TEMPOL and Insulin Resistance: A study of the underlying mechanisms behind changes in glucose metabolism, lipid accumulation and cellular stress

Gihani Manodara

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Under the supervision of Dr Kristine McGrath, Professor Alison Heather, Professor Michael Davies & Professor Sheila Donnelly

School of Life Sciences
Faculty of Science
University of Technology Sydney

Certificate of Original Authorship

I, Gihani Manodara, declare that this thesis is submitted to fulfil the requirements for the award of Doctor of Philosophy in the School of Life Science, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my work, conducted under the supervision of my principal supervisor Dr Kristine McGrath. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution; however, results have been presented in scientific meetings and submitted for publication as informed. The Australian Government Research Training Program supports this research.

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Statements

In accordance with the University of Technology Sydney thesis committee 'Graduate Research Candidature Management, Thesis Preparation and Submission Procedures (Version 1.10, 2021)', this PhD thesis is presented by compilation. It is comprised of one original Systematic Review study and three original Research studies to be submitted to peer-reviewed journals of which I am the first author. I hereby declare that I have contributed significantly to these studies.

For the Systematic study, I procured all the data, performed a systematic analysis of the papers, and drafted the first copy of the review paper.

For the Research studies, I carried out all experimental procedures, data analysis and drafted the first copy of the three research papers.

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Publications

The following publications have arisen directly from the work contained within this thesis.

TEMPOL as an antioxidant & anti-inflammatory therapy for type 2 diabetes mellitus, obesity and non-alcoholic fatty liver disease: A scoping review.

Authors: Gihani Manodara, Alyssa Borra, Michael J. Davies, Alison K. Heather, Kristine C. McGrath

Intend to submit: Liver International

Exploring the therapeutic potential of TEMPOL: Reducing insulin resistance in C57BL/6 mice via enhanced lipid metabolism adiposity reduction and adipose tissue inflammation suppression.

Authors: Gihani Manodara, David Van Reyk, Michael J. Davies, Alison K. Heather, Kristine C. McGrath

Intend to submit: Obesity.

TEMPOL reduces hepatic lipid accumulation and inflammation in high-fat diet-fed mice

Authors: Gihani Manodara, Scott Genner, Claire Rennie, David Van Reyk, Michael J.Davies, Alison K. Heather, Kristine C. McGrath

Intend to submit: Journal of Hepatology.

TEMPOL ameliorates palmitate-induced insulin resistance in MIN6 pancreatic beta cells.

Authors: Gihani Manodara, Michael J. Davies, Alison K. Heather, Kristine C. McGrath

Intend to submit: Journal of Diabetes Investigation.

Conference Proceedings

New Horizon, 2017

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Inhibitory effect of TEMPOL on palmitic acid-induced steatosis and inflammation in HepG2 hepatocytes, 2017; Perth; Australia.

ANZOS: Australian and New Zealand Obesity Society, 2018

Gihani Manodara, Claire Rennie, Alison K. Heather, Kristin C. McGrath.
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Award: Best Oral presentation

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Abbreviations

ACAB	Acetyl Co-A carboxylase b
ACC	Acetyl- CoA carboxylase
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATF6	Activating transcription factor 6
BMI	Basal Metabolic Index
CEBP α	CAAT-enhancing binding protein α
CHREBP	Carbohydrate response element binding protein
CKD	Chronic kidney disease
CPT-1	Carnitine palmitoyltransferase - I
CVD	Cardiovascular disease
Cyt C	Cytochrome C
DNL	De novo lipogenesis
DCFH-DA	2′, 7′-dichlorodihydrofluorescein diacetate
DEXA	dual-energy x-ray absorbance
DMEM	Dulbecco's Modified Eagle Medium
EMA	European Medicine Agency
ER	Endoplasmic reticulum
ERK1/2	Extracellular-related kinase 1/2
ETC	Electron transport chain
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FDA	Federal drug administration
FFA	Free fatty acid
FXR	Farnesoid X nuclear receptor
GLUT 4	Glucose transporter- 4
GOI	Gene of interest
GPX	Glutathione peroxidases
GSK	Glycogen Synthase Kinase
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HepG2	Human hepatoma cells
HFD	High fat diet
HNO	Nitroxyl anion
HOMA-IR	Homeostatic model assessment of insulin resistance
IKK β	Inhibitor of nuclear factor Kappa B kinase
IL-1 β	Interleukin- 1 β
IL-8	Interleukin - 8
IL6	Interleukin - 6
IRE1 α	inositol-requiring transmembrane kinase endonuclease -1 α
ipGTT	Intraperitoneal glucose tolerance test
ip ITT	Intraperitoneal insulin tolerance test
IRS	Insulin receptor substrate
JNK	c-Jun-N -terminal kinase

LPL	Lipoprotein lipase
MAPK4	Mitogen-activated protein kinase-4
MCP-1	Monocyte chemoattractant protein- 1
NADPH	Nicotinamide dinucleotide phosphatase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
NFkB	Nuclear factor kappa B
NRF2	Nuclear factor erythroid 2-related factor 2
StD	Standard chow diet
O_2^-	Superoxides
OH	Hydroxyl ions
ONOO	Peroxyl nitrites
PAI-1	Plasminogen activator inhibitor- 1
PBS	Phosphate buffer saline solution
PDX -1	Pancreatic and duodenal homeobox-1
PERK	Protein kinase R-like ER kinase
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein Kinase C
PPAR α	Peroxisome proliferator-activated α
Retn	Resistin
ROS	Reactive oxygen species
RQI	RNA quality indicator
SAA- 1	Serum amyloid A-1
SEM	Standard error mean
Ser/Thr	Serine/threonine
SOC-3	Suppressor of cytokine signalling-3
SOD	Superoxide dismutase
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TNF	Tumour necrosis factor
UPR	Unfolded protein response
VLDL	Very-low-density lipoprotein
XBP-1	X-box binding protein-1
β-OHB	β-Hydroxybutyrate

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Abstract

Obesity is increasing in prevalence and is now considered an epidemic in many developed populations. Obesity is a multifactorial disease in which genetics and environmental factors interact. For example, in rare cases of childhood obesity, a single gene can have a major impact on determining the occurrence of obesity [1]. However, when considering the worldwide epidemic, there is much agreement that environmental factors are the major contributors to obesity [2]. Overnutrition-induced obesity can lead to chronic oxidative stress and inflammation, both of which contribute to the development of insulin resistance. There are currently no pharmaceutical treatments available to address the chronic cellular stresses that underpin insulin resistance and the downstream clinical sequelae that include type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). However, TEMPOL, a potent antioxidant, could hold the key.

The nitroxide, TEMPOL, has been shown in cell and whole animal systems to suppress obesity-induced adipokine secretion and oxidative stress. Therefore, given the need for a therapeutic agent for obesity and its related syndrome, insulin resistance, in Chapter 3, current biomedical research describing TEMPOL's antioxidative and anti-inflammatory properties, from 2011-2021 were summarised in a scoping review. The data from this investigation demonstrated that TEMPOL is a potent agent for reducing oxidative stress and inflammation. This scoping review also revealed that TEMPOL treatment directly suppresses lipid accumulation in tissues and cells. Together, the evidence provided in the scoping review led to the hypothesis of this thesis that TEMPOL through its ability to suppress excessive lipid build-up as well as its antioxidant and anti-inflammatory effects would reverse insulin resistance and thus provide protection against T2DM and NAFLD. Given that TEMPOL reduces lipid accumulation, it was further hypothesized that TEMPOL has the potential to decrease body mass gain normally induced by a high-fat diet.

To explore the hypothesis that TEMPOL acts as an antioxidant, anti-inflammatory and anti-obesity agent, a C57BL/6 high-fat diet-fed mouse model was used. The *in vivo* mouse data was reinforced, and molecular mechanisms were further delineated, using supplemental *in vitro* cell culture models.

In Chapter 4, the study investigates TEMPOL's effects on lipid accumulation induced by palmitic acid in both *in vitro (*adipocyte 3T3L-1 cells using palmitic acid) and *in vivo* (C57BL/6 mouse fed a high-fat diet) models. Results indicated that TEMPOL supplementation reduced body mass gain, altered lipid metabolism, decreased adipogenesis markers, mitigated inflammatory cytokine expressions, reduced mitochondrial oxidative stress, and improved insulin sensitivity in high-fat diet (HFD) mice. *In vitro* findings show similar effects, suggesting TEMPOL's potential as a promising anti-obesity drug by suppressing adipose inflammation and oxidative stress.

In Chapter 5, it was investigated whether TEMPOL mitigates hepatic insulin resistance induced by a HFD in mice, a well-characterised model for insulin resistance. TEMPOL was administered to HFD-fed mice resulted in a significant reduction in hepatic lipid accumulation, improved glucose tolerance, and insulin sensitivity. Additionally, TEMPOL-treated mice exhibited decreased hepatic inflammation, oxidative stress, fibrosis, and body mass. These findings were corroborated by *in vitro* experiments using HepG2 cells exposed to palmitic acid-induced cellular stress. Chapter 5 concludes that TEMPOL holds promise in improving hepatic insulin sensitivity and suggests further exploration of its clinical application in treating early insulin resistance or type 2 diabetes.

Chapter 6 investigates the potential protective effects of TEMPOL, against lipotoxicity-induced pancreatic β-cell dysfunction, a significant contributor to diabetes. Using both *in vitro* (MIN 6 β-cells exposed to palmitic acid) and *in vivo* (C57BL/6 mice fed a HFD) models, the lipotoxic conditions led to hyperlipidaemia, mitochondrial and endoplasmic reticulum stress, decreased insulin sensitivity, and insulin secretion. However, TEMPOL exposure reversed these effects, restoring the cellular profile to closely resemble that of the untreated control group. Mice on HFD administered TEMPOL also showed improved glucose tolerance and insulin sensitivity. These findings suggest that TEMPOL could be developed into a pharmaceutical therapy for addressing pancreatic dysfunction associated with diabetes.

In summary the findings presented in this thesis show that TEMPOL does attenuate oxidative stress and inflammation in all three key tissue types that underpin insulin resistance. Notably, the protective effects of TEMPOL on reducing cellular stress associated with suppressing lipid accumulation. While this study is limited by mouse and cellular studies, the data obtained does now provide a strong impetus to follow up with a pre-clinical or proof-of-principal pilot clinical

study. TEMPOL is used in the clinical arena for other medical issues and thus re-purposing the drug for insulin resistance may be an option that could be fast-tracked as some safety and efficacy data in humans would already exist.

Chapter 1: Introduction

Chapter Summary

This chapter provides the background needed to understand insulin resistance and type 2 diabetes mellitus (T2DM). This chapter also provides the possible mechanistic link between lipid accumulation and T2DM. A potential new therapy for insulin resistance is introduced.

1.1 Obesity: Definition

Obesity is a complex multifactorial disease caused by excessive energy consumption and little to no energy expenditure (e.g., physical activity) by an individual. The characteristic lipid accumulation in adipose tissue and other organs in an obese patient leads to an increased risk of detrimental health problems and reduced life expectancy [3]. Obesity can drive the development of several chronic diseases such as diabetes mellitus, cardiovascular disease, non-alcoholic liver disease and cancer [4-7].

The most accurate methods of measuring body fat are underwater body weight measurements, dual-energy x-ray absorbance (DEXA), magnetic resonance or computed tomography. However, to identify overweight and obese individuals, the most used and accepted by health professionals and organisations is the body mass index (BMI). It is a simple screening calculation that is easily deployed in the initial stage of obesity assessment [8]. BMI is calculated using an individual's height in meters squared (m^2) and weight (kg). The formula used is:

$$\mathbf{BMI = kg / m^2}$$

As shown in Table 1, the range of BMI values indicates the health risk, where a BMI below 18.5 and BMI above 40 are both considered extremely high risk to an individual's health. Individuals with a BMI greater than 30 are considered obese and associate with an increased health risk of fat-related disorders [9]. The severity of obesity is classified into classes I to III, as shown in Table 1.

Table 1: Classification of overweight and obesity by BMI [10]

Category	**BMI (kg/ m^2)**	**Health Risk**
Underweight	< 18.5	Low
Normal range	18.5 – 24.9	Average
Overweight	25.0 – 29.9	Mildly increased
Obese	≥ 30	
Obese class I	30.0 – 34.9	Moderate
Obese class II	35.0 – 39.9	Severe
Obese class III	≥ 40	Very severe

1.1.1 Epidemiology of Obesity

Over the past several decades, the rate of obesity among adults and children has not waned and reports have shown that its prevalence has tripled since 1975 [11]. Obesity has become one of the core threats to global health issues as it parallels the development of socioeconomic statuses in every continent of the world. Nearly two-thirds of the population in the United States of America is considered obese, and nearly half of its population lives with prediabetes or diabetes. Several trends were significant in the years 2013-2014, such as the prevalence of overweight (BMI ≥ 25- 30 kg/ m^2) plateaued whilst the population of extreme obesity (BMI ≥ 40 kg/ m^2) underwent a nine-fold increase from the year 1960 to 2014 [12]. In Australia, 25% of the population is considered obese, which will double by 2030 [13]. Additionally, the global growth of childhood obesity is also a risk factor for adulthood obesity, as reports have noted a tripling of obesity rates among children in the United States. Overall global trends in BMI recorded 1.3 billion adults as overweight in 2016, a six-fold increase from 1975 to 2016 [14].

The global prevalence of obesity is thought to be due to the development of technology and changes in socioeconomic lifestyles [15]. For example, the advancing economies of work life, leading to a more sedentary lifestyle with less leisure time, increased wealth to purchase processed foods and more meals eaten outside [16, 17]. These global trends in overweight and obesity have become a threat, an economic burden to the health care system, and an additional cost for treatment demands [18, 19]. In 2014, in the USA alone, the health cost incurred by a single obese individual was $1901 per annum; when extrapolated, it cost $149.4 billion at the

national level [20]. Besides health care costs of lost workdays, lower work productivity, mortality, permanent disability, and mental health disorders (e.g., depression), obesity also results in lost productivity and foregone economic growth [21-23].

Additionally, obesity is the fifth leading risk factor for morbidity and mortality from non-communicable diseases. Specifically, 44% have T2DM, 23% have cardiovascular disease, and 41% have cancer related to obesity or overweight [24, 25]. The rates of T2DM are currently on the rise, primarily in Asian and South Asian populations [26]. Over the past three decades, the most significant increase in obesity has occurred in developing countries, particularly among the urbanised group in Oceania, Latin America and North America [27]. To deal with obesity and obesity-related diseases, there is a need to provide adequate health care and develop innovative and alternative solutions to reverse current trends.

1.1.2 Pathogenesis of Obesity

Obesity is primarily caused by a longstanding energy imbalance between calories consumed and expended. The evolution of humans and their predecessors were forced to endure periods of undernutrition, and selection pressure mostly likely resulted in genotypes that favour overeating and inactivity [28]. In the last few years, overnutrition has emerged as a more significant health threat (i.e., people are now dying more from obesity and overweight than from undernutrition). Thus, obesity research to better understand the underlying mechanism in order to develop new interventions to help people achieve and maintain healthy body weight is becoming increasingly urgent.

Adipose tissue, a hormonally active endocrine organ, comprises adipocytes, which accumulate triglycerides (TG), and secretes cytokines, chemokines and adipokines, which are involved in regulating feeding behaviour and immunity [29]. In overweight or obese individuals, adipocyte hypertrophy rather than hyperplasia is primarily responsible for the increase in fat mass [30]. An increase in intracellular lipids in adipocytes leads to the release of adipokines. Increased levels of circulating adipokines, in turn, are associated with various organ dysfunctions such as hepatic steatosis and chronic diseases such as insulin resistance.

Adipocyte hypertrophy triggers the accumulation of macrophages, resulting in macrophage phenotypic modifications and inflammatory status of adipose tissue [31]. For example,

monocyte chemoattractant protein- 1 (MCP-1) expression levels are directly associated with adipose tissue expansion [32]. MCP-1 receptor is highly expressed in those adipose tissue regions where macrophages proliferate [33, 34], with a 7.2 -fold increase in MCP-1 expression levels in obese mice compared to the control group reported [35]. Further to this MCP-1 levels are directly proportional to macrophage infiltration and are higher in diet-induced obese and insulin resistance mouse models [36, 37]. Adiponectin and classical cytokines such as interleukin-6 (IL-6) and tumour necrosis factor (TNF) are also produced during adipose hypertrophy and are linked with circulating MCP-1 and increased BMI [32, 38].

The increase in classical cytokines, particularly TNF, is directly related to the increased macrophage numbers [39]. TNF production requires activations of both the IKB kinase - nuclear factor kappa β (IKKβ-NF-κB) pathway and the c-Jun-N -terminal kinase (JNK) - mitogen-activated protein kinase-4 (MAPK4) signalling pathway [40]. TNF can directly impact insulin signalling in skeletal muscle cells by triggering serine/threonine (Ser/Thr) phosphorylation of the insulin receptor substrate (IRS) [41]. In addition to the marked increased in TNF, it has been reported that adipocyte hypertrophy leads to a 100-fold increase in other cytokines such as IL-6 [42].

It is now well-recognised that the inflammatory changes underlie, at least in part insulin resistance in obese patients [43, 44]. For example, activated JNK pathway is the mediator of obesity-associated disruption of the metabolic homeostasis [45]. TNF activation of MAPK and IKKβ can lead to an inflammatory cascade that activates phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt) pathways [46, 47]. Both kinases in turn, then drive Ser phosphorylation of insulin receptor substrate -1 (IRS-1), the hallmark characteristic of insulin resistance. IL-1β and IL-6 have also been shown to impair insulin signalling through their effects on the extracellular-related kinase 1/2 (ERK1/2) and the activation of P13K, Akt and glycogen synthase kinase (GSK) pathways [48, 49].

Increased oxidative stress, impaired antioxidant defence and augmented conditions of hyperlipidaemia and hyperinsulinemia are reported endemic in adipocytes from insulin-resistant obese subjects [50]. Oxidative stress occurs when there exists an imbalance between endogenous antioxidant levels and reactive oxygen species (ROS) generation [51]. Obesity induces systematic oxidative stress through the chronically activated inflammatory pathways

described above [50, 52]. It is noteworthy that oxidative stress is intricately linked to the redox regulation of the transcription factor NF-κB. NF-κB, an upstream regulator of TNF, plays a crucial role in promoting and regulating many of the activities attributed to TNF. This connection further strengthens the understanding of the mechanisms involved, as discussed in the preceding pages regarding TNF and its impact on obesity-induced oxidative stress. For example, the link between oxidative stress and transcriptional regulation of cells can be linked to the redox regulation of transcription of factor NF-κB. Studies have shown antioxidants blocking the induction of TNF apoptosis mediated by NF-κB [53]. These findings highlight the importance, but also the complexities, of redox control of transcription factors in healthy and pathologic circumstances. Furthermore, an increase in oxidant level results in impairment in proteasome activity, resulting in protein oxidation and protein misfolding that, in turn, contributes to insulin resistance has been shown in mouse models [52].

A dysfunctional mitochondrial electron transport chain (ETC) has been reported in response to the excess free fatty acids (FFAs) in plasma [54]. Mitochondrial dysfunction can lead to cellular apoptosis [55]. A diet-induced mouse model by Wang et al. demonstrated a low proliferation rate of regulatory T-cells (white blood cells that regulate the immune system) and an enhancement in apoptosis due to a reduced mitochondrial transmembrane potential [56]. Disruption of the membrane potential of the mitochondria can trigger apoptotic proteins that will activate apoptotic pathways [57].

Obesity-led lipid accumulation in adipose tissue drives inflammation and mitochondrial dysfunction that increases ROS levels leading to cellular dysfunction, and eventually apoptosis. Systemically, the secreted adipokines and cytokines lead to defective insulin signalling in other tissues leading to insulin resistance.

1.1.3 Current therapeutic agents and interventions for Obesity

The challenge of controlling excess body fat is one of the most pressing healthcare issues today [58]. Since lifestyle and behavioural interventions have moderate effectiveness, pharmacological and surgical interventions are increasingly recognised as obesity treatment strategies. Currently, bariatric surgery remains the most effective approach for losing body weight. The improvement in laparoscopic procedures and decrease in hospitalisation time have

increased the popularity of this approach and with increasing numbers, it has shown that the life expectancy of an obese patient has increased by three years [59]. However, with the obesity epidemic, surgical interventions simply cannot meet the medical demand.

Anti-obesity drugs have been trialled but, to date, have faced a series of regulatory problems, as they demonstrate adverse side effects including drug dependence, abuse (methamphetamine) [60] and cardiovascular problems (fenfluramine, dexfenfluramine, sibutramine) [61, 62]. The variety of drugs that have been investigated ranges from sympathomimetics, gastrointestinal-deprived peptides that are chemically optimised, lipase inhibitors, mitochondrial uncouplers and cannabinoid receptor antagonists [63, 64]. However, at clinical dosage levels, all drugs tested show only a weight loss of 10% or less of initial body weight and the weight loss was only achievable because of the reported adverse effects [63].

Despite their failings, there are approved drugs to manage obesity used together with behavioural modifications. For example, dopamine and norepinephrine reuptake inhibitor (bupropion), GLP-1 receptor agonists (liraglutide & semaglutide), and pancreatic lipase inhibitor (orlistat), are registered and promoted in the United States of America and Europe [65]. Bupropion, a norepinephrine and dopamine inhibitor, is used with naltrexone, an opioid agonist that does not affect weight loss; instead, it blocks the β-endorphins that are released by the hypothalamus, inhibiting feeding stimulus [66, 67]. Similarly, GLP-1 receptor agonists induce satiety by releasing the incretin hormone, GLP-1, resulting in glucose-lowering actions such as stimulation of glucose-induced insulin secretion. It was found that exogenous GLP-1 infusion in humans resulted in reduced caloric intake, reduced appetite and effects on the reward system without direct changes in energy expenditure [68, 69]. The first GLP-1-based anti-obesity drug introduced in the USA is liraglutide, also used to control T2DM [70]. However, gastrointestinal side effects such as nausea and vomiting have been reported. Currently, the most recent FDA-approved anti-obesity drug is semaglutide at a dosage of 2.4 mg, which can decrease body weight by 15% over a period of 68 weeks. The drug has, however, been reported to cause gastrointestinal side effects [65].

There are many challenges confronting the development of anti-obesity drugs. One challenge includes the heterogeneity and environmental risk factors that may affect an individual's response to specific pharmacotherapies [71, 72]. Another factor is the neuroendocrine

hormones that regulate food intake, a system evolutionary designed to defend against undernutrition [73]. In addition, the imbalance of insulin and leptin levels in obese patients leads to desensitisation, causing impairment in the responsiveness of the homeostatic system and obstructing the effectiveness of the weight loss pharmacology [74]. Pharmacology that focuses not solely on weight loss, but on improving cardiometabolic parameters such as lowering serum triglycerides, raising high-density lipoprotein (HDL) cholesterol and controlling blood pressure can progressively enhance weight loss.

1.2 Non-alcoholic liver disease (NAFLD): Definition

Non-Alcoholic Fatty Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH) represent two stages of a liver disease continuum. NAFLD, characterised by the accumulation of fat in the liver unrelated to alcohol consumption, ranges from simple steatosis to the more severe form known as NASH. NASH involves inflammation, hepatocyte injury, and potential fibrosis, posing a higher risk of progressing to advanced liver disease. A clinical diagnosis of NASH requires a liver biopsy, which reveals a histological indicator such as steatosis, inflammation, and hepatocellular injury. Additionally, clinical markers such as elevated liver enzymes and imaging evidence of inflammation may contribute to diagnosis. Accurate differentiation between conditions is crucial for appropriate diagnosis, management, and prognosis in individuals with liver conditions associated with fat accumulation [75, 76]. Clinically NAFLD is first established using radiological imaging techniques to detect $\geq$ 5% hepatic fat accumulation without preclinical issues that might cause fatty liver, e.g. extreme alcohol consumption, viral diseases, steatogenic medications and autoimmunity [77]. Accumulating excess lipids in the liver may lead to insulin resistance and chronic inflammation [78], increasing the risk of advanced liver disease with fibrosis and cirrhosis and the risk of hepatocellular carcinoma [79].

There is growing evidence that NAFLD is a multisystem disease that impacts many organs and gives rise to chronic diseases such as T2DM, chronic kidney disease (CKD) and cardiovascular disease (CVD). However, among these co-morbidities, T2DM seems to be the highest risk factor for having NAFLD and a common predictor of adverse outcomes such as NASH, hepatic fibrosis and cirrhosis. Recent meta-analysis studies have shown that NAFLD increased mortality by 57% in patients with CVD and increased the incidence of T2DM by two-folds [80].

1.2.1 Epidemiology of NAFLD

NAFLD is becoming the world's most common chronic liver disease with a global prevalence now estimated to be 25.5% [81]. NAFLD accounts for 75.1% of chronic liver disease cases in the USA, 42% in Southeast Asia and 13% in Africa [81-83]. This development parallels the changing socioeconomic status and lifestyle trends [84]. In addition, NAFLD/NASH is one of the top three indications for liver transplantations in the USA. The prevalence of hepatic fibrosis is 40 - 50% in NASH patients, and current statistics show that 30 - 40% of patients develop NASH from NAFLD [85], thus, accounting for nearly ~10% of NASH patients undergoing liver transplantations in the USA [86]. The prevalence of NAFLD in Australia is projected to increase from 25% to 76.3% by 2030, and the incidence of death is estimated to be 85% [87].

NAFLD is strongly associated with obesity, insulin resistance and T2DM. Among the published studies, the diagnosis of NAFLD has been shown to increase the risk of developing T2DM by 64% [88]. Meta-analysis data presents a prevalence of 22.51% of T2DM patients among NAFLD and 43.63% among NASH patients [81]. In addition, patients with NAFLD who also have T2DM are more likely to develop hepatocellular carcinomas, experience liver-related mortality and suffer an overall worsening of health outcomes [89, 90]. In a recent analysis, the increased incidence of diabetes and obesity in the USA was projected to increase the disease burden related to NASH and its complications [91]. Therefore, it is apparent that patients with NAFLD appear to have a higher disease burden and progression if T2DM is a comorbidity.

1.2.2 Pathogenesis of NAFLD

NAFLD is a complex disease, with steatosis developing hepatic inflammation and fibrogenesis occurring simultaneously or sequentially according to the severity of the disease. Presently, the progression of NAFLD to fibrosis and its different phenotypes is shown using the multiple-hit hypothesis [92]. This hypothesis states that myriad factors work in parallel and synergistically in an individual. Even though the progression from NAFLD to NASH is generally considered a sequential process, the marked heterogeneity in the clinical outcome of these two subgroups has led to the hypothesis that NAFLD and NASH may represent two distinct conditions [92].

Numerous signalling pathways are involved in regulating hepatic lipid homeostasis, including hormones, transcription factors, and nuclear receptors, as well as insulin signalling [93]. The primary pathophysiology of NAFLD is an imbalance between the synthesis and utilization of triglyceride (TG). The development of insulin resistance in patients with obesity or T2DM leads to a substantial increase in lipolysis in the adipose tissue, causing an excessive influx of non-esterified fatty acids (NEFAs) into the liver, which is facilitated by the fatty acid transporters in hepatocytes [94]. In addition, chronic overnutrition and de novo lipogenesis (DNL) are the other two contributing factors to hepatic fat accumulation. It has been estimated that 60% of plasma fatty acid from adipocyte lipolysis accounts for hepatic TG, while 25% of lipid from DNL (threefold higher in NAFLD patients) and 15% dietary contribution [95, 96]. Fatty acid synthesis in hepatocytes is aided by two enzymes, fatty acid synthase (FAS) and acetyl- CoA (ACC), and their expressions are controlled by insulin and glucose levels [95, 97, 98]. Thereby the lipogenesis process in the liver is markedly increased in NAFLD by hyperinsulinemia and an increased intake of simple sugars such as fructose [99].

Poor TG utilization results in hepatic TG accumulation; this can be caused by disturbed hepatic fatty acid oxidation, impaired very-low-density lipoprotein (VLDL) production or altered synthesis. In hepatocytes, fatty acid oxidation occurs in the mitochondria and peroxisome, resulting in higher energy production. It is unclear how mitochondrial fatty acid β-oxidation might interfere with the development of NAFLD because conflicting studies have reported reduced and increased rates of fatty acid (FA) oxidation in NAFLD, and some studies have shown an impairment with progression to NASH [100]. On the other hand, reports indicate that VLDL production in NAFLD is increased; however, its production does not compensate for the increased rate of TG synthesis [100, 101].

In obese individuals, insulin action in adipocytes is impaired, which increases the risk of lipolysis suppression, adipocyte stress, macrophage recruitment and infiltration into the adipose tissue. Consequently (as discussed in section 1.1.2), the release of proinflammatory cytokines such as IL-6, TNF, resistin (Retn) and MCP-1 trigger the NFkβ and JNK pathway disrupting the insulin signalling pathway creating a vicious cycle of promoting insulin resistance in insulin-sensitive tissues such as the liver [102]. Studies have reported that levels of protective adipokines such as adiponectin are lowered in NAFLD patients, however, therapies that target activation of AMP-activated protein kinase (AMPK) and peroxisome

proliferator-activated α (PPAR α) pathways have been shown to improve insulin sensitivity and steatosis in murine NAFLD models [103, 104].

Fatty acid (FA) accumulation in hepatocytes leads to lipotoxicity whereby physiologically adaptive mechanisms in the liver are overwhelmed by excessive ROS production and endoplasmic reticulum (ER) stress [105, 106]. Contributing to ROS overload is the mitochondrial dysfunction [107]. The excessive ROS leads to disrupted phospholipid membranes, DNA damage, and proinflammatory cytokines release [107, 108]. ER stress results in misfolded proteins that, in turn, trigger the unfolded protein response (UPR) and other stress proteins including activating transcription factor 6 (ATF6), inositol-requiring transmembrane kinase endonuclease -1 α (IRE1α) and protein kinase R-like ER kinase (PERK) that regulate triggering the autophagy pathways. The combined effect leads to hepatocellular injury [109, 110].

Chronic oxidative stress and inflammation ultimately lead to the activation of cell death pathways, which is a distinctive pathological feature of NAFLD and NASH [111, 112]. There are two types of cell death: an intentional process involving intact plasma membranes (apoptosis) and an accidental process involving lysis of the cellular membranes (necrosis). This process is also known as lipoapoptosis when it occurs in the context of lipotoxicity. Lipoapotosis and the necro-inflammatory form of the NAFLD disease confers risk of progression towards more advanced liver fibrosis, cirrhosis and HCC [113, 114].

1.2.3 Current therapeutic agents and interventions for NAFLD

Lifestyle interventions including modification to diet and physical activity are the first line of treatment to initiate body weight loss to improve insulin sensitivity. Diets based on real-food, healthy eating such as the Mediterranean diet are highly recommended to reduce body weight, insulin resistance and hepatic lipid accumulation [115]. Studies have shown that long-term exercise improves the phagocytic capacity of the liver, thus reducing liver inflammation and fibrogenesis along with a decrease in blood pressure, insulin resistance and hepatic fat accumulation [116-118]. However, lifestyle and diet changes are not long-term solutions, as patients often struggle to sustain and maintain body weight loss and adherence to lifestyle

interventions is often poor. Therefore, there is a critical need to develop pharmacologic therapies for NAFLD.

Currently, there are no specific pharmacologic therapies that have been approved by Food and Drug Administration (FDA) or by the European Medicine Agency (EMA) to treat NAFLD [119]. The current pharmacologic therapies for NAFLD are anti-obesity (cilofexor, liraglutide) [120] and anti-diabetic drugs (canagliflozin, dapagliflozin, and empagliflozin) [121]. For example, recommended pharmacotherapies such as Vitamin E and thiazolidinediones (pioglitazone) are used for NAFLD patients with and without diabetes [77, 122]. However, there have been some safety concerns with Vitamin E, as it may increase the risk of prostate cancer [123, 124] and pioglitazone tends to induce weight gain and post-menopausal bone loss over long-term use [125]. A few of the new drugs that are in the advanced stage of clinical trials (phase 3) are farnesoid X nuclear receptor (FXR) agonists (cilofexor) [126, 127], GLP-1 receptor agonists (liraglutide) [128, 129], PPAR agonists (elafibranor & fenofibrate) and apoptosis inhibitors (Selonsertib) [130-132]. Most phase 3 new drugs have recorded adverse effects ranging from headaches, myalgia, pruritus and appetite suppression leading to anorexia [119].

As the incidence of NAFLD is rising along with associated obesity and T2DM, ideally, pharmacological therapies should not only be effective for liver disease but also reduce the risk of comorbidities. Therefore, despite numerous clinical trials, there is a pressing need for definitive treatment for NAFLD and its commodities.

1.3 Type 2 Diabetes Mellitus: Definition

Diabetes mellitus (DM) is characterised by hyperglycaemia that results in defects in insulin action, secretion, and insulin resistance in insulin-sensitive tissues. Chronic hyperglycaemia affects several vital organs including the kidneys, heart, liver and eyes [133]. Most DM cases fall into Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). By far, the more prevalent is T2DM resulting from insulin resistance in targeted organs and inadequate compensatory insulin secretion [134]. T2DM patients are diagnosed with different degrees of insulin deficiency and obesity-related insulin resistance, which can lead to nephropathy NAFLD and cardiovascular disease [135, 136]. T2DM has quadrupled in prevalence and

incidence between 1980 and 2004 due to obesity, sedentary lifestyles, and an ageing population [137].

1.3.1 Epidemiology of T2D

An unprecedented increase in T2DM cases has been attributed to obesity, physical inactivity, and energy-dense diets worldwide. There were 415 million patients diagnosed with DM in 2015, and more than 90% were T2DM [138]. In the same year, T2DM was labelled as the sixth leading cause of mortality [138]. It is estimated that by 2040, the number of DM cases will reach 642 million, placing a significant burden on socioeconomic pressures on individuals and global health costs [139].

The prevalence of T2DM has been increasing since the 1980s. However, it varies from region to region and is most common in low to middle-income countries [137]. The increase in T2DM cases in developing countries is in parallel with the increase in urbanisation, accompanied by diets and sedentary lifestyles [140]. In the years 2020-2021, it was reported that 1.3 million Australians were diagnosed with diabetes with 85-95% as T2DM following the global trend [141]. Notably, it was also reported that indigenous populations, such as in Australia, had a four-fold increase in T2DM incidence relative to the non-indigenous population 2008-2011 [142]. Patients with T2DM are noted to have a 15% increased risk of mortality compared to non-diabetic patients, and it is twice as high in patients less than 55 years of age. The annual health burden cost for those with diabetes and obesity is AU $3131 per person, including additional subsidies, which is $8,085 per person. It has been calculated that the direct cost and subsidies were 46% higher for obese diabetic patients than for average-weight diabetic patients, regardless of the patient's diabetic status [143].

T2DM can be affected by genetic and environmental factors. Heritability is estimated to be between 25 % - 80%, where the lifetime risk of developing T2DM is 40% for an individual with one parent with T2DM and a 70% chance for an individual whose both parents are diagnosed with T2DM [144]. There is no doubt T2DM is affected by genetic factors however the collision of social determinants and environmental factors has changed the pathogenesis of the disease rapidly over the decades. A traditional high-energy-burning lifestyle has given way to a Western sedentary lifestyle with little or no activity and a high-energy diet. Meanwhile,

genetic characteristics evolve at a slower rate across generations and tend to favour the selection of "energy-saving thrifty genotypes," which may have aided individuals living in times of insecurity by storing energy in times of plenty [145]. Therefore, at current, diet modifications and increased physical activity are the first line of therapy suggested by many medical professionals [146]. The initial stages of T2DM are characterised by hyperinsulinemia, which diet interventions can control; however, pharmaceutical therapy will be required along with insulin injections as the disease progresses.

1.3.2 Pathogenesis of T2DM

T2DM is associated with numerous pathophysiologic abnormalities. Insulin resistance is characterised by a reduced peripheral glucose uptake (mostly muscle) accompanied by increased endogenous glucose production. Increasing lipolysis, elevating FFA levels, and accumulating intermediary lipid metabolites contribute to increased glucose output, decreased peripheral glucose utilisation and impairment of pancreatic β-cell function. One of the main contributors to T2DM is adipocyte insulin resistance, inflammation, and the presence of NAFLD is an integral part of insulin resistance patients.

Glucose homeostasis depends upon the secretory response of insulin and the function of pancreatic β cells and the sensitivity of the insulin-sensitive tissues, independent of hyperglycemia and hyperinsulinemia. Upon glucose ingestion, insulin is secreted, and glucagon release is inhibited. The high insulin: glucogen ratio, signals the liver to stop hepatic glucose output. However, if the liver is in a state of chronic insulin resistance, the high insulin: glucogen ratio will not be properly sensed and gluconeogenesis will continue alongside glucose uptake via the gastrointestinal tract, leading to systemic hyperglycemia. In T2DM subjects, basal endogenous glucose production is elevated at ~ 0.5 mg/kg. Min (compared to non-T2DM subjects) and continues even while sleeping. This leads to an additional 35 g of glucose produced by the liver leading to a high fasting glucose level (140-200 mg/dl, > 7.8 mmol/l) [147-150].

Recent animal studies show insulin's direct and indirect effects (such as elevated FFA's), on endogenous glucose production [151]. Results indicate that adipose tissue lipolysis impacts insulin regulation, where an increase in systemic FFA's due to an increase in lipolysis will impair insulin signalling and its sensitivity, a common pathogenesis among obese T2DM

patients. These factors negatively impact glucose homeostasis by impairing β-cell function and worsening insulin resistance. Mice studies have shown the impact of lipotoxicity on insulin sensitivity by interfering with insulin signalling via PKC-induced serine phosphorylation of insulin receptor substrate (IRS-1) [152]. IRS-1 is the first downstream member of the insulin signalling cascade, and T2DM persons are characterised by enhanced phosphorylated serine residues of IRS-1 and decreased insulin-stimulated tyrosine-phosphorylated IRS-1[153, 154]. In addition, lipotoxicity induces the proinflammatory pathway IKKβ-NF-κB resulting in an increase of inflammatory cytokines (e.g. TNF and IL-6), which will interfere with the insulin signalling pathway [155]. Alternatively, FFA-induced lipotoxicity results in oxidative stress [156] and malonyl Co-A inhibiting fatty acid β-oxidation resulting in further insulin resistance in insulin-sensitive tissues [157].

Additionally, chronic hyperglycemia leads to progressive deterioration of β-cell function and viability. One of the proposed mechanisms is gluco-lipotoxicity (glucotoxicity and lipotoxicity). This concept implies an abnormal increase in glucose and FFA's will directly lead to β-cell exhaustion and cell death. T2DM model studies have shown that impaired β-cell function occurs after insulin secretion rates have increased to rates that are not sustainable to reach the hyperglycaemic demands [158, 159]. Whatever the mechanism, however, and maybe that most mechanisms contribute, the result of sustained high glucose levels and chronically activated insulin secretion leads to loss of β-cells.

In summary, increased blood glucose (hyperglycemia) and excess fatty acids (hyperlipidaemia) are typical of both obesity and insulin resistance. The glucolipotoxic environment triggers oxidative stress, inflammation and impaired insulin signalling in insulin-sensitive tissues such as the liver and the pancreas.

1.3.3 Current therapeutic agents and interventions for T2DM

As 90% of T2DM patients are either obese or overweight [160], the aetiology of T2DM is linked to overnutrition and insufficient energy expenditure. Currently, treatments aim to reduce hyperglycemia in T2DM patients by improving insulin secretion and decreasing insulin resistance [161].

After first diagnosis of T2DM, the recommendation to treat is that of lifestyle interventions [162, 163]. This involves diet modification and where possible, to increase physical exercise. Both can reduce peripheral insulin resistance and T2DM-associated complications [164]. In combination with lifestyle interventions, T2DM patients can be prescribed metformin [165]. Metformin acts in several ways to improve insulin and glucose signalling. First, it decreases peripheral insulin resistance by increasing insulin-mediated IRS-1 tyrosine phosphorylation activity improving insulin sensitivity [166]. Secondly, metformin increases insulin secretion by stimulating postprandial secretion of glucagon-like peptide 1 (GLP-1) [165, 166]. Thirdly, metformin acts at the level of the liver to decrease hepatic glycogenolysis and fourthly, it delays digestion [167]. Currently, metformin is the gold standard treatment for T2DM, however, if ineffective then patients are prescribed sulfonylureas and meglitinides. These two treatments act as insulin secretagogues and are only effective for short-term use [167]. The use of these alternatives is complicated by adverse side effects, such as weight gain and hypoglycaemia.

Failure of these first-line medications leads to the treatment of T2DM patients with insulin. As for T1DM, this involves multiple daily subcutaneous injections and its use is associated with side effects such as weight gain, hypoglycaemia and increased risk of colorectal cancer [168]. Another option for T2DM patients is bariatric surgery, however, this is restricted to those of whom are severely obese (BMI $\geq$ 40), and this procedure is only considered when other options have not been effective in weight reduction [169]. Despite the benefits of this procedure, it is costly and is not always successful for weight loss. Post-surgery complications include iron and B12 deficiency, vomiting and dumping syndrome [170].

1.4 Causational and mechanistic link between obesity and T2D

The prevalence of obesity is rising, and with it the comorbidities of T2DM, dyslipidaemia, hypertension and cardiovascular diseases. Together, this suite is known as the “21st-century epidemic” [171]. Due to the similarity of the statistical trend between obesity and T2DM, the term “diabesity” has been coined by Zimmet et al. to exemplify their interdependency [172].

1.4.1 Lipid metabolism

Obese patients show increased levels of visceral and ectopic fat tissue relative to the normal weight population [173]. Imaging techniques such as magnetic imaging resonance (MRI) and computed tomography (CT) scans have specifically shown, regardless of gender, that visceral adipose tissue (VAT) accumulation is associated with insulin resistance [174]. Furthermore, insulin resistance is proportional to VAT mass independent of the BMI [175]. It has been proposed that excess FFAs from VAT enter the hepatic portal circulation to induce hepatic insulin resistance by enhancing gluconeogenesis. For example, Gastaldelli et al. showed gluconeogenesis flux positively correlated with visceral fat in T2DM patients [176]. They further found that VAT accumulation led to a significantly worse glycaemic profile via reduced peripheral insulin sensitivity and enhanced gluconeogenesis.

Several transgenic mouse model studies have shown increased circulating FFAs and triglyceride levels mediating insulin resistance [177]. Studies done by Kim et al. with transgenic mice overexpressing lipoprotein lipase in muscle [178] and Oakes et al. presented a high fat-fed rat model [179] impairing insulin signalling pathway. Furthermore, to gain insight into whether lipid metabolism has an impact on insulin signalling Dobbins et al. presented a rat model that was given a carnitine palmitoyltransferase - I (CPT-1) inhibitor (Etomoxir) [180]. CPT-1 is a crucial enzyme required for fatty acid β-oxidation, a multi-step process that breaks down fat and converts them to energy. The study showed inhibition of this enzyme caused defects in insulin signalling due to the increase in lipid accumulation [180]. Interestingly human studies showed lipid deprivation caused reduced body mass with a parallel improvement of insulin sensitivity [181, 182]. It is plausible that FFA accumulation in human and animal studies may be due to an increase in the uptake of FA and/or due to reduced fatty acid β-oxidation.

1.4.2 Inflammation

The hypothesis that inflammation underlies, at least in part, the pathologies of insulin resistance was first published by Hotamisligil et al. in 1993 [183]. This eminent study showed the continuous expansion of adipose tissue expresses increased levels of proinflammatory cytokines including TNF and that neutralisation of TNF in obese (*ob/ob*) mouse models

reversed insulin resistance [183]. Since the first study, human studies have confirmed that TNF expression levels from adipose tissue decreased with an associated weight loss. Further to this, a significant correlation has been demonstrated between BMI and TNF levels [184, 185]. Mechanistically, TNF inhibits the auto-phosphorylation of tyrosine residues of insulin receptor substrate (IRS-1) and promotes phosphorylation of serine residues that results in defective insulin signalling in adipocytes [186]. Further to this TNF suppresses the expression of the insulin receptor itself [187].

Beyond TNF, IL-6 inhibits insulin transduction in hepatocytes in a similar fashion to the suppressor of cytokine signalling-3 (SOC-3) [188]. SOC3 is a protein associated with the insulin receptor that inhibits tyrosine residue phosphorylation and blocks the association with the p85 phosphoinositide-3-kinase (PI-3-K) subunit. Previously, it has been shown that SOC-3 is a mediator of leptin resistance in obese patients and follows that high levels of IL-6 could have similar effects.

Excess macronutrient intake in obese individuals has been shown to induce inflammation in various cells via oxidative stress and activation of the inflammatory transcription factor, NFκB [189, 190]. NFκB is one of the transcriptional pathways linked to the production of pro-inflammatory cytokines in obesity and insulin resistance, along with the c-Jun NH_2- terminal kinase (JNK) pathway. Overnutrition associates with, the activation of transcription pathways in hypertrophic adipose tissue that, in turn, drives the production of proinflammatory cytokines such as TNF, IL6, interleukin-8 (IL-8), and interleukin 1β (IL-1β), serum amyloid A-1 (SAA-1), monocyte chemoattract protein -1 (MCP-1) and resistin. Resistin is particularly interesting as it has recently emerged as an adipocyte inflammatory adipokine detected at elevated levels in obese patients [191, 192]. Delineation of the association further has shown that resistin administration induces insulin resistance in obese mice [193].

Elevated FFA's and glucose increased plasma cytokines and lipid peroxidation levels within the first 3 hours, indicating an increased inflammation and oxidative stress [189, 194]. Increased mitochondrial oxidative stress in cells causes superoxide radicals to activate the redox-sensitive pro-inflammatory transcription factor, NFκB causing a feed-forward mechanism in the production of proinflammatory cytokines. Thus, in response to excess macronutrients pro-oxidant and pro-inflammatory effects act together in obese patients.

1.4.3 Oxidative Stress

Oxidative stress is another inducer of insulin resistance. An increase in endogenous reactive oxygen species (ROS) results in increased oxidative stress, which damages cells and their signalling pathways [195]. ROS include hydrogen peroxide (H_2O_2), superoxides (O_2^-), peroxyl nitrites ($ONOO^-$) and radical hydroxyl ions (OH^-). Physiologically, all of these are produced at low levels and are involved in normal cellular signalling and function [196, 197]. Pathological levels of ROS, however, can damage cells which, in turn, predisposes to the development and progression of insulin resistance and T2DM [198].

Overnutrition results in hyperglycemia and hyperlipidaemia, that in turn raises circulating insulin levels in the body. Sustained insulin leads to the downward regulation of glucose uptake, thereby perpetuating the problem. A consequence of high glucose and lipid levels is chronic oxidative stress [199] because high levels of either energy source increase substrate availability for mitochondria [200]. Hyperactive mitochondria, in turn, produce more ROS as by-products of metabolism. With regards to cellular signalling, mitochondrial ROS activate a number of key inflammatory and oxidative stress response pathways including NFκB, JNK and MAPK [201-203]. Further to this, ROS induce mitochondrial fission, a process that has been shown to cause muscle insulin resistance [204].

Radical scavenging has long been thought of as a potential treatment for insulin resistance. This involves the administration of antioxidants that essentially mop up the oxidants. Several studies have shown that vitamins such as ascorbic acid and tocopherols have therapeutic potential [205, 206]. However, to date, most have failed to in clinical settings due to their dosage dependency, bioavailability and/ or pharmacokinetics. As oxidative stress is central to the pathology of insulin resistance, targeting ROS accumulation at the molecular level remains a major avenue for the treatment of this disorder.

1.4.4 Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is the site of protein and lipid synthesis. In the ER, native proteins undergo folding and exportation to their site of action or exocytosis in vesicles. The ER itself is very sensitive to cellular stress due to the activation of the inflammatory pathway and /or increase ROS levels, ultimately leading to misfolded proteins that upon accumulation

become toxic to the cell [207, 208]. To prevent subsequent cellular apoptosis and to re-establish ER homeostasis, ER relies on a cellular cascade named the unfolded protein response (UPR) to try and restore order in the cell [209]. The three ER- resident proteins: activating inositol requiring enzyme-1 (IRE-1), activating transcription factor 6 (ATF6) and protein kinase RNA-like endoplasmic reticulum (PERK) [210] are the proximal sensors of the presence of ER stress, that activate UPR. UPR is initiated whenever there is protein misfolding in the ER, which could be induced by altered metabolic conditions e.g., hyperglycemia. The UPR transmits information about the protein-folding state in the ER lumen to the nucleus and cytoplasm. When cells are subjected to irreversible ER stress, this route destroys injured cells by apoptosis, implying the existence of systems that integrate information about the duration and intensity of stress stimuli [211-213].

ER stress underlies the pathogenesis of insulin resistance [214]. Not surprisingly, ER stress is triggered by activated NFκB/IKKβ and JNK pathways, in response to high levels of glucose and lipids. In particular, these two pathways activate IRE-1 and PERK UPR-dependent pathways [215]. Activation of these UPR pathways results in serine phosphorylation of IRS, inhibiting the insulin signalling pathway [216]. The association between UPR, inflammation and oxidative stress in insulin-sensitive cells was effectively demonstrated by Xue et al., where induction of ER stress in fibrosarcoma L929 cells was activated by cytokine TNF, in a ROS-dependent manner [217]. Furthermore, *in vivo* and *in-vitro* studies have shown that activation of ER stress underlies the emergence of the stress and inflammatory responses in obesity and the deterioration of glucose homeostasis [216, 218, 219].

1.4.5 Protein Kinase C

Protein Kinase C (PKC) is a multifunctional enzymatic family with a central role in regulating lipid-induced insulin resistance [211]. Depending on the activating stimuli, different PKC isoforms can act as mediators or inhibitors in the insulin signalling pathway [211]. Obese individuals show increased circulating levels of FFA that are associated with activated PKC proteins. Delineating the mechanism, studies have shown that inflammatory cytokines such as TNF increase the esterification of FFA into diacylglycerol (DAG) [220] and it is DAG that is the activator of PKC, particularly the isoforms PKC θ and PKCε [211]. These isoforms act to serine phosphorylate IRS1, driving insulin resistance, demonstrated by reduced insulin-

stimulated glucose transport activity [221]. The dependence on PKC θ was determined by in PKC θ knockout mice that were protected from the FFA- induced insulin signalling disruption. Together the findings show so far that inflammation itself is not the direct regulator of PKC but that cytokines associated with the lipid-induced inflammation and oxidative stress regulate DAG-dependent PKC activation.

1.5 TEMPOL as a therapy for obesity-induced insulin resistance

TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a cell-permeant soluble nitroxide best known for its antioxidant properties (Figure 1) [222]. Apart from the dismutation of superoxide anions (O_2^-), in a similar fashion to SOD, TEMPOL is known to metabolise or prevent the generation of many ROS, including hydrogen peroxide, peroxynitrite ($OONO^-$), nitroxyl anion (HNO) and peroxyl radicals (^-OOR). With one and two electron transfer processes, TEMPOL can shuttle between the nitroxide radical, the reduced hydroxylamine, and the oxidised oxoammonium cation form (Figure 2) [223]. This is aided by its conformational "boat and chair" change, resulting in a rapid catalytic activity and water miscibility [222]. Due to the nature of its activity, TEMPOL has been reported to be more effective than other "antioxidants" and vitamins [224]. TEMPOL is water soluble and is considered an ampholyte, therefore it has a high capacity to permeate through cell membranes, the blood-brain barrier and the gastrointestinal tract [222]. Moreover, sequential kinetic studies using an electron spin resonance (ESR) spectrometer have shown that TEMPOLs' half-life depends on the reduced activity and the redox states of the organ, e.g. TEMPOL injected via the tail vein of rats has a half-life of 30.5 ± 1.5 sec compared to kidney 14.5 ± 1.0 sec [225]. The study performed by Ueda et al, 2003 suggests that the resultant half-life of TEMPOL in the liver and kidney is due to its main reduction site, mitochondria, as the kidneys are composed of a relatively higher content of mitochondria than the liver [225]. Furthermore, TEMPOL has been shown to effectively suppress inflammatory signalling pathways, such as NF-κB and JNK [226-228].

Clinical trials involving the nitroxide TEMPOL, have explored where there is potential for its use as a therapy targeting a broad range of medical conditions. Notably, such studies have delineated efficacy, safety and tolerability for several different administrations including doses and treatment times. For example, for cardiovascular disease, TEMPOL is effective at mitigating oxidative stress and inflammation [229, 230], with the most efficacy shown for hypertension [231-233]. Furthermore, the evidence that TEMPOL is a potent antioxidant has

led to its use in pre-clinical and clinical trials, such as extensive studies on complications arising from radiation therapy [234, 235]. For example, a consequence of head and neck radiotherapy can have complications for healthy, normal tissues in radiation therapy, salivary glands in particular resulting in oral dryness (xerostomia). Several pre-clinical tumour mice models showed TEMPOL protecting salivary glands, supporting further development for human clinical trials in the future [235, 236]. Moreover, in 2004, a Phase I trial showed the safety profile of TEMPOL used as a topical cream, to protect against radiation-induced alopecia in patients who were treated with whole-brain radiotherapy. Results showed the topical application of TEMPOL (0.4 to 3.1 μM/L) protected the area of application and full scalp hair retention [234]. This trial led to the Phase IIB trial in 2009, which uses TEMPOL in a gel formulation (https://classic.clinicaltrials.gov/ct2/show/NCT00801086) and the aim is to determine the extent of lessening hair loss. Additionally, TEMPOL is currently under investigation in clinical trial phase II/III as an oral medication for its effectiveness in treating COVID-19 infection, by downregulating multiple inflammatory cytokines and preserving the lung architecture [237, 238]. These COVID-19 trials add a timely dimension to the research associated with the clinical use of TEMPOL, underscoring its versatility and potential effectiveness in addressing emerging health challenges. The outcome of these trials, documented in scholarly articles and clinical trial registries, collectively contribute to our understanding of the potential clinical benefits of TEMPOL.

