). They are fundamental for post-transcriptional gene expression regulation and regulate multiple biological functions, including proliferation, cellular metabolism, differentiation, and apoptosis (Satake *et al*., 2018). MiRNAs suppress targeted gene expression by binding to messenger RNA (mRNA) via their 3′-untranslated regions (3′-UTR), whereas other miRNAs promote the expression of targeted genes (O'Brien *et al*., 2018). Targeted gene expression is suppressed by mRNA degradation, induction of "decapping" (the process of eliminating the m7GpppN mRNA cap), triggered adenylation, mRNA sequestration, and altered binding of cap proteins (Mohr & Mott, 2015; Charenton & Graille, 2018). These effects may change the expression of insulin or insulin receptors in target tissues, leading to T2DM development and diabetic complications (Hall *et al*., 2014).

MiRNAs are abundant in tissues and are present in trace amounts in biological fluids, implying that miRNA may be a helpful biomarker (Necula *et al*., 2019). Therefore, these molecules may be used in disease screening instead of specific protein biomarkers with low specificity and sensitivity. Nearly 4000 human miRNAs have been identified, and new ones are continuously being discovered (Kilic *et al*., 2018; Satake *et al*., 2018). These miRNAs have been shown to regulate most protein-coding genes, thereby regulating various developmental and cellular processes in the eukaryotic organism (Pordzik *et al*., 2019). Additionally, miRNAs have been

shown to regulate hundreds of mRNAs. Approximately 200 different mRNAs can be regulated by a single miRNA, and each act on a different signalling pathway; similarly, a single mRNA can be controlled by multiple miRNAs (Oliveto *et al*., 2017). Thus, it is unsurprising that miRNAs are involved in nearly every biological process and that their dysregulation can result in various diseases, including diabetes and its associated complications (Soifer *et al*., 2007).

2.7.3 Biogenesis

The biogenesis, maturation, function, and secretion of miRNAs is a highly complex molecular mechanism still being investigated (Sebastiani *et al*., 2017). The biogenesis of miRNAs begins with the transcription of the miRNA gene in the nucleus, generating long primary miRNA transcripts (pri-miRNA) with the help of RNA polymerase II or RNA polymerase III (Figure 2) (Ha & Kim, 2014; Mansoori *et al*., 2019). The pri-miRNA is approximately 1000 bases long with a cap structure and poly (A) tails, distinctive characteristics of class II gene transcripts (Lee *et al*., 2003). The pri-miRNA then folds into a 60–70 nucleotide long hairpin structure and is cleaved by the microprocessor complex, called *Drosha* (ribonuclease III enzyme) and DiGeorge Syndrome Critical Region 8 *(DGCR8)* to produce the precursor-miRNA (pre-miRNA) (Church *et al*., 2017; Astamal *et al*., 2020). The pre-miRNA has an imperfect stem-loop structure (Lee *et al*., 2003). The maturation step is performed outside of the nucleus; therefore, this short 60–70 bp long pre-miRNA is exported out of the nucleus to the cytoplasm by exportin-5 in the presence of a Ras-related nuclear protein-guanosine-5' triphosphatase *(Ran-GTPase)* (Wu *et al*., 2018). *Exportin-5* is a Karyopherin family member involved in the nuclear transport of structured RNA molecules, such as transfer RNA (tRNA), pre-tRNAs with introns, and pre-miRNAs (Köhler & Hurt, 2007). In the cytoplasm, an RNase III enzyme called Dicer cleaves the loop of pre-miRNA, resulting in an asymmetrical miRNA duplex of 20–25 nucleotides (Dastmalchi *et al*., 2020). After cleavage by Dicer, miRNA duplexes unwind and produce the mature miRNA guiding strand and the miRNA passenger strand. The mature guiding strand is then incorporated with an RNA-induced Silencing Complex (RISC), which is driven by the *Argonaute 2 (AGO2)* protein (Gregory *et al*., 2005; Fan *et al*., 2019). The miRISC complex can bind to the 3’ untranslated region (UTR) to target a specific messenger RNA (mRNA) for inhibition of translation or degradation while the passenger strand is degraded (Jonas & Izaurralde, 2015). However, it has been shown that miRNAs can bind to the 5’ end of the coding sequence and repress the mRNA by inhibiting translation (Lytle *et al*., 2007). As a result, increased miRNA levels result in decreased expression of their target gene(s) and potentially reduced protein levels. Moreover, decreased miRNA levels also result in increased levels of their target gene(s)/protein(s) (Friedman *et al*., 2009).

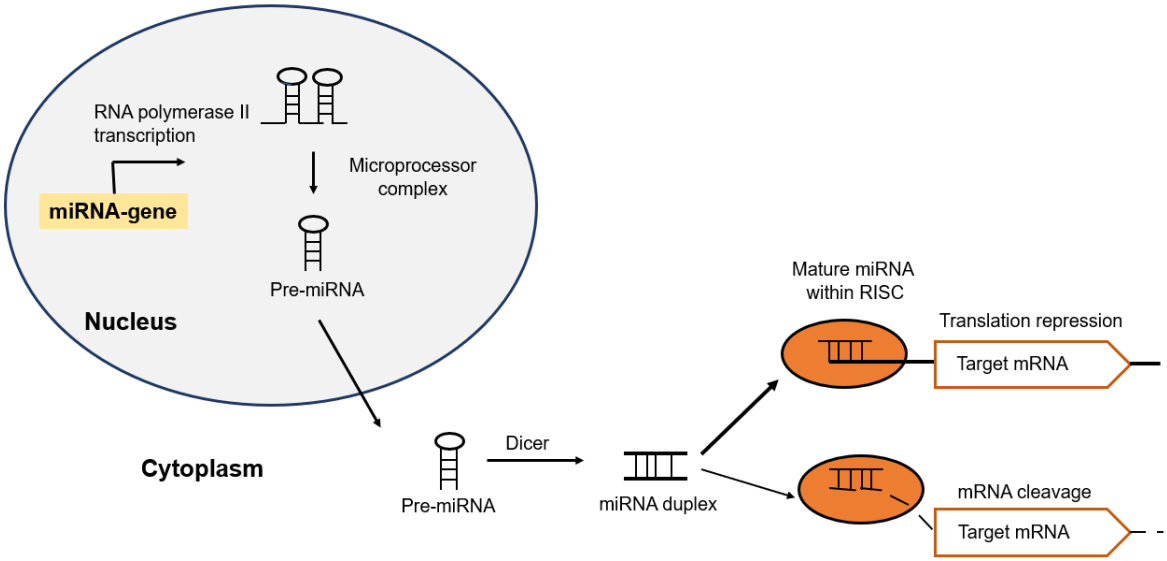

Figure 2.2: Biogenesis of miRNAs. It begins with the transcription of the miRNA gene into pri-miRNAs in the nucleus by Pol II. Microprocessor complexes cleave pri-miRNAs to form pre-miRNAs. Thereafter, exportin-5 transports pre-miRNA into the cytoplasm, cleaving it by dicer to create an asymmetrical miRNA duplex. The passenger strand is released and degraded, while the mature strand is incorporated into RISC, inhibiting translation and increasing mRNA degradation. (*Image created by author*)

2.7.4 MicroRNAs and Diabetes

2.7.4.1 Role of circulating microRNAs

MiRNAs are a new class of signalling molecules that mediate intercellular communication and are released into the bloodstream or expressed in blood cells. Furthermore, miRNA profiles may change under different pathophysiological conditions, affecting the pathogenesis and progression of diseases, such as diabetes (Zhang *et al*., 2018). Circulating miRNAs may be isolated from various cell-free matrices, including blood (serum and plasma), saliva, urine, cerebrospinal fluid (CSF), faeces, follicular fluid, synovial fluid, pancreatic juice, bile, and gastric juice (Grillari *et al*., 2021; Godoy *et al*., 2018). The discovery of these miRNAs has expanded their use as disease biomarkers and created the possibility of using them for therapeutic purposes in the future (Condrat *et al*., 2020; Song *et al*., 2018). It has been shown that extracellular miRNAs are highly stable even under adverse conditions, surviving RNase digestion and adverse conditions, such as boiling, several freeze-thaw cycles, and high (pH=13) or low (pH=1) pH; as a result, they are ideal materials for monitoring disease progression (Chen *et al*., 2008; Kroh *et al*., 2010; Chien *et al*., 2015). Studies suggested that once released, miRNAs remain in circulation in a stable condition and reflect the underlying pathological/physiologic processes (Gilad *et al*., 2008; Ai *et al*., 2010; Mendell, 2005). Serum and plasma can be stored at -20 ºC or -80 ºC for several months without any notable degradation of miRNAs (Mraz *et al*., 2009). This also suggests that these non-coding RNAs can be used as biomarkers. This remarkable stability of circulating miRNAs may be due to protective mechanisms against ubiquitous extracellular RNases. First, miRNAs are enveloped

in a lipid membrane coating within extracellular vesicles (EVs), such as exosomes, microvesicles, or apoptotic bodies (Gallo *et al*., 2012; Iftikhar & Carney, 2016). Second, miRNAs may be complexed with proteins and protein particles, particularly AGO-2 (Gallo *et al*., 2012), high-density lipoprotein (HDL) (Vickers *et al*., 2011; Tabet *et al*., 2014), or nucleophosmin 1 (NPM1) (Arroyo *et al*., 2011; Turchinovich *et al*., 2012), which aid in preventing degradation.

In vitro studies have shown that miRNAs transported by exosomes or HDL can be transferred to recipient cells in an active form, allowing them to regulate the translation of target genes, implying that circulating miRNAs can act as extracellular communicators (Vickers *et al*., 2011; Valadi *et al*., 2007; Creemers *et al*., 2012). Circulating miRNAs may be used as biomarkers because of their stability, and this possibility is now being investigated in various diseases, including diabetes (Chien *et al*., 2015; Guay & Regazzi, 2013). These circulating miRNAs possess several characteristics which make them useful as biomarkers: 1) they can be detected in blood and different biological fluids, such as urine, saliva, amniotic fluids, and breast milk; 2) they are detectable using quantitative real-time PCR; and 3) they are highly conserved (Weber *et al*., 2010). Due to these properties, circulating miRNAs have become popular over recent years.

2.7.4.2 Role of microRNAs in diabetes pathogenesis

The dysregulation of miRNAs has been linked with several disorders, including DM (Dumortier *et al*., 2013; Guay & Regazzi, 2013). MiRNAs are broadly distributed throughout the human body, except for miR-375, which is highly enriched in pancreatic islets and controls the expression of genes involved in hormone secretion and beta-cell expansion in response to insulin resistance (Poy *et al*., 2009). MiRNA profiles are altered in beta cells and tissues affected by insulin in T1DM and T2DM patients, which results in impaired function under disease states (Kumar *et al*., 2012; Shantikumar *et al*., 2012). A study of non-obese diabetic (NOD) mice, a model of T1DM, demonstrated elevated levels of several miRNAs, such as miR-21, miR-34a, miR-29, and miR-146a, which affect beta-cell function (Roggli *et al*., 2010; Roggli *et al*., 2012). In a mouse model, the expression of miR-29 and miR-34a was elevated in tissues targeted by insulin, which contributes to insulin resistance (Herrera *et al*., 2010; Trajkovski *et al*., 2011). The dysregulation of miR-143 and miR-802 has been identified in insulin-targeted tissues in ob/ob mice, obese, and diabetic Goto-Kakizaki rat models. These miRNAs have also been linked to the development of insulin resistance (Trajkovski *et al*., 2011; Jordan *et al*., 2011; Herrera *et al*., 2010;Kornfeld *et al*., 2013). Changes in miRNA profiles have also been linked to the development of diabetes. In human skeletal muscle biopsy samples of patients with T2DM, over 600 differentially expressed miRNAs have been detected, in which two miRNAs, miR-206 and miR-1388a, were downregulated and miR-143 was upregulated.

Around 15% of miRNAs have been altered in individuals with impaired glucose tolerance, indicating that miRNAs are active in the early stages of disease (Gallagher *et al*., 2010).

2.7.4.3 MicroRNAs as diabetes Biomarkers

Biomarkers are molecules or conditions that can be used to predict, diagnose, and monitor the pathological state of a disease (Vaishya *et al*., 2018). Many types of biomarkers, such as proteins and mRNAs, are used to diagnose various conditions, including T2DM, cancer, and neurological disorders (Graves & Haystead, 2002). Researchers are still exploring the potential of miRNAs as biomarkers and have high hopes for discoveries that could use miRNAs in theranostic applications (Chaudhary *et al*., 2018; Chong *et al*., 2020). Cancer research was the first to introduce and demonstrate the use of miRNAs as disease biomarkers. Several new studies have been published following the initial research linking blood circulating miRNAs and cancer status. Some of these studies attempt to discover a signature associated with diabetes (Guay & Regazzi, 2013; Chen *et al*., 2008; Mitchell *et al*., 2008). Previous research has linked abnormally expressed miRNA signatures to various serious diseases, including cancer and cardiovascular and cerebrovascular diseases (Wang *et al*., 2017; Khanmi *et al*., 2015). Several studies have suggested that certain circulating miRNAs may be linked to diabetic conditions (Vasu *et al*., 2019). Circulating miRNAs were indicated as possible biomarkers for diabetes when it was observed that miRNA profiles were dysregulated in various biological fluids of patients with diabetes compared with healthy controls. There has been an observation in differential miRNA profiles between T2DM patients and healthy controls in whole blood (Karolina *et al*., 2011; Zhou *et al*., 2013), serum (Kong *et al*., 2011; Liu *et al*., 2014; Yang *et al*., 2014), and plasma (Zhang *et al*., 2013). Previous research has shown that miRNAs can be beneficial biomarkers for DM since they are often tissue-specific and remain stable in circulation for a long time (Romaine *et al*., 2015). It is now clear that altered miRNA expression plays a role in the development and progression of DM.

2.7.4.4 Glucose Metabolism and microRNAs

Maintaining normal blood glucose levels requires a delicate balance with normal insulin secretion and action. The dysfunction of glucose metabolism significantly impacts other cells, tissues, and processes, all of which contribute to the development of diabetes. MiRNAs have become known as novel regulators of these phenomena and are thus appropriately referred to as "ribo-regulators of glucose homeostasis" (Gauthier & Wollheim, 2006; Pandey *et al*., 2009). Numerous let-7 target genes are found to be involved in the regulation of glucose metabolism in various tissues (Frost & Olson, 2011). Zhu *et al.* discovered that overexpression of *Lin-28a/b* inhibited the activity of let-7 and increased the movement of the insulin-phosphatidylinositol 3-kinase (PI3K)-mechanistic target of rapamycin (mTOR) pathway and insulin sensitivity, implying that it can promote glycaemic stability and DM resistance (Zhu *et al*., 2011). PI3K/AKT signalling can increase glucose uptake by promoting glucose transporter

4 (Glut4) translocation, while mTOR signalling can increase glucose uptake by altering Glut4 expression and translocation (Brugarolas *et al*., 2003; Buller *et al*., 2008). Further, Zhu *et al*. showed that let-7 and lin-28a/b co-ordinately regulated the insulin-PI3K-mTOR pathway, influencing glucose metabolism (Zhu *et al*., 2011). It was also reported that miR-223 can regulate glucose uptake in muscle tissue by inhibiting glucose transporter 4 (GLUT4). Additionally, miR-33a and miR-33b have been shown to regulate the insulin pathway through the insulin receptor substrate 2 (IRS2), sirtuin 6 (SIRT6), and AMP-activated protein kinase 1 (AMPK1) pathways (Lu *et al*., 2010; Dávalos *et al*., 2011). Glucose tolerance was shown to be improved by miR-130a and miR-204 through the inhibition of Growth Factor Receptor Bound Protein 10 (GRB10) and glucagon-like peptide-1 receptor (GLP1R), respectively (Xiao *et al*., 2014; Jo *et al*., 2018). Furthermore, the liver plays a fundamental role in maintaining glucose homeostasis through the regulation of glucose metabolism. One key player in this process is the enzyme glucokinase (GCK), which holds a prominent position, and its involvement in the glycolytic process underscores its significance in regulating hepatic glucose production (Irwin & Tan, 2014). Previous research has discovered that in the Goto-Kakizaki rat islets, GCK protein levels were decreased in correlation with the increased expression of miR-130a, miR-130b, and miR-152 (Ofori *et al*., 2017). The discovery of miRNAs as regulators of GCK levels may represent a different strategy and hold more therapeutic promise (Mirra *et al*., 2018). Additionally, the regulatory function of miR-182-5p in maintaining glucose homeostasis has been suggested by targeting Forkhead box O1 (FOXO1), a gene involved in gluconeogenesis (Kaur *et al*., 2020). Nonetheless, it is undeniable that abnormal expression and function of miRNAs disrupt glucose homeostasis, resulting in pathogenic conditions (Feng *et al*., 2016; Hashimoto & Tanaka, 2017). Therefore, understanding the role of miRNAs in glucose metabolism may provide valuable insight for developing therapeutic strategies to manage diabetes.

2.7.4.5 Inflammation and miRNAs linked to diabetes.

MiRNA disruption can cause chronic inflammation in patients with diabetes, pancreatic cell dysfunction, and insulin resistance in metabolic tissues (McClelland & Kantharidis, 2014). Chronic inflammation in insulin-responsive tissues causes insulin resistance by increasing reactive oxygen species (ROS) levels, which activate stress-related signalling pathways, in turn activating protein kinases like c-Jun N-terminal kinases (JNKs), Protein kinase C (PKC), glycogen synthase kinase-3 (GSK-3), and Nuclear factor kappa B (NF-κB) (Henriksen *et al*., 2011; Zeinali *et al*., 2020). MiR-122 is predominantly expressed in the liver and has been shown to play a role in the accumulation of lipids. Upregulation of miRNA-122 expression aided in the resolution of inflammation in a human liver organoid model through the suppression of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) levels (Sendi *et al*., 2018). MiR-126, which targets the inflammatory protein Sprouty-related EVH1 domain containing 1

(SPRED1), may be a critical factor in ameliorating inflammation. Increased SPRED1 expression promotes the production of IL-6, TNF- α, and ROS in people with diabetes due to miR-126 suppression, resulting in endothelial cell dysfunction (Li *et al*., 2016). Therefore, further studies are needed to investigate the link between miRNAs and inflammation, which will therefore aid in developing therapeutic targets.

2.8 Resveratrol (RES)

Resveratrol (RES; 3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin that has anti-inflammatory, anti-platelet aggregation, anti-carcinogenic, cartilage-protective, and anti-aging properties (Timmers *et al*., 2013; Zhu *et al*., 2017). In 1939, Michio Takaoka was the first to isolate RES from *Veratrum grandiflorum* (Takaoka, 1939). RES is present in approximately 72 species; plants synthesize it as a protective mechanism against harmful environmental factors, including ultraviolet radiation and attacks from pathogens, such as fungi, bacteria, and viruses (Gerszon *et al*., 2014). RES is found in high concentrations in the skins of red grapes. It was reported that berries, nuts, apples, and dark chocolate contain RES at varying concentrations (Figure 3) (Catalgol *et al*., 2012; Shaito *et al*., 2020). It is also found in low concentrations in grape-derived products like red wine and grape juices, as shown in Table 2 (Weiskirchen & Weiskirchen, 2016).

Figure 2.3: Sources of Resveratrol (https://www.istockphoto.com/photos/resveratrol).

Table 2.2: The most common resveratrol sources and their estimated concentrations (Mukherjee *et al*., 2010; Oyenihi *et al*., 2016).

Sources of resveratrol	Resveratrol concentration
Grapes	0.16–3.54 µg/g
Dry grape skin	~24.06 µg/g
100% Natural peanut butter	~0.65 µg/g
Bilberries	~16 ng/g
Blueberries	~32 ng/g
Boiled peanuts	~5.1 µg/g
Cranberry raw juice	~0.2 mg/L
Peanut butter	0.3–1.4 µg/g
Peanuts	0.02–1.92 µg/g
Pistachios	0.09–1.67 µg/g
Ports and sherries	<0.1 mg/L
grape juice	~0.50 mg/L
Red wines	0.1–14.3 mg/L
Roasted peanuts	~0.055 µg/g
White grape juice	~0.05 mg/L
White wines	<0.1–2.1 mg/L
Cocoa powder	~1.85 μg/g
Dark chocolates	~0.35 μg/g

2.8.1 Structure and Composition

RES has the same molecular properties as oestrogen diethylstilbesterol, with a formula of $C_{14}H_{12}O_3$ and a molecular weight of 228.25 g/mol (Chan *et al*., 2019). RES is a polyphenolic compound with three hydroxyl groups and two aromatic rings bound by a methylene bridge in its structure (Figure 4A). This structure enables RES to donate electrons to various free radicals (FR), attenuating biomolecule damage (García-Martínez *et al*., 2021). It has been reported that RES exists in both *cis*- and *trans*-stereoisomeric forms (Figure 7B and C). Although *cis*-RES is less prevalent and extremely unstable, the *trans*-form is more biologically active and provides the most potent therapeutic benefits (Cottart *et al*., 2010); Catalgol *et al*., 2012; Wu & Liu, 2013). A styrene double bond connects two phenol rings to form 3,4',5-trihydroxystilbene. Under ultraviolet (UV) light, the trans-isomer can be converted to the *cis*-form. *Trans*-RES is commercially available and is relatively stable when protected from high pH and light (Soleas *et al*., 1997). The trans-isomer displays absorbance at 307 nm, while the cis-isomer displays absorbance at 288 nm, allowing for isolation and detection of the two isomers using High-Performance Liquid Chromatography (HPLC) and a C18 reverse-phase column.

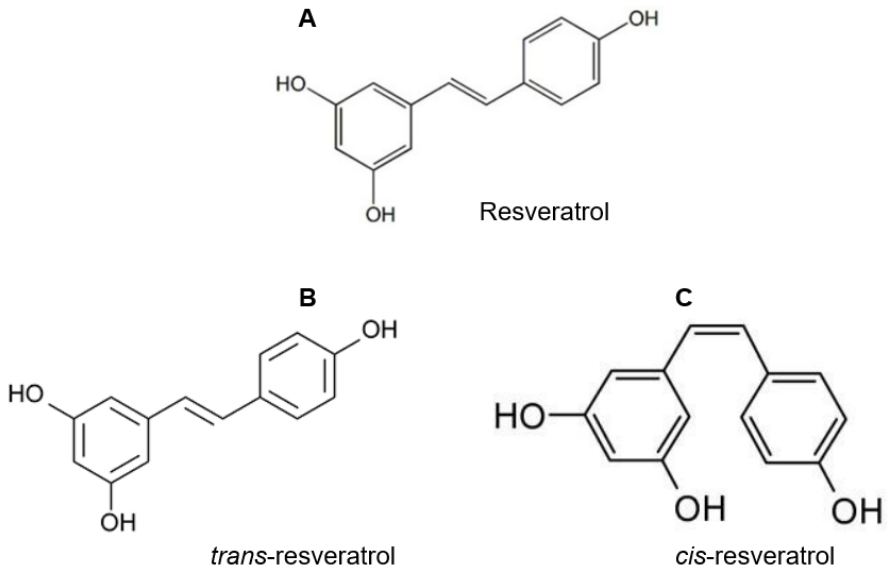

Figure 2.4: The chemical structure of (A) resveratrol, (B) *trans*-resveratrol, (C) *cis*-resveratrol.

2.8.2 Resveratrol biological activities

2.8.2.1 Anti-diabetic effect of resveratrol

Several researchers have demonstrated the protective role of RES in DM in several studies. For example, RES reportedly lowers glucose levels in human and rodent obesity models (Oyenihi *et al*., 2016). Resveratrol beneficially impacts both the action of insulin and pancreatic β cells and the prevention of disease complications (Szkudelski & Szkudelska, 2015). Furthermore, anti-diabetic studies have demonstrated that RES can lower fasting hyperglycemia and hemoglobin A1c in patients with diabetes or age-related glucose impairment, as well as prevent and improve systemic insulin resistance and lowering glucose levels (Brasnyó *et al*., 2011; Timmers *et al*., 2011; Bhatt *et al*., 2012; Crandall *et al*., 2012; Szkudelski & Szkudelska, 2015). Although studies have demonstrated that RES has beneficial effects on DM, there has been a conflict of results. Possible causes include RES dosage and absorption (Su *et al*., 2022). Further research is essential to fully understand the effect of RES on DM and its potential as a therapeutic option.

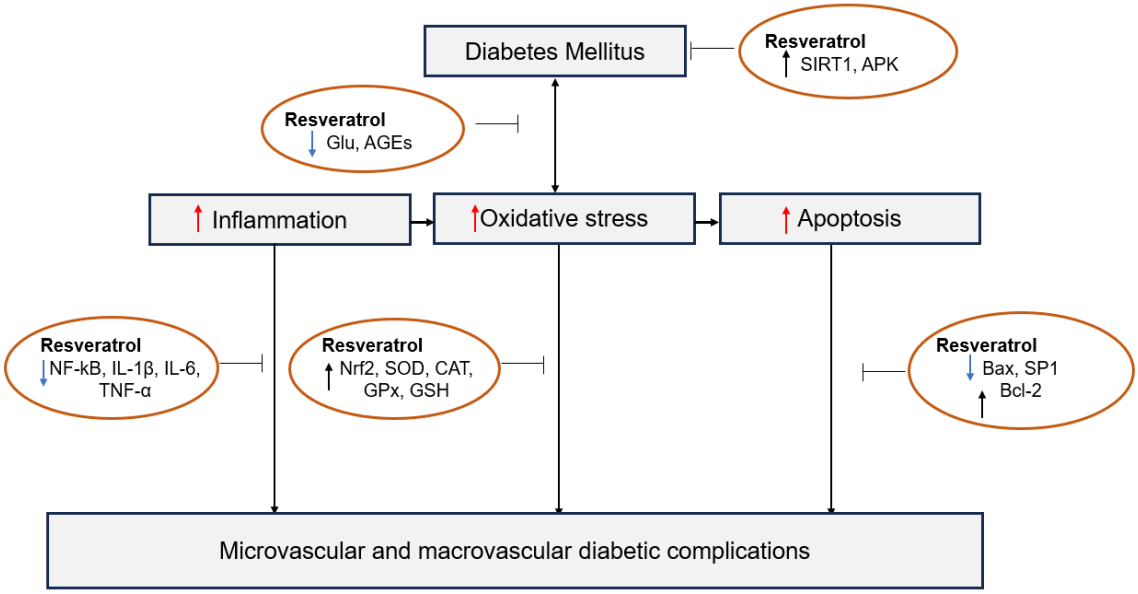

Figure 2.5: A schematic diagram illustrating how resveratrol protects against diabetic complications. Glu, glucose; AGEs, advanced glucose end-products; SIRT1, Sirtuin 1; AMPK, adenosine monophosphate-activated kinase; Nrf2, nuclear factor erythroid 2-related factor 2; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GPx, glutathione peroxidase; NF-κB, nuclear factor-kappa B; IL, interleukin; TNF-α, tumor necrosis factor; SP1, specificity protein 1; Bax, bcl-2-like protein 4; Bcl-2, B-cell lymphoma 2 (*Image created by author).*

2.8.2.2 Anti-inflammatory effect of resveratrol

Inflammation occurs due to numerous pathological stimuli and tissue injuries associated with diabetes, which is thought to contribute to the development of diabetes by causing insulin resistance (Huang *et al*., 2020). When exacerbated in hyperglycemia, inflammation can potentially cause long-term diabetic complications (Olatunji *et al*., 2018; Ni *et al*., 2019). Studies have established that resveratrol has significant anti-inflammatory properties (Meng *et al*., 2020). Resveratrol demonstrates anti-inflammatory effects in patients with DM, owing primarily to its inhibition of the nuclear factor kappa B (NF-κB) pathway (Sadeghi *et al*., 2017). NF-κB controls proinflammatory cytokine expression and the apoptosis cascade (Lawrence, 2009; Huang *et al*., 2020). Resveratrol inhibited inflammation through the downregulation of high mobility group box 1 (HMGB1) and inhibition of NF-κB and Janus kinase (JAK) and activator of transcription (STAT) signalling pathways (Meng *et al*., 2020; Ma *et al*., 2015). Additionally, RES prevented the activity of NF-κB and reduced elevated pro-inflammatory protein levels, resulting in decreased neuroinflammation and protection of diabetic neuropathy patients from functional and behavioral deficiencies (Kumar & Sharma, 2010). Resveratrol's anti-inflammatory effect has also been linked to Nrf2 (Huang *et al*., 2020). RES administration activates AMPK, which accelerates Nrf2 nuclear translocation and prevents the production of proinflammatory cytokines. Heme oxygenase (decycling)-1 (HO-1) is an antioxidant enzyme activated by RES and regulated by Nrf2. Inhibiting HO-1 can counteract the inhibitory effect of RES on proinflammatory cytokine production (Soeur *et al*., 2015); Iwasaki *et al*., 2013; Hassan

et al., 2019). Lastly, RES was shown to inhibit the activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2), resulting in elevated Myeloid differentiation factor 88 (MyD88) Short expression, a negative regulator of inflammation (Andrews *et al*., 2016).

2.8.2.2.1 The Nuclear factor-kB pathway

Nuclear factor-kB is involved in various biological processes, including inflammation, immune response, survival, and apoptosis (Girard *et al*., 2009). The NF-kB family comprises five distinct members, namely P50, p52, RelA (p65), RelB, and c-Rel (Moynagh, 2005; Hoffmann *et al*., 2006). Nuclear factor-kB is activated by different stimuli, such as chemicals produced by pathogens, intercellular inflammatory cytokines, and many enzymes (Pasparakis *et al*., 2006; Basak *et al*., 2007). The presence of IkB proteins in the cytoplasm prevents the activity of NF-kB under normal or basal physiological conditions (Kadhim *et al*., 2001). PRRs activate IkappaB kinase (IKK) through similar signalling pathways. IKK consists of two kinase subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ). It controls NF-kB pathway activation by phosphorylating IkB (Lawrence, 2009). The proteasome degrades IkB when phosphorylated, releasing NF-kB for nuclear translocation and gene transcription activation (Hayden & Ghosh, 2012). This pathway contributes to the inflammatory response by producing pro-inflammatory cytokines and recruiting inflammatory cells (Chen *et al*., 2018).

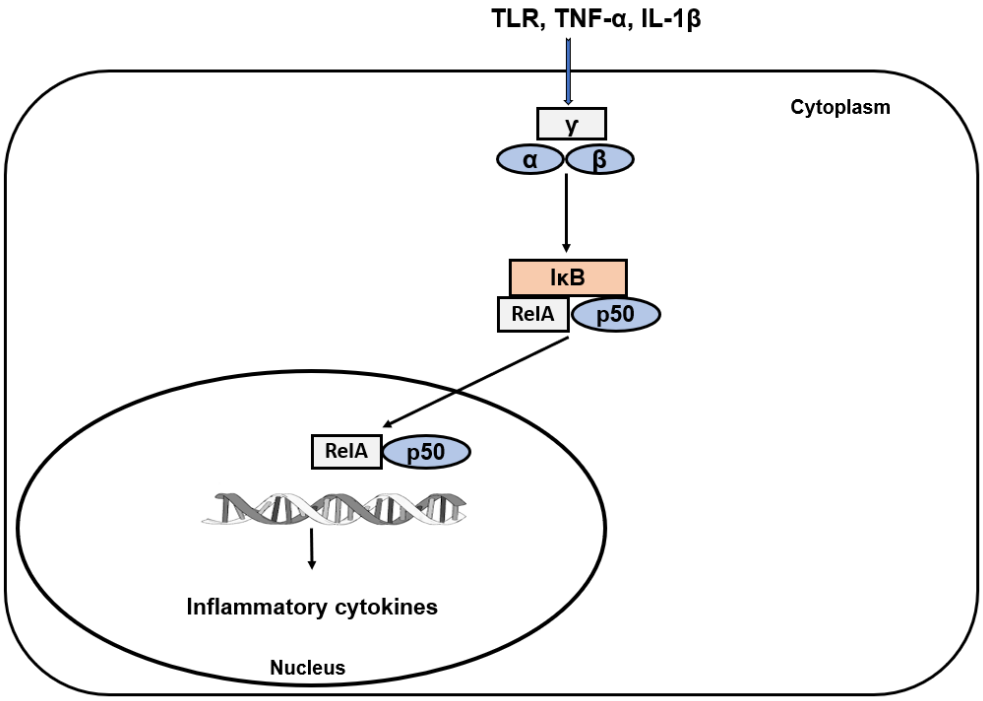

Figure 2.6: The NF-κB signalling pathway (*image created by the author*).

TLRs and inflammatory like TNF-α and IL-1 kickstart a pathway. This pathway activates RelA/p50 complexes, which control the production of more inflammatory molecules. To get this pathway going, IKK subunits are needed. They control the pathway by phosphorylating IκB.

2.8.2.3 The antioxidant effect of resveratrol.

Oxidative stress is a frequent and significant factor linking hyperglycemia and diabetes complications. It is an imbalance between ROS production and antioxidant defence (Huang *et al*., 2020; Galiniak *et al*., 2019). Reactive oxygen species encompass oxygen-derived radicals, such as the hydroxyl radical (OH•), superoxide anion ($O_2^{\bullet-}$), and peroxynitrite ($ONOO^-$). Additional derivatives of oxygen that are not classified as radicals are also recognized as ROS, including hydrogen peroxide (H_2O_2), owing to its ability to readily produce free radicals. ROS are produced as a result of regular metabolic processes in a cell and play a significant role in various biological functions. Reactive oxygen species play a crucial role in sustaining life; however, their inherent chemical reactivity renders them capable of causing harm to macromolecules such as lipids, proteins, and nucleic acids. Consequently, cellular defence mechanism is initiated to regulate the generation of ROS and prevent oxidative damage. Most defence mechanism against ROS consist of enzymes that effectively eliminate surplus ROS. These enzymes include Superoxide Dismutase (SOD), Catalase (CAT), peroxiredoxins, thioredoxins, and glutathione peroxidase (Burgos-Morón *et al*., 2019).

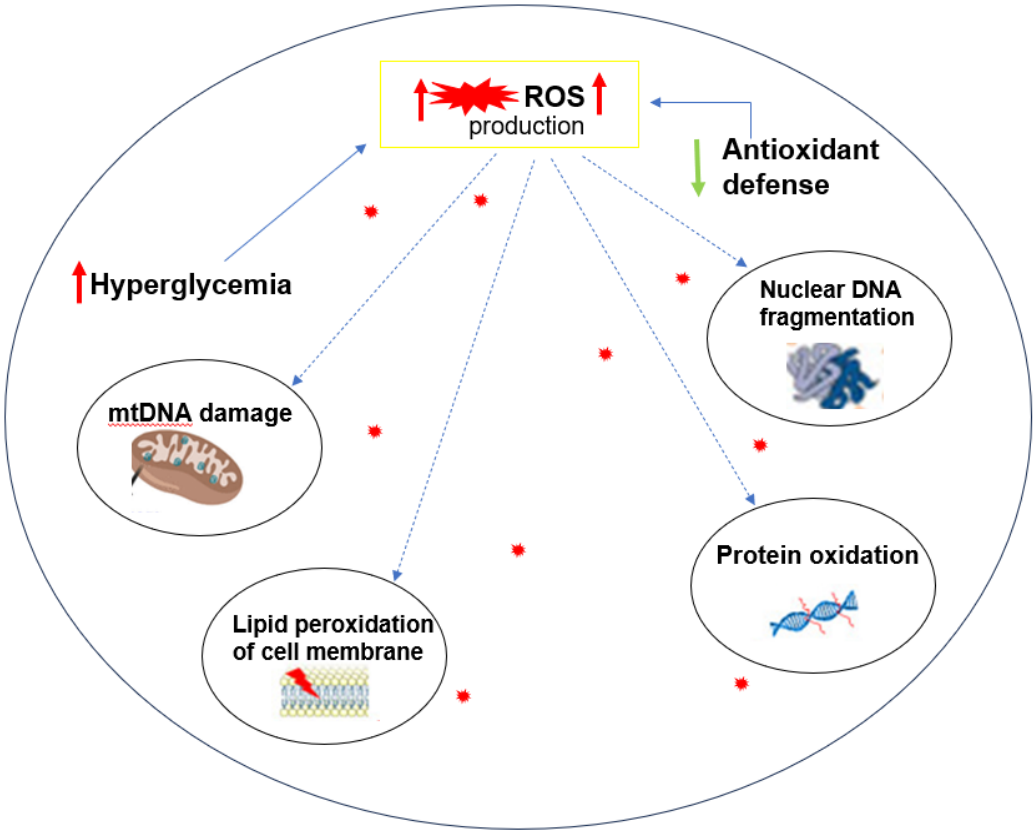

Figure 2.7: Mechanism of oxidative stress in diabetes (*image created by author*).
Hyperglycemia prompts the overproduction of ROS, overwhelming the antioxidant defense enzymes. This imbalance results in damage to various cellular components, including mitochondrial DNA (mtDNA), lipid membranes, proteins, and nuclear DNA.

Resveratrol has been shown to defend against oxidative stress, a fundamental cause of many diseases (Meng *et al*., 2020). Resveratrol was found to possess antioxidant activity in diabetic animal models as determined by variations in oxidative stress markers, including lipid peroxidation, malondialdehyde, and antioxidant enzymes, such as SOD, CAT, and Glutathione (GSH). Antioxidant enzymes are the backbone of the antioxidant defence system, scavenging excess free radicals and reversing oxidative stress (Sadi *et al*., 2018; Prabhakar, 2013; Sadi & Konat, 2015); (Huang *et al*., 2020). RES increased the expression of phosphatase and tensin homolog (PTEN), inhibiting the phosphorylation of Akt, resulting in an increase in the levels of antioxidant enzymes, including CAT and SOD (Inglés *et al*., 2014). Additionally, resveratrol may strengthen the antioxidant defence system by regulating antioxidant enzymes and preventing ROS-induced activation of extracellular signal-regulated kinase (ERK) (Singh & Vinayak, 2017). Nuclear factor erythroid 2-related factor 2 is critical for alleviating oxidative and electrophilic stress in cells, where it controls the redox state and energy metabolism (Zhang *et al*., 2018; Liao *et al*., 2017). RES may protect against oxidative stress through Nrf2 pathway regulation. Nrf2 is a transcription factor that controls the activity of several antioxidant enzymes, including HO-1, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH), and quinine oxidoreductase-1 (NQO1). Moreover, Nrf2 is now recognized as a key RES mediator in reducing oxidative stress damage (Zhang *et al*., 2018; Liao *et al*., 2017; Yadav *et al*., 2018; Huang *et al*., 2020). Resveratrol activity on Nrf2 requires the activation of several cellular signalling pathways, including PI3K/Akt and AMPK pathways (Iwasaki *et al*., 2013). These pathways are activated by RES, resulting in the nuclear translocation of Nrf2, thereby activating antioxidant enzyme expression and increasing SOD (Hui *et al*., 2018; Shen *et al*., 2016).

2.8.2.3.1 Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway

Nuclear factor erythroid 2-related factor 2 controls the production of antioxidant genes and aids in protecting cells against detrimental effects caused by oxidative stress (Gu *et al*., 2021). The antioxidant properties arise from activating genes that contain antioxidant response elements (AREs) (Teixeira *et al*., 2017). Without external stimulation, Nrf2 resides in the cytoplasm, interacting with the inactive Kelch-like ECH-associated protein 1 (KEAP1). When ROS accumulation occurs, KEAP1 undergoes conformational changes, causing it to detach from Nrf2 and enter the nucleus (Menshchikova *et al*., 2013). The musculoaponeurotic fibrosarcoma (Maf) protein and Nrf2 form a heterodimer, which then interacts with the ARE to promote phase II antioxidant gene expression, resulting in the production of antioxidant enzymes (Kou *et al*., 2013). When the Nrf2/ARE pathway is activated, the proteins eliminate ROS and exogenous/endogenous harmful substances (Gu *et al*., 2021)

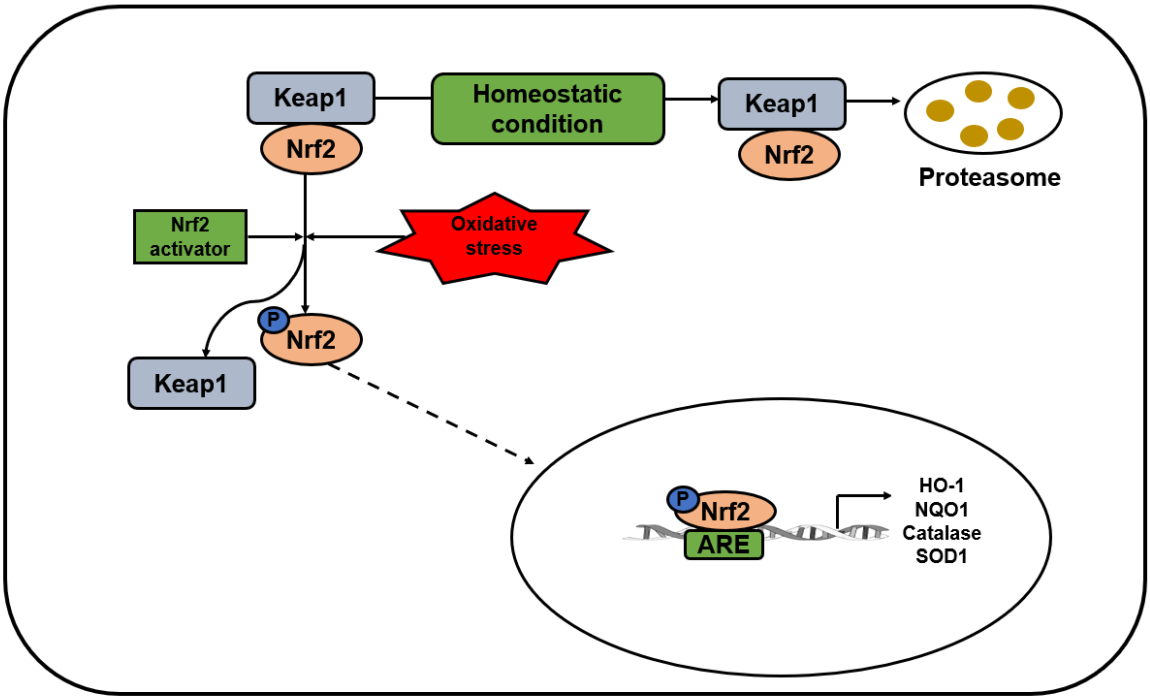

Figure 2.8: The Nrf2 signaling pathway (*image created by the author*).
An illustration of how reactive oxygen species (ROS) activate the antioxidant response element (ARE) pathway through nuclear factor erythroid 2-related factor 2 (Nrf2). Under normal circumstances, Nrf2 is constitutively linked to the protein Keap1 in the cytoplasm. Keap1 inhibits Nrf2 signaling pathway by promoting Nrf2 ubiquitination and subsequent degradation through the proteasomal pathway. Mild oxidative stress and Nrf2 activators result in the dissociation of Nrf2-Keap1 complex, phosphorylation of Nrf2, and nuclear translocation. In the nucleus, Nrf2 binds to the ARE in the target gene promoter regions to facilitate the transcriptional activation of detoxifying and antioxidant enzymes.

2.8.2.4 Anti-apoptotic effects of RES

Previous research has provided evidence that RES could effectively alleviate oxidative stress and apoptosis in various types of cells (Liu *et al*., 2014; Kitada & Koya, 2013; Hoca *et al*., 2021; (Do *et al*., 2012). Apoptosis is a programmed cell death initiated in response to prolonged stress. Apoptosis in mammalian cells is facilitated by two distinct pathways: (i) the Extrinsic pathway, which is alternatively referred to as the death-receptor mediated pathway; and (ii) the Intrinsic pathway, which is also denoted as the Bcl-2 regulated or mitochondrial pathway (Wali *et al*., 2013) (Figure 9). The activation of the extrinsic pathway occurs when ligands from the TNF super-family, such as FasL, bind to cell surface death receptors like Fas or TNFR. This leads to the recruitment of FAS-associated death domain (FADD), followed by the recruitment of caspase-8 and the subsequent activation of downstream effector caspases-3, 6, and 7. Ultimately, this process leads to the activation of proteases, fragmentation of DNA, and subsequent cell death (Hotchkiss *et al*., 2009; Strasser, 2005; Wali *et al*., 2013). The activation of the intrinsic pathway occurs in response to a range of cellular stresses, including exposure to radiation and withdrawal of growth factors (Wali *et al*., 2013). The regulation of this pathway

is determined by the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family. The BH3-only proteins, which are members of the pro-apoptotic family, are characterized by possessing a single Bcl-2 homology domain. Various cellular stresses elicit the activation of distinct BH3-only proteins within a tissue, exhibiting specificity towards the particular stimulus. The anti-survival proteins, include Bcl-2, Bcl-xl, Bcl-w, and Mcl-1 (Lee *et al*., 2014). The activation of the pro-apoptotic Bcl-2 family members and the suppression of anti-apoptotic proteins is triggered by cellular stress, leading to the translocation of Bax and Bak to the outer mitochondrial membrane. This translocation ultimately leads to the formation of pores. This process induces the translocation of cytochrome c from the mitochondria to the cytoplasm, leading to the subsequent activation of caspase-9 and subsequent activation of caspase-3,6, and 7, ultimately resulting in the initiation of apoptosis (Hotchkiss *et al*., 2009; Strasser, 2005; Thomas *et al*., 2009). Bcl-2 is an anti-apoptotic protein that prevents apoptosis by inhibiting Bax/Bak oligomerization, which enhances mitochondrial membrane permeability and inhibits the Cyto-C release. Previous research has demonstrated reduced Bcl2 expression in response to diabetes stimulus (Ren *et al*., 2020). Resveratrol has been shown to effectively inhibit apoptosis in retinal Müller cells exposed to high glucose. The protective effect was shown to be mediated by miR-29b. Furthermore, the expression levels of Bax and specificity protein 1 (SP1) were reduced upon RES treatment, whereases the Bcl-2 expression was increased (Zeng *et al*., 2017). Further investigations is required to explore the anti-apoptotic effect of RES.

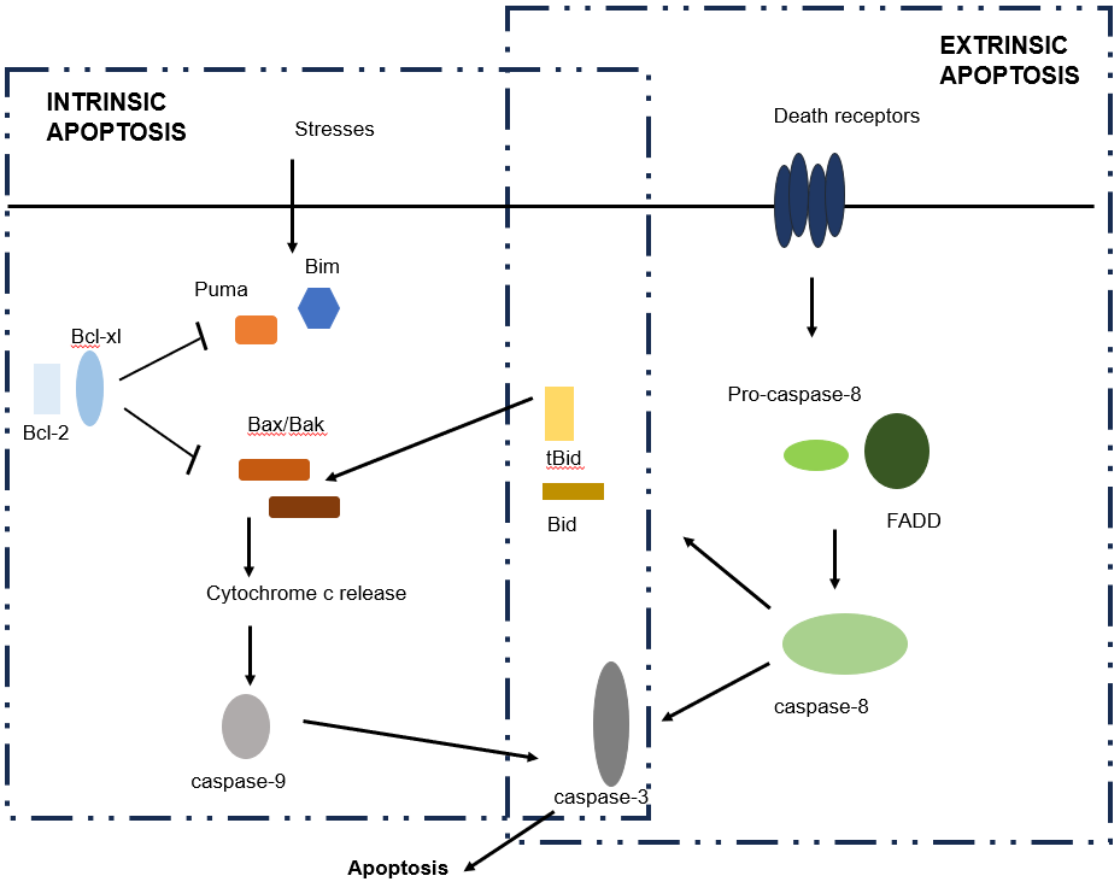

Figure 2.9: Apoptosis pathways (*image created by the author*).

The two pathways of apoptosis. Intrinsic pathway: This is triggered by cellular stresses like high glucose or growth factor deprivation. BH-3only proteins like Bim and Puma initiate signaling by binding to pro-survival Bcl2 proteins. This leads to mitochondrial outer membrane permeabilization, cytochrome c release, and activation of caspases, culminating in apoptosis. Extrinsic pathway: Initiated by activation of death receptors like Fas, containing intracellular death domains. This leads to the formation of death-inducing signalling complexes, activating initiator caspase-8 through FAS-associated death domain (FADD). This activates the caspase cascade and induces apoptosis. Bid protein facilitates cross-talk between the two pathways.

2.8.2.5 Resveratrol effect on glucose uptake and metabolism

Individuals with DM demonstrated impairment in glucose metabolism. Maintaining optical glucose metabolism is imperative to preserve the body's physiological equilibrium (Su *et al*., 2022). Skeletal muscles are the primary contributors to the body's glucose metabolism balance (Schram *et al*., 2004; Karaman *et al*., 2012). There is enough evidence that glucose transporter 4 (GLUT4) is essential for glucose uptake in skeletal muscle cells (Su *et al*., 2022). The enhancement of glucose uptake induced by RES primarily depends on the expression of GLUT4 and the translocation of GLUT4. In fat and muscle cells, GLUT4 is primarily translocated from the intracellular to the cell membrane (Chi *et al*., 2007; Tan *et al*., 2012; Chen *et al*., 2011). Research has shown that RES-fed db/db mice greatly increased glucose absorption, boosting the level of GLUT4 (Do *et al*., 2012). Furthermore, RES has been

demonstrated to enhance the phosphorylation of AMPK through the activation of binding to an estrogen receptor (ER), leading to improved GLUT4 expression and translocation, thus affecting glucose uptake by skeletal muscle cells (Rogers *et al*., 2009; Klinge *et al*., 2008). In addition, the liver also plays a fundamental role in regulating glucose homeostasis by regulating multiple glucose metabolic pathways, including glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis (Han *et al*., 2016). These metabolic pathways are often disrupted in individuals with DM. Previous research has demonstrated that RES can enhance nutrient-sensing systems and improve renal function in elderly patients with T2DM. Additionally, RES was found to reduce the production and activity of glucose-6-phosphatase (G6P), a gluconeogenesis enzyme (Ma & Zhang, 2022). It is important to understand the mechanism by which RES confers protection against disrupted glucose homeostasis and its potential role in modulating the activity of enzymes implicated in glucose metabolism. Hence additional investigation is required to explore the precise functions of RES in glucose metabolism, to develop an innovative therapeutic approach that effectively targets and addresses impaired glucose metabolism.