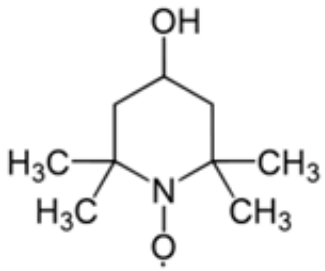

Figure 1: Structure of the cyclic nitroxide TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

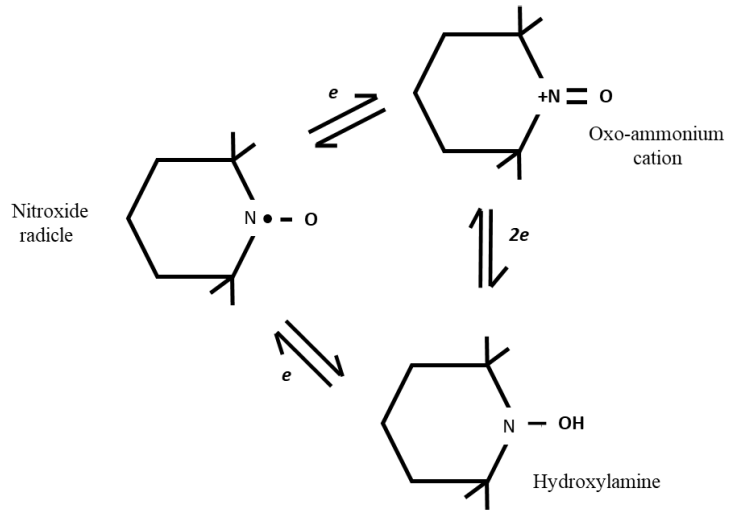

Figure 2: TEMPOLs interconversion between oxidized and reduced nitroxide forms

With respect to this current research project, it was of major interest that TEMPOL treatment via food (10 mg/g in standard chow) or water (2.9 - 58 mM final concentration in drinking water) reduced body mass in C3H mouse model [239]. At a concentration of 10 mg/g, TEMPOL showed no impact on food intake, but mice lost 40.7% of body mass, compared to their vehicle-treated (sucrose) controls. In addition, mice given the highest concentration of TEMPOL (58 mM) in drinking water tolerated the treatment for 75 weeks and did not gain body mass compared to the control group that consumed sucrose only in drinking water. Further to this dramatic effect on body mass, the TEMPOL group showed elevated leptin levels, an increase in lifespan (123 weeks vs 92.3 weeks for the vehicle control) and decreased tumour incidence. In separate studies, TEMPOL has been shown to increase urinary excretion of metabolites generated by bacteria in the gut, suggesting TEMPOL might have an effect on gut microbial metabolism [240, 241]. Studies following this have shown and suggested TEMPOL decrease obesity and insulin resistance by remodelling the intestinal microbiota that changes bile acids composition and inhibition of farnesoid X receptor (FXR) signalling [242, 243]. FXR plays a key role in lipid and glucose metabolism and FXR $^{-/-}$ mice models have shown improvement in glucose homeostasis and agonist of FXR has shown aggravation of lipid metabolism and insulin resistance in obese mouse models [244, 245]. Although the mechanism of FXR signalling is not fully understood, the given action of TEMPOL

manipulating this signalling pathway is one probable mechanism in improving obesity in HFD-fed mouse models.

Limited studies have previously investigated the role of TEMPOL in protecting against insulin resistance. For example, an insulin resistance Wistar rat model showed TEMPOL treatment of 1 mM, injected for 14 days reduced systemic oxidative stress and improved the HOMA index of insulin resistance (HOMA-IR) [246], a measure used to quantify the severity of insulin resistance. Furthermore, the antihypertensive effect of TEMPOL has been extensively studied on animal models, revealing TEMPOL's action in cell signalling pathways. Hypertensive kidney models have shown TEMPOL protective role by acting as a vasodilator in small arteries in visceral adipose tissue (VAT) in obese patients [247].

Given the antioxidant and anti-inflammatory properties of TEMPOL, coupled with the studies showing improvement in insulin resistance associated with reduced body mass gain in rodent models, this project sought to delineate the underlying protective pathways that were targeted by TEMPOL as well as provide further evidence in an alternate rodent model of insulin resistance that TEMPOL administration is able to improve insulin signalling and preserve pancreatic β-cell function. The overarching hypothesis was that TEMPOL would improve insulin resistance in mice fed a high-fat diet via antioxidant and anti-inflammatory effects that in turn downregulate ER stress and PKC activation in major insulin-sensitive tissues including the liver, adipose tissue and pancreas. In addition, the project sought to evaluate the lipid accumulation-sparing effect of TEMPOL because in reducing FFA accumulation this provides another pathway in which the agent is protective against FFA-induced ER stress and PKC pathways. Ultimately, the goal is to provide fundamental mechanistic research to understand more fully the potent protective effects of TEMPOL because such an understanding is needed to provide impetus to explore TEMPOL as a therapeutic option for the treatment of insulin resistance.

1.6 Thesis Aims and Outline

The research undertaken in this project was designed to investigate the effect of TEMPOL on reversing the pathogenic mechanisms that underlie high fat-diet-associated insulin resistance, a state that drives obesity-induced T2DM.

The aims of this project are:

Aim 1: This study aimed to investigate the use of TEMPOL as an antioxidant and anti-inflammatory therapy for obesity and related risk factors, particularly T2DM and NAFLD. **Chapter 3** of this thesis is a scoping review analysing the biomedical research outcomes of TEMPOL on obesity and obesity associated T2DM and NAFLD. In doing so, gaps in our knowledge of the underlying mechanisms of TEMPOL, and its potential as a therapeutic option for the growing burden of obesity, emerged.

Aim 2: This study aimed to explore the potential role of TEMPOL on saturated fatty acid-induced oxidative stress and inflammation in adipose tissue. **Chapter 4** examines the anti-inflammatory, anti-oxidative and anti-obesity properties of TEMPOL in an *in vitro* model of 3T3L-1 adipocytes and an *in vivo* model of a high fat diet-fed C57BL/6 mouse model. In doing so, TEMPOL's effects on lipid metabolism and adipogenesis were explored as well as any preventative role in body mass gain. TEMPOL's effects on suppression of adipose inflammation and oxidative stress were also investigated.

Aim 3: This study aimed to investigate the antioxidant, anti-inflammatory and anti-obesity properties of TEMPOL in the setting of insulin resistance and the clinical sequelae of saturated fatty acid induced NAFLD. **Chapter 5** examines the effect of TEMPOL on NAFLD by using a high fat-diet-fed C57BL/6 mouse model and TEMPOL's mechanism of action using a HepG2 *in vitro* model. In doing so, TEMPOL's effects on lipid accumulation, oxidative stress, and inflammation were explored as well as its preventative role in liver damage. Finally, TEMPOL's ability to improve insulin sensitivity was reported.

Aim 4: This study aimed to investigate TEMPOL's effect on preventing pancreatic β-cell dysfunction induced by lipotoxicity. **Chapter 6** examines this effect by using MIN6 β -cell exposed to palmitic acid (PA), an *in vitro* model for diet-induced β-cell stress, and in high-fat

diet-fed C57BL/6 mice as an *in vivo* model of insulin resistance. In doing so, TEMPOL's antioxidant effects on lipotoxicity-induced mitochondrial dysfunction and endoplasmic reticulum stress in MIN6 β -cells were explored. Further to this, the effect of TEMPOL on improving glucose and insulin sensitivity *in vivo* were reported.

Chapter 2 : Materials and Methods

Chapter Summary

This chapter presents a general description of the Materials and Methods used to perform the research described in this project. More specific methods, relevant to the separate aims will be provided in subsequent chapters in the form of a Method and Material section in scientific articles submitted for publication.

2.1 In vivo model

Five-week-old male C57BL/6 mice supplied by Australian BioResources (Moss Vale, NSW, Australia) were housed in a temperature-controlled (22 ± 2 ºC) and circadian rhythm-adjusted room. Following 1 week of acclimatisation, mice were ear notched for identification and were placed on standard chow diet (StD, 6% fat, Gordon Specialty Stock feeds) and water *ad libitum*. Animal housing and care were carried out by the staff in the Ernest Facility, UTS.

The total study period was 16 weeks. The number of mice proposed for each group (n=10) were the minimum number of animals required to provide statistical power difference to measure glucose metabolism. At eight weeks of age, the mice were randomly divided into two main groups, one to receive a specialised bacon-flavoured StD (n = 10; 63% carbohydrate, 4.1% fat, Biological Associate; Envigo Teklad) and the other a bacon-flavoured high-fat diet (n = 40; HFD, 46.2% carbohydrate, 29.0% fat, Biological Associate; Envigo Teklad) enriched with trans fatty acids, mono and polyunsaturated acids. On the eighth week of the study, The StD group was then further subdivided, with one subset continuing the StD chow (n = 10) and another other fed StD supplemented with TEMPOL (n = 10; StDT). Similarly, the HFD group was subdivided into (i) 10 continuing HFD, (ii) StD (n = 10; HFDStD), (iii) StD with TEMPOL (n = 10; HFDStDT) and (iv) HFD with TEMPOL (n = 10; HFDT). TEMPOL supplementation (10 mg kg^{-1}) was made using the cold-pressed method [248]. The TEMPOL dosage regimen was chosen as per study conducted by Mitchell et al, 20003, that did not observe any potential adverse reactions or toxicity [239].

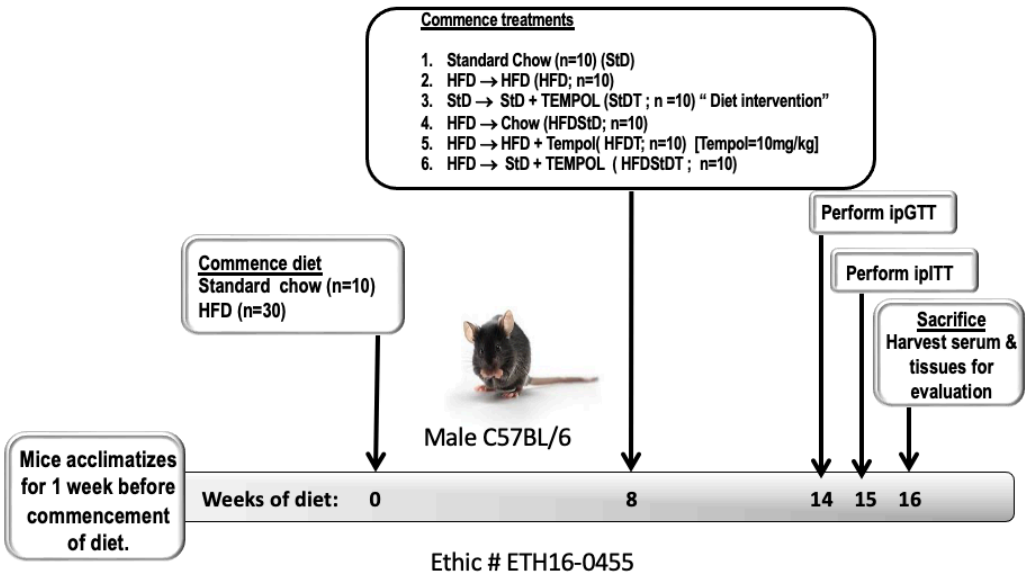

Figure 3: A timeline summary of the high-fat diet C57BL/6 *in vivo* study. After acclimatizing, the mice were divided into two groups to receive either standard diet chow (StD; n=10) or high fat diet (HFD; n=10). Diet intervention with or without TEMPOL was commenced on the 8th week. In week 14 and 15, intraperitoneal glucose tolerance test (ipGTT) or insulin tolerance test (ipITT) was completed, respectively. In week 16, all animals were sacrificed for blood and tissue collection.

The body mass of mice and food intake was weighed and monitored weekly. Food and water were available *ad libitum*, except during periods of planned fasting, where only water was available. At weeks 14 and 15, the intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT) were performed, as described [249]. On the 16th week, mice were euthanised, and tissues were harvested and stored at -80°C until processed. All experiments were approved by the University of Technology Sydney Animal Care and Ethics Committee, Sydney, NSW, Australia (Ethics approval number: UTS ACEC ETH16-0455) and conducted according to guidelines described by the Australian National Health and Medical Research Council Code of Conduct of Animals.

2.2 Glucose and insulin tolerance tests

An intraperitoneal glucose tolerance test (ipGTT) and an intraperitoneal insulin tolerance test (ipITT) were performed on all mice to measure diabetes and insulin resistance, respectively. These experiments examine how well mice can maintain glucose homoeostasis following a bolus dose of exogenous glucose or insulin and provide an indication of glucose or insulin tolerance. If mice can sustain glucose homoeostasis, it means they are glucose/insulin tolerant. If the blood glucose level remains high in the ipGTT or low in the ipITT, it shows that insulin sensitivity has been lowered.

In brief, the ipGTT was conducted in mice after a 5 h fast, whereas the ipITT was performed in non-fasted mice. A commercial glucometer (Roche ® Diagnostic, CA, USA) was used to take a basal glucose reading (time = 0) from a drop of blood at the tip of the tail. The mice were subsequently given a bolus of sterile D-glucose (2 g kg^{-1} weight) or insulin (1 I.U kg^{-1} weight, Insulin glulisine, Apidra SoloStar ®, USA) in the peritoneum. The concentration of glucose in the blood was then tested at 15, 30, 60, 90, and 120 minutes after injection.

2.3 Tissue harvest and blood collection

On the 16th week, animals were euthanised with isoflurane in an induction chamber and then placed on a nose cone until no reflex to pedal or ocular stimulation was observed. Before making a cut in the belly to expose the peritoneum, the skin of the abdomen was generously sprayed with 70% v/v ethanol. A cardiac puncture was conducted into the left ventricle to collect roughly 1 mL of blood with an 18-gauge heparin-coated syringe (100 I.U mL^{-1} DBL Heparin Sodium Injection, Hospira, Vic, Australia), to prevent blood clotting. Following collection, the whole blood sample was immediately centrifuged (13, 500 RCF, 5 min). The plasma layer was removed and stored at - 80°C. Blood glucose was measured using the True Track^{TM} Smart System glucometer (Nipro Diagnostics Inc., Fort Lauderdale, FL, USA).

To confirm death, cervical dislocation was performed, before tissue samples were collected. Tissues such as heart, liver, skeletal muscle, pancreas, and visceral adipose tissue were harvested. Each group's tissue was stored in paraformaldehyde (later transferred to 70% v/v ethanol) and snap-frozen in liquid nitrogen (later stored at -80°C until needed).

2.4 Biochemical Analysis of Plasma

Alanine aminotransferase

Alanine aminotransferase (ALT) levels are an indicator of liver damage [250, 251]. ALT activity of plasma was measured using a commercially available ALT assay kit (Cat # 105134; Abcam, Cambridge, MA, USA). Using the manufacturer's protocol, a 100 µL/well master mix was prepared by combining 86 µL assay buffer, 2 µL OxiRed Probe, 2 µL enzyme mix and 10 µL ALT substrate. Samples (10 µL of plasma) were diluted with 10 µL assay buffer. Samples and the master mix were then combined and aliquoted to a 96-well plate to give 120 µL reaction mix per well. The plate was incubated at room temperature for 10 mins (T1), protected from light and the absorbance (A1) was read at 570 nm using M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria). The plate was incubated for the second time (T2) for 60 min at 37°C, protected from light and absorbance (A2) was read again.

As ALT catalyses the reaction of α-ketoglutarate + alanine ⇌ glutamate + pyruvate, the production of pyruvate formed in the reaction determines the activity of ALT. The background of the assay was adjusted by subtracting A1 from A2. The resultant absorbance (A_F) was used to determine the concentration of pyruvate from the standard curve (B). ALT activity was then calculated using the formula below:

$$ALT\ activity\ (mU/mL) = \frac{B}{(A2 - A1)\ X\ Sample\ volume}$$

Aspartate aminotransferase

Aspartate aminotransferase (AST) was measured using a commercially available AST activity kit (Cat # 105135 Abcam, Cambridge, MA, USA). As per the manufacturer protocol, 100 µL master mix was made containing, 80 µL assay buffer, 10 µL of the substrate, 8 µL of developer and 2 µL of enzymes mix. Samples (10 µL of plasma) were diluted with 10 µL assay buffer. 120 µL of sample and master mix was aliquoted to each well of the 96-well plates. The plate was incubated at room temperature for 10 mins (T1), protected from light and the absorbance (A1) was read at 450 nm using M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria). The plate was incubated for the second time (T2) for 60 min at 37°C, protected from light and absorbance (A2) was read again.

The background of the assay was adjusted by subtracting A1 from A2. The resultant absorbance (A_F) was used to determine the concentration of glutamate from the standard curve (B). AST activity was then calculated using the formula below:

$$AST\ activity\ (mU/mL) = \frac{B}{(A2 - A1)\ X\ Sample\ volume}$$

Beta-hydroxybutyrate

The β-Hydroxybutyrate (β-HB) levels in plasma were measured using a commercially available β-HB kit (Cat # 83390, Abcam, Cambridge, MA, USA). As per the manufacturer's protocol, a 50 µL master mix per well was prepared, containing 46 µL β-HB assay buffer, 2 µL β-HB substrate and 2 µL β-HB enzyme mix. Samples (plasma) were prepared by doing 1:5 dilution with assay buffer, and 50 µL was added to each well of a 96-well plate followed by 50 µL master mix to give a total volume of 100 µL per well. The plate was then incubated for 30 min at room temperature, protected from light. Absorbance at 450 nm was read using M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria).

Non-esterified Fatty Acid assay

Non-esterified fatty acids (NEFA) were quantified using the Lab Assay NEFA kit (Cat # 633-52001; WAKO diagnostic; Osaka, Japan). Using the manufacturer's protocol, a standard curve (0 – 2.5 mEq L^{-1}) was generated by pipetting NEFA standard solution, Oleic acid (1 mEq L^{-1}), supplied, followed by pipetting 4 µL of plasma sample to each well of a 96-well plate. Reagent A (80 µL) was added to the standards and sample wells and was incubated for 10 mins at 37°C. Reagent B (160 µL) was added and incubated for further 10 mins at 37°C. The plate was then cooled down to room temperature, and absorbance was read at 550 nm using an M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria).

Determination of neutral triglyceride and lipids in liver tissue.

To quantify lipid accumulation in liver tissue, an Oil-Red O assay was performed. Liver tissue weighing ~100 mg was homogenised in 1 mL Oil-Red O (ORO) working solution (1:1.5 ratio of ORO stock [ProsciTech, QLD, Australia]: 1% w/v dextrin [Sigma-Aldrich, CA, USA]) with zirconium oxide beads (1.4 mm, Precellys 24, France) using a homogenising machine (Minilys, Bertin Technologies, France). Samples were washed by adding 60% v/v isopropanol, centrifuged and the resultant supernatant discarded. Extraction of the ORO stain was done by washing the sample thrice with a 90% v/v isopropanol and discarding the supernatant. On the fourth wash, 100 µL of the sample supernatant was collected and transferred to a 96-well plate. Absorbance was read at 520 nm on a plate reader (Tecan Sunrise, Grödig, Austria).

2.5 Histological analysis

Preparation of tissue fixation, processing, embedding, and cutting

Liver and adipose tissue were fixed in 4% v/v paraformaldehyde (Sigma-Aldrich, MO, USA) overnight, then rinsed with phosphate-buffered saline (PBS) and stored in 70% v/v ethanol for one week. Tissues were then placed in cassettes and processed using the ExcelsiorTM AS Tissue Processor (Thermo Fisher Scientific, MA, USA) for 16 h overnight routine composed of two changes of formalin, increasing grades of ethanol, followed by xylene and three changes of paraffin wax. A microtome (Epredia, MICH, USA) was used to cut sagittal sections of embedded tissues after processing them in paraffin wax. A set of six duplicates (slides 1- 6) of liver and adipose tissues were cut at a thickness of 5 µm and mounted on Platinum PRO adhesive glass slides (Trajan, VIC, AU) and air dried before placing in racks to dry overnight at 37°C.

Dewaxing and re-hydration of paraffin-embedded sections

For staining purposes, section slides were dewaxed by immersing into two changes of xylene for 10 mins each. Progressive rehydration of the slides was done by two changes in 100% ethanol for 2 mins each, followed by two changes in 95% v/v ethanol (2 mins each) and the final step in 70% v/v ethanol. Sectioned slides were then washed under a running tap for at least 2 mins.

Haematoxylin and Eosin staining (H & E)

Three prepped sectioned slides (slides #1, #3, #5) were chosen to be stained with Harris' Haematoxylin for 2-3 mins followed by washing the slides under a running tap until the water colour is clear. To remove excess blue colouration, the slides were dipped three times in an acid alcohol differentiator (conc. HCL diluted in 1: 400 in 70% v/v ethanol). Slides were then submerged in Scotts's Blueing solution for 30 sec and were checked under the light microscope to determine whether nuclei were clearly differentiated. If not, the slides are re-washed under running tap water and repeat staining starting from Harriss' Haematoxylin.

Once the stain clearly differentiates the nuclei the slides sections were washed under running tap water and then dipped in 70% v/v ethanol for 30 sec. Next, the slides were stained with Eosin, by dipping into two changes of eosin at 30 sec each. Sections slides were washed in two changes of 95% v/v alcohol (30 sec each) followed by two washes in 100 % ethanol (30 sec each). Slides were then immersed in two changes of histolene (10 mins each) before being mounted with DPX (mixture of distyrene, plasticizer and xylene; Sigma-Alrdich) and cover slipped. The slides were then visualised using ZEISS AxioScan Digital Slide Scanner (Carl Zeiss Microscopy, Germany) at a magnification of x20.

Hepatic F4/80 immunohistochemical staining for the identification of macrophage infiltration.

Three slides of prepped liver tissue (slides #2, #4, #6), as described above, were chosen to be stained for the macrophage marker F4/80. Rehydrated slides were placed in pressure for 15 mins to retrieve antigens and were stained using the Agilent Technologies CSA II kit (CA, USA). The slides were incubated for 5 mins in the peroxide block provided in the kit, followed by washing of the slides composed of 3 lots of 3 mins washing by PBST. Slides were incubated for a further 5 mins in the peroxide block then incubated overnight at 4 °C with the F4/80 antibody diluent (Table 2). The following day, slides were washed and incubated in CSA Rabbit Link for 15 mins followed by another wash and incubated for 15 min in the amplification reagent, protected from the light. Following this, slides were washed and incubated in the provided anti-fluorescein-HRP, washed and stained in the provided DAB solution for 1-2 mins. Slides were then washed with PBST and water, for 3 mins each and were counterstained with Mayer's haematoxylin for 30 sec and was washed under running water for

5 mins. Slides were blue in Scott's blue, then washed under running water for 2 mins and the dehydration process was done before mounting and cover-slipping the slides.

Pico Sirius red staining

Three slides of prepared liver tissue were stained with Pico Sirius Red Stain for fibrosis. Following the manufacturer's protocol, the Pico Sirius Red Stain Kit (Cat # 150681 Abcam, UK). The slide was rehydrated and incubated in Pico Sirius Red stain for 60 mins at room temperature. Slides were washed twice in supplied acetic acid, followed by a wash in 100 % ethanol. Slides were dehydrated by washing twice in 100 % ethanol, and two changes in xylene and coverslipped.

Table 2: Antibodies used in immunostaining of tissues.

Target	**Dilution**	**Company**	**Catalogue Number**
F4/80	1:200	Abcam, UK	ab 111101
488-fluorescent secondary	1:1000	Invitrogen, CA, USA	A11008
568-fluorescent secondary	1:500	Invitrogen, CA, USA	A11011

Oil-Red O Staining

Oil Red-O (ORO) was used to visualise lipid build-up in liver tissue, and the methodology described by Mehlem et al. [252], was followed with minor alterations for the acquisition of representative histological pictures. Liver tissue sections were made by embedding frozen tissue in Tissue-Tek (Sakura® Finetek, USA), sectioning at 8-10 nm, and air drying for 10 mins. Slides were stained with ORO working solution for 10 mins at room temperature followed by a 30 mins wash by placing the slides under running water. Slides were mounted (Histoline, Italy) and coverslipped for imaging using a BX51 Upright Fluorescence microscope (Olympus, USA).

2.6 Palmitic Acid and TEMPOL treatment preparation for *in vitro* model

Albumin bound-palmitic acid (PA: 20 mM) was prepared by dissolving the palmitic acid powder (Cat# PO500; Sigma Aldrich, MO, USA) in Dulbecco's Modified Eagles Medium (DMEM) containing 1% w/v free fatty-acid free (FFA- free) BSA and 150 mM NaCl at 70 °C. The stock PA was diluted in DMEM containing 1% w/v FFA-free BSA to yield a 10 mM solution for treatment.

A working solution of TEMPOL (100 mM) was prepared by weighing 17 mg of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; Cat # 176141; Sigma Aldrich) and dissolved in 1 mL of sterile phosphate buffer saline solution (PBS). The working solution was discarded after each use. For TEMPOL treatments, the concentration used was 250, 500 or 1000 µM for a further 24 h unless otherwise stated.

2.7 Cell culture *In vitro* model

The *in vitro* models consist of three key insulin-sensitive cell types, which will aid in determining the effect of TEMPOL on fatty acid-induced inflammation, oxidative stress and insulin resistance. The cells are murine 3T3L-1 (Chapter 4), human HepG2 cells (Chapter 5), and MIN6 β-cells (Chapter 6).

HepG2 cells

HepG2 cells [ATCC HB-8065] are a line of immortalised cells derived from the liver tissue of a 15-year-old Caucasian male with hepatocellular carcinoma. HepG2 cells can proliferate indefinitely in culture and are commonly used in the laboratory as a liver cell model. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, CA, USA) containing L-glutamine (4 mM), glucose (5.5 mM) and supplemented with 10% v/v Fetal Bovine Serum (FBS, Sigma Aldrich, MO, USA) in T75 cm^2 flasks (Corning Costar, NY, USA) at 37°C in a humidified incubator under 20% O_2 /5% CO_2. For subculture, cells that had reached 80-90% confluency were briefly washed with phosphate-buffered saline (PBS, Astral Scientific, NSW, AUS) and incubated with 0.05% w/v trypsin EDTA (ThermoFisher, MA, USA) for 5-6 mins in the incubator to allow cells to detach from the flask surface. Trypsin-EDTA was then inactivated by adding an equal volume of media containing 10% v/v FBS, followed by centrifugation of the cell suspension at 400 x g for 5 mins in a 50 mL centrifuge

tube (Falcon, BD Biosciences, NJ, USA). After centrifugation, the supernatant was discarded, and the pelleted cells were resuspended in a fresh medium. One-third of the cell suspension was then transferred to a new culture flask. Cells were passaged up to a maximum of 25 times. Unless otherwise stated, HepG2 cells were incubated for 24 h with PA (0.5 mM) and then exposed to TEMPOL 200, 500 or 1000 µM for 5 h. Control cells were treated with 1% w/v fatty-acid-free BSA.

3T3L-1 cells

All experiments performed in this study were with differentiated 3T3L-1 cells. Murine 3T3L-1 fibroblast cells [ATCC# CL-173] were cultured in basal DMEM, containing glucose (4.5 g L^{-1}) and 10% v/v fetal bovine serum (FBS; Scientifix, USA) supplemented with 1% v/v penicillin-streptomycin (ThermoFisher, MA, USA). Cells were seeded in 6-well plates (Corning Costar, NY, USA) at a 2 x 10^5 cells/mL density. The cells were cultured until they reached 80% confluence (designated "day 0") before the commencement of the first stage of differentiation ("DFI") by culturing the cells for three days with a basal medium supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1- methylxanthine (IBMX; Sigma Aldrich, MO, USA) and 5 µg mL^{-1} insulin. The second stage of differentiation ("DFII") commenced on day four, wherein the supplementation of the basal medium was only with insulin. On day 8, the media was changed to basal DMEM until lipid droplets were visible under a light microscope (3-4 days). The differentiated 3T3L-1 adipocytes were exposed to PA at a concentration of 0.5 mM for 24 h, followed by post-TEMPOL treatment (250, 500 or 1000 µM) for a further 24 h. Control cells were treated with 1% w/v free fatty-acid-free BSA.

Min6 β-Cells

Murine pancreatic MIN6 β-cells (gifted by Prof. Ann Simpson, UTS) were cultured in DMEM containing 25 mM glucose, supplemented with 10% v/v fetal bovine serum (FBS) and 5% penicillin-streptomycin (ThermoFisher, MA, USA). The cells were incubated in humidified 5% CO_2 at 37°C in humid conditions with 5% carbon dioxide. When MIN6 β-cells reached 80% confluency, they were passaged by treatment with 0.05% v/v trypsin. MIN6 β-cells from passages 25-28 were used for this study. Harvested MIN6 β-cells were plated (2 x 10^5 cells/well) on six-well plates and were incubated with PA at a concentration of 0.5 mM for 24 h, followed by post-TEMPOL treatments (250, 500 or 1000 µM) for a further 24 h.

2.8 Biochemical analysis

Methylthiazolydiphenyl-tetrazolium bromide (MTT) assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted, to assess the effect of TEMPOL on cell metabolic activity following PA treatment. MTT is a positively charged tetrazolium compound that quickly penetrates eukaryotic cells and can detect metabolically active viable cells. Viable cells with active metabolism will convert MTT to a purple stain [253]. Briefly, blue MTT powder (Cat # M2003; Sigma Aldrich, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to generate the MTT working solution (5 mg mL^{-1}). After the treatment of cells, 20 µL of the MTT solution was added to each well that contained 200 µL media. The plates were incubated for 3-4 h in the dark at 37°C. Once the purple-coloured formazan crystals were observed under a microscope, media and dye were aspirated. Dissolution of the MTT dye was done by adding 200 µL of DMSO to each well. The absorbance at 570 nm was then determined by a plate reader (TECAN i-Control, Grödig, Austria). The results are expressed as a percentage increase from the control group.

2'7'-Dichlorofluoerscein (DCFH-DA) assay

Intracellular oxidant (ROS) levels were measured using the fluorogen 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, DCFH-DA (Cat# 35845; Sigma Aldrich, MO, USA) was dissolved in DMSO to make a stock solution of 100 µM. Next, a working solution was made to 10 µM using PBS. Following the treatment of cells, the media was aspirated, and cells were washed with 100 µL PBS (x2). Next, a DCFH-DA working solution (100 µL/well) was added to each well; plates were then covered by aluminium foil and incubated for 15 min at 37 ºC. After incubation, the DCFH-DA solution was aspirated, and cells were washed twice with PBS (100 µL/well). ROS levels were then determined by fluorescence intensity (λ_{ex} 488 nm; λ_{em} 520 nm) using the infinite M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria). The results are expressed as a percentage increase from the control.

Cytochrome C assay

To assess mitochondrial oxidative stress, cytochrome C release into the cytoplasm [254] was determined using cell lysates. This assay was conducted using the cytochrome C release assay kit as per the manufacturer's protocol (Cat # ab65311; Abcam).

Triglyceride assay

Cell lysates were used to determine TEMPOL's effect on triglyceride. This assay was conducted using the Triglyceride assay kit as per the manufacturer's protocol (Cat # ab65336; Abcam).

Human IL-8 ELISA

To determine the levels of interleukin-8 (IL-8) secreted, the conditioned media of HepG2 hepatocytes was collected following treatment. The inflammatory cytokine IL-8 in the conditioned media was subsequently measured by an IL-8 enzyme-linked immunosorbent assay (ELISA) kit as per the manufacturer's instructions (Cat# KHC0081; Invitrogen). The absorption of IL-8 ELISA was read using an M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria).

Glycogen Assay

A commercially available glycogen assay kit (Cat# ab65620; Abcam) was used to detect glycogen content in HepG2 cells. The kit utilises glucose amylase, which hydrolysis the glycogen in the cells to glucose. This latter oxidises to produce a product that reacts with an Oxi-red probe to generate a red/pink colour. Briefly, the treated cells were washed with cold PBS, lysed by adding cold deionised water (110 µL) and scraped with a rubber policeman. The lysate was then transferred to a 1.5 mL tube and boiled for 10 min at 100 ºC to inactivate intracellular enzymes. After centrifugation (18,000 rpm, 10 min, 4 ºC), the supernatant was collected, and glycogen content was measured according to the manufacturer's protocol. The absorbance reading of the reaction mix was read at 570 nm using an M1000 PRO microplate reader (Tecan Sunrise, Grödig, Austria).

2.9 Quantification of gene expressions by RT- qPCR & ddPCR

Total RNA extraction

To extract total RNA from the HepG2, 3T3L-1 and MIN6 β-cells (Chapter 4, 5, 6), media was first aspirated off, and wells were washed twice with PBS. To lyse the cells, 1 mL of TRIsure™ (Bioline, MA, USA) was added to each well on a 6-well plate and was repeatedly washed before transferring to a clean 1.5 ml tube containing 100 µL of 1-bromo-chloropropane (Cat #9673; Sigma Aldrich). The samples were vortexed and centrifuged at 12,000 RCF for 15 min at 4°C. The clear aqueous phase was transferred to a clean 1.5 mL tube, and 500 µL of isopropanol was added. The samples were briefly vortexed and incubated at -20°C overnight to precipitate the RNA. Following the incubation, the samples were centrifuged (12,000 RCF for 15 min, 4°C). The supernatant was removed, and the RNA pellet was washed with 1 mL of ice-cold 70 % v/v ethanol, followed by another centrifugation (12,000 RCF for 10 min, 4°C); this process was repeated twice to ensure to increase in the quality of the RNA extracted. After removing the ethanol wash, the RNA pellet was air-dried for at least 5 min before resuspending in approximately 30 µL of nuclease-free PCR water (Bioline, UK). The RNA samples were stored at -80°C until required.

Total RNA was extracted from the visceral adipose tissue (Chapter 4) and liver tissue (Chapter 5), using the Isolate II RNA/DNA/Protein Kit (Phenol free) (Bioline, UK). All components were extracted from tissue following the manufacturer's instructions. Briefly, for RNA extraction, the entire tissue was weighed and lysed using TRI-sure, and the lysate was loaded into a column. The RNA flow-through was washed with ethanol and loaded onto the RNA/protein column. RNA bound to the column was washed and eluted in the RNA elution buffer. RNA was stored at -80°C until used.

RNA quality check

The integrity of the extracted RNA was assessed using an Experion™ RNA standard Sensitivity Analysis Kit (Bio-Rad, CA, USA) and the Experion™ Automated Electrophoresis Station (Bio-Rad, CA, USA), as per the manufacturer's instructions, to ensure that the RNA was of satisfactory quality and purity for downstream qPCR analysis. An electropherogram and virtual gel (Figure 4) were produced, displaying distinct peaks/bands corresponding to 18S and 28S ribosomal RNA, respectively, when aligned with the ladder provided in the sample preparation kit. An RNA Quality Indicator (RQI) was also generated, which indicates the

quality of the sample tested. An RQI of 10 indicates intact RNA, and an RQI of 1 indicates completely degraded RNA, with samples with an RQI above 7 deemed acceptable for further analysis. Furthermore, the NanoDrop 2000 spectrophotometer (Thermofisher Scientific, MA, USA) was used to test the quality of the RNA. Absorbance maxima 260/280 indicate the purity of the RNA, and a ratio of 1.8 - 2.0 is accepted as pure.

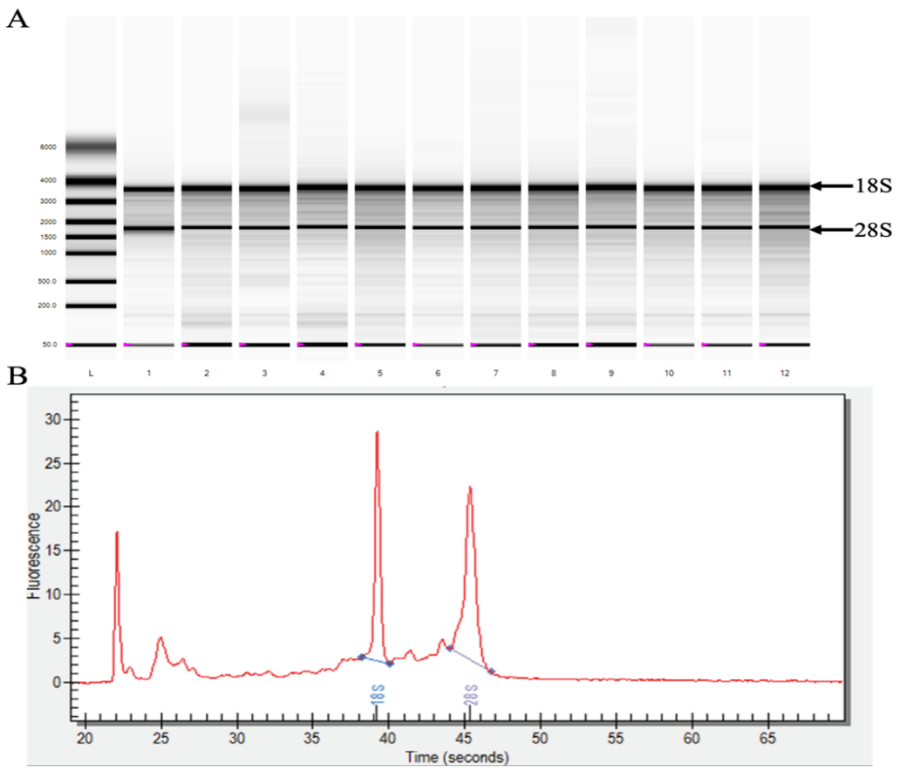

Figure 4: The Experion RNA StdSens Analysis Kit developed a representative virtual gel and electropherogram. (A) A virtual gel with two bands representing 18S and 28S ribosomal RNA. (B) An electropherogram displaying two peaks that correspond to 18S and 28S ribosomal RNA.

cDNA synthesis using reverse transcription.

After the integrity was assessed, the RNA was normalised to 220 ng/µL using the NanoDrop 2000 spectrophotometer (Thermofisher Scientific, MA, USA). Following this, the samples were reverse transcribed in duplicate using the Tetro cDNA Synthesis Kit (Cat # BIO- 65043; Bioline, UK). Briefly, a master mix was prepared to contain 0.5 µL each random hexamer, 10 mM dNTP mix, RiboSafe RNase Inhibitor and Tetro Reverse Transcriptase, 2 µL of 5x RT buffer and 4.7 µL of PCR water. Total RNA (1.3 µL) was added to give 1 µg of RNA per 10

µL reaction. Samples were incubated in the T100TM Thermocycler (Bio-Rad, CA, USA) for 10 min at 25 °C, followed by 30 min at 45 °C. The reaction was terminated by incubating at 85°C for 5 min. Duplicate samples were then pooled and diluted 1:3 with nuclease-free water and stored at -20°C until testing was completed.

Real-Time PCR (RT- qPCR)

Gene expression levels were quantified *via* real-time PCR (qPCR) using the SensiFast SYBR No-ROS mix (Bioline, UK). Each reaction mix comprised 5 µL of 2x SensiFAST SYBR No-ROS mix, 0.4 µL each of the forward and reverse primer (primer sequences shown in Table 4.7; 20 pmol μL^{-1}) and 1.2 µL of PCR water. The cDNA (3 µL) was then added to give approximately 100 ng of cDNA in a 10 µL reaction. The PCR plate was covered using a Microseal "B" PCR plate sealing film (BioRad, CA, USA), followed by brief centrifugation. Samples were then amplified in the CFX96 thermocycler (Bio-Rad, CA, USA) at 95°C for 2 mins for polymerase activation, 40 cycles of 5 secs at 95°C for denaturation, 10 seconds at 60°C for annealing, and 10 seconds at 72°C for the extension. A melt curve was generated by incubating the cDNA at 65°C for 5 seconds, then increasing the temperature to 95°C in 0.5°C increments. After each qPCR run, melt curve analysis was conducted to assess the specificity and purity of the amplified products derived from each specific primer sets.

Calculation of fold change in the gene of interest.

Relative changes in mRNA levels were determined using the comparative analysis method ΔΔCq. The difference between the quantification cycle (Cq) value of the gene of interest (GOI) and the reference gene is given by ΔCq. The baseline ΔCq is set as the ΔCq for the W/T Saline administered mice. The ΔΔCq calculation was then determined by subtracting each sample's ΔCq and the baseline ΔCq. The Cq is exponentially related to the copy number. Therefore, the absolute value for the relative expression level is given by the formula $2^{-\Delta\Delta}$Cq. The reference genes for the current study were β actin and 18s [255, 256].

Digital droplet PCR (dd PCR)

A digital droplet PCR reaction is prepared by adding 11.5 µL QX200 Evergreen Supermix (Cat # 186-4033; Bio-Rad, CA, USA), 1 µL forward and reverse primer mix at 4.6 µM, 1 µL of cDNA of interest (prepared as mentioned above) and 9.5 µL nuclease-free water to give a total volume of 23 µL, per reaction. Each sample was prepared in an Eppendorf tube and transferred

into an opaque PCR 96-well plate. The plate was thermally sealed and centrifuged before being placed in the QX200 droplet generator (Cat # 1864002, Bio-Rad, CA, USA) to generate droplets as per Maziaka and Homsy [257]. The ddPCR amplification was determined using a QX200 droplet digital PCR system (Cat # 1864001, Bio-Rad, CA, USA). The analysis of data was done as per manufacturer protocol, by using QuantaSoft™ Analysis Software (version 1.7; Instruction manual # 10031906) and designating the samples under experiment type, QX200 Evergreen Supermix, as the super mix and target type Ch1 [257]. After data acquisition, samples were selected to be analysed and an automatic threshold was applied. The concentration was reported as copies/ µL per 1x dd PCR reaction.

Table 3: PCR Primer Sequence for the *in vitro* experiments

Species	Gene ID	Description	F/R	Primer Sequence (5'-3')	Accession number *
Mouse	18 S	18S RNA Gene	F	AACTTTCGATGGTAGTCGCCGT	NM_146819.2
			R	TCCTTGGATGTGGTAGCCGTTT	
	Adipo	Adiponectin	F	GGCAGGAAAGGAGAACCTGG	NM_009605.5
			R	AGCCTTGTCCTTCTTGAAGA	
	ATF-6	Activating transcription factor 6	F	AGCTGTCTGTGTGATGATAG	NM_001081304.1
			R	GTGATCATAGCTGTAACTGTC	
	CAT	Catalase	F	CTCCATCAGGTTTCTTTCTTG	NM_009804.2
			R	CAACAGGCGTTTTTGATG	
	CPT-1	Carnitine palmitoyltransferase 1	F	GGGAGGTACATCTACCTG	NM_013495.2
			R	GMGACGTAGGTTTGAGTTC	
	GLUT 4	Glucose transporter 4	F	GATTCTGCTGCCCTTCTGTC	NM_009204.2
			R	ATTGGACGCTCTCTCTCCAA	
	GPX-1	Glutathione Peroxidase 1	F	ATTGCCAAGTCGTTCTACGA	NM_030677.2
			R	GTAGGACAGAAACGGATGGA	
	IL-1β	Interleukin-1β	F	GCTCAGGGTCACAAGAAACC	NM_008361.4
			R	CATCAAAGCAATGTGCTGGT	
	IL-6	Interleukin - 6	F	GAACAACGATGATGCACTTGC	NM_031168.2
			R	TCCAGGTAGCTATGGTACTCC	
	IL-8	Interleukin - 8	F	ACTTTTGGCTCAACCCTGTG	NM_009909.3
			R	ACGCAGTACGACCCTCAAAC	
	IL-10	Interleukin - 10	F	ATAACTGCACCCACTTCCCA	NM_010548.2
			R	GGGCATCACTTCTACCAGGT	
	IL-13	Interleukin - 13	F	GCAGCATGGTATGGAGTGTG	NM_008355.3
			R	TGGCGAAACAGTTGCTTTGT	
	MCP-1	Monocyte Chemoattractant Protein -1	F	GCTCAGCCAGATGCAGTTAA	NM_011333.3
			R	TCTTGAGCTTGGTGACAAAAACT	
	mTOR	Mammalian target of rapamycin	F	CTTCACAGATACCCAGTACC	NM_020009.2
			R	AGTAGACCTTAAACTCCGAC	
	PPAR α	Peroxisome Proliferator Activator Receptor α	F	ATTCGGCTGAAGCTGGTGTAC	NM_011144.6
			R	CTGGCATTTGTTCCGGTTCT	
	PDX-1	Pancreases duodenum homeobox 1	F	GATGAAATCCACCAAAGCTC	NM_008814.4
			R	TAAGAATTCCTTCTCCAGCTC	
	PKC	Protein kinase C	F	GTCTCAGAGCTAATGAAGATG	NM_011101.3
			R	TTGGCTTTCTCAAACTTCTG	
	SAA-1	Serum Amyloid A-1	F	AGCGATGCCAGAGAGGCTGT	NM_011315.3

			R	ACCCAGTAGTTGCTCCTCTT	
	TNF	Tumour Necrosis Factor	F	CTGTGAAGGGAATGGGTGTT	NM_013693.3
			R	CTCCCTTTGCAGAACTCAGG	
	UCP2	Uncoupled protein 2	F	ACCTTTAGAGAAGCTTGACC	NM_011671.6
			R	TTCTGATTTCCTGCTACCTC	
Human	β2M	β-2-Microglobulin	F	CATCCAGCGTACTCCAAAGA	NM_004048.4
			R	GACAAGTCTGAATGCTCCAC	
	IKK β	Inhibitor of nuclear factor κB	F	CAGATGGTCAAGGAGCTG	NM_020529.3
			R	CAAGTGCAGGAACGAGTC	
	IL-1β	Interleukin-1β	F	GCTTATTACAGTGGCAATGAGG	NM_000576.3
			R	AGTGGTGGTCGGAGATTCG	
	IL-6	Interleukin- 6	F	TGTGGTTGGGTCAGGGGTGGT	NM_000600.5
			R	CAATGAGGAGACTTGCCTGGTGA	
	IL-8	Interleukin- 8	F	CGGAAGGAACCATCTCACTGT	NM_000584.4
			R	GGTCCACTCTCAATCACTCTCA	
	NFκB	Nuclear factor-κB	F	TCCGAGACAGTGACAGTG	NM_003998.4
			R	GTTGAGAGTTAGCAGTGAGG	
	PEPCK	Phosphoenolpyruvate carboxykinase	F	ATTCTGGGTATAACCAACCC	NM_002591.4
			R	GTTGATGGCCCTTAAATGAC	
	TNF	Tumour necrosis factor	F	CTCTTCTGCCTGCTGCACTTTG	NM_000594.4
			R	ATGGGCTACAGGCTTGTCACTC	

**Accession numbers are indicative of correct primer sequence design*

Table 4: PCR Primer Sequence for the *in vivo* experiments

Species	Gene ID	Description	F/R	Primer Sequence (5'-3')	Accession Number*
Mouse	ACAB	Acetyl- CoA Carboxylase Beta	F	GCATGAAGGACATGTATGAG	NM_001403528.1
			R	AGGGATGTAGATGAGAATGG	
	Axin 1	Axin 1	F	AAATCACAAAGAAAGGCAGG	NM_024405.2
			R	AAAGTAGTATCTGTAGCTCCC	
	CEBPα	CAAT/ Enhancing Binding Protein α	F	AAGGGTGTATGTAGTAGTGG	NM_001287514.1
			R	AAAAAGAAGAGAAGGAAGCG	
	FABP4	Fatty Acid Binding Protein 4	F	GTAAATGGGGATTTGGTCAC	NM_024406.4
			R	TATGATGCTCTTCACCTTCC	
	FGF10	Fibroblast Growth Factor 10	F	AGTGTCCTGGAGATAACATC	NM_008002.5
			R	AGGTGTTGTATCCATTTTCC	
	GPX2	Glutathione Peroxidase 2	F	TTCGCACTTCTCAAACAATG	NM_002083.4
			R	CTCCATCAGGTTTCTTTCTTG	
	Insr	Insulin Receptor Substrate	F	AAGACCTTGGTTACCTTCTC	NM_010568.3
			R	GGATTAGTGGCATCTGTTTG	
	LPL	Lipoprotein Lipase	F	GAGACTCAGAAAAAGGTCATC	NM_008509.2
			R	GTCTTCAAAGAACTCAGATGC	
	NRF2	Nuclear Factor Erythroid -2-related Factor 2	F	GGGGACAGAATCACCATTTG	NM_008686.3
			R	GATGCAGGCTGACATTCTGA	
	PPAR *δ*	Peroxisome Proliferator Activator Receptor *δ*	F	CTGACAGATGAAGACAAACC	NM_011145.4
			R	CTTCCTCTTTCTCCTCTTCC	
	Retn	Resistin	F	CCTCCTTTTCCTTTTCTTCC	NM_001204959.1
			R	ATTTGGAAACAGGGAGTTG	
	TAZ	Tafazzin	F	CAGAAGGGAAAGTAAACATGAG	NM_181516.6
			R	ATCATTCATTCCAACATGCC	

**Accession numbers are indicative of correct primer sequence design*

2.10 Protein Extraction

Whole protein lysate was extracted from HepG2 cells, 3T3L-1 cells, MIN6 β-cells, liver and adipose tissue using RIPA lysis buffer (150 mM sodium chloride, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1 % w/v sodium dodecyl sulphate, 50 mM Tris pH 8.0) supplemented 1/10 volume of protease inhibitor cocktail (Sigma Aldrich MO, USA) containing 1.4 mm Zirconium oxide beads (Precellys 24, France) using a Minilys (Bertin Technologies, France). The samples were homogenised and incubated in the buffer on ice for 30 mins before being centrifuged at 12,000 RCF to form a pellet. The extracted protein's supernatant was collected and stored at -80°C until required. The concentration of the extracted protein was quantified with a Pierce BCA Protein Assay Kit (Thermofisher Scientific, MA, USA, cat. 23225).

Immunoblot Analysis

For immunoblot analysis, protein extracts (25 µg for the visceral adipose and liver tissue) were denatured by heating at 95 °C for 5 mins, then mixed with a 1:1 ratio of Laemmli loading buffer (BioRad, CA, USA). The samples were loaded into wells on a 4-15% Mini Protean TGX stain-free protein gel (Bio-Rad, CA, USA). Electrophoresis was performed in the 10x Tris/Glycine/SDS running buffer (BioRad, CA, USA) for 80 mins at 110 v. The Precision Plus Protein KaleidoscopeTM ladder (BioRad, CA, USA) was included in each gel run. Resolved protein samples were transferred to a mini PVDF membrane (BioRad, CA, USA) using the Trans-Blot® TurboTM Transfer System (BioRad, CA, USA), then blocked with skim milk (5% v/v in 1 x TBST) for 2 h at room temperature. Blots were incubated with primary antibodies (Table 5) overnight at 4°C. After washing with TBST (3x), the membranes were incubated with appropriate secondary antibodies for 2 h at room temperature; antibodies are listed in Table 5. Proteins were then visualised using ECL substrate (Bio-Rad, CA, USA) and a Bio-Rad Chemidoc Imaging System™. All samples (n= 4-5) were assessed by immunoblot analysis. Protein band densities were analysed using Image J 1.53a software by normalising against β-actin expression and one way ANOVA and Post-Hoc analysis was used to determine significance.

Table 5: Antibodies used for immunoblotting analysis.

Protein	Dilution	Company	Catalogue
pNFκB	1:100	Cell Signalling Technology, MA, USA.	76778
NFκB	1:100	Cell Signalling Technology, MA, USA.	8248
pSAPK/JNK	1:100	Cell Signalling Technology, MA, USA.	9251
SAPK/JNK	1:100	Cell Signalling Technology, MA, USA.	9252
Cytochrome C	1:100	Cell Signalling Technology, MA, USA.	4727
pAKT	1:100	Cell Signalling Technology, MA, USA.	4060
AKT	1:100	Cell Signalling Technology, MA, USA.	9272
pAMPK	1:100	Cell Signalling Technology, MA, USA.	50081
AMPK	1:100	Cell Signalling Technology, MA, USA.	5831
Fatty Acid Synthase	1:100	Cell Signalling Technology, MA, USA.	3189
pIRS (Ser)	1:100	Cell Signalling Technology, MA, USA.	2381
IRS	1:100	Cell Signalling Technology, MA, USA.	2382
IRE1 α	1:100	Cell Signalling Technology, MA, USA	3294
PDI	1:100	Cell Signalling Technology, MA, USA	3501
PERK	1:100	Cell Signalling Technology, MA, USA	5683
Perilipin- 1	1:100	Cell Signalling Technology, MA, USA	9349
β-Actin	1:100	Abcam, UK	8226
Anti-rabbit Secondary	1:10,000	Cell Signalling Technology, MA, USA	7074P2
Anti-mouse Secondary	1:10,000	Cell Signalling Technology, MA, USA	NXA931-1ML

2.11 Statistical Analysis

All data are expressed as mean ± SEM. The data was checked for normal distribution (Gaussian) distribution by column data analysis using Shaprio-Wilks normality test. When normality was determined, then parametric (one-way ANOVA) statistical analysis was performed. If determined to be not normal, then non-parametric tests (Kruskal-Wallis) with post-hoc multiple comparison analysis was performed. GraphPad Prism v8.00 (Graph-Pad, CA, USA) was used to complete all analyses. analyse the results. Results with $p < 0.05$ were considered significant.

Chapter 3 : TEMPOL as an antioxidant & anti-inflammatory therapy in type 2 diabetes mellitus, obesity and non-alcoholic fatty liver disease: a scoping review.

Submitted as:

Gihani Manodara, Michael J. Davies, Alison K. Heather, David Van Reyk, Kristine C. McGrath. TEMPOL as an antioxidant & inflammatory therapy in type 2 diabetes mellitus, obesity and non-alcoholic fatty liver disease: a scoping review. *Liver International*

Chapter Summary:

The well-studied antioxidant TEMPOL has generated an increased scientific interest due to its protective effects in obesity-related conditions, such as reduction in body mass and modulation of signalling pathways implicated in metabolic disorders such as NAFLD. This scoping review reveals gaps in the available literature and emphasises the need for additional research to fully understand the therapeutic potential of TEMPOL and its unique mechanisms of action in insulin-sensitive tissues. This chapter has been written in accordance with the guidelines of the journal, Liver International, to which this manuscript will be submitted.

TEMPOL as an antioxidant & anti-inflammatory therapy for type 2 diabetes mellitus, obesity and non-alcoholic fatty liver disease: a scoping review.

Gihani Manodara [a], Alyssa Borra [a], Michael J. Davies [b], Alison K Heather [c], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand*

Authors contribution:

For this paper, the PhD candidate, GM, completed all the literature searches, screened all the relevant studies, charted data from studies and wrote the manuscript. AB assisted with the literature searches, screened and charted data from identified studies. KM conceptualised the study and supervised GM. AH, MD and KM helped with the interpretation and edited the manuscript.

Gihani Manodara	Production Note: Signature removed prior to publication.
Alyssa Borra	Production Note: Signature removed prior to publication.
Michael J. Davies	Production Note: Signature removed prior to publication.
Alison K. Heather	Production Note: Signature removed prior to publication.
Kristine C. McGrath	Production Note: Signature removed prior to publication.

TEMPOL as an antioxidant & anti-inflammatory therapy for type 2 diabetes mellitus, obesity and non-alcoholic fatty liver disease: a scoping review

Gihani Manodara [a], Alyssa Borra [a], Michael J. Davies [b], Alison K Heather [c], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand*

Keywords: nitroxide, hepatic treatment, NAFLD, adipose tissue, oxidative stress, inflammatory response

Corresponding Author:
Dr Kristine McGrath
University of Technology Sydney
PO Box 123, Broadway
Ultimo, NSW, 2007, Australia
Ph: 612 9514 9773
Email: kristine.mcgrath@uts.edu.au

Key points:

- The global prevalence of obesity, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD) have reached concerning levels, creating serious health challenges.
- A comprehensive search was conducted across multiple electronic databases to determine the potential of TEMPOL to treat T2DM, obesity and NAFLD.
- TEMPOL exhibited protective effects in obesity-related conditions, such as reducing weight gain and modulating signalling pathways implicated in metabolic disorders like NAFLD.
- TEMPOL demonstrated promising results in mitigating disease in T2DM-specific models, including attenuating oxidative stress and inflammation.
- This scoping review identifies gaps in the existing literature and highlights the need for further research to fully understand TEMPOL's therapeutic potential and its specific mechanisms of action in insulin-sensitive tissues.