2.8.3 Resveratrol and microRNAs

Although RES may be beneficial for managing diabetes and its symptoms, including hyperglycemia, its effect on hyperglycemia-related miRNAs has yet to be thoroughly researched. Previous research has demonstrated that the expression of certain miRNAs, including miR-133a-3p, miR-188- 5p, miR-206-3p, miR-18a-5p, miR-382-5p, miR541-5p, and miR-714, changed after RES treatment in the renal cortex of db/db mice. Furthermore, they reported that miR-18a-5p was significantly upregulated after RES treatment in db/db mice. Overexpression of miR-18a-5p in podocytes resulted in significant inhibition of cleaved-caspase-3 protein and increased the ratio of LC3-II/LC3, suggesting that kidney protection by RES occurred via upregulation of miR-18a-5p (Xu *et al*., 2017). Furthermore, researchers observed a decrease in pro-inflammatory TNF-α and IL-6 cytokine expression as well as miRNAs involved in inflammatory response regulation (i.e., miR181b, miR21, miR30c2, miR34a, miR155, and miR663) in 35 hypertensive patients with T2DM-associated coronary artery disease (Tomé-Carneiro *et al*., 2013).

Further research is required to investigate the effect of RES on miRNAs associated with DM. A key target of resveratrol's effects on diabetes may be regulating miRNA expression. Consequently, gaining knowledge about the specific miRNAs influenced by RES could offer valuable understanding regarding its potential therapeutic advantages for individuals with DM.

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CHAPTER 3: The effect of resveratrol on hyperglycemia-related microRNAs in HepG2 cells (Submitted to *Biochimica et Biophysica Acta – Molecular Basis of Disease*, currently under review).

The effect of resveratrol on hyperglycemia-related microRNAs in HepG2 cells

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Abstract

Background: The disruption in the normal functioning of specific microRNAs (miRNAs), namely miR-30a-5p, miR-126-3p, and miR-182-5p, is closely linked to the initiation and advancement of type 2 diabetes mellitus. Therapeutic interventions involving endogenous and exogenous substances can restore miRNA regulation. This study examines the effect of high glucose (HG) on HepG2 cells and assesses the effects of resveratrol (RES), a polyphenol phytoalexin, on HG-induced miRNA dysregulation.

Methods: We investigated the expression levels of three miRNAs (miR-30a-5p, miR-126-3p, and miR-182-5p) and their target genes (*SPRED1, FOXO1, G6P, Neurod1*) in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 and 50 µM). The expression levels of the miRNAs and mRNAs were measured using qPCR, after 48 and 72 hours (h).

Results: Exposure to HG for 48 and 72 h decreased the expression of miR-126-3p and increased the expression of its target gene *SPRED1* in HepG2 cells. Conversely, RES treatment increased the expression of levels of miR-126-3p and reduced the expression of *SPRED1*. The HG treatment decreased miR-182-5p and enhanced the expression of *FOXO1* and *G6P*. RES treatment increased miR-182-5p expression, concomitant with a reduction of *FOXO1* and *G6P* levels. Furthermore, HG decreased miR-30a-5p levels, which subsequently increased *Neurod1* expression. Conversely, treatment with RES increased miR-30a-5p while simultaneously reducing *Neurod1*.

Conclusion: Treatment with resveratrol showed a potential therapeutic application by increasing miR-30a-5p, miR-126-3p, and miR-182-5p expression levels. In addition, resveratrol may mitigate the effects exerted by miRNAs on their respective target genes.

Keywords: Hyperglycemia, MicroRNAs, Resveratrol, miR-126-3p, miR-182-5p, miR-30a-5

1. Introduction

Diabetes mellitus (DM) is a metabolic condition in which disruptions in insulin production or function, resulting from genetic and environmental factors, lead to a disease affecting glucose metabolism (Huang *et al*., 2020). In Africa, an estimated 24 million individuals were living with diabetes in 2021, and this is projected to rise to 55 million by 2045 (Sun *et al*., 2022). In South Africa, the prevalence of diabetes increased from 4.5% in 2010 to 12.7% in 2019. Furthermore, in 2019, 4.58 million individuals aged between 20-79 were estimated to have diabetes, of which 52.4% were undiagnosed (International Diabetes Federation, 2021). Notably, low-income countries face an even higher percentage of undiagnosed cases at 50.5%, while the global average of middle- and low-income countries remains at 87.5% (International Diabetes Federation, 2021).

Unfortunately, current diabetes treatments suffer from serious side effects, such as weight gain, hypoglycemia, gastrointestinal discomfort, or contraindications that restrict their use (Öztürk *et al*., 2017; Su *et al*., 2022). Improper treatment or diet control diminishes the quality of life for patients with diabetes and often leads to serious complications, including diabetic nephropathy, neuropathy, retinopathy, and cardiomyopathy (Dow *et al*., 2018). Early diagnosis and effective treatment of diabetes are both critical components to improving patient outcomes. Therefore, researchers must aim to create safer drugs that enhance the quality of life while decreasing the risks associated with this condition.

Drug therapy remains the most effective means of delaying T2DM progression (Guo & Smith, 2021). Anti-diabetic medications are designed to target specific tissues, such as fat and muscle, to decrease insulin resistance, regulate liver glucose production, or stimulate pancreatic release of insulin (Chaudhury *et al*., 2017). Although existing diabetes medications effectively treat symptoms, they do not completely halt the progression of the condition (Dahlén *et al*., 2022). Specific microRNAs (miRNAs) that regulate genes involved with insulin resistance and pancreatic beta cell function may serve as promising pharmacological targets to treat diabetes (Chen *et al*., 2014).

Recent advancements in diabetes research have demonstrated the role of miRNAs circulating in the blood as crucial in its onset and progression. Hence, they possess immense therapeutic and diagnostic potential. MiRNAs, which are intrinsic noncoding RNAs, function to regulate gene expression at the posttranscriptional stage by either promoting the degradation of messenger RNAs (mRNAs) or preventing their translation into proteins (Bartel, 2004; Lewis *et al*., 2005). MiRNAs are highly stable molecules present in tissues and trace amounts in biological fluids (Necula *et al*., 2019). Numerous research endeavors focusing on miRNAs have been conducted in Africa. For example, Weale *et al*. showed that dysregulation of certain

miRNAs, such as miR-30a-5p, miR-126-3p, and miR-182-5p, is associated with both the development and progression of T2DM in a South African population (Weale *et al*., 2020; Weale *et al*., 2021). These miRNAs were identified as being significantly overexpressed in those with prediabetes or T2DM, suggesting that they may serve as useful biomarkers for the early detection of pre-diabetes or T2DM in patients. This study served as a foundational framework for our research.

Furthermore, the dysregulation of these miRNAs has been linked to diabetes-related pathways. For example, miR-182 has been shown to influence glucose metabolism, primarily by targeting Forkhead box protein O1 (FOXO1) (Karolina *et al*., 2011; Zhou *et al*., 2014) FOXO1 can stimulate the transcription of downstream genes involved in gluconeogenesis, such as glucose-6-phosphate (G6P) (Barthel *et al*., 2005; Gross *et al*., 2008) Therefore, decreased miR-182 levels can cause hyperglycemia, as it allows the FOXO1 to increase G6P transcription and thus increase gluconeogenesis. Mir-126 plays a key role in regulating vascular development and homeostasis by targeting specific mRNAs, including CXCL12, VCAM-1, SPRED-1, and PIK3R2, which contributes to the endothelial dysfunction linked to diabetes and its complications (Wang *et al*., 2008; Sessa *et al*., 2012). Additionally, miR-30a-5p targets protein kinase B to promote chondrocyte apoptosis in osteoarthritis patients (Fu *et al*., 2018). Further research must be conducted to develop therapeutic strategies targeting miRNAs that are dysregulated in those living with DM.

Treating diabetes involves lowering blood glucose levels through lifestyle adjustments and anti-diabetic medication. Clinical practice medications commonly used include insulin secretagogues, metformin, sodium-glucose transporter 2 (SGLT-2) inhibitors, GLP1 receptor agonists, and a-glycosidase inhibitors (Accili *et al*., 2023). However, many drugs containing insulin come with unwanted side effects, including hypoglycemia, digestive disorders, and urinary infections. There is an urgent need to identify safe and effective drugs that provide anti-hyperglycemic benefits while protecting against complications of diabetes (Gupta *et al*., 2017).

Natural products have long been acknowledged for their therapeutic effects. Resveratrol (RES) is a polyphenol phytoalexin, known as trans-3,4,5-trihydroxystilbene, that is found in many plants, such as grapes, peanuts, and berries (Summerlin *et al*., 2015). Numerous studies have investigated the role of RES in managing diabetes and its complications (Soufi *et al*., 2012; Turan *et al*., 2012; Singh *et al*., 2013; Vallianou *et al*., 2013) and researchers have demonstrated that RES can reduce blood glucose levels (Oyenihi *et al*., 2016; Brasnyó *et al*., 2011; Bhatt *et al*., 2012; Crandall *et al*., 2012; Szkudelski & Szkudelska, 2015). Although RES may be beneficial for managing diabetes and its symptoms, including hyperglycemia, its effect on HG-related miRNAs has yet to be thoroughly researched. Resveratrol's effects on diabetes may involve modulating miRNA expression, which regulates gene expression and, in turn,

protein expression, thus contributing to disease onset and progression. Therefore, this study aimed to determine miR-30a-5p, miR-125-3p, and miR-182-5p expression levels in HepG2 cells exposed to HG concentrations and observe the effects of RES on these miRNAs during HG and control conditions. Moreover, we aimed to determine the effect of glucose-induced miRNA dysregulation on specific genes (mRNA expression) associated with glucose metabolism and the onset and progression of DM.

2. Materials and Methods

2.1. Materials

Tissue culture consumables and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). HepG2 cells were kindly donated by Prof JL Marnewick (Cape Peninsula University of Technology, South Africa). D-glucose and resveratrol were purchased from Sigma–Aldrich. PCR primer mixtures, kits, and reagents used for miRNA extraction and real-time quantitative Polymerase Chain Reaction (qPCR) were purchased from Qiagen (Hilden, Germany). Reagents used for gene expression were purchased from Bio-Rad (Hercules, CA, USA), and primer sequences were manufactured by Inqaba Biotechnical Industries (Pretoria, South Africa). Primers were verified for specificity BLAST database.

2.2. Methods

2.2.1. Study design

Cells were categorized into six groups: Control (cultured in normal complete culture medium (CCM)), Low resveratrol (LR; cultured in normal CCM + 25 μM RES), High resveratrol (HR; cultured in normal CCM + 50 μM RES), High glucose (HG; cultured in normal CCM + 40 mM glucose), LR+HG (cultured in normal CCM + 25 μM RES + 40 mM glucose), and HR+HG (cultured in normal CCM + 50 μM RES + 40 mM glucose). A literature search was conducted to determine the concentrations and exposure periods for the glucose and resveratrol treatments. For the resveratrol treatment, studies by Baselga-Escudero *et al.,* and Khan *et al.* reported the use of 50 μM and 25 μM resveratrol, respectively (Baselga-Escudero *et al*., 2014; Raghubeer *et al*., 2015; Khan *et al*., 2013) .Similarly, several studies reported the use of 40 mM glucose to represent “hyperglycemic” or high glucose (HG) conditions (Chu *et al*., 2011; Leinninger *et al*., 2004; Varma *et al*., 2005; Kapoor & Kakkar, 2012).

2.2.2. Cell culture

HepG2 cells were cultured in 25 cm^3 flasks, in a monolayer (10^6 cells per flask), using Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penstrepfungizone (PSF), and 1% L-glutamine. The cells were cultured in a 37 °C humidified

incubator (5% CO_2). Cells were washed with 0.1 M phosphate-buffered saline (PBS). Once 70-80% confluent, cells were treated with RES (25 µM and 50 µM) and HG (40 mM) and incubated for 48 and 72 h. Thereafter, cells were removed using trypsin and counted using the trypan blue exclusion method of cell counting. Briefly, cell suspensions were diluted (1:5) with 60 µL CCM + 20 µL cell suspension + 20 µL trypan blue solution and incubated at room temperature for 5 min. Then, a 22 x 22 cm coverslip was placed on a clean hemocytometer, 10 µL of a well-mixed counting solution was dispensed into the middle bar of the hemocytometer, and capillary action was used to move the suspension between the coverslip and the hemocytometer. The number of living cells was then determined using a microscope. The cell viability was determined using the standard equation (Live cell average x 5 (dilution factor) x 10,000 = cells/mL).

2.2.3. Resveratrol treatments

Resveratrol stock solutions (20 mM) were prepared in 100% dimethyl sulphoxide (DMSO). RES treatment concentrations were determined based on a 50% inhibitory concentration (IC_{50}) from previous studies (Baselga-Escudero *et al*., 2014; Raghubeer *et al*., 2015 (Khan *et al*., 2013)

2.2.4. miRNA isolation

Total RNA, encompassing miRNA, was isolated using the miRNeasy Tissue/Cells Advanced Minikit (Qiagen) following the provided guidelines. The extracted total RNA was quantified using Nanodrop spectrometry (Nanodrop one C, Thermo Fisher Scientific, Wilmington, DE, USA). For cDNA synthesis, the miRCURY LNA RT kit (Qiagen) was employed per the manufacturer's instructions. Once cDNA synthesis was completed successfully, the samples were stored at -20 °C until they were needed for qPCR assays.

2.2.5. microRNA analysis

The quantification of miRNA expression was performed using the miRCURY LNA SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol using Applied Biosystems™ QuantStudio™ 7 Flex (Thermo Fisher Scientific). Pre-designed primers for each miRNA were used to quantify target miRNAs. Primers used were hsa-miR-182-5p (CAT no: YP00206070), hsa-miR-30a-5p (CAT no: YP00203695), and hsa-miR-126-3p (CAT NO: YP00204227) (Qiagen). Data were obtained as Ct values and normalized against an endogenous control (U6 snRNA). The miRNA expression level in each sample was determined using the $2^{-\Delta Ct}$ method, and the $2^{-\Delta\Delta Ct}$ value was used to compare the miRNA expression level in each sample to the control (Livak & Schmittgen, 2001).

2.2.6. RNA extraction and Gene expression analysis

Total RNA was isolated using a Trizol reagent according to the manufacturer's protocol. The isolated total RNA was quantified using Nanodrop spectrometry (Nanodrop one C, Thermo Fisher Scientific, Wilmington, DE, USA). The iScript cDNA synthesis kit (Bio-Rad) was utilized for cDNA synthesis by the manufacturer's guidelines. Once cDNA conversion was completed successfully, the amplification of mRNA was performed using Applied BiosystemsTM QuantStudioTM 7 Flex (Thermo Fisher Scientific, USA) with the following reaction mixture: 5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 1.5 µL cDNA, 0.5 µL forward and reverse primers, and 2.5 µL nuclease-free water, resulting in a reaction volume of 10 µL. The primers (purchased from Inqaba Biotechnical Industries) used in this study are shown in Table 1. *Beta actin* was utilized as a housekeeping gene, with three replicates per treatment. The mRNA expression level in each sample was determined using the $2^{-\Delta Ct}$ method, and the $2^{-\Delta\Delta Ct}$ value was used to compare the mRNA expression level in each sample to the control **(Livak & Schmittgen, 2001)**.

Table 31: Primers used in this study.

Gene name	Forward	Reverse
Beta actin	5′ TGACGGGTCACCCACACTGTGCC CAT 3′	5′CTAGAAGCATTTGCGGTGGACGA TGGAGGG 3′
Neurdod1	5′ CTCCGGGGTTATGAGATCGTCAC 3′	5′ GCCTTCATGCGCCTTAATTT 3′
FOXO1	5′ AAGCTCCCAAGTGACTTGGATG 3′	5′ CTGCTCACTAACCCTCAGCCTGA 3′
G6P	5′ TTTCCCCACCAGGTCGTGGCT 3′	5′ CCCATTCTGGCCGCTCACAC 3′

2.2.7. Statistical analysis

All data analyses were conducted using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California, USA). The statistical methods employed included the Student's *t*-test and one-way analysis of variance (ANOVA). All experiments were conducted in triplicates, and statistical significance was determined at a threshold of $p<0.05$.

3. Results

3.1. Expression levels of miR-126-3p and *SPRED1* in HepG2 cells treated with high glucose and resveratrol

HepG2 cells were exposed to HG (40 mM) for 48 and 72 h. Thereafter, RNA was isolated, and cDNA was synthesized. Real-time PCR was used to measure the expression levels of miR-126-3p and *SPRED1* mRNA expression. We observed that miR-126-3p showed a significant decrease compared to controls after exposure to HG over 48 and 72 h (Figure 1 A and B) (p<0.0001). The findings of our study showed that high levels of glucose may lead to a decrease in the expression of certain miRNAs, which may contribute to the development of DM. Furthermore, we investigated the effect of two different RES concentrations (25 and 50 μM) on the expression of miR-126-3p after exposure over 48 and 72 h. miR-126-3p showed no significant differences after exposure to LR (25 μM) over 48 and 72 h as compared to controls; however, miR-126-3p was significantly decreased over 48 and 72 h, respectively, after exposure to HR (50 μM) (p<0.001; p=0.0015) (Figure 1 A and B). Since HG exposure reduced miR-126-3p expression levels, we aimed to determine if RES could reverse the effects of HG on this miRNA. Cells were treated with HG+LR and HG+HR over 48 and 72 h (Figure 1 A and B). The qPCR results show that the expression levels of miR-126-3p increased significantly after exposure to HG+LR and HG+HR over 48 (p<0.001) and 72 h (p=0.0001 and p=0.0008) (Figures 1 A and B) as compared to HG alone.

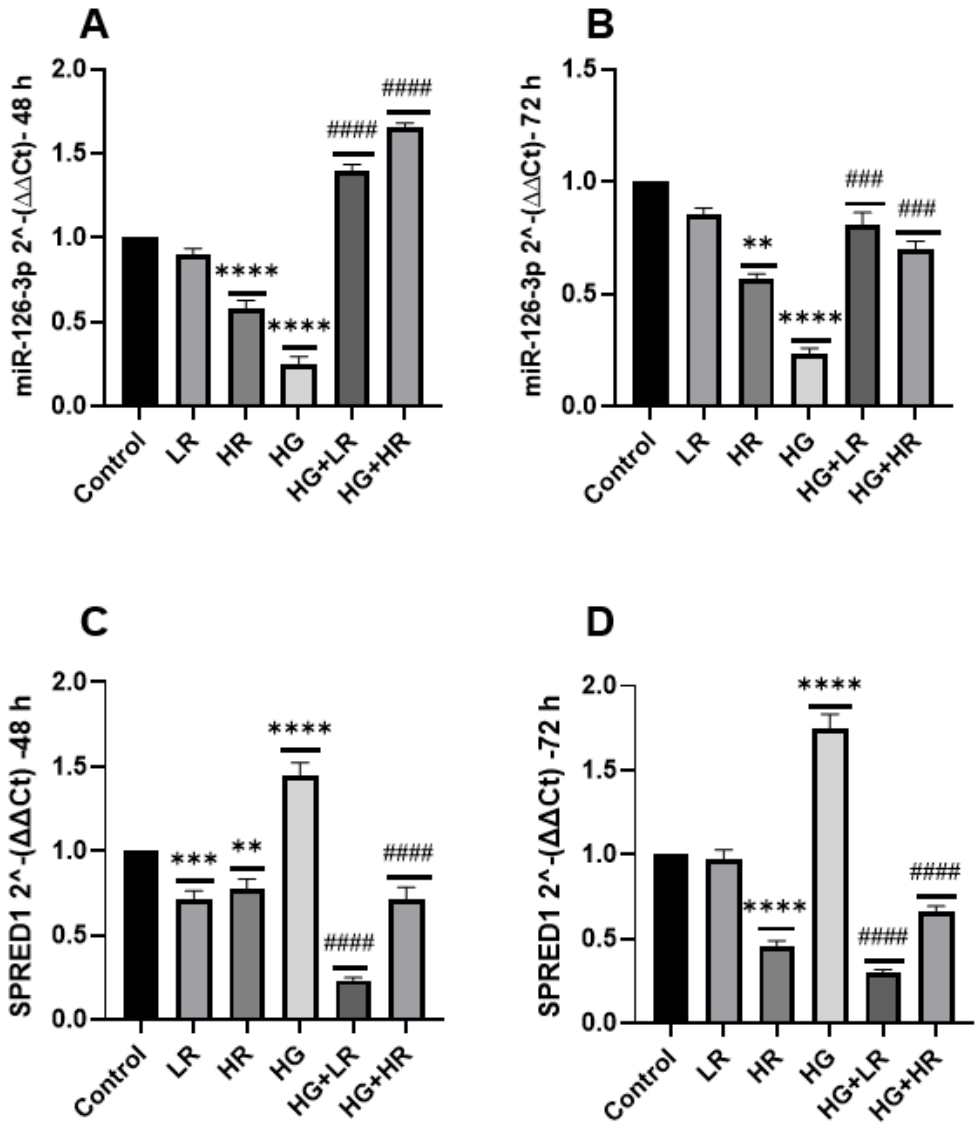

Figure 3.1: Expression of miR-126-3p (A and B) and *SPRED1 (*C and D) in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 h and 72 h. The expression levels of miR-126-3p and *SPRED1* were quantified using qPCR. The expression of the miRNAs was normalized to the relative expression of U6 snRNA and the housekeeping gene (*Beta actin*) was used to normalize *SPRED1*. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; SPRED1, Sprouty-related EVH1 domain containing 1.

It is widely recognized that miRNAs carry out their biological role by repressing the expression of specific target genes. The miRDB-microRNA target prediction Database shows that miR-126-3p targets SPRED1 (http://www.mirdb.org/). In the present study, we investigated the mRNA expression of *SPRED1*. The mRNA expression levels of *SPRED1* exhibited a statistically significant increase following treatment of cells with HG over 48 and 72 h (Figure 1 C and D), as compared to controls (p<0.0001). The findings of our study indicated that reduction in miR-126-3p significantly enhances the mRNA expression of *SPRED1* in HepG2

cells. When cells were treated with LR and HR alone for 48 h, the mRNA expression of *SPRED1* decreased significantly (p=0.002; p=0.0019). When treated for 72 h with LR and HR, no significant expression was observed in cells treated with LR; however, a significant reduction was observed in cells treated with HR (p<0.0001) as compared to controls. Interestingly, when cells were treated with HG+LR and HG+HR over 48 and 72 h, the mRNA expression of *SPRED1* was significantly decreased (Figure 1 C and D) (p<0.0001) as compared to HG alone. These results provide additional evidence that elevated levels of miR-126-3p are associated with a reduction in the expression of *SPRED1*.

3.2. Expression of miR-182-5p and its target gene (*FOXO1*) in HepG2 cells

It has been observed that the treatment of cells with HG significantly reduced the expression level of miR-182-5p (p<0.0001) while increasing the mRNA expression of *FOXO1* over 48 and 72 h (p=0.0141; p<0.0001, respectively) as compared to controls (Figure 2). When cells were treated with LR and HR alone for 48 h, LR showed no significant influence on miR-182-5p expression, while HR significantly decreased miR-182-5p expression (p<0.0001) as compared to the control. When treated for 72 h with LR and HR alone, there was a significant reduction in miR-182-5p expression (p=0.0003; p<0.0001, respectively) as compared to the control (Figure 2 A and B). A notable decrease in *FOXO1* expression was observed when cells were treated with LR and HR over 48 and 72 h (p<0.0001) as compared to the control (Figure 2 C and D). To determine the effect of resveratrol in the presence of HG on the expression of miR-182-5p and FOXO1, HepG2 cells were treated with HG+LR and HG+HR for over 48 and 72 h. A significant increase in miR-126-3p expression was observed when cells were treated with HG+LR and HG+HR for over 48 and 72 h, respectively (p<0.0001) as compared to the HG group alone (Figure 2 A and B). *FOXO1* also showed a significant increase when cells were treated with HG+LR and HG+HR over 48 (p=0.0475; p=0.0002, respectively) and 72 h (p<0.0001) as compared to the HG group alone (Figure 2 C and D). Our study shows that increased miR-182-5p resulted in decreased expression of *FOXO1* mRNA expression (Figure 2).