ABSTRACT

The prevalence of obesity has reached alarming levels globally, highlighting the urgent need for effective therapeutic interventions. This scoping review assessed the literature on the potential of TEMPOL, a nitroxide radical with antioxidative and anti-inflammatory properties, as a therapy for obesity and associated pathologies, particularly type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). Relevant research articles were acquired from Medline, PubMed, Embase, Web of Science, Scopus, and Cochrane Library. After screening, 35 journal articles that met all inclusion criteria were identified and included in this review. The selected studies predominantly utilized laboratory-based *in vitro* or *in vivo* models to investigate the effects of TEMPOL. China and the USA showed the highest number of publications for research on TEMPOL from 2011 to 2021. Core information was extracted from the articles and categorized into four themes with TEMPOL's effects on pathologies of T2DM, obesity and NAFLD in T2DM and HFD models. Analysis of the data from these studies indicated that TEMPOL could attenuate oxidative stress and the inflammatory response, possibly by repressing excessive lipid accumulation and subsequently offering protective effects in T2DM, obesity and NAFLD. Notably, clinical presentations such as increased weight gain could be reduced by TEMPOL. These findings underscore the importance of investigating TEMPOL as a potential therapeutic option for the growing burden of obesity, T2DM and NAFLD. However, further research is warranted to fully understand the mechanisms underlying TEMPOL's effects and its translation into clinical applications.

INTRODUCTION

The global prevalence of obesity has significantly increased since 1975, with the United States experiencing a tripling of obesity rates, affecting over two-thirds of its population [1]. However, of even greater concern are the associated health conditions it contributes to, particularly type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). T2DM stands out as a global pandemic characterized by impaired insulin sensitivity and secretion. At the time of T2DM diagnosis, approximately 90% of patients are typically either obese or overweight [2, 3]. The incidence of T2DM is projected to surpass 590 million patients by the year 2035 [4, 5]. Additionally, NAFLD frequently occurs as a consequence of a T2DM and obesity phenotype, where lipid accumulation in the liver is caused by elevated levels of free fatty acids (FFA's). Nearly 70% of T2DM patients develop NAFLD, which often progresses to non-alcoholic steatohepatitis (NASH), liver cirrhosis and subsequent hepatocellular carcinoma (HCC). The burden on healthcare systems is substantial, as evidenced by the staggering financial costs, where the US alone bears an estimated annual financial burden of $190 billion due to obesity and its associated diseases [6]. Addressing the complex interplay between obesity, T2DM and NAFLD is therefore paramount.

The first line of therapy for managing obesity, T2DM and NAFLD involves lifestyle and behavioural changes, but its efficacy has been moderate. While bariatric surgery remains the most effective method for weight loss in obese patients, its capacity to meet global demand is limited. Over the past two decades, there has been a growing interest in the development of anti-obesity medication (AOM). However, regulatory-approved AOMs have faced continuous failure primarily due to adverse effects such as cardiovascular disease (CVD), depression and abuse of certain AOMs such as from sympathomimetic (fenfluramine & sibutramine) and liver injury from pancreatic lipase inhibitors (orlistat) [7-9]. Currently, the long-term FDA-approved AOMs used for obesity management are based on glucagon-like peptide 1 receptor (GLP1R) agonism, specifically semaglutide and liraglutide [10, 11]. However, although subjects using GLP1R treatments have maintained and achieved more than 10% body fat loss (particularly when combined with exercise programs [12-14]), which may improve the severity of comorbidity, the efficacy falls short when compared to bariatric surgery [15-18]. In treating T2DM, the standard approach involves a combination of lifestyle intervention and metformin to reduce hepatic glycogenolysis and peripheral insulin resistance [19]. If this regime is ineffective, insulin secretagogues such as sulfonylureas and meglitinides are commonly

prescribed. However, their use is limited to short-term treatment due to side effects like weight gain and hypoglycaemia [19, 20]. If these approaches fail, daily insulin therapy through subcutaneous injections effectively lowers plasma glucose. Unfortunately, this therapy risks inducing hypoglycaemia, weight gain and an increased incidence of colorectal cancer [20, 21]. For NAFLD there are currently no FDA-approved drugs specifically for this condition, and although there are > 25 potential treatment options currently being evaluated in clinical trials [22], the best options considered so far are the improvement of insulin resistance via lifestyle changes, T2DM medications and surgical interventions.

To develop an effective pharmacotherapy for obesity induced T2DM and NAFLD, it is important to consider intracellular activity, receptors and the synergistic interactions of the molecular pathways common to these conditions. Obese patients with T2DM and/or NAFLD often show increased lipid accumulation in insulin-sensitive tissues, such as the liver resulting in insulin resistance in the liver, adipose tissue and muscle, as well as an elevated risk of T2DM [23-25]. Thus, this metabolic imbalance can lead to the progression from NAFLD to NASH and cirrhosis [26]. Additionally, hyperlipidaemia contributes to hepatic insulin resistance, while systemic inflammation can exacerbate disease progression in obese individuals with T2DM and NAFLD [23, 27]. Moreover, excessive nutrient supply stimulates heightened mitochondrial activity, leading to an overproduction of reactive oxidant species (commonly called "ROS") and a decrease in endogenous antioxidants. This imbalance increases oxidative damage (oxidative stress), a shared pathology among obese individuals with T2DM and NAFLD [28, 29]. Therefore, targeting these three aspects - hyperlipidaemia, inflammation, and oxidative stress - with pharmacological therapy could effectively treat T2DM and NAFLD and potentially reduce weight gain in obese patients.

Nitroxides, a group of stable free radicals, possess unique characteristics that make them suitable for medical applications [30]. Their low molecular weight enables efficient cellular permeability, and they can mimic the actions of superoxide dismutase, protecting against oxidative stress-induced cellular damage [31, 32]. Moreover, nitroxides exhibit a wide range of antioxidative and anti-inflammatory properties [32] as well as inhibition of some oxidant-generating enzymes [33, 34]. Among the various types of nitroxides, the most well-known is the piperidine nitroxide, TEMPOL [4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl] [31]. While there is extensive evidence documenting the efficacy of TEMPOL in various body systems, such as the renal system, however particularly in the context of obesity, T2DM,

NAFLD and related diseases, have received less attention. Given the key role of the liver in the pathogenesis of NAFLD and T2DM, this scoping examines how the liver in particular is protected by TEMPOL against antioxidative and anti-inflammatory effects. Furthermore, it identifies gaps in the existing literature that is required to be filled as we move towards developing potential TEMPOL-based treatments.

METHOD

This scoping review adheres to the framework outlined by Arksey and O'Malley [35]. The framework consisted of (1) outlining the research question, (2) determining the relevant studies, (3) selection of studies, (4) charting data from included studies, and (5) collating, summarising, and reporting results.

Identifying the Research Question

This review was conducted to examine the existing evidence supporting the use of TEMPOL as an antioxidative and anti-inflammatory agent for obesity and T2DM, as well as their associated pathophysiology NAFLD, with a particular focus on its ability to protect against the hepatic system. This review also aims to explore the benefits and limitations of TEMPOL as a proposed therapy to guide further scientific research.

Identifying the Relevant Studies

A set of keywords based on the PICOS (Population/ Problem, Intervention, Comparison, Outcomes and Study setting) framework was established to facilitate the search strategy. As per our research question, only Population, Intervention and Outcome were applied (Table 1). Original, peer-reviewed research articles were acquired from six electronic databases: Medline, PubMed, Embase, Web of Science, Scopus, and Cochrane Library. All literature searches were conducted by authors GM and AB from September to October 2021 using a search strategy developed with the complete set of keywords presented in **Table 1**.

Table 1: PICOS framework for determining the eligibility of review questions and keywords chosen.

Criteria	Determinants	Keywords
Population	*In-vitro* and *in vivo* studies on TEMPOL in treating obesity and T2DM.	*In vivo* OR Murine OR Rodents OR mice OR i*n vitro* OR cells OR humans.
Intervention	Does TEMPOL reduce obesity and obesity-associated comorbidities that include T2DM?	Nitroxide OR TEMPOL OR 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.
Outcome	TEMPOL's effect on obesity induced NAFLD.	Oxidative stress OR antioxidants OR insulin sensitivity OR hepatic steatosis.

Inclusion and Exclusion Criteria

To be eligible for inclusion in this scoping review, studies had to meet the following criteria: they were identified through the search strategy based on the predetermined keywords (Table 1); they were published in English between the year 2011 to 2021; they were either original research or a clinical trial. Studies conducted on the renal system were excluded from the review, as were articles on disorders independent of oxidative stress and those unrelated to the benefits of TEMPOL.

The selected research studies were imported into EndNote software [36], to remove duplicates and then transferred to JBI SUMARI for screening. Authors GM and AB then performed title and abstract screening for the applicability to this review before progressing to full-text screening. During the two screening stages, any conflicts between reviews were resolved through discussion until a final consensus was reached.

Charting the Data

Data from the final selection of studies, identified as suitable for this review, were extracted and charted using Microsoft Excel Software. The data recorded from each study included the author(s), publication year, the study aim, country of origin, sample sizes, administration method, the concentration of TEMPOL used, parameters measured and key findings. Following data collection, the studies were categorized based on their similarities and differences and organized into themes that align with our research question (**Table 2**). Furthermore, the studies were classified according to their respective research methods.

RESULTS

Characteristics of Included Studies

To identify relevant papers, two independent investigators, GM and AB, searched across six databases using the methodology's specified search terms, resulting in the identification of 7178 papers (Fig. 1). Duplicates (4588 papers) were removed, leaving 2590 papers, which were screened based on their titles and abstracts. As a result, 2517 papers that did not meet the inclusion criteria were excluded. After a full-test assessment of the remaining 73 papers, 38 articles were excluded for various reasons, such as studies related to cancer, radiology and nephrology research, as well as studies where full text could not be retrieved. The remaining studies included in this research were published between 2011- 2021 and their origin countries (17) were widespread (Fig. 2). The USA was the most prevalent country (8 using TEMPOL [37-44]. The next most prevalent countries were China & Japan (4) [45-51], Argentina, Brazil, Canada and Korea (2) [52-59]. Of the 35 studies included in the analysis, 26 studies used *in vivo* murin*e* models only, six studies used *in vitro* models only, one article utilized both *in vivo* and *in vitro* study models and two were human studies.

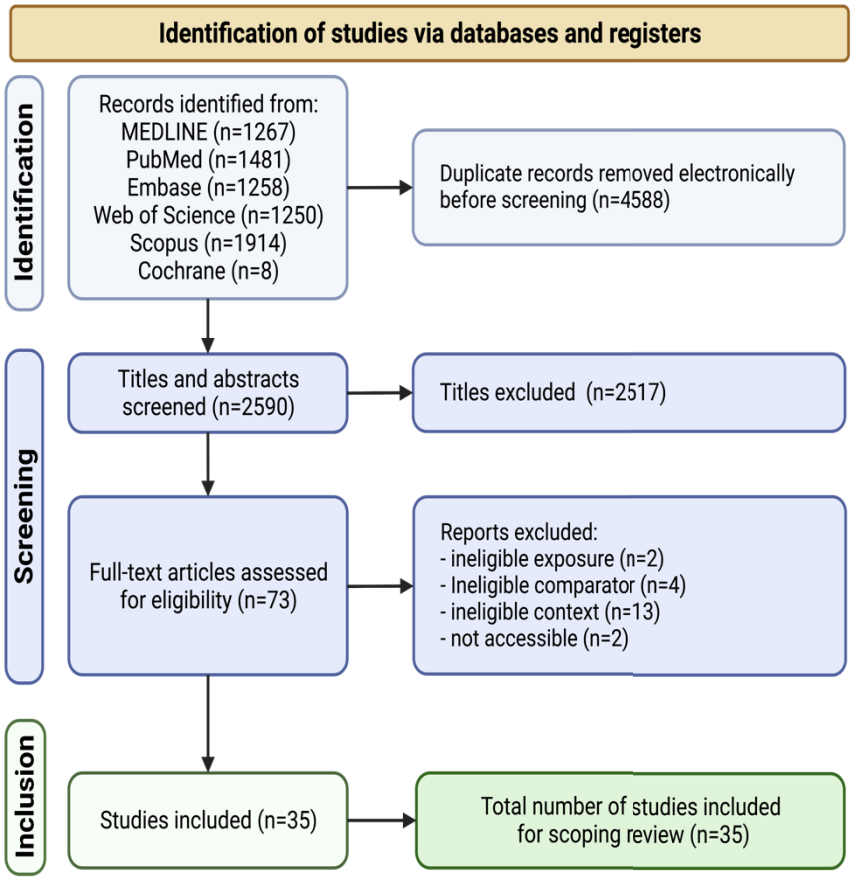

Figure 1: Flowchart depicting the article selection process for the scoping study generated using PRISMA software.

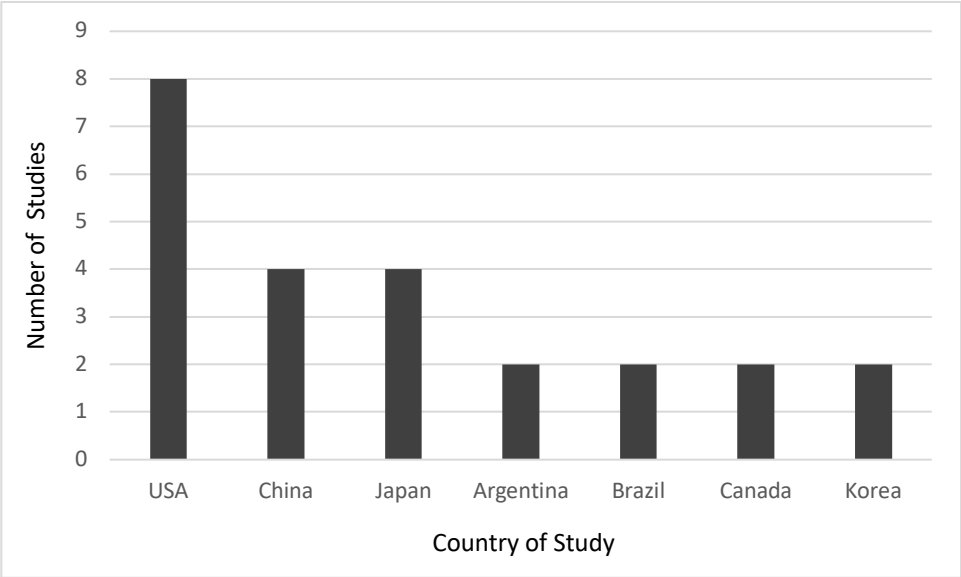

Figure 2: Distribution of studies using TEMPOL as a therapeutic option for T2D and obesity-induced hepatic pathologies from 2011-2021. USA (n= 8), China (n=4), Japan (n=4) Argentina (n=2), Brazil (n=2), Canada (n=2) and Korea (n=2). The following countries were not represented in the figure (n=1): Australia, Egypt, Finland, France, Germany, Iran, Jordan, Netherlands, Saudi Arabia, and Turkey.

The anti-inflammatory and antioxidant properties of TEMPOL contribute to the alleviation of T2D clinical features.

Of the 35 studies that met the inclusion criteria, seven studies showed that TEMPOL administration could alleviate clinical indicators associated with T2DM and obesity by exerting antioxidative and anti-inflammatory responses (**Table 2**). Exposure to the food additive carrageenan, commonly found in processed food, is known to induce inflammation and impair insulin signalling [60]. Two studies showed exposure to TEMPOL inhibited carrageenan-induced phosphorylation of IκB in HepG2 cells resulting in a reduction of inflammation [37, 39]. Moreover, in a follow-up study, the same research group showed a carrageenan-induced increase in phosphorylated serine residues (Ser (P) ^{307}) in insulin receptor substrate 1 (IRS1) and protein kinase B (Akt), indicating disrupted insulin signalling that was reversed with TEMPOL [38]. This positive impact on insulin signalling is corroborated by findings that insulin inhibition by 3- nitropropionic acid (3-NPA) in islet cells could be recovered by TEMPOL [61].

TEMPOL's antioxidative and anti-inflammatory responses have also been demonstrated in various diseases related to obesity and TD2M. For example, obstructive sleep apnoea syndrome (OSAS) is an important risk factor for insulin resistance, independent of obesity. Even more, chronic intermittent hypoxia (CIH) in OSAS is associated with inflammation and oxidative stress [62, 63]. Two independent articles (Lu et al., 2019; Wang et al., 2019) show that CIH in obstructive sleep apnoea leads to increase inflammatory cytokines such as tumour necrosis factor (TNF), interleukin-6 (IL-6) and interleukin-8 (IL-8) in liver and adipose tissue of rat models stimulated with hypoxic chambers [51, 64]. Moreover, increased C-reactive protein (CRP) and malondialdehyde (MDA; a non-specific indicator of lipid oxidation) were observed, indicating concomitant increased oxidative stress. Notably, these two studies showed a reduction of inflammatory cytokines and oxidative stress in the liver and adipose tissue with TEMPOL treatment. Furthermore, Wang et al.,2019 found that changes to insulin signalling mediated by CIH could be recovered using TEMPOL [51].

Similarly, a study conducted by Yamato and colleagues presented the potential of TEMPOL consumption to improve outcomes of age-related diseases characterized by inflammation. TEMPOL-treated mice showed a reduction in mortality, normalized levels of oxidative stress markers and an increased proportion of CD4 lymphocytes in the blood compared to aged mice that did not receive TEMPOL [46]. This suggested that the beneficial effects of TEMPOL on mortality reduction could be attributed to its antioxidant properties, which help alleviate inflammation and oxidative stress

Table 2. Included journal articles that studied TEMPOL for its effect on the pathological mechanisms that underlie obesity, T2DM and liver dysfunction.

TEMPOL and its effects on underlying pathological mechanisms of T2DM (2012-2019; n = 7)			
TEMPOL Dose & Experimental Model	**Effects**	**Mechanisms**	**Reference**
HepG2 cells were treated with λ- carrageenan (1 mg/L) for 20 h in media with serum, and a further 4h with λ- carrageenan (1 mg/L) in serum-free media followed by 100 nM/L TEMPOL	↓Phospho (S32)-IκBα* ↓Phospho (Ser307)-IRS1*	↓ Inflammation Blocked inhibition of insulin signalling	Bhattacharyya et al., 2012, [37]
6 mM TEMPOL in drinking water on 20-23 months old C57BL/6N mice for 20 months	↑Ascorbic acid*** ↓CD4+ lymphocytes Normalized c reactive proteins (CRP)* and plasma thiobarbituric acid-relative substances (TBARS)**	↓ Oxidative stress ↓ Inflammation ↓ Mortality	Yamato et al., 2014 [46]
HepG2 cells treated with 100 nM/L TEMPOL together with λ- carrageenan (1 mg/L) for 24 h	Inhibited BCL10 ↓ Ser(P)^{307}-IRS1 Partially normalised Ser(P)^{473}-AKT	↓ Inflammation ↑ Insulin signalling	Bhattacharyya et al., 2015 [38]
1 mM/L TEMPOL treatment of, isolated pancreatic islets from adult C57BL/6N mice stimulated with 3-Nitropropionic acid (3-NPA, 1 mM/L)	Recovery from compromised insulin secretion ↓ Hydrogen peroxide	↓ Oxidative stress	Edalat et al., 2015 [61]
An intraperitoneal injection containing 10% of 100 mg/kg TEMPOL for six weeks, using Sprague Dawley rat model exposed to hypoxic chambers intermittently for 90 secs	Recovered SOD* ↓TNF-α*, IL-8*, IL-6* and MDA* Inhibited P-p38 MAPK pathway	↓ Inflammation ↓ Oxidative stress	Lu et al., 2019 [64]

Table 2 (*continued*)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
Drinking water containing 1 mM TEMPOL daily for 12 weeks using Wistar rat model stimulated with intermittent hypoxia for 60 secs for 12 days	Suppressed MAPK pathways ↓ NF-κB pathway ↓ Bax/Bcl-xl ratio ↓ TNF-α***, IL-6***, IL-1ß***, PGE2***, NO*** ↓ iNOS and COX-2 levels ***	Restored insulin levels** ↓ Inflammation ↓ Pancreatic injury ↓ Apoptosis	Wang et al., 2019 [51]
100 nM TEMPOL for 24 h treatment on HepG2 cells pre-exposed to either λ-carrageenan (1 mg/L) in media with serum for 20 h or 200 µM palmitic acid in media without serum for 4 h	↓ Phospho (Ser32)-IκBα and macrophage ↓ NF-κB, IL-8, TNF-α	↓ Inflammation	Bhattacharyya et al., 2019 [39]

TEMPOL and its reported effects in T2DM Models (2011-2019; n= 8)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
20 mg/kg (bw, i.v) TEMPOL in saline, once a day for 15 days using STZ-induced (ip 60 mg/kg dose for 15 days) hyperglycaemic Wistar rat model	↓ ROS*, OH radicals, Bax/Bcl-xl ratio* ↓ Lipid peroxidation (LPO)	↓ Oxidative Stress	Frances et al., 2011 [52]
20 mg/d TEMPOL in drinking water for 16 weeks, using Otsuka Long- Evans Tokushima Fatty (OLETF) rat model	↓ Blood glucose levels*, ↓ LPO, ↓ 8-OHdG (8-hydroxy-2'-deoxyguanosine) ↓ Apoptotic beta cells ↓ Islet fibrosis ↑ Blood insulin levels ($p<0.05$) ↑ β-cell mass	↓ Oxidative stress ↓ Pancreatic apoptosis	Lee et al., 2011 [56]

Table 2 (*continued*)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
2.41 μM kg^{-1} min^{-1} of TEMPOL for 48 h via cannulation using Wistar rats	↓ eIF2α phosphorylation, ↓ Xbp1 mRNA*** ↓ Chop mRNA	↓ Endoplasmic Reticulum (ER) stress ↓ Uncoupled protein response (UPR)	Tang et al., 2012 [54]
10 mg 4-OH TEMPOL dissolved in 0.1M PBS using STZ-induced Sprague-Dawley albino rat model	Dose-dependent inhibition of peroxynitrite formation ↓ Blood glucose levels	↓ Oxidative stress	Van Dyke et al., 2014 [41]
500 mg/kg body mass/daily TEMPOL intraperitoneal injection for 28 days using $P58^{IPK-/-}$ C57BL/6J mice model	Restored glucose tolerance test (GTT) after 42 days	Protected against β-cell failure	Han et al., 2015 [42]
Pre-treated with TEMPOL (0.5 mM) for 12 h using palmitate + oleate induced insulin resistance in in isolated satellite cells from eight healthy non-smoking men	Normalized glucose uptake in insulin-resistant myotubes Counteracted increased mitochondrial ROS levels	↓ Oxidative stress ↑ Glucose uptake	Makinen et al., 2017 [65]
Oral administration of 100 mg/kg of TEMPOL in 2 ml normal saline daily for 28 days using STZ-induced (single intraperitoneally dose of 55 mg/kg body mass of STZ) diabetes Wistar strain rat model	↓ Blood glucose ↓ AST & ALT ↓ LPO* ↑ Total antioxidant capacity (TAC) ↑ Total thiol group (TTG)*	↓ STZ-induced oxidative stress. ↑ Cellular GSH and prevent lipoperoxidation. ↑ Hepatoprotective effect	Ghadermazi et al., 2018 [66]
TEMPOL pre-treatment (0.5mM/L & 1mM/L) for 24 h on STZ-induced (0.5mM/L) oxidative stress in β -cells	Recovered reduced cell viability induced by STZ*** ↓ STZ-induced caspase-3 activity and β-cell death	↓ Oxidative stress	Lee et al., 2019 [57]

Table 2 (*continued*)

TEMPOL and its effects on the pathogenic processes associated with High Fat Diet Models (2011-2019; n=10).			
TEMPOL Dose & Experimental Model	**Effects**	**Mechanisms**	**Reference**
30 min incubation with 1mM/L TEMPOL treatment on small arteries isolated from visceral fat from obese human patients	↓ Superoxide radical anion levels* Restored vessel's relaxation response to nitric oxide	↓ Oxidative stress increase nitric oxide	Virdis et al., 2011 [67]
TEMPOL 1 mg/kg body mass/ day given to fructose fed ApoE- deficient mice for 4 weeks	↓ Vascular NAD(P)H oxidase activity	↓ Oxidative stress	Cannizzo et al., 2012 [53]
1.5 mM/kg TEMPOL body mass/day was given oral gavage given to high-fat high-sucrose (HFHS) diet fed Sprague-Dawley rat model for 4 weeks	↓ Blood pressure & heart rate ↓ Body mass ↓ NAD(P) oxidase activity ↓ iNOS expression ↑ eNOS expression ↑ Glucose transport activity by preventing the reduction of glucose trasnporter 4 (GLUT 4) protein expression	↓ Oxidative stress ↓ Inflammation	Bourgoin et al., 2013 [55]
TEMPOL 0.064 % (w/v) was given in drinking water to intestine-specific *Fxr*-null and wild type C57BL/6N mice on a high-fat diet for 16 weeks	↓ Liver/body mass ratio ↓ Triglyceride content by 50% ↑ Bile acids ↑ Glycogen	↓ Lipogenesis	Jiang et al., 2015 [43]
10 mg/g TEMPOL treatment infused on a fat HFD of ApoE-deficient mice for 12 weeks	↓ Weight gain**, ↓ IL-6*, SAA*, MCP-1, myloperoxidase (MPO)*, ↓ Triglyceride*, total cholesterol, HDL-C* and LDL-C* ↑ Adiponectin ↓ p47phox, TNF-α and iNOS ↓ Vascular superoxide levels	↓ Inflammation ↓ Oxidative stress	Kim et al., 2015 [30]

Table 2 (*continued*)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
100 µM TEMPOL treatment on fructose-activated hepatocytes *in vitro* model for 24 h	↑ SIRT1 expression	Improved fructose-induced oxidative stress	Sodhi et al., 2015 [44]
50 mg/kg/day TEMPOL was given in drinking water to young and old mice on a HFD for ten weeks	Enhanced glucose tolerance in old mice ↓ TG and VLDL in plasma in young and old mice ↓ AST* and ALT* in young mice only	Scavenging of superoxides	Nunes-Souza et al., 2016 [58]
30 mM TEMPOL in drinking water using HFD-induced obese C57BL6N & 6J mice model for 8 weeks	↑ NADP/NADH ratio ↓ Total body fat percentage ↓ Superoxide levels	Modulates ROS scavenging and redox cycling system	Yamato et al., 2016 [48]
50 µM TEMPOL using HFHS diet induced obese Wistar rat model for 12 h	↓ ROS generation and lipid accumulation	↓ Oxidative stress	Braud et al., 2017 [68]
TEMPOL 30 mg/kg gavage daily for 30 days using HFD fed LDLr $^{-/-}$ mice model	Prevented lipid accumulation*by ↓ TG, VLDL & LDL ↓ Average body mass	↓ Oxidative stress ↓ Adiposity	Viana Goncalves et al., 2017 [59]
TEMPOL and its effect on liver pathology (2012-2019; n = 10)			
TEMPOL Dose & Experimental Model	**Effects**	**Mechanisms**	**Reference**
1 mM/L TEMPOL and palmitic acid (250 µM/L) for 24 h using HepG2 *in vitro* model	↓ Lipid deposition* ↓ Superoxide anion levels	↓ Adiposity ↓ Oxidative stress	Ma et al., 2012 [45]
5 mM TEMPOL for 15 mins on hepatic stellate cells prior to stimulation by an oxide anion radical ($O_2^{\bullet-}$)	Normalised TGF-β1* and collagen levels* Scavenge superoxide ions	↓ Fibrosis ↓ Oxidative tress	Den Hartog et al., 2013 [69]

Table 2 (*continued*)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
25, 50, 75 or 100 mM TEMPOL treatment for 2 mins of whole blood from healthy human donors	↓ Carbonyl formation* ↓ TBARS formation*	↓ Oxidative damage	Mustafa et al., 2015 [70]
TEMPOL 20 mg/kg/day for 3 days was given for 3 days after single administration of carbon tetrachloride (CCl_4; 0.5 mg/kg) on Albino mice model	↓ AST, ALT, bilirubin ↓ Malondialdehyde (MDA) ↓ NF-κB, TNF-α ↓ Caspase-3 ↑ Bcl-2 ↑ GSH and catalase activity	↓ Oxidative stress ↓ Inflammation ↓ Liver apoptosis ↓ Hepatoxicity	Abouzied et al., 2015 [71]
42.5 mg/kg/day orally administered for 4 weeks on C57BL/6 mice fed choline define amino acid (CDAA) diet	↓ Collagen mRNA expression* ↓ MPO*, Ly6C ($p < 0.05$), F4/80, IL-1B and NLRP3***	↓ Oxidative stress ↓ Inflammation ↓ Fibrosis	Eguchi et al., 2015 [47]
Pre-treatment with 20 mg/kg of TEMPOL orally administered daily for 14 days followed by one dosage of CCL_4 (1 ml/kg) using Albino mice model	↓ ALT***, AST*** ↓ MDA*** ↓ Bilirubin*** ↑ GSH***, and catalase * ↓ TNF-α and caspase-3	↓ Oxidative stress ↓ Inflammation ↓ Apoptosis ↓ Hepatoxicity	Abdel-Hamid et al., 2016 [72]
30 mg/kg TEMPOL for 10 days using Wistar albino rat model stimulated with one intraperitoneal dosage of Methotrexate (MTX, 20 mg/kg)	↓ MDA*, ↓ MPO*, ↓ Bax*, caspase 3* ↓ AST*, ALT* ↓ Necrosis ↑ Catalase*, SOD*, GPX* ↑ Bcl-2*,	Inhibit superoxides and increase nitric oxide ↓ Oxidative stress ↓ Inflammation ↓ Apoptosis ↓ Hepatoxicity	Pinar et al., 2018 [73]
Pre-treatment with 0.01, 0.05, 0.1 & 0.5 mM of TEMPOL treatment for 30 mins before stimulated with H_2O_2 (0.5 mM) for 1h in primary hepatocytes (Hepa 1-6 cells) from 4-month C57BL/6N mice.	↓ ROS ↓ NAD^+ levels ↓ Caspase-3 ↓ JNK & MAPK activation	↓ Oxidative stress ↓ DNA damage ↓ Apoptosis	Shinto et al., 2018 [49]

Table 2 (*continued*)

TEMPOL Dose & Experimental Model	Effects	Mechanisms	Reference
0.064 % TEMPOL in drinking water for 10 days in hepatocyte specific *Depdc5* and *Tsc1* double knockout (DKO) C57BL/6 mice model	↓ Liver/body weight ratio, ↓ AST*, ALT****, ALP*** and bilirubin* ↓ Fibrotic lesions ↓ ER stress ↓ mTORC1 activation	Scavenge superoxide radicals ↓ Hepatoxicity ↓ Fibrosis ↓ Oxidative stress	Cho et al., 2019 [40]
Pre-treatment with 20 mg/kg TEMPOL orally for 7 days followed by single dose of Acetaminophen (APAP, 300 mg/kg) C57BL/6 mice model	↓ ALT*, AST**, ↓ MDA***, ↓ Caspase-3*** and Bax* ↑ SOD***, catalase***, GSH** ↑ Bcl-2**	Activate PI3K/Akt/Nrf2 pathway ↑ Antioxidant response ↓ Hepatoxicity ↓ Oxidative stress ↓ Apoptosis	Ge et al., 2019 [50]

Inhibitors of nuclear factor kappa-B kinase, IκBα; Insulin receptor substrate, IRS; Thiobarbituric acid reactive substances, TBARS; protein Kinase B, AKT; malondialdehyde, MDA; inducible nitric oxide synthase, iNOS; Cyclooxygenase enzyme 1, COX-2; Mitogen-activated protein kinase, MAPK. STZ, Streptozotocin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TTG, total thiol groups; TAC, total antioxidant capacity; RAS, Renin-angiotensin system, ROS, Reactive oxygen species. NADPH, nicotinamide dinucleotide phosphate; eNOS, endothelial nitric oxide synthase; Fxr, farnesoid X receptor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SIRT1, sirtuin. CDAA, choline-deficient amino acid defined; GPX, glutathione peroxidase; DKO, *Depdc5* and *TSC1* interbreeding mice; ALP, alkaline phosphatase.

Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Antioxidative Effects of TEMPOL in Type 2 Diabetes Model

Given the beneficial effects of TEMPOL on T2DM clinical markers (see above), it was therefore not surprising that of the 35 studies, eight studies showed TEMPOL was highly effective in reducing oxidative stress exacerbated by T2DM. Key findings from TEMPOL treatment of T2DM-specific models are outlined in **Table 2.** The majority of the *in vivo* studies investigating the effects of TEMPOL on T2DM used streptozotocin (STZ) induced pancreatic β cell death to induce diabetes in rodents. TEMPOL was administered at various doses across the range of 10-100 mg/kg [41, 52, 66]. Overall, it was repeatedly demonstrated that TEMPOL administration reduced ROS levels induced by STZ and restored the total antioxidant capacity (TAC), leading to an improvement in the diabetic status of the animal's [41, 52, 66]. Additionally, it was demonstrated that TEMPOL treatment led to lower lipid peroxidation (LPO) levels in Otsuka Long Evans Tokushima Fatty (OLETF) rats [56, 66].

The susceptibility of β-cells to apoptosis induced by T2DM-induced oxidative stress was also decreased by TEMPOL. Several studies reported TEMPOL decreased the Bax/ Bcl-xl ratio, which when elevated signifies cell vulnerability to apoptosis [42, 52, 56]. Additionally, studies reported TEMPOL not only reduced β-cell death but also decreased caspase-3 activity, which is commonly elevated in STZ-induced models [57]

TEMPOL inhibits inflammation and oxidative stress in high-fat diet-fed rodent models.

High-fat diet (HFD) animal models are commonly used to study the effects of excessive dietary fat intake on various physiological processes. When animals are fed with a HFD, it can lead to obesity, insulin resistance and metabolic dysfunctions such as systemic inflammation and oxidative stress, mimicking, at least in part, human obesity and T2DM. Of the 35 studies, 10 studies investigated the effects of TEMPOL in HFD-fed and/or high-fat high sucrose-fed (HFHS) animal models (**Table 2**). Five of the 10 research studies showed that body mass was markedly reduced by TEMPOL treatment [30, 48, 55, 59]. Moreover, Viana Goncalves et al.,2017 and Yamato et al.,2016 showed that the improvement in average body mass change was concomitant with improved outcomes, including decreased serum triglycerides, lipoproteins, and improved glucose tolerance. It was further reported that a TEMPOL-induced decrease in body mass correlated with decreased liver weight [43]. As HFD is known to provoke oxidative stress and in turn systemic inflammatory response [74], the beneficial effect of TEMPOL reducing body mass in aforementioned studies attributes to its ability to ability to

scavenge superoxides and reduce oxidative stress, facilitating the recovery from the imbalance-of the systemic homeostasis, induced by HFD.

Therapeutic and its effects on liver dysfunction associated with obesity related NAFLD.

Extensive literature supports the association between NAFLD and metabolic syndrome, primarily attributed to hyperlipidaemia and hyperglycemia, which remains the strongest risk factor for developing NAFLD [75-77]. However, there is currently no specific therapeutic treatment for NAFLD targeting hyperlipidaemia and hyperglycemia-induced inflammation and oxidative stress in the liver. Nevertheless, the hepatoprotective effects of TEMPOL have been examined by several research groups, where five rodent studies showed a reduction of AST and ALT levels with TEMPOL treatment [40, 50, 71-73] (**Table 2**). Measurement of AST and ALT liver enzymes is a common predictor of liver dysfunction, where an AST: ALT ratio > 1 is a predictor of liver dysfunction such as NAFLD or severe state of the disease such as cirrhosis [78]. Furthermore, two rodent studies [40, 47] and one *in vitro* study [69] showed the reversal of fibrosis by TEMPOL in the liver induced by high caloric diets and in HepG2 cells induced by oxide anion radical. Notably, TEMPOL showed promising results in lowering abnormal lipid levels, reducing carbonyl formation in human plasma, and restoring liver function in rodents and hepatocyte models [45, 50, 70, 73]. Therefore, it is probable that a reduction in body mass and liver weight would have aided in the reversal of hepatoxicity (AST: ALT ratio) induced by hyperlipidaemia.

Given oxidative stress and inflammation are common pathologies of NAFLD, **Table 2** shows TEMPOL's potent antioxidant properties reducing oxidative stress *in vivo* [40, 47, 50, 71-73] and *in vitro* [45, 49, 69] and interestingly in human blood by one human study [70]. In addition to its effects on oxidative stress TEMPOL attenuated the inflammatory response in rodent models [40, 47, 71-73] and in primary hepatocytes [49]. Further, TEMPOL treatment reduced liver cell death stimulated by stress responses in both rodent models [71-73]and *in vitro* [49]. These studies showed promising hepatoprotective effects specifically on reducing oxidative stress and inflammatory responses by TEMPOL.

DISCUSSION

Using the methodological framework developed by Arksey and O'Malley [35], this scoping review aimed to comprehensively map the potential of TEMPOL to reduce the pathophysiology associated with obesity, T2DM and NAFLD. To the best of our knowledge,

this is the first scoping review that has identified the extent, range and nature of research illustrating the different models used, outcomes measured and geographical distribution where research focused on the use of TEMPOL as an antioxidative and anti-inflammatory agent in the context of obesity and T2DM, with a specific emphasis on its potential therapeutic effects within the hepatic system.

The majority of the TEMPOL research, to date, has primarily been conducted in countries with a high prevalence of T2DM and obesity, with the United States of America (USA) and China being the top contributors. However, it is crucial to acknowledge that T2DM is a global health issue that affects both developed and developing countries [79]. The rapid increase in T2DM prevalence is observed across various nations [80], highlighting the need for a comprehensive understanding of the disease and potential therapeutic interventions. The inclusion of affluent countries like the USA and China in TEMPOL research can be attributed to factors such as advanced research facilities and healthcare systems. In contrast, developing countries often face challenges in allocating sufficient budgets for research and development, especially for long-term research projects that are required to show translational applications [81].

Of the studies included in this review, 74% of the studies were composed of *in vivo* models, while three studies employed both *in vitro* and *in vivo* murine models. Additionally, six *in vitro* studies were included, and notably, two of these studies involved cell isolation from human subjects. The substantial number of *in vivo* and *in vitro* studies, including those using primary human cell lines, supports the assessment of TEMPOL's safety and the absence of cellular toxicity in biological systems. Collectively, these studies indicate that the properties of TEMPOL make it a potential therapeutic option for treating obesity, T2DM and its associated pathologies. For example, research conducted by Bhattacharyya et al., 2012, and Han et al., 2015, exemplifies TEMPOL's ability to mitigate the effects of oxidative stress and inflammation, resulting in protecting β cell failure and improved insulin signalling [37, 42]. Furthermore, TEMPOL has demonstrated hepatoprotective mechanisms in pathologies induced by high-fat diets [57]. Studies done by Li et al., 2013 and Choudhuri et al., 2022 presents the plausibility of TEMPOL's anti-obesity effect by inhibiting farnesoid X receptor (FXR) as they revealed TEMPOL changing the composition of bile acids in both liver and serum of HFD treated mice models [82, 83]. In addition to this and despite all 35 research articles emphasizing the significance of TEMPOL as a potential therapeutic treatment, there is

still a gap in our understanding of the specific mechanism by which TEMPOL exerts its beneficial effects.

Diet and lifestyle interventions are typically the first-line therapy for obesity and T2DM, however, what delineates an effective treatment for these two diseases is the lack of pharmaceutical therapies that target common pathways. Oxidative stress, inflammation, and insulin resistance are associated not only with obesity but also with T2DM and resultant co-morbidities such as NAFLD. Furthermore, ER stress plays a main role in the pathogenesis of T2DM due to activation by three proteins (PERK, IRE1α, ATF 6) that lead to the initiation of unfolded protein response (UPR) stress signalling, contributing to insulin resistance. Seven studies included in our analysis demonstrated the antioxidant effects of TEMPOL in T2DM models, showcasing its ability to normalize indicators of oxidative stress, including reactive oxidants (ROS) and total antioxidant capacity (TAC) [41, 52, 54, 56, 57, 61, 66]. For instance, Makinen et al.,2017 showed that TEMPOL reduced mitochondrial oxidant production and restored insulin-stimulated glucose uptake in impaired insulin signalling and ER stress myotubes, while Tang et al., 2012, revealed that TEMPOL's downregulation of UPR signalling proteins contributed to the prevention of oxidative stress and β-cell damage [65]. Notably, metformin, the currently recommended medication for insulin resistance [84, 85], appears to have some similar activities to TEMPOL by targeting the activation of AMP-activated protein kinase (AMPK) protein to attenuate ER stress markers. It should be noted, however, that some of the markers and methods used to measure oxidant generation, oxidative damage and antioxidant levels in these studies are not ideal with the value of some of these assays subjected to criticism [86, 87]. Thus, there appears to be a need for further research, using state-of-the-art and validated assays to examine TEMPOL's activities, particularly in complex systems. In addition, measurements of the concentration of TEMPOL in the systems under study would be of considerable value in terms of assessing its effects, particularly whether they achieve the high concentrations required to act as a direct radical scavenger. If only low concentrations are present, it is more likely to act via alternative pathways, such as via enzyme inhibition (including those that may generate oxidants, see earlier), via receptor interactions, or modulation of signalling pathways.

In addition to its potential antioxidant effects, TEMPOL has also demonstrated significant anti-inflammatory properties in both *in vitro* and *in vivo* models of T2DM. In *in vitro* models,

TEMPOL reduced factors associated with suppression of the inflammatory response as a reduction in the inhibitor of NFκB, IκB, whilst also reducing insulin resistance as indicated with a decrease in Ser(P)^{473}-IRS1 [37-39]. In *in vivo* models, TEMPOL administration appears to dampen the cellular inflammatory response as indicated by a decrease in proinflammatory cytokines (IL8, TNF and IL-6), reduced cellular stress response as indicated by the attenuation of MAPK [51, 64] and suppression of oxidative stress as indicated by a reduction in superoxide radical anion generation and lipid peroxidation levels [52, 53, 56]. These data on TEMPOL's effects on oxidative stress and β-cell damage, combined with its potential to modulate inflammatory pathways and reduce insulin resistance, highlights its promising role as a multifaceted therapeutic approach in reducing and managing T2DM.

The effectiveness of TEMPOL as a multifaceted therapeutic approach in the reduction and management of T2DM is further evidenced by its impact on high-fat diets and obesity, as the accumulation of fat depots, particularly in obese individuals, is known to contribute to inflammation. In eleven studies exploring TEMPOL's effects on various types of high-fat diets resulting in obesity, it was consistently demonstrated that TEMPOL effectively mitigates the increased oxidative stress and inflammatory response induced by such diets. Two of these studies highlighted TEMPOL's ability to decrease pro-inflammatory markers, including NF-κB, SAA, IL-6, IL-8, TNF-α, and the oxidant-generating heme enzyme myeloperoxidase (MPO) [30, 39]. Moreover, chronic hyperlipidaemia triggers a synergistic relationship between inflammation and oxidative stress, as suggested by three studies showing that TEMPOL's anti-inflammatory activities are associated with a decrease in NAD(P)H oxidase activity, iNOS expression and superoxide radical generations [48, 53, 55], while three additional studies highlight TEMPOL's capacity to decrease oxidant levels in murine high-fat diet models, by exhibiting a potent superoxide radical scavenging ability akin to that of the endogenous superoxide dismutase enzymes (SODs) [44, 48, 58] Subsequently, TEMPOL has been shown to attenuate the increase in body mass and liver weight induced by high-fat diets [30, 43, 55, 59]. Therefore, there is potential for TEMPOL as a therapeutic treatment for obesity, given its anti-inflammatory and antioxidant effects.

In addition to the use of TEMPOL for the treatment of obesity and T2DM, the compound also shows significant potential for use to help with hepatic dysfunction. We identified ten studies that showed TEMPOL inhibits hepatic steatosis, leveraging its anti-inflammatory and

antioxidant properties to target the underlying pathologies of NAFLD. Moreover, TEMPOL counteracted hepatic fibrosis induced by oxidative stress in hepatic stellate cells [69]. Eguchi et al., 2015 used novel redox nanoparticles (RNPs) incorporating TEMPOL and showed an attenuated leukocyte increase and oxidative stress with a marked reduction in liver fibrosis in a NASH-specific mouse [47]. Pinar et al., 2018 also demonstrated TEMPOL's efficacy in mitigating oxidative stress and inflammatory mediators induced by methotrexate, thus preventing the development of hepatic steatosis and cirrhosis [47, 73]. Overall, the reviewed studies consistently demonstrate TEMPOL's capacity to scavenge superoxide radicals, reducing ROS production and modulation of the inflammatory response. These effects ultimately improve hepatic function.

Before considering further clinical applications of TEMPOL, specifically to target obesity, T2DM, and NAFLD, it will be necessary to assess the toxicity of TEMPOL in pre-clinical and pilot clinical studies. It is likely that TEMPOL will be safe to use as numerous *in vivo* studies confirm TEMPOL's safety and efficacy profile as an antioxidant. For example, in macular surgery, the dye indocyanine green (ICG) is toxic to retinal cells. TEMPOL mitigated the ICG-induced retinal damage and showed no toxicity at concentrations up to 1 mM [88, 89]. Furthermore, TEMPOL at a dosage of 50 mg/kg has demonstrated efficacy in treating neuronal death in permanent cerebral ischemia. Studies in mice with permanent middle cerebral artery occlusion revealed substantial motor and behavioural improvement, along with reduced injured tissue volumes in both short and long-term stroke assessments [90]. TEMPOL has been also studied for its protective effects against radiation-associated toxicities such as xerostomia and alopecia. A topical formulation of TEMPOL is currently in phase IIB clinical trial as a protection against radiation-induced alopecia [91]. Moreover, TEMPOL has garnered attention in the context of COVID-19, with clinical trials investigating its potential therapeutic role in managing the disease. Trials have explored TEMPOL's effects against viral infections [92, 93]. These COVID-19 trials add a timely dimension to TEMPOL’s research landscape, underscoring its versatility and potential relevance in addressing emerging health challenges.

There are several limitations inherent in our scoping review. Firstly, our search method relied solely on electronic databases, potentially excluding relevant studies from manual searches and grey literature. Additionally, a substantial number of studies meeting the criteria for T2DM, and obesity had to be excluded due to not meeting the specified timeframe, which was implemented to ensure the most recent research was captured. Finally, the results focus

primarily on animals. models and do not include human studies as at the time of study capture, there were no clinical trials to report on. In addition, some of the techniques employed do not reflect the state-of-the-art with regard to the measurement of oxidant production and damage.

CONCLUSIONS

This review sheds light on the potential of TEMPOL as a therapeutic intervention for obesity and T2DM. Antioxidant and anti-inflammatory properties of TEMPOL have been consistently reported in various *in vivo* and *in vitro* models, showcasing its ability to attenuate inflammation, oxidative stress, and pathologies associated with high-fat diets and NAFLD. However, to translate these promising findings into clinical applications, it is crucial to further investigate TEMPOL's effects, especially in insulin-sensitive tissues. Understanding its mechanisms of action will provide valuable insights for future clinical trials and the development of targeted interventions. Nevertheless, the reduction in oxidative stress and inflammation observed in obesity, T2DM and NAFLD models does emphasize a need for continued research to fully explore TEMPOL's therapeutic potential. By addressing the existing gaps in knowledge and conducting rigorous investigations, TEMPOL may emerge as a valuable tool in managing and treating obesity, T2DM and its associated pathologies.

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Chapter 4 : Exploring the therapeutic potential of Tempol: Reducing insulin resistance in C57BL/6 mice via enhanced lipid metabolism, adiposity reduction and adipose tissue inflammation suppression.

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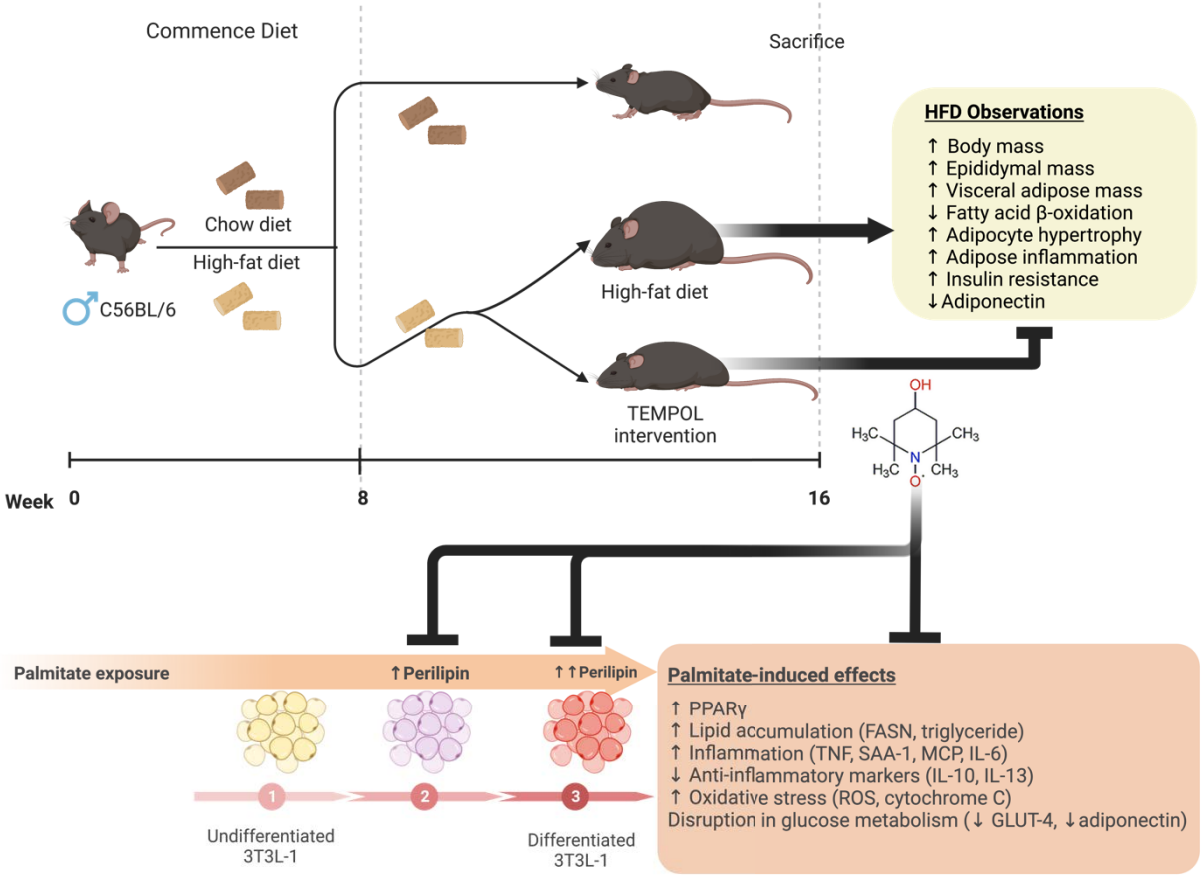
Chapter Summary:

Obesity is reaching epidemic proportions worldwide, and its management is challenging due to increased obesity-related risk factors such as insulin resistance. A pharmacological agent that focuses not solely on body mass loss but on improving and targeting obesity-led lipid inflammation and mitochondrial dysfunction is in great need. Given that TEMPOL has anti-inflammatory, anti-oxidative, and anti-obesity properties, this chapter evaluates TEMPOL's ability to inhibit lipid accumulation in an *in vitro* model of palmitic acid-treated 3T3L-1 adipocytes and HFD-fed C57BL/6 *in vivo* model. This chapter has been written in accordance with the guidelines of the journal, Obesity, to which this manuscript will be submitted.

GRAPHICAL ABTRACT



Exploring the therapeutic potential of TEMPOL: Reducing insulin resistance in C57BL/6 mice via enhanced lipid metabolism, adiposity reduction and adipose tissue inflammation suppression

Gihani Manodara [a], Michael J. Davies [b], Alison K Heather [c], David Van Reyk [a], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand*

Author Contribution

For this paper, the PhD candidate, GM, completed the majority of the experiments, data analysis and interpretation and wrote the manuscript. MD & DR contributed to manuscript editing. KM supervised GM and contributed to data acquisition. AH and KM conceptualised the study, helped with data analysis and interpretation, and edited the manuscript. All authors contributed to the execution and approved the final version of the manuscript.

Gihani Manodara	Production Note: Signature removed prior to publication.
David Van Reyk	Production Note: Signature removed prior to publication.
Michael J. Davies	Production Note: Signature removed prior to publication.
Alison K. Heather	Production Note: Signature removed prior to publication.
Kristine C. McGrath	Production Note: Signature removed prior to publication.

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Gihani Manodara [a], Michael J. Davies [b], Alison K. Heather [c], David Van Reyk [a], Kristine C. McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand*

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Corresponding Author:

Dr Kristine McGrath
University of Technology Sydney
PO Box 123, Broadway
Ultimo, NSW, 2007, Australia
Ph: 612 9514 9773
Email: kristine.mcgrath@uts.edu.au

Abstract

Obesity is an expensive epidemic disease that affects 13% of the adult population worldwide. Obesity is linked to adipose tissue hypertrophy and hyperplasia, disrupted lipid metabolism, and pathologic endocrine changes in adipose tissue, such as local and chronic systemic low-grade inflammation. Furthermore, this inflammatory state is a risk factor for metabolic syndrome and insulin resistance. TEMPOL, a nitroxide radical, has been shown to have anti-inflammatory, anti-oxidative, and anti-obesity properties. Administration of TEMPOL, to mice fed a high-fat diet has been previously reported to prevent excessive body mass gain. Based on these observed fat-sparing effects, we aimed to assess the capacity of TEMPOL to block lipid accumulation induced by palmitic acid (PA) in an *in vitro* model (adipocyte 3T3L-1 cells) and an *in vivo* high-fat diet mouse (C57BL/6) model. The data obtained indicate that TEMPOL supplementation of the high-fat diet mice reduced (when compared to non-treated mice) the increase in body mass, with associated alterations in lipid metabolism, reduced adipogenesis markers, inflammatory cytokine gene expressions and mitochondrial oxidative stress, and improved insulin sensitivity. Data from the *in vitro* cell model showed that TEMPOL reduced expressions of adipogenesis markers, diminished oxidative stress and expression of inflammatory markers, and decreased lipogenesis and lipolysis. These findings suggest that TEMPOL plays a preventative role in weight gain accompanied by the suppression of adipose inflammation and oxidative stress, indicating that TEMPOL may have potential as an anti-obesity drug.

Introduction

Lifestyle changes, including overnutrition and reduced physical activity, contribute to the increasing incidence of obesity worldwide. Associated with the rise in obesity levels, there is an increased prevalence of metabolic diseases, including hypertension, cardiovascular disease, type 2 diabetes mellitus (T2DM) and non-alcoholic liver disease (NAFLD) [1]. The first line of therapy to treat the early stages of such metabolic diseases includes changes such as increasing physical activity and improved diet [2]. However, adherence to these changes is poor and pharmacological or surgical interventions (e.g., bariatric surgery) are often required to reduce or maintain a loss of body mass [2]. Pharmacological therapy generally targets fat absorption or appetite suppression [3-5].