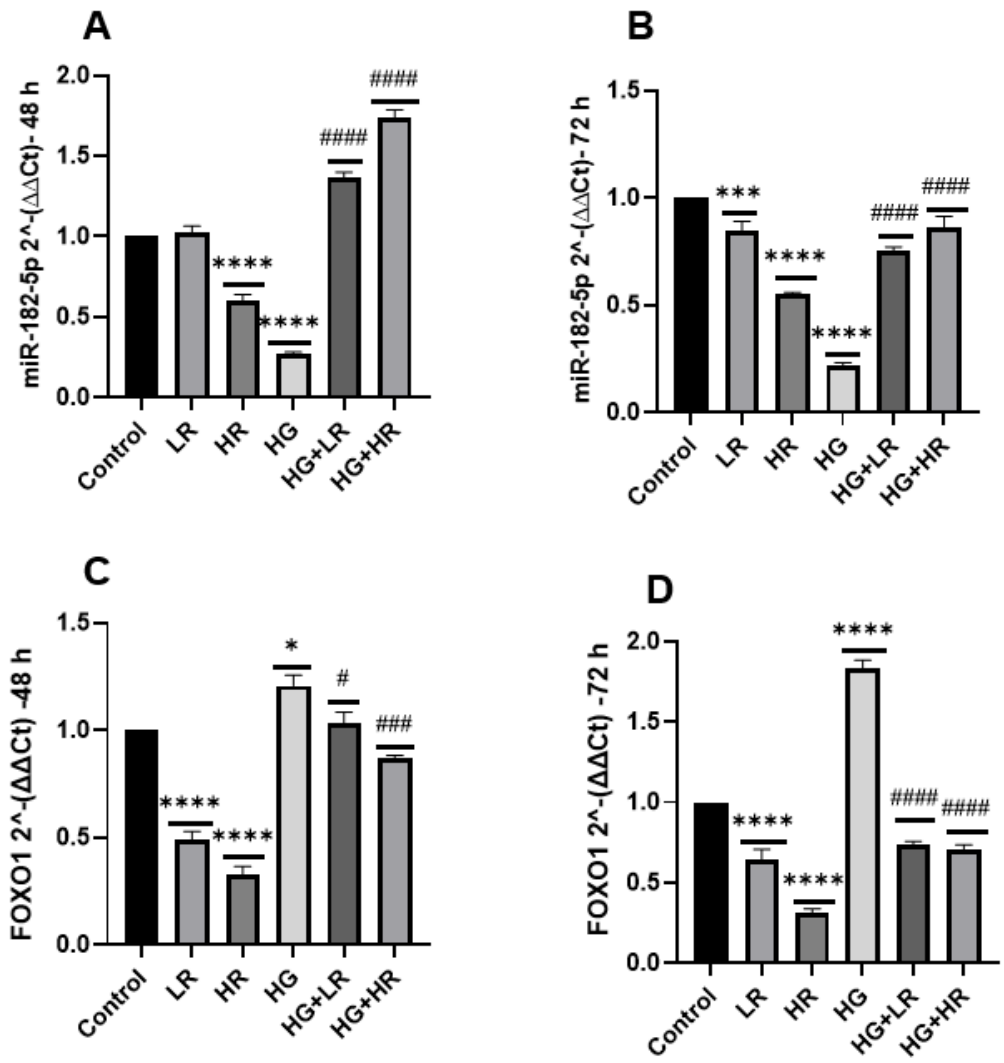

Figure 3.2. Expression of miR-182-5p (A and B) and *FOXO1* (C and D) in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 and 72 h. The presence of high glucose in HepG2 cells led to a reduction in the expression of miR-182-5p and an increase in the expression of *FOXO1*. However, when resveratrol was introduced in the presence of HG, there was an increase in the expression of miR-182-5p and a decrease in the expression of *FOXO1*. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; FOXO1, Forkhead box O1.

3.3. Increased mRNA expression of *FOXO1* increases the mRNA expression of *G6P* in HepG2 cells.

FOXO1 governs the transcription of downstream enzymes integral to gluconeogenesis, including PEPCK and G6P (Barthel *et al*., 2005; Gross *et al*., 2008). In this study, we investigated the mRNA expression of *G6P*. *G6P* showed a significant increase when cells were treated with HG over 48 and 72 h (*p*<0.0001) as compared with the control (Figure 3). When cells were treated with LR and HR alone over 48 and 72 h, we observed a significant decrease (*p*<0.0001) as compared to the control. Furthermore, when cells were treated with HG+LR and

HG+HR over 48 and 72 h, a remarkable decrease in the expression of *G6P* was observed (p<0.001) as compared to the HG group alone (Figure 3). Our study shows that increased expression of *FOXO1* (Figure 2 C and D) resulted in increased expression of *G6P* (Figure 3) while decreased expression level of *FOXO1* resulted in decreased expression of *G6P*.

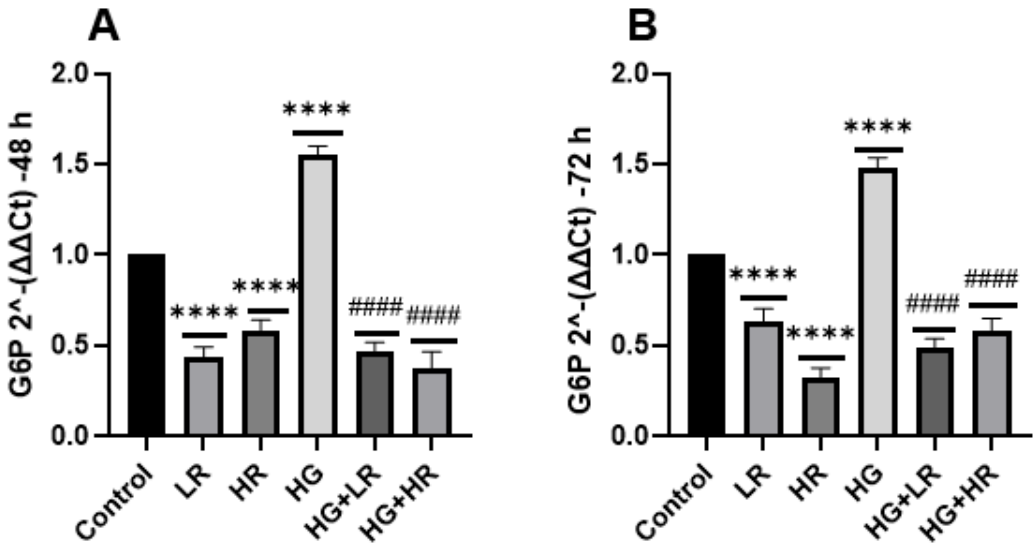

Figure 3.3: The mRNA expression of *G6P* in HepG2 cells treated with high glucose (40 Mm) and resveratrol (25 µM and 50 µM) over 48 (A) and 72 h (B). High glucose increases the expression of *G6P,* whereas resveratrol decreases the expression of *G6P*. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; G6P, Glucose-6-phosphatase.

3.4. Expression of miR-30a-5p and *Neurod1* in HepG2 cells.

The miRDB-microRNA target prediction Database shows that miR-30a-5p targets Neurod1 (http://www.mirdb.org/). We investigated the effect of high glucose and resveratrol on the expression of miR-30a-5p and Neurod1 in HepG2 cells. We observed that cells treated with HG significantly decreased miR-30a-5p over 48 and 72 h (p<0.0001) as compared to the control (Figure 4 A and B). *Neurod1* was significantly increased when cells were treated with HG for 72 h (p<0.0001), however, we did not observe any statistical significance when treated over 48 h (Figure 4 C And D). When cells were treated with LR and HR alone for 48 h, the LR group did not show any statistical difference in the expression of miR-30a-5p; however, HR significantly reduced miR-30a-5p (p=0.0002) as compared to controls (Figure 4 A and B). *Neurod1* significantly decreased when cells were exposed to LR and HR alone over 48 and 72 h (p<0.0001) as compared to controls (Figure 4 C and D). miR-30a-5p increased significantly when cells were treated with HG+LR and HG+HR over 48 and 72 h (p<0.0001) as compared to HG alone (Figure 4 A and B). A significant reduction in *Neurod1* mRNA expression was

observed in cells treated with HG+LR and HG+HR over 48 and 72 h (p<0.0001) as compared to HG alone. The findings of our study indicate that a decrease in miR-30a-5p levels led to an increase in *Neurod1* expression, while an increase in miR-30a-5p resulted in a significant reduction of *Neurod1* mRNA expression.

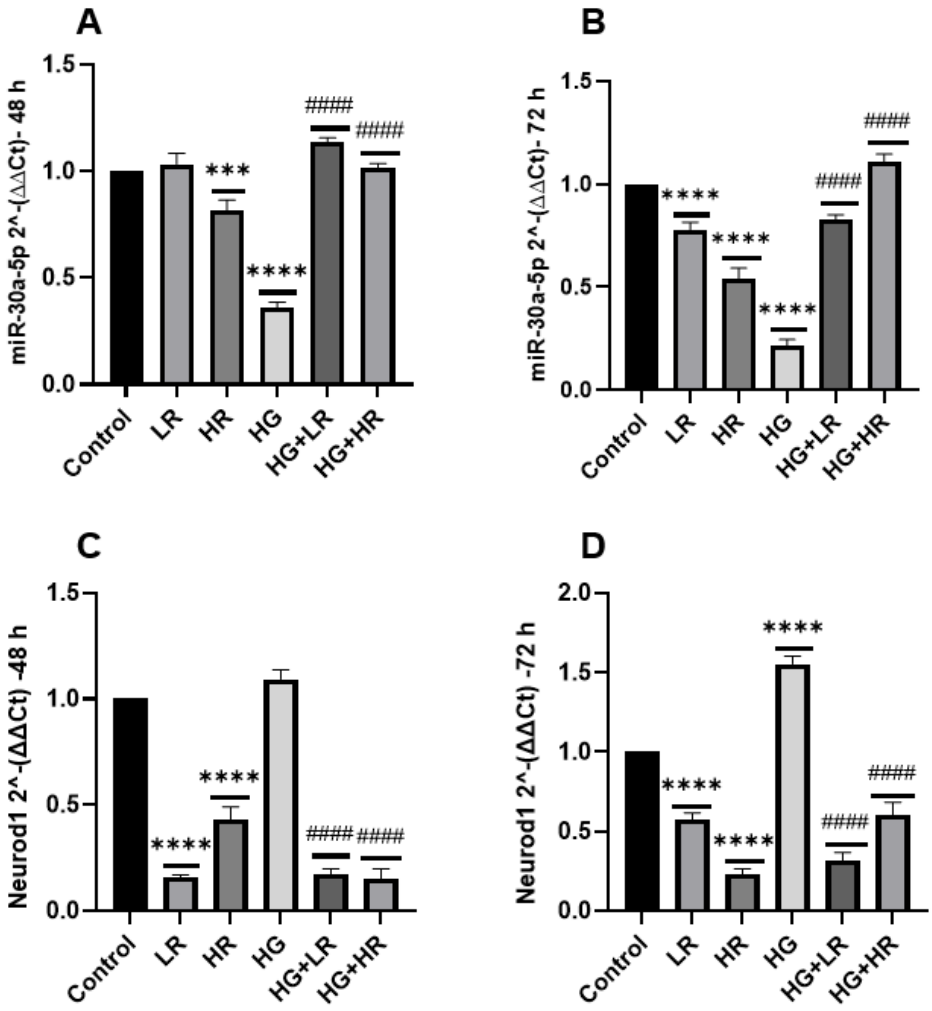

Figure 3.4: Expression of miR-30a-5p (A and B) and *Neurod1* (C and D) in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 and 72 h. High glucose significantly decreased the expression of miR-30a-5p while increasing *Neurod1* mRNA expression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; Neurod1, Neuronal differentiation 1.

4. Discussion

Despite recent advancements in understanding and managing DM, the disease prevalence continues to rise, with debilitating and life-reducing consequences for the global population. Due to their involvement in various biological processes, the aberrant expression of miRNAs may contribute to various pathophysiological conditions, making them promising targets for prevention or intervention of DM. Here, we assessed miR-126-3p, miR-182-5p, and miR-30a-

5p expression levels and their target genes in HepG2 cells exposed to high glucose (HG) and determined the effects of resveratrol (RES) exposure.

Our findings revealed significant downregulation of miR-126-3p, miR-182-5p, and miR-30a-5p expression levels in HepG2 cells following high glucose (HG) exposure for 48 and 72 h. These results align with previous research demonstrating reduced expression of miR-126-3p and miR-182-5p in conditions associated with diabetes (Arevalo-Martinez *et al*., 2021; Nyawo *et al*., 2021). MiR-126-3p has been identified as an essential regulator of vascular development and homeostasis, and its downregulation is associated with endothelial dysfunction in diabetes (Weale *et al*., 2021; Ait-Aissa *et al*., 2020). MiR-182-5p has been implicated in type 2 diabetes mellitus pathogenesis (Weale *et al*., 2021). However, our study revealed that miR-30a-5p downregulation is not widely researched regarding diabetes-related mechanisms. Additional investigation of this gene will likely help elucidate its precise role.

Resveratrol (RES), a polyphenol phytoalexin, has been studied for its potential therapeutic effects in diabetes (El-Sayed *et al*., 2022). We demonstrated that treatment with RES reversed the downregulation of miR-126-3p, miR-182-5p, and miR-30a-5p caused by high glucose (HG) in HepG2 cells, suggesting it may protect against miRNA dysregulation associated with diabetes. Furthermore, previous research demonstrated the effects of RES against oxidative stress, apoptosis, and glucose metabolism associated with diabetes. However, further investigation is required to identify how RES may impact miRNA dysregulation in diabetes.

Zeinali *et al.* (Zeinali *et al*., 2021) reported that miR-126-3p levels are associated with inflammation in patients suffering from pre-diabetes and T2DM. Their studies demonstrated that this miRNA is significantly lower in patients with pre-diabetes or T2DM compared with healthy individuals. Zampetaki *et al.* (Zampetaki *et al*., 2010) found that the levels of miR-126 in the plasma of diabetes patients had decreased, suggesting that endothelial dysfunction and inflammation were contributing factors. MiR-126 influences angiogenesis signalling and has been shown to target SPRED1 (Zeinali *et al*., 2021). Increasing SPRED1 expression in diabetes patients by suppressing miR-126 expression would promote IL-6, TNF-α, and ROS production, resulting in endothelial dysfunction (Li *et al*., 2016). This study aimed to conduct a further investigation into the mRNA expression of SPRED1. It has been observed that high glucose levels have a substantial impact on the mRNA expression of *SPRED1*. Conversely, treatment with RES has been shown to reduce the expression of *SPRED1*. Our study agrees with prior research, which indicates that a decrease in miR-126-3p expression leads to an increase in *SPRED1* expression. Additionally, augmented expression of miR-126-3p led to a decrease in the expression of *SPRED1*.

Based on these findings, the upregulation of miR-126-3p by RES in the present study suggests a plausible mechanism by which RES exerts its beneficial effects in diabetes. Resveratrol has

been noted for its anti-inflammatory properties and modulation of miRNA expression. A study by Mahjabeen *et al*. demonstrated a significant upregulation in the expression of miR-126 among individuals with T2DM following supplementation with resveratrol (Mahjabeen *et al*., 2022). Therefore, RES' upregulation of miR-126-3p may contribute to its anti-inflammatory benefits and potentially improve endothelial function in diabetes patients.

Additionally, the upregulation of miR-182-5p by RES is noteworthy, given its association with T2DM development (El-Sayed *et al*., 2022). While miR-182-5p's role in inflammation and diabetes remains poorly understood compared to that of miR-126-3p, its upregulation by RES suggests a possible underlying mechanism in treating diabetes.

The regulatory function of miR-182-5p in maintaining glucose homeostasis is thought to occur via *FOXO1*, a gene involved in gluconeogenesis (Kaur *et al*., 2020). MiR-182-5p may contribute to hyperglycemia in diabetes by increasing the expression of FOXO1, leading to upregulated gluconeogenesis. This study investigated the mRNA expression of *FOXO1* and *G6P*. Intriguingly, our findings demonstrated that reduced miR-182-5p expression correlated with increased *FOXO1* expression as well as heightened *G6P* mRNA expression, accentuating gluconeogenesis. Furthermore, the present study revealed that RES treatment significantly increased miR-182-5p expression. This elevation, in turn, precipitated a reduction in *FOXO1* mRNA expression thereby reducing *G6P* mRNA expression, ultimately dampening gluconeogenesis. This suggests that RES may play an essential role in glucose homeostasis by modulating miR-182-5p and its target genes, such as *FOXO1*. Our observations of an upregulated miR-182-5p by RES correlate well with previous research by Zuo *et al*. (Zuo *et al*., 2021), who demonstrated that miR-182-5p directly targets and suppresses FOXO1 expression, leading to decreased hepatic lipid accumulation due to alcohol-related liver disease (ALD). Their results demonstrate that RES may modulate miR-182-5p and its downstream targets to improve glucose metabolism and homeostasis in diabetes patients.

MiR-30a-5p upregulation by RES is particularly interesting as its involvement has been implicated in diabetes pathogenesis and beta-cell dysfunction (Kim *et al*., 2013). Glucotoxicity, associated with the gradual degradation of beta cell function characteristic of T2DM, has been found to upregulate miR-30a-5p expression in beta cells. *In vitro* studies have revealed that excessive expression of miR-30a-5p results in dysfunctional beta cells characterized by reduced insulin levels and weakened insulin secretion stimulated by glucose. The induction of miR-30a-5p, triggered by glucotoxicity-induced beta cell dysfunction, inhibits Beta2/NeuroD gene function regulation in beta cell therapy. The findings of our study indicate that high glucose levels resulted in increased *Neurod1* mRNA expression. Our study showed that RES treatment increased expression levels for miR-30a-5p while decreasing the mRNA expression of *Neurod1* in HepG2 cells. Although further investigation of the specific implications of miR-

30a-5p upregulation by RES in diabetes requires further study, this evidence indicates that RES may alter miR-30a-5p expression and may influence beta cell function and glucose homeostasis. Further research is required to determine the exact mechanism underlying RES function, as well as the therapeutic applications in diabetes.

Overall, our findings suggest that RES could effectively regulate miRNA dysregulation in hyperglycemia-related diseases. By countering HG-induced downregulation of miR-126-3p, miR-182-5p, and miR-30a-5p and potentially contributing to their restoration, RES may regulate miRNA levels and assist in managing or preventing complications related to diabetes. Moreover, RES demonstrated the capability to counteract the impact imposed by miRNAs on their target genes. It is important to note that our study was conducted *in vitro,* and further research must be performed in animal models, such as diabetes-induced mice, to validate these findings. Furthermore, RES may be included in a community study with diabetes patients to determine if RES improves glucose management. Our findings illustrate the potential of RES as a therapeutic agent to manage diabetes by modulating miRNA expression and its target genes. Future studies may help validate the mechanisms of RES underlying miRNA regulation in both *in vitro* and *in vivo* models. Furthermore, novel therapeutic strategies targeting miRNA dysregulation may offer new avenues to prevent and treat diabetes and its arising complexities.

This study has several strengths and limitations. First, an *in vitro* model allows for controlled experimental conditions and focused analysis. Second, these findings align with previous research, adding to our existing knowledge of how miRNAs may become dysregulated during diabetes-related conditions. Regarding the limitations, our study focused solely on HepG2 cells. Therefore, it may not adequately represent the complexity of miRNA regulation within diabetes pathophysiology. Additionally, this study used 40 mM as a high glucose concentration, other studies may use 25 mM or 30 mM as a high glucose concentration to better reflect the clinical relevance for diabetes patients. Furthermore, no investigation was made into the functional implications of downregulated miRNAs or their specific roles within diabetes-related mechanisms; however, this research will be conducted in subsequent studies by our research group. Further investigations using diverse cell models and experimental methods are recommended to gain a comprehensive understanding of the roles played by these miRNAs within the pathophysiology of diabetes.

5. Conclusions

This study provides evidence supporting the role of three specific miRNAs, namely miR-126-3p, miR-182-5p, and miR-30a-5p, as potential biomarkers for diabetes. The downregulation of these microRNAs in HepG2 cells exposed to high glucose levels indicates their involvement in the development or progression of diabetes. Moreover, this study presents supplementary findings that support the notion that miR-126-3p affects *SPRED1* expression, miR-182-5p

affects *FOXO1*, and miR-30a-5p influences *Neurod1*. This suggests that the dysregulation of miRNA expression caused by high glucose levels may contribute to the disruption of metabolic processes associated with diabetes. Moreover, the findings highlight the therapeutic potential of resveratrol. To our knowledge, this study is the first attempt to study the link between resveratrol and diabetes-specific miRNAs in HepG2 cells. Interestingly, resveratrol was found to reverse the downregulation of microRNAs induced by high glucose, indicating its potential as a therapeutic approach for treating diabetes. This study not only improves our understanding of the molecular mechanisms underlying diabetes but also uncovers a promising avenue for therapeutic intervention through the modulation of miRNA expression. The identification of these specific miRNAs as potential biomarkers for diabetes provides valuable insights for the development of diagnostic tools and personalized treatment strategies. Further research is warranted to explore the precise mechanisms by which these miRNAs and resveratrol exert their effects and to evaluate their clinical applicability in the management of diabetes. The intricate interplay between glucose metabolism, microRNA regulation, and resveratrol's therapeutic effects may pave the way for novel therapeutic approaches in combating diabetes and its complications.

Author Contributions: Conceptualization, Shanel Raghubeer; Data curation, Abegail Tshivhase; Formal analysis, Abegail Tshivhase; Funding acquisition, Tandi Matsha; Investigation, Abegail Tshivhase and Shanel Raghubeer; Methodology, Abegail Tshivhase and Shanel Raghubeer; Project administration, Shanel Raghubeer and Tandi Matsha; Supervision, Shanel Raghubeer and Tandi Matsha; Visualization, Abegail Tshivhase; Writing – original draft, Abegail Tshivhase; Writing – review & editing, Shanel Raghubeer and Tandi Matsha.

Funding: This research project was supported by a grant from the South African Medical Research Council (SAMRC), with funds from the National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant no. 115450). Department of Science and Innovation (DSI)-Inter-bursary Support Programme-Council for Scientific and Industrial Research (CSIR) (awarded to AMT).

Declaration of Interest: The authors declare no conflict of interest.

Acknowledgments: The authors would like to thank Prof JL Marnewick for donating HepG2 cells to us for the experimental work.

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CHAPTER 4: Resveratrol attenuates high glucose-induced inflammation and improves glucose metabolism in HepG2 cells

(Published in *Scientific Reports,* https://doi.org/10.1038/s41598-023-50084-6).

Resveratrol attenuates high glucose-induced inflammation and improves glucose metabolism in HepG2 cells

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Abstract

Diabetes mellitus (DM) is characterized by impaired glucose and insulin metabolism, resulting in chronic hyperglycemia. Hyperglycemia-induced inflammation is linked to the onset and progression of diabetes. Resveratrol (RES), a polyphenol phytoalexin, is studied in diabetes therapeutics research. This study evaluates the RES effect on inflammation and glucose metabolism in HepG2 cells exposed to high glucose. Inflammation and glucose metabolism-related genes were investigated using quantitative polymerase chain reaction (qPCR). Further, inflammatory genes were analyzed by applying enzyme-linked immunoassay (ELISA) and Bioplex. High glucose significantly increases inhibitory-kB kinase alpha (IKK-α), IkB-alpha (IKB-α), and nuclear factor-kB (NF-kB) expression than the controls. NF-kB's increased expression was followed by increased expression of pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin-1 beta (IL-β), and cyclooxygenase-2 (COX2). RES treatment significantly reduced the expression of NF-kB, IKKα, and IKB-α, as well as pro-inflammatory cytokines. High glucose levels reduced the expression of transforming growth factor-beta 1 (TGFβ1), while treatment with RES increased the expression of TGFβ1. As glucose levels increased, Phosphoenolpyruvate carboxylase (PEPCK) expression was reduced, and glucokinase (GCK) expression was increased in HepG2 cells treated with RES. Further, HepG2 cells cultured with high glucose showed significant increases in Kruppel-like factor 7 (KLF7) and hypoxia-inducible factor 1 Alpha (HIF1A) but decreased in Sirtuin 1 (SIRT1). Moreover, RES significantly increased SIRT1 expression and reduced KLF7 and HIF1A expression levels. Our results indicated that RES could attenuate high glucose-induced inflammation and enhance glucose metabolism in HepG2 cells.

1.Introduction

Diabetes Mellitus (DM) has imposed a significant burden on global healthcare systems due to its increasing incidence and prevalence, a trend projected to rise in the future (Lin *et al*., 2023); (Goyal, 2018). Type 2 DM (T2DM) is a metabolic condition characterized by chronic hyperglycemia resulting from relative insulin deficiency (Wu *et al*., 2014; Mukai *et al*., 2022). It accounts for about 90-95% of all diabetes cases. Chronic hyperglycemia often results in microvascular complications, such as nephropathy, neuropathy, and retinopathy (Faselis *et al*., 2019).

To fulfil the energy needs of vital organs and maintain a healthy metabolism, glucose homeostasis is strictly regulated. In this regard, the liver plays a vital role by regulating multiple glucose metabolic pathways, including glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis (Han *et al*., 2016). Research has demonstrated that abnormal glucose metabolism in the liver is one of the primary causes of T2DM. Individuals with diabetes often have disrupted glycogenesis and glycogenolysis, with glycogenesis playing a particularly important role (Rines *et al*., 2016). Enzymes responsible for gluconeogenesis and glycogenesis are often elevated in hyperglycemic livers, whereas glycolysis enzymes are attenuated (Zhou *et al*., 2015). Phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G6P) are the main enzymes in the liver that regulate the conversion of non-sugar substances into glucose in the process of gluconeogenesis (Zhu *et al*., 2021). The elevated expression of these enzymes is linked to increased gluconeogenesis (Rui, 2014). Glycolysis is the pathway by which glucose is broken down into pyruvate/lactate after glucose uptake by the cells and glucose phosphorylation. Glucokinase (GCK) is an important regulatory enzyme in glycolysis (Guo *et al*., 2012). The reduced activity of GCK has been associated with individuals with T2DM (Caro *et al*., 1995; Clore *et al*., 2000; Basu *et al*., 2001). Therefore, understanding the regulation of GCK and PEPCK activity and their function in glycolysis and gluconeogenesis is essential for the development of efficient treatment for individuals with T2DM.

Previous research has shown inflammation to be a critical factor in the pathogenesis of T2DM (Phillip James White and André Marette, 2008). Nuclear factor-kB (NF-kB) activation is linked with inflammatory response activation (Jeon *et al*., 2010). It regulates the expression of pro-inflammatory genes. Tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, interleukin (IL)-8, interleukin-1 beta (IL-1β), and cyclooxygenase-2 (COX2) are mediated by NF-kB (Tak & Firestein, 2001). NF-kB is activated by high glucose levels, which activates the expression of inflammatory cytokines, including TNF-α and IL-6 (Yun *et al*., 2011; Miao *et al*., 2004). To mitigate the ongoing inflammatory response, the strategic inhibition of pro-inflammatory cytokine production and secretion has been postulated as a prospective approach to halt the

onset of diabetes (Tsalamandris *et al*., 2019). Previous research has shown that transforming growth factor-beta 1 (TGFβ1) has demonstrated substantial regulatory characteristics within inflammation (Li & Flavell, 2008). Prior research has established that TGFβ1 possesses anti-inflammatory characteristics by neutralizing pro-inflammatory cytokines (Park *et al*., 2000).

Sirtuin 1 (SIRT1), an extremely conserved NAD+-dependent deacetylase, is a critical enzyme in aging and metabolism, including adapting gene expression and metabolism to the cellular energy state(Rai *et al*., 2012; Takeda-Watanabe *et al*., 2012). Furthermore, SIRT1 functions as a suppressor of NF-kB activity. It inhibits transcription by deacetylating the NF-kB subunit RelA/p56 at lysine 310 (Yeung *et al*., 2004; Yang *et al*., 2012). Kruppel-like factor 7 (KLF7), the first discovered transcriptional factor amongst the KLF family, has been reported to play a fundamental role in regulating glucose and lipid metabolism and inflammation (Qiu *et al*., 2022) KLF7 can promote pro-inflammatory IL-6 cytokine expression and prevent glucose metabolism in human Islet and HepG2 cells (Kawamura *et al*., 2006Hafidi *et al*., 2019) Hypoxia-inducible factor 1 Alpha (HIF1A) is another transcriptional factor involved in inflammation and glucose metabolism. It is vital in regulating pro-inflammatory gene expression and cytokine production (Fitzpatrick *et al*., 2018) Therefore, a natural compound with the capability to regulate these transcriptional genes may be valuable in managing inflammatory diseases and metabolic disorders.

Currently, there are several chemical agents for glycemic control utilized in T2MD therapy. However, they are associated with severe side effects such as hypoglycemia and weight gain or contraindications that restrict their use which necessitates the search for an effective T2DM treatment method (Bain *et al*., 2016; Zhu *et al*., 2017). In this regard, natural compounds with anti-diabetic activity and fewer side effects can be effective for T2DM treatment (Ku *et al*., 2015). Several indigenous plants have been utilized for the management or treatment of diabetes. Some have been investigated, and their active ingredients have been isolated (Li *et al*., 2013).

Resveratrol (RES) is a polyphenol phytoalexin known as trans-3,4,5-trihydroxystilbene. Studies have shown that RES has an antihyperglycemic effect resulting in improved blood glucose parameters, inflammation, and insulin resistance (Imamura *et al*., 2017). Due to this, RES has been implicated in the management of T2DM. This study aims to evaluate the effects of resveratrol on glucose metabolism and inflammation in high glucose-induced HepG2 cells. Understanding its potential as a treatment for diabetes and comprehending the basic molecular pathway may aid in developing novel strategies to combat glucose dysregulation and inflammation in diabetes.

2. Methods

2.1. Study design

Cells were categorized into six groups: Control (cultured in normal complete culture medium (CCM)), Low resveratrol (LR; cultured in normal CCM + 25 µM RES), High resveratrol (HR; cultured in normal CCM + 50 µM RES), High glucose (HG; cultured in normal CCM + 40 mM glucose), LR+HG (cultured in normal CCM + 25 µM RES + 40 mM glucose), and HR+HG (cultured in normal CCM + 50 µM RES + 40 mM glucose). A literature search was employed to determine the concentrations and exposure periods for glucose and resveratrol treatments. For resveratrol treatment, research conducted by Baselga-Escudero *et al*. and Raghubeer *et al*. reported the use of 50 µM and 25 µM resveratrol, respectively (36; 37). Similarly, several research demonstrated the use of 40 mM glucose to represent "hyperglycemic" or high glucose (HG) conditions(Chu *et al*., 2011; Leinninger *et al*., 2004; Varma *et al*., 2005; Kapoor & Kakkar, 2012). Therefore, in this study, 40 mM was used as a high glucose concentration, and for resveratrol, 25 µM, and 50 µM were used. Resveratrol was prepared in 100% dimethyl sulphoxide (DMSO).

2.2. Cell culture

The HepG2 cell line was purchased from Merck (Darmstadt, Germany; catalogue number 85011430). Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penstrepfungizone (PSF), and 1% L-glutamine was utilized for culturing HepG2 cells in 25 cm3 flasks in a monolayer (106 cells per flask) in a 37 °C humidified incubator (5% CO2). Phosphate-buffered saline (PBS) (0.1 M) was used to wash the cells. Cells were treated with RES (25 µM and 50 µM) and HG (40 mM) upon reaching 70-80% confluent and incubated for 48 and 72 h. Afterward, trypsin was used to remove the cells, and cells were counted using the trypan blue exclusion method of cell counting. Briefly, 60 µL CCM + 20 µL cell suspension + 20 µL trypan blue solution was incubated for 5 minutes at room temperature. A coverslip (22 x 22 cm) was placed on a clean hemocytometer. Then 10 L of well-mixed counting solution was distributed into the middle bar of the hemocytometer. The number of living cells was then determined using a microscope. The cell viability was evaluated using the standard equation (Live cell average x 5 (dilution factor) x 10,000 = cells/mL).

2.3. RNA isolation and gene expression analysis

Total RNA was isolated using a Trizol reagent according to the manufacturer's protocol. The isolated total RNA was quantified using Nanodrop spectrometry (Nanodrop one C, Thermo Fisher Scientific, Wilmington, DE, USA). The iScript cDNA synthesis kit (Bio-Rad) was utilized for cDNA synthesis by the manufacturer's guidelines. Once cDNA was completed successfully, the amplification of mRNA was performed using Applied Biosystems™ QuantStudio™ 7 Flex (Thermo Fisher Scientific, USA) with the following reaction mixture: 5 µL SsoAdvanced™

Universal SYBR® Green Supermix (Bio-Rad), 1.5 µL cDNA, 0.5 µL forward and reverse primers, and 2.5 µL nuclease-free water was made up to 10 µL. The primers (purchased from Inqaba Biotechnical Industries (Pretoria, South Africa)) used are shown in Table 1. *GAPDH* was utilized as a housekeeping gene, with three replicates per treatment. The mRNA expression level in each sample was determined using the $2^{-\Delta Ct}$ method, and the $2^{-\Delta\Delta Ct}$ value was used to compare the mRNA expression level in each sample to the control (Livak & Schmittgen, 2001).

Table 4.1: Primers used in this study.

Gene names	Forward	Reverse	Annealing temperature °C
GAPDH	5' TCCACCACCCTGTTGCTGTA 3'	5' ACCACAGTCCATGCCATCAC 3'	
SIRT1	5' TGCCGGAAACAATACCTCCA 3'	5' AGACACCCCAGCTCCAGTTA 3'	55
IkB-α	5' TGCACTTGGCCATCATCCAT 3'	5' TCTCGGAGCTCAGGATCACA 3'	60
NFk-B	5' ATGTGGAGATCATTGAGCAG C 3'	5' CCTGGTCCTGTGTAGCCATT 3'	58
IKKα	5' GGCTTCGGGAACGTCTGTC 3'	5' TTTGGTACTTAGCTCTAGGCGA 3'	60
COX2	5' TAAGTGCGATTGTACCCGGA C 3'	5' TTTGTAGCCATAGTCAGCATTG T 3'	55
IL-6	5' ACTCACCTCTTCAGAACGAAT TG 3'	5' CCATCTTTGGAAGGTTCAGGTT G 3'	55
TNF-α	5' GCTGCACTTTGGAGTGATCG 3'	5' TCACTCGGGTTCGAGAAGA 3'	55
GCK	5' TGGACCAAGGGCTTCAAGGC C 3'	5' CATGTAGCAGGCATTGCAGCC 3'	55
PEPCK	5' CTTTTTCGGTGTCGCTCCTG 3'	5' GACACCTGAAGCTAGCGGCT 3'	55
HIF1A	5' GAACGTCGAAAAGAAAAGTC TCG 3'	5' CCTTATCAAGATGCGAACTCAC A 3'	55
KLF7	5' GGTGAGCCAGACAGACTGAC AA 3'	5' GAAGTAGCCGGTGTCGTGGA 3'	55

2.4. Enzyme-linked Immunosorbent Assay (ELISA)

The culture supernatant was collected 48 and 72 h after treatment of HGR, LR, HR, LR+HG, and HR+HG. The ELISA kits used to detect human TNF-α (CAT no: DY210-05) and IL-1β (CAT no: DY201-05) were purchased from R&D Systems Biotechnology Company

(Minneapolis, Minnesota, United States). The assay was performed in accordance with the manufacturer's protocol.

2.5. Multiplex cytokines assay

The supernatant collected after 48 and 72 h treatments were used in the Bio-Plex 200 system (Bio-Rad) to detect the concentration of the cytokines. The Bio-plex Pro Human Cytokine Grp 1 Panel 27-Plex (Bio-Rad, USA) was used per the manufacturer's protocol. In this study, only two cytokines (IL-6 and IL-1β) were analyzed using Bio-plex Manager Software.

2.6. Statistical analysis

All data analyses were conducted using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California, USA). The statistical methods employed included the Student's t-test and one-way analysis of variance (ANOVA). All experiments were conducted in triplicate, and statistical significance was determined at a threshold of $p<0.05$.

3.Results

3.1. Resveratrol reversed the increased pro-inflammatory cytokines caused by high glucose in HepG2 cells.

HepG2 cells were cultured under various conditions for 48 and 72 h, and the mRNA expression levels of TNF-α, IL-6, and COX2 were analyzed using qPCR (Figure 1). Furthermore, human TNF-α and IL-1β ELISA were performed using collected supernatant (Figure 2). Interestingly, as shown in Figure 1, the expression patterns of the three inflammatory cytokines were similar. In HepG2 cells cultured with LR and HR for 48 h and LR for 72 h, no statistical difference was observed in the expression levels of TNF-α, and IL-6 as compared to control cells (Figure 1A, C, and D). The TNF-α and IL-6 mRNA expression levels, were significantly reduced ($p<0.00001$; $p=0.0109$, respectively) when cells were cultured with HR for 72 h. When cells were cultured with LR and HR over 72 h, the expression of COX2 was significantly decreased as compared to control group ($p=0.0008$ and $p<0.0001$, respectively) (Figure 1F). In ELISA results, when cells were cultured with LR and HR over 48 and 72 h, no statistical difference was observed in the concentration of TNF-α and IL-1β as compared to the control (Figure 2). The mRNA expression levels of TNF-α, IL-6, and COX2 were increased significantly ($p<0.0001$) in the HG group compared to the control group (Figure 1). Similar to ELISA's results, the expression levels of TNF-α and IL-1β increased significantly in the HG group as compared to control cells ($p<0.0001$) (Figure 2). These results indicate that high glucose levels can lead to an increase in pro-inflammatory cytokine expression.

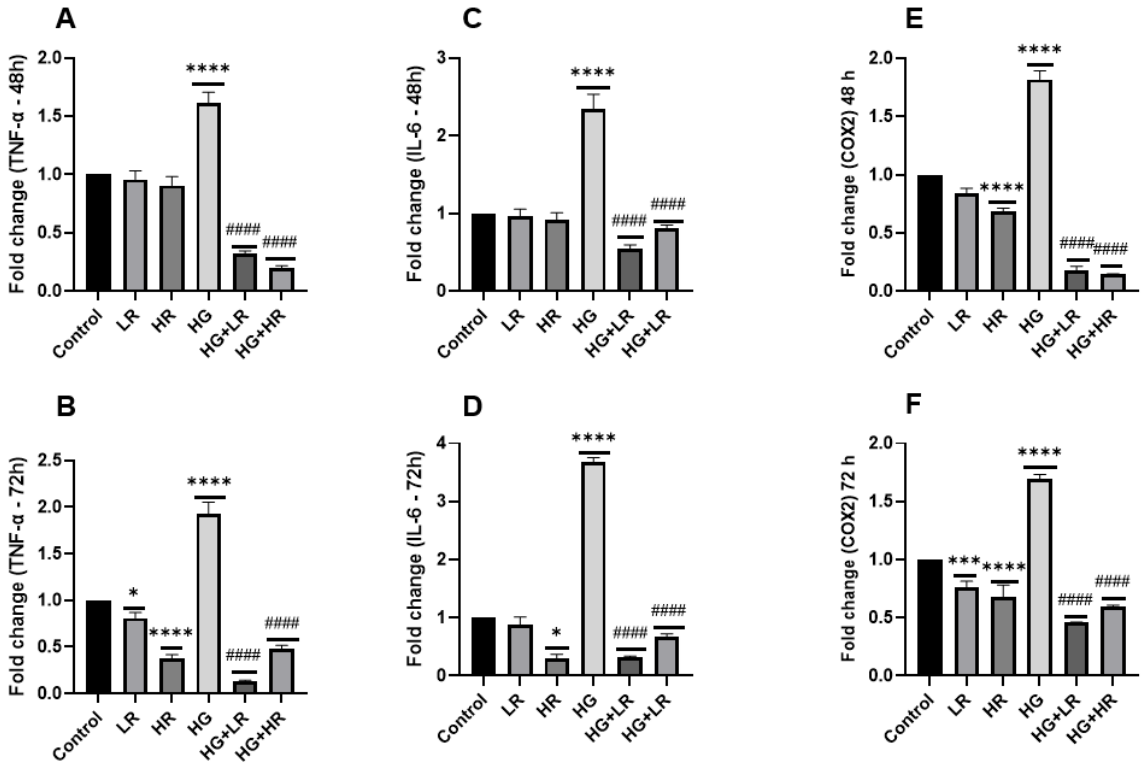

Figure 4.1: The expression levels of pro-inflammatory cytokines in HepG2 cells cultured with high glucose (40 mM) and resveratrol (25 µM AND 50 µM) over 48 and 72 h. (A) Expression of TNF-α cultured over 48 h. (B) Expression of TNF-α cultured over 72 h. (C) Expression of IL-6 cultured over 48 h. (D) Expression of IL-6 cultured over 72 h. (E) Expression of COX2 cultured over 48 h. (D) Expression of COX2 cultured over 72 h. GAPDH was utilized as the housekeeping gene. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; TNF-α, Tumor necrosis factor alpha; IL-6, interleukin-6; COX2, Cyclooxygenase-2.

According to our qPCR and ELISA findings, HG levels increased the expression of pro-inflammatory cytokines in HepG2 cells. To investigate the anti-inflammatory effect of RES, HepG2 cells were exposed to HG in the presence of RES. The mRNA expression levels of TNF-α, IL-6, and COX2 were significantly decreased (p<0.0001) when HepG2 cells were cultured with LR+HG and HR+HG as compared to HG alone (Figure 1). Similarly, to the qPCR results, in our Elisa results, we observe that the concentration of TNF-α and IL-1β were significantly decreased when cells were cultured with LR and HR in the presence of HG as compared to HG alone (p<0.0001). These results suggest that RES has a potential anti-inflammatory effect on HepG2 cells exposed to HG. Furthermore, the significant decrease in TNF-α, IL-6, COX2, and IL-1β expression levels indicates that RES may have a role in mitigating the pro-inflammatory response induced by HG levels in HepG2 cells.

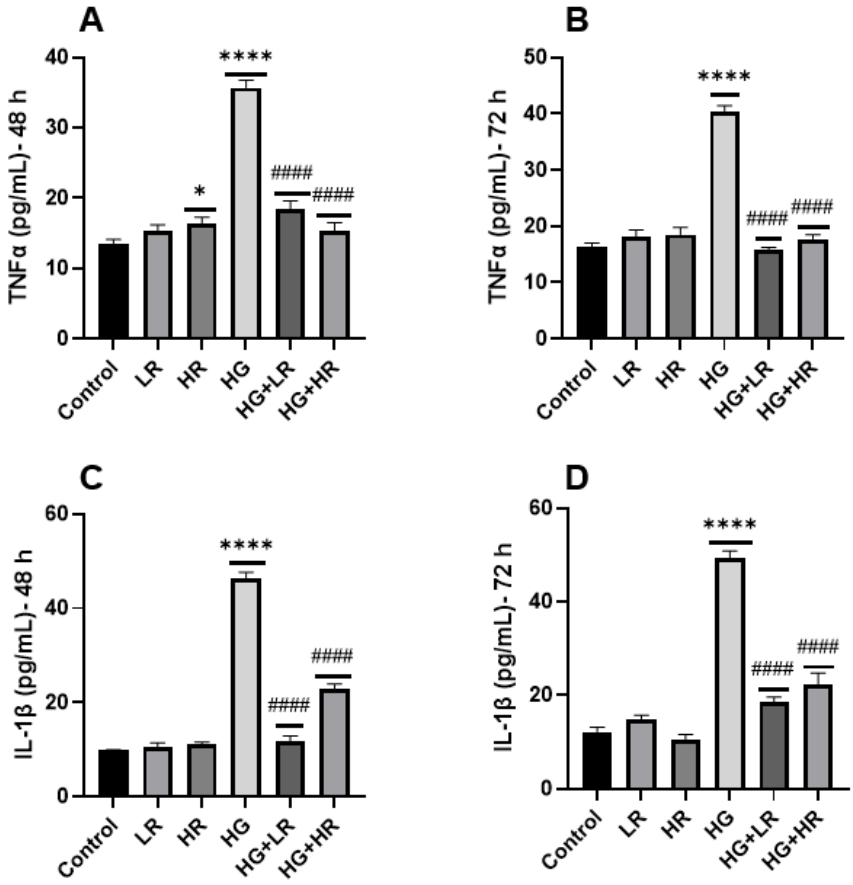

Figure 4.2: The ELISA for TNF-α (A and B) and IL-β (C and D) after high glucose treatment (40 mM), Low resveratrol (25 μM), High resveratrol (50 μM), High glucose + Low resveratrol (40 mM + 25 μM), and High glucose + High resveratrol (40 mM + 50 μM) treatments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.
LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; TNF-α, Tumor necrosis alpha; IL-1β, Interleukin-1 beta

3.2. IL-6 and IL-1β cytokines levels

In agreement with ELISA and qPCR results, the Bio-Plex assay revealed that when HepG2 cells were cultured with HG, the concentration of IL-6 and IL-1β cytokines were significantly higher as compared to control cells over 48 h (p<0.0001) and 72 h (p<0.0001; p=0.0109) (Figure 3). IL-6 was significantly reduced when HepG2 cells were cultured with HR in the presence of HG over 48 and 72 h (p=0.206; p=0.0013, respectively); however, no statistical difference was observed when HepG2 cells were cultured with LR+HG over 48 and 72 h (Figure 3A and B). We observed that IL-1β was significantly reduced when HepG2 cells were cultured with both LR and HR in the presence of HG over 48 h (p<0.0001) and 72 h (p=0.0435) as compared to the HG group alone (Figure 3C and D).

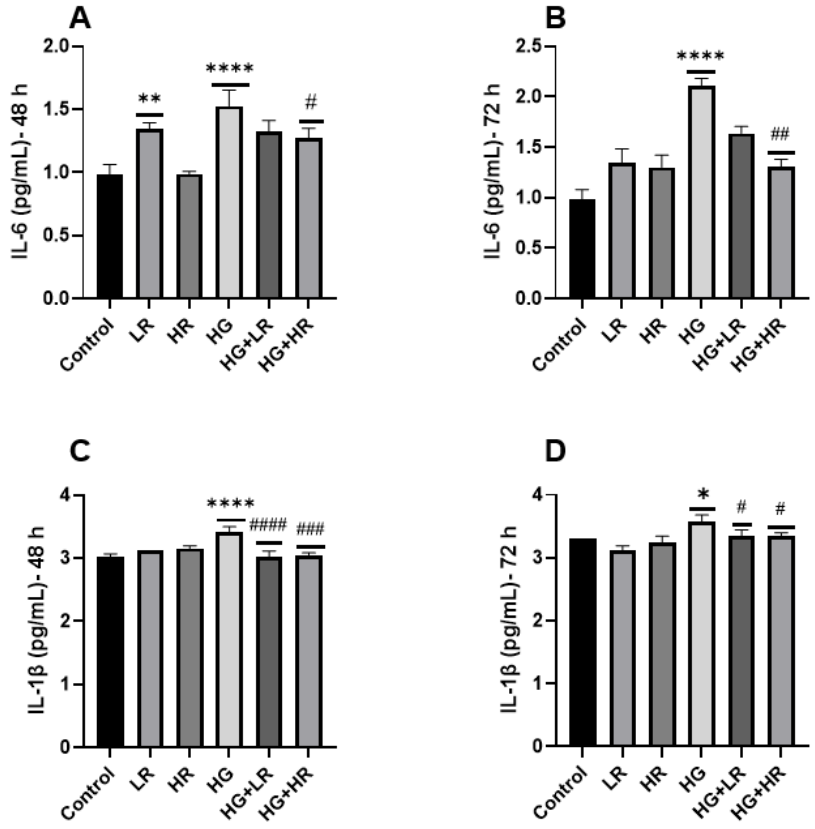

Figure 4.3: The Bio-Plex cytokines assay. A) IL-6 48h, B) IL-6 72 h, C) IL-1β 48 h and D) IL-1β 72 h. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol.

3.3. The expression levels of NF-kB, IKKα, and IkB-α in HepG2 cells

As shown in Figure 4 E and F, when HepG2 cells were cultured with HG, the NF-kB mRNA expression was significantly increased compared to control cells (*p*<0.0001). Moreover, the mRNA expression of IKKα and IkB-α was significantly increased in the HG group (*p*<0.0001; Figure 4 A, B, C, and D), suggesting activation of NF-kB signaling pathway. To explore the anti-inflammatory effect of RES, HepG2 cells were cultured with LR and HR concentrations in the presence and absence of HG. Interestingly, NF-kB, IKKα, and IkB-α mRNA expressions were significantly decreased when exposed to LR and HR in the presence of HG over 48 and 72 h (*p*<0.0001). When cells were exposed to LR and HG in the absence of HG, no statistical difference was observed in the expression of NF-kB as compared to control cells. IKKα and IkB-α did not show any statistical difference when cells were cultured to LR and HR over 48 h; However, IKKα was significantly decreased when cells were cultured with LR and HR (*p*<0.0001; *p*=0.0255, respectively). A significant decrease was also observed when cells were cultured with HR over 72 h (*p*<0.0001). The decrease in NF-kB, IKKα, and IkB-α expression with RES treatment indicates its potential to modulate the NF-kB signaling pathway.

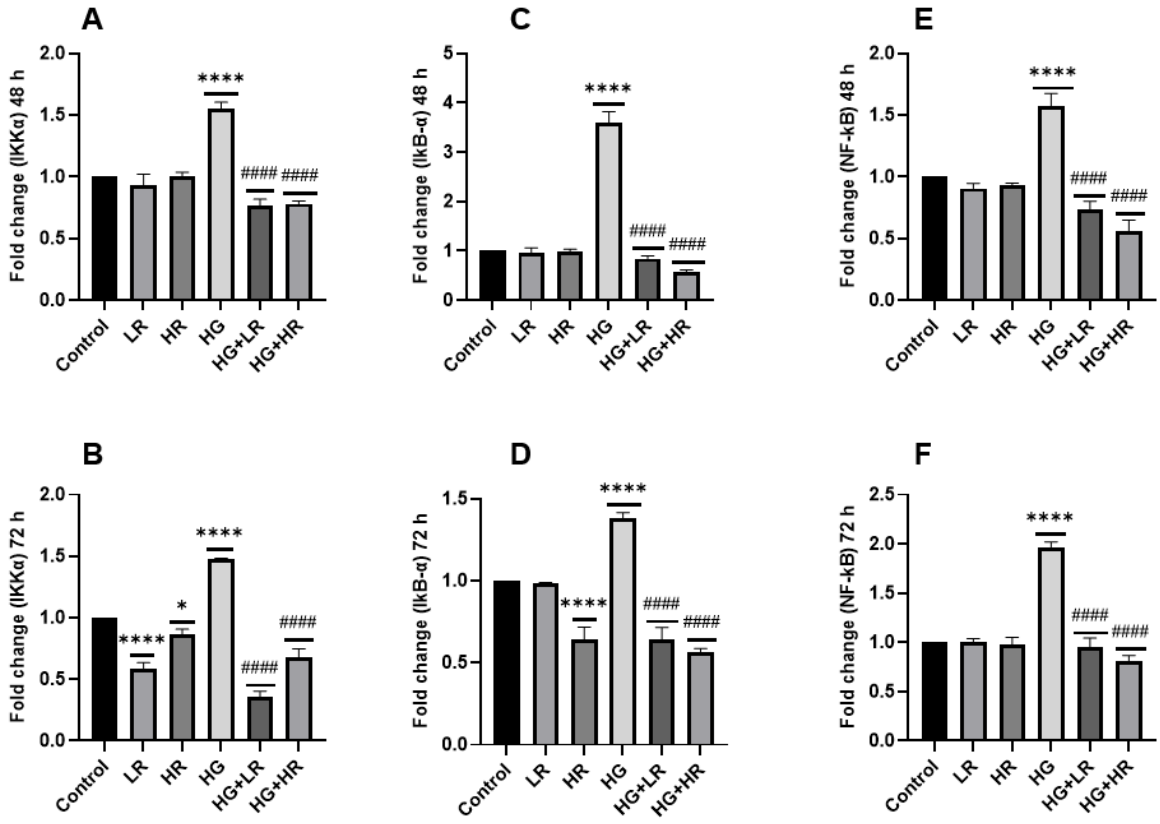

Figure 4.4: The mRNA expression of IKKα (A and B), IkB-α (C and D), and NF-kB (E and F) after high glucose (40 mM) and resveratrol (25 µM and 50 µM) treatment over 48 and 72 h. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG. LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; IKK-α, inhibitory-κB kinase alpha; IKB-α, IkB-alpha; NF-kB, nuclear factor-kB.