Adipocyte differentiation involves an integrated signalling cascade coordinated by peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT- enhancer binding protein (C/EBP) [6]. PPARγ- and C/EBP regulate the expression or activity of a number of proteins involved in lipid homeostasis, triglyceride (TG) accumulation and lipolysis [6-8]. PPARγ also plays a critical role in carbohydrate metabolism, with anti-diabetic drugs such as thiazolidinediones (TZD) acting as synthetic PPARγ ligands to improve insulin sensitivity and reduce hyperglycaemia [9].

Obesity has been linked with dysregulated redox signalling in adipocytes, whereby exposure to excess free fatty acids (FFA) leads to the overproduction of oxidants (commonly referred to as reactive oxygen species, ROS), including superoxides radical anion ($O_2^{\cdot-}$) [10, 11]. High oxidant levels have been reported to disrupt adipocyte differentiation by downregulating PPARγ expression [12, 13]. Excess FFA also promotes the release of adipokines, such as monocyte chemoattractant protein- 1 (MCP-1) and tumour necrosis factor (TNF) [10, 14, 15], both of which activate an adipocyte inflammatory response. Together, the oxidative stress and inflammatory responses drive dysregulated insulin-signalling in this cell type.

TEMPOL (4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl), a nitroxide radical with reported antioxidant properties against oxidative damage induced by hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$, and peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH) [16, 17]. Previous mouse studies have demonstrated that TEMPOL treatment blocked body mass gain when animals were fed a high-fat diet (HFD) [18, 19]. Follow-up studies demonstrated that TEMPOL decreases obesity and

insulin resistance by altering the intestinal microbiota, which changes bile acid composition and inhibits the farnesoid X receptor (FXR) signalling [20, 21]. In HFD mouse models, TEMPOL's ability to manipulate FXR signalling is one mechanism that may improve obesity, although the underlying mechanism of FXR signalling is not well understood. Given the reported involvement of elevated oxidant levels (ROS) in the dysregulation of insulin signalling in adipocytes, we hypothesized that TEMPOL treatment might improve insulin sensitivity in a mouse model of a high-fat-fed diet via its ability to reduce the oxidative stress and inflammatory responses associated with lipid accumulation. These TEMPOL-mediated effects were expected to correlate with reduced body mass gain.

Materials and methods

Animals

Four-week-old male C57BL/6 mice (Australian BioResources, MossVale, NSW, Australia) were housed in temperature-controlled and circadian rhythm-adjusted rooms. They were fed standard chow (StD) and water *ad libitum* until eight weeks of age when the mice were randomly divided into two groups to receive StD (n = 20, 13.1% kCal fat, Biological Associate; Envigo Teklad) or a high-fat diet (n = 40; HFD, 52.4% kCal fat, Biological Associate; Envigo Teklad) enriched with 8.7% palmitic acid and 26% trans-fats. The StD group was then further subdivided, with one subset continuing the StD chow (n = 10) and another fed StD supplemented with 10 mg/kg of TEMPOL (n = 10; StDT). Similarly, the HFD group was subdivided into (i) 10 continuing HFD, (ii) diet switched to StD (n = 10; HFDStD), (iii) StD with TEMPOL (n = 10; HFDStDT) and (iv) HFD with TEMPOL (n = 10; HFDT). All diets continued for a further eight weeks. The diet containing TEMPOL was made using the cold-pressed method [22].

Mice were weighed weekly. At weeks 14 and 15, an intraperitoneal glucose tolerance test (ipGTT) or intraperitoneal insulin tolerance test (ipITT) was performed, as described [23]. At week 16, mice were euthanized, and visceral adipose tissues (VAT) were harvested and stored at -80◦C. All experiments were approved by the University of Technology Sydney Animal Care and Ethics Committee, Sydney, NSW, Australia (Ethics approval number: UTS ACEC

ETH16-0455) and conducted according to guidelines described by the Australian National Health and Medical Research Council Code of Conduct of Animals.

Palmitate preparation, 3T3-L1 cell culture and treatment

Albumin bound-palmitic acid (PA; 20 mM) was prepared by dissolving the palmitic acid powder (Sigma Aldrich; MO, USA) in Dulbecco's Modified Eagle's Medium (DMEM ThermoFisher; CA, USA) containing 1 % w/v free fatty-acid free bovine serum albumin (BSA) and 150 mM NaCl at 70 ºC.

Murine 3T3-L1 fibroblast cells (ATCC# CL-173) were cultured in basal medium comprising DMEM, glucose (4.5 g L^{-1}), 10% v/v fetal bovine serum (FBS; Scientifix, USA) and 5% penicillin-streptomycin (ThermoFisher, CA, USA). The 3T3-L1 cells were seeded in 6-well plates at a density of 2 x 10^5 cells mL^{-1}. The cells were cultured until 80% confluent (day 0; "D-I") before differentiation ("D-II") was initiated by culturing the cells for 3 days with basal medium supplemented with 1 μM dexamethasone (Sigma Aldrich; MO, USA), 0.5 mM of 3-isobutyl-1- methylxanthine (IBMX; Sigma Aldrich; MO, USA) and 5 μg mL^{-1} of insulin. The next stage of differentiation (D-III) commenced on day 4, where the basal medium was supplemented only with insulin (5 μg mL^{-1}). On day 6 (D-IV), the media was changed to basal DMEM until lipid droplets were visible under a light microscope. The matured 3T3-L1 adipocytes (fully differentiated stage, DF) were exposed to 0.5 mM PA for 24 h before treatment with TEMPOL (250, 500 or 1000 μM).

Quantitative and qualitative analysis of lipid levels in matured 3T3L-1 adipocytes.

Oil Red O staining

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) before being fixed with 10% w/v neutral formalin for 30 min. The cells were then rinsed with PBS, 70% v/v ethanol and stained with 10% v/v Oil-Red-O (ORO) stain for 45-60 min. Cells were washed with PBS before being counterstained with hematoxylin for 10 min before being visualized with light microscopy (x20 magnification; Omax, USA). To quantify the staining, the cells were washed twice with PBS, then 70% v/v ethanol before extraction with 4% v/v non-idet P-40 (Sigma Aldrich; MO, USA). Extracts were measured by absorbance at 520 nm (Tecan i-control infinite 200 Pro, Tecan Switzerland).

Triglyceride assay.

Triglyceride (TG) levels were measured using a commercially available kit as per the manufacturer's protocol (Abcam; ab65336; MA, USA).

Cellular metabolic activity assay

Thiazolyl blue tetrazolium bromide powder (Sigma Aldrich; MO, USA) was dissolved in DMSO to generate the MTT solution (5 mg mL^{-1}). 20 µL MTT solution was added to each well of cultured adipocytes and incubated for 3-4 h before the media was replaced with 200 µL of DMSO to extract and dissolve the formazan product. This was quantified by its absorbance at 570 nm (Tecan i-control infinite 200 Pro, Tecan Switzerland).

Inflammatory gene expression measurements

Total RNA (1000 µg) from mature 3T3L-1 cells and visceral adipose tissue (VAT) was extracted using TRI-sure reagent (Bioline; Meridian Biosciences, OH, USA). Extracted RNA was normalized to 200 ng μL^{-1} (Nanodrop Technologies, USA), and first-strand cDNA was synthesized (Tetro cDNA synthesis kit, Bioline; Meridian Biosciences, OH, USA). For 3T3L-1 cells, the SensiFAST SYBR No-ROX kit (Bioline; Meridian Biosciences, OH, USA) with 20 pmol primer pairs (**Table S1**) was used in a Bio-Rad thermocycler (Bio-Rad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to determine the change in mRNA gene expression levels relative to those of the reference gene, 18S [24]. For VAT, QX200 Bio-Rad digital droplet PCR (ddPCR) was used with 100 µM oligonucleotide primer pairs in single-plex reactions (Bio-Rad, CA, USA).

Immunoblot analysis.

Whole-cell protein lysates were extracted from 3T3L-1 cells and VAT from mice using radioimmunoprecipitation assay (RIPA) buffer as previously described previously [25]. Protein lysates (20 µg) were heat denatured for 5 min before being separated using SDS-PAGE and then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA, USA). Membranes were blocked in 1x tris-buffered saline with Tween (TBST) buffer containing 5% (w/v) non-fat milk for 1 h before being exposed overnight at 4 ºC to 1:1000 dilution of primary antibody for fatty acid synthetase (FASN), PPARγ and perilipin-1 (Cell signalling, Gene Search; **Table S2**). Membranes were then washed three times with 1xTBST (5 min each) before incubating with anti-rabbit IgG (1:1000) conjugated to horseradish

peroxidase for 2 h at room temperature (Cell signalling, Gene Search; **Table S2**). Proteins were then visualized using a Chemiluminescent HRP Substrate reagent (BioRad, CA, USA) and a BioRad Chemi-Doc imaging system (Chemi-Doc MP #12003154 BIO-RAD, CA USA). Densitometry was performed using Image J software (https://imagej.nih.gov/ij/). Results obtained were normalized to β-actin protein bands.

Detection of reactive oxygen species

Intracellular oxidant levels were measured using the fluorogen 2′, 7′-dichlorodihydrofluorescein diacetates. Dichlorodihydrofluorescein diacetate (DCFH-DA) assay detects total ROS in adherent cells including hydroxyl radicals and nitrogen dioxide, there is no specific species but an overall ROS species. Following the treatment of 3T3L-1 cells, media was aspirated, and wells were washed twice with 100 µL PBS. Next, 10 µM DCFH-DA solution (100 µL well^{-1}) was added, and plates were covered by aluminium foil and incubated for 15 min at 37 ºC. DCFH-DA solution was aspirated, and cells were washed twice with PBS (100 µL well^{-1}). Oxidant levels were determined by fluorescence intensity at λ_{ex} 488 nm and λ_{em} 520 nm (Tecan i-control infinite 200 Pro, Tecan Switzerland).

Mitochondrial Oxidative Stress: cytochrome c assay.

Cytochrome c was measured using the cytochrome c oxidase assay kit as per the manufacturer's protocol (Abcam, ab 65311, MA, USA).

Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA with Bonferroni's *post-hoc* correction test using GraphPad PRISM software was used to determine statistical significance, with p-value < 0.05 was considered significant.

Results

In vivo experiments

TEMPOL is as effective as dietary intervention in reducing body mass and decreasing VAT in mice

The changes in mouse body mass, epididymal fat and visceral adipose fat content during the 16-week study are shown in **Table 1**. Food intake was not significantly reduced upon intervention with TEMPOL (Table 1), with the average food intake difference between weeks 0-8 and weeks 9-16 being 15.53 g for HFDStDT and 13.4 g for the HFDT group compared to the HFD group (17.56 g) and StD control group (17.39 g). Table 1 and Fig. 1A show that the HFD-fed mice increased body mass by 4.5% ($p < 0.05$) compared to StD mice. TEMPOL reduced body mass in HFDStDT by 13% and HFDT by 24% ($p < 0.05$), compared to the HFD group. Significantly, HFDStDT and HFDT led to a substantial reversal of body mass gain by 41.2% and 67.5%, respectively ($p < 0.05$), reaching a level measured for StD control group. This is unlikely to be due to a toxicity effect, as the StDT group maintained the same body mass as the StD group.

The epididymal tissue and VAT mass corresponded with body mass change (Fig.1 B, C). HFD mice had higher epididymal and VAT mass when compared to the StD group. TEMPOL decreased epididymal mass by ~ 54% in the HFDStDT group, and ~ 63% in the HFDT group ($p < 0.001$ vs HFD), with this decrease being consistent with the reduced levels achieved by dietary intervention alone (i.e., the HFDStD). Similarly, TEMPOL significantly decreased VAT mass by ~ 40% in HFDStDT ($p < 0.05$) and ~ 63% in HFDT ($p < 0.001$) compared to the HFD group. Moreover, TEMPOL supplementation effectively reverted the VAT and epididymal mass to a level comparable to those on the StD diet.

Table 1: Anthropometry of mice after 16 weeks of HFD *in vivo* murine model.

	StD	**StDT**	**HFD**	**HFDStD**	**HFDStDT**	**HFDT**
Body mass change (g)	8. 0 ± 1.0	4.7 ± 0.4*	10.6 ± 0.8	7.1 ±1.7$^{\#}$	4.7 ± 0.3$^{*\#}$	2.6 ± 0.5$^{*\#}$
Body mass change (%)	32 . 0 ± 3.8	18.8 ± 1.6 *	44.3 ± 3.2*	29.0 ± 2.2$^{\#}$	18.8 ± 1.1$^{*\#}$	10.7 ± 2.7$^{*\#}$
Food intake (g)	17.4 ± 0.9	16.0 ± 0.3	17.6 ± 1.5	22.4 ± 1.7$^{*\#}$	15.5 ± 0.6	13.4 ± 0.9
Epididymal fat (g)	1.0 ± 0.2	0.6 ± 0.04	1.5 ± 0.1	0.8 ± 0.1$^{\#}$	0.7 ± 0.04$^{\#}$	0.6 ± 0.05$^{\#}$
Epididymal fat (%)	103 ± 13.1	59.6 ± 4.0*	127.9 ± 17.7*	75.9 ± 9.3$^{\#}$	70.2 ± 3.8$^{\#}$	53.7 ± 7.3$^{*\#}$
Visceral adipose fat (g)	0.5 ± 0.1	0.3 ± 0.03*	0.5 ± 0.04	0.3 ± 0.04*	0.3 ± 0.02	0.2 ± 0.01$^{*\#}$
Visceral adipose fat (%)	101.3 ± 12.1	56.3 ± 5.8*	103.8 ± 7.9	62.7 ± 8.9$^{*\#}$	62.8 ± 5.1$^{*\#}$	38.3 ± 3.1$^{*\#}$

Data are presented as means ± SEM, * $p < 0.05$ Vs StD, # $p < 0.05$ Vs HFD (n = 9-10).

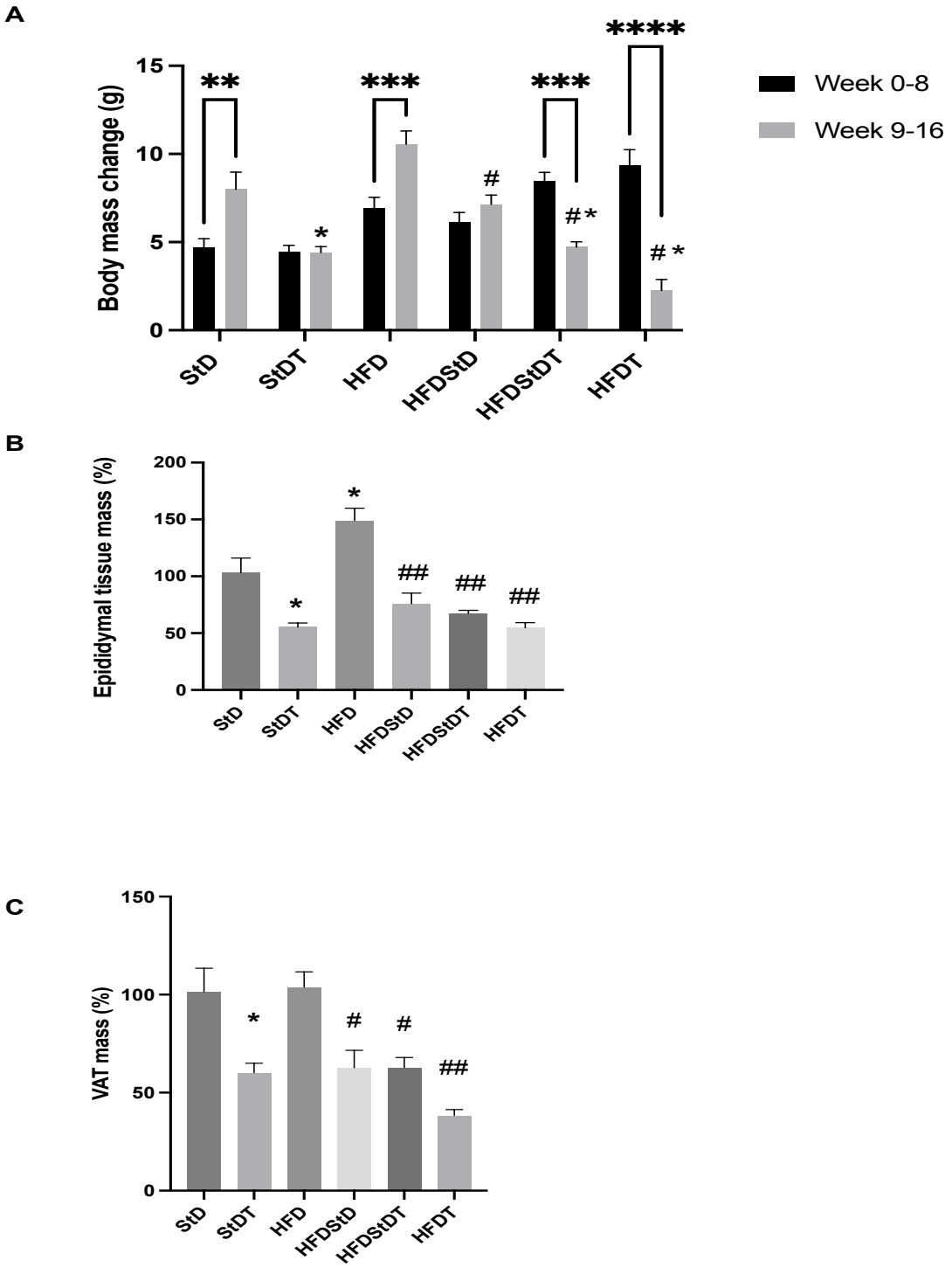


Figure 1: TEMPOL reduces body mass and tissue weight of high fat-fed mice.

(A) Body mass measured from weeks 0-8 and 9-16 after the diet intervention with TEMPOL supplementation (n= 9-10; ** $p < 0.05$; *** $p < 0.001$, **** $p < 0.0001$). Once completion of the 16-week study (B) epididymal tissue and (C) VAT mass were weighed. Data are presented as means ± SEM. * $p < 0.05$ & ** $p < 0.001$ vs StD group and # $p < 0.05$ & ## $p < 0.001$ vs HFD group.

TEMPOL is as effective as dietary intervention in modulating key mediators involved in lipogenesis, lipolysis and fatty acid β-oxidation in VAT.

To attempt to understand the mechanism by which TEMPOL uses to reduce body mass and VAT, we investigated its effect on the mRNA levels of key regulators of lipogenesis, lipolysis and fatty acid β-oxidation in VAT extracted from the mice. The expression of sterol regulatory binding protein 1c (SREBP-1c), signal transducer beta-2-adrenergic receptor (ADRB2), fatty acid synthase (FASN), lipoprotein lipase (LPL), acetyl-CoA carboxylase beta (ACAB) and proliferator-activated receptor (PPAR δ) were quantified, together with plasma triglyceride levels.

SREBP-1c is a transcription factor that induces lipogenic target genes, including ADRB2 [26]. The HFD resulted in a significant increase in SREBP-1c compared to StD ($p < 0.05$; Fig. 2AI). However, a significant decrease in SREBP-1c expression was measured with TEMPOL supplementation of the HFD (HFDT) by 39% ($p < 0.05$) when compared to the standard diet intervention (HFDStD). HFD diet increased ADRB2 mRNA levels by 77% relative to the StD group ($p < 0.05$) (Fig. 2A II). Whilst there was a significant difference between the HFD and StD group ($p < 0.05$), TEMPOL supplementation showed reduction. Interrogation of the data suggests only a trend towards reduction of HFD-induced ADRB2 mRNA levels by 96% in HFDStDT and 77% in HFDT. This is in keeping with SREBP-1C activation, however, more would be required to fully define the mechanism.

ACAB encodes the malonyl-CoA enzyme, an allosteric inhibitor of the fatty acid ß-oxidation [27]. FASN encodes the enzyme fatty acid synthase which catalyses the conversion of malonyl CoA to saturated fatty acid, palmitate [28]. HFD induced a trend in the increase of ACAB by 48% and FASN mRNA levels by 23% compared to the StD group (Fig. 2A: III, IV). TEMPOL supplementation decreased ACAB significantly (by 47% HFDStDT and 22% HFDT; $p < 0.001$) and FASN (by 55% HFDStDT; $p < 0.05$, and 31% HFDT) compared to HFD.

These mRNA analyses suggested that TEMPOL downregulates the expression of key enzymes involved in lipogenesis. Plasma TG levels, therefore, were measured to assess if this correlates with phenotypic changes (Fig. 2AV). HFD increased TG content by 48%. Compared to the StD group, TEMPOL decreased plasma TG levels by 34% in HFDStDT and 45% in HFDT compared to the HFD group. This reduction is consistent with that seen with the dietary switch

intervention (HFDStD; $p < 0.001$ vs HFD). TEMPOL reduced plasma TG in StDT chow-fed mice by 65% compared to the StD group. Overall TEMPOL's supplementation showed a discernible trend in reducing lipogenesis regulators to levels measured for the StD group.

Given the positive effects (decreases) of TEMPOL on lipogenesis, we next explored for effects on lipolysis. Lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4) were examined as LPL is a rate-limiting enzyme catalysing the hydrolysis of TG [29] and FABP4 is a lipid chaperon that blocks inhibitors of lipolytic enzymes, thus an increase in FABP4 increases lipolysis [30]. We showed that HFD induced an increase in LPL by 64% ($p < 0.001$; Fig. 2BI) and FABP4 mRNA levels by 90% compared to the StD group ($p < 0.05$; Fig. 2BII). TEMPOL reduced LPL (by 63% for HFDStDT and 94% for HFDT; $p < 0.05$) and FABP4 (by 26% for HFDStDT and by 75% for HFDT; $p < 0.05$) compared to the HFD group. The StDT groups also showed a trend in decreasing LPL by 81% and FABP4 by 51% compared to the HFD group.

Given the positive decrease of ACAB expressions, the effect of TEMPOL on fatty acid ß-oxidation was also quantified. PPAR δ promotes fatty acid ß-oxidation by positively regulating the gene expression in this pathway. Fig. 2C shows the trend in decrease by 86% of PPAR δ with HFD compared to the StD chow. TEMPOL significantly increased PPAR δ in the HFDStDT group ($p < 0.05$) and a trend in increase in the HFDT group compared to the HFD group. The increase measured with TEMPOL was similar to that of dietary intervention (HFDStD).

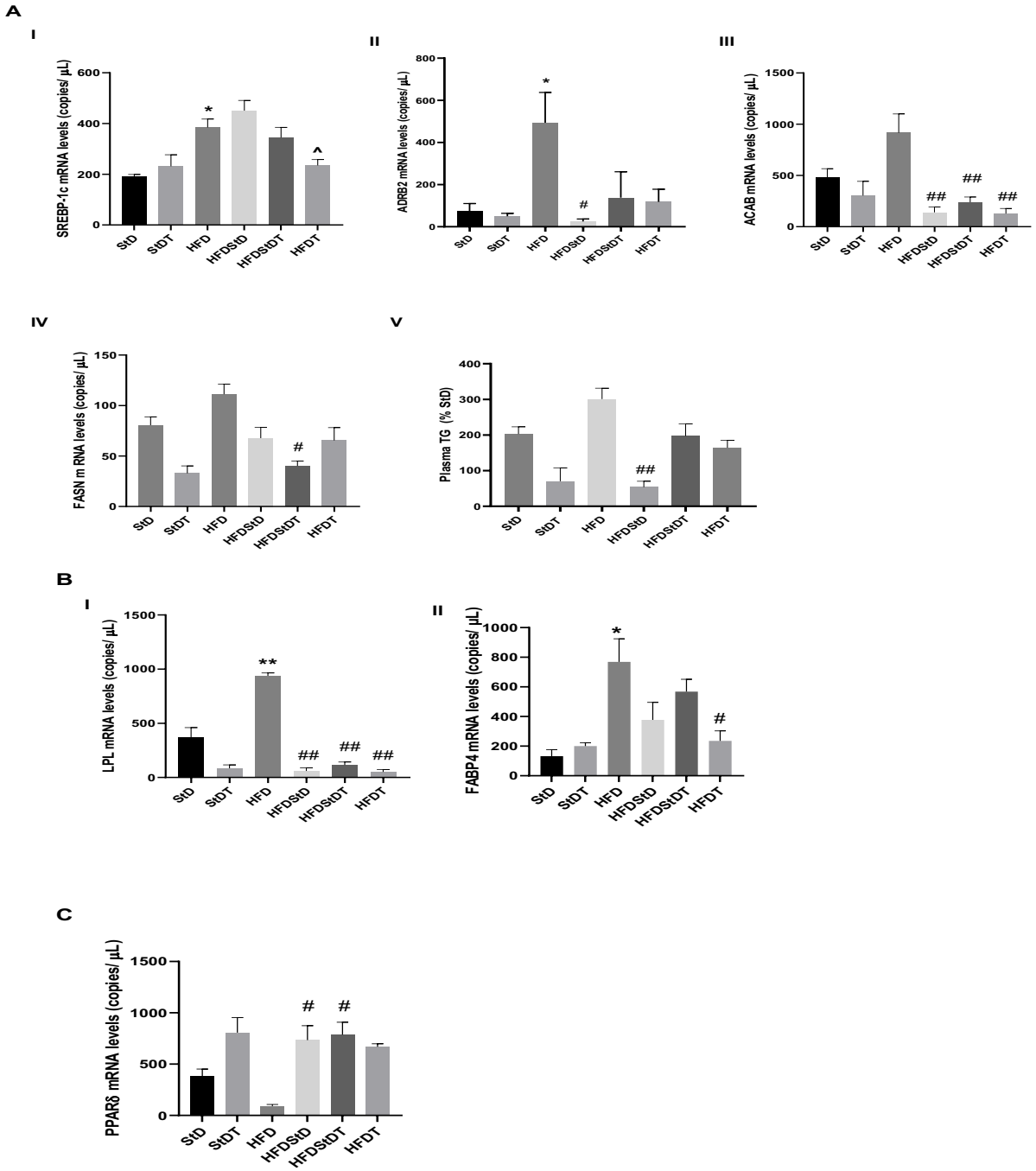


Figure 2: TEMPOL decreased lipid accumulation and facilitated key regulators in lipid metabolism HFD *in vivo* model.

TEMPOL reduced HFD induced (A I-V) lipogenesis and (B) lipolysis in VAT, while TEMPOL increased (C) fatty acid β-oxidation. (A) Digital droplet qPCR (dd PCR) analysis of (I) SREBP-1c, (II) ADRB2 (III) ACAB, (IV) FASN mRNA expressions in VAT and (V) plasma TG levels measured. (B) Lipolytic activity was assessed by quantifying (I) LPL and (II) FABP4 gene expression in VAT. (C) dd-qPCR analysis of PPARδ gene expression showed that TEMPOL supplementation had an impact on fatty acid β-oxidation. Data are presented as mean ± SEM, (n = 9-10). * $p < 0.05$ & ** $p < 0.001$ vs StD group. # $p < 0.05$ & ## $p < 0.001$ vs HFD group.

The effect of TEMPOL on adipogenesis is similar to that of dietary intervention

Excessive lipid accumulation promotes an increase in new adipocytes (hyperplasia, also known as adipogenesis) [31]. To understand if TEMPOL supplementation suppresses adipogenesis, the expression of two key regulators of adipogenesis regulators, CCAAT enhancer binding protein α (C/EBPα) and fibroblast growth factor 10 (FGF10) were measured. C/EBPα induces adipocyte genes directly to initiate adipogenesis, and FGF10 is a structural protein secreted in adipose tissues during the early stages of adipogenesis [32, 33]. Adipocyte cell size was also examined. Fig. 3A I & II shows that HFD induces mRNA levels of C/EBPα by ~ 75% ($p < 0.05$) and FGF10 by ~ 89% ($p < 0.001$) compared to the StD group. TEMPOL showed a trend in decreased C/EBPα by 19% HFDStDT and by 61% in the HFDT group ($p < 0.05$ vs HFD). Similarly, TEMPOL decreased FGF10 significantly by 81% HFDStDT ($p < 0.001$) and by 84% HFDT ($p < 0.001$). These decreases exceed that of dietary intervention alone (HFDStD). Histological analysis of VAT showed increased adipocyte cell size by (91%, $p < 0.05$) for the HFD group, compared to the StD (Fig. 3B, C). TEMPOL supplementation decreased cell size by 14% in HFDT ($p < 0.05$) compared to the HFD and was more effective than HFDStD (by ~ 26%) and equally effective as the StD group. TEMPOL supplementation alone (StDT) showed no decrease in adipocyte cell size relative to the StD group.

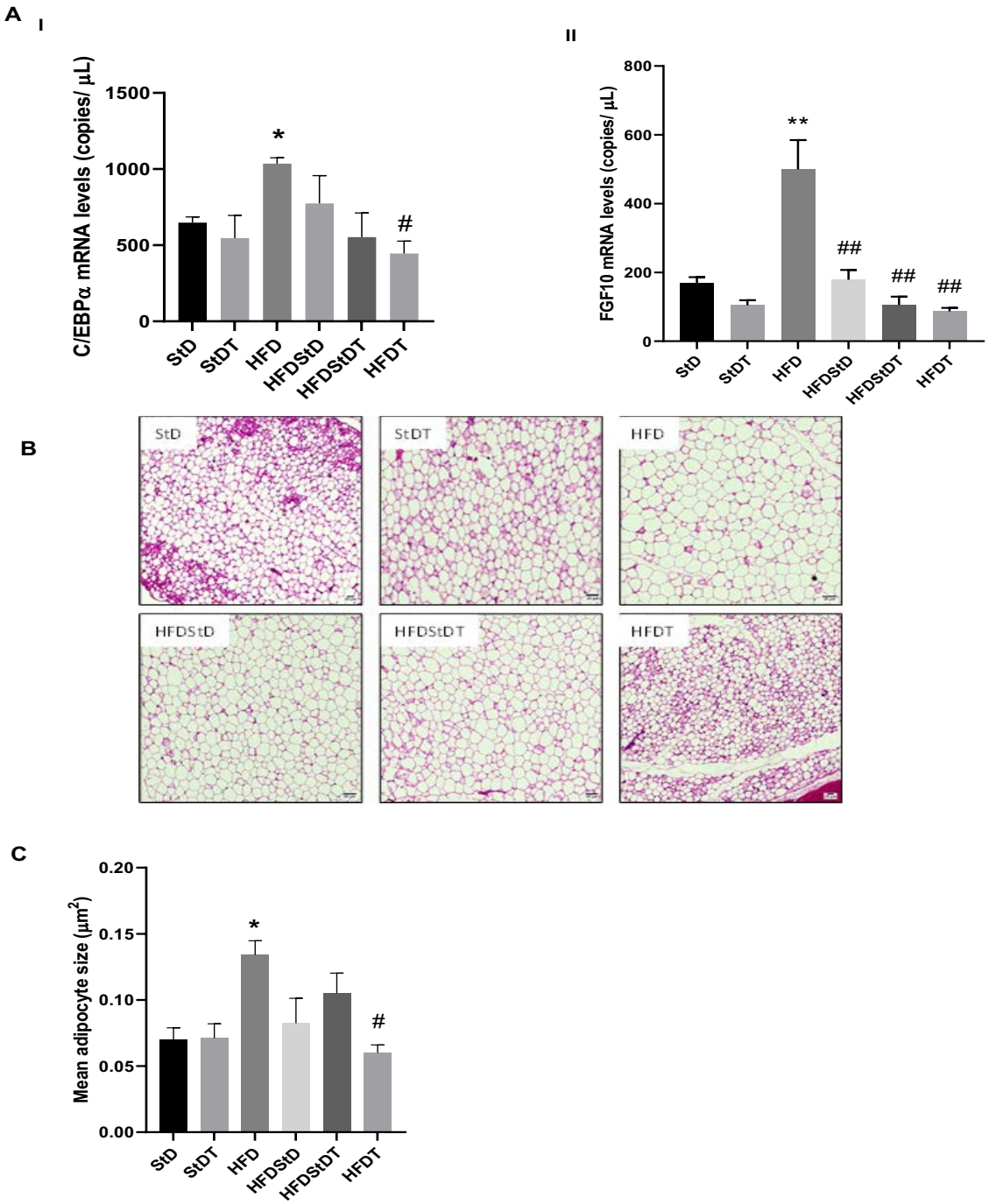


Figure 3: TEMPOL reduces adipocyte hypertrophy and adipogenesis by decreasing the expression of differentiation genes in HFD in vivo model.

(A) To indicate the TEMPOL effect in adipogenesis ddPCR analysis was done to measure mRNA levels of (I) CEBPα and (II) FGF10 from VAT. (B) To assess the effect of dietary TEMPOL supplementation on adipocyte size, histological assessment was done on VAT stained with hematoxylin and eosin (H&E) (Scale bar: 20 µm). (C) Mean adipocyte cell size in VAT. *In vivo,* data are presented as means ± SEM (n = 9-10). * $p < 0.05$, ** $p < 0.001$ vs StD group. # $p < 0.05$, ## $p < 0.001$ vs HFD group.

The effect of TEMPOL on visceral inflammation is similar to diet intervention

Lipid accumulation in adipose tissue induces an inflammatory response in obese patients [34]. As TEMPOL reduced lipid accumulation in HFD VAT, potential suppression of the inflammatory response by TEMPOL was examined by measuring resistin and peroxisome proliferator-activated receptor α (PPARα) levels. Resistin is an adipose-secreted circulatory protein whose levels shown to correlate with systemic inflammation [35]. Fig. 4A shows a trend in HFD increasing resistin mRNA levels by 27% compared to the StD group. TEMPOL supplementation showed a trend in reducing resistin levels by 8% in HFDT and 72% with HFDStDT ($p < 0.001$) and was more effective than the standard diet intervention (HFDStD) by ~ 50%. TEMPOL (StDT) also presented a trend in reducing resistin levels by 64% compared to the StD group. PPARα inhibits the inflammatory response and is suppressed by HFD compared to the StD group ($p < 0.001$; Fig. 4B) [36, 37]. Fig. 4B shows that TEMPOL significantly increased PPARα mRNA levels by 126% in HFDT and by 188% in HFDStDT relative to the HFD group ($p < 0.05$). This significant increase was greater than that generated by the standard diet intervention (HFDStD).

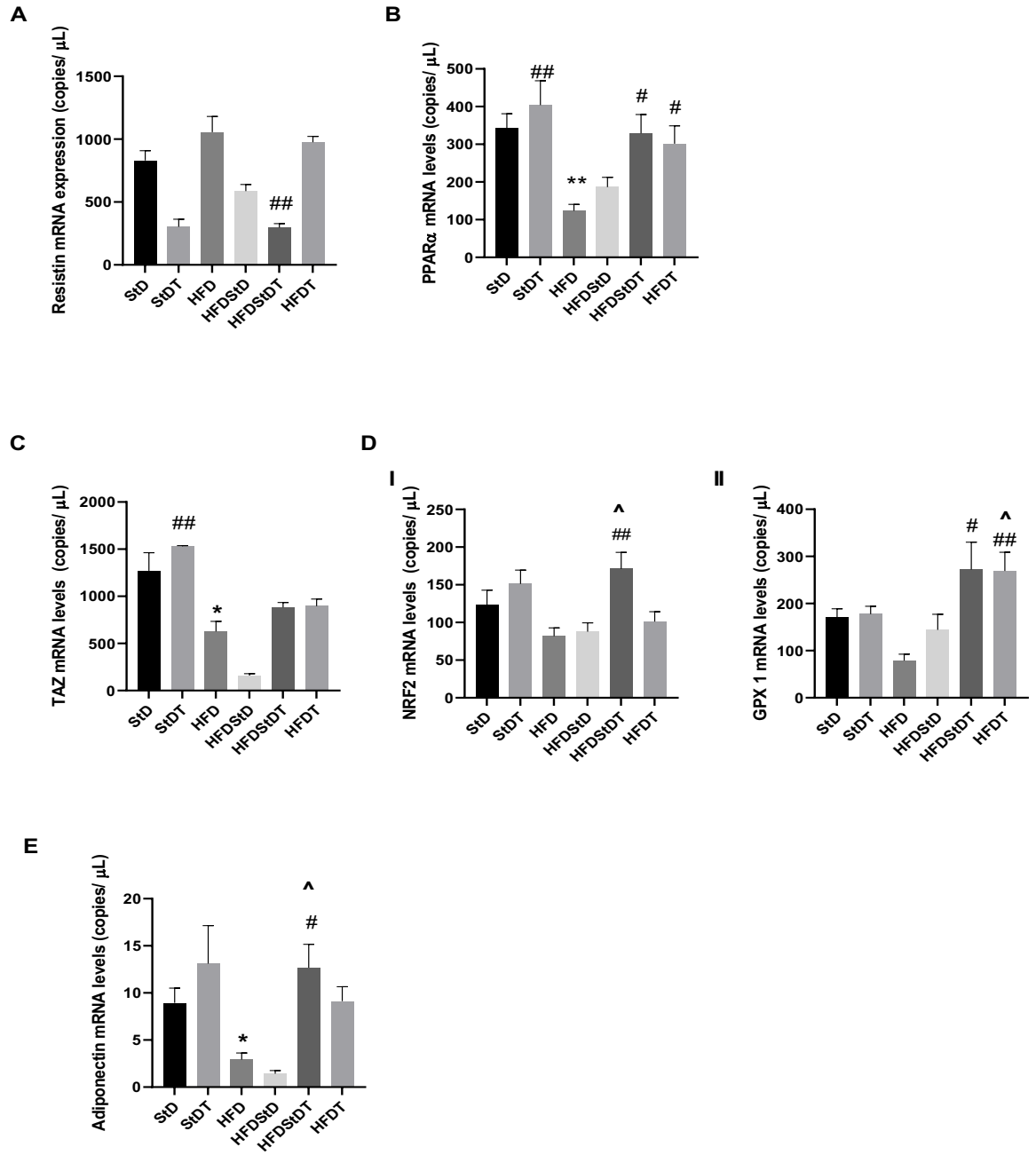


Figure 4: TEMPOL attenuated inflammation, oxidative stress and doing so improved insulin sensitivity in HFD *in vivo* model.

To analyse TEMPOL's effect on inflammatory markers (A) ddPCR analysis of resistin mRNA expression in VAT was analysed, (B) To analyse TEMPOL's effect on anti-inflammatory markers, PPARα mRNA gene expression in VAT was measured. (C) Using ddPCR analysis, mRNA levels of mitochondrial structural protein TAZ from VAT, and (D) antioxidants (I) NRF2 and (II) GPX1 were measured. To analyse mRNA expressions that enhance insulin sensitivity, ddPCR was used to obtain absolute quantification of (E) Adiponectin mRNA expressions. *In vivo*, data are presented as means ± SEM, (n = 9-10). * $p < 0.05$, **$p < 0.001$ vs StD group. # $p < 0.05$, ## $p < 0.001$ vs HFD group. ^ $p < 0.05$ vs HFDStD group.

The effect of TEMPOL or dietary intervention on oxidative stress in VAT.

Tafazzin (TAZ) is a mitochondrial protein that controls the function of the phospholipid cardiolipin, which maintains mitochondrial function. Tafazzin dysfunction consequentially reduces ATP levels and results in defective electron transport chain (ETC) activity and ultimately enhances mitochondrial oxidative stress via oxidant (ROS) production. Therefore, defects in TAZ levels will correlate with cardiolipin function and vice versa [38, 39]. Fig. 4C shows that HFD significantly reduced TAZ mRNA levels by 51% ($p < 0.05$ vs StD). TEMPOL reversed this effect by 47% in HFDStDT and 43% in HFDT and was more effective than standard diet intervention (HFDStD). StDT also showed increased TAZ mRNA levels by 20% ($p < 0.001$ vs HFD).

Nuclear factor erythroid2 p45-related factor 2 (NRF2) also protects cells from oxidative stress by acting as a transcription factor for multiple defence genes including members of the GPX family [40]. These enzymes are central players in the detoxification of hydrogen peroxide and (GPX4) lipid hydroperoxides [41]. Fig. 4DI & II show that TEMPOL increased NRF2 levels (by 12.6% in HFDT and by 78% in HFDStDT, $p < 0.001$) and a significant increase of GPX1 (by 59% for HFDStDT and 65% for HFDT, $p < 0.001$) compared to the HFD group. These data indicate that TEMPOL enhances the cellular defences against oxidants, and more efficiently than the standard diet intervention ($p < 0.05$).

TEMPOL increases circulating adiponectin levels.

Adipose tissue plays a critical role in maintaining whole-body glucose homeostasis. Hyperlipidaemia, as occurs in the obese state, perturbs insulin signalling and contributes to insulin resistance. Adiponectin, an adipokine secreted from the adipose tissues, is known to enhance insulin sensitivity and suppress inflammation [42, 43]. Fig. 4E shows that HFD decreases adiponectin expression by ~ 71% compared to the StD group ($p < 0.05$). TEMPOL (HFDT & HFDStDT, $p < 0.01$) increased adiponectin relative to HFD by ~ 6 folds. The increase in adiponectin mRNA levels was better with TEMPOL supplementation (HFDStDT) than with standard diet intervention ($p < 0.05$).

In vitro Experiments

The effect of TEMPOL on 3T3L-1 adipocyte cellular metabolic activity and differentiation

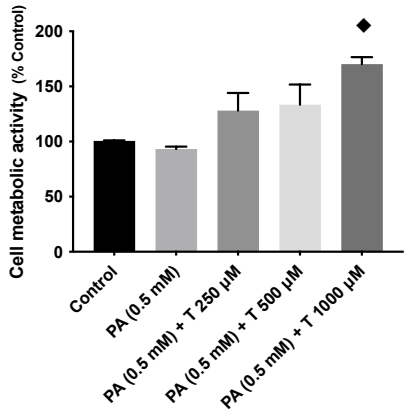
To examine the potential cytotoxic effects of TEMPOL, an MTT assay was performed. Fig. 5A shows that incubation with 0.5 mM PA for 24 h decreased 3T3L-1 adipocyte cell metabolic activity by 13.5% compared to the control group. TEMPOL treatment showed a dose-dependent protective effect on restoring, with 1000 µM TEMPOL showing a significant enhancement (to 94%) compared to PA ($p < 0.05$).

As discussed above PPARγ is the master adipogenesis regulator and critical for adipocyte differentiation [44, 45]. Perilipin-1 is a marker of adipocyte differentiation and an intracellular neutral lipid surface protein involved in regulating lipid hydrolysis [46]. As the differentiation progresses, perilipin-1 levels increase, as mature adipocytes contain larger lipid droplets with a perilipin-1 coating [47]. Fig. 5B shows, as expected, a gradual increase in perilipin-1 levels with differentiation ($p < 0.05$). TEMPOL reduced perilipin-1 levels across all differentiation stages, with this being significant at 1000 µM when compared to differentiated 0.5 mM PA-treated cells ($p < 0.001$). Fig. 5CI & II shows that TEMPOL treatment also decreased PPARγ protein ($p < 0.001$) and mRNA ($p < 0.05$) levels in a dose-dependent manner compared to the PA-treated group.

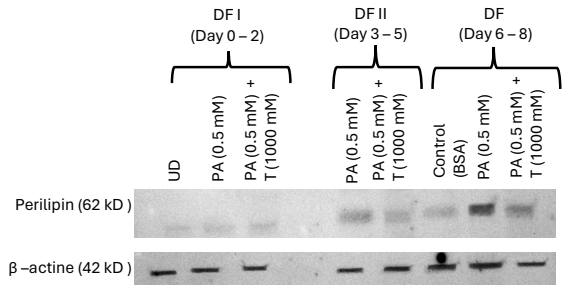
TEMPOL reduces lipid accumulations and lipogenesis in cultured 3T3L-1 adipocytes

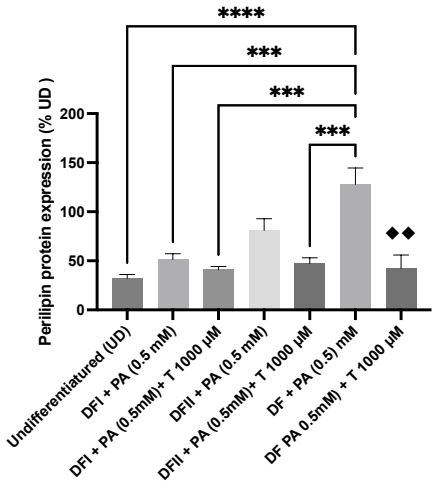
TEMPOL treatment (1000 µM) significantly decreased PA-induced lipid accumulation by 22% ($p < 0.001$ vs the PA group; Fig. 6A). To examine the effect of TEMPOL on lipogenesis, FASN and perilipin-1 protein levels were measured. PA presented a trend in increased FASN levels by ~ 57% with TEMPOL treatment resulting in a significant decrease (by 77%) at the 500 µM dose ($p < 0.05$; Fig. 6B). Perilipin-1 levels were measured to analyse the effect of TEMPOL on lipolysis (Fig. 6C). PA exposure presented a trend in increased perilipin-1 levels by 23% which was decreased significantly by TEMPOL at all doses ($p < 0.05$; Fig. 6C). Fig. 6D shows that 0.5 mM PA presented a trend in increased TG levels by 61% compared to the control group. TEMPOL decreased TG levels significantly at 250 µM ($p < 0.05$) & 1000 µM TEMPOL ($p < 0.05$) concentrations compared to the PA-treated group.

A

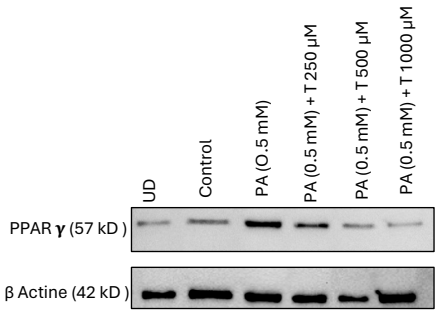

Cell metabolic activity (% Control)
200
150
100
50
0
◆
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

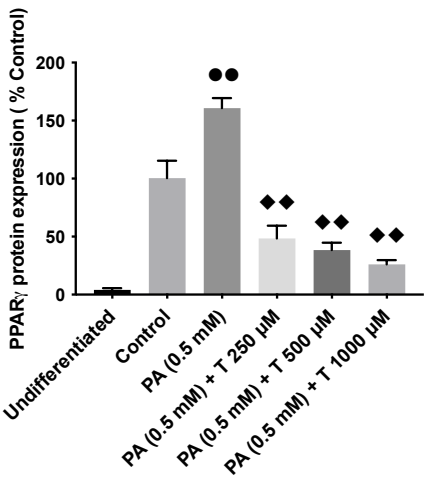
B


DF I
(Day 0 – 2)
DF II
(Day 3 – 5)
DF
(Day 6 – 8)
UD
PA (0.5 mM)
PA (0.5 mM) + T (1000 mM)
PA (0.5 mM)
PA (0.5 mM) + T (1000 mM)
Control (BSA)
PA (0.5 mM)
PA (0.5 mM) + T (1000 mM)
Perilipin (62 kD)
β –actine (42 kD)


****
***
***
***
Perilipin protein expression (% UD)
200
150
100
50
0
◆◆
Undifferentiatured (UD)
DFI + PA (0.5 mM)
DFI + PA (0.5mM)+ T 1000 µM
DFII + PA (0.5 mM)
DFII + PA (0.5mM)+ T 1000 µM
DF + PA (0.5) mM
DF PA 0.5mM) + T 1000 µM

C I


UD
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM
PPAR γ (57 kD)
β Actine (42 kD)


PPARγ protein expression (% Control)
200
150
100
50
0
●●
◆◆
◆◆
◆◆
Undifferentiated
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

II

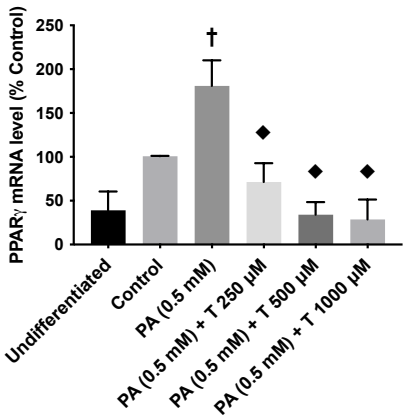
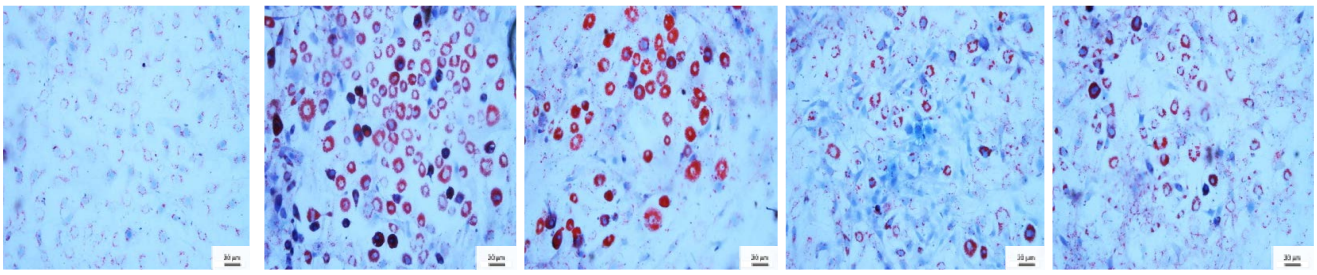
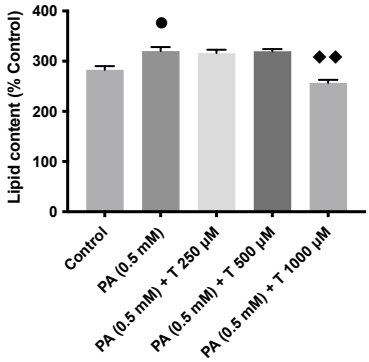


Figure 5: TEMPOL treatment does not have significant effects on the metabolic activity of 3T3L-1 adipocytes and attenuated adipogenesis.

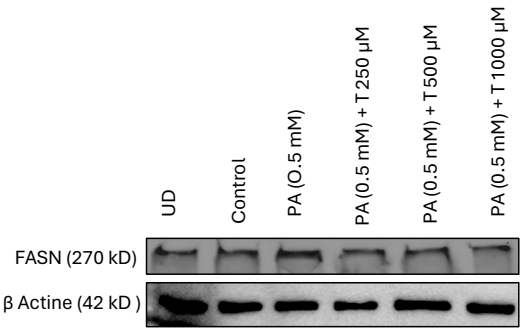
(A) A cell metabolic activity test was done on 3T3L-1 adipocytes that were pre-incubated with PA (0.5 mM) for 24 h followed by post-TEMPOL treatment (250, 500 or 1000 µM) for a further 24 h. (B) The effect of TEMPOL treatment on each differentiation stage of 3T3L-1 adipocyte was analysed with probed perilipin-1, using immunoblot analysis. Each differentiating (DF) stage (DFI, DFII & DF) was compared with 0.5 mM PA incubation for 24 h followed by TEMPOL treatment at 1000 µM (1 mM), the highest concentration used in this study (n=4-5; *** $p < 0.001$, **** $p < 0.0001$). (C) To understand if TEMPOL impacts the main adipogenesis regulator PPARγ (I) protein expression and its (II) mRNA expression levels analysed (n = 4-6). Data are presented as means ± SEM, ● $p < 0.05$ & ●● $p < 0.001$ vs control group. ◆ $p < 0.05$ & ◆◆ $p < 0.001$ vs PA group, ✝ $p < 0.05$ vs undifferentiated group.

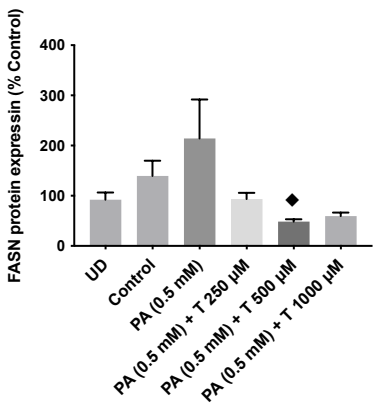
A


Control
PA (0.5 mM)
TEMPOL (250 µM)
TEMPOL (500 µM)
TEMPOL (1000 µM)

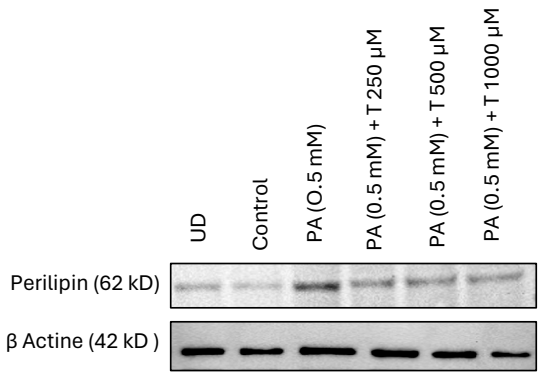

Lipid content (% Control)
400
300
200
100
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

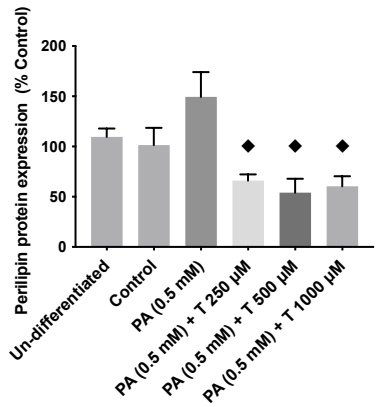
B


UD
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM
FASN (270 kD)
β Actine (42 kD)


FASN protein expressin (% Control)
400
300
200
100
0
UD
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

C


UD
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM
Perilipin (62 kD)
β Actine (42 kD)


Perilipin protein expression (% Control)
200
150
100
50
0
Un-differentiated
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

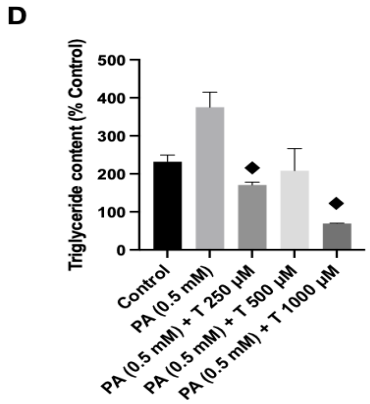


Figure 6: TEMPOL attenuated lipid accumulation induced by PA exposure and altered lipid metabolism in 3T3L-1 adipocytes.

(A) Oil red O (ORO) quantification and image analysis of 3T3L-1 cells treated with 0.5 mM PA for 24 h, followed by TEMPOL treatments (250, 500 or 1000 µM) 24h. Lipid droplets (stained red) were observed under a microscope at x20 magnification (Scale bar: 20 µm). Following ORO staining, the dissolution of the stain was conducted, and the ORO stain accumulated was analysed to measure lipid content. TEMPOLs' impact on lipogenesis was analysed by probing 3T3L-1 protein expression with (B) FASN and TEMPOLs' impact on lipolysis was assessed by quantifying protein expression probed with (C) perilipin 1. The analysis was compared to the β-Actin antibody. (D) The effect of TEMPOL on TG content in 3T3L-1 cell lysate was analysed using a TG assay. Data are presented as means ± SEM, (n = 4-6). ● $p < 0.05$ & ●● $p < 0.001$ vs control group. ◆ $p < 0.05$ & ◆◆ $p < 0.001$ vs PA group.