3.4. Resveratrol effectively mitigated decreased expression of TGFβ1 induced by high glucose in HepG2 cells.

HG levels reduced the expression of TGFβ1 in HepG2 cells. No statistically significant differences were observed when HepG2 cells were exposed to HG for 48 h compared to the control group (Figure 5A). However, when the exposure was extended 72 h, a significant reduction in the expression of TGFβ1 was observed (p=0.0043, Figure 5B). The HepG2 cells were exposed to LR and HR treatment for 48 and 72 h, respectively. The levels of TGFβ1 expression exhibited a significant increase in HepG2 cells following exposure to LR (p<0.0001) and HR (p<0.0001; p=0.0016) for 48 and 72 h. The expression levels of TGFβ1 were significantly increased when HepG2 cells were exposed to RES in the presence of HG for 48 and 72 h as compared to HG alone (p<0.0001).

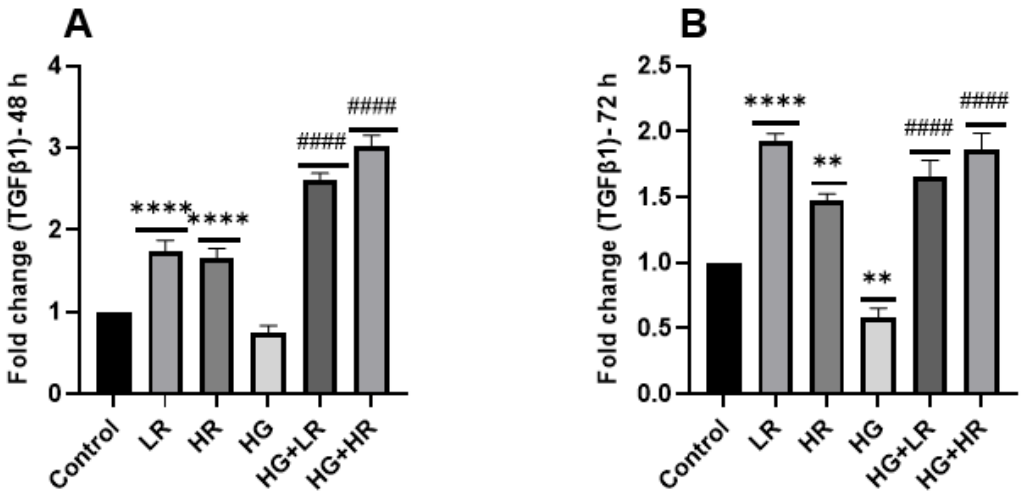

Figure 4.5: The mRNA expression of TGFβ1 exposed to high glucose and resveratrol over 48 and 72 h. High glucose decreased the expression levels of TGFβ1, whereas resveratrol treatment increased the expression levels of TGFβ1. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.

LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; TGFβ1, Transforming growth factor beta 1.

3.5. Effect of high glucose and resveratrol on the expression of GCK and PECK.

The effect of HG and RES on the expression of glycogenesis and gluconeogenesis-related genes in HepG2 liver cells was evaluated. When compared to the control group, HepG2 cells cultured with HG over 48 and 72 h showed a significant decrease in the expression of *GCK* (*p*=0.0002 and *p*=0.0001, respectively) (Figure 6 A and B). A significant increase in the mRNA expression levels of *PEPCK* was observed in HepG2 cells culture with HG over 48 and 72 h as compared to the control group (*p*<0.0001; *p*=0.0003) (Figure 6 C and D). These results indicate impaired glucose metabolism in HepG2 cells. HepG2 cells were also treated with LR and HR alone. When compared to the control group, no statistical difference was observed in the expression of *GCK* when cells were cultured with LR and HR over 48 h and with HR over 72 h; however, a statistical difference was observed when HeG2 cells treated with LR over 72 h (*p*<0.0001) (Figure 6 A and B). *PEPCK* showed no significant difference when HepG2 cells were cultured with HR over 48 h compared to the control group; however, a significant decrease was observed when cells were cultured with LR for 48 h (*p*<0.0001) and when cultured with LR and HR for 72 h (*p*=0.0034; *p*=0.0003, respectively) (Figure 6 C and D).

To detect the efficiency of RES on the expression of *GCK* and *PECK*, HepG2 cells were cultured with RES in the presence of HG. The mRNA expression of *GCK* was significantly increased in HepG2 cells treated with both LR and HR in the presence of HG (*p*<0.0001) (Figure 6 A and B) as compared to HG alone. Furthermore, *PEPCK* showed a significant decrease when HepG2 cells were treated with LR+HG and HR+HG (*p*<0.0001) (Figure 6 A

and B), as compared to HG group. The increased *GCK* and reduced *PEPCK* expression level may indicate the potential role of RES in regulating glucose metabolism in liver cells under HG conditions.

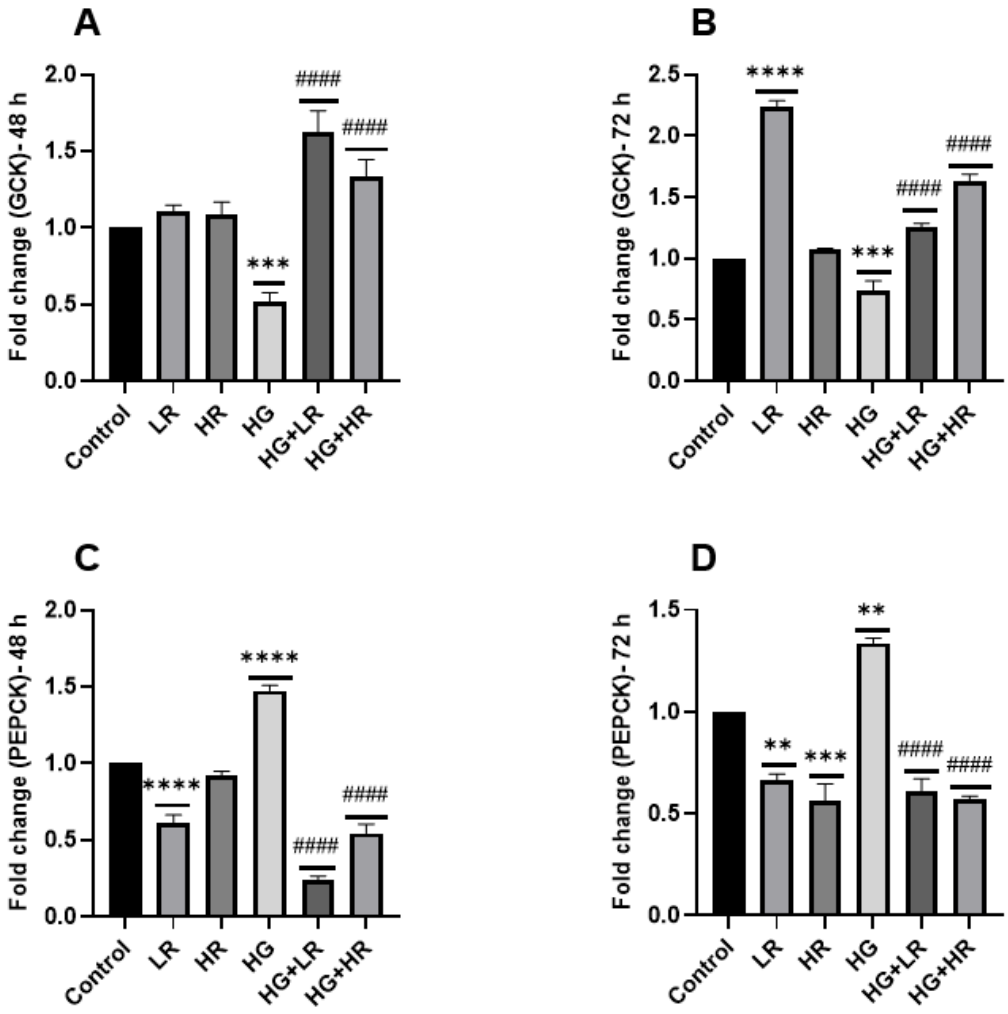

Figure 4.6: High glucose significantly reduced the expression of *GCK* and increased the expression of *PEPCK* in HepG2 cells. Resveratrol treatment increased *GCK* and decrease *PEPCK* expression levels. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.

LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; *GCK*, Glucokinase; *PEPCK*, Phosphoenolpyruvate carboxylase.

3.6. The expression of glucose metabolism and inflammation-related genes

KLF7, *HIF1A*, and *SIRT1* are involved in glucose metabolism and inflammation. In this study, we explored the effect of HG on these genes (Figure 7). The qPCR results show that when HepG2 cells were exposed to HG over 48 and 72 h, the expression levels of *KLF7* and *HIF1A* were significantly increased (*p*<0.0001) (Figure 7 A, B, C, and D). The expression levels of SIRT1 were significantly decreased when cells were exposed to HG over 72 h (*p*=0.0003; Figure 7E); however, we did not observe any statistical difference when cells were exposed to

HG over 48 h (Figure 7F). To investigate the involvement of RES in the expression of these mRNAs, HepG2 cells were cultured with RES in the presence and absence of HG. When HepG2 cells were cultured with LR and HR over 48 and 72 h, no statistical difference was observed in the expression of *KLF7* compared to the control; however, we observed a significant decrease when exposed to HR over 48 h (Figure 7 A and B). The expression of *HIF1A* was significantly decreased when cells were exposed to LR and HR over 72 h (p=0.0001 and p<0.0001, respectively) and when exposed to LR over 48 h (p=0.0287); however, no statistical difference was observed when cells were exposed to HR over 48 h (Figure 6 C and D). *SIRT1* showed a significant increase when cells were exposed to LR and HR over 48 h *(*p=0.0004 and p<0.0001, respectively) and HR over 72 h (p=0.0002); however, when cells were exposed to LR for 72 h, the mRNA expression of SIRT1 decreased slightly, but no statistical difference was observed. When HepG2 cells were exposed to LR and HR in the presence of HG over 48 and 72 h, the expression levels of *KLF7* and *HIF1A* were significantly decreased (p<0.0001) compared to the HG group (Figure 7 A, B, C, and D). *SIRT1* showed a significant increase when cells were exposed to LR and HR in the presence of HG (p<0.0001) as compared to HG alone (Figure 7 E and F).

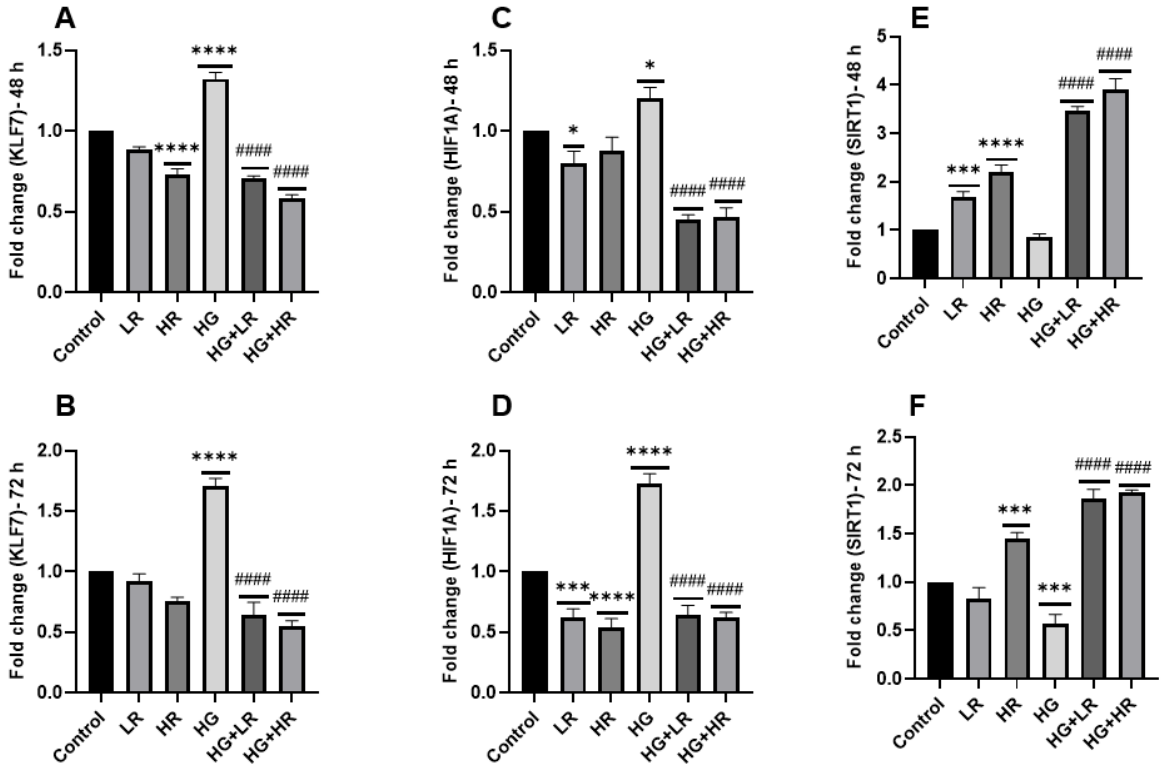

Figure 4.7: KLF7, HIF1A, and SIRT1 expression in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 and 72 h. High glucose significantly the expression of *KLF7* and *HIF1A* over 48 and 72 h, whereas the expression level of SIRT1 was significantly decreased following exposure to high glucose. Resveratrol reversed the dysregulation caused by high glucose. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.

LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; *KLF7*, Kruppel-like factor 7; *HIF1A*, hypoxia-inducible factor-1 Alpha; *SIRT1*, Sirtuin 1.

4. Discussion

Impaired glucose metabolism and associated inflammation results in chronic hyperglycemia leading to the development and progression of T2DM. Resveratrol (RES), a polyphenol phytoalexin, is a natural compound with anti-diabetic effects (Imamura *et al*., 2017). In this study, the role of RES in glucose metabolism and inflammation in high glucose-induced HepG2 cells was examined.

In hyperglycemic conditions, the NF-kB signaling pathway is mainly implicated in the inflammatory response (Baker *et al*., 2011). This study demonstrated that high glucose activates the NF-kB pathway, as evidenced by elevated mRNA expression of IKKα and IkB-α (Figure 4). These results are consistent with those of Ramana *et al*. where they studied vascular smooth muscle cells (Ramana *et al*., 2004). The activation of NF-kB triggers the expression of pro-inflammatory cytokines. Herein, a significant increase was observed in the expression of TNF-α, IL-6, COX2, and IL-1β in HepG2 cells exposed to high glucose (Figure 1-3). Panahi *et al*. also reported similar results. They observed high glucose levels significantly increased the expression of TNF-α and IL-6 in HepG2 cells (Panahi *et al*., 2018). From these findings, it can be inferred that high glucose may induce inflammation in liver cells resulting in the development of diabetes. Furthermore, targeting the NF-kB pathway may be a therapeutic potential to manage high glucose-induced inflammation.

Interestingly, the current study revealed that RES reduced the expression of IKKα and IkB-α, thereby decreasing NF-kB activity (Figure 4). It was also demonstrated that treating HepG2 cells with RES in the presence of high glucose significantly reduced the expression of pro-inflammatory cytokines (Figure 1-3). These results align with previous research. One study demonstrated a significant reduction in TNF-α and IL-6 in diabetic rats upon treatment with RES (Prabhakar, 2013). Another study reported similar results in diabetic mice wherein RES treatment decreased the expression of TNF-α and IL-1β while inhibiting the NF-kB activity (Zhang *et al*., 2021). These findings provide further evidence that RES has significant anti-inflammatory effects in diabetic conditions, by decreasing the expression of pro-inflammatory cytokines and preventing NF-kB activity. Therefore, RES might be a promising therapeutic agent for treating inflammation in patients with diabetes.

The expression of TGFβ1 mRNA was also investigated. TGFβ1 is a versatile cytokine that plays a role in various cellular processes, including cell growth, migration, proliferation, differentiation, and apoptosis (Ghadami *et al*., 2000). In addition, studies have shown that TGFβ1 exhibits anti-inflammatory properties by inhibiting the expression of TNFα or

counteracting the pro-inflammatory effects of IL-1b and IFNγ (Park *et al*., 2000). The present study observed that treatment with RES resulted in the upregulation of TGFβ1 expression in high glucose-induced HepG2 cells (Figure 5). It was also correlated with the downregulation of pro-inflammatory cytokines.

This study further demonstrated that gluconeogenesis *PEPCK* gene was significantly increased and glycolysis gene *GCK* was significantly decreased in HepG2 cells treated with high glucose (Figure 6). These findings agree with previous research by Zhu *et al*., which demonstrated similar results in the liver tissue of STZ-diabetic mice (Zhu *et al*., 2017). These findings imply that high glucose levels can result in elevated gluconeogenesis and reduced glycolysis in liver cells. This dysregulation of glucose metabolism may contribute to developing hyperglycemia and insulin resistance. However, when HepG2 cells were treated with RES in the presence of high glucose, the expression level of *PEPCK* was significantly reduced while the expression of *GCK* increased (Figure 6). This indicates that RES treatment can potentially restore the balance between gluconeogenesis and glycolysis in the liver cells exposed to high glucose, implying that RES could have therapeutic potential in treating hyperglycemia and insulin resistance associated with dysregulated glucose metabolism.

SIRT1, abundant in mammals, is implicated in fundamental biological processes such as stress response, glucose metabolism, and inflammation (Zhang *et al*., 2015; Lu *et al*., 2023). Patients with poor glycemic control have consistently lower SIRT1 levels than those with good glycemic control (Balestrieri *et al*., 2013). The protein expression of SIRT1 was observed to be significantly reduced in mouse microvascular endothelial cells following high glucose exposure (Arunachalam *et al*., 2014). Furthermore, SIRT1 has been shown to mediate NF-kB deacetylation and inhibit its function (Jia *et al*., 2015). This study demonstrated that high glucose significantly reduced the mRNA expression of *SIRT1* in HepG2 cells (Figure 7 E and F). This further supports the role of SIRT1 in mediating the effects of high glucose on cellular processes. Additionally, the reduction of SIRT1 in HepG2 cells may have implications for NF-kB activity and its role in inflammation. Our results further demonstrate that RES treatment significantly increased the expression of *SIRT1* in HepG2 cells (Figure 7 E and F), as shown by previous research (Goh *et al*., 2014). Increased expression of SIRT1 by RES treatment highlights its potential as a therapeutic intervention for mitigating the detrimental effects of high glucose on cellular function.

This study also explored the effect of high glucose and RES on the expression of *KLF7* and *HIF1A* in HepG2 cells. KLF7 and HIF1α play a crucial role in regulating inflammation and glucose metabolism (Qiu *et al*., 2022; Matoba *et al*., 2013). Shao *et al.* found that the levels of HIF1α in serum of patients with T2DM were significantly increased compared to the control

group (Shao *et al*., 2016). Additionally, the protein and mRNA expressions of HIF1A have been shown to increase in hyperglycemic conditions (Isoe *et al*., 2010). Consistent with these findings, the current study demonstrated that high glucose significantly increased the expression of *HIF1A* in HepG2 cells (Figure 7 A and B). A significant increase in the mRNA expression of *KLF7* was also observed (Figure 7 C and D). Upon treatment with RES, mRNA expression of *HIF1α* and *KLF7* was reduced. This suggests that RES may have potential therapeutic effects in reducing the expression of KLF7 and HIF1α in hyperglycemic conditions. The current findings highlight the importance of exploring the role of RES in the regulation of KLF7 and HIF1A expression and its potential implications for managing inflammatory diseases and metabolic disorders.

There are few limitations of this study. Firstly, it relied on an *in vitro* model to establish controlled experimental conditions and enable focused analysis. It would be beneficial to extend our investigations by using appropriate animal models, such as diabetes-induced mice. Secondly, our research was confined to examining genes associated with inflammation and glucose metabolism, protein expression analysis was not conducted. Future studies should consider assessing functional protein expression to establish potential correlations with gene expression. Quantifying protein expression can provide insights into the intricate process of genes transforming into functional proteins, and it can also help explore the influence of various factors on protein synthesis.

Our findings suggest that resveratrol has multifaceted therapeutic potential for diabetes. It can mitigate inflammation, restore balanced glucose metabolism, enhance SIRT1 expression, and reduce the expression of key transcriptional factors. Although these results are promising, further research is necessary to fully understand the underlying mechanism and practical implications of using resveratrol as a treatment for diabetes. The diverse effects of resveratrol on glucose metabolism and inflammation make it a valuable tool in the fight against the global diabetes epidemic.

Acknowledgments: The authors would like to thank Prof JL Marnewick for donating HepG2 cells to us for the experimental work. I would also like to thank the Department of Science and Innovation (DSI) - Inter-bursary Support Programme - Council for Scientific and Industrial Research (CSIR) for providing me with the bursary.

Author Contributions: Experimental design, statistical analysis, interpretation of data, and writing original draft: A.M.T. Conceptualized, Experimental design, Interpretation of the data, revised and proofread the manuscript, supervision: S.R. Supervision, revised and proofread

the manuscript: T.E.M. All authors have read and agreed to the published version of the manuscript.

Funding statement: This research project was supported by a grant from the South African Medical Research Council (SAMRC), with funds from the National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant no. 115450).

Ethics approval statement: The proposal for the current study was approved by the Ethics Committee of Cape Peninsula University of Technology (CPUT/HW-REC 2021/H6).

Informed Consent Statement: Not applicable

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5: The protective role of resveratrol against high glucose-induced oxidative stress and apoptosis in HepG2 cells

(Published in *Food Science & Nutrition*, https://doi.org/10.1002/fsn3.4027).

The protective role of resveratrol against high glucose-induced oxidative stress and apoptosis in HepG2 cells

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Abstract

High glucose concentrations result in oxidative stress, which may cause damage to cellular components like DNA, proteins, and lipids, ultimately resulting in apoptosis. Resveratrol, a polyphenol phytoalexin, has been studied for its potential therapeutic effects on diabetes. This study examined the effect of high glucose (HG) on HepG2 cells and assessed the resveratrol effect on high-glucose-induced oxidative stress and apoptosis. HepG2 cells were treated for 48 and 72 hours with high glucose (40 mM), low resveratrol (25 µM), high resveratrol (50 µM), high glucose + low resveratrol, and high glucose + high resveratrol. After exposure, oxidative and apoptosis-related gene expression was assessed using real-time quantitative polymerase chain reaction (qPCR), and Lactate dehydrogenase (LDH) release was measured using supernatant. In cells treated with high glucose, all antioxidant enzymes (SOD, Superoxide dismutase; GPx1, Glutathione peroxidase 1; CAT, Catalase; Nrf2, Nuclear factor erythroid 2–related factor 2 and NQO1, NAD(P)H quinone oxidoreductase 1) were significantly reduced; however, when HepG2 cells were treated with resveratrol (25 µM and 50 µM) in the presence of high glucose, the expression levels of all antioxidant enzymes were increased. Anti-apoptotic gene (B-cell lymphoma 2; *Bcl2*) and the DNA repair gene (Oxoguanine glycosylase-1, *OGG1*) were significantly decreased following high glucose exposure to HepG2 cells. Surprisingly, the expression of *Bcl2* and *OGG1* significantly increased after resveratrol treatment. Furthermore, high glucose increased the LHD release in HepG2 cells, whereas resveratrol treatment reduced the LDH release. Our results demonstrate that resveratrol protects against high glucose-induced oxidative stress and apoptosis in HepG2 cells. Therefore, resveratrol may serve as a promising strategy to combat antioxidant response

dysfunction caused by high glucose levels characteristic of diabetes conditions and metabolic disorders.

Keywords: Hyperglycemia, high glucose, Oxidative stress, apoptosis, resveratrol

1. Introduction

Diabetes mellitus (DM) is a group of metabolic conditions characterized by increased blood glucose levels and inadequate insulin production or action (Solis-Herrera *et al*., 2018). According to the International Diabetes Federation (IDF), 537 million people worldwide had diabetes in 2021, expected to increase to 643 million by 2030 and 745 million by 2045 (Sun *et al*., 2022). This has become an urgent public health threat due to its rising incidence. Hyperglycemia is accompanied by micro- and macrovascular complications and multi-organ damage (Kapoor and Kakkar, 2012). Hence, it is severely essential for hyperglycemia to be treated effectively to inhibit these complications and improve the patient's outcome.

The liver serves as the primary organ responsible for glucose metabolism and regulation. Consequently, it is of considerable interest to investigate the impacts of hyperglycemia on liver cells cultured in vitro. The HepG2 cell line, derived from human hepatoma, has been widely employed in vitro to investigate hyperglycemia (Chandrasekaran *et al*., 2010). Moreover, HepG2 cells exhibit certain physiological characteristics akin to those of the human liver (Knowles *et al*., 1980). In Hyperglycemia, high glucose levels can increase reactive oxygen species (ROS), overwhelming the body's antioxidant defense and producing oxidative stress (Bhatti *et al*., 2022). Oxidative stress has been implicated in the onset and progression of DM (Subramaniyan & Kumar, 2017). Aging, obesity, and poor diet are common risk factors that initiate an oxidative environment that can change insulin sensitivity by either elevating insulin resistance or lowering glucose tolerance (Hamed *et al*., 2011). The most reactive oxygen species that cause damage to cells during oxidative stress are superoxide anion radicals (O2 -), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), and singlet oxygen ($^1O_2$) (Nimse and Pal, 2015). These species are volatile and can react and cause damage to various cellular components such as lipids, proteins, and DNA (Iside *et al*., 2020).

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that controls the expression of anti-electrolyte and antioxidant genes (Kovac *et al*., 2015**;** Valenzuela *et al*., 2017). During normal conditions, Keap1 attaches Nrf2 within the cytoplasm. During oxidative stress, Nrf2 is detached from the complex and translocated to the nucleus, where it binds to the antioxidant response element (ARE), activating gene transcription for NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) among others (Slocum *et al*., 2016). Furthermore, under normal conditions, the body has a potent endogenous antioxidant system consisting of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), Glutathione Peroxidase (GPx), and Glutathione Reductase (GR) that protects it from the deleterious effects

of excessive production of reactive ROS (Bhattacharyya *et al*., 2014**;** Hong & Lee, 2009). These antioxidants work together to neutralize and eliminate ROS, preventing oxidative damage to cells and tissues.

Oxidative stress-induced DNA damage results from an imbalance between DNA repair and DNA damage (Tao *et al*., 2021**;** Pang *et al*., 2012). This imbalance can accumulate DNA lesions and impair the cell's ability to maintain genomic stability. 8-oxo-7,8-dihydroguanine (8-oxoG) is the most studied DNA oxidation product, which is repaired by the 8-oxoG glycosylase1-initiated base excision repair (BER) pathway (OGG1-BER) (Wang *et al*., 2018)**.** The OGG1-BER pathway plays a vital role in maintaining genomic stability by repairing DNA damage caused by oxidative stress. Dysregulation of this pathway can result in the accumulation of 8-oxoG lesions, leading to increased susceptibility to DM complications.

Research has shown that ROS may be a signal molecule to promote cell proliferation and apoptosis (Finkel, 1998). Thus, apoptosis is one of the cellular responses to oxidative stress and ROS production that results from high glucose (Sun *et al*., 2012; Xu *et al*., 2012). Apoptosis, also known as programmed cell death, plays a significant role in maintaining cellular homeostasis. It serves as a protective mechanism to eliminate damaged or dysfunctional cells, preventing the spread of potential harm throughout the organism. Apoptosis is controlled by the balance involving pro- and anti-apoptotic proteins. The BCL2 family comprises pro-apoptotic proteins (Bax and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-x). These proteins cooperate to determine whether cells undergo apoptosis or survival (Hagenbuchner *et al*., 2012). This process is essential in maintaining cellular homeostasis and inhibiting disease development. Bcl-2 is an anti-apoptotic protein that prevents apoptosis by inhibiting Bax/Bak oligomerization, which enhances mitochondrial membrane permeability and inhibits the Cyto-C release. Previous research has demonstrated reduced Bcl2 expression in response to diabetes stimulus (Ren *et al*., 2020). Thus, decreased expression of Bcl-2 may increase an apoptosis process due to the reduced ability to inhibit Bax/Bak oligomerization following the release of Cyto-C from the mitochondria. Understanding the regulation of Bcl-2 expression in response to diabetic stimuli could provide valuable insights into the development and progression of diabetes-related complications.

Managing hyperglycemia with chemical drugs or insulin causes numerous complications, including insulin-induced fatty liver (Zhang & Liu, 2011; Kandhare *et al*., 2012). Therefore, silencing or quenching excess ROS using various natural antioxidants may be a cost-effective and efficient method for better hyperglycemia management. These natural antioxidants are risk-free and readily absorbed by cellular systems.

Resveratrol (RES), a natural polyphenol found in various fruits and plants, has been extensively studied for its anti-diabetes, anti-obesity, anticancer, anti-inflammatory, antioxidant, and cardioprotective properties, and potential health benefits (Faal *et al*., 2022; Elshaer *et al*., 2018). Previous research has demonstrated that RES can effectively mitigate oxidative stress and apoptosis in different cell types (Liu *et al*., 2014; Kitada and Koya, 2013; Hoca *et al*., 2021; Do *et al*., 2012). However, its protective effects against high glucose-induced damage in HepG2 cells have not been fully elucidated. Therefore, this study seeks to fill this knowledge gap and present valuable perceptions of the therapeutic potential of RES in managing oxidative stress and apoptosis. In addition, the findings may aid in creating novel strategies for preventing or treating diabetic complications.

2. Materials and Methods

2.1. Materials

Tissue culture consumables and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). HepG2 cells were kindly donated by Prof JL Marnewick (Cape Peninsula University of Technology, South Africa). D-glucose and resveratrol were purchased from Sigma–Aldrich. All quantitative polymerase chain reaction (qPCR) consumables and reagents were purchased from Bio-Rad (Hercules, CA, USA), and primer sequences were manufactured by Inqaba Biotechnical Industries (Pretoria, South Africa).

2.2. Methods

2.2.1. Study design

The glucose and resveratrol treatment concentrations were selected based on the literature. Previous studies established hyperglycemia at 50 mM and 40 mM (Chandrasekaran *et al*., 2010; Chu *et al*., 2011; Leinninger *et al*., 2004; Varma *et al*., 2005; Kapoor and Kakkar, 2012). In this study, we used 40 mM to establish hyperglycemia. The resveratrol (RES) concentrations were determined using previous studies (Poonprasartporn and Chan, 2022; Cheng *et al*., 2012; Khan *et al*., 2013). This study used 25 µM and 50 µM concentrations for RES prepared in 100% dimethyl sulphoxide (DMSO). Cells were categorized into six groups: Control groups were cultured in normal complete culture medium (CCM), Low resveratrol (LR; cultured in normal CCM with 25 µM RES), High resveratrol (HR; cultured in normal CCM with 50 µM RES), High glucose (HG; cultured in normal CCM with 40 mM glucose), LR+HG (cultured in normal CCM with 25 µM RES and 40 mM glucose), and HR+HG (cultured in normal CCM with 50 µM RES and 40 mM glucose).

2.2.2. Cell culture

The human hepatoma G2 (HepG2) cell line is derived from human hepatoma tissue. Many studies have used the HepG2 cell line to study hyperglycemia in *vitro* (Chandrasekaran *et al*., 2010; Zhou *et al*., 2021; Shokrzadeh *et al*., 2016). Moreover, they are reliable, easy to culture and well characterized. Our study used the HepG2 cell line to establish hyperglycemia. HepG2 cells were cultured in monolayers (10^6 cells per flask) in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penstrepfungizone (PSF), and 1% L-glutamine in 25 cm^3 flasks. The cells were cultured in a humidified incubator at 37 °C with 5% CO_2. The cells were washed with phosphate-buffered saline (PBS) containing 0.1 M phosphate. After the cells reached 70-80% confluence, they were treated with RES (25 µM and 50 µM) and HG (40 mM) and incubated for 48 and 72 hours (h). The cells were then removed using trypsin and counted using the trypan blue exclusion technique. Cell suspensions were diluted (1:5) with 60 µL CCM + 20 µL cell suspension + 20 µL trypan blue solution and incubated for 5 min at room temperature (RT). Then, a 22 x 22 cm coverslip was placed on a clean hemocytometer, and 10 µL of a well-mixed counting solution was dispensed into the hemocytometer. Using a microscope, the number of living cells was determined using the standard equation (Live cell average x 5 (dilution factor) x 10,000 = cells/mL).

2.2.3. RNA isolation and cDNA synthesis

Total RNA was isolated using a QIAzol extraction reagent (Qiagen, Hilden, Germany) and a previously published protocol based on the manufacturer's instructions. Briefly, a 1:1 ratio of QIAzol and PBS was added to the flask, followed by incubation at RT for 2 min. Cells were then scraped off the surface of the flask using a cell scraper, transferred into a microcentrifuge tube, and frozen at -80 °C. Next, samples were thawed, and 100 µL of chloroform was added to each tube. The tubes were shaken vigorously for 15 seconds, incubated for 2-3 min at RT, and centrifuged (15 min, 4 °C, 12 000 × *g*). Thereafter, the aqueous phase was transferred to a new tube, 250 µL of isopropanol was added, and tubes were mixed and incubated overnight at -80 °C. Next, samples were thawed and centrifuged (4 °C, 12 000 × *g*, 20 min), and the pellets were retained. Pellets were washed using 500 µL of cold 75% ethanol. The tubes were then flicked to loosen the pellet and centrifuged at 4 °C, 7400 × *g* for 15 min. Ethanol was removed using a pipette without agitating the pellet. The samples were allowed to dry for 1.5 h. The pellet was then resuspended in 15 µL nuclease-free water and incubated for 2-3 min at RT. Thereafter, the purity and integrity of the RNA samples were evaluated using the Nanodrop system (Nanodrop Technologies, Wilmington, USA), and the A260/A280 ratio was used to evaluate RNA integrity. For cDNA synthesis, the iScript cDNA synthesis kit (Bio-Rad) was employed as per the manufacturer's instructions (4 µL 5× iScript reaction mix, 1 µL iScript reverse transcriptase, 14 µL nuclease-free water, and 1 µL of each RNA sample). After cDNA synthesis, 80 µL of nuclease-free water was added to each tube, and the samples were stored at -20 °C until they were needed for qPCR.

2.2.4. Quantitative polymerase chain reaction (qPCR)

Gene expression was assessed using qPCR. A reaction mixture of 5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 1.5 µL cDNA, 0.5 µL forward and reverse primers, and 2.5 µL nuclease-free water was made up to 10 µL. All primers were acquired from Inqaba Biotechnical Industries (Pty) Ltd. The mRNA expression of *Gpx1* (Forward 5' AAGGTGCTGCTCATTGAGAATG 3'; reverse 5' CGTCTGGACCTACCAGGAACTT 3'), *CAT* (forward5'ACGAGATGGCACACTTTGACAG 3'; reverse 5' TGGGTTTCTCTTCTGGCTATGG 3'), *SOD* (forward 5' AGGATTAACTGAAGGCGAGCAT 3'; reverse 5' TCTACAGTTAGCAGGCCAGCAG 3'), *Nrf2* (forward 5' AGTGGATCTGCCAACTACTC 3'; reverse 5' CATCTACAAACGGGAATGTCTG 3'), *BCL-2* (forward 5' TGTGGAGAGCGTCAACCGGGAG 3'; reverse 5' ATCAAACAGAGGCCGCATGCTG 3'), *NQO1* (forward 5' GAAGAGCACTGATCGTACTGGC 3'; reverse 5'GGATACTGAAAGTTCGCAGGG 3'), and *OGG1* (forward 5' GCATCGTACTCTAAGCCTCCAC 3'; reverse 5' AGGACTTTGCTCCCTCCAC 3') were investigated. GAPDH (forward 5' TCCACCACCCTGTTGCTGTA 3'; reverse 5' ACCACAGTCCATGCCATCAC 3') was used in this assay as a house-keeping gene, with three replicates per treatment. The initial denaturation occurred at 95 °C (2 min). This was followed by 40 cycles of denaturation (95 °C; 15 s), annealing (40 sec; *CAT*, *S0D*, *GPx*, *OGG1*—60 °C; *Nrf2*, *NQO1*, Bcl-2—55 °C), and extension (72 °C; 30 s). Changes in relative mRNA expression were determined using the Livak and Schmittgen method, where $2^{-\Delta\Delta Ct}$ represents the observed fold change in mRNA expression (Livak and Schmittgen, 2001).

2.2.5. Lactate dehydrogenase (LDH) assays

The LDH cytotoxicity Detection Kit (Roche, Mannheim, Germany) evaluated the extracellular LDH released levels. A 96-well microtiter plate was filled with 100 µL of control and treated cell supernatants in triplicate. The substrate mixture (100 µL) was added to the supernatant and incubated for 25 min at room temperature with a catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate). The optical density at 490 nm was measured using the Elisa microplate reader (Thermo Fisher Scientific; USA). The findings are presented in the form of mean optical density.

2.2.6. Statistical analysis

All data analyses were done using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA, USA). The statistical methods employed were One-way analysis of variance (ANOVA) and Student's t-test were employed. All experiments were conducted in triplicates, and $p<0.05$ was considered statistically significant.

3. Results

3.1 High glucose decreased the expression of antioxidant enzymes.

Oxidative stress results from the excessive accumulation of ROS caused by high glucose concentrations in the cells. SOD and CAT are the two main antioxidant enzymes that protect cells from damaging ROS-mediated effects (Wang *et al*., 2020). This study evaluated the mRNA expression of *SOD*, *GPx1*, and *CAT* using qPCR. HepG2 cells were exposed to high glucose (HG) (40 mM) for 48 and 72 h. Results showed that when HepG2 cells were exposed to HG for 48 h, SOD, GPx1, and CAT expression levels significantly decreased (p=0.0046; p=0.0045 and p=0.0011, respectively) (Figure 1A; 1C and 1E). When cells were exposed to HG for 72 h, we observed that the expression levels of *SOD* and *GPx1* were significantly decreased (p=0.0017 and p=0.043, respectively) (Figure 1B and 1D), whereas no statistical difference was observed in the expression level of CAT as compared to the normal cells (p=0.3358; Figure 1F). The results suggest that the effects of HG on antioxidant enzymes are time-dependent, with longer exposure resulting in greater decreases in *SOD*, *GPx1*, and *CAT* expression levels, indicating that prolonged exposure to HG conditions will gradually reduce the antioxidant potential of cells and allow ROS and RNS to accumulate and damage cellular components. These findings may provide information about oxidative stress-related cellular damage observed in patients with diabetes.

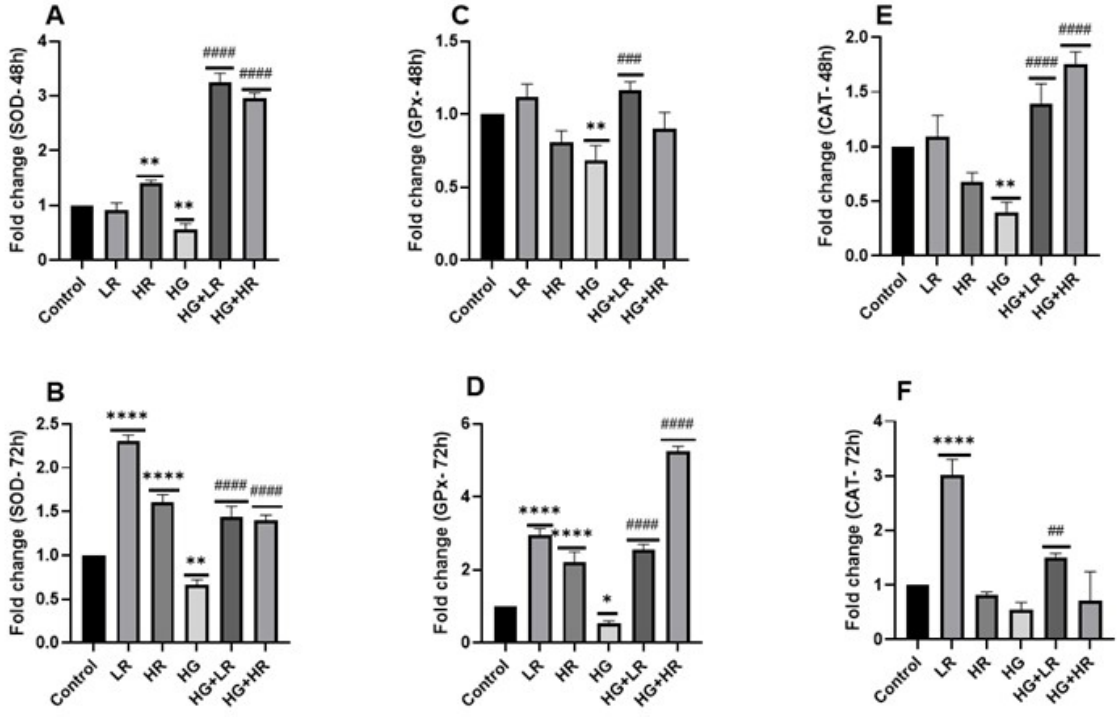

Figure 5.1: Expression of antioxidant enzyme genes in HepG2 cells treated with high glucose (40 Mm) and RES (25 µM and 50 µM) over 48 and 72 h. The mRNA expression was quantified using qPCR. (A)

Expression of *SOD* over 48 h. (B) Expression of *SOD* over 72 h. (C) Expression of *GPx* over 48 h. (D) Expression of *GPx1* over 72 h. (E) Expression of *CAT* over 48 h. (F) Expression of *CAT* over 72 h. High glucose significantly decreases all the antioxidant enzymes. Conversely, resveratrol increases the expression levels of all the antioxidant enzymes. GAPDH was used as the housekeeping gene. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.

LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; *SOD*, superoxide dismutase; *GPx1*, glutathione peroxidase 1; *CAT*, Catalase

3.2. Resveratrol increased gene expression of antioxidant enzymes.

Since exposure to high glucose decreased the expression of *SOD*, *GPx1*, and *CAT*, we investigated whether RES could reverse the effects of high glucose on these mRNAs. HepG2 cells were treated with HG+LR and HG+HR over 48 and 72 h (Figure 1). The qPCR results show that the expression levels of *SOD* increased significantly after exposure to HG+LR and HG+HR over 48 and 72 h (Figure 1A and 1B) (p<0.0001) as compared to HG alone. *GPx1* increased significantly after exposure to both HG+LR and HG+HR over 72 h (p<0.0001) (Figure 1D); however, a significant difference was not observed after exposure to HG+HR over 48 h (p=0.0525) as compared to HG (Figure 1C). We observed that *CAT* was significantly increased after exposure to both HG+LR and HG+HR over 48 h (p<0.0001) (Figure 1E) and when exposed to HG+LR for 72 h (p=0.0069); however, a significant difference was not observed after exposure to HG+HR over 72 h (p=0.9700) as compared to HG (Figure 1F). These findings suggest that RES has the potential to reverse the negative effects of high glucose on the expression of these antioxidant enzymes.

We investigated the effect of two RES concentrations (25 and 50 μM) on the expression of *SOD*, *GPx1*, and *CAT* (Figure 1) after exposure over 48 and 72 h. The expression levels of *GPx1* and SOD were significantly increased after exposure to LR (25 μM) and HR (50 μM) over 72 h as compared to the control (p<0.0001) (Figure 1B, 1D, and 1E). We did not observe any statistical difference in GPx1 and CAT expression levels when exposed to LR and HR over 48 h compared to the control (Figure 1B and 1C). However, SOD was significantly increased when treated with HR for 48 h (p=0.0066) (Figure 1A).

3.3. qPCR analysis of antioxidant response element.

The mRNA expression of *Nrf2* and *NQO1* was evaluated using qPCR. When treated with HG for 48 and 72 h, the expression of *Nrf2*, an antioxidant defense regulator, was significantly decreased (p<0.0001 and p=0.0010, respectively) relative to the control (Figure 2A and 2B). *Nrf2* showed no significant difference when treated with LR and HR over 48 h; however, *Nrf2* appeared to increase when treated with LR over 72 h (p<0.0001) compared to the control (Figure 2A and 2B). HG+LR and HG+HR significantly increased *Nrf2* expression over 48 h

compared to HG alone (p<0.0001) (Figure 3A). Nrf2 significantly increased when treated with HG+LR and HG+HR for 72 h (p=0.0125 and p<0001, respectively) (Figure 2B). High glucose significantly decreased the expression of *NQO1* over 48 h (p=0.0055); however, no statistical difference was observed when treated with HG over 72 h (p=0.3578) (Figure 2C and 2D). *NQO1* showed no statistical difference when treated with LR over 48 h; however, *NQO1* significantly increased when treated with LR and HR for 72 h compared to the control (p=0.0042 and p<0.0001) (Figure 2C and 2D). When treated with HG+LR and HG+HR over 48 and 72 h, the *NQO1* expression level significantly increased compared to HG alone (p<0.0001; Figure 2C and 2D)

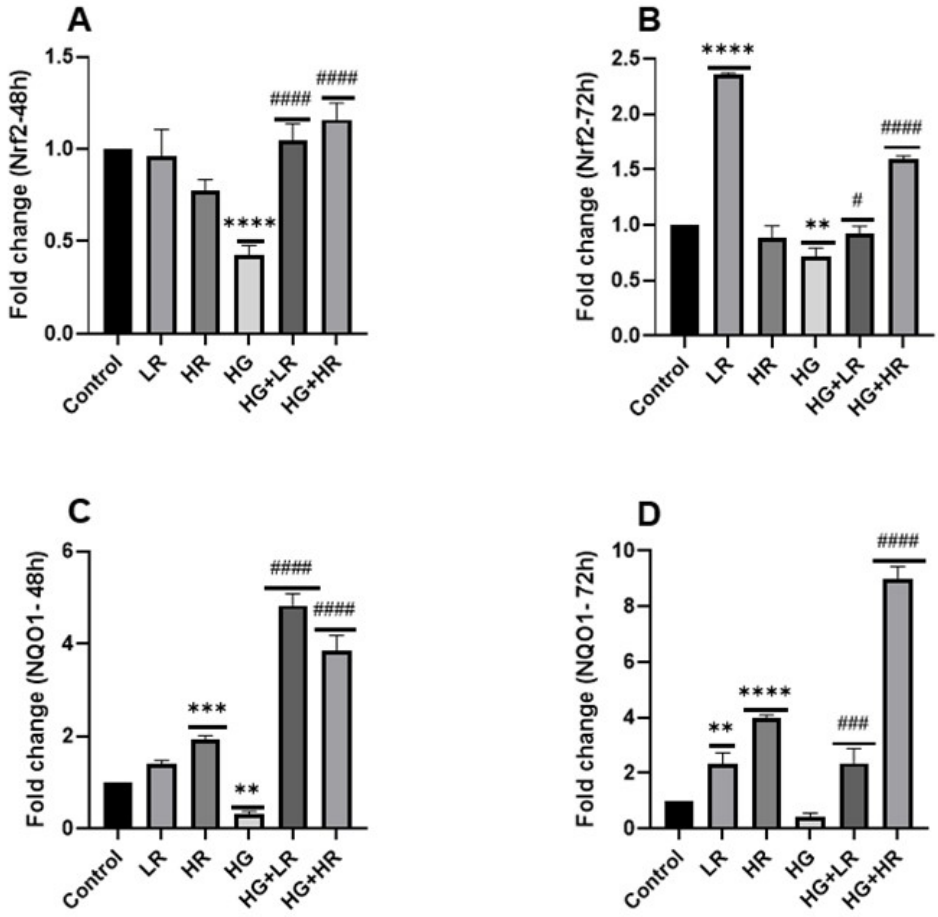

Figure 5.2: Expression of *Nrf2* and *NQO1* in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 and 72 h. High glucose significantly decreased the expression of *Nrf2* and *NQO1*; however, when treated with resveratrol, the expression of *Nrf2* and *NQO1* was significantly increased. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.

LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; *Nrf2*, Nuclear factor erythroid 2-related factor 2; *NQO1*, NAD(P)H quinone oxidoreductase 1

3.4. Effect of high glucose and resveratrol on BCL2 and OGG1 genes.

The expression levels of the Bcl-2 gene related to apoptosis were explored using qPCR. The qPCR results showed that the expression of Bcl-2 was significantly decreased when treated with high glucose over 48 h (p<0.0001) but not significantly expressed when treated over 72 h (p=0.1279) as compared to the control (Figure 3A and 3B). These findings indicated that high glucose levels may have a negative impact on the regulation of Bcl-2 in HepG2 cells. Bcl-2 increased when treated with HG+LR and HG+HR over 48 h (p<0.0001) compared to HG. When treated with HG+HR for 72 h, Bcl-2 was significantly increased (p=0.005); however, no statistical difference was observed during exposure to HG+LR over 72 h compared to HG (Figure 3A and 3B). In addition, no statistical difference was observed in Bcl-2 when treated with HG+HR over 48 and 72 h as compared to HG. These findings suggest that resveratrol may have a protective effect on HepG2 cells by increasing Bcl-2 levels.

The involvement of a DNA repair-related gene was also explored. The qPCR results showed that when HepG2 cells were exposed to HG over 48 and 72 h, the expression levels of OGG1 were significantly decreased (p=0.0108 and p=0.0003, respectively) (Figure 3C and 3D). This suggests that exposure to HG may lead to impaired DNA repair mechanisms, specifically affecting the expression of OGG1. OGG1 was significantly increased when treated with HG+LR and G+HR over 48 72 h (p<0.0001) compared to HG alone (Figure 3C and 3D). This finding suggests that resveratrol may positively impact OGG1 levels, potentially indicating its role in DNA repair and oxidative stress response.

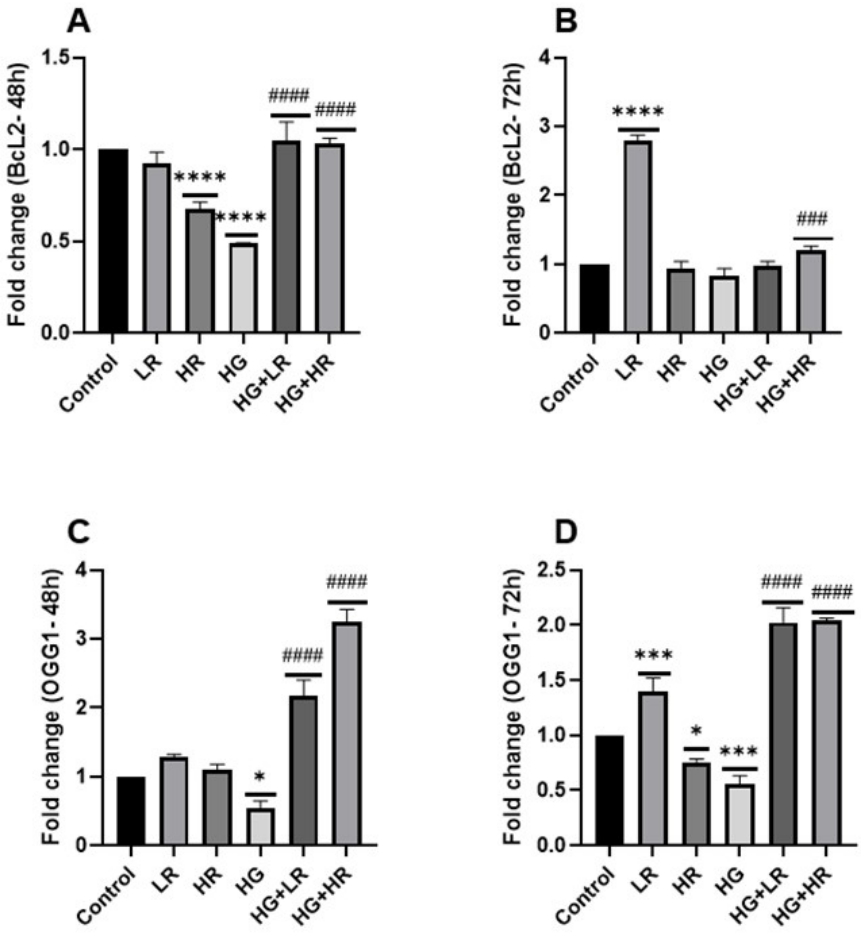

Figure 5.3: Expression of *Bcl-2* and *OGG1* in HepG2 cells treated with high glucose (40 mM) and resveratrol (25 µM and 50 µM) over 48 and 72 h. (A) *Bcl-2* expression during 48 h exposure. (B) *Bcl-2* expression during 72 h exposure. (C) *OGG1* expression during 48 h exposure. (D) *OGG1* exposure during 72 h exposure. High glucose reduced the expression of *Bcl-2* and *OGG1*, whereas resveratrol increased the expression level of *Bcl-2* and *OGG1*. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p$<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}p$<0.001, $^{\#\#\#\#}p$<0.0001 versus HG.
LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol; *Bcl-2*, B-cell lymphoma 2; *OGG1*, Oxoguanine glycosylase-1

3.5. LDH activity-based cytotoxicity assay

The measurement of LDH release using culture supernatant was employed to determine the integrity of cell membranes. Compared to the control cells, we did not observe any statistical in the LDH released in HepG2 cells cultured with HG, LR, HR, HG+LR, and HG+HR over 48 h (Figure 4A). Furthermore, no significant difference was observed after exposure to LR and HR over 72 h compared to the control cells. However, a significant increase was observed when HepG2 cells were treated with HG over 72 h compared to control cells (p<0.0001) (Figure 4B). Consequently, our results suggest prolonged exposure to high glucose in HepG2 cells may lead to increased cell damage and death. Interestingly, a significant decrease in the LDH

released was observed when HepG2 cells were treated with HG+LR and HG+HR over 72 h as compared to HG alone ($p<0.0001$ and $p=0.0314$, respectively) (Figure 4B). These results suggest that RES may protect against high glucose-induced cell damage and death in HepG2 cells.