The effect of TEMPOL on inflammatory cytokines, anti-inflammatory markers and oxidative stress in PA-treated 3T3l-1 adipocytes

Ectopic lipid accumulation in adipose tissue promotes a microenvironment in which the adipocytes secrete pro-inflammatory cytokines such as TNF, SAA-1, MCP-1 and IL-6 [48, 49]. These proinflammatory markers are a characteristic indicator of inflammation in obese individuals, particularly the enhanced levels of TNF in adipocytes [50]. Figure 7A: I- IV shows a trend in an increase of the mRNA levels of each of these species by 56%, 77%, 52 % ($p < 0.05$ vs control) and 55%, respectively with PA incubation. Treatment with TEMPOL decreased the levels of these species with significance shown for all cytokines with 500 and 1000 µM TEMPOL treatments ($p < 0.05$ vs PA group).

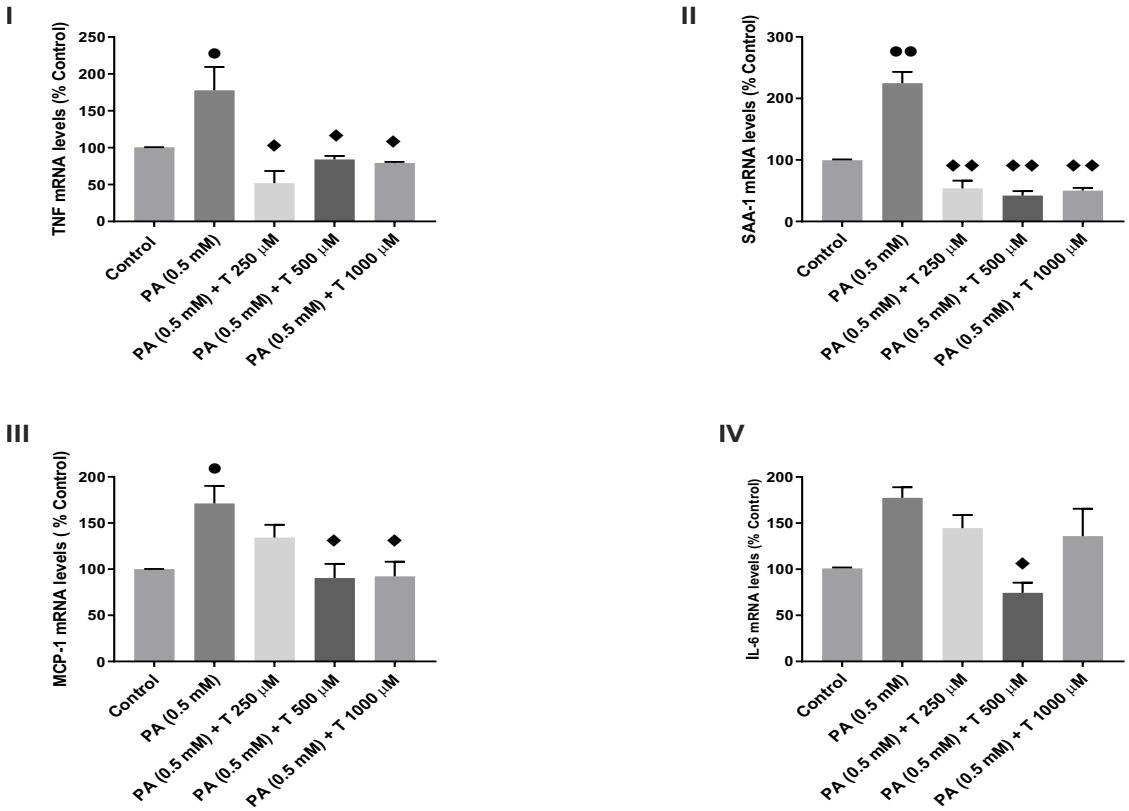
To investigate if TEMPOL enhances systemic anti-inflammatory interleukin levels, we measured the mRNA levels of interleukin-10 (IL-10), interleukin-13 (IL-13) and their central regulator PPARα. Fig. 7B: I-III shows PA exposure presented a trend in decreasing mRNA

levels of IL-10, IL-13 and PPARα by 28%, 52%, and 19%, respectively. Treatment with TEMPOL restored the mRNA levels in a dose-dependent manner.

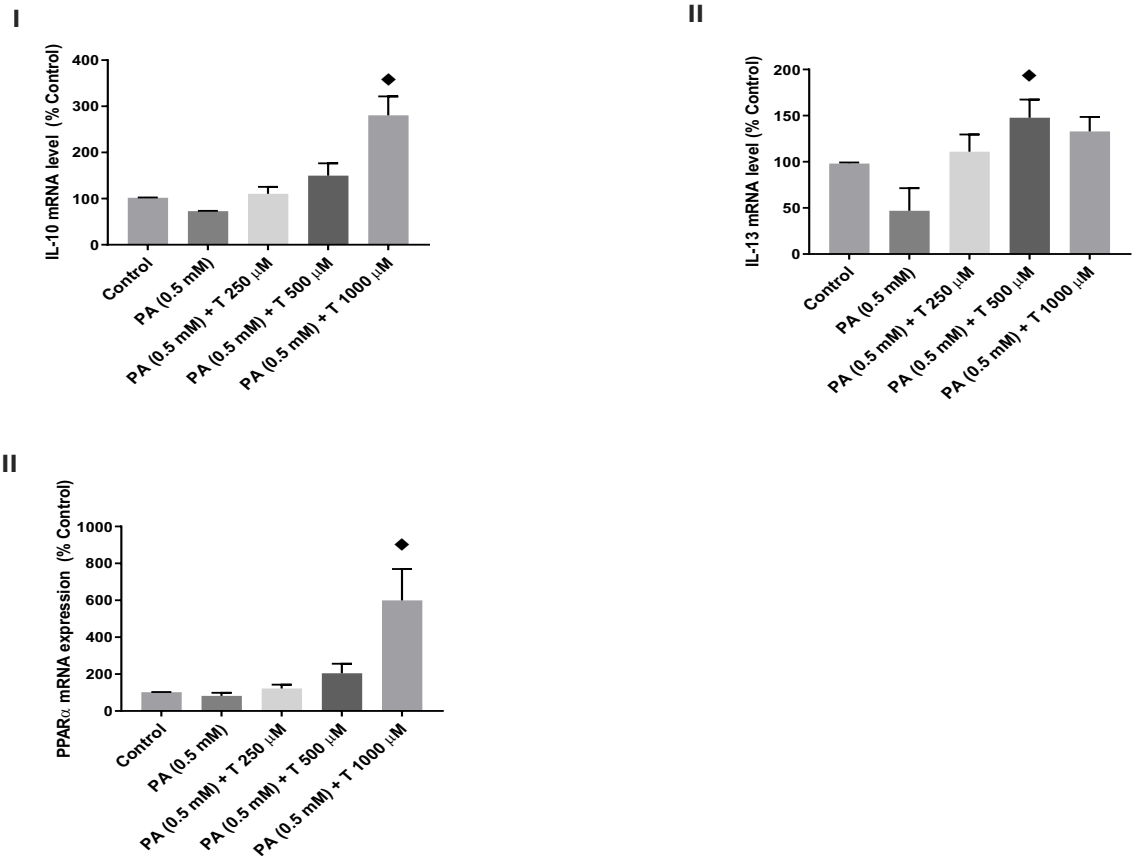
The impact of TEMPOL on PA-induced oxidative stress in adipocytes. Fig. 7CI shows that 0.5 mM PA presented a trend in increased oxidant levels (as determined by DCFH fluorescence, a generic marker of oxidation) by 14% compared to the control group. TEMPOL treatment diminished this increase in a dose-dependent manner ($p < 0.05$). To determine if TEMPOL has any effect on mitochondrial damage, cytosolic cytochrome c (cyt c) levels were measured (Fig. 7CII). The PA group showed a 72% increase in cyt c protein levels ($p < 0.001$ vs control), which was decreased by TEMPOL treatment.

The potential of TEMPOL to act as an insulin sensitizer in PA-treated 3T3L-1 adipocytes.
The effect of TEMPOL on adiponectin and glucose transport 4 (GLUT4), key players that modulate insulin sensitivity, was also examined. GLUT 4 is an insulin-regulated transporter of glucose into adipose and skeletal muscle [51]. Fig. 8A & B show that PA treatment induced a 16% and 24% reduction in GLUT4 and adiponectin levels ($p < 0.05$), respectively, possibly indicating PA-induced inhibition of glucose transport and insulin signalling. TEMPOL, particularly at 1000 μM, restored GLUT4 ($p < 0.05$) and adiponectin mRNA expressions to basal levels.

A


I
TNF mRNA levels (% Control)
250
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM
II
SAA-1 mRNA levels (% Control)
300
200
100
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM
III
MCP-1 mRNA levels (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM
IV
IL-6 mRNA levels (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM

B


I
IL-10 mRNA level (% Control)
400
300
200
100
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM
II
IL-13 mRNA level (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM
III
PPARα mRNA expression (% Control)
1000
800
600
400
200
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 μM
PA (0.5 mM) + T 500 μM
PA (0.5 mM) + T 1000 μM

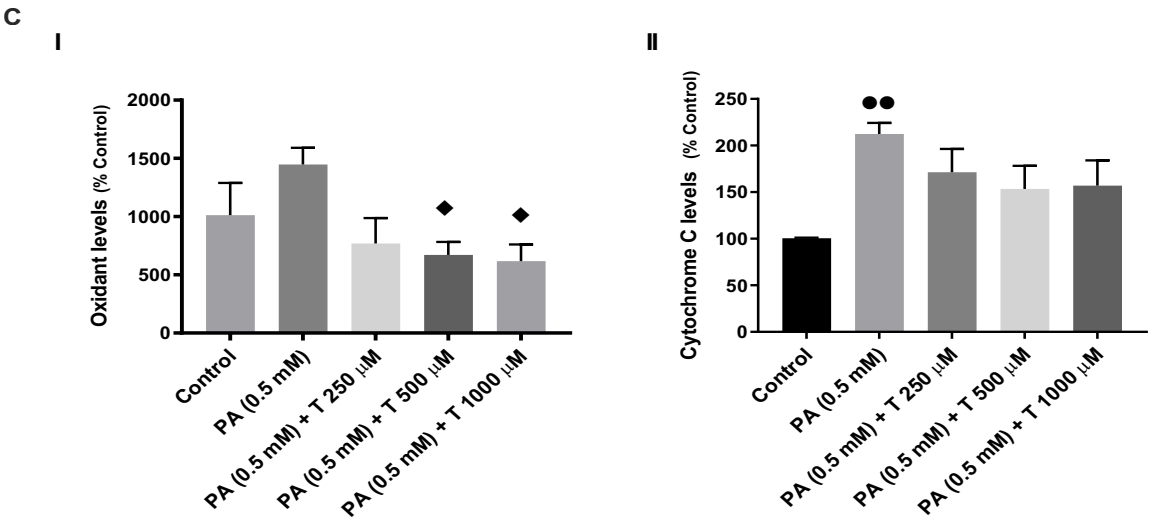


Figure 7: TEMPOL attenuates inflammation and oxidative stress in 3T3l-1 adipocytes induced by PA exposure.

(A) (I) TNF, (II) SAA-1, (III) MPC-1 and (IV) IL6 mRNA expressions derived from 3T3L1 adipocytes that were treated with 0.5 mM PA for 24h, followed by TEMPOL treatments (250, 500 or 1000 µM) for 24h. (B) RT-qPCR was used to analyse the anti-inflammatory effect of TEMPOL (I) interleukin-10 (IL-10), (II) IL13 and (III) PPARα mRNA expressions. To investigate the TEMPOL antioxidant effect, (C) (I) intracellular ROS levels and (II) and the generation of ROS promoter proteins cytochrome c (cyt c) were measured. Data are presented as means ± SEM, (n = 4-6). ● $p < 0.05$ & ●● $p < 0.001$ vs control group. ◆ $p < 0.05$ & ◆◆ $p < 0.001$ vs PA group.

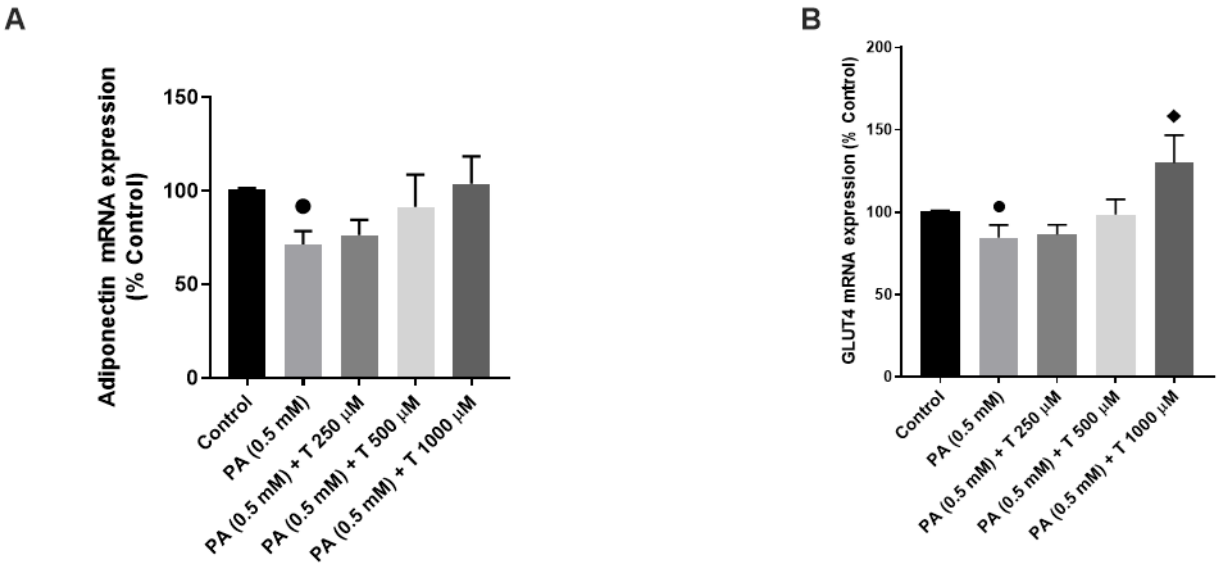


Figure 8: TEMPOL increases insulin sensitivity in 3T3l-1 adipocytes that were impaired by PA exposure.

RNA extracted from 3T3L-1 adipocytes were treated with 0.5 mM PA for 24 h, followed by TEMPOL treatments (250, 500 or 1000 µM) for 24 h was analysed for (A) Adiponectin and (B) GLUT4 mRNA expression levels. Data are presented as means ± SEM, (n = 4-6). ● $p < 0.05$ vs control group. ◆ $p < 0.05$ vs PA group.

Discussion

Increased adipose tissue is a common metabolic failure in obesity, resulting from increased lipogenesis and adipogenesis with a concurrent defect in lipolysis. Potential pharmacological strategies target all these pathways in an attempt to decrease hyperlipidaemia and excess body fat. To date, drug development has been largely unsuccessful because of adverse side effects on metabolically relevant organs. This study examined the hypothesis that TEMPOL, a potent and well-described anti-inflammatory agent and antioxidant suppresses lipogenesis, lipolysis and adipogenesis in both *in vivo* model and an *in vitro* model to delineate the underlying protective mechanisms. The data obtained indicate that TEMPOL effectively retards lipid accumulation and that is associated with protection from saturated fat-induced inflammatory and oxidative stress responses and positive effects on fatty acid β-oxidation. These findings provide a strong foundation and impetus to explore TEMPOL as a drug to reverse adiposity and its comorbidities in obesity.

One of the most significant findings of this study was that TEMPOL reduced HFD-associated hyperlipidaemia and that TEMPOL has an overall better effect than a standard dietary modulation intervention. Eight-week dosing with TEMPOL resulted in a lower ectopic lipid accumulation, as evidenced by a lower body mass, which correlated with decreased VAT and epididymal tissue. The dose of TEMPOL used in the current study is within the range Mitchell et al. used for a one-year murine study model, with no reported toxicity [18], and also shorter term studies [19]. Further data on the safety and efficacy of TEMPOL are provided by human clinical trials, where it is being used to protect against radiation-induced alopecia at doses (0.4 to 3.1 μM) equivalent to the mice study [52]. The current studies showed an absence of toxicity in the animal study and also standard cellular metabolic activity assays.

The action of TEMPOL in reducing hyperlipidaemia is most likely due to its effect on three major cellular pathways, lipolysis, lipogenesis and adipogenesis. Notably, enhanced lipolysis, or the release of fatty acids from adipose triglyceride reserves, leads to the elevated circulation of fatty acids and "ectopic" lipid deposition in other tissues [53]. Novel therapies that target the inhibition of enzymes involved in lipolysis are under consideration. For example, inhibition of FASN is such a target, as its decreased expression and/or activity would suppress lipogenesis and consequently reduce fat storage [54, 55]. The FASN inhibitor is C75, which has been

shown to suppress appetite and enhance fatty acid β-oxidation in mice [56, 57]. Our data showed that TEMPOL reduces FASN alongside ACAB, ADRB2 and SREBP-1c, all proteins that orchestrate lipogenesis. The multifactorial actions of TEMPOL may be more effective than the specific actions of C75 and should be explored further. Notably, TEMPOL suppression of key regulators of lipogenesis is associated with a phenotypic endpoint of decreased plasma TG.

A recognised therapeutic target for enhancing lipolysis is LPL [29], however, to date, there is no targeted medication that specifically targets LPL activation. This is likely because its main mode of mechanism has yet to be defined. Interestingly, our study showed a reduction in LPL expression by TEMPOL. Moreover, TEMPOL reduced FABP4 levels, an effect known to improve lipid markers and reduce cardiometabolic outcomes in men [58-60]. Studies with a specific inhibitor of FABP4, BMS309403 [61] show that targeting these key proteins is an effective anti-obesity drug therapy. Our findings that TEMPOL suppresses both LPL and FABP4 suggest that TEMPOL could have more pronounced effects on lipolysis than the use of these single protein inhibitors.

Studies have shown a reciprocal relationship between fatty acid β-oxidation and body mass gain [62]. Given the importance of altered fatty acid metabolism in the aetiology of obesity and its comorbidities, therapeutic approaches to modify fatty acid metabolism are increasingly being considered. Medications that target fatty acid β-oxidation include fibrates and metformin [63, 64] and their target genes include PPARδ and ACAB. Studies using ACAB knock-out mice studies have shown the reduction of malonyl CoA, increases in fatty acid β-oxidation and diminished diet-induced body mass gain [27]. Our data suggests, indirectly, that TEMPOL also increases fatty acid β-oxidation as TEMPOL increased PPARδ and decreased ACAB expression levels in VAT. The study by Mitchell et al. reported that mice treated with TEMPOL were more active than the control groups [18], and a possible explanation for this phenomenon was the enhanced ATP levels resulting from increased fatty acid β-oxidation.

Currently, bariatric surgery is the most successful way of reducing body fat. This is a highly invasive, expensive, and unsustainable approach for treating the growing epidemic. A pharmacological approach may be more practical and cost-effective. Modulating regulators of adipogenesis is a potential therapeutic target for controlling or preventing obesity. The major transcription factors of adipogenesis, C/EBPα and PPARγ, are stimulated by C/EBP β during

the intermediate stage of adipogenesis. In the last stage of differentiation, PPARγ and C/EBPα work together to promote differentiation and the induction of various adipocyte-specific genes, including perilipin-1. Tanaka et al. showed adipogenesis is substantially reduced in C/EBP β-null animals [65]. As a result, C/EBP β inhibition could be a possible target for preventing or treating obesity because lowering early adipogenic transcription factors limits subsequent transcriptional cascades and suppresses terminal adipogenic development. Furthermore, PPARγ is an established target for anti-obesity and anti-diabetic drugs (e.g., TZDs and orlistat) [9, 66, 67]. In this study, we have shown TEMPOL reduced C/EBPα and PPARγ expression with the phenotypic result of reduced differentiation as evidenced by decreased perilipin-1 and reduced adipocyte cell number. Such findings provide impetus to further explore the potential of TEMPOL.

Consideration should also be given to the links between obesity and other chronic conditions such as T2DM and cardiovascular disease. The metabolic syndrome is linked to obesity via adipose inflammation and oxidative stress. The effective reduction of lipid accumulation and adipogenesis by TEMPOL was associated with reduced adipocyte tissue inflammation and oxidative stress. Van der Heijden et al. have shown, using a HFD-fed C57LB/6 mice model, that an increase in lipid accumulation in adipose tissue triggered increased production of the pro-inflammatory cytokines such as MCP-1 and TNF and that, in turn, this led to the progression and the development of metabolic inflammation resulting in hepatic inflammation and insulin resistance [68]. Similarly, Esser et al. showed that saturated fat shake ingestion in 18 obese individuals led to increased levels of pro-inflammatory cytokine IL-8 [69]. Our study showed that HFD-fed mice and PA treatment of cultured adipose cells resulted in elevated inflammatory status with high levels of resistin, SAA-1, L-6, TNF, and MCP-1. Moreover, SAA-1, an acute-phase protein, which can bind to HDL, displacing ApoAI at high expression levels, and disrupting HDL's lipid transport function [70]. Consequently, TEMPOL's capacity to reduce SAA-1 gene expression may contribute to regulating lipid transport. All in all, given the strong link between lipid-induced inflammation and insulin resistance, it is not surprising that pharmacological approaches have already targeted inflammation cascades. Liraglutide, a currently recommended anti-obesity drug, has been shown to reduce inflammation in a 10-week osteoarthritis *in vivo* murine model [71]. The current study shows that TEMPOL has strong anti-inflammatory effects of TEMPOL; potentially increasing its potential to be used in the prevention of comorbidities [19].

Obesity is also associated with enhanced oxidative stress and dysfunctional antioxidant mechanisms. Adipose tissue from obese patients shows increased secretion of adipokines that can enhance oxidant production, impair antioxidant capacity, and trigger the production of pro-inflammatory cytokines. TEMPOL is a highly effective membrane-permeable antioxidant in both *in vivo* and *in vitro* models, and may there act via such a mechanism [16, 72, 73]. Moreover, TEMPOL has been shown to enhance natural antioxidant defence mechanisms (catalase, SOD, and glutathione) in diabetic mice with ischemic kidney injury, with these linked with an improvement in diabetic kidney disease [74]. Our data showed TEMPOL treatment reduced oxidative stress in VAT from HFD-fed mice and in PA-treated 3T3L-1 cells. In addition, our mice data show that TEMPOL can upregulate endogenous antioxidant systems via NRF2, as determined by GPX1 levels, with this being a more effective approach than dietary intervention. Interestingly, the current anti-obesity drug, orlistat, also shows antioxidant properties in a murine vascular oxidative stress model [75] and liraglutide has been shown to activate the NRF2 / heme oxygenase- 1 (HO-1) pathway and thereby protect against cerebral ischemia injury in a diabetic rat model [76].

Studies by Barazzoni et al. (2006) and Tsuruta et al. (2010) showed that increased oxidative stress in VAT is associated with the expressions of pro-inflammatory cytokines and insulin resistance [77, 78]. Given our observation that TEMPOL reduces lipid accumulation in adipose tissue and also alleviates high levels of inflammation and oxidative stress, we examined whether TEMPOL improved adipose insulin sensitivity. Adiponectin is a promising target in treating obesity-related diseases, such as T2DM, as it is known to reverse hyperlipidaemia and hyperglycemia. Current lipid-lowering drugs found to increase adiponectin levels include statins (pravastatin, atorvastatin, simvastatin), fibrates (fenofibrate) and thiazolidinedione [79]. Our *in vivo* data showed TEMPOL increased adiponectin levels to a greater extent than the standard diet intervention, and these effects were confirmed in our 3T3L-1 *in vitro* model. Furthermore, TEMPOL increased GLUT4 mRNA in a dose-dependent manner. Interestingly, studies have shown an inverse relationship between GLUT4 and adiponectin with pro-inflammatory cytokines such as IL-6 and TNF [80]. Given TEMPOL reduced inflammation and increased adiponectin, this compound may be a promising insulin sensitizer. TEMPOL also increased PPARδ levels significantly in our *in vivo* model, with PPARδ a recognized marker of improved insulin sensitivity [81, 82]. Furthermore, the PI3K/AKT/mTOR pathway, which regulates cellular growth, survival, and metabolism, is subject to modulation by oxidative stress [83, 84]. Chronic oxidative stress and dysregulation of the PI3K/AKT/mTOR

pathway contribute to insulin resistance. Although not assessed in this study, it may be that TEMPOL impacts the lower arm of the insulin signalling pathway instead of, or in addition to the upstream arm. Further work is necessary to fully evaluate TEMPOL's exact mechanism of action and it is likely given the potent antioxidant nature of TEMPOL that effects will be multifaceted.

In summary, our research shows that TEMPOL reduces hyperlipidaemia in adipocytes by impacting both arms of lipid metabolism arms, thereby limiting lipid accumulation. Its potent anti-inflammatory and antioxidant properties, along with a significant impact on improving adiponectin levels, suggest potential insulin sensitisation. These positive findings lay the groundwork for further research to explore TEMPOL's potential anti-obesity actions in humans. Since TEMPOL is already in clinical trials for other indications, utilising targeted or untargeted mass spectrometry in lipidomic analysis could enhance our understanding of its influence on novel pathways, supporting the highlighted study findings and potentially expediting TEMPOL's clinical application.

Declarations

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Conflicts of interest
MJD declares commercial consultancy contracts with Novo Nordisk A/S. This funder has no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish these results. The other authors declare no conflict of interest with regard to the data presented.

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Supplementary Table 1: Murine PCR primer and primer sequence used in this study.

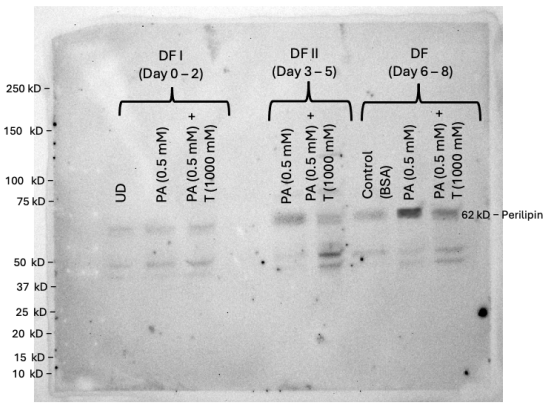
Primer (Cell signalling: Gene works)	**Primer Sequence (5'-3')**		**Accession Number**
Reference Gene			
18S	F	AACTTTCGATGGTAGTCGCCGT	NM_146819.2
	R	TCCTTGGATGTGGTAGCCGTTT	
Lipid metabolism Genes			
Acetyl- CoA Carboxylase Beta (ACAB)	F	GCATGAAGGACATGTATGAG	NM_001403528.1
		AGGGATGTAGATGAGAATGG	
Fatty Acid Synthase (FASN)	F	GGAGGTGGTGATAGCCGGTAT	NM_007988.3
	R	TGGGTAATCCATAGAGCCCAG	
Lipoprotein Lipase (LPL)	F	GAGACTCAGAAAAAGGTCATC	NM_008509.2
	R	GTCTTCAAAGAACTCAGATGC	
Fatty Acid Binding Protein 4 (FABP4)	F	GTAAATGGGGATTTGGTCAC	NM_024406.4
	R	TATGATGCTCTTCACCTTCC	
Peroxisome Proliferator Activator Receptor δ (PPAR δ)	F	CTGACAGATGAAGACAAACC	NM_011145.4
	R	CTTCCTCTTTCTCCTCTTCC	
Adipogenesis Genes			
Fibroblast Growth Factor 10 (FGF10)	F	AGTGTCCTGGAGATAACATC	NM_008002.5
	R	AGGTGTTGTATCCATTTTCC	
CAAT/ Enhancing Binding Protein α (CEBPα)	F	AAGGGTGTATGTAGTAGTGG	NM_001287514.1
	R	AAAAAGAAGAGAAGGAAGCG	
Inflammatory Genes			
Resistin (Retn)	F	CCTCCTTTTCCTTTTCTTCC	NM_001204959.1
	R	ATTTGGAAACAGGGAGTTG	
Interleukin - 6 (IL-6)	F	GAACAACGATGATGCACTTGC	NM_031168.2
	R	TCCAGGTAGCTATGGTACTCC	
Interleukin- 8 (IL-8)	F	ACTTTTGGCTCAACCCTGTG	NM_009909.3
	R	ACGCAGTACGACCCTCAAAC	
Interleukin-1β (IL-1β)	F	GCTCAGGGTCACAAGAAACC	NM_008361.4
	R	CATCAAAGCAATGTGCTGGT	
Serum Amyloid A-1 (SAA-1)	F	AGCGATGCCAGAGAGGCTGT	NM_011315.3
	R	ACCCAGTAGTTGCTCCTCTT	

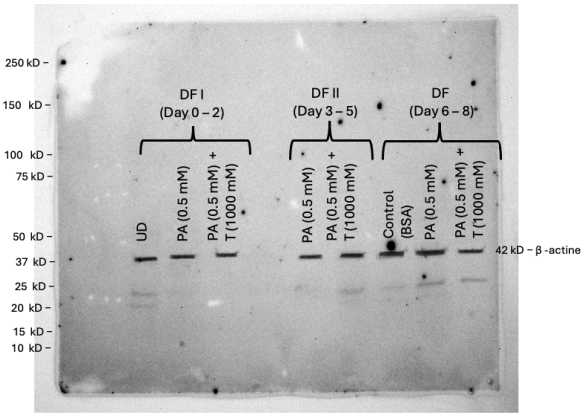
Monocyte Chemoattractant Protein -1 (MCP-1)	F	GCTCAGCCAGATGCAGTTAA	NM_011333.3
	R	TCTTGAGCTTGGTGACAAAAACT	
Anti-inflammatory Genes			
Interleukin- 10 (IL-10)	F	ATAACTGCACCCACTTCCCA	NM_010548.2
	R	GGGCATCACTTCTACCAGGT	
Interleukin-13 (IL-13)	F	GCAGCATGGTATGGAGTGTG	NM_008355.3
	R	TGGCGAAACAGTTGCTTTGT	
Peroxisome Proliferator Activator Receptor α (PPAR α)	F	ATTCGGCTGAAGCTGGTGTAC	NM_011144.6
	R	CTGGCATTTGTTCCGGTTCT	
Oxidative Stress and Antioxidant Genes			
Tafazzin (TAZ)	F	CAGAAGGGAAAGTAAACATGAG	NM_181516.6
	R	ATCATTCATTCCAACATGCC	
Glutathione Peroxidase 1 (GPX1)	F	TTCGCACTTCTCAAACAATG	NM_002083.4
	R	CTCCATCAGGTTTCTTTCTTG	
Nuclear Factor Erythroid -2- related Factor 2	F	GGGGACAGAATCACCATTTG	NM_008686.3
	R	GATGCAGGCTGACATTCTGA	
Apoptotic Genes			
Axin -1	F	AAATCACAAAGAAAGGCAGG	NM_024405.2
	R	AAAGTAGTATCTGTAGCTCCC	
Gluconeogenic Gene			
Insulin receptor substrate (Insr)	F	AAGACCTTGGTTACCTTCTC	NM_010568.3
	R	GGATTAGTGGCATCTGTTTG	
Glucose Transporter -4 (GLUT4)	F	GATTCTGCTGCCCTTCTGTC	NM_009204.2
	R	ATTGGACGCTCTCTCTCCAA	
Adiponectin	F	GGCAGGAAAGGAGAACCTGG	NM_009605.5
	R	AGCCTTGTCCTTCTTGAAGA	

Supplementary Table 2: Antibodies used for immunoblotting analysis.

Specificity	**Source/Isotype**	**Cell Signalling, Cat #**
Perilipin -1	Rabbit IgG	9349
Fatty acid synthase	Rabbit IgG	3189
Peroxisome proliferator-activated receptor γ (PPARγ)	Rabbit IgG	2435
β-Actin	Rabbit IgG	4970
Anti-rabbit IgG HRP – linked Antibody	Goat	7074

Appendix





Full immunoblot images for Figure 5 B

Chapter 5 : TEMPOL reduced hepatic lipid accumulation and inflammation in high-fat-diet fed mice.

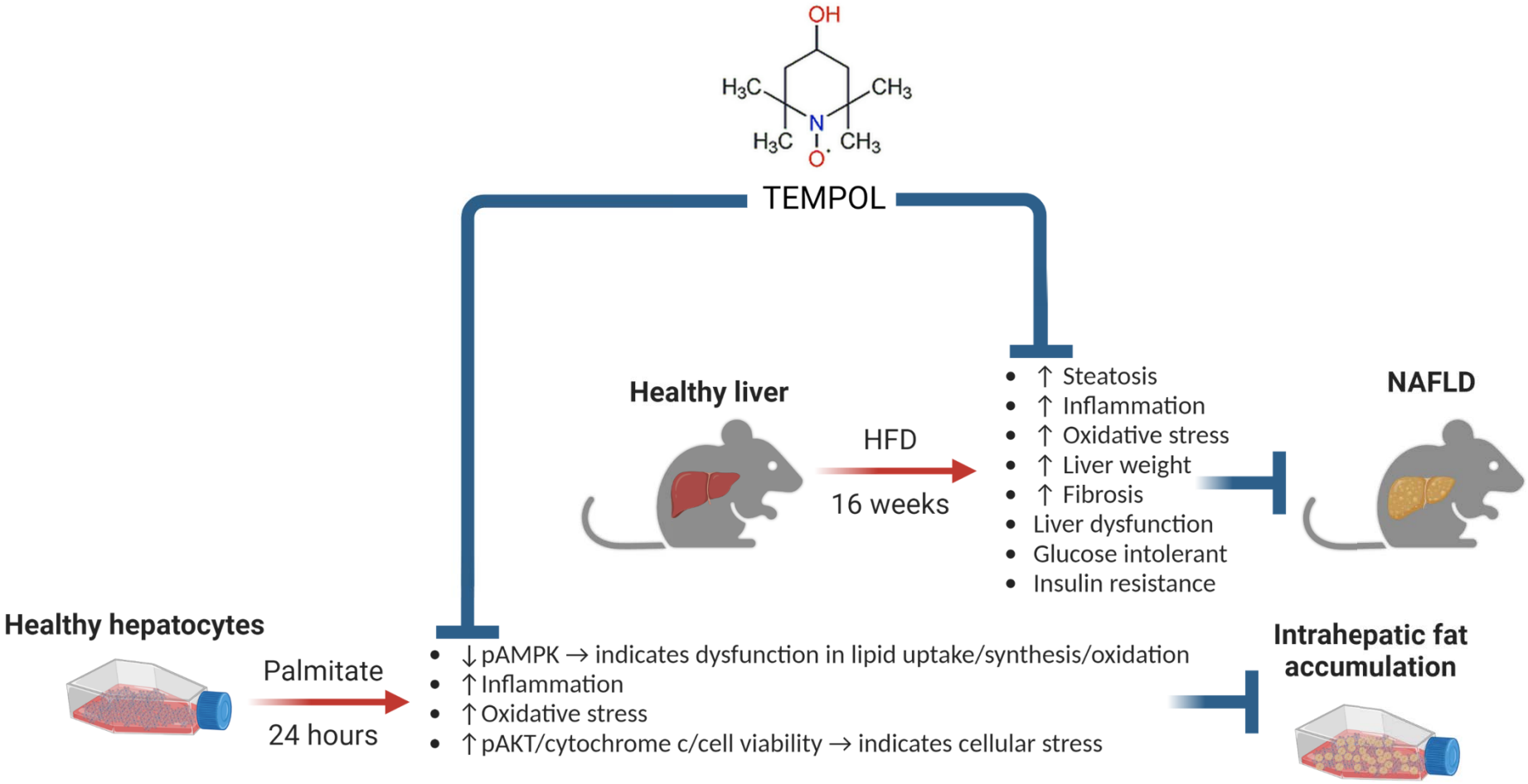
Submitted as:

Gihani Manodara, Scott Genner, Claire Rennie, David Van Reyk, Michael J. Davies, Alison K. Heather, Kristine C. McGrath. TEMPOL reduced hepatic lipid accumulation and inflammation in high-fat- diet-fed mice. *Journal of Hepatology.*

Chapter Summary:

At present, there are no approved pharmaceutical therapies for the treatment of NAFLD. As a result, there is an urgent need to find new NAFLD treatment alternatives, especially given the magnitude of the growing diabetes epidemic. This study hypothesised the administration of TEMPOL, a synthetic antioxidant, could be utilised to treat NAFLD by reducing lipid accumulation in hepatocytes, decreasing hepatic oxidative stress and inflammation, and improving glucose tolerance and insulin sensitivity. The study was conducted using a high-fat diet (HFD) fed C57BL/6 mice model and HepG2 hepatocytes to understand TEMPOL's mechanism of action. This chapter has been written in accordance with the guidelines of the Journal of Hepatology, to which this manuscript will be submitted. Furthermore, due to the nature of the guidelines by the Journal of Hepatology, detailed information on the methods and materials used in this study is provided in the supplementary section of this chapter.

GRAPHICAL ABSTRACT



TEMPOL reduces hepatic lipid accumulation and inflammation in high-fat diet-fed mice

Gihani Manodara [a], Scott Genner [b], Claire Rennie [a], David Van Reyk [a], Michael J. Davies [c], Alison K Heather [d], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Ramaciotti Centre for Genomics, Faculty of Science, University of New South Wales, NSW, Australia*
c. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej, 3B, Denmark*
d. *Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand*

Author contribution:

For this paper, the PhD candidate, GM, completed the majority of the experiments, data analysis and interpretation and wrote the manuscript. SG performed part of the *in vitro* experiments and analysed data. CR performed histological staining and analysis. MD & DR contributed to manuscript editing. KM supervised GM and contributed to data acquisition. AH and KM conceptualised the study, helped with data analysis and interpretation, and edited the manuscript. All authors contributed to the execution and approved the final version of the manuscript.

Gihani Manodara	Production Note: Signature removed prior to publication.
Scott Genner	Production Note: Signature removed prior to publication.
Claire Rennie	Production Note: Signature removed prior to publication.
David Van Reyk	Production Note: Signature removed prior to publication.
Michael J. Davies	Production Note: Signature removed prior to publication.
Alison K. Heather	Production Note: Signature removed prior to publication.
Kristine C. McGrath	Production Note: Signature removed prior to publication.

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a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Ramaciotti Centre for Genomics, Faculty of Science, University of New South Wales, NSW, Australia*
c. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej, 3B, Denmark*
d. *Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand*

Keywords: Liver, Nitroxide, Steatosis, Antioxidant, Insulin resistance, fatty acid β oxidation

Corresponding Author:

Dr Kristine McGrath
University of Technology Sydney
PO Box 123, Broadway
Ultimo, NSW, 2007, Australia
Ph: 612 9514 9773
Email: kristine.mcgrath@uts.edu.au

Highlights:

- High-fat diet and palmitic acid-induced lipid accumulation, inflammation, oxidative stress, and reduced insulin sensitivity in the liver and HepG2 cells, respectively.
- Administration of a nitroxide, TEMPOL, improved fatty acid beta-oxidation, cleared hyperlipidaemia, and increased insulin sensitivity in the liver of C57BL/6 mice fed a high-fat diet.
- In vitro experiments using HepG2 cells stimulated with palmitic acid further confirmed the findings.
- TEMPOL may be a therapeutic strategy for the treatment of insulin resistance and NAFLD.

Lay summary:

We show that TEMPOL reduced fat build-up in the liver, improved insulin sensitivity, reduced inflammation, and even led to weight loss. These findings suggest that TEMPOL could potentially be used as a treatment for insulin resistance and non-alcoholic fatty liver disease.

Impact and implications:

NAFLD (non-alcoholic fatty liver disease) is a prevalent comorbidity in type 2 diabetes mellitus (T2DM), with a current global prevalence of 25%. There are presently no approved medications. Therefore, there is an urgent need to develop new treatment options for NAFLD, especially considering the extent of the ongoing diabetes epidemic. Our findings show that TEMPOL, a synthetic antioxidant, could be used to treat NAFLD by inhibiting lipid accumulation in hepatocytes, decreasing hepatic oxidative stress and inflammation, and enhancing glucose tolerance and insulin sensitivity. We anticipate that our findings will be of significant interest to the medical community including patients.

Abstract

Background & Aims: Hepatic insulin resistance arises, at least in part, because of hepatic lipid accumulation that induces localised and systemic inflammation and oxidative stress. The nitroxide, TEMPOL, has reported anti-inflammatory, anti-oxidative and anti-obesity effects, although whether this extends to protect against hepatic stress and insulin resistance remains unknown. We aimed to identify if TEMPOL administered to high fat diet (HFD)-fed mice, a well-characterised mouse model of insulin resistance, would block hepatic lipid accumulation, suppress hepatic inflammation and oxidative stress, and thereby preserve insulin sensitivity.

Methods: C57BL/6 mice were fed standard chow diet or HFD for a total of 16 weeks. A cohort of mice were supplemented with TEMPOL at 10 mg/kg in their food. HepG2 cells were used to study TEMPOL's mechanism of action. Histological techniques (H&E, Picrosirius red, F4/80 immunohistochemistry) were employed to assess hepatic fibrosis, lipid accumulation and macrophage infiltration). Western blot analysis and RT-qPCR measured inflammation and oxidative stress. Commercial kits and reagents assessed biochemical (glucose, insulin, ALT, AST) and various markers (ROS, IL-8 and glycogen levels).

Results: We show that lipid accumulation in HFD-fed mice treated with TEMPOL was markedly decreased and this was associated with i) improved glucose tolerance and insulin sensitivity (ii) reduced hepatic inflammatory cytokine gene expression, (iii) reduced hepatic oxidative stress, (iv) reduced hepatic fibrosis and (v) reduced body mass. Our *in vivo* observations were further confirmed using *in vitro* culture of HepG2 cells stimulated with palmitic acid to induce a state of cellular stress.

Conclusions: Our findings show TEMPOL improves hepatic insulin sensitivity. Further experimentation is now warranted to explore the potential clinical application of TEMPOL in the treatment of early insulin resistance or type 2 diabetes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the leading chronic liver diseases in the world, with a current global prevalence of 25% and estimated to be projected to be 100.9 million by 2030 in the United States of America alone[1, 2]. Recent studies showed that more than 80% of patients suffer from hepatic steatosis, a hallmark of NAFLD. These patients are often overweight or obese, and 44% of this population are diagnosed with type 2 diabetes mellitus (T2DM) [1]. With increasing steatohepatitis levels, the number of patients waiting for liver transplants has risen dramatically risen and is expected to increase relative to hepatitis C and liver carcinoma [3, 4]

The escalating number of steatohepatitis patients coincides with the higher rates of obesity and is often associated with high-calorie high-fat diets (HFD) and little physical activity [5]. In obese patients, ectopic lipid accumulation in the liver is often observed as a hepatic steatosis [6]. If left untreated, hepatic steatosis progresses to steatohepatitis associated with hepatic insulin resistance and subsequently, systemic insulin resistance [7].

Ectopic lipid accumulation in the liver triggers inflammatory and oxidative stress pathways that, in turn, lead to insulin resistance [8]. Studies have shown that levels of antioxidant defence are often compromised, while reactive oxygen species are increased, in liver biopsies of NAFLD patients [9, 10]. Oxidative stress reduces liver function and is commonly reported in obese patients with NAFLD [11, 12]. Modulating lipid accumulation, oxidative stress and inflammation in the liver may prevent the progression to systemic insulin resistance.

TEMPOL (4- hydroxy-2,2,6,6, -tetramethylpiperidine-1-oxyl), a cell-permeable synthetic antioxidant, has emerged as an anti-obesity drug. Use of TEMPOL for 10 months at 2.9-14.8 mM concentration has led to body mass loss in a C3H murine *in vivo* model, comparable to the caloric-restricted mice group [13]. Further research demonstrated that TEMPOL had a comparable effect on the body mass of mice fed an HFD [14]. In each of these investigations, no adverse effects were detected in mice given TEMPOL. Subsequent research suggests TEMPOL anti-obesity effect was due to an altercation of gut microbiome resulting in inhibiting intestinal farnesoid X receptor (FXR) agonists [15].

TEMPOL mimics the action of SOD by scavenging superoxide radical anions (O_2^-) thereby, protecting cells from radical oxidative damage [13, 16]. In a murine nephrotoxicity model,

TEMPOL demonstrated anti-inflammatory effects by reducing kidney levels of tumour necrosis factor (TNF) and nuclear factor-kB (NFκB) levels [17]. We aimed to show whether TEMPOL administration to C57BL/6 mice fed a high-fat diet reduced insulin resistance and whether this effect was associated with hepatic anti-inflammatory and/or antioxidant effects. To validate our *in vivo* findings, additional studies employed an *in vitro* HepG2 cell culture model.

Methods and Materials

Animal experiments

Male *C57BL/6* mice were seven weeks old when obtained from Australian Bioresources (MossVale, NSW, Australia) and housed in temperature-controlled rooms with a 12 h dark/light cycle. Following acclimatisation, the mice were randomly assigned to either a standard chow diet (StD; n = 10, 68.3% carbohydrate, 4.1% fat) or high-fat diet (HFD; n = 30, 52.4% kCal fat, Biological Associate; Envigo Teklad) enriched with 8.7% palmitic acid and 26% trans-fats) purchased from Biological Associates (Gladesville, NSW, Australia) (**Table S1)**. At 8 weeks of age, mice in the StD group remained on StD (control group) whilst HFD-fed mice were randomly subdivided into three groups (n = 10 per group): (1) continuing the HFD (n = 10), (2) diet intervention with HFD switched to StD (HFDStD; n = 10) or (iii) HFD supplemented with TEMPOL (HFDT; n = 10). Diets supplemented with TEMPOL (10 mg/g) were prepared using the cold-pressed method [18], and *ad libitum* access to food and water was ensured throughout the study. Body weight was monitored weekly. To assess glucose tolerance and insulin sensitivity, an intraperitoneal glucose tolerance test (ipGTT) and an intraperitoneal insulin tolerance test (ipITT) were performed at weeks 14 and 15 (**SI Material and Methods Section)**. At 16-weeks of age, animals were euthanized. Liver samples were either fixed in 4% v/v paraformaldehyde or snap-frozen in liquid nitrogen and stored at -80◦C. All experiments were conducted in accordance with the guidelines outlined in the Australian National Health and Medical Research Council Code of Conduct of Animals and carried out under a protocol approved by the University of Technology Sydney Animal Care and Ethics Committee, Sydney, NSW, Australia (UTS ACEC ETH16-0455).

Plasma biochemical analysis, histological assessment and measurement of lipid content in liver tissue samples

Details are in **SI Material and Methods Section.**

***In vitro* experiments and palmitate acid (PA) preparation**

Detailed descriptions of PA preparation, HepG2 cell culture and analysis of cells are provided in **SI Material and Methods Section**. Unless otherwise stated, HepG2 cells were incubated for 24 h with PA (0.5 mM) and then exposed to TEMPOL (200, 500 or 1000 μM) for 5 h. Control cells were treated with 1% w/v fatty acid-free BSA (g/v).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Prism Software;MA, USA). checked for normal distribution (Gaussian) distribution by column data analysis using Shaprio-Wilks normality test. When normality was determined, then parametric (one-way ANOVA) statistical analysis was performed. If determined to be not normal, then non-parametric tests (Kruskal-Wallis) with post-hoc multiple comparison analysis was performed. GraphPad Prism v8.00 (Graph-Pad, CA, USA) was used to complete all analyses. analyse the results. Results with $p<0.05$ were considered significant.

Results

TEMPOL treatment reversed the insulin-resistant state of HFD-fed mice and PA-treated HepG2 cells.

The HFD group showed increased blood glucose levels (by 54%, $p < 0.05$) and impaired insulin response (by 20%) when compared to the StD group (Table 1). TEMPOL treatment (10 mg kg $^{-1}$ of food) of HFD mice for 8 weeks induced a marked decrease in blood glucose levels (by 33%; ($p < 0.05$) and improved insulin sensitivity (by 18.6%; $p < 0.05$), relative to the HFD group and the control StD group.

Table 1: Insulin Sensitivity and plasma insulin secretion of C57BL/6 mice from the HFD model.

	StD	HFD	HFDStD	HFDT
ipGTT blood glucose level (mM/L)	1635 ± 126	2515 ± 63*	2216 ± 125	1558 ± 89#
ipITT Blood glucose level (mM/L)	767 ± 34	923 ± 58	831 ± 30	787 ± 26
Plasma insulin secretion (ng/ml)	104 ± 9	82 ± 7 *	117 ± 10	107 ± 12#

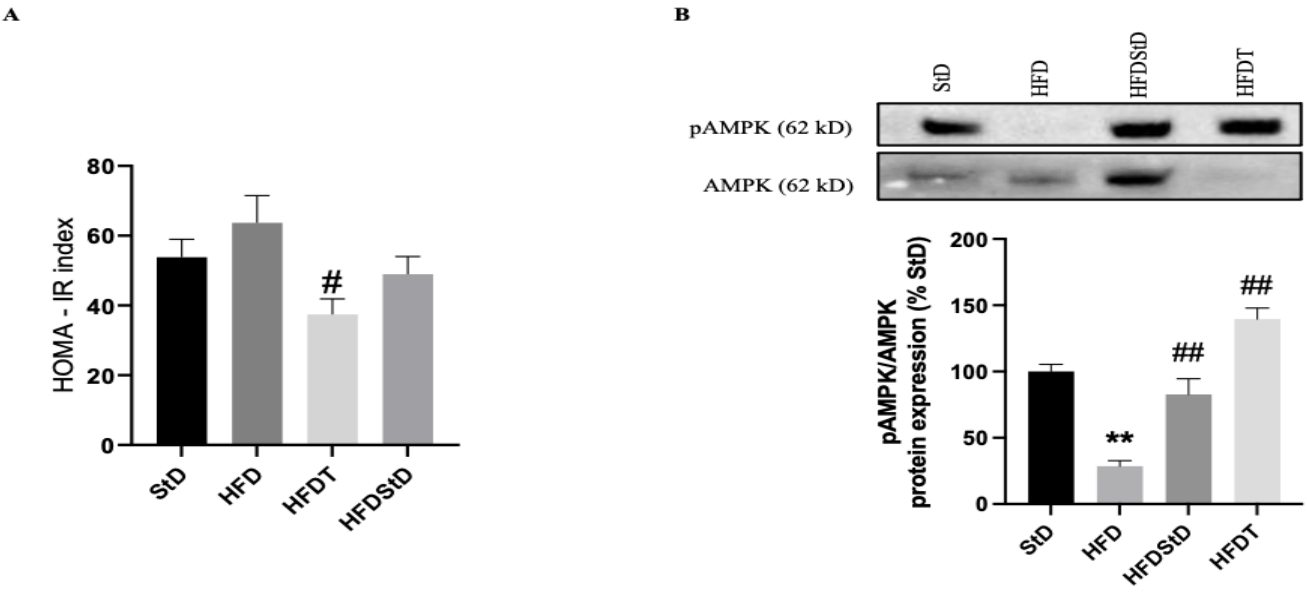
Values are mean ± SEM (n = 8-10). * p < StD, # p < 0.05 vs HFD

The HOMA-IR index, an indicator of insulin resistance, was calculated using fasting blood glucose levels and fasting insulin plasma levels [19], with the HFD group showing an 18% increase in trend in this index (Fig. 1A). Treatment with TEMPOL decreased the HOMA-IR by 41% ($p < 0.05$) relative to the HFD group and 23% relative to the HFDStD group. The final HOMA-IR index of the HFDT was not significantly different to the StD group.

To explore the effect of TEMPOL on insulin sensitivity, phosphorylated AMP-activated protein kinase (pAMPK) protein levels in liver tissues were assessed by immunoblotting. Low pAMPK protein levels indicate insulin resistance and are used as a marker of T2DM [20]. Fig. 1B shows a 41% reduction of pAMPK protein levels in the HFD group ($p < 0.001$) compared to the StD group. HFD has been previously reported to lower AMPK [21] and this is in keeping with what is shown in Fig 1B. We show that TEMPOL treatment to HFD- fed mice resulted in partially normalised AMPK levels, although not the levels observed in those that transitioned from a HFD to a standard diet. What is intriguing is that TEMPOL supplementation leads to a trend in increasing pAMPK levels by 39% compared to the StD. Additionally, TEMPOL treatment of HFD mice completely abrogated this effect, with pAMPK levels brought back to the StD mice levels ($p < 0.001$ vs HFD). It is worth noting, that there appears to be a marked impact level of (native) AMPK in the TEMPOL group. This indicates that the overall amount of AMPK protein in the cells may have decreased as a result of degradation or reduced synthesis whilst the existing pool of AMPK is being activated as indicated by the higher level of pAMPK.

In vitro experiments in PA-treated HepG2 cells were used to elucidate further TEMPOL's effects on insulin resistance. Incubation of HepG2 cells with PA (0.5 mM, 24 h) induced a a trend in decreased pAMPK protein expression levels (by 38%) relative to the vehicle group (Fig. 1C). Exposure of PA-treated cells to 200, 500 or 1000 µM TEMPOL for 5 h, restored pAMPK protein levels relative cell treated with PA alone. Serine phosphorylated IRS-1 is a marker of insulin resistance due to the inability of the insulin receptor substrate-1 (IRS-1) to bind insulin [22]. PA treatment (0.5 mM, 24 h) resulted in an increase of serine phosphorylated IRS-1 protein levels by 81% ($p < 0.001$), compared to the controls. Five hours of exposure of PA-treated HepG2 cells to 200, 500 or 1000 µM TEMPOL ablated the effect of PA ($p < 0.001$; Fig. 1C).

In hepatocytes, glucose is converted to glycogen via an insulin-dependent process [23], with this being defective in T2DM patients [24]. Fig. 1D shows insulin treatment (1 nM for 3 h) increased glycogen levels in treated HepG2 cells by 48% ($p < 0.05$) relative to the vehicle group. In contrast, PA treatment (0.5 mM, 24 h) of cells decreased insulin-activated glycogen synthesis by 18% ($p < 0.05$), an effect that was also ablated by co-treatment with TEMPOL at 200, 500 or 1000 µM for 5 h ($p < 0.001$) compared to PA treatment.


A
HOMA - IR index
80
60
40
20
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#
StD
HFD
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HFDStD
B
StD
HFD
HFDStD
HFDT
pAMPK (62 kD)
AMPK (62 kD)
pAMPK/AMPK protein expression (% StD)
200
150
100
50
0
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StD
HFD
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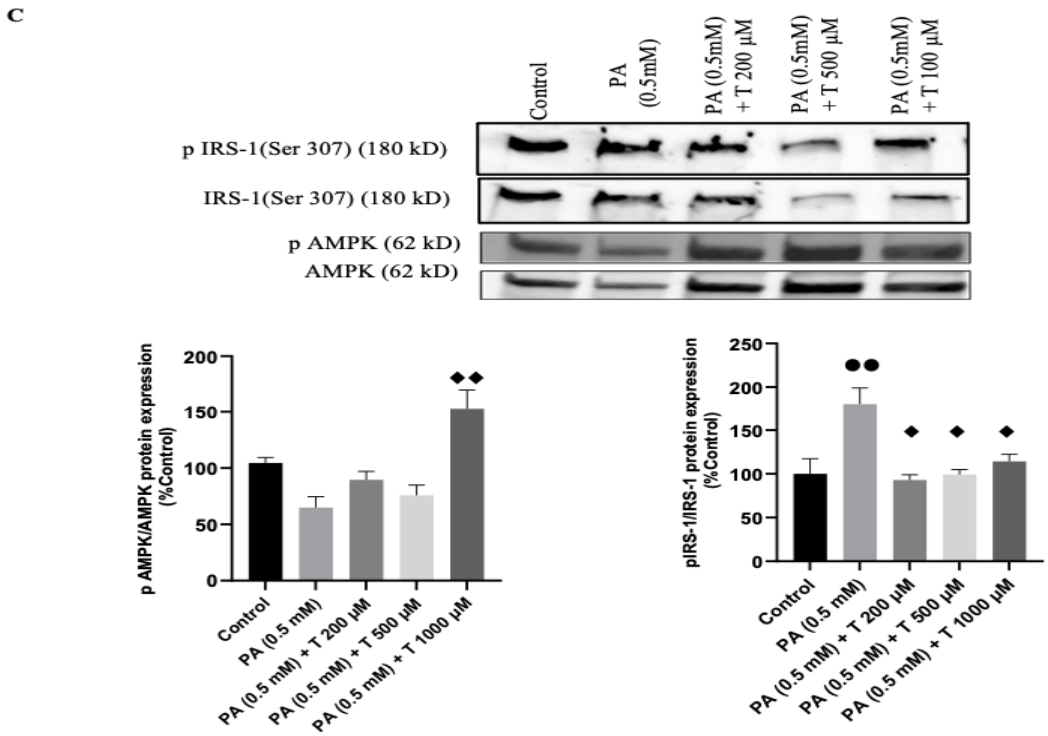
HFDT


C
Control
PA (0.5mM)
PA (0.5mM) + T 200 µM
PA (0.5mM) + T 500 µM
PA (0.5mM) + T 100 µM
p IRS-1(Ser 307) (180 kD)
IRS-1(Ser 307) (180 kD)
p AMPK (62 kD)
AMPK (62 kD)
p AMPK/AMPK protein expression (%Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 200 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM
pIRS-1/IRS-1 protein expression (%Control)
250
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 200 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

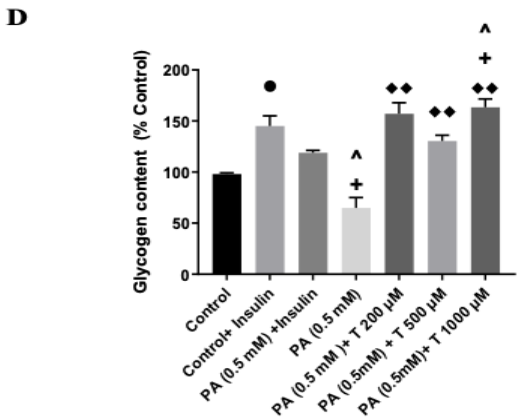

D
Glycogen content (% Control)
200
150
100
50
0
Control
Control+ Insulin
PA (0.5 mM) +Insulin
PA (0.5 mM)
PA (0.5 mM)+ T 200 µM
PA (0.5mM) + T 500 µM
PA (0.5mM)+ T 1000 µM

Fig. 1: TEMPOL improves insulin sensitivity in liver tissues and HepG2 cells.

From the HFD *in vivo* model, (A) the HOMA-IR index was calculated using the fasting insulin and blood glucose levels. (B) pAMPK protein expression level was assessed by immunoblot analysis of whole liver lysates. AMPK antibody was used as a reference (n= 8–10). Using the HepG2 *in vitro* steatosis model, (C) protein expression levels of phospho- IRS-1 (Ser 307) and pAMPK were assessed by immunoblot analysis (n= 8–10). Native AMPK and IRS antibodies were used as a reference. (D) The effect of TEMPOL in reversing insulin resistance by enhancing glycogenesis. Following treatment, intracellular glycogen levels were measured using a glycogen assay kit (n= 3-4). Results are mean ± SEM. * $p < 0.05$ & **$p < 0.001$ vs StD group, # $p < 0.05$ & ## $p < 0.001$ vs HFD group, ● $p < 0.05$ & ●● $p < 0.001$ vs control group, ♦ $p < 0.05$ & ♦♦ $p < 0.001$ vs PA.

Effect of TEMPOL treatment on hepatic inflammation

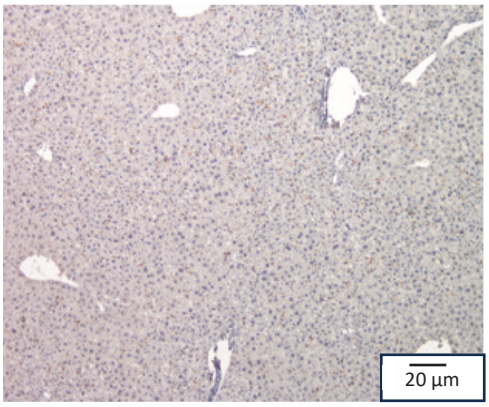
Fig. 2A shows a 35% trend in increase in macrophage cell count in liver tissue from HFD relative to the StD group, consistent with enhanced hepatic inflammation, as commonly detected in the late stages of T2DM [25]. TEMPOL treatment of HFD mice lowered macrophage numbers by 53% ($p < 0.001$) compared to the HFD group. Notably a comparable decrease (by 48%; $p < 0.01$) was observed when HFD mice were returned to StD.

These data suggest that TEMPOL might suppress the inflammatory response induced by HFD. Key proteins that trigger and/or regulate the inflammatory response in hepatocytes include NFκB and pSAPK/JNK [26, 27]. Changes in the native and phosphorylated (transcriptionally active forms of these proteins, lead to altered inflammatory signalling pathways and its response. Elevated native protein levels may indicate heightened readiness for activation, while decreased levels could suggest reduced synthesis or increased degradation. Conversely, increased phosphorylated protein levels indicate activation of the inflammatory signalling pathway, leading to inflammation or immune activation. Decreased phosphorylated protein levels signal pathway inhibition most likely through increased degradation or dephosphorylation, resulting in attenuated cellular responses. Interrogating liver tissue using immunoblot analysis showed the HFD group had 58% higher NFκB and 219% higher pSAPK/JNK protein levels ($p < 0.05$) compared to the StD group (Fig. 2B). TEMPOL-treated HFD mice showed lowered NFκB and pSAPK/JNK (76 and 74 %, respectively), compared to the HFD group ($p < 0.001$), with levels restored to the StD control. In keeping with the effect of dietary intervention on macrophage numbers, HFDStD mice showed lower NFκB and SAPK/JNK ($p < 0.05$) levels that were not significantly different from the StD group.

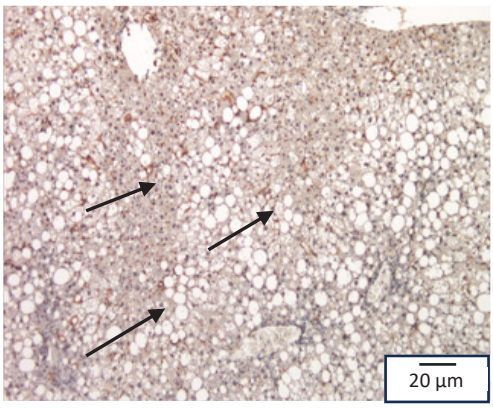
To delineate the anti-inflammatory effects of TEMPOL further, we explored TEMPOL's effect on PA-treated HepG2 cells. We explored TEMPOL's regulation of NFκB. Fig. 2 CI & CII shows PA-treated HepG2 cells (0.5 mM, 24 h) treated with TEMPOL (at 200, 500 or 1000 μM for 5 h) decreased NFκB mRNA and pNFκB/NFκB protein ratio, compared to PA-treated cells ($p < 0.001$), with levels restored to baseline controls.

Given the effects of TEMPOL on reducing HFD or PA-induced NFκB and pSAPK/JNK pathway activation, we examined if this led to reduced pro-inflammatory cytokine expression. Major cytokines associated with the inflammatory response in HepG2 cells are interleukin 8 (IL8), interleukin 6 (IL-6), interleukin 1β (IL-1β) and tumour necrosis factor (TNF) and accordingly, levels of these cytokines are high in the liver of T2DM patients with liver disease [28]. PA-treated HepG2 cells showed a trend in increased expressions of these inflammatory cytokines relative to vehicle controls (Fig. 2D), and TEMPOL exposure of PA-treated HepG2 cells reduced IL-8, IL-6, TNF, IL-1β and IL-8 mRNA levels, relative to PA alone with expression levels similar to the controls group. IL-8 protein levels showed a similar trend (Fig. 2E).

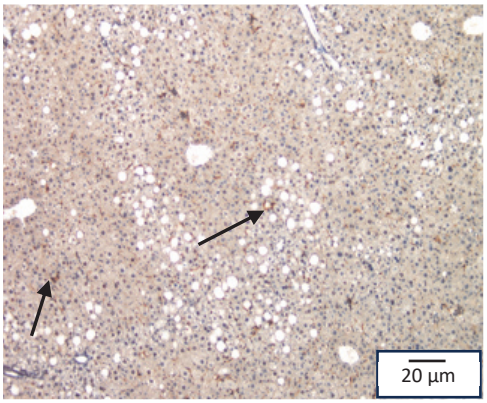
A



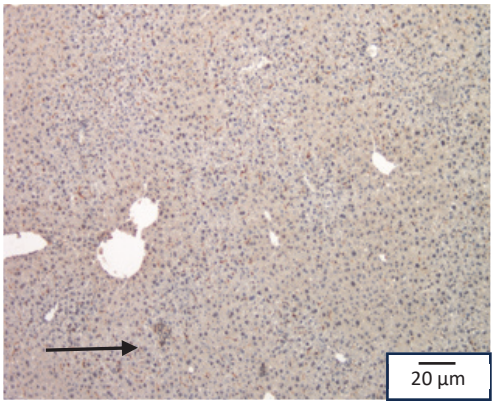
StD



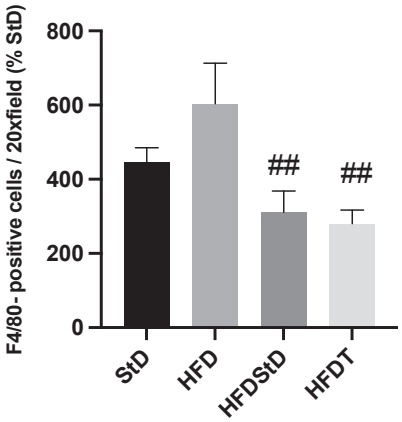
HFD



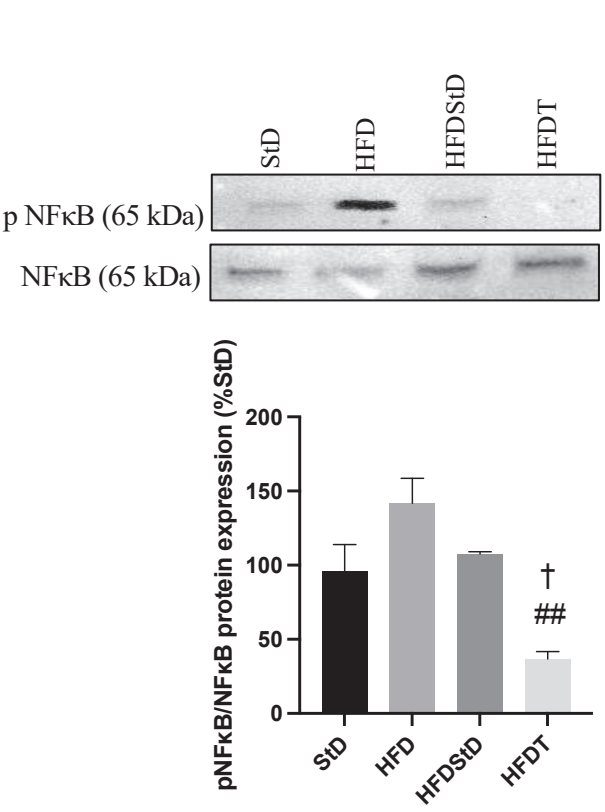
HFDStD

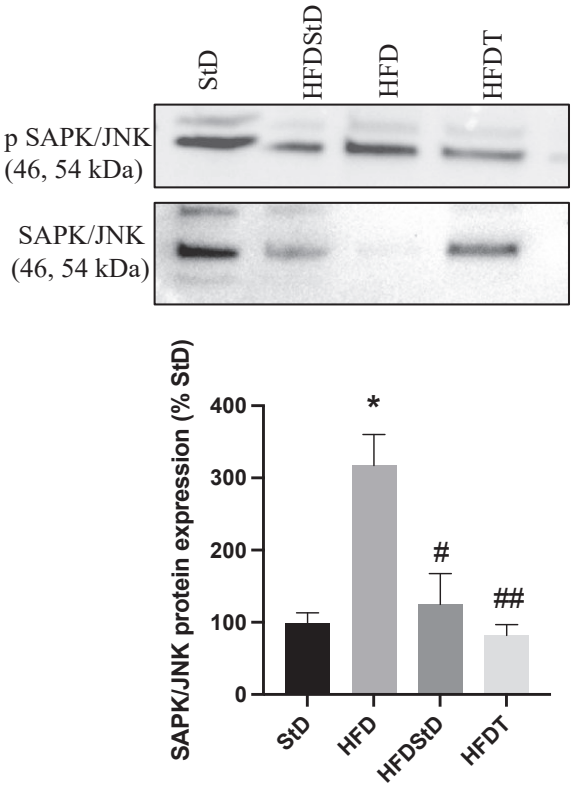


HFDT



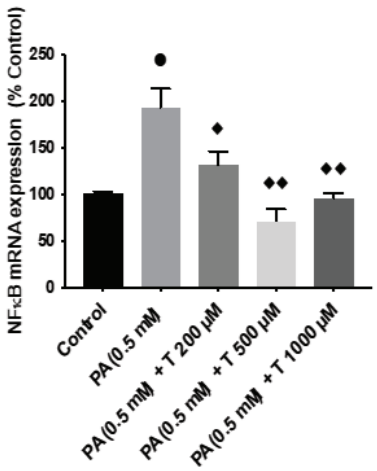
B


StD
HFD
HFDStD
HFDT
p NFκB (65 kDa)
NFκB (65 kDa)
pNFκB/NFκB protein expression (%StD)
200
150
100
50
0
†
##
StD
HFD
HFDStD
HFDT

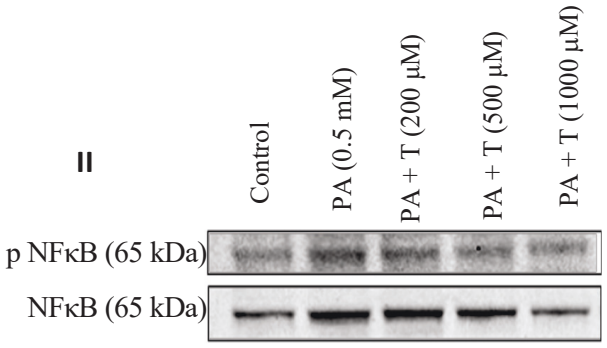

StD
HFDStD
HFD
HFDT
p SAPK/JNK
(46, 54 kDa)
SAPK/JNK
(46, 54 kDa)
SAPK/JNK protein expression (% StD)
400
300
200
100
0
*
#
##
StD
HFD
HFDStD
HFDT

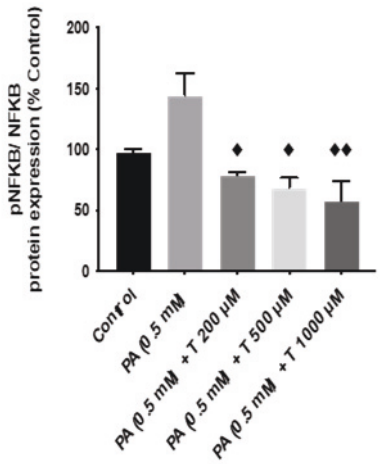
C

I


NFκB mRNA expression (% Control)
250
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 200 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

II


Control
PA (0.5 mM)
PA + T (200 µM)
PA + T (500 µM)
PA + T (1000 µM)
p NFκB (65 kDa)
NFκB (65 kDa)


pNFKB/ NFKB
protein expression (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 200 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

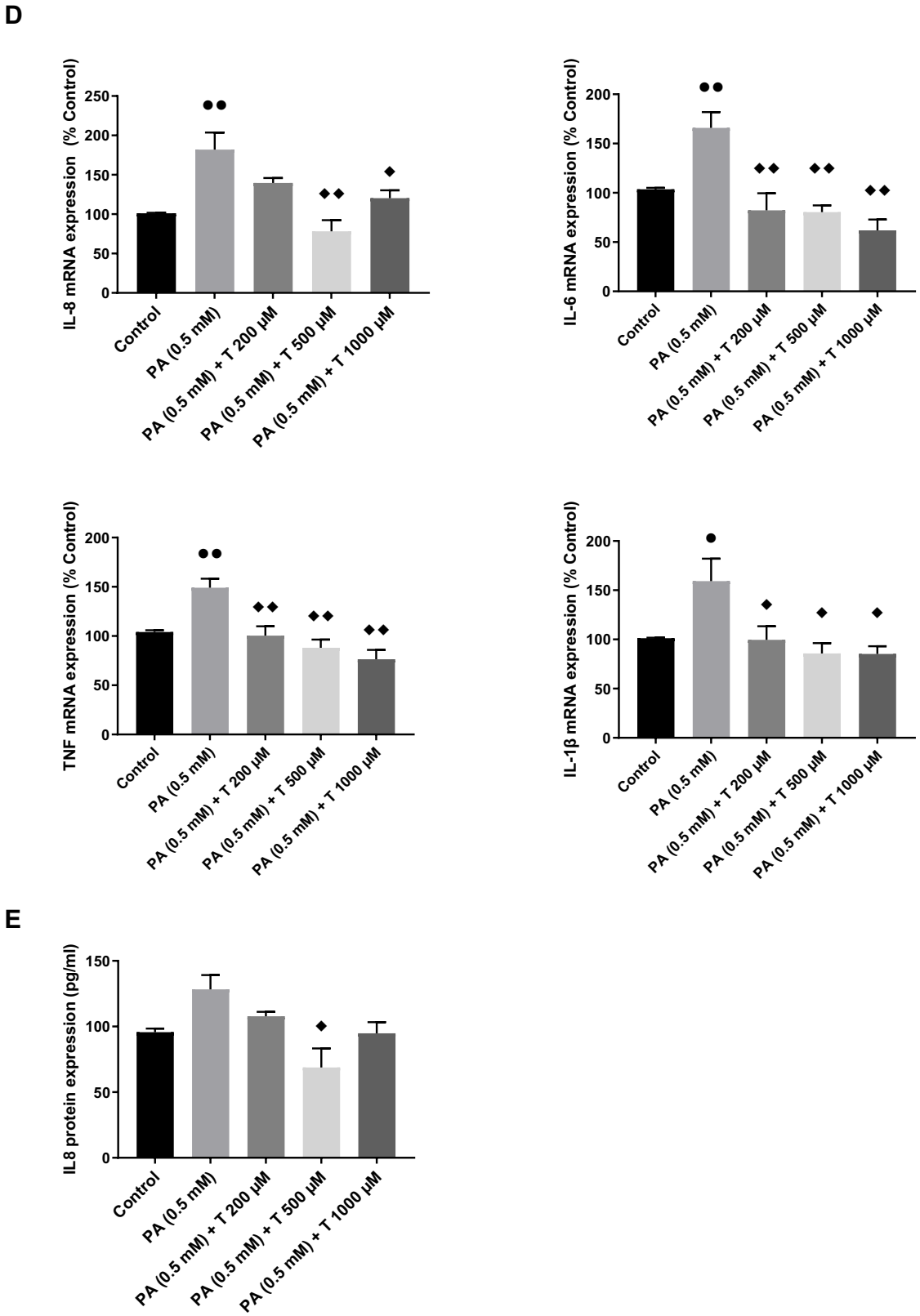


Fig 2: TEMPOL prevents chronic hepatic inflammation in the murine HFD model and in HepG2 *in vitro model*.

(A) Representative F4/80 staining of liver tissue to detect macrophage infiltration (black arrows) induced by HFD. Scale bar: 20 µm (n= 4-5 mice per group). (B) pNFκB and

pSAPK/JNK protein expressions were assessed by immunoblot analysis in whole liver lysates. NFκB and SAPK/JNK antibodies were used to calculate the ratio (n= 8–10). (C-E) HepG2 *in vitro* model was used to determine TEMPOL's effect on the pro-inflammatory transcription factor NFκB pathway in hepatic steatosis. (C) RT-qPCR was conducted to analyse (I) NFκB mRNA expression levels and (II) protein expressions assessed using immunoblot analysis. NFκB (P65) antibody was used as a reference to calculate the ratio (n= 4-5). (D) RT-qPCR was conducted to analyse IL-8, IL-6, TNF, and IL-1β mRNA expressions derived from HepG2 cells treated with PA-TEMPOL treatment protocol (n= 5-6). (E) The effect of TEMPOL on IL-8 cytokine release was determined using an IL-8 cytokine-specific ELISA (n= 5–6). Results are mean ± SEM. * $p < 0.05$ & ** $p < 0.001$ vs StD group, # $p < 0.05$ & ## p 0.001 vs HFD group, ✝ $p < 0.05$ vs HFDStD, ● $p < 0.05$ & ●● $p < 0 .001$ vs control group, ♦ $p < 0.05$ & ♦♦ $p < 0.001$ vs PA, ^ $p < 0.05$ vs control +insulin, + $p < 0.05$ vs PA + insulin.

Post-TEMPOL treatment protects PA-induced oxidative stress in HepG2 cells

PA-treated HepG2 (0.5 mM, 24 h) showed increased oxidant levels (by 30%) when compared to vehicle-treated cells ($p < 0.001$, Fig. 3A). TEMPOL treatment reduced these levels back to control values ($p < 0.001$). TEMPOL exposure of PA-treated cells reduced mitochondrial protein cytochrome c (cyt c) levels. An increase in cyt c levels is a well-recognised marker of mitochondrial oxidative stress and apoptosis [29], as confirmed by PA treatment shown in Fig. 3B. TEMPOL exposure to PA-treated cells reduced ($p < 0.001$, Fig. 3B) mitochondrial protein cyt c levels.

Effect of post-TEMPOL treatment on cellular apoptosis

As acute inflammatory responses, coupled with elevated oxidant levels, are strongly associated with apoptosis [30], we reasoned that TEMPOL might reduce hepatocyte apoptosis. Protein levels of pAKT and FOXO1, which are key apoptotic signalling proteins, were assessed with Fig. 4A showing a higher pAKT and FOX01 protein expression levels in the liver of HFD, compared to the control (StD) group ($p < 0.05$). TEMPOL treatment significantly reduced pAKT protein levels (by 65%; $p < 0.001$ vs HFD); FOXO1 levels were also diminished (by 36%) but this decrease was not statistically significant.

PA-treated cells (0.5 mM, 24 h) when exposed to 1000 μM TEMPOL (5 h) had significantly lower pAKT levels ($p < 0.05$, Fig. 4B), relative to PA-controls. To determine if the pAKT result correlated with improved HepG2 cell metabolic activity and representative viable cells, an MTT assay was performed (Fig. 4C). HepG2 cells exposed to only to TEMPOL (at 200, 500 or 1000 μM, 24 h) had increased metabolic activity ($p < 0.001$) compared to the PA-treated group.

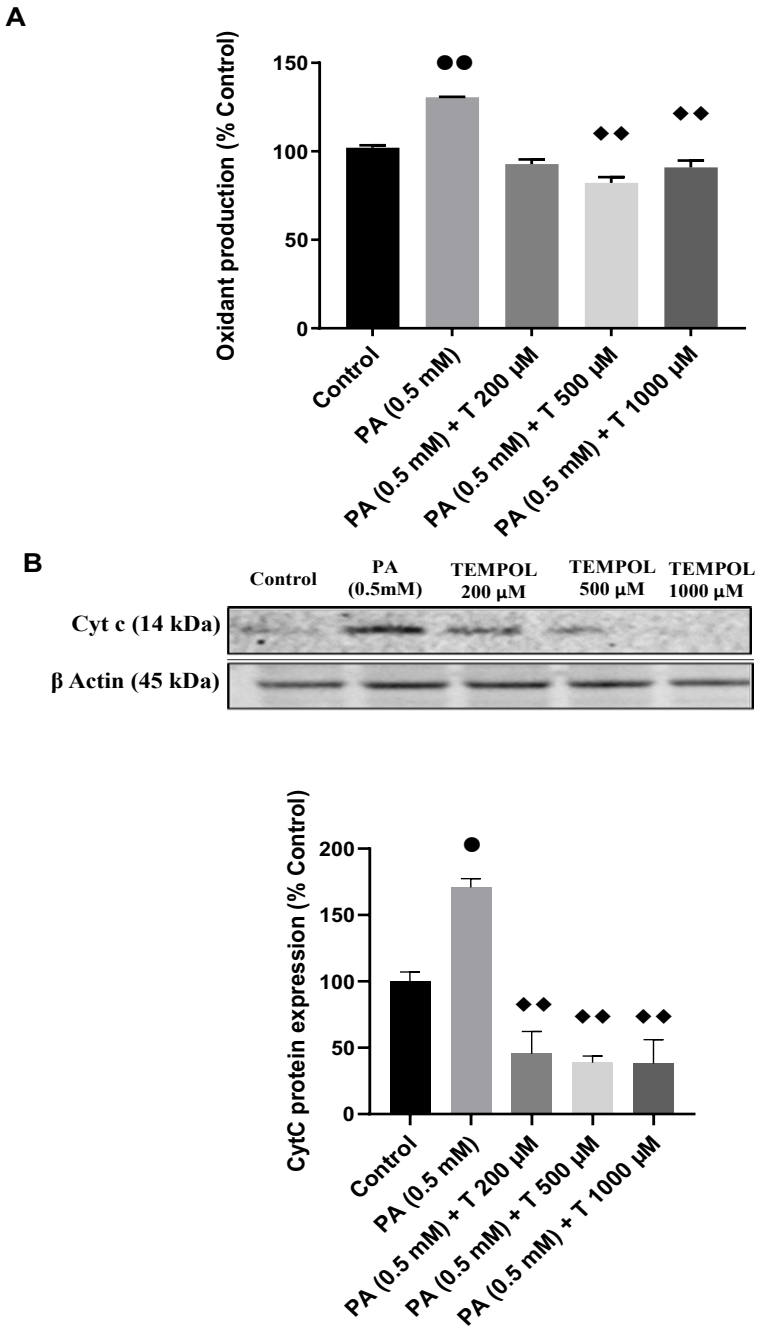


Fig 3: TEMPOL attenuates mitochondrial oxidative stress in HepG2 cells.
(A-B) HepG2 cells were treated with PA (0.5 mM) for 24 h, followed by TEMPOL treatments (200, 500, or 1000 µM) for 5 h. (A) Intracellular oxidant levels were assessed with a DCFH-DA assay (n = 4-5). (B) The effect of TEMPOL on the generation of mitochondrial ROS promoter proteins such as cytochrome c protein level was assessed using immunoblot analysis. β-Actin antibody was used as a reference to calculate the ratio (n= 4–5). Results are mean ± SEM. ● $p < 0.05$ & ●● $p < 0.001$ vs control group, ♦♦ $p < 0.001$ vs PA.

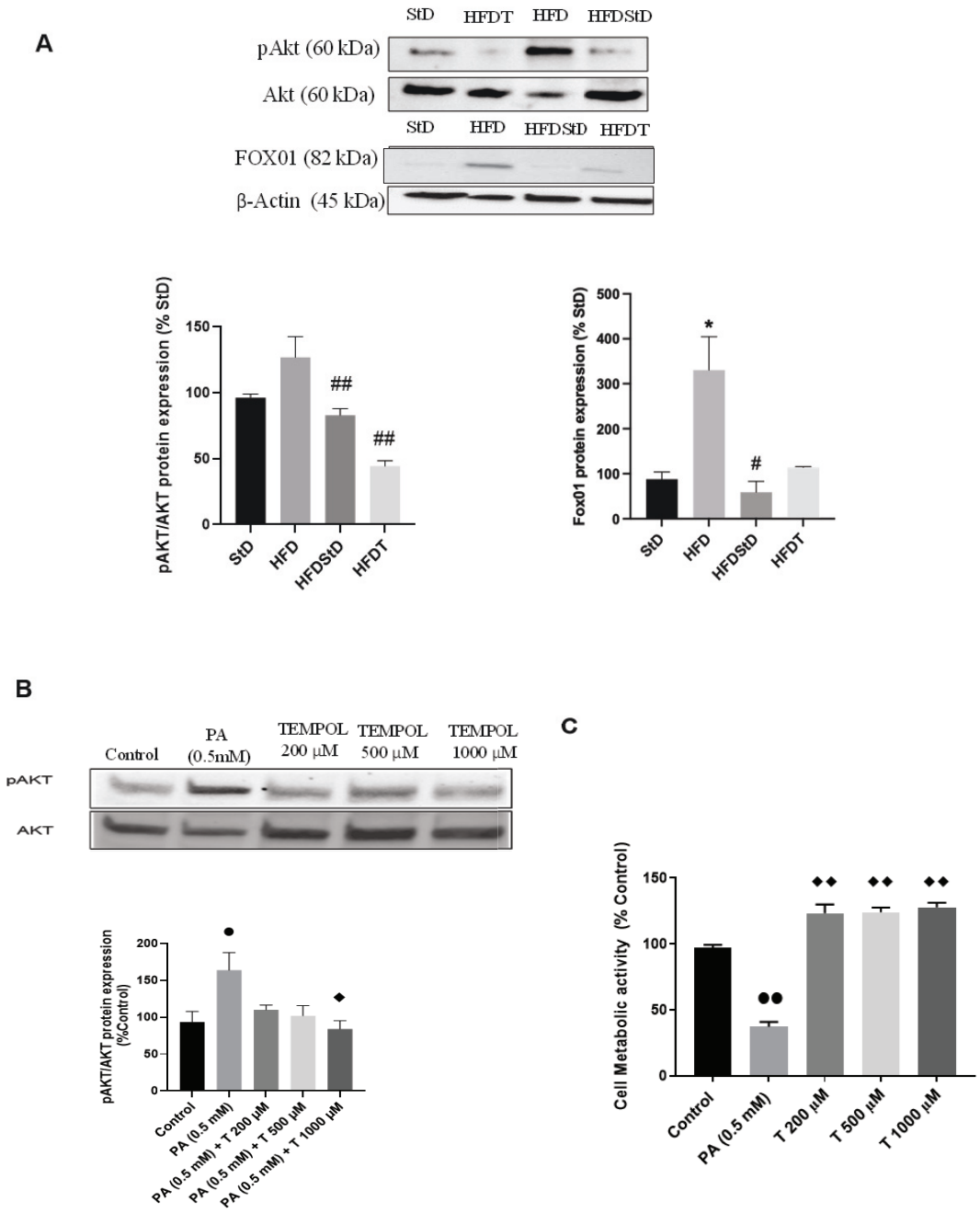


Fig 4: TEMPOL hinders cellular apoptosis in liver tissues and in HepG2 cells.
(A) pAKT and FOX01 apoptotic mediators were assessed using immunoblot analysis derived from the whole liver tissue lysate from the HFD model. β-Actin antibody was used as a reference to calculate the ratio of FOX01. Native AKT was used as a reference to calculate the ratio with pAKT (n = 8–10). (B) pAKT protein expression levels derived from HepG2 cells treated with PA-TEMPOL treatment protocol (200, 500, or 1000 µM). Native AKT was used as a reference to calculate the ratio (n= 4-5). (C) An MTT assay was used to assess the cell metabolic activity of HepG2 cells (n= 4-5). Results are mean ± SEM. * $p < 0.05$ & ** $p < 0.001$ vs StD group, # $p < 0.05$ & ## $p < 0.001$ vs HFD group, ● $p < 0.05$ & ●● $p < 0.001$ vs control group, ♦ $p < 0.05$ & ♦♦ $p < 0.001$ vs PA group.

Effect of post-TEMPOL on hepatic lipid accumulation

Lipid accumulation is often associated with elevated inflammatory responses, oxidative stress and hepatocytes apoptosis [31]. Consequently, we examined if TEMPOL decreased lipid accumulation in the liver of HFD-fed mice. Fig. 5A shows that the intracellular lipid content of liver tissues, as measured by Oil Red O (ORO) staining, was significantly reduced in the TEMPOL-treated HFD group ($p < 0.001$). This decrease in lipid accumulation correlated with a 28% reduction in liver mass ($p < 0.001$, Fig. 5B), and an overall reduction in body mass by 71% ($p < 0.05$, Fig. 5C), relative to the HFD group.

ORO staining of HepG2 cells treated with PA-treated (0.5 mM, 24 h) showed a 27% increase in cytosolic lipid droplets (Fig. 5D), compared to the vehicle control group ($p < 0.001$). TEMPOL (5 h) significantly reduced the number of lipid droplets in a dose-dependent fashion ($p < 0.001$).

Effect of post-treatment of TEMPOL on liver function and histology

HFD and its associated lipid pathology drive liver dysfunction, which is commonly measured by the ratio of aspartate aminotransferase (AST) and alanine aminotransferases (ALT), with the former being localised to mitochondria and the cytosol of hepatocytes, whereas ALT is solely cytosolic. An AST: ALT ratio >1 indicates liver dysfunction [32], as detected for the HFD group ($p < 0.05$ vs StD; Fig. 6A). Treatment with TEMPOL restored this ratio to < 1 in keeping with the value measured for the StD group ($p < 0.05$ vs HFD). In contrast, the AST: ALT ratio remained > 1.0 in the diet intervention group.

β-hydroxybutyrate (β-OHB) is an additional indicator of liver dysfunction, with the liver releasing β-OHB as an alternative fuel source to glucose. Patients with hepatic steatosis and non-alcoholic liver disease (NAFLD) have lower β-OHB levels as fat accumulation in the liver suppresses β-oxidation resulting in lowered ketone production, hence lowering plasma β-OHB levels [33]. Fig. 6B shows that HFD mice had lower plasma β-OHB levels; these were restored (by 59%) by TEMPOL treatment when compared to the HFD ($p < 0.05$).

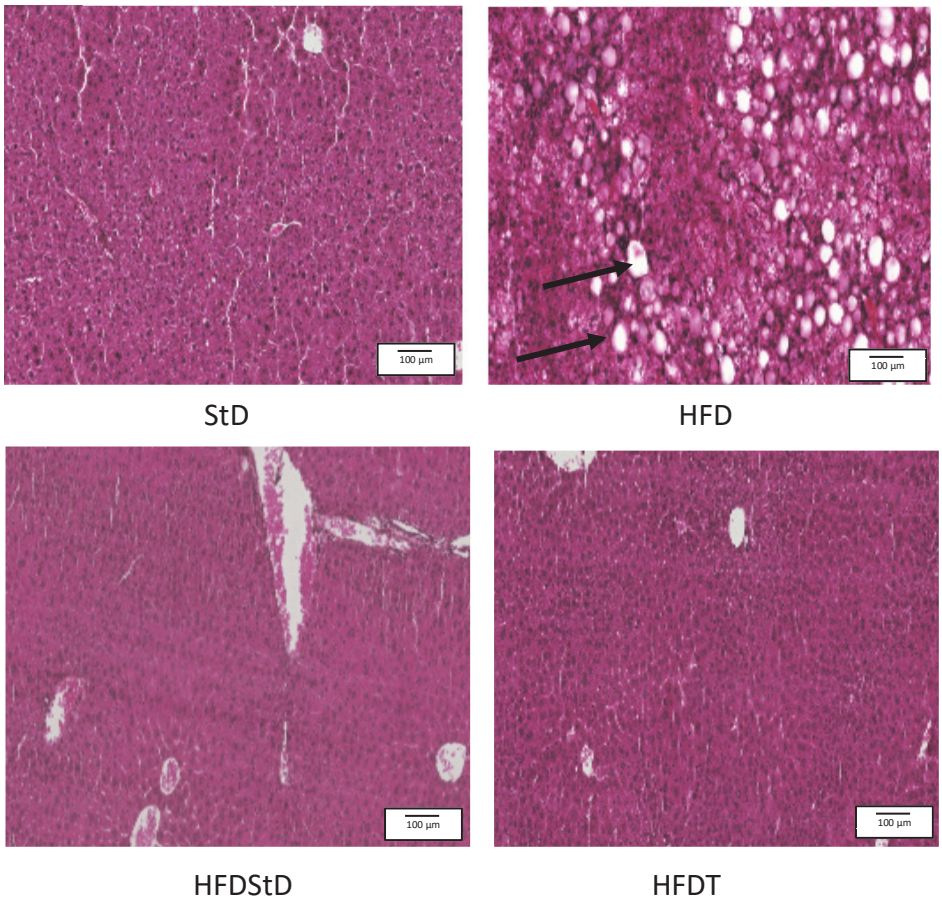
Liver fibrosis is a commonly reported adverse effect of chronic HFD. Fig. 6C shows the relative area of fibrosis and the histology of the liver tissues stained by Sirius Red stain. The stain highlights fibrotic regions by distinct alterations in histoarchitecture indicative of collagen deposition. These fibrotic regions appear thicker and more densely packed compared to surrounding tissue, creating a mesh-like network that disrupts the normal hepatic architecture.

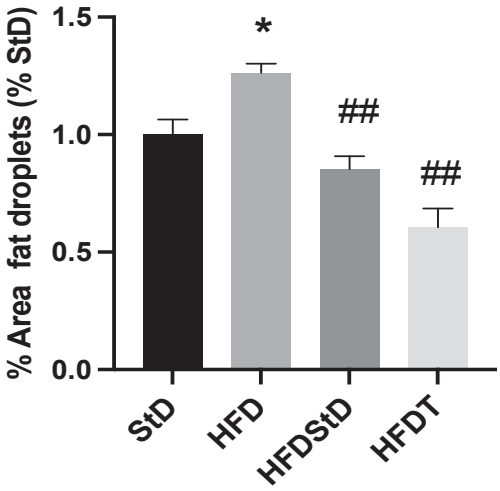
This is noted in both the HFD and HFDStD groups which consequently show a significant increase in fibrotic area relative to the StD group (139% and 182%, respectively; $p < 0.05$). TEMPOL treatment of the HFD-fed mice markedly reduced the fibrotic area by 44% compared to the HFD or HFDStD mice ($p < 0.05$).

Effect of pre-treatment of TEMPOL

The above data indicate that TEMPOL can modulate inflammatory and oxidative stress responses in HepG2 cells pre-activated by PA treatment (0.5 mM, 24 h). To explore whether TEMPOL could block PA-induced insulin resistance, inflammatory and oxidative stress responses, HepG2 cells were pre-exposed to TEMPOL (500 μM, 1 h) before PA exposure (0.5 mM, 5 h). Fig. 7A shows that PA-treated HepG2 cells had a trend in reduced insulin-induced glycogen levels (although not significantly different to the no PA-insulin-induced controls), and that pre-exposure of TEMPOL before PA-treatment restored glycogen levels to those measured for the insulin-only treated group. Further, cells pre-exposed to TEMPOL showed 47% lower mRNA levels of the rate-limiting enzyme of the gluconeogenesis pathway, phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 7B). Pre-exposure of cells to TEMPOL before PA treatment also prevented increased IL-8 mRNA levels (Fig. 7C). Furthermore, oxidant levels were diminished by extensive pre-treatment with TEMPOL before PA treatment ($p < 0.001$) (Fig. 7D).

A


100 µm
StD
HFD
HFDStD
HFDT


% Area fat droplets (% StD)
1.5
1.0
0.5
0.0
*
##
##
StD
HFD
HFDStD
HFDT

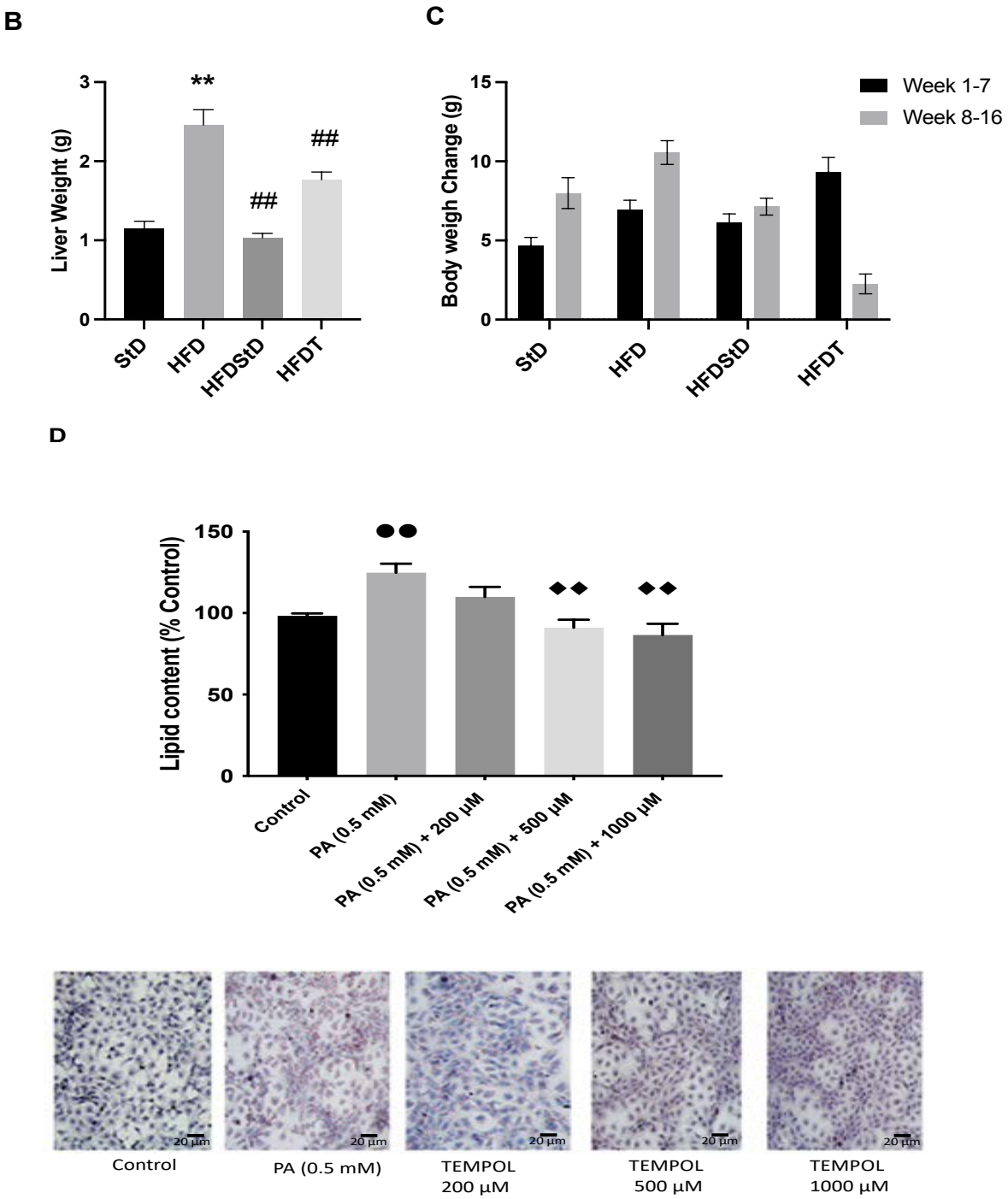


Fig 5: TEMPOL reduces lipid accumulation in liver tissue and in HepG2 cells.
(A) Representative H&E staining of liver tissue to detect and assess hepatic steatosis and its reversal/ protective effect by TEMPOL. Black arrows indicate lipid storage. Scale bar: 100 µm. (n=9-10). (B) Liver weights were then recorded at the end of the 16th week of the study. (C) The body weight change of C57BL/6 mice was recorded before and after the diet intervention (n = 8–10). From the in vitro HepG2 model (D) Representative Oil red O (ORO) staining was done to assess lipid droplets (stained red). Scale bar: 10 µm was. Dissolution of the ORO stain was conducted, to quantify lipid content in HepG2 cells. Results are mean ± SEM (n = 5-6). * $p < 0.05$ & ** $p < 0.001$ vs StD group, # $p < 0.05$ & ## $p < 0.001$ vs HFD group, ● $p < 0.05$ & ●● $p < 0.001$ vs control group, ♦ $p < 0.05$ & ♦♦ $p < 0.001$ vs PA group.

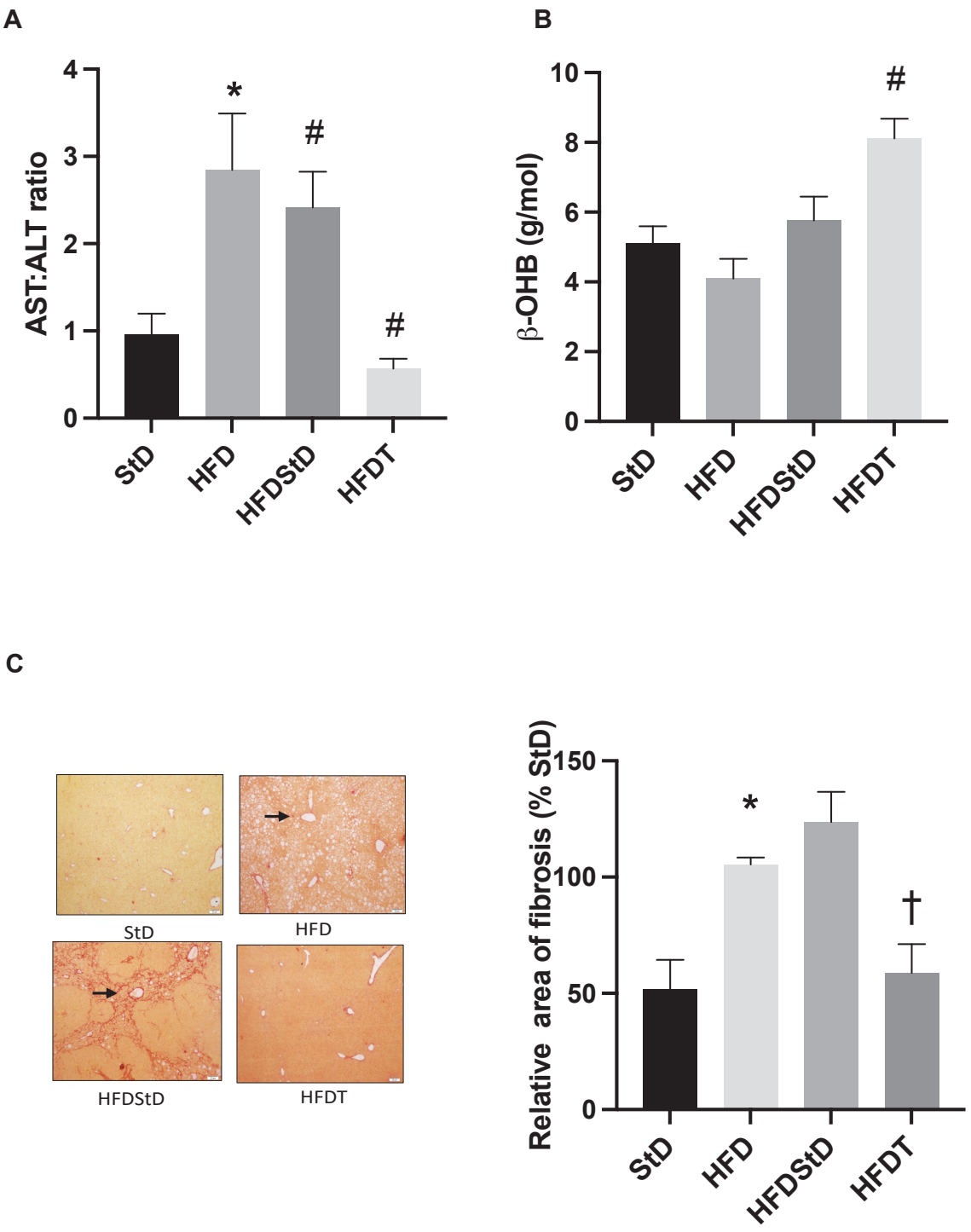


Fig 6: TEMPOL prevents chronic hepatic damage induced by HFD.
Serum harvested on the 16th week of the study was used to quantify (A) AST: ALT ratio and (B) β hydroxybutyrate levels to assess HFD-induced hepatocellular damage and its impact on ketogenesis (n= 8-10). (C) Representative Sirius red staining to assess the relative area of fibrosis. Scale bar: 20 µm. (n= 8-10) The black arrow indicates liver fibrosis. Results are mean ± SEM. * $p < 0.05$ vs StD group, # $p < 0.05$ vs HFD group, † $p < 0.05$ vs HFDStD.

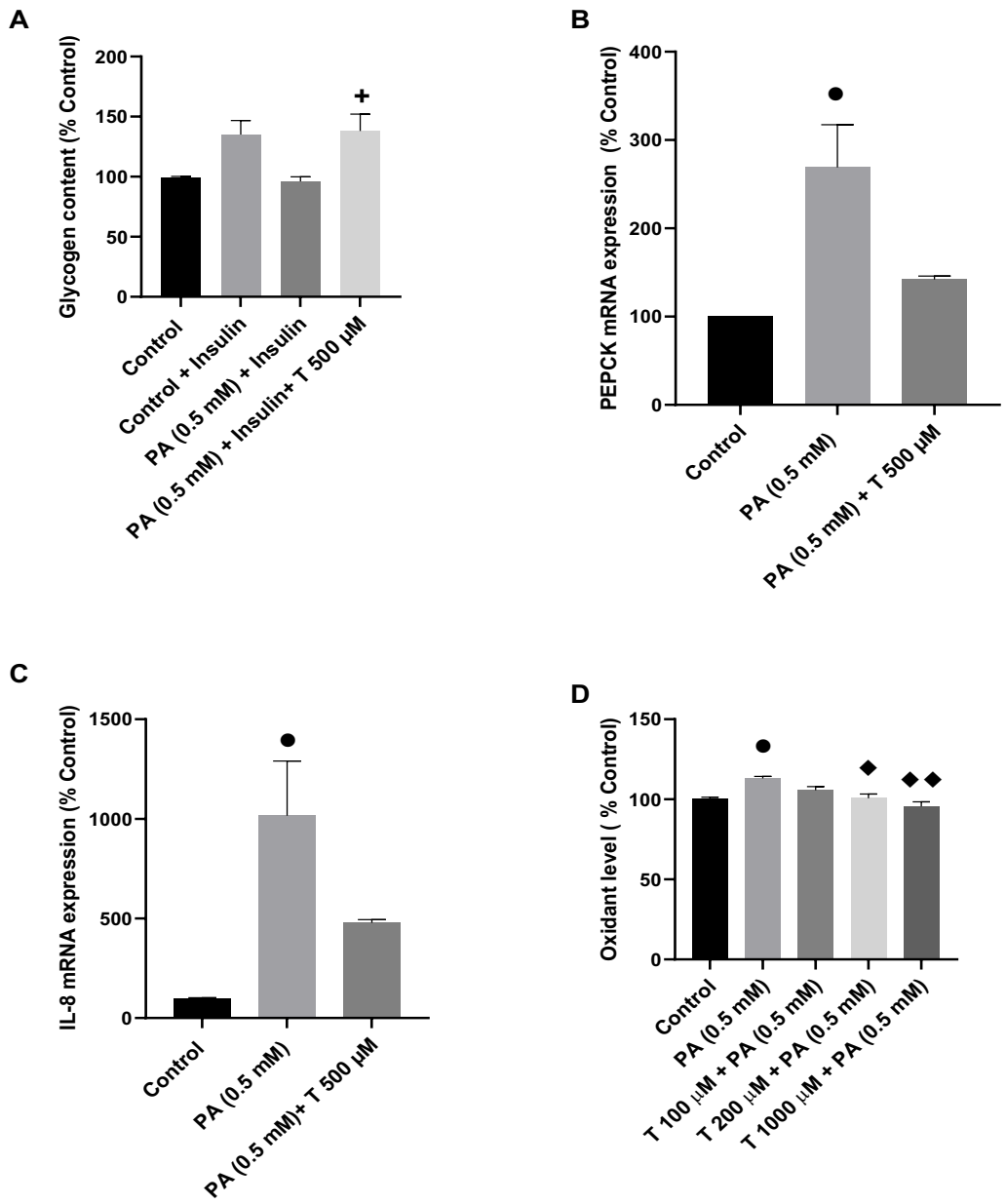


Fig 7: To determine if TEMPOL has pre-clinical relevance in treating insulin resistance in hepatocytes.

HepG2 cells were pre-treated with TEMPOL (500 or 1000 µM for 1 h) followed by PA (0.5 mM) + insulin treatment for 24 h. (A) Intracellular glycogen levels were measured using a glycogen assay kit (n= 4-5). Total RNA was extracted from HepG2 cells pre-treated with TEMPOL (500 µM for 1 h) + PA (0.5mM) for 5 h. RT-PCR was performed to analyze (B) PEPCK and (C) IL-8 mRNA expression levels of (n= 4-5). (D) Oxidant production levels of pre-treated HepG2 cells (TEMPOL 100, 200, or 1000 µM for 16 h) followed by PA (0.5 mM) treatment for 3 h (n= 4-5). Results are mean ± SEM. ● $p < 0.05$ vs control group, ♦♦ $p < 0.001$ vs PA, + $p < 0.05$ vs PA + insulin.

Discussion

This study shows that TEMPOL, an antioxidant, anti-inflammatory and anti-obesity agent, reduces insulin resistance in an HFD mouse model, and indicates that TEMPOL achieves these effects via its ability to suppress the inflammatory and oxidative response in hepatic tissue *in vivo*, and hepatocytes *in vitro*. The data clearly demonstrate that TEMPOL protects against insulin resistance not only via its anti-inflammatory, antioxidant and anti-apoptotic effects but also by blocking hepatic lipid accumulation in hepatocytes, a known trigger for cellular stress. Together, our findings provide a strong impetus for further study of TEMPOL as a treatment for insulin resistance and hepatic steatosis, especially the pathology associated with a high-fat Western diet.

A significant finding of this study is that TEMPOL treatment improves HFD-associated insulin resistance. In our HFD mouse model, 8 weeks of TEMPOL treatment completely abrogated the insulin resistance induced as shown by the ipGTT and ipITT data, as well as the lowered HOMAR-IR index. The dose used here is consistent in with those used *in vivo* to show the anti-obesity and cancer treatment effects [13, 14]. The improvement of TEMPOL on insulin resistance is similar to that shown in HFD mouse models treated with the gold standard, metformin [34] and other clinical drugs, including such as thiazolidinediones and sulphonylureas [35].

The TEMPOL treatment improvement in insulin resistance was most likely mediated by increased levels of phosphorylated pAMPK with a concomitant reduction in serine phosphorylation of IRS-1. Current pharmacological and hormonal therapies specifically target pAMPK to increase insulin sensitivity [36, 37]. Our findings suggest a potential use of TEMPOL as an insulin sensitiser. Further to this, excess nutrient level-driven obesity induces S6 kinase 1 (S6K1) activity leading to phosphorylated IRS receptor and subsequent inhibition of the insulin signalling pathway [38]. Our findings for TEMPOL with respect to IRS-1 suggest it could be developed towards an effective therapeutic to inhibit the negative feedback role of Ser/Thr IRS-1 activation [39].

TEMPOL's effects on restoring glycogen synthesis and reducing PEPCK levels could also underlie improved insulin resistance. Defective glycogen synthesis (up to 50%) is seen in patients with T2DM resulting in hyperglycemia [40]. Gao *et al*. has previously demonstrated that insulin-stimulated glycogen storage is impaired in PA-treated HepG2 cells [41]. Our data

extended this finding and show that the effects of PA can be reversed by both pre-and post-TEMPOL treatment. PEPCK, a rate-controlling gluconeogenic enzyme associated with hyperglycemia in diabetic and obese patients [42, 43], was also lowered in PA-treated cells by TEMPOL.

TEMPOL, in addition to restoring insulin sensitivity and glucose metabolism, also markedly reduced hepatic lipid accumulation. Our study showed that TEMPOL treatment for 8 weeks reduced the body mass of HFD-fed mice. Previously, TEMPOL has been evaluated for toxic adverse effects in mice, with no issues recognised [13]. The decrease in body mass induced by TEMPOL supplementation is associated with reduced liver mass and lipid droplet content in liver tissues compared to the HFD group, and *in vitro* we showed reduced lipid dysregulation in HepG2 cells. Our findings are supported by Jiang *et al.* who detected reduced hepatic lipid droplets in TEMPOL-treated mice fed an HFD [44]. In this study, the bile acid composition was altered, with a significant increase in conjugated bile acid metabolites that reduced intestine farnesoid X receptor (FXR) activation, which is known to modulate lipid metabolism. Similarly, Li et al. showed TEMPOL therapy modifies the gut microbiome by lowering *Lactobacillus*, hence decreasing bile salt hydrolase resulting in the rise of tauro- β-muricholic acid, a substrate of bile salt hydrolase and a farnesoid X receptor antagonist. TEMPOL was thought to exert its anti-obesity benefits by blocking in-intestinal FXR via elevated tauro- β-muricholic acid level [15]. This provides a mechanism for the altered lipid metabolism that is independent of the antioxidant / anti-inflammatory effects of TEMPOL. Together, the findings support an anti-obesity effect of TEMPOL, at least with respect to lipid accumulation in liver tissue.

In keeping with the diminished lipid accumulation, TEMPOL also stimulated β-OHB levels. β-OHB is an emerging therapeutic target for preventing liver injury and body mass loss due to its anti-lipogenic effect [45]. β-OHB also mimics caloric restriction, increasing life span and modulating age-related disease in rodents and non-vertebrates [46]. Increased levels of β-OHB can increase the expression of carnitine palmitoyltransferase-1 (CPT-1), a key regulatory enzyme in fatty acid β-oxidation [46]. Therefore, increasing the levels of β-OHB can have a beneficial anti-lipogenic effect in the liver, as it has been shown to reduce lipid accumulation in both *in vivo* and *in vitro* studies [46]. Our data showed a significant increase in the levels of

β-OHB in the TEMPOL-treated mice group, which may explain why this group had decreased body mass without caloric restrictions.

Reduced hepatic lipid accumulation in response to TEMPOL reduced stress pathways, including inflammatory and oxidative stress responses as well as hepatocyte apoptosis. Previous *in vivo* and i*n vitro* studies have shown that an increase in systemic saturated fatty acid is a major initiator of hepatic inflammation [26, 47]. Wang et al. reported exacerbation in obesity-induced inflammation and insulin resistance in Sprague Dawley rats within 8 weeks on a HFD diet [47]. Moreover, Eser et al. reported an increase in the pro-inflammatory cytokine IL-8 levels in 18 obese men who consumed shakes containing saturated fats [48]. An association between inflammatory cytokine status and insulin resistance is common in humans [49, 50]. Inflammation is driven by an unbalanced activation of inflammatory transcription factors, such as NFκB and its activator IKK-β, which together activate the expression of a multitude of pro-inflammatory cytokines [25, 27]. Yuan et al reported that heterozygous deletion of IKK $^{-/-}$ in HFD-fed Lep$^{ob/ob}$ mice protected them from insulin resistance [51]. Our HFD *in vivo* and HepG2 *in vitro* data reveal that both HFD and PA treatment increased inflammatory status via upregulation of NFκB, SAPK/JNK, and IKK-β, followed by the augmented expression of IL-8, IL-6, TNF, and IL1-β. TEMPOL decreased the expression of these inflammatory transcription factors and pro-inflammatory cytokines expression levels in both our models, with the additional information obtained from the *in vitro* model, that both post- and pre-treatment with TEMPOL had anti-inflammatory effects. Other common anti-diabetic drugs, including metformin, thiazolidinedione (TZD), and salicylate [52], are effective, at least in part, through anti-inflammatory effects on NFκB associated pathways.

Enhanced oxidant formation can damage cells and stimulate the NFκB and JNK pathways, triggering inflammation and defective insulin signalling [53, 54]. TEMPOL mimics the key antioxidant enzyme SOD and is highly membrane-permeable in both *in vitro* and *in vivo* models [55-58]. In our *in vitro* HepG2 model, pre- and post-TEMPOL treatment reduced oxidant levels, as measured by the generic, non-specific, oxidation marker DCF-DA. Furthermore, TEMPOL is known to mitigate oxidative damage to mitochondrial components, including lipids, proteins, and DNA, which can occur during oxidative phosphorylation [59]. It follows then that TEMPOL treatment inhibits various biomarkers associated with oxidative stress. This includes reducing lipid peroxidation markers such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), preventing protein carbonylation, lowering levels of DNA oxidation biomarkers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), and maintaining

mitochondrial membrane potential [60, 61]. Overall, TEMPOL's ability to scavenge ROS contributes to the preservation of mitochondrial function and cellular homeostasis under conditions of oxidative stress [62]. Our results show with post-TEMPOL treatment reduced cytochrome C (cyt C) levels, a critical protein that controls redox signalling in mitochondrial oxidative phosphorylation [63-65]. Studies have shown that increased oxidant formation can lead to the dephosphorylation of cyt C, which then binds to apoptotic protease activating factor-1 (APAF1), leading to the activation of caspase 3 and 9 pathways initiating cellular apoptosis [66]. We showed that TEMPOL effectively suppressed such redox signalling. TEMPOL has been reported to diminish oxidant levels in hypertensive rat models [67, 68] and inflammatory injuries in mouse models [69]. Ranjbar et al. [70] reported a decrease in endogenous antioxidants (catalase, SOD and glutathione) in diabetic mice with ischemic kidney injury, which was reversed by TEMPOL treatment, resulting in an improvement in diabetic kidney function.

Oxidative stress can induce cellular apoptosis [71]. In the current study, phosphorylated protein kinase B (pAKT) and forkhead box protein -1 (FOXO1) protein expression levels increased in the liver tissue of the HFD-fed mice. Phosphorylated AKT triggers cellular stress signalling, initiating a feed-forward signalling loop that promotes increased mitochondrial oxygen consumption and oxidant production [72]. Moreover, AKT activation leads to oxidant-mediated cellular apoptosis via the FOXO1 pathway [73, 74]. The FOXO1 pathway induces apoptosis by regulating Bim, BCL-6 and Fas Ligand transcription genes. TEMPOL supplementation lowered pAKT and FOXO1 protein expression levels, thus inhibiting this apoptosis signalling cascade. Similar results were observed in the HepG2 *in vitro* model, where the PA-induced pAKT protein expression levels were lowered by TEMPOL treatment.

In our study, TEMPOL effectively reduced hepatic lipid accumulation, inflammation, oxidative stress and apoptosis and improved liver functions. The AST and ALT ratios in the *in vivo* HFD group were markedly increased (ratio > 1), indicating structural liver damage, with the TEMPOL treatment reversing the effect (ratio < 1). Furthermore, histological assessments showed that TEMPOL treatment reduced HFD-induced hepatic fibrosis. Currently, metformin and TZD are used clinically to treat liver damage [75]. Metformin is widely used, with its main site of action being the liver, due to its ability to reduce gluconeogenesis and activate pAMPK, resulting in the switching off of lipogenesis and increasing fatty acid β-oxidation [76]. TEMPOL appears to have similar properties, including reducing gluconeogenesis by reducing

PEPCK levels, increasing pAMPK levels and increasing β-oxidation in the liver. Together, these data suggest that TEMPOL has potential beneficial roles in preventing or treating liver damage in T2DM patients while simultaneously modulating insulin resistance.

In summary, TEMPOL reverses HFD-induced insulin resistance and could be considered for development as a therapy to increase insulin sensitivity and treat hepatic steatosis. TEMPOLs' has been used to reduce radiation-induced hair loss in humans [77] and is in clinical trials to treat COVID-19 [56], prostate cancer [78, 79] and fibrocystic disease in humans [80]. Our results, although pre-clinical, suggest that this drug, which is already in clinical use and has existing safety data, could be used to treat insulin resistance.

Declarations

Funding statement

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Conflicts of interest

MJD declares commercial consultancy contracts with Novo Nordisk A/S. This funder has no. role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish these results. The other authors declare no conflict of interest with regard to the data presented.

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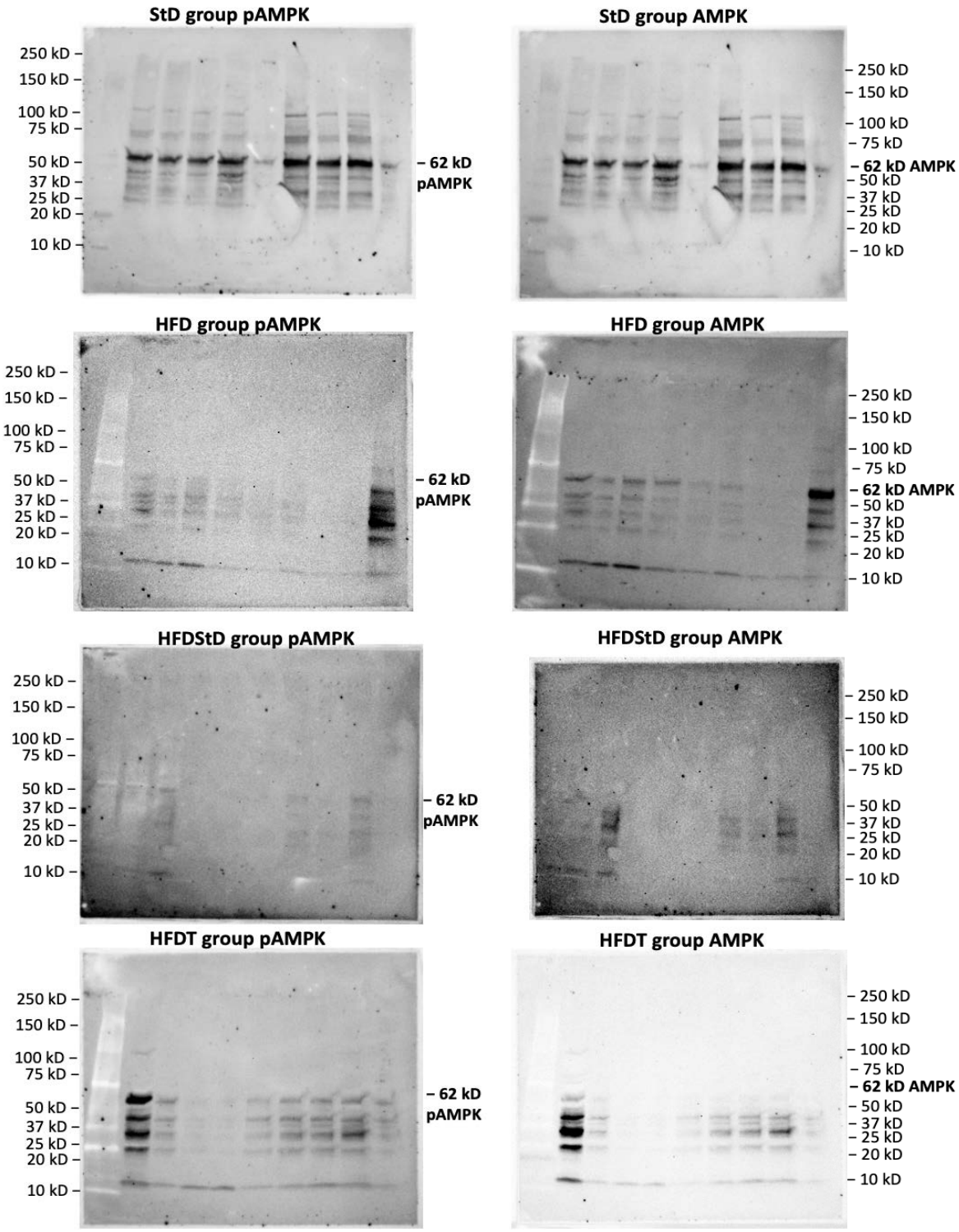
Appendix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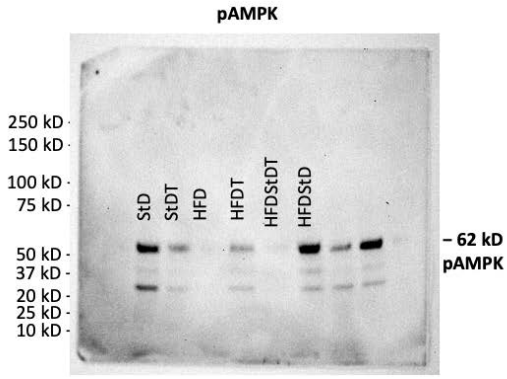

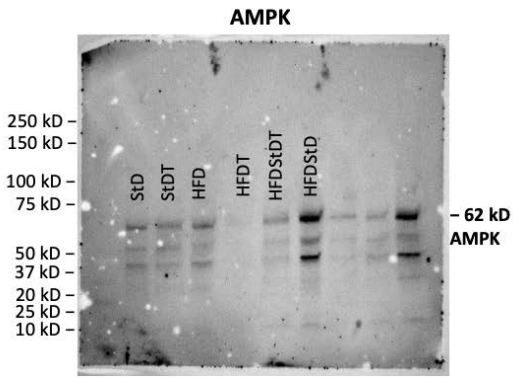

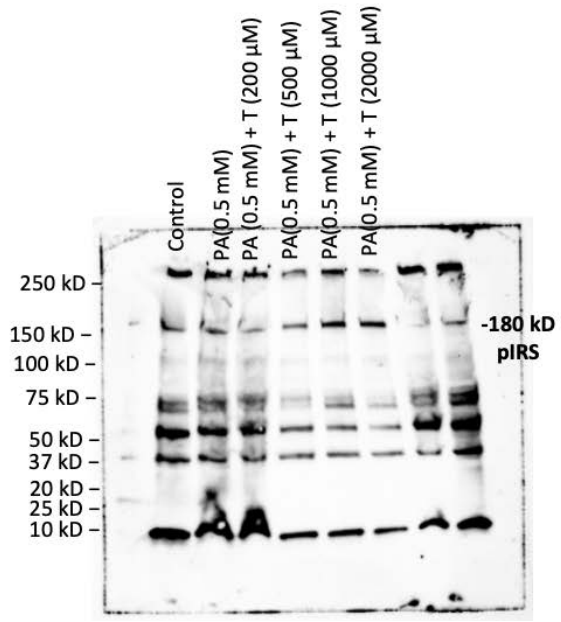

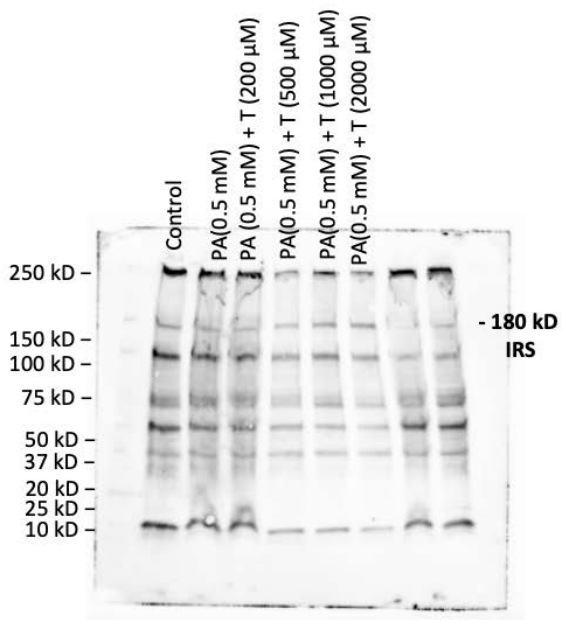

Supplementary Information

TEMPOL reduces hepatic lipid accumulation and inflammation in high fat diet fed mice

Gihani Manodara [a], Scott Genner [b], Claire Rennie [a], David Van Reyk [a], Michael J. Davies [c], Alison K. Heather [e], Kristine C. McGrath [a]

a. *School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia*
b. *Ramaciotti Centre for Genomics, Faculty of Science, University of New South Wales, NSW, Australia*
c. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej, 3B, Denmark*
d. *Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand*

Keywords: Liver, Nitroxide, Steatosis, Antioxidant, Insulin resistance, fatty acid β oxidation

Corresponding Author:

Dr. Kristine McGrath
University of Technology Sydney
PO Box 123, Broadway
Ultimo, NSW, 2007, Australia
Ph: 612 9514 9773
Email: kristine.mcgrath@uts.edu.au

SECTION S1. MATERIALS AND METHODS

Glucose tolerance and insulin tolerance tests

Intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT) were performed as previously described [1] but with glucose concentration (2 g/kg) and insulin (1 IU/kg, Apidra SoloStar®, Insulin glulisine) administered to animals that were fasted only for five hours (i.e. 8 a.m. to 1 p.m.). Prior to administration of glucose or insulin, basal glucose levels were obtained using a glucometer (Accu Chek, Roche Diagnostics, Castle Hill, NSW, Australia) from the tip of the tail. Glucose levels were then measured at 15, 30, 60, and 120 minutes after glucose or insulin administration.

Plasma biochemical analysis

Plasma samples were assessed for non-esterified fatty acid (NEFA) following the protocol by Lab Assay NEFA kit (WAKO diagnostic; Osaka, Japan). Absorbance was read at 550 nm on Infinite® 200 PRO plate reader (Tecan Switzerland). Plasma alanine transaminase (ALT), aspartate aminotransferase (AST) and β-Hydroxybutyrate (β-OHB) were determined using commercial kits according to the respective protocols provided (Abcam, Cambridge, MA, USA).

Histological assessment and measurement of lipid content in liver tissue samples

Liver tissue samples were formalin-fixed and paraffin-embedded and sectioned at 5 µm. Slides were rehydrated and stained for identification of hepatic fibrosis, lipid accumulation and macrophage infiltration, before being mounted and coverslipped. Briefly, for the identification of fibrosis, slides were stained with Picrosirius red (Abcam, Cambridge, UK) according to the manufacturer's instructions. For lipid accumulation, sections were stained with routine haematoxylin and eosin, and unstained globular areas were deemed to be areas of fat. Finally, tissue sections were stained for macrophage infiltration *via* immunohistochemical staining using macrophage marker F4-80 (Cat # Ab111101, Abcam, Cambridge, UK). Briefly, rehydrated sections underwent antigen retrieval in citrate buffer (0.01 M, pH 6), were blocked with PBG protein block (2% normal goat serum, 0.2% v/v Trition-X, PBS), and were incubated with F4-80 (1:200) overnight at 4°C. Slides were then washed with PBST and incubated in an anti-mouse IgG secondary antibody (AF488; Invitrogen, MA, USA). Slides were then washed, mounted and coverslipped. Stained slides were scanned using the slide scanner (ZEISS AxioScan, Jena, Germany) at x20 magnification and analysed using ZEISS software.

The accumulation of neutral lipids, which includes triglyceride and cholesterol esters, in liver samples were assessed by quantitatively measuring the Oil Red-O staining intensity in tissue extracts as previously described [1].

Palmitate Acid (PA) preparation, HepG2 cell culture and treatment

Human hepatoma HepG2 cells (ATCC, Manassas, VA) were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies; CA, USA) supplemented with 10% (w/v) fetal bovine serum (FBS) (Sigma Aldrich, MO, USA) and 5% (v/v) penicillin-streptomycin (Life Technologies; CA, USA). Cells were maintained in a 37ºC humidified incubator at 5% CO_2.

Albumin bound-palmitic acid (20 mM) was prepared by dissolving the palmitic acid powder (Sigma Aldrich, MO, USA) in DMEM containing 10% (v/v) FBS, 1% (v/v) fatty acid-free-BSA (FFA-BSA) and 150 mM NaCl at 70 ºC. The stock palmitic acid (PA) was diluted in DMEM containing 1% (w/v) FFA-BSA to yield a 10 mM solution for treatment. Unless otherwise stated, HepG2 cells (2 x 10^5 cells/ml density) were incubated for 24 h with PA (0.5 mM) and then exposed to TEMPOL 200 µM, 500 µM or1000 µM for 5 h. Control cells were treated with 1% FFA-BSA (w/v).

Quantitative and qualitative analysis of lipid content in HepG2 cells

HepG2 cells were seeded into 6-well plates at a 2 x 10^5 cells/ml density. After treatment, the cells were washed three times with cold phosphate-buffered saline (PBS) before fixation with 10% neutral formalin for 30 min. The cells were washed with PBS, followed by 70% (v/v) ethanol before staining with Oil Red O (ORO) for 45-60 min. Cells were then processed for imaging or analysed by quantitation of ORO as an indication for lipid content in cells. For imaging purposes, cells were washed with PBS, counterstained with haematoxylin for 10 min before visualisation by light microscopy (Omax 40x-200x, USA). For quantitative purposes, cells were washed twice with PBS, once with 70% (v/v) ethanol before ORO-stained intracellular lipid was extracted by the addition of 4% IGEPAL® CA-630 (Sigma Aldrich, MO, USA) and quantified by measuring absorbance at 520 nm on the Infinite® 200 PRO plate reader (Tecan, Mannedorf, Switzerland).

Assessment of reactive oxygen species levels, cytotoxicity, IL-8 and glycogen levels in HepG2 cells

Intracellular oxidant (ROS) levels and cytotoxicity levels were measured using the fluorescent derivative 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) or thiazolyl blue tetrazolium bromide powder (MTT reagent, Sigma Aldrich; MO, USA), respectively, as previously described [2]. To determine interleukin-8 (IL-8) levels, conditioned media of HepG2 hepatocytes were assessed using an IL-8 enzyme-linked immunosorbent assay (ELISA) kit as per the manufacturer's instructions (Invitrogen; MA USA). To measure glycogen levels, treated HepG2 cells were washed with ice-cold PBS and lysed by the addition of ice-cold deionised water (110 µL). The lysate was then boiled for 10 min (100ºC) to inactive enzymes and centrifuged (18,000 rpm, 10 min, 4 ºC) to remove insoluble material. The supernatant was then

collected, and glycogen content measured using a glycogen assay kit (Cat # ab65620, Abcam, Cambridge, UK), according to the manufacturer's protocol. The absorbance was read at 570 nm on the Infinite® 200 PRO plate reader (Tecan, Mannedorf, Switzerland).

Immunoblot analysis

Protein lysate was extracted from HepG2 cells and liver tissue using RIPA lysis buffer (1% v/v non-idet P-40, 0.1% v/v SDS, 0.5% v/v deoxycholate, 150 mM NaCl, 50 mM Tris, pH 8) as previously described [1]. Protein lysates (20 µg) were then denatured for 5 mins (100°C), before resolving on a 4-10% gradient SDS-polyacrylamide gel (Bio-Rad, CA, USA). Separated proteins were then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA, USA). The membranes were then rinsed with Tris-buffered saline-0.1% Tween-20 (TBST), blocked with 5% (w/v) skim milk for 1 h before a final rinse with TBST (3X 5 min). The membrane was then incubated with a primary antibody recognising phospho–NFκB (p65), NFκB p65, mitogen- stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK), protein kinase B (AKT), Forkhead box protein 01 (FOX01), AMP-activated protein kinase (AMPK), cytochrome c (cyt c) or β-actin antibodies at 1:1000 dilution (Cell signalling, Gene Search; **Table S2**) overnight at 4°C. Following incubation, the membranes were washed with TBST (3X 5 min) before incubating with anti-rabbit IgG (1:1000) conjugated to horseradish peroxidase (Cell signalling, Gene Search). Proteins were then visualised by Chemiluminescent HRP Substrate reagent (BioRad, CA, USA) and membranes were directly digitised using a Chemi-Doc imaging system (BioRad, CA, USA). Densitometry was performed using Image J software (ImageJ, NIH). Results obtained were normalised with β-actin reference protein bands.

Analysis of inflammatory gene expression

Total RNA was extracted using TRI-Sure reagent (Bioline, Meridian Biosciences) and concentration normalised to 100 ng/µL using a UV spectrophotometer (Nanodrop Technologies, USA). Reverse transcription-polymerase chain reaction (RT-qPCR) was then performed as previously described [2] using the primers listed in **Table S3**. The β-2-microglobulin (β2M) gene was used as baseline control.

Supplementary Table 1: List of ingredients in the high-fat diet used for *in vivo* murine model in this study.

Ingredients
Trans fatty acids
Saturated fatty acids
Polyunsaturated fatty acids
16:0 palmitic acid
18:0 stearic acid
18:1 n9T elaidic acid
18:1 n9C oleic acid
18:1 n7C vaccenic acid
18:1 other cis isomers
18:2 n6 linoleic acid
18:2 other trans isomers
19:0 nonadecanoic acid
20:0 arachidic acid

Supplementary Table 2: Antibodies used for immunoblotting analysis.

Specificity	Source/Isotype	Cell Signalling, Cat #
pAMPK	Rabbit IgG	50081
AMPK	Rabbit IgG	5831
pAKT	Rabbit IgG	4060
AKT	Rabbit IgG	9272
Cytochrome C	Rabbit IgG	4272
Fox 01	Rabbit IgG	2599
pIRS	Rabbit IgG	2381
IRS	Rabbit IgG	2382
pNFκB	Rabbit IgG	76778
NFκB	Rabbit IgG	8248
pSAPK/JNK	Rabbit IgG	9251
SAPK/JNK	Rabbit IgG	9252
β-Actin	Rabbit IgG	4970
Anti-rabbit IgG HRP – linked Antibody	Goat	7074

Supplementary Table 3: PCR primers and primer sequences used in the study.

Gene ID	Description	F/R	Primer Sequence (5'-3')	Accession Number
β2M	β-2-Microglobulin	F	CATCCAGCGTACTCCAAAGA	NM_004048.4
		R	GACAAGTCTGAATGCTCCAC	
IKK β	Inhibitor of nuclear factor κB	F	CAGATGGTCAAGGAGCTG	NM_020529.3
		R	CAAGTGCAGGAACGAGTC	
IL-1β	Interleukin-1β	F	GCTTATTACAGTGGCAATGAGG	NM_000576.3
		R	AGTGGTGGTCGGAGATTCG	
IL-6	Interleukin- 6	F	TGTGGTTGGGTCAGGGGTGGT	NM_000600.5
		R	CAATGAGGAGACTTGCCTGGTGA	
IL-8	Interleukin- 8	F	CGGAAGGAACCATCTCACTGT	NM_000584.4
		R	GGTCCACTCTCAATCACTCTCA	
SAA-1	Serum Amyloid A-1	F	AGCGATGCCAGAGAGGCTGT	NM_011315.3
		R	ACCCAGTAGTTGCTCCTCTT	
NFκB	Nuclear factor-κB	F	TCCGAGACAGTGACAGTG	NM_003998.4
		R	GTTGAGAGTTAGCAGTGAGG	
PEPCK	Phosphoenolpyruvate carboxykinase	F	ATTCTGGGTATAACCAACCC	NM_002591.4
		R	GTTGATGGCCCTTAAATGAC	
TNF	Tumour necrosis factor	F	CTCTTCTGCCTGCTGCACTTTG	NM_000594.4
		R	ATGGGCTACAGGCTTGTCACTC	

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Chapter 6 : TEMPOL ameliorates palmitate-induced insulin resistance in MIN6 pancreatic cells.

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Chapter Summary

In this chapter, we focus in lipotoxicity-induced deleterious consequences of oxidative stress and endoplasmic reticulum (ER) stress on β-cell dysfunction and apoptosis with a special emphasis on T2DM. Given the reported protective antioxidant effect of TEMPOL, this study aimed to determine whether TEMPOL could mediate a protective effect on MIN6 β-cells exposed to palmitic acid (PA), an *in vitro* model for diet-induced β-cell stress, and in high fat diet-fed (HFD) C57BL/6 mice as in an *in vivo* model of diabetes. This chapter has been written according to the guidelines of the Journal of Diabetes Investigation, which the manuscript will be submitted to.

TEMPOL ameliorates palmitate-induced insulin resistance in MIN6 pancreatic beta cells

Gihani Manodara [a], Michael J. Davies [b], Alison K Heather [c], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, the University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand*

Author Contributions:

For this paper, the PhD candidate, GM, completed the majority of the experiments, data analysis and interpretation and wrote the manuscript. MD contributed to manuscript editing. KM supervised GM and contributed to data acquisition. AH and KM conceptualised the study, helped with data analysis and interpretation, and edited the manuscript. All authors contributed to the execution and approved the final version of the manuscript.

Gihani Manodara	Production Note: Signature removed prior to publication.
Michael J. Davies	Production Note: Signature removed prior to publication.
Alison K. Heather	Production Note: Signature removed prior to publication.
Kristine C. McGrath	Production Note: Signature removed prior to publication.

TEMPOL ameliorates palmitate-induced insulin resistance in MIN6 pancreatic beta cells

Gihani Manodara [a], Michael J. Davies [b], Alison K Heather [c], Kristine C McGrath [a]

a. *School of Life Sciences, Faculty of Science, the University of Technology Sydney, Broadway, NSW, Australia*
b. *Panum Institute, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3B, Denmark*
c. *Department of Physiology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand*

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Lipotoxicity, pancreatic β cells, oxidative stress, ER stress, apoptosis, inflammation, insulin sensitivity, TEMPOL, antioxidant

Corresponding Author:
Dr Kristine McGrath
The University of Technology Sydney
PO Box 123, Broadway
Ultimo, NSW, 2007, Australia
Ph: 612 9514 9773
Email: kristine.mcgrath@uts.edu.au

ABSTRACT

Lipotoxicity induces mitochondrial oxidative stress and endoplasmic reticulum stress, leading to pancreatic β-cell dysfunction, a common pathological determinant of diabetes. TEMPOL, a superoxide dismutase mimetic, has displayed antioxidant effects in both *in vitro* and *in vivo* models. Therefore, this study aimed to determine whether TEMPOL could mediate a protective effect in MIN 6 β-cells exposed to palmitic acid (PA), an *in vitro* model for diet-induced β-cell stress, and in high fat diet-fed (HFD) mice as an *in vivo* model of diabetes. The data revealed PA-induced hyperlipidaemia, mitochondrial and endoplasmic reticulum (ER) stress whilst decreasing insulin sensitivity and insulin secretion in *in vitro*. TEMPOL exposure significantly reversed these effects, with the profile of treated cells more closely resembling that of the untreated control group. Similarly, C57BL/6 mice on an HFD supplemented with TEMPOL displayed improved glucose tolerance and insulin sensitivity. These results indicate that TEMPOL may be a useful pharmaceutical therapy for improving pancreatic dysfunction and its loss in people with diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is a major public health concern worldwide, with its prevalence rising due to modern lifestyle factors such as over-nutrition and lower levels of physical activity [1]. In response to increased calorie intake, the insulin-secreting pancreatic β-cells in individuals with T2DM face increased pressure to support the metabolic demand for insulin [2]. Over time, the sustained compensatory increases in insulin secretion induce β-cell stress, which leads to β-cell dysfunction and apoptosis [3]. Over-nutrition is strongly associated with chronic dyslipidaemia (lipotoxicity) and high plasma glucose levels, which can trigger several pathological mechanisms in β-cells, including inflammation, mitochondrial oxidative stress, and endoplasmic reticulum (ER) stress. Thus, in addition to sustained insulin secretion causing β-cells harm, the same cells are exposed to chronic cellular stressors (often termed glucolipotoxicity) that ultimately all contribute to β-cell dysfunction [4, 5].

Mitochondrial oxidative stress arises in β-cells from an imbalance between the generation of reactive oxidants (often termed reactive oxygen species, ROS) and endogenous antioxidants and protective systems. The latter include both enzymatic systems (such as superoxide dismutase (SOD), catalase, peroxiredoxins, glutathione peroxidases, glutaredoxins and the thioredoxin system) and low molecular mass antioxidants (e.g., glutathione, ascorbic acid, tocopherols, carotenoids and flavonoids) [6-8]. Clinical studies have shown that T2DM patients have elevated levels of oxidant formation and that markers of oxidative stress correlate with the degree of glucose-stimulated insulin secretion (GSIS) [9]. Additionally, an increase in insulin synthesis due to hyperglycaemia results in the accumulation of unfolded proteins in the ER, which triggers the unfolded protein response (UPR) [10]. The UPR is a cellular response to alleviate ER stress resulting from the presence of higher levels of damaged or misfolded proteins. It consists of a triad of stress-sensing proteins: inositol-requiring enzyme 1α (IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [11]. UPR protein components, such as PERK, are known to lower insulin biosynthesis, and IRE1α directly co-operates with the c-Jun amino-terminal kinase stress kinase (JNK) pathway linking ER stress to inflammation and apoptosis [12-14]. Therefore, two pathological pathways, oxidative stress and ER stress are intertwined and trigger an inflammatory response, leading to defects in insulin sensitivity and insulin secretion from β-cells [15]. It follows that targeting both pathways with novel therapeutic compounds could improve β-cell dysfunction.

One potential therapeutic agent is TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a long-lived radical and cellular permeable SOD mimetic agent [16]. *In vitro* studies have shown that TEMPOL can protect cells against oxidative damage induced by hydrogen peroxide (H_2O_2), superoxide radical anions (O^{2-}), and peroxynitrite/peroxynitrous acid ($ONOO^-$/ ONOOH) [17]. In addition, TEMPOL has been shown to scavenge free radicals and inhibit lipid peroxidation, thus preventing oxidative damage in cells and tissues [17, 18]. These *in vitro* experiments with TEMPOL have been complemented by several *in vivo* models of oxidative stress [16, 19-21], including an experimental acute necrotizing pancreatitis model in rats, which showed that TEMPOL treatment reduced oxidative stress, inflammation and decreased pancreatic tissue damage [19]. Similarly, TEMPOL treatment demonstrated cardioprotective effects and reduced oxidative stress, inflammation and tissue damage in the pancreas in a cerulein-induced pancreatitis rat model [20]. Moreover, in a rat model of T2DM, TEMPOL decreased oxidant levels, ER stress and inflammation in pancreatic β-cells, leading to a decrease in β-cell apoptosis [21].

In light of these reported protective effects of TEMPOL, and its ability to decrease β-cell apoptosis associated with reduced cellular oxidative stress in a rat model of T2DM, we hypothesised that TEMPOL might abrogate the chronic activation of cellular stress pathways induced by lipotoxicity. In order to test this hypothesis, we have used both *in vitro* and *in vivo* models to discern which lipid-induced molecular pathways are modulated by the presence of TEMPOL in cultures of β-cells, and whether TEMPOL-mediated protection against β-cell stress and dysfunction is associated with an improvement in insulin sensitivity in an *in vivo* model of high-fat diet (HFD)-fed mice. By examining the impact of TEMPOL on these lipotoxicity-induced models, the study aims to gain a deeper understanding of the mechanisms by which TEMPOL protects β-cells from damage and apoptosis, potentially leading to the development of novel therapeutic strategies for T2DM.

Method and Materials

Animals

Male C57BL/6 mice were obtained from Australian BioResources (Moss Vale, NSW, Australia) and housed in temperature-controlled rooms with an adjusted circadian rhythm. During acclimatization, mice were provided with standard chow (StD) and water *ad libitum*. At eight weeks of age, the mice were randomly assigned to two cohorts, one of which received

a standard chow diet (n= 10; StD, Biological Associate; Envigo Teklad) and the other a high-fat diet (n= 30; HFD, Biological Associate; Envigo Teklad) enriched with trans fatty acids, mono and polyunsaturated acids. After eight weeks, the StD-fed animals were retained on this diet, while the HFD-fed mice were randomly divided into three subgroups (n= 10 each); one continued on the HFD, one was switched back to StD (HFDStD), and one received HFD supplemented with TEMPOL (HFDT) at a dose of 10 mg g^{-1}, prepared using the cold-pressed method as described [22]. The mice were maintained for a further 7 weeks, during which time an intraperitoneal glucose tolerance test (ipGTT) and an intraperitoneal insulin tolerance test (ipITT) were performed as previously described [23]. The experiment was concluded on the 16th week of the diet, at which point, animals were euthanized, and tissues were harvested and stored at -80◦C until further processing. This study was approved by the University of Technology Sydney Animal Care and Ethics Committee, Sydney, NSW, Australia (Ethics approval number: UTS ACEC ETH16-0455) and conducted according to guidelines described by the Australian National Health and Medical Research Council Code of Conduct of Animals.

Palmitic acid preparation

Albumin bound-palmitic acid (PA: 20 mM) was prepared by dissolving the palmitic acid powder (Sigma Aldrich, MO, USA) in Dulbecco's Modified Eagles Medium (DMEM; Life Technologies; CA, USA) containing 1% w/v fatty-acid free (FFA-free) BSA and 150 mM NaCl at 70°C. The resulting stock of albumin bound-PA was diluted in DMEM containing 1% w/v free fatty acid-free BSA to yield a 10 mM solution, which was then stored at -20°C until used.

MIN6 β-cell culture and treatment

Murine pancreatic MIN6 β-cells were cultured in DMEM containing 25 mM glucose (ThermoFisher, MA, USA), supplemented with 10% v/v fetal bovine serum (FBS; Sigma Aldrich, MO, USA) and 5% v/v penicillin-streptomycin at 37°C under humidified 20% O_2 / 5% CO_2. The MIN6 β-cells were passaged at 80% confluency with passages 25-28 used for this study. To model diet-induced lipotoxicity, MIN6 β-cells were plated (2 x 10^5 cells/well) on 6 well plates and incubated with PA at a concentration of 0.5 mM for 24 h, followed by TEMPOL treatments (250, 500, or 1000 μM) for an additional 24 h. The concentration of PA used was based on previous studies in which it was reported this concentration to be optimal for modelling inducing diet-induced diabetes *in vitro* [24].

Quantitative and qualitative analysis of lipid content in MIN6 β-cells

To determine the lipid content in MIN6 β-cells following treatment, cultured cells were washed thrice with ice-cold phosphate-buffered saline (PBS) and fixed with 10% v/v neutral formalin for 30 min. After fixation, the cells were rinsed four times with PBS, followed by a 70% v/v ethanol wash before staining with 10 % v/v Oil-Red-O (ORO) in Milli-Q water for 45-60 mins at room temperature. For imaging studies, the cells were washed with PBS and counterstained with hematoxylin for 10 min before examination by light microscopy at x 20 magnification (Omax 40x-200x, USA). For quantification purposes, the cells were washed twice with PBS, once with 70% v/v ethanol and the stain was subsequently extracted by adding 4% v/v non-idet P-40 (Sigma Aldrich, MO, USA). The ORO-stained intracellular lipids were quantified by absorbance measurement at 520 nm using a plate reader (Tecan i-control infinite 200 Pro, Tecan Switzerland).

Cell metabolic activity assay

To assess the effect of TEMPOL on cell metabolic activity (an approximate measure and proxy of cellular viability) following PA incubation, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted to assess potential effects of TEMPOL following PA incubation. MTT is a positively charged tetrazolium compound that quickly penetrates eukaryotic cells and is subsequently reduced by mitochondrial reductive enzymes to an insoluble purple precipitate (formazan), which can subsequently be redissolved and quantified by its optical absorbance [25]. Briefly, thiazolyl blue tetrazolium bromide powder (Sigma Aldrich, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to generate the MTT solution (5 mg mL^{-1}). After the treatment of cells with PA + TEMPOL, 20 µL of the MTT solution was added to each well containing 200 µL media. The plates were then incubated for 3-4 h before removing the media. To extract and redissolve the precipitate formazan, 200 µL of DMSO was then added to each well, and the absorbance at 570 nm was determined using the Infinite® 200 PRO plate reader (Tecan, Mannedorf, Switzerland).

Quantification of gene expression

Total RNA from treated MIN6 β-cells was extracted using TRI-sure reagent (Bioline; Meridian Biosciences). Extracted RNA samples were normalised to 200 ng μL^{-1} using a UV spectrophotometer (Nanodrop Technologies, USA), and first-strand cDNA was synthesised using the Tetro cDNA synthesis kit (Bioline; Meridian Biosciences). Gene expression levels were then determined by real-time PCR (qPCR) using the SensiFAST SYBR No-ROX kit

(Bioline; Meridian Biosciences) containing 20 pmol specific oligonucleotides primer pairs (**Table S1**) using a Bio-Rad thermocycler (BioRad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to determine the change in mRNA gene expression levels relative to the reference gene 18S [26].

Quantification of protein abundance in cells using immunoblotting

After treatment, whole-cell protein lysate was extracted from MIN6 β-cells using a radioimmunoprecipitation assay (RIPA) buffer [23]. Protein lysates (20 µg) were denatured by boiling for 5 min before separating them using SDS-PAGE. Separated proteins were electro-transferred to a polyvinylidene fluoride membrane (Bio-Rad, CA, USA). The membranes were then blocked in 1x tris-buffered saline with Tween (TBST) containing 5% w/v non-fat milk for 1 h before incubation with a 1:1000 dilution of primary antibodies recognising protein kinase B (Akt), protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1α, (IRE1α,), protein disulphide isomerase (PDI), insulin receptor substrate-1 (IRS-1) and AMP-activated protein kinase (AMPK) (**Table S2**) overnight at 4ºC. Following incubation, the membranes were washed thrice with 1 x TBST (5 min each) before incubation with anti-rabbit IgG (1:1000) conjugated with horseradish peroxidase for 2 h at room temperature (Cell signalling; Gene Search). Proteins were then visualised using chemiluminescent HRP Substrate reagent (Bio-Rad, CA, USA) and a Bio-Rad Chemi-Doc imaging system (BioRad, CA, USA). Densitometry was performed using Image J software (ImageJ, NIH, https://imagej.nih.gov/ij). Results obtained were normalised with β-actin reference protein bands.

Measurement of reactive oxygen species

Intracellular oxidant levels were measured using the pro-fluorescent substrate 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA). A stock solution of 100 µM was made by dissolving DCFH-DA (Sigma Aldrich; MO, USA) in DMSO, followed by making a 10 µM working solution using PBS. Following the treatment of MIN6 β-cells, the media was aspirated, and the wells were washed with 100 µL PBS (x2). Next, DCFH-DA working solution (100 µL/well) was added to each well; plates were covered by aluminium foil and incubated for 15 min at 37 ºC. After incubation, the DCFH-DA solution was aspirated, and cells were washed twice with PBS (100 µL/well). The fluorescence arising from DCFH-DA oxidation was then determined using a λ_{ex} 488 nm and λ_{em} 520 nm using the Infinite® 200 PRO plate reader (Tecan, Mannedorf, Switzerland).

Mitochondrial Stress determined using cytochrome C release

To assess mitochondrial damage, cytochrome C release into the cytoplasm [27] was determined in cell lysates. This assay was conducted by using the cytochrome c oxidase assay kit per the manufacturer's protocol (Abcam, Cambridge, UK).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Prism Software; MA, USA). Data are expressed as mean ± SEM and analysed by one-way ANOVA with Bonferroni's post-hoc correction test, with $p < 0.05$ considered significant.

Results

TEMPOL attenuates PA-induced lipid accumulation in MIN6 β-cells.

To induce lipotoxicity, MIN6 β-cells were incubated with palmitic acid (PA) for 24 h, followed by post-TEMPOL treatment (250, 500 or 1000 μM) and incubated for a further 24 h. Intracellular lipid accumulation was visualised using ORO stain (Fig. 1A) and measured quantitatively by extraction of intracellular lipids (Fig. 1B). While this analysis showed an increase in lipid accumulation in β-cells exposed to PA as compared to the control cells, this difference did not reach statistical significance. Nonetheless, the addition of TEMPOL reversed this increase, with average levels of PA-induced-intracellular lipids returning to that seen in the untreated control β-cells.

To determine whether the reduction of lipid accumulation by TEMPOL was due to enhanced fatty acid β-oxidation, the expression of carnitine palmitoyltransferase 1 (CPT-1) was measured. CPT-1 is an essential enzyme involved in the transport of long-chain fatty acids in mitochondria for β-oxidation. Reflecting the increase in lipid accumulation (Fig. 1B), cells treated with PA had a trend in reducing CPT-1 mRNA levels by 18% compared to the control group (Fig. 1C). In contrast, adding TEMPOL significantly increased the expression of CPT-1 mRNA in a concentration-dependent manner ($p < 0.05$, $p < 0.001$ vs PA).

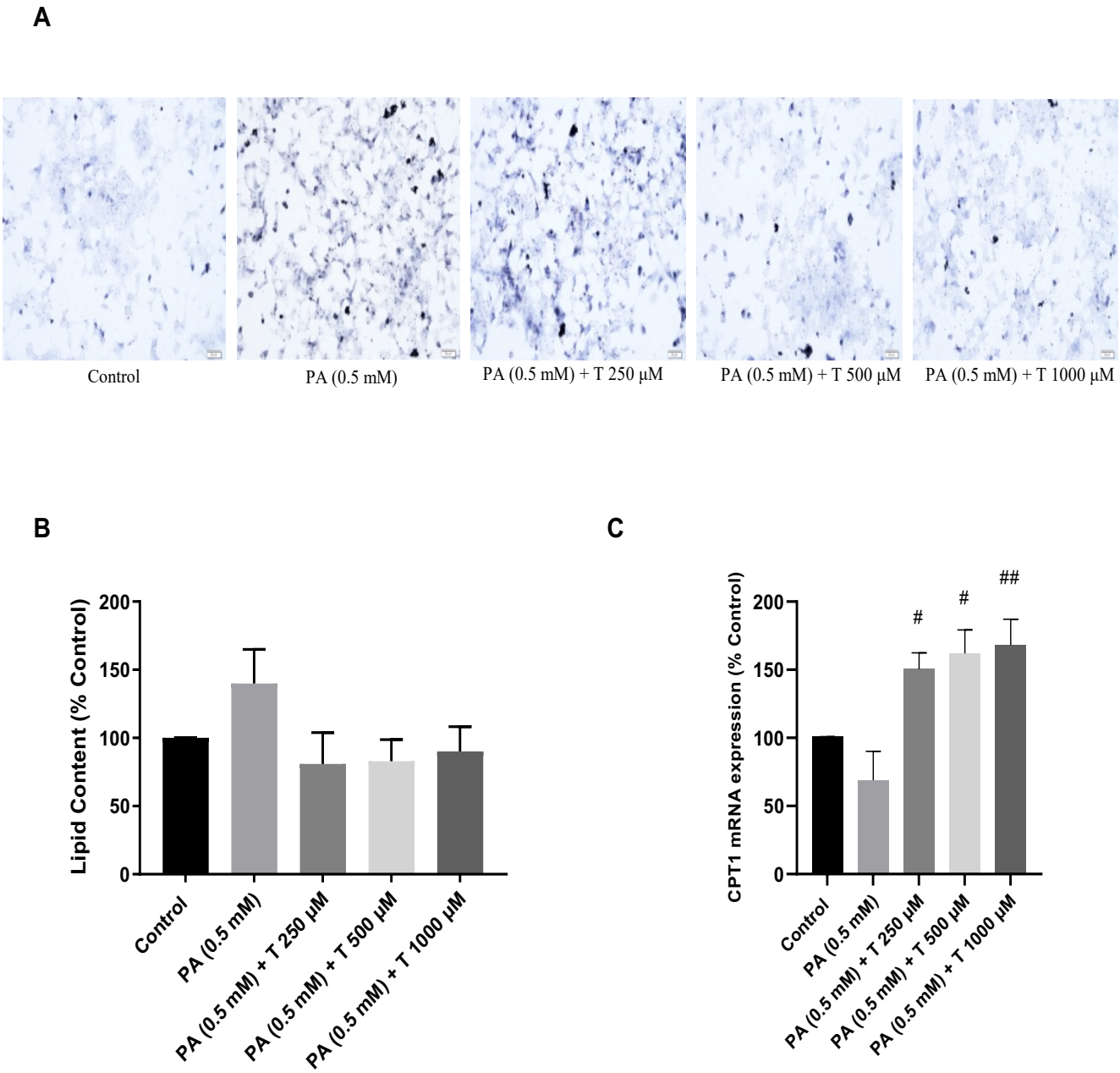


Figure 1: TEMPOL reduces lipotoxicity induced by palmitic acid in MIN6 β-cells.

MIN6 β-cells were incubated in 0.5 mM PA for 24 h followed by TEMPOL treatments (250, 500 or 1000 µM) for a further 24h. (A) Representative image of cells stained with Oil Red O (x20 magnification; scale bar 20 µm). (B) Quantification of intracellular lipid accumulation from PA + TEMPOL treated cells. (C) Carnitine palmitoyltransferase-1 (CPT-1) mRNA gene expression was measured using RT-qPCR to analyse TEMPOLs' impact on fatty acid β-oxidation. Results are mean ± SEM (n= 5–6). * $p < 0.05$ & ** $p < 0.001$ vs control. # $p < 0.05$ & ## $p < 0.001$ vs PA.

TEMPOL attenuates MIN6 β-cell metabolic activity induced by lipotoxic conditions.

Chronic exposure to circulating free fatty acids (FFA's) can contribute to defects in the function and survival of pancreatic β-cells [28]. FFA can be absorbed by the pancreas and accumulate as intracellular triglycerides, resulting in lipotoxicity [29]. The mammalian target of rapamycin (mTOR) is a crucial regulator of nutrient sensing, metabolism and energy in cells that aids in maintaining their survival and growth. Consequently, chronic activation of mTOR due to excessive lipid accumulation can cause β-cell failure and death [30, 31]. Our results show that despite no significant change in the expression of mTOR following treatment with PA, post-TEMPOL treatment significantly decreased mTOR mRNA expression levels at 500 μM and a maximal inhibition was reached when the TEMPOL dose was increased at 1000 μM ($p < 0.001$ vs PA group). Additionally, assaying cellular metabolic activity as determined using the MTT assay (Fig. 2B) demonstrated a trend in lowering effect on the metabolic activity of MIN6 β-cells with 24 h PA incubation, whilst a significant increase was observed in the presence of 250 μM and 500 μM TEMPOL treatment ($p < 0.05$ vs PA). These results suggest that TEMPOL can protect MIN6 β-cells from lipotoxic-induced defects in metabolic activity.

TEMPOL attenuates inflammation induced by lipotoxic conditions in MIN6 β-cells.

Saturated fatty acids, such as PA, can induce inflammation in a variety of cultured cells [32]. This occurs due to an increased expression of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor (TNF). Consistent with previous reports, Fig. 3 show the expression of IL-6 and TNF were significantly increased in MIN6 β-cells on incubation with PA relative to the control group ($p < 0.05$). This effect was reversed by the addition of TEMPOL, with the expression of both cytokines significantly reduced compared to the PA group ($p < 0.05$) and at comparable levels to the control group.

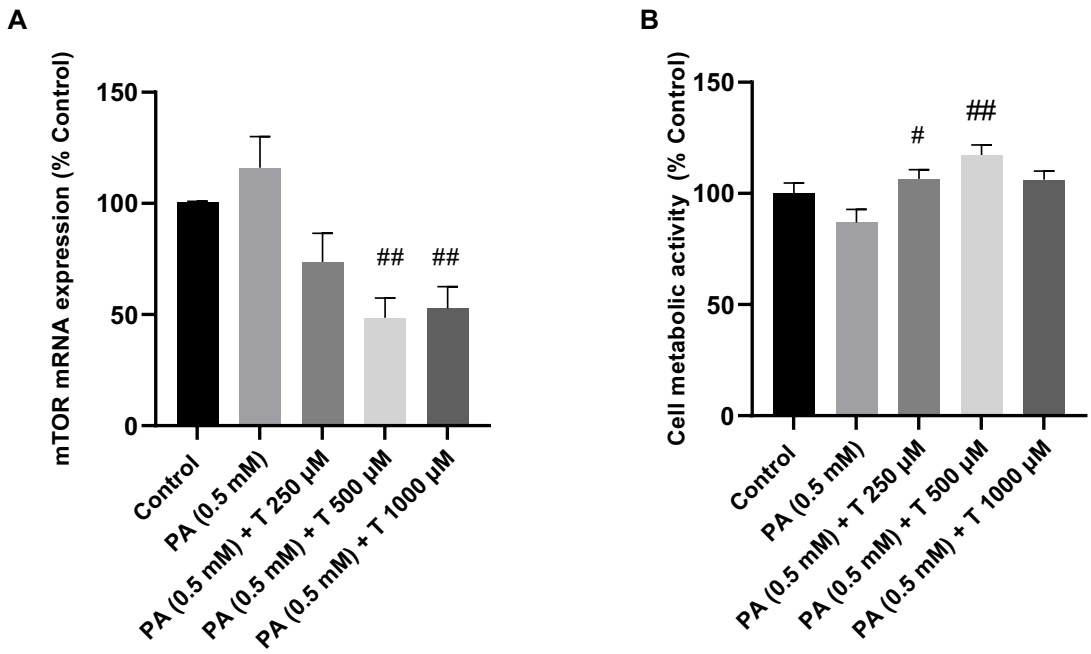


Figure 2: TEMPOL restrained cellular apoptosis induced by lipotoxicity in MIN6 β-cells.

(A-B) MIN6 β-cells were incubated in 0.5 mM PA for 24 h followed by TEMPOL treatments (250, 500 or 1000 µM). (A) RT-qPCR was conducted to analyse mTOR mRNA expression levels. (B) An MTT assay was used to assess TEMPOL's effect on cell metabolic activity (n= 5 - 6). Results are mean ± SEM (n= 5-6). * $p < 0.05$ & ** $p < 0.001$ vs control. # $p < 0.05$ & ## $p < 0.001$ vs PA.

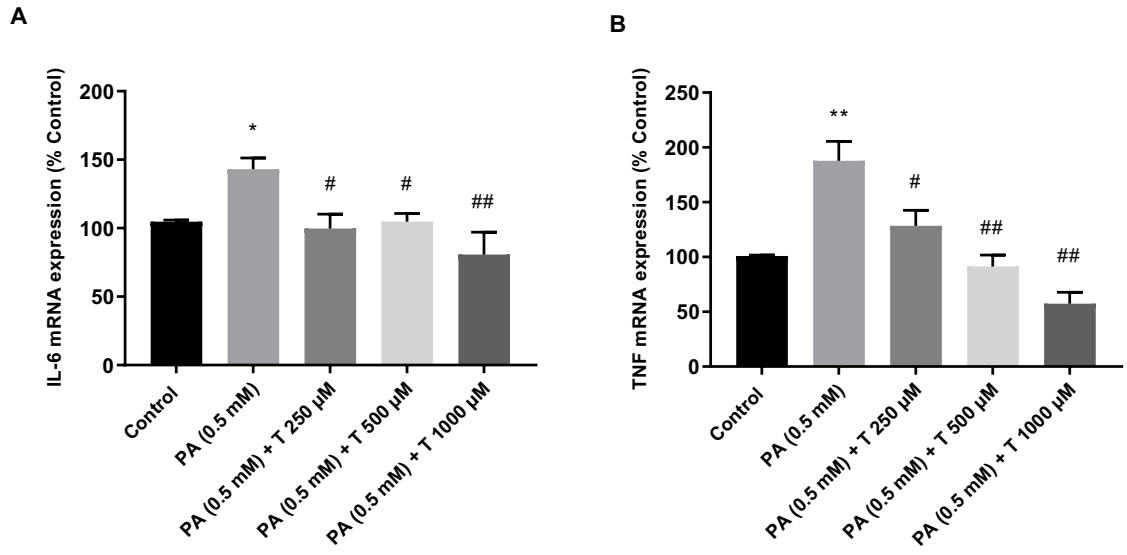


Figure 3: TEMPOL reduces PA-induced inflammation in MIN6 β-cells.

(A-B) MIN6 β-cells were incubated in 0.5 mM PA for 24 h followed by TEMPOL treatments (250, 500 or 1000 µM). RT-qPCR was used to analyse (A) IL-6 and (B) TNF mRNA expression levels Results are mean ± SEM (n =5–6). * $p < 0.05$ & ** $p < 0.001$ vs control, # $p < 0.05$ & ## $p < 0.001$ vs PA.

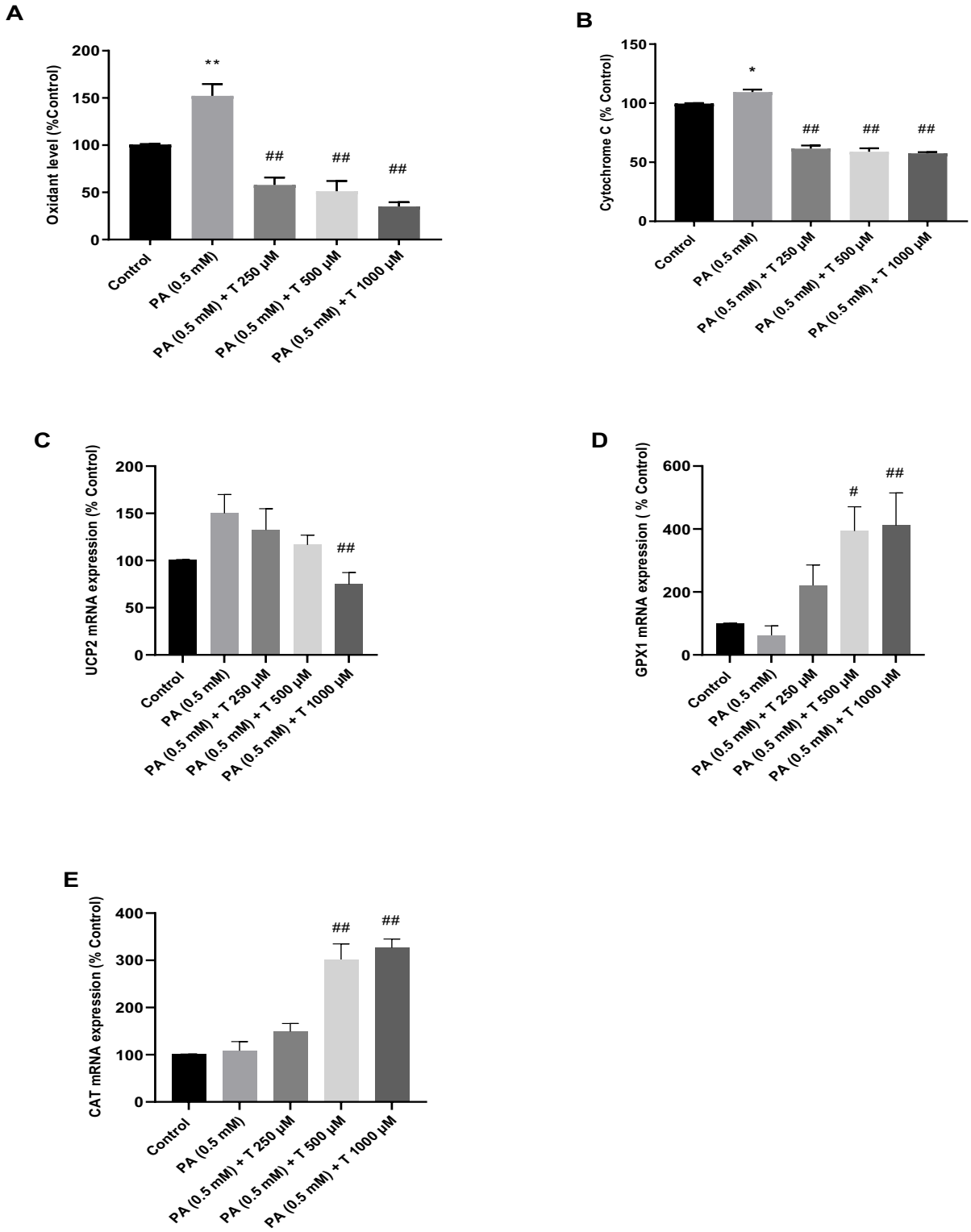


Figure 4: TEMPOL reduces oxidative stress induced by PA in MIN6 β-cells and increases endogenous antioxidant levels.

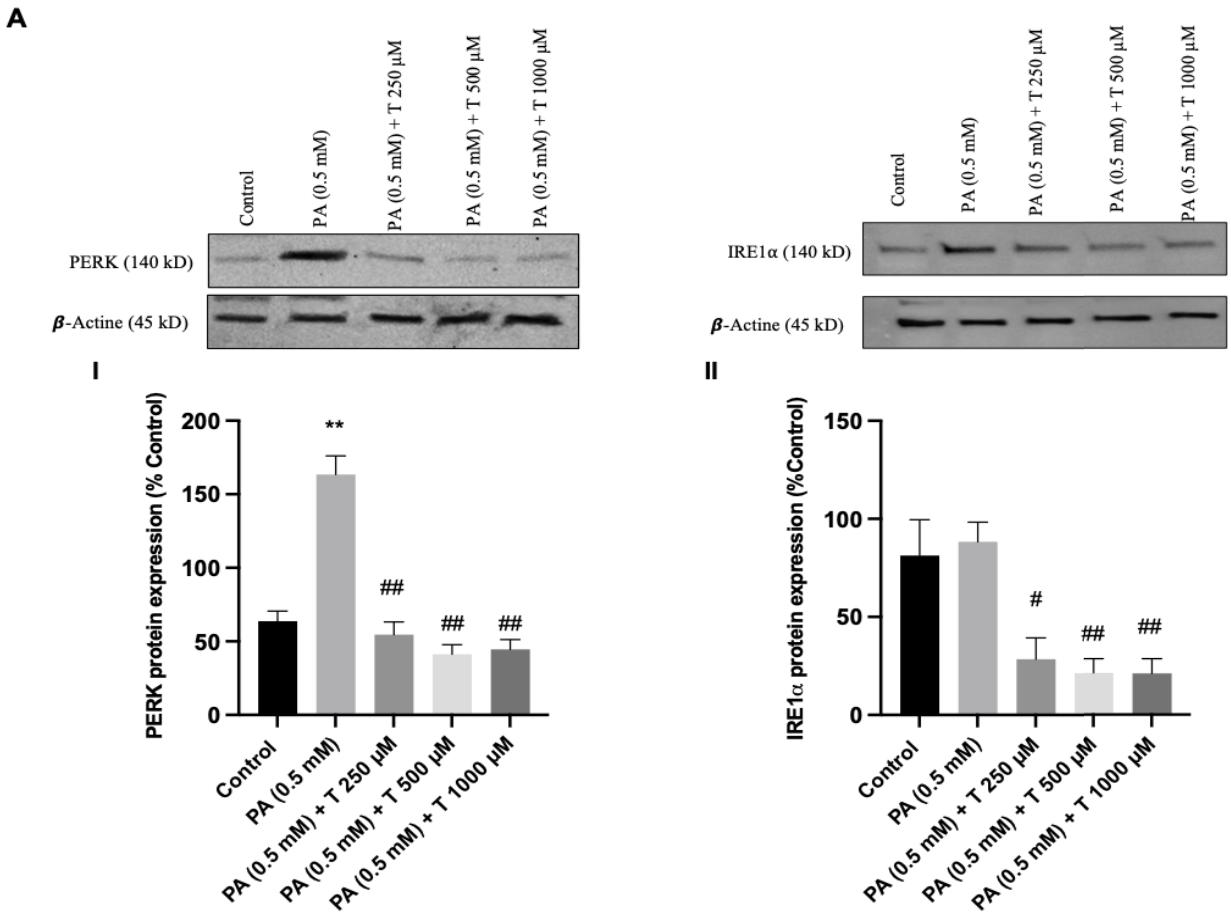
(A) Intracellular oxidant levels were assessed with a DCFH-DA assay. (B) The effect of TEMPOL on the generation of mitochondrial ROS promoter proteins such as (C) cytochrome

c (cyt c) and (D) UCP2 levels were analysed. To investigate TEMPOL's effect on endogenous antioxidants, mRNA expression levels of antioxidants (D) GPX1 and (E) CAT were also quantified. Results are mean ± SEM (n =5–6). * $p < 0.05$ & ** $p < 0.001$ vs control, # $p < 0.05$ & ## $p < 0.001$ vs PA.

TEMPOL protects MIN6 β-cells from lipotoxic-induced mitochondrial oxidative stress.

Given TEMPOL's ability to mitigate PA-induced lipotoxicity, we next investigated whether TEMPOL also reduced PA-induced mitochondrial damage [33, 34]. MIN6 β-cells incubated with PA (24 h, 0.5 mM) showed a ~ 51 % increase in DCFH fluorescence, which has been ascribed to increased oxidant formation ($p < 0.001$) when compared to the control cells (Fig. 4A). TEMPOL abrogated this increase ($p < 0.001$ vs PA).

As elevated oxidative stress may be driven by dysregulation of mitochondrial complex proteins, cyt c and uncoupling protein 2 (UCP2) [35, 36], the levels of these proteins were assessed. Consistent with the increase in DCFH fluorescence, PA enhanced cyt c levels by 10% ($p < 0.05$) and showed a trend in increased expression of UCP2 by 49 %, compared to the controls (Fig. 4B, C). TEMPOL treatment reversed these changes in a dose-dependent manner ($p < 0.05$ vs PA). For UCP2 expression significance was only attained at a concentration of 1000 μM TEMPOL ($p < 0.001$ vs PA). To further explore TEMPOL-mediated effects on cellular antioxidant defences, the expression of glutathione peroxidase 1 (GPX-1) and catalase (CAT) were quantified, as these play a key role in oxidant removal. Although treatment of β-cells with PA failed to induce significant changes in GPX-1 and CAT expression, the addition of TEMPOL, significantly increased their expression at both 500 μM and 1000 μM concentrations ($p < 0.05$ vs PA; Fig. 4D, E).


A
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM
PERK (140 kD)
β-Actine (45 kD)
IRE1α (140 kD)
β-Actine (45 kD)
I
PERK protein expression (% Control)
II
IRE1α protein expression (%Control)
B
ATF6 mRNA expressions (% Control)

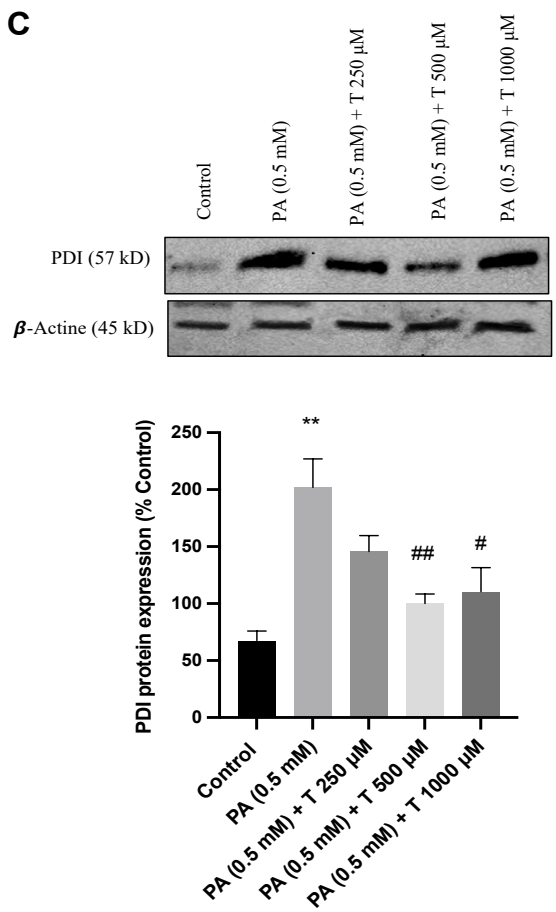


Figure 5: TEMPOL reduces ER stress induced by PA in MIN6 β-cells.

(A) TEMPOL treatments (250, 500, or 1000 µM) attenuate protein markers of ER stress in MIN6 β-cells incubated in 0.5 mM PA for 24 h.(A) Relative densitometry presented fold changes of (I) PERK (140 kDa) and (II) IRE 1α (130 kDa); β-Actin was used as a reference in loading control. (B) RNA was extracted from MIN 6 β-cells after PA + TEMPOL treatment and RT-qPCR was conducted to analyse ATF6 mRNA expression levels. (C) Relative densitometry presented as fold changes in PDI protein expression levels. β-Actin was used as a reference in loading control. Results are mean. Results are mean ± SEM (n =5–6). * $p < 0.05$ & ** $p < 0.001$ vs control, # $p < 0.05$ & ## $p < 0.001$ vs PA.

TEMPOL protects MIN6 β- cells from lipotoxic-induced endoplasmic reticulum stress.

Further experiments analysed whether TEMPOL modulated ER stress, as this pathway can activate signalling pathways that induce the expression of pro-apoptotic proteins and trigger programmed cell death, via the UPR [11, 15]. Exposure of MIN6 β-cells to PA increased PERK expression levels by ~ 160% ($p < 0.001$ vs control), which was abrogated by the treatment with TEMPOL in a dose-dependent manner (Fig. 5AI). Similarly, TEMPOL significantly decreased IRE 1α expression ($p < 0.001$ vs PA) compared to PA-treated cells (Fig. 5AII). PA also increased ATF 6 mRNA levels (by ~ 60%; $p < 0.05$ vs control, Fig. 5B), which was reversed with 1000 μM TEMPOL treatment ($p < 0.05$ vs PA). The effects of TEMPOL treatment on the UPR was also examined by quantifying protein disulfide isomerase (PDI), an ER-resident chaperone involved in regulating the activity of IRE1 α and ATF 6 activity [37]. Min 6 β-cells exposed to PA showed a significant increase in PDI protein levels by ~200% ($p < 0.001$ vs control, Fig. 5C), which was diminished by TEMPOL treatments at 500 μM ($p < 0.05$) and 1000 μM ($p < 0.001$) when compared to the PA group.

TEMPOL restores MIN6 β-cell function.

Lipotoxicity is negatively associated with β-cell functions such as insulinogenic index, early glucose-stimulated insulin secretion, β-cell glucose sensitivity, and rate sensitivities (adjusted for insulin resistance state) [38-40]. These effects may arise from disruption of the insulin transcription factors, such as pancreatic and duodenal homeobox 1 (PDX-1) which has been shown to induce pancreatic agenesis [41]. PDX-1 is therefore required for normal pancreatic β-cell function and its reduction impairs glucose sensing and insulin secretion [42]. Fig. 6A shows PA exposure results in a trend in the reduction of PDX-1 mRNA levels by ~23%. The presence of TEMPOL significantly enhanced PDX-1 mRNA (1000 μM; # $p < 0.05$ vs PA) in a dose-dependent manner to levels that were markedly greater than those observed in control cells, or after PA treatment.

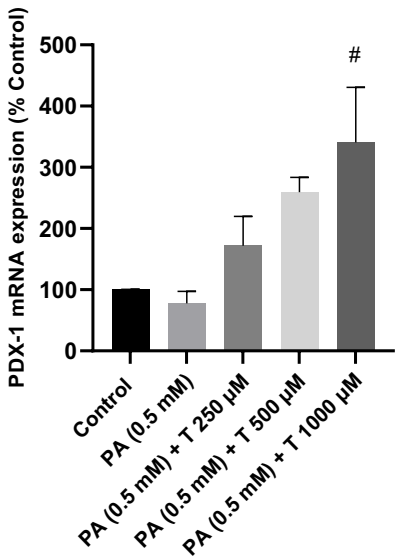
Glucose is the main stimulator of insulin release, though this is also dependent on protein kinase C (PKC). PKC directly stimulates insulin exocytosis, and inhibitors such as chronic lipid accumulation in β cells can suppress PKC, blocking insulin release [43, 44]. Fig. 6B shows that exposure of MIN6 β-cells to PA decreased PKC mRNA levels by ~77%, compared to the controls ($p < 0.001$ vs control), whilst TEMPOL reverted this effect significantly with all doses ($p < 0.001$ vs PA).

To delineate whether and how TEMPOL protects against insulin resistance, we measured phosphorylated serine residues of insulin receptor substrate (IRS-1). Increased phosphorylated serine IRS-1(PserIRS-1) is a well-characterised marker of insulin resistance as phosphate groups impair IRS-1 binding to insulin [45]. AMP-activated protein kinase (AMPK) levels were also quantified as low AMPK levels have been associated with insulin resistance and postulated as a potential therapeutic target [46, 47]. Incubation of MIN6 β-cells with PA showed a trend in increased PserIRS-1protein levels by ~22%, an effect that was reversed in a dose-dependent manner by TEMPOL (Fig. 6CI). Similarly, a trend of 16% reduction in pAMPK expression induced by 24 h of PA exposure, was reversed by TEMPOL, with protein levels increasing in a dose-dependent manner (Fig. 6CII).

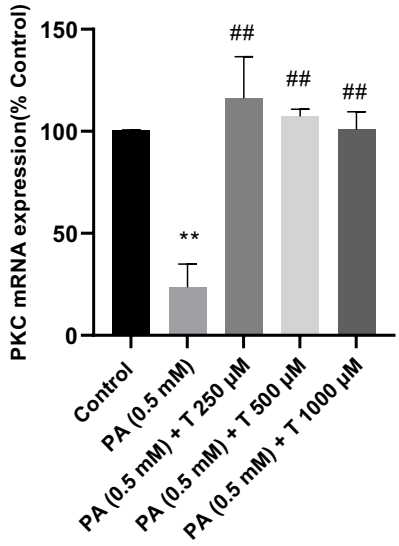
Effects of TEMPOL on insulin resistance in high-fat diet (HFD) fed C57BL/6 mice

C57BL/6 mice were fed a high-fat diet (HFD) or normal chow for 16 weeks before the assessment of insulin tolerance (ipITT) and glucose tolerance (ipGTT) tests (Fig. 6D, E). The HFD-fed mice had a significant increase in blood glucose levels compared to standard chow-fed mice ($p < 0.05$; Fig. 6D). Administration of TEMPOL to the HFD-fed mice significantly reduced blood glucose levels relative to the untreated HFD mice, with the final levels detected in these animals being comparable to those detected in the chow-fed mice ($p < 0.05$; Fig. 6D, E). A diet intervention control where mice fed an HFD for 16 weeks were subsequently switched to a chow diet for 8 weeks also improved insulin sensitivity, however, the TEMPOL-treated group showed a greater level of improvement ($p < 0.05$ vs HFD).

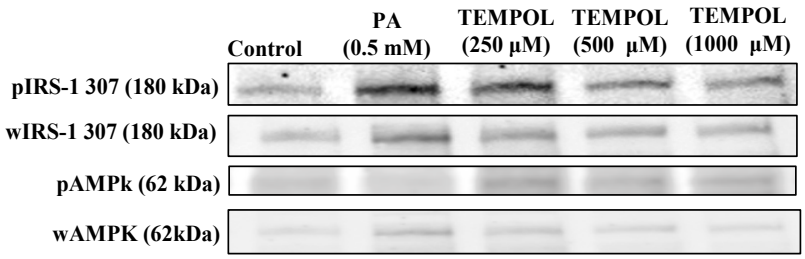
A


PDX-1 mRNA expression (% Control)
500
400
300
200
100
0
#
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

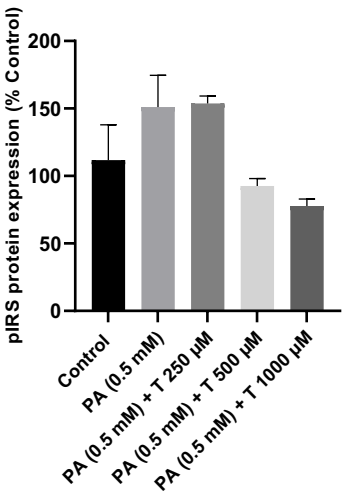
B

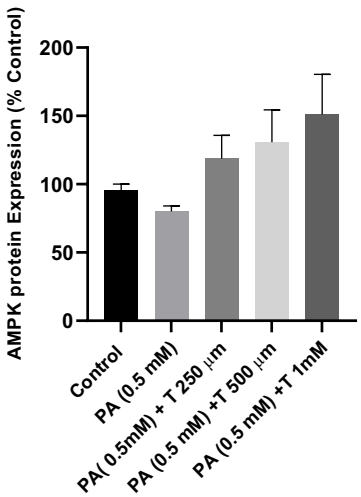

PKC mRNA expression(% Control)
150
100
50
0
**
##
##
##
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM

C


Control
PA (0.5 mM)
TEMPOL (250 µM)
TEMPOL (500 µM)
TEMPOL (1000 µM)
pIRS-1 307 (180 kDa)
wIRS-1 307 (180 kDa)
pAMPk (62 kDa)
wAMPK (62kDa)

II


pIRS protein expression (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA (0.5 mM) + T 250 µM
PA (0.5 mM) + T 500 µM
PA (0.5 mM) + T 1000 µM


AMPK protein Expression (% Control)
200
150
100
50
0
Control
PA (0.5 mM)
PA(0.5mM) + T 250 µm
PA (0.5 mM) +T 500 µm
PA (0.5 mM) +T 1mM

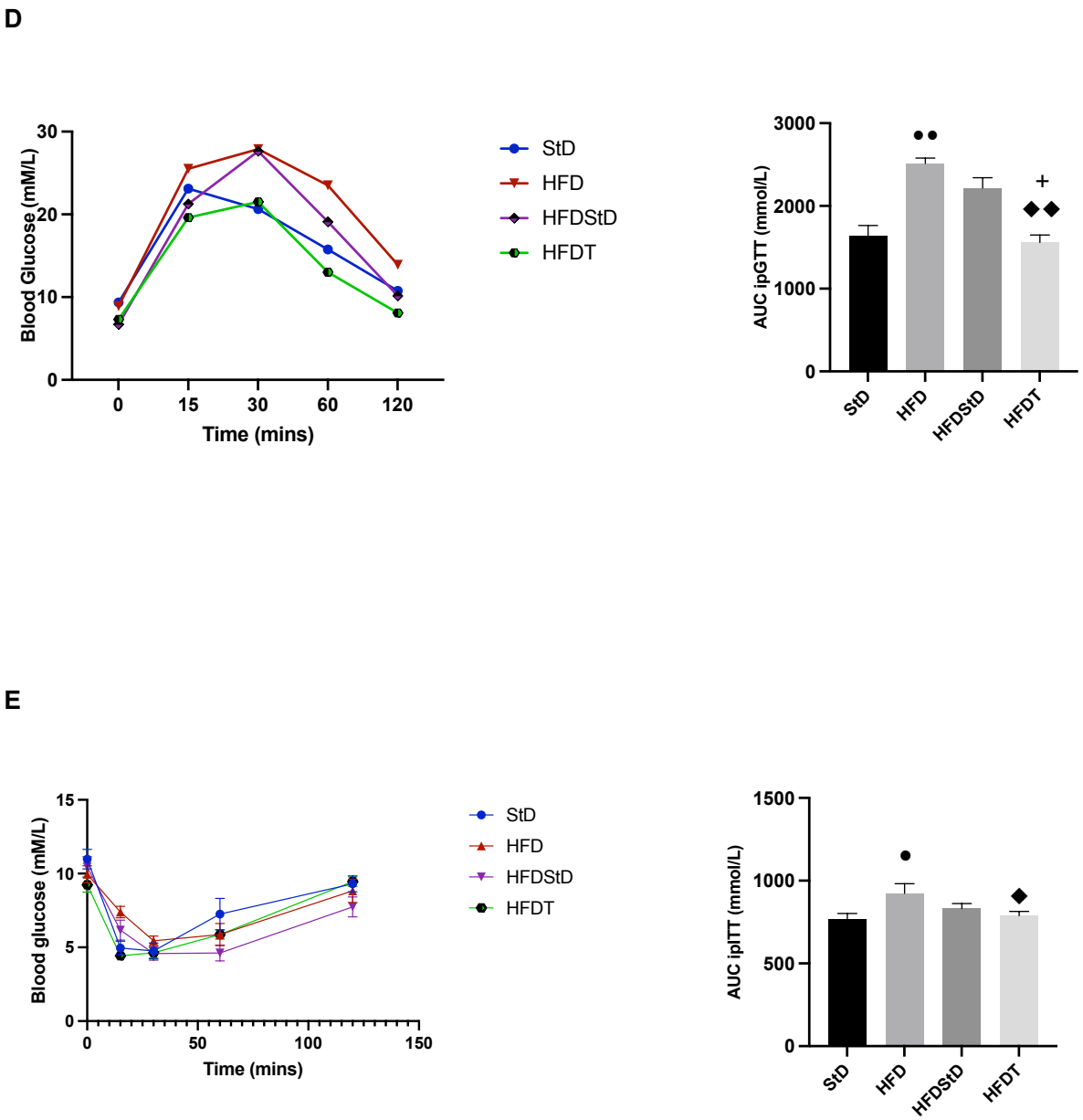


Figure 6: TEMPOL increased insulin sensitivity and insulin secretion
(A) RT-qPCR was conducted to analyse (A) PDX-1 and (B) PKC-1 mRNA expression levels which are involved in insulin secretion. (C) Protein expression levels of phospho- IRS-1 (Ser 307) and pAMPK were analysed using immunoblot. The native AMPK, and IRS antibodies were used as a reference. From the HFD *in vivo* model (D) ipGTT and (E) ipITT were calculated. Results are mean ± SEM (n =5–6). * $p < 0.05$ & ** $p < 0.001$ vs control, # $p < 0.05$ & ## $p < 0.001$ vs PA. ● $p < 0.05$; ●● $p < 0.001$ vs StD group. ♦ $p < 0.05$; ♦♦ $p < 0.001$ vs HFD group, + $p < 0.05$ vs HFDStD group.

Discussion

These data indicate that long-lived nitroxide radical TEMPOL is a potent protector against lipotoxic effects on model pancreatic β-cells, reversing both dysfunction and loss of metabolic function. Specifically, we show that TEMPOL abrogated inflammatory, mitochondrial damage and ER stress in cultured pancreatic β-cells. Furthermore, these *in vitro* protective effects of TEMPOL were mirrored by improved insulin sensitivity in an *in vivo* HFD-fed mouse model.

A critical stage in the development of insulin resistance is β-cell dysfunction. This loss of function and associated cell death can lead to decreased circulating insulin levels in response to a glucose load [48]. Given the importance of β-cell insulin secretion in maintaining glucose homeostasis, the major finding of this study is that TEMPOL treatment can protect pancreatic β-cells from lipid-induced apoptosis. It is well established that prolonged exposure of model β-cells to saturated fatty acids affects their function with the onset of β-cell death occurring 24 h to 48 h after exposure, under typical cell culture conditions [49, 50]. Current medications including metformin and insulin therapy fail to maintain pancreatic β-cell mass in people with T2DM, however, recent studies have reported that liraglutide (a GLP-1 receptor agonist) can increase pancreatic cell mass in diabetic rodents and restore to pre-diabetic levels [51]. Although our results are primarily *in vitro*, the protective effects of TEMPOL against lipotoxic effects on model pancreatic β-cells, as well as the reduction in oxidative stress and inflammation reported in other studies [16-21], are very likely to underpin, at least in part, the reversal of insulin resistance in TEMPOL-administered HFD-mice. In our study, TEMPOL treatment was more effective than diet intervention and this may be attributed to the stabilization and maintenance of β-cell mass, though this was not measured in this current study. This aspect would be worthy of examination in future studies.

β-cell dysfunction in response to overnutrition is primarily driven by the activation of the mTOR pathway [52]. If the mTOR activation is disrupted, it allows for β-cell expansion and therefore a sustained β-cell mass with associated maintenance or restoration of normal insulin secretion. Clinically, human pancreatic β-cells isolated from T2DM patients show higher mTOR activation, relative to non-diabetic, age- and sex-matched controls [53-55]. Current medications, such as sirolimus or the NovoMedix compound NMX2, are used to treat cardiac complications in T2DM patients with these drugs being effective at blocking of mTOR activation [56]. Our *in vitro* data clearly show that TEMPOL administration to cultured MIN6 β-cells suppresses mTOR levels (Fig. 7). Although activity was not measured, it is likely that

this decrease would result in suppression of mTOR pathway activation. The use of TEMPOL in cancer patients means that there is already some human safety and efficacy data for this compound [57], although further data are required (e.g. dosages, routes of administration or frequency of treatment) would be needed before TEMPOL could be employed in this manner. In keeping the β-cell mass increase, TEMPOL also had a positive effect on insulin secretion mediators. Not surprisingly, β-cells protected from lipid-induced cell death pathways showed improvement in PDX-1 expression, PKC activity, pAMPK levels and reduced PserIRS-1 after TEMPOL treatment. Maintenance of PDX-1 levels is a well-established target for treatment with anti-diabetic drugs including DA -1241, tectorigenin and liraglutide [58-62]. PKC is directly involved in the insulin secretion pathway promoting exocytosis of the hormone, however, drugs that target PKC directly have not been developed as PKC belongs to a large family of related proteins and therefore off-target effects are possible [43, 44, 63]. pAMPK is a target for the current anti-diabetic drug, metformin, however, studies have shown that metformin overdosage can lead to β-cell toxicity [64]. Our finding that TEMPOL has effects on all these key pathways that modulate β-cell insulin secretion and/or sensitivity is promising. However, whether chronic exposure to TEMPOL or dosage effects as seen with metformin, lead to long-term β-cells dysfunction requires further evaluation.

The improvement in β-cell function, despite exposure to high levels of FFAs, as used in the current study, may arise from the reported actions of TEMPOL as an antioxidant and anti-inflammatory agent [19, 20, 65, 66]. High cytokine levels and oxidative stress are key inducers of β-cell dysfunction and apoptosis [67] with β-cell dysfunction being detectable prior to the onset of T2DM in a high-fat diet-fed mouse model [68]. TEMPOL treatment of MIN6 β-cells decreased PA-induced IL-6 and TNF expression levels, decreased oxidation (as measured by DCFH fluorescence), and associated ER stress as evidenced by suppression of UPR protein levels (Fig. 7). A possible mechanism for these strong protective effects is its effect on promoting the expression of endogenous antioxidants, such as GPX1 and catalase. The degree of oxidative and inflammatory stress reduction is in keeping with other anti-diabetic drugs, including metformin, thiazolidinedione (TZD) and salicylate [69-71]. Further work is warranted to directly compare TEMPOL against these gold-standard treatments for T2DM.

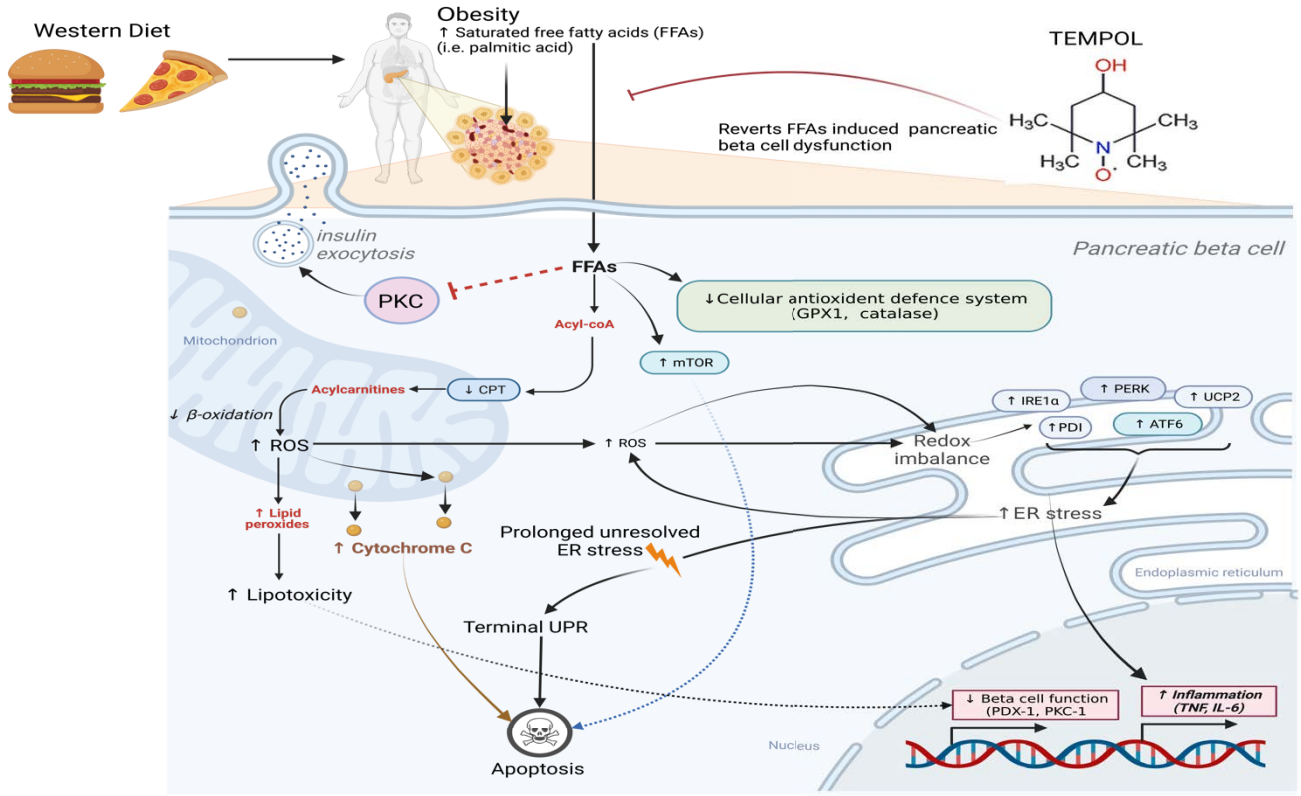


Figure 7. Schematic diagram of TEMPOL's underlying mechanisms in preventing MIN 6 β cell dysfunction induced by saturated free fatty acids.
Image generated using BioRender (BioRender.com)

The effect of TEMPOL on the UPR is a novel finding, with this compound affording protection against activation of the 3 key facilitators: PERK, IRE-1, and ATF-6. Of these, PERK is the most well-studied and it is accepted that directly targeting PERK activation leads to toxic effects in cells [72]. An alternative approach that has been suggested is to target a protein chaperone, PDI, of PERK. If PDI levels are suppressed, as seen in this study, this inhibits PERK activation and subsequently enhances cell survival [73, 74].

In summary, TEMPOL has been demonstrated to have potent anti-inflammatory and protective actions for pancreatic β-cells against ER stress and metabolic dysfunctions [19, 20, 65]. TEMPOL also improved insulin signalling pathways and *in vivo* reversed HFD-induced insulin resistance [21, 75]. The underlying mechanism(s) for TEMPOLs' effects in diminishing lipid-induced β-cell dysfunction may involve either its antioxidant properties or its ability to block lipid accumulation in cells [66, 76]. Both effects were confirmed in the present study for β-cells. Our demonstration that TEMPOL has such strong protective effects against β-cell dysfunction *in vitro* may act as a catalyst to research its potential as a therapeutic option to preserve β-cell function in insulin-resistant states including early T2DM. Such translation to humans may be aided by the use of TEMPOL to reduce radiation-induced hair loss in humans [57, 77] and is in clinical trials to treat COVID-19 [78], prostate cancer [79, 80] and fibrocystic disease [81].

Declarations

Funding statement

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Conflicts of interest

MJD declares commercial consultancy contracts with Novo Nordisk A/S. This funder has no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish these results. The other authors declare no conflict of interest with regard to the data presented.

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Table S1: Murine forward and reverse primer sequences**.**

Primer	Primer Sequence		Accession Number
Reference Gene			
18S	F	AACTTTCGATGGTAGTCGCCGT	NM_146819.2
	R	TCCTTGGATGTGGTAGCCGTTT	
Glucolipotoxicity Genes			
Carnitine palmitoyltransferase 1 (CPT-1)	F	GGGAGGTACATCTACCTG	NM_013495.2
	R	GMGACGTAGGTTTGAGTTC	
Apoptotic Genes			
Mammalian target of rapamycin (mTOR)	F	CTTCACAGATACCCAGTACC	NM_020009.2
	R	AGTAGACCTTAAACTCCGAC	
Inflammatory Genes			
Interleukin- 6 (IL-6)	F	GAACAACGATGATGCACTTGC	NM_031168.2
	R	TCCAGGTAGCTATGGTACTCC	
Interleukin- 8 (IL-8)	F	ACTTTTGGCTCAACCCTGTG	NM_009909.3
	R	ACGCAGTACGACCCTCAAAC	
Interleukin-1β (IL-1β)	F	GCTCAGGGTCACAAGAAACC	NM_008361.4
	R	CATCAAAGCAATGTGCTGGT	
Tumour necrosis factor (TNF)	F	CTGTGAAGGGAATGGGTGTT	NM_013693.3
	R	CTCCCTTTGCAGAACTCAGG	
Oxidative Stress and Antioxidant Genes			
Uncoupled protein 2 (UCP2)	F	ACCTTTAGAGAAGCTTGACC	NM_011671.6
	R	TTCTGATTTCCTGCTACCTC	
Glutathione Peroxidase 1 (GPX1)	F	ATTGCCAAGTCGTTCTACGA	NM_030677.2
	R	GTAGGACAGAAACGGATGGA	
Catalase (CAT)	F	CTCCATCAGGTTTCTTTCTTG	NM_009804.2

	R	CAACAGGCGTTTTTGATG ′	
ER stress genes			
Activating transcription factor 6 (ATF6)	F	AGCTGTCTGTGTGATGATAG	NM_001081304.1
	R	GTGATCATAGCTGTAACTGTC	
Insulin secretion aiding genes			
Pancreases duodenum homeobox 1 (PDX-1)	F	GATGAAATCCACCAAAGCTC	NM_008814.4
	R	TAAGAATTCCTTCTCCAGCTC	
Protein kinase C (PKC)	F	GTCTCAGAGCTAATGAAGATG	NM_011101.3
	R	TTGGCTTTCTCAAACTTCTG	

Table S2: Antibodies used for immunoblot analysis

Specificity	**Host**	**Supplier and Cat #**
PERK	Rabbit mAB	Cell Signalling: 5683
IRE 1 α	Rabbit mAB	Cell Signalling: 3294
PDI	Rabbit mAB	Cell Signalling: 3501
pIRS (ser)	Rabbit mAB	Cell Signalling: 2381
β Actine	Rabbit mAB	Cell Signalling: 13E5
Anti- Rabbit IgG- HRP link antibody	Rabbit	Cell Signalling: 7074

Chapter 7 : Conclusion and Future Directions

Chapter Summary

The research undertaken in this PhD project focused on investigating the effect of nitroxide TEMPOL on protecting against the progression of obesity-induced insulin resistance and associated T2DM. Overall, the results presented provide strong foundation evidence for the potential development of TEMPOL as a pharmacological therapy for insulin resistance, especially that associated with obesity. In this chapter, I will summarise the key findings, discuss experimental limitations and explore future directions that could help bring this nitroxide, TEMPOL to the clinic.

7.1 Hyperlipidaemia

One of the major goals of the research of this thesis was to explore the effects of TEMPOL on hyperlipidaemia. Hyperlipidaemia is central to the obese state and is a common pathology amongst obese T2DM patients. High levels of FFAs are well recognised to be not a consequence, but causal of insulin resistance, and indeed commonly used pharmacological approaches target anti-inflammatory pathways [258-260]. Given the primary role of hyperlipidaemia in driving insulin resistance, it is of major importance that results reported in Chapters 4, 5 and 6 all highlight the ability of TEMPOL to markedly reduce hyperlipidaemia. Significantly in Chapter 4, it was shown that the decrease in lipid accumulation in adipose tissue (*in vivo*) and adipose cells (*in vitro*) was associated with lowered body mass. This finding is in keeping with Mitchell et al. (2003), who also showed this anti-obesity effect of TEMPOL [239]. Chapter 4 shows the reduction in mean adipocyte cell size, indicating that TEMPOL reduces adipose hypertrophy. Further to this, the underlying mechanism/s to explain the decrease in lipid accumulation involved both decreased lipogenesis and lipolysis. Together, the previous and present data provide impetus to further explore the option of TEMPOL as a weight-reducing agent for obese patients.

The liver tissue studies of Chapter 5 confirmed TEMPOL's effect in blocking lipid accumulation. Further to that, the decreased lipid accumulation is associated strongly with reduced hepatic inflammation and oxidative stress, very likely associated strongly with reduced ER stress. Many studies have reported that hepatic inflammation drives insulin resistance and the finding that TEMPOL can suppress dominant cellular stress pathways demonstrates the utility of this drug as an antioxidant and anti-inflammatory.

It was hypothesised that the mechanism that underlies the anti-obesity effect of TEMPOL is its capacity to improve lipid-suppressed rates of fatty acid β-oxidation that results in higher uptake of FFA's [261, 262]. Obesity and its associated insulin-resistant state are reported to markedly disturb fatty acid metabolism [263, 264], therefore any agent able to restore fatty acid β-oxidation is likely to be of clinical benefit. In Chapters 4, 5 and 6, it was shown that TEMPOL increases the expression of critical enzymes involved in fatty acid β-oxidation, including CPT-1 and PPAR δ and from previous studies, there is a strong link between CPT-1 expression and enhanced rate of fatty acid β-oxidation[265, 266]. Thereby TEMPOL raising CPT-1 levels is in keeping with the finding that TEMPOL increases the expression of CPT-1 regulator PPAR

δ in 3T3l-1 adipocytes (Chapter 4). Others have shown that PPAR δ agonists (e.g., GW501516, GW0742) promote expressions of CPT-1 [267, 268]. indeed, modulating PPAR δ activity has become an attractive target for obesity-related metabolic disease.

The data provided in this thesis, together with the known literature, allows for a much stronger recognition of TEMPOL's anti-obesity effect and the likely involvement of improved fatty acid β-oxidation. Further studies are now necessary to explore the wider effect of TEMPOL on the dietary pathway for example, if TEMPOL impact on satiety and energy expenditure. Mitchell et al. (2003) reported the increased exercise of mice on TEMPOL supplementation with no impact on food intake. The energy hormones, leptin and ghrelin affect satiety and energy level homeostasis and thus the impact of TEMPOL on the cellular pathways regulated by both hormones would be of interest, especially if TEMPOL leads to raised leptin levels. Additionally, whether extended treatment times provide further benefits and/or whether periodisation of TEMPOL treatment (e.g., on/off regime) is also effective could be worth exploring (given the mice had unlimited access to food sources, it is likely that treatment persisted throughout their waking hours). It would also be worth evaluating for sustained effects of post-treatment and how long any such sustained effect persists. Such studies will provide a deeper understanding of TEMPOL's mode of action at the molecular level in reducing hyperlipidaemia as well as beginning to understand safety and efficacy at the pre-clinical level.

Given that TEMPOL is already in clinical trials for other indications [234, 269-271], the mice used in the present study remained healthy throughout the eight weeks of TEMPOL treatment, with no change in food intake and no indication of liver toxicity presented by the liver enzymes levels (AST & ALT) data in Chapter 5. Mitchell et al. (2003) also reported no adverse effects of TEMPOL on mice in their 25–52 week study. The safety of TEMPOL as a treatment was further shows the current research with cell viability data *in vitro* models of 3T3L-1 adipocytes (Chapter 4), HepG2 hepatocytes (Chapter 5) and MIN6 pancreatic β cells (Chapter 6).

7.2 Inflammation

Chronic systemic inflammation is present in individuals who progressed to T2D and is proposed to be involved in the pathogenic process of obesity-induced T2DM [272]. Population studies have shown the relationship between inflammatory markers and lipid metabolism abnormalities in obesity and T2DM [273-275]. Although there are several drugs (e.g. aspirin,

calcineurin inhibitors) available to directly target components of the inflammatory system [276, 277], patients with obesity-induced T2DM and associated states such as NAFLD, may experience an improvement in the inflammatory pathways. Similar to these anti-inflammatory drugs, the data shown in this thesis provides strong evidence for TEMPOL as a potential anti-inflammatory drug. Both the *in vivo* and *in vitro* data show that TEMPOL suppressed FFA-induced inflammatory transcription factor expression and reduced inflammatory cytokine expression.

The two main inflammatory transcription factor signalling pathways are nuclear factor kappa B (NFκB) and c-Jun NH_2 terminal kinase (JNK) [278, 279]. NFκB is activated by an inhibitor of NFκB (IkB) kinase B (IKK β), which regulates the transcription of multiple inflammatory cytokines such as TNF, IL-6, IL-1 β, resistin and MCP-1 [280]. By contrast, activation of the JNK pathway directly impacts the insulin signalling pathway. Genes knock-out studies show that the inhibition of the JNK increases insulin sensitivity [281], while activation of the JNK pathway as initiated by excess FFA drive phosphorylation of the insulin receptor substrate (IRS), thereby inhibiting the insulin signalling transduction pathway and promotes insulin resistance [282]. The anti-inflammatory effects of TEMPOL were shown in the current study to suppress FFA-activated NFκB and JNK showing the double prolonged approach that treatment with TEMPOL would create the data that is now needed if prolonged suppression of both inflammatory pathways leads to longer-term adverse consequences.

If we consider an alternate anti-inflammatory agent now in practice, salsalate, by comparison to TEMPOL, then suppression of NFκB has a very potent effect on sensitising the insulin signalling pathways. In T2DM patients, salsalate treatment showed decreased systemic inflammation associated with improved glucose metabolism in T2DM [283, 284]. Salsalate also demonstrated sensitised insulin pathway signalling in obese rodents via inhibiting the NFkB pathway. In Chapter 5, it was demonstrated that TEMPOL suppressed HFD-induced phosphorylated NFκB and JNK protein levels that were associated with a reduction of macrophage infiltration in liver tissue. TEMPOL also reduced MCP-1, IL-6, IL-1 β and TNF, the main cytokines involved in obesity-induced T2DM [285]. IL-6 & IL-1 β are reported to increase the risk of developing T2DM by threefold [286]. TNF is hyperexpressed in obesity and known to impair insulin signalling [183], and IL6 and MCP-1 are known to promote inflammation and macrophage recruitment [287, 288]. Salsalate is in Phase III clinical trial and it follows based on the similarity in underlying mode of mechanism between salsalate and

TEMPOL that there is a foundation of investigating the potential of TEMPOL in sensitising the insulin response via suppressed inflammation in insulin-resistant patients.

The anti-inflammatory properties of TEMPOL are well-recognised for other medical indications. For example, TEMPOL protects against cisplatin (CP)-induced acute kidney injury (AKI) and acute pancreatitis models [289, 290]. In summation, the current study only supports the potent anti-inflammatory properties of TEMPOL that are increasingly recognised.

7.3 Oxidative Stress

The correlation between high oxidative stress levels and insulin resistance has been reported previously [291, 292]. ROS development can be a vicious cycle in obese patients, as overconsumption of macronutrients, such as glucose and FFA, increases substrate availability for ATP production. This results in mitochondria being hyperactive, ultimately producing more ROS. Increased ROS levels directly stimulate stress responses such as ER stress, systemic inflammation, and cellular apoptosis. Further to this, excess FFA leads to high mitochondrial fissions in adipocytes that also increase ROS production. No matter the source, ROS disrupts the insulin transduction pathway [293, 294]. Chapter 4 examines the antioxidant potency of TEMPOL in visceral adipose tissue (VAT) from the HFD *in vivo* model and 3T3L-1 adipocytes from the PA incubated *in vitro* model. TEMPOL supplementation increased Tafazzin expression, an enzyme that supports mitochondrial function to ensure less disruption to the electron transport chain (ETC). In turn, this stops elevated ROS production. Additionally, TEMPOL treatment of PA-induced 3TL-1 cells showed decreased cytochrome C (cyt C) levels, a mitochondrial protein that can actively increase endogenous ROS levels. Similar effects were reported in Chapters 5 and 6 for liver and pancreatic cells, respectively. Overall, our findings with other support the powerful antioxidant effects of TEMPOL.

TEMPOL not only acted as a direct antioxidant but also stimulated the expression of their antioxidant proteins and enzymes. TEMPOL treatment of HFD-fed mice increased adipose tissue levels of nuclear factor erythroid 2-related factor 2 (NRF2) and glutathione peroxidase 1 (GPX1) expression. These enzymes neutralise the final redox products of endogenous radical-scavenging antioxidants [295]. Furthermore, the results presented in Chapter 5 showed that TEMPOL enhanced expressions of other endogenous antioxidants such as catalase (CAT). An increase in endogenous oxidative stress can damage protein turnover in cells, leading to

damaged protein accumulation, causing ER stress [209, 296]. TEMPOL treatment suppressed inositol response element-1 (IRE-1), activating transcription factor (ATF)-6 and protein kinase-like ER kinase (PERK) showing that not only does TEMPOL reduce oxidative stress levels but can also improve downstream consequences including ER stress.

Although the underlying mechanism of TEMPOL's antioxidant effect is not fully elucidated, the evidence shows direct and indirect antioxidant effects in a number of cell types. This has a downstream impact on reducing inflammation and improving insulin sensitivity. More work remains to show full mechanistic action. The studies here imply the involvement of mitochondrial function, however, to fully elucidate studies of oxygen consumption rate (OCR) in mitochondria of live cells to unequivocally show the protective effect of TEMPOL on these organelles is now warranted. Additionally, a combination of TEMPOL and other antioxidants (curcumin, resveratrol, coenzyme Q10 and α-lipoic acid) that is known to reduce oxidative stress (OS) in the progression of diabetes [297-299] could be tested to determine if there are synergistic beneficial effects of different combinations. Further to this, to enhance the efficacy of TEMPOL in treating insulin resistance, exploring drug delivery methods involving microparticles, nanoparticles, or liposomes is warranted. While the current clinical trial delivery mechanisms could be initially replicated for proof-of-principle studies, consideration could also be given to targeted delivery, such as utilizing Mito-TEMPO, to specifically target organelles. This approach would allow for a more tailored and potentially more effective delivery of TEMPOL to specific cellular compartments involved in insulin resistance.

It is worth noting that Tempol’s potential may extend beyond diabetes. Considering the oxidative mechanisms involved in pancreas pathology in conditions such as COPD and ARDS [300], TEMPOL could prove effective for these conditions. While mechanistic studies are now warranted to more fully delineate TEMPOL's protective cellular actions, the overall potential of TEMPOL to improve insulin sensitivity justifies expediting its progressing to a pilot clinical trial.

7.4 Insulin sensitivity

Insulin resistance develops when there is a defect in the insulin signalling pathway. Primarily there is decreased autoactivation of insulin receptors in insulin-sensitive tissues, lowering their insulin sensitivity [301]. Hyperlipidaemia, inflammation, and oxidative stress are the leading

causes of disruption to the pathway. The auto-activation of insulin receptors is inhibited when there is excess serine phosphorylation of insulin receptor substrate -1 (IRS-1). Tyrosine phosphorylation is required for normal insulin signalling [154, 302]. Studies have shown the inactivation of the insulin receptor in adipose tissues of T2DM patients [303]. These effects were also observed in this current study where in, Chapter 4, it is shown that VAT from the HFD-fed mice shows a decrease in the insulin sensitiser, adiponectin. Additionally, in the PA-incubated 3T3L-1 *in vitro* model, where adiponectin and glucose transporter- 4 (GLUT4) expressions were increased with TEMPOL treatment. Normal GLUT4 function is central to responsive insulin sensitivity.

Hepatic steatosis is often associated with decreased hepatic insulin sensitivity. Chapter 5 investigated the effect of TEMPOL on the hepatic insulin sensitivity of the liver. One of the significant results presented in this chapter was the TEMPOL's effect on AMP-activated protein kinase (AMPK) protein expression levels. AMPK is a marker of insulin resistance and is reported to show low levels in T2DM patients. An increase in AMPK associates with increased glucose uptake and glycogen synthesis and decreased lipolysis and FA synthesis [304, 305]. Due to its critical role in hepatic insulin sensitivity, it is the target of current anti-diabetic drugs. In the current study, TEMPOL increased AMPK expression and this was linked to pronounced improvement in insulin resistance as evidenced by HOMA-IR in the TEMPOL-supplemented group.

Defective insulin secretion is reported for obese and NAFLD patients. In Chapter 6, upon exposure to the saturated fatty acid, PA, the MIN6 β-cell in vitro model demonstrated decreased protein kinase C (PKC) and pancreatic and duodenal homeobox-1 (PDX-1) expression. Decrease of both of these proteins is common to defective insulin secretion. TEMPOL treatment blocked the decrease in PDX-1 and PKC expression against demonstrating the insulin sensitising properties of TEMPOL.

In summary, TEMPOL has strong antioxidant, anti-inflammatory and insulin-sensitising properties across the three major cell types, liver, adipose and pancreatic, central to insulin resistance and the development of type 2 diabetes. Moreover, TEMPOL is effective as an anti-obesity drug blocking lipid accumulation by acting to increase lipolysis and reduce lipogenesis. Together, the results of this study provide impetus to drive the study of TEMPOL and its possible therapeutic use for the treatment of obesity-related insulin resistance and the

subsequent progression to type 2 diabetes. Its analogous mechanisms of action to other commonly used agents including anti-diabetic, anti-inflammatory and antioxidant drugs coupled with the existing use of TEMPOL for other medical indications provides a strong foundation for testing TEMPOL in the clinical arena. While some mechanistic studies remain warranted to explain all of its protective cellular actions more fully, the overall potential of TEMPOL to improve insulin sensitivity is worth fast-tracking the drug to a pilot clinical trial.

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