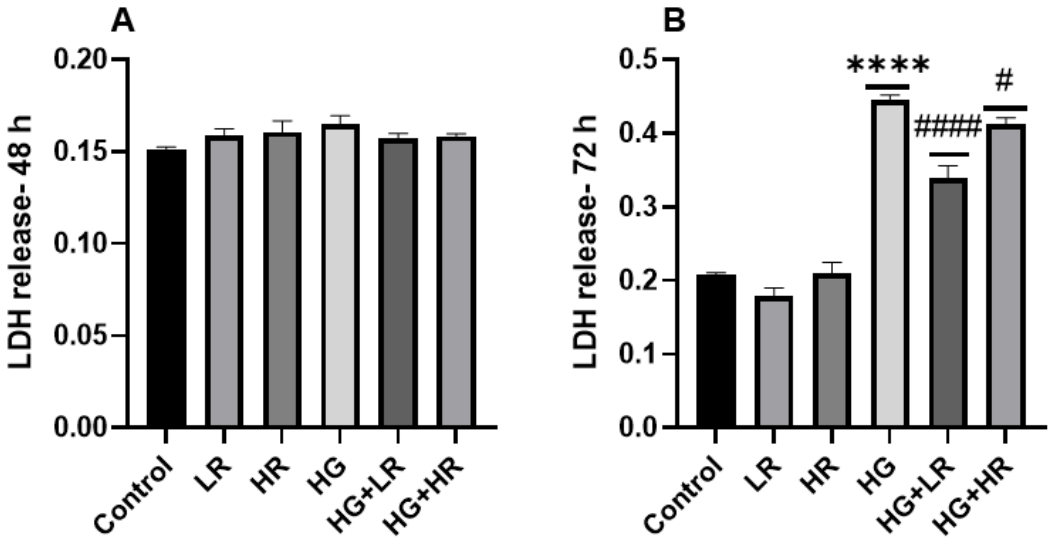

Figure 5.4: Lactate dehydrogenase release after HepG2 cells were cultured with high glucose (40 mM), Low resveratrol (25 µM), High resveratrol (50 µM), High glucose + Low resveratrol (40 Mm + 25 µM), and High glucose + high resveratrol (40 mM + 50 µM). The results were expressed as the fold change relative to untreated cells. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus controls and $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ versus HG.
LR, Low resveratrol; HR, High resveratrol; HG, High glucose, RES, Resveratrol

4. Discussion

Hyperglycemia-induced oxidative stress has been demonstrated to play a vital role in the development and progression of diabetes. In this study, we investigated the expression of oxidative stress and apoptosis-related genes in HepG2 cells exposed to high glucose and assessed the effect of resveratrol (3,5,4′-trihydroxy-trans-stilbene), a polyphenol phytoalexin, on these genes.

Oxidative stress is the imbalance between oxidant and antioxidant substances (Francisqueti *et al*., 2017). The overproduction of ROS is linked with the onset of several metabolic diseases, including diabetes (Shradha *et al*., 2010). In this study, we measured the mRNA expression of CAT, SOD, Gpx1, Nrf2, and NQO1 antioxidant. Our findings showed a significant reduction in the mRNA expression of *SOD*, *CAT*, *GPx1*, *Nrf2*, and *NQO1* in HepG2 cells following high glucose exposure for 48 and 72 h. These results align with previous research demonstrating reduced expression of *SOD*, *CAT*, *GPx*, *Nrf2*, and *NQO1* (Ahmadvand *et al*., 2023;

Subramaniyan and Kumar, 2017; Wang and Guo, 2019). Antioxidant defenses such as SOD, CAT, and GPx are known to neutralize superoxide anions, lipids, and hydroperoxides in order to protect cells from oxidative stress (Shi *et al*., 2020). Nrf2 is thought to play a fundamental role in regulating the antioxidant defense system due to its ability to bind to the antioxidant response element on active antioxidant enzymes (Shi *et al*., 2020). Furthermore, NQO1 is implicated in the detoxification of quinones and defense against oxidative stress. The reduced expression of these antioxidant enzymes and Nrf2 suggest a potential impairment in the cellular defense against oxidative damage caused by high glucose levels.

Resveratrol, a polyphenol phytoalexin, has been studied for its potential therapeutic effects in diabetes (El-Sayed *et al*., 2022). Our results demonstrated that treatment with resveratrol reversed the reduction of *SOD*, *CAT*, *GPx1*, *Nrf2*, and NQO1 mRNA expression caused by high glucose in HepG2 cells treated with resveratrol, suggesting that it may protect against high glucose-induced oxidative stress in a variety of diseases. Previous research has also demonstrated increased SOD, CAT, GPx, and Nrf2 expression after resveratrol treatment (Hu *et al*., 2022; Bagul *et al*., 2012). These findings suggest that resveratrol's ability to upregulate antioxidant enzymes and activate the Nrf2 signaling pathway may also contribute to its potential therapeutic effects in other oxidative stress-related diseases. Furthermore, the observed reversal of the high glucose-induced reduction in antioxidant enzyme expression highlights the potential of resveratrol as a protective agent against oxidative stress in diabetes and other conditions associated with elevated glucose levels. However, further investigations are required on the mechanism underlying resveratrol's effects on antioxidant enzymes and its potential clinical application.

It has been previously shown that oxidative stress-induced DNA damage is the main cause of cell death. 8-oxo-7,8-dihydroguanine (8-oxoG) is the most studied DNA oxidation product, which is repaired by the 8-oxoG glycosylase1-initiated base excision repair (BER) pathway (OGG1-BER) (Wang *et al*., 2018). Previous research has demonstrated a reduction the in the expression of *OGG1* in the presence of high glucose. This finding provides a mechanism for the DNA damage caused by oxidative stress in diabetes (Simone *et al*., 2008). Moreover, high glucose inhibited OGG1 expression in *vivo* and in *vitro* studies (Xie *et al*., 2020). Consistent with previous research, our findings demonstrated that high glucose reduced the expression of *OGG1*. Intriguingly, *OGG1* mRNA levels increased significantly after treatment with resveratrol, demonstrating enhanced DNA repair. According to our findings, resveratrol may be a potential therapeutic agent for diabetic complications by enhancing DNA repair by upregulating OGG1 expression.

Apoptosis is a programmed cell death triggered by prolonged stress and is strongly controlled by several signaling pathways, including the B-cell lymphoma 2 (Bcl-2) family and mitochondrial pathways (Liu *et al*., 2011). The BCL2 family, including the pro-and anti-apoptotic genes (Bax, Bak, Bcl-2, and Bcl-x1), are important regulators of apoptosis (Rojas-Rivera, 2010). Our study assessed the mRNA expression of *Bcl-2* in HepG2 cells. Our findings revealed that *Bcl-2* was significantly reduced when HepG2 cells were exposed to high glucose. These results align with the previous research demonstrating that *Bcl-2* expression was reduced in HepG2 cells exposed to high glucose (Jiang *et al*., 2015). Our study further revealed that in HepG2 cells treated with resveratrol in the presence of high glucose, the expression of *Bcl-2* was significantly increased. Based on these findings, the increased expression of *Bcl-2* by resveratrol could be its ability to regulate apoptotic pathways. Additionally, our results revealed that the LDH activity was significantly increased in HepG2 cells over 72 h, whereas when HepG2 cells were in high glucose over 48 h, no statistical difference was observed. Previous research also demonstrated increased LDH activity following exposure to high glucose (50 mM) to HepG2 cells (Chandrasekaran *et al*., 2010). Our results suggest that exposure to HepG2 in a high glucose concentration for prolonged periods, i.e., 72 h is toxic to HepG2 cells. Surprisingly, HepG2 cells treated with resveratrol in the presence of high glucose significantly decreased the LDH released compared to the high glucose group alone. These findings demonstrated the protective effect of resveratrol against the toxicity induced by high glucose in HepG2 cells. Further studies are needed to elucidate this protective effect's underlying mechanisms and explore the potential therapeutic applications of resveratrol in managing high glucose-induced toxicity in liver cells.

Our research demonstrates that resveratrol displays antioxidant and antiapoptotic properties. This research has certain limitations. This study was conducted in *vitro* using HepG2 cells and would benefit from further investigations using appropriate animal models, such as diabetes-induced mice. Moreover, this study only focused on genes implicated in oxidative stress and apoptosis. Consequently, it is imperative to assess functional protein expression in order to determine a correlation with gene expression. Studies in humans, such as clinical trials or dietary supplementation, may provide further insights into resveratrol's antioxidant and antiapoptotic functions. Future research must investigate the potential clinical applications of resveratrol in humans. In addition, it would be intriguing to investigate the synergistic effects of resveratrol when combined with other antioxidants or antiapoptotic agents.

5. Conclusion

The findings of this study demonstrated the potential of resveratrol to mitigate oxidative stress injury and apoptosis in HepG2 cells induced by high glucose levels. The antioxidative stress-protective effect of resveratrol was observed through its ability to enhance intracellular antioxidants. In the interim, it impeded the process of apoptosis triggered by elevated glucose

levels through upregulation of Bcl-2 mRNA expression. Furthermore, the administration of resveratrol resulted in an upregulation of the DNA repair gene known as OGG1. Hence, it is plausible that RES could be a viable approach in addressing the impairment of antioxidant response resulting from elevated glucose levels commonly observed in diabetes and metabolic disorders.

Acknowledgments: The authors would like to thank Prof JL Marnewick for donating HepG2 cells to us for the experimental work. This research project was supported by a grant from the South African Medical Research Council (SAMRC), with funds from the National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant no. 115450). I would also like to thank the Department of Science and Innovation (DSI) - Inter-bursary Support Programme - Council for Scientific and Industrial Research (CSIR) for providing me with the bursary.

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CHAPTER 6: DISCUSSION

This chapter presents a comprehensive overview of the key findings derived from our study. Additionally, it delves into the study's limitations and provides recommendations for future research endeavors.

6.1 Summary of Manuscript Findings

6.1.1 Manuscript 1

This section examined the impact of high glucose and resveratrol treatment on HepG2 cells. Specifically, we quantified the expression levels of three established miRNAs (miR-126-3p, miR-182-5p, and miR-30a-5p) previously associated with diabetes. Through quantitative PCR analysis, we observed a significant reduction in the expression levels of miR-126-3p, miR-182-5p, and miR-30a-5p when HepG2 cells were exposed to high glucose conditions. Intriguingly, our findings revealed that the dysregulation induced by high glucose was effectively countered by resveratrol treatment. This suggests a promising role for resveratrol in regulating miRNA expression patterns implicated in diabetes.

It is widely recognized that miRNAs carry out their biological role by repressing the expression of specific target genes. In this study, we used an online database (miRDB-microRNA target prediction Database, http://www.mirdb.org/) to identify target genes of miR-126-3p, miR-182-5p, and miR-30a-5p. miR-126-3p reportedly targets SPRED1. Specifically, we observed that a decrease in miR-126-3p levels, induced by high glucose, resulted in an increase in *SPRED1* gene expression. Conversely, an increase in miR-126-3p expression due to resveratrol treatment, led to a significant reduction in *SPRED1* gene expression. Similarly, the downregulation of miR-182-5p due to high glucose treatment led to an upregulation of its corresponding target gene, *FOXO1*. The upregulation of *FOXO1* led to a concomitant upregulation of *G6P* expression. On the contrary, the upregulation of mi-182-5p led to a decrease in the expression of *FOXO1* and *G6P*. Furthermore, it has been demonstrated that miR-30a-5p exhibits targeting capabilities towards *Neurod1*. Decreased miR-30a-5p led to an increase in the expression of the *Neurod1* gene, while increased miR-30a-5p resulted in a significant reduction in the expression of *Neurod1* gene. These findings shows that the dysregulations of miRNAs can have a significant impact on the expression levels of their target genes. Moreover, our study suggest that resveratrol could potentially serve as a therapeutic agent for mitigating the dysregulation of miRNAs, thereby restoring the normal expression of their target genes.

6.1.2 Manuscript 2

Herein, we presented compelling evidence showcasing the mitigating effects of resveratrol on inflammation induced by high glucose levels and its potential to enhance glucose metabolism in HepG2 cells. Employing quantitative PCR (qPCR), we investigated the NF-kB pathway at the genetic level. NF-kB is a pivotal player in activating inflammatory responses (Jeon *et al*., 2010). Our investigation demonstrated a substantial rise in the mRNA expression of NF-kB, IKKα, and IkB-α under high glucose conditions. Notably, resveratrol administration markedly reduced NF-kB, IKKα, and IkB-α levels. Moreover, we assessed the expression profiles of TNF-α, IL-6, COX2, and IL-1β using a combination of qPCR, ELISA, and Bio-Plex techniques. We observed a significant surge in the expression levels of these pro-inflammatory cytokines in response to high glucose stimulation. However, the introduction of resveratrol led to a significant decline in the levels of these cytokines. Furthermore, we investigated the mRNA expression of *TGFβ1*, an anti-inflammatory cytokine. We observed that high glucose reduced the expression of *TGFβ1*; however, resveratrol recovered its expression. These findings further substantiate resveratrol's potential as an anti-inflammatory agent, particularly concerning diabetes, by modulating pro-inflammatory cytokine expression and curtailing NF-kB activity. Consequently, resveratrol is a promising therapeutic candidate for alleviating inflammation in individuals with diabetes.

The research underscores abnormal glucose metabolism in the liver as a primary instigator of type 2 diabetes (T2DM). Notably, disrupted glycogenesis and glycogenolysis play pivotal roles, with glycogenesis assuming particular significance (Rines *et al*., 2016). Our investigation unveiled an increase in gluconeogenesis *PEPCK* gene expression and a concurrent decline in glycolysis gene *GCK* expression in HepG2 cells under high glucose conditions. In contrast, resveratrol administration yielded a reduction in *PEPCK* expression coupled with an elevation in *GCK* expression within HepG2 cells. These revelations suggest the potential therapeutic utility of resveratrol in addressing hyperglycemia and insulin resistance linked to disrupted glucose metabolism.

In addition, we investigated the expression of *KLF7*, *HIF1A*, and *SIRT1* related to glucose metabolism and inflammation. We observed that high glucose increased the expression of *KLF7* and *HIF1A* and reduced the expression of *SIRT1*. Conversely, resveratrol increased the expression of *SIRT1* and reduced the expression of *KLF7* and *HIF1A*. These findings exhibited a putative protective influence by elevating the expression of *SIRT1* while suppressing the expression of *KLF7* and *HIF1A,* thereby potentially ameliorating the adverse consequences of elevated high glucose levels.

6.1.3 Manuscript 3

This study scrutinized the protective role of resveratrol against oxidative stress and apoptosis induced by high glucose in HepG2 cells. Our approach involved utilizing qPCR to assess the expression of key genes involved in the oxidative stress response (*SOD*, *CAT*, *GPx*, *Nrf2*, and *NQO1*), apoptosis (*BcL2*), and DNA repair (*OGG1*). Nrf2, a transcription factor orchestrating the expression of antioxidant and anti-electrolyte genes, holds relevance in this context (Kovac *et al*., 2015; Valenzuela *et al*., 2017). Our investigation unveiled a significant reduction in the gene expression of *Nrf2* and antioxidant enzymes (*SOD*, *CAT*, *GPx*, and *NQO1*) under high glucose conditions. Remarkably, the introduction of resveratrol led to an elevation in Nrf2 expression, subsequently triggering an increase in genes associated with antioxidant enzymes (*SOD*, *CAT*, *GPx*, and *NQO1*). These observations highlight resveratrol's potential in activating the Nrf2 signaling pathway at the transcription level and enhancing antioxidant enzyme expression, potentially extending its therapeutic application to other oxidative stress-related ailments.

Additionally, we explored the impact of resveratrol on apoptotic pathways at the transcriptional level. High glucose exposure notably decreased *BcL-2* expression, whereas resveratrol treatment significantly increased *BcL-2* expression. This augmentation in *BcL-2* levels may signify resveratrol's involvement in apoptotic pathway regulation.

Employing the LDH cytotoxicity Detection Kit, we evaluated LDH release. Notably, prolonged exposure of HepG2 cells to high glucose (72 h) led to a substantial increase in LDH release. Intriguingly, resveratrol administration reduced LDH release, underscoring its protective effect against high glucose-induced toxicity in HepG2 cells.

Furthermore, the gene expression of *OGG1* was assessed. High glucose exposure reduced *OGG1* expression, while resveratrol treatment significantly increased *OGG1* mRNA levels. This upregulation indicated enhanced DNA repair potential. These findings imply resveratrol's prospective therapeutic application in managing diabetic complications by fostering DNA repair through *OGG1* upregulation.

6.2 MiR-126-3p and Inflammation

The potential role of miR-126, which targets SPRED1, in inflammation resolution has been explored (Zeinali *et al*., 2021). Research indicates that pro-inflammatory agents can disrupt insulin-signaling pathways integral to glucose metabolism regulation, ultimately contributing to type 2 diabetes (Tsalamandris *et al*., 2019). Enhancing SPRED1 expression by suppressing miR-126 may incite IL-6, TNF-α, and ROS production, thereby causing endothelial dysfunction (Li *et al*., 2016). Our study reports suppressive effects of high glucose levels on miR-126-3p expression, leading to an elevation in SPRED1. Increased SPRED1 expression led to an

elevation in the levels of pro-inflammatory cytokines, TNF-α and IL-6. This sheds light on the potential role of miR-126-3p dysregulation in fueling the pro-inflammatory state observed in diabetes. Consequently, miR-126-3p is a therapeutic target for mitigating inflammation in diabetes patients. Resveratrol treatment augmented miR-126-3p expression, correlating with decreased SPRED1 and the pro-inflammatory cytokines, TNF-α and IL-6 expression. These insights suggest the viability of resveratrol-mediated miR-126-3p targeting to alleviate inflammation in diabetes.

6.3 MiR-182-5p and Glucose Metabolism

MicroRNAs (miRNAs) have emerged as pivotal regulators of hepatic glucose metabolism (Mirra *et al*., 2018). Prior studies have underscored their role in modulating insulin signaling, glucose uptake, glycogen synthesis, and gluconeogenesis in the liver (Mirra *et al*., 2018). Notably, miR-182 has been implicated in the modulation of glucose homeostasis, predominantly through its interaction with FOXO1 (Karolina *et al*., 2011); (Zhou *et al*., 2014); (Zhang *et al*., 2016). FOXO1 governs the transcription of downstream enzymes integral to gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Barthel *et al*., 2005; Gross *et al*., 2008). Dysregulation of metabolic flexibility and glucose homeostasis can result from elevated FOXO1 levels triggered by miR-182 downregulation (Zhang *et al*., 2016).

Our investigation highlighted the downregulation of miR-182-5p under high glucose conditions. Consistent with previous studies, diminished miR-182 levels correlated with FOXO1 upregulation, activating gluconeogenesis enzymes, PEPCK and G6P. Our findings demonstrated that reduced miR-182-5p expression correlated with heightened gene expression of *PEPCK* and *G6P*, accentuating gluconeogenesis. These insights highlight the potential therapeutic relevance of targeting miR-182-5p to ameliorate impaired glucose metabolism. However, further investigations are imperative to validate these initial findings.

Prior research has showcased the positive impact of miR-182 overexpression on glucose metabolism (Zhang *et al*., 2016). In alignment with this trajectory, our study unearthed resveratrol's capability to elevate miR-182-5p expression within HepG2 cells. This elevation, in turn, precipitated a reduction in FOXO1, PEPCK and G6P expression, ultimately dampening gluconeogenesis. Our results posit that resveratrol enhances glucose metabolism by modulating miR-182-5p expression in HepG2 cells. These observations suggest that harnessing resveratrol-mediated miR-182-5p modulation could be a prospective therapeutic strategy to rectify glucose metabolism impairment. It is important to note that further investigations are warranted to corroborate these preliminary findings.

6.4 Strengths and Limitations of the Study

The current study boasts a blend of strengths and limitations that warrant consideration. Our *in vitro* model provides a controlled platform conducive to meticulous experimentation and precise analyses. Moreover, our findings are consistent with established research, improving the existing understanding of potential miRNA dysregulation in diabetes-related conditions. Notably, prior investigations have underscored the presence of compromised glucose metabolism, escalated pro-inflammatory markers, heightened oxidative stress, and augmented apoptosis rates in individuals with diabetes.

However, certain limitations should be considered. Our study's exclusive focus on HepG2 cells, therefore, it may not adequately represent the complexity of miRNA regulation within diabetes pathophysiology. Due to severe budget constraints, our exploration was confined to genetic expression, and we were unable to perform certain proteomic analyses. Notably, protein expression analysis was not in the scope of this particular study and will be considered in future research.

6.5 Recommendations for Future Studies

Subsequent research endeavors should prioritize an expanded repertoire of cell models and experimental methodologies to comprehensively unravel the multifaceted roles enacted by these microRNAs within the realm of pathophysiology. The present study was performed in *vitro* employing HepG2 cells; it would be advantageous to conduct additional investigations utilizing suitable animal models, such as diabetes-induced mice. This expansion would offer a more holistic view of the intricate mechanisms associated with diabetes and its associated complications.

Additionally, to establish a substantive linkage between gene and protein expression, future investigations should incorporate the assessment of functional protein expression. This interplay is vital in deciphering the underlying mechanisms governing diverse biological processes. Quantifying protein expression can offer profound insights into the intricate transformation of genes into functional proteins while also investigating the impact on protein synthesis.

Achieving translational significance involves human-centric studies, such as clinical trials or dietary supplementation studies, to glean deeper insights into resveratrol's antioxidant, anti-inflammatory, and antiapoptotic properties, as well as its effects on glucose metabolism. Exploring the clinical implications of resveratrol in human trials will provide important insights and may produce potential therapeutic targets.

Moreover, investigating the potential synergistic interplay between resveratrol and other compounds boasting antioxidant, anti-inflammatory, and antiapoptotic traits is an interesting avenue. These synergistic explorations may highlight further therapeutic applications for resveratrol that may surpass its standalone attributes.

Taken together, these prospective investigations, marked by diverse models, intricate protein-gene relationships, human-centric approaches, and exploration of synergistic dynamics, may reveal novel therapeutic applications for resveratrol, improving scientific understanding and the practical application in clinical settings.

6.6 Conclusion

This study offers compelling evidence that resveratrol effectively counteracts the dysregulation of miR-126-3p, miR-182-5p, and miR-30a-5p induced by high glucose conditions. This interesting discovery highlights a promising avenue for therapeutic intervention, hinging on the modulation of miRNA expression through resveratrol administration. However, it is important to recognize that our study has certain limitations, necessitating cautious interpretation of the data and underscoring the need for further investigation.

Furthermore, our research substantiates the multifaceted attributes of resveratrol, reinforcing its capacity for counteracting inflammation, harnessing antioxidant potential, mitigating apoptosis, and regulating glucose metabolism within the intricate framework of diabetes. The intricate interplay between glucose metabolism, inflammation, oxidative stress microRNA regulation, and resveratrol's therapeutic effects could pave the way for novel therapeutic approaches in combating diabetes and its complications.

6.6 References

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APPENDIX

APPENDIX A: ETHICS CERTIFICATE

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HWS-REC)
Registration Number NHREC: REC-230408-014

P.O. Box 1906, Bellville 7535
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13 June 2023
REC Approval Reference No: CPUT/HWS-REC 2021/H6

Faculty of Health and Wellness Sciences Dear Ms. A Tshivhase

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms. A Tshivhase for ethical clearance. This approval is for research activities related to research for Ms. A Tshivhase at Cape Peninsula University of Technology.

TITLE: **Biochemical analysis and microRNA profiling in a diabetic in vitro model**

Supervisor: **Prof Tandi Matsha-Erasmus**

Comment:

Approval will not extend beyond 14 June 2024. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an annual progress report that should be submitted to the CPUT HWS-REC in December of that particular year, for the CPUT HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Ms. Carolynn Lackay
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences