The drug interaction between N-acetylcysteine, ascorbic acid-2 phosphate and metformin

by
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Declaration

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ABSTRACT

Background: Type 2 diabetes (T2DM) is a glucose metabolism disorder. Its prevalence is increasing rapidly in sub-Saharan Africa with it negatively impacting global health systems and the economy. The South African Department of Health management objectives for the treatment of T2DM is to relieve symptoms, prevent acute metabolic and long-term complications, improve quality of life and productivity of patients and reduce the economic burden on individuals, family, and community. However, despite achieving glucose control, >80% of T2DM patients still develop co-morbidities because of persistent oxidative stress and inflammation. Adjuvant treatments using antioxidants are effective at counteracting oxidative stress, it is however unclear if these treatments will interfere with the insulin-sensitizing function of metformin, which is available as the first-line oral medication for the treatment of T2DM. This study therefore investigated if there is any drug interaction between metformin and the antioxidants, N-acetylcysteine (NAC) and ascorbic acid-2-phosphate (AAP).

Methods: *In vitro* experiments were performed using C2C12 skeletal muscle myoblasts under different treatment periods and pretreatments to determine if the antioxidants NAC and AAP alters glucose uptake and thus the requirement for Metformin.. Cellular growth and viability under low and high glucose culture conditions were assessed over a period of 48 hours. Additionally, a dose response experiment over a period of 6 days exposing cells to 5 different concentrations of NAC and AAP was performed to determine the optimal and non-toxic concentration of the specific antioxidants. Glucose uptake was assessed using fluorescent microscopy and the 2NBDG assay under various conditions (insulin, metformin) following pretreatment (24h) with either NAC and/or AAP. The beneficial effect(s) of combination therapy was also determined by assessing the Total Antioxidant Capacity (colorimetric assay) and reactive oxygen species (ROS) (fluorometric assay) within the culture supernatants with and without NAC and/or AAP pretreatment (24h).

Results: Over the 48-hour period, cells cultured in high glucose conditions had a significantly ($p<0,0001$) higher cell number per $10mm^2$ plate surface area when compared to cells cultured in low glucose conditions. The optimal concentrations of NAC and AAP was determined to be 3.75mM and 0.6mM, respectively. The combination of AAP, NAC and metformin treatment significantly decreased ROS levels (2-fold, $p<0.05$) and increased the total antioxidant capacity ($p<0.01$) (11.57±5.66 U/mL) when compared to metformin treatment on its own (0.81±2.48 U/mL). The pretreatment (24h) of cells with a combination of AAP/NAC prior to glucose starvation (2h) and exposure to either insulin (30min) and/or metformin (2h) significantly increased glucose uptake compared to cells without pre-treatment.

Conclusion: There is a comparable effect between metformin, NAC and AAP when used in combination with each other, which reduces oxidative stress *in vitro*. Additionally, the combination of metformin, NAC and AAP improves glucose uptake in C2C12 mouse myoblasts *in vitro* that resulted in altered glucose profiles. Thus, patients taking adjuvant antioxidants may require glucose monitoring and changes in metformin requirements. This study warrants further investigation to determine the precise mechanism of action underlying the synergistic effect observed between NAC, AAP and metformin affecting glucose uptake. Screening the efficacy of other anti-diabetic agents and antioxidants that target both glucose homeostasis and oxidative stress within a diabetic microenvironment as well as its associated comorbidities is furthermore recommended.

OPSOMMING

Agtergrond: Tipe 2 diabetes (T2DM) is ‘n glukose metabolisme siekte. Daar is ‘n vinnige groei in gevalle in sub-Sahara Afrika wat wêreldwyd ‘n negatiewe invloed het op albei gesondheids-en-ekonomiese stelsels. Die Suid-Afrikaanse Departement van Gesondheid se doelwitte met betreking tot die beheer en behandeling van T2DM is om simptome te verlig, lang termyn en akute metaboliese komplikasies te verhoed, om die lewensgehalte en produktiwiteit van die pasiënt te verbeter asook om die ekonomiese uitdagings op die individu, hul familie en gemeenskap te verminder. Alhoewel daar beheer van glukose onder pasiënte is, word daar steeds komorbiditeite aangetref onder >80% van T2DM pasiënte as gevolg van langdurige oksidatiewe stres en inflamasie. Die gebruik van antioksidante as wyse van behandeling is effektief en werk teen oksidatiewe stres, maar dit is egter onduidelik of hierdie manier van behandeling enige invloed het op die komponent van metformien wat ‘n rol speel in die insulien-sensitiwiteit van pasiënte het, wat as ‘n medikasie beskikbaar is en die eerste opsie aan pasiënte vir die behandeling van T2DM is. Hierdie studie het ondersoek of daar enige interaksie is tussen metformien en die antioksidante N-asetielsistieën (NAC) en askorbiensuur-2-fosfaat (AAP).

Metodes: In vitro eksperimente is gedoen en daar is van C2C12 skeletspier mioblaste gebruik gemaak om vas te stel of die antioksidante NAC en AAP die opname van glucose beïnvloed en dus ook die die gebruik van metformien. Sellulêre groei en lewensvatbaarheid is oor ‘n tydperk van 6 dae onder hoë sowel as lae glukose-konsentrasie kulture gemeet. ń Dosis-reaksie eskperiment is ook oor ‘n tydperk van 6 dae gedoen waartydens selle aan 5 verskillende konsentrasies van NAC en AAP blootgestel is om vas te stel wat die ideale dosis is en hoe om toksisiteit te verhoed rakende die spesifieke antioksidante. Die opname van glukose onder verskeie omstandighede is getoets deur middel van fluoresserende mikroskopie asook die 2DG-toets (Insulien, Metformien) ná die toediening van NAC en/of AAP behandeling. Die voordelige effek(te) van die kombinasie-terapie is bepaal deur die Totale Antioksidant Kapasiteit (kleurmetriese toets) en reaktiewe suurstof spesie (ROS) (flourometriese toets) te assesseer binne die kultuur supernante met en sonder NAC of AAP.

Resultate: Oor ‘n tydperk van 48-uur het selle in hoë glukose-konsentrasie omstandighede ($p<0,0001$) aansienlike hoër selnommers per $10mm^2$ opervlakte getoon as dié wat in lae glukose-konsentrasie omstandighede gekweek is. Die optimale konsentrasies van NAC en AAP is vasgestel as 3.75mm en 0.6mm onderskeidelik. Deur NAC, AAP en Metformien te kombineer het die behandeling die ROS-vlakke aansienlik gedaal (2-voudig, $p<0.05$) en het die Totale Antioksidant Kapasiteit ($p<0.01$) (11.57 +/-5.66 U/ml) verhoog invergelyking met Metformien as alleenlike behandeling (0.81+/-2.48 U/ml). Deur selle vooraf vir 24-uur met ‘n

kombinasie van NAC/AAP te behandeling, gevolg deur 2 ure in die afwesigheid van glukose en uiteindelik blootstelling aan insulien (30min) en/of metformien (2 ure), het die glukose opname van die selle beduidend verhoog in vergelyking met selle wat nie die vooraf behandeling ontvang het nie.

Gevolgtrekking: Die kombinasie van Metformien, NAC en AAP toon ‘n sinergistiese effek wat oksidatiewe stres verminder in vitro. Verder, verbeter die kombinasie van metformien, NAC en AAP die glukose opname in C2C12 muis mioblaste in vitro en het dit gelei tot veranderde glukose profiele. Dus, mag pasiënte wat ekstra antioksidante neem dalk glukose-monitering sowel as veranderinge in Metformien vereistes benodig. Hierdie studie wil verdere ondersoek regverdig om vas te stel wat die presiese meganisme van aksie is wat lei tot die singergistiese effek wat waargeneem is tussen NAC, AAP en metformien en glukose opname beïnvloed. ‘n Siftingstoets wat die doeltreffendheid van ander anti-diabetiese agente en antioksidante wat beide glukose opname en homeostase teiken sowell as oksidatiewe stress binne a diabetiese mikro-omgewing en die ko-morbiditeite wat daarmee geassosieer word ondersoek, word verder voorgestel.

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ABBREVIATIONS

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose)
AAP	Ascorbic acid-2 phosphate
ACC	Acetyl-CoA carboxylase
AGEs	Advanced glycation end products
AMPK	Adenosine monophosphate kinase
ANT2	Adenine nucleotide translocase 2
ATP	Adenosine triphosphate
COXs	Cyclooxygenases
CVD	Cardiovascular diseases
CYPOR	Cytochrome P450 reductase
Cys	Cysteine
DAG	Diacylyglycerol
DCF	Oxidized dichlorofluorescein
DCFH	Dichlorofluorescein
DCFH-DA	2,7-dichlorofuorescin diacetate
DHA	Dehydroascorbic acid
DM	Diabetes mellitus
DPP4i	Dipeptidyl peptidase-4 inhibitor
eNOS	Endothelial NO synthase
FBS	Foetal bovine serum
FFA	Free fatty acids
Flav1	5,7,4´-trihydroxy-3,6,3´,5´-tetramethoxyflavone
G6P	Glucose-6-phosphate
GCL	Glutamate cysteine ligase
GLP-1a	Glucagon-like peptide-1 receptor agonist
GLUT	Glucose transporters
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Disulfide oxidized GSH
H_2O_2	Hydrogen peroxide
hADMSCs	Human adipose tissue-derived mesenchymal stem cells
HbA1c	Glycated haemoglobin
HIF-1α	Hypoxia-inducible factor-1α

HIV/AIDS	Human immunodeficiency virus/ acquired immunodeficiency syndrome
HO	Hemeoxygenases
HOCl	Hydrochlorous acid
hsCRP	High-sensitivity C-reactive protein
IAPP	Islet amyloid polypeptide
ICAM1	Intercellular adhesion molecule-1
INSR	Insulin receptor
IL-6	Interleukin- 6
IL-17	Interleukin-17
IL-1β	Interleukin-1β
Il-23	Interleukin-23
IR	Insulin resistance
IRS	Insulin receptor substrates
IV	Intravenous
JNK	C-Jun N-terminal kinase
kg	Kilogram
LHE	Lowest human equivalent
MAPK	P38 Mitogen-activated protein kinase
MATE	Multidrug and toxin extrusion
MATE1	Multidrug and toxin extrusion 1 transporter
MATE2	Multidrug and toxin extrusion 2 transporter
MCP-1	Monocyte chemotactic protein-1
MFI	Mean fluorescent intensity
mg	Milligram
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-p-benzoquinoneimine
NF-kB	Nuclear factor-κB
NFκB p65	Nuclear factor kappa B p65
nm	Nanometre
NO	Nitric oxide
NO_2	Nitrogen dioxide

NOX	Nicotinamide adenine nucleotide phosphate oxidase
O_2^-	Superoxide
OCT1	Organic cation transporter 1
OCT2	Organic cation transporter 2
OCTs	Organic cation transporters
OGTT	Oral glucose tolerance test
OH	Hydroxyl
$ONOO^-$	Non-radical peroxynitrite
PAI-1	Plasminogen activator inhibitor-1
PARP	ROS/Poly ADP-ribose polymerase
PBS	Phosphate buffered solution
PI3K-Akt	Phosphoinositide-3-kinase-protein kinase B
PKC	Protein kinase C
PMN	Polymorphonuclear
Protein kinase B	Akt
RBP4	Retinol binding protein 4
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEMDSA	Society for Endocrinology, Metabolism and Diabetes of South Africa
SADoH	The South African Department of Health
SGM	Standard growth medium
SGLT2i	Sodium glucose cotransporter-2 inhibitor
SINHIB	
SOD	Superoxide dismutase
sTNFr2	Soluble tumour necrosis factor receptor 2
SVCT1	Sodium-dependent Vitamin C transporter 1
SVCT2	Sodium-dependent Vitamin C transporter 2
T2DM	Type 2 diabetes
T-AOC	The antioxidant capacity
TB	Tuberculosis
TBHP	Tert-Butyl hydroperoxide
TGF-ß	Transforming growth factor beta
TGF-α	Transforming growth factor alpha
TNF	Tumour necrosis factor
TNF-a	Tumour necrosis factor alpha

UDP-GlcNAc	UDP-N-Acetyl glucosamine
WHO	World health organisation
XO	Xanthine oxidase
µg	Microgram
µL	Microlitre
µM	Micromolar

1 INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder, characterized by hyperglycaemia (elevated blood glucose levels (Nasri and Rafieian-Kopaei, 2014). There are three distinct types of DM, with type 2 diabetes (T2DM) accounting for approximately 90% of the total diabetes cases (Saeedi et al., 2019). Insulin release and action are essential processes for maintaining glucose homeostasis. The molecular mechanisms involved in the synthesis and release of insulin, as well as in its binding and downstream signalling are closely regulated. In obesity-associated T2DM, defects occur in the mechanisms related to the response of insulin-sensitive tissues (at the onset of the disease) and impaired insulin secretion by pancreatic β-cells in later stages of the disease (Galicia-Garcia et al., 2020). T2DM is ranked among the top 10 causes of death in adults, with the prevalence of disease increasing rapidly in sub-Saharan Africa (Atun et al., 2017).

In 2019, it was estimated that 463 million people suffer from DM globally. This is predicted to rise to 578 million by 2030 and 700 million by 2045 (Saeedi et al., 2019). In addition, there is a higher prevalence of the disease among high-income countries and middle-income countries such as South Africa, compared to low-income countries (Atun et al., 2017). Urbanisation and the adoption of a Western diet, containing processed, high-calorie foods in addition to physical inactivity have contributed to the increase of obese individuals in South Africa's population, which is a known risk factor for T2DM (Debroy et al., 2019, Pheiffer et al., 2021).

T2DM risk factors encompass a complex combination of genetic, metabolic, and environmental factors that interact with one another contributing to its increased prevalence. Individuals may be predisposed to non-modifiable risk factors (ethnicity and family history), which have a strong genetic basis. However, mounting evidence from epidemiological studies suggests that T2DM is also a negative consequence of modifiable factors such as sedentary living and high-energy diets (Galicia-Garcia et al., 2020, Saeedi et al., 2019). T2DM has major negative impacts on the well-being of patients, their families, the local and global health systems as well as the economy (Atun et al., 2017, Saeedi et al., 2019), as these patients develop multiple comorbidities.

Oxidative stress is one of the major contributors of diabetes complications (Yaribeygi et al., 2020). The persistent high glucose levels associated with T2DM cause the formation of advanced glycation end products (AGEs), which in turn increases reactive oxygen species (ROS) production by mitochondria through the electron transport chain, and increases the release of pro-inflammatory cytokines, which all perpetuates oxidative stress and secondary tissue damage (Burgos-Moron et al., 2019, Kuzan, 2021, Nowotny et al., 2015). Cells consume oxygen to generate energy in the form of adenosine triphosphate (ATP) and in the

process, produce free radicals that are predominantly reactive oxygen and nitrogen species (RNS). At low concentrations, ROS and RNS have beneficial effects on cellular responses and immune function, however excessive production or accumulation of these free radicals cause oxidative stress and cellular damage (mitochondrial dysfunction and DNA damage) (Johansen et al., 2005). Oxidative stress is defined as an imbalance between production and accumulation of intracellular ROS and the inability of a biological system to detoxify these reactive products (Dal and Sigrist, 2016).

Oxidative stress therefore plays a significant role in the development of chronic and degenerative diseases (Pizzino et al., 2017, Pham-Huy et al., 2008). It leads to a reduction of peripheral insulin sensitivity, impaired glucose uptake in muscle and adipose tissue, pancreatic β-cell dysfunction and endothelial dysfunction, which leads to widespread microvascular complications and thus disease progression. It has been shown that β-cells display high endogenous production of ROS and lower antioxidative capacity making them susceptible to oxidative stress-induced cellular damage (Eguchi et al., 2021). Similarly, vascular endothelial cells are sensitive to glucose toxicity and is one of the main targets of hyperglycaemic damage. The subsequent endothelial dysfunction amplifies the inflammatory response (leukocytes migration and adhesion, platelet aggregation) and is characterized by impaired vasodilatation and vascular wall remodelling (Burgos-Moron et al., 2019) leading to tissue ischaemia and secondary damage.

The South African Department of Health (SADoH) management objectives in the treatment of T2DM is to relieve symptoms, prevent acute metabolic and long-term complications with reduction in premature morbidity and mortality, improve quality of life and productivity of patients and reduce the economic burden on individual, family, and community (SADoH, 2014). The clinical treatment goal for managing T2DM patients is to prevent the onset of co-morbidities by focusing on glycaemic control, however, recent data have demonstrated that 88.5% of diabetic patients have more than one co-morbidity (Iglay et al., 2016). This is due to the complex interactions between hyperglycaemia-induced oxidative stress and sterile metabolic inflammation, which intensify and aggravate each other leading to the development of secondary complications (Luc et al., 2019, Yaribeygi et al., 2020)

To achieve effective glycaemic control in T2DM, both non-pharmacological and pharmacological interventions are prescribed. Non-pharmacological treatment includes diabetes awareness and education as well as lifestyle modification (appropriate eating and weight loss plans). Pharmacological treatment includes three main types of glucose lowering agents (biguanides, sulphonylureas, and insulin). In South Africa, the biguanide, metformin, is available as the first line oral medication used for the treatment of T2DM, it is thought to contain

both glucose-lowering as well as anti-inflammatory properties (SADoH, 2014). Nonetheless, secondary complications remain prevalent in T2DM patients and there is thus a need for alternative complementary strategies to protect against the pathological microenvironment to prevent secondary tissue damage.

An antioxidant is a chemical substance derived from the active components of plant and animal extracts, including macromolecular proteins, peptides and polysaccharides and some small molecule peptides, phenols and flavonoids (Pham-Huy et al., 2008). These substances are responsible for controlling the redox state by limiting and/or slowing down the oxidation of ROS/RNS and scavenges free radicals to limit cellular damage (Zhang et al., 2020b). Endogenous and exogenous antioxidants therefore possess protective effects against cellular dysfunction and lower the risk of developing comorbidities (Pham-Huy et al., 2008). In diabetic patients, the endogenous antioxidant enzymes are however diminished, leaving tissues vulnerable to oxidative damage (Adeshara et al., 2020). Various preclinical studies from our lab and others, have however demonstrated the protective effect of antioxidants, such as N-acetylcysteine (NAC) and ascorbic acid against diabetes induced hepatopathy, nephropathy, retinopathy, myocardial injury and stem/progenitor cell impairment (Genc and Peker, 2021, Liu et al., 2021, Maartens et al., 2021, Mehrbani Azar et al., 2018, Wang and Kang, 2020, Yalçın and Gürel, 2021). Similarly, there is increasing evidence from animal studies indicating improved insulin signal transduction and glucose homeostasis following supplementation with natural plant-based antioxidants (Bagul et al., 2012, Hininger-Favier et al., 2009).

Healthcare systems in countries in sub-Saharan Africa are unable to manage with the current burden of diabetes and its complications (Atun et al., 2017). This coupled with the rapidly increasing prevalence of diabetes in sub-Saharan Africa, demands interconnected, simple, inexpensive, and broad-based alternative preventative strategies to limit T2DM progression and delay the onset of complications (Atun et al., 2017). This taken together with the supporting literature, warrants the potential clinical use of antioxidants as an adjuvant therapy in the treatment of metabolic disorders such as T2DM.

It is however unclear if these antioxidants will interfere with the insulin sensitizing function of metformin and studies are thus required to investigate possible drug interactions and/or synergistic effects. Thus, this research project investigated if the antioxidants namely NAC and ascorbic acid-2 phosphate (AAP) alters glucose uptake and thus the requirement for metformin treatment in an *in-vitro* skeletal muscle model. The glucose lowering and insulin sensitizing effect of metformin in the presence of NAC and AAP as well as the beneficial effect(s) of combination therapy were assessed in this research project.

This thesis comprises of a literature review (Chapter 2) that provides an overview of the pathogenesis of T2DM, its association with oxidative stress and the common pharmacological interventions used. The subsequent chapters give a detailed description of the aims and objectives (Chapter 3), methodological approach used (Chapter 4) and results obtained from experimental laboratory work (Chapter 5). Finally, a discussion and conclusion (Chapter 6 and 7) give insight on the relevance of this study.

2 LITERATURE REVIEW

2.1 OVERVIEW OF TYPE 2 DIABETES MELLITUS: EPIDEMIOLOGY, CAUSES, DIAGNOSIS.

Diabetes mellitus is a group of metabolic disorders with heterogeneous causes, in which the blood glucose levels are characteristically higher than normal and interferences of carbohydrate, fat and protein metabolism result from defects in insulin secretion and/or action (Nasri and Rafieian-Kopaei, 2014, Webb, 2018). There are 3 distinct types of DM, namely, Type 1, Type 2 and gestational diabetes with T2DM accounting for approximately 90% of the total diabetes cases (Saeedi et al., 2019). Refer to Table 1 for a summary of the three main types of DM and their associated characteristics.

Table 2.1. Types of DM and their associated characteristics.

Diabetic type	Onset	Characteristics	Reference
Type 1 diabetes mellitus	Usually during childhood or adolescence	Insulin deficiency due to early pancreatic β-cell loss and impaired insulin secretion	Katsarou et al. (2017)
Type 2 diabetes mellitus	Adolescence and adulthood	Early event: insulin resistance Late event: compromised β-cell function leading to impaired insulin secretion	DeFronzo et al. (2015)
Gestational diabetes	Women during 2nd or 3rd trimester of pregnancy	Insulin resistance or chronic glycaemic intolerance occurs due to pancreatic β-cell alterations during gestation	Shamsad et al. (2023)

Footnote: The table above describes the three main types of diabetes. The types of DM are differentiated based on their characteristics and can occur at different stages of a person's life.

DM is ranked among the top 10 causes of death in adults, with the prevalence of disease increasing rapidly in sub-Saharan Africa (Atun et al., 2017). South Africa is a unique country that is not only plagued by high economic and health inequalities but also by a quadruple disease burden characterised by high rates of communicable diseases primarily human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (TB), non-communicable diseases (DM, cardiovascular diseases (CVD), cancers and chronic respiratory diseases), maternal and childhood mortality and injury-related disorders (Pheiffer et al., 2021). Thus, DM poses a serious health and economic burden to South Africa which has an already overburdened and under-resourced health system.

In 2016 and 2017 evidence suggested, that DM was the second leading, underlying cause of death in South Africa (StatsSA, 2017). South Africa has experienced a rapid increase in the prevalence of DM, from 4.5% in 2010 to 12.7% in 2019. It was further estimated in 2019, that of the 4.58 million people between the ages 20 and 79 with diabetes 52.4% were undiagnosed (Grundlingh et al., 2022). In South Africa, T2DM is most common amongst the Asian (30%) and mixed ancestry (13%) populations, who seem to have a genetic predisposition with equal prevalence among the black and white (8%) populations (Pheiffer et al., 2021). Additionally,

T2DM prevalence is higher in South African females compared to South African males (Pheiffer et al., 2021). T2DM exists in all sectors of society, with a similar prevalence in rural informal and urban formal settlements (SEMDSA, 2017).

Diabetes has major negative impacts on the well-being of patients, their families, the local and global health systems as well as the economy, (Atun et al., 2017, Saeedi et al., 2019). T2DM is associated with a number of specific long-term adverse health complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases (atherosclerosis, hypertension) as well as cerebro- and peripheral vascular diseases (SEMDSA, 2017). In addition to the acute metabolic and long term complications DM has an immense economic burden. In 2017, it was estimated that the global health expenditure on DM was USD727 billion (Saeedi et al., 2019) while in 2015, it was estimated that the overall cost of DM in Sub-Saharan Africa was USD19.45 billion. About USD10.81 billion (55·6%) of the Sub-Saharan Africa cost arose from direct costs such as, DM medication, hospital stays, and treatment of complications (Atun et al., 2017). It is further estimate that the total DM-related cost will increase to between USD35.33 billion and USD59.32 billion by 2030 (Atun et al., 2017). To address the socio-economic impact of DM, it is thus essential to better understand the causes and risk factors associated with disease onset and progression.

T2DM risk factors and aetiology encompass a complex combination of genetic, metabolic, and environmental factors that interact with one another contributing to its increased prevalence. Individuals may be predisposed to non-modifiable risk factors (ethnicity and family history), which have a strong genetic basis however, mounting evidence from epidemiological studies suggests that T2DM is a negative consequence of modifiable risk factors such as the rapid increase in urbanization and obesogenic environments (sedentary living and high-energy diets) (Saeedi et al., 2019, Galicia-Garcia et al., 2020). These modifiable risk factors lead to T2DM pathophysiological changes that are characterized by hyperglycaemia, insulin resistance, accumulation of AGEs leading to oxidative stress, cellular/tissue damages and persistent inflammation (DeFronzo et al., 2015).

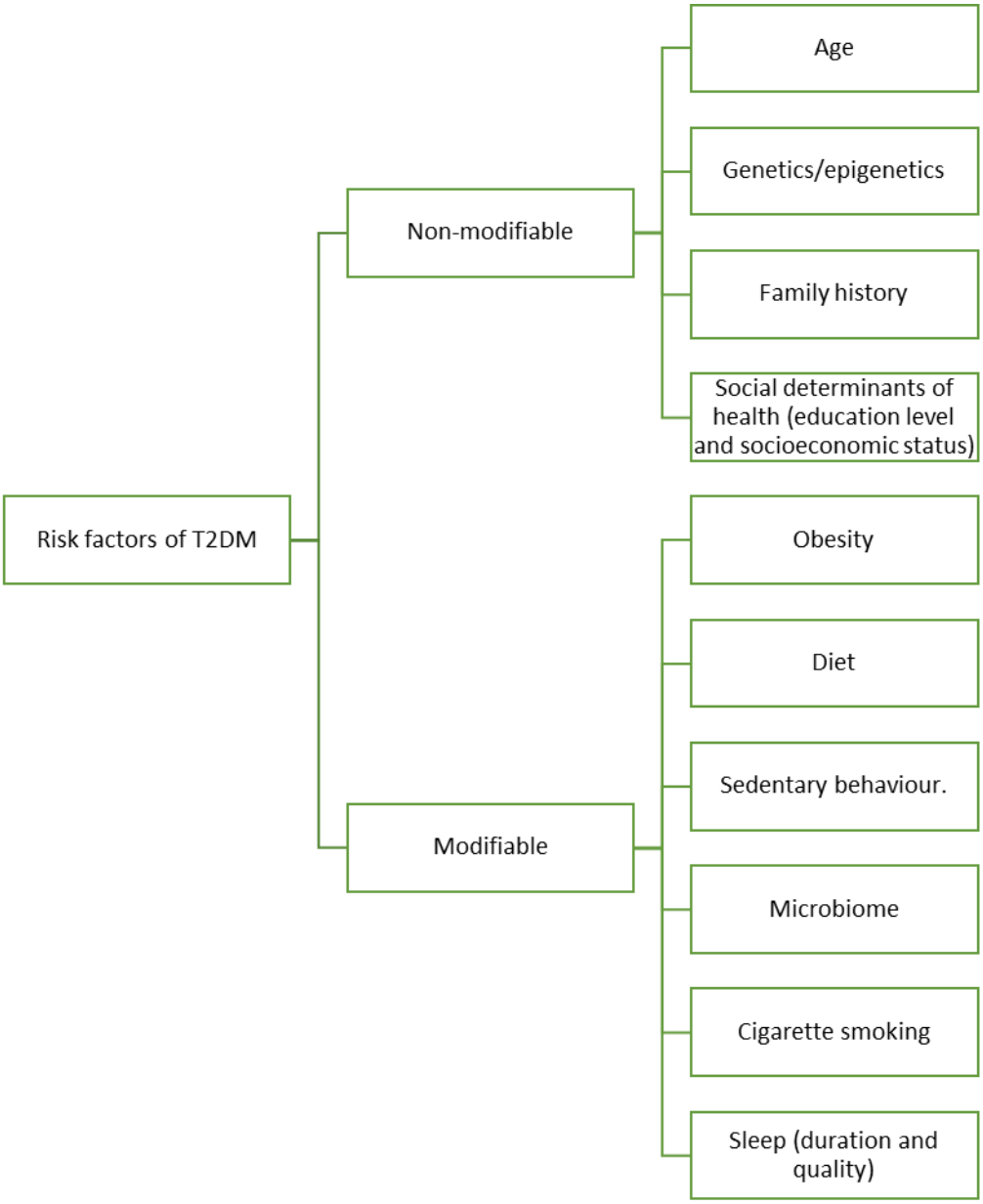


Figure 2.1: Non-modifiable and modifiable risk factors of T2DM. There are numerous non-modifiable (age, genetics/epigenetics, family history and social determinants of health) and modifiable (obesity, diet, the microbiome, cigarette smoking, sleep) risk factors of T2DM. **Abbr:** T2DM, type 2 diabetes. Image adapted from Salzberg (2022)

Symptoms of T2DM and specifically hyperglycaemia include polyuria, polydipsia, blurred vision, weight loss, rapid breathing, flushing, confusion, drowsiness and fruity breath odour (Nasri and Rafieian-Kopaei, 2014). A combination of hyperglycaemia tests may be performed to confirm the diagnosis. The oral glucose tolerance test (OGTT) is currently the gold standard for diagnosing diabetes. The World Health Organisation (WHO) recommends using a 75g anhydrous glucose (or 90g monohydrous glucose) glucose load dissolved in 250mL water, that is ingested over 5 minutes. A positive diabetes diagnosis is confirmed when the two-hour plasma glucose (2-h PG) during OGTT is ≥ 11.1mmol/L. A positive diabetes diagnosis may additionally be confirmed, if a patient presents with random blood glucose ≥ 11mmol/L and/or

fasting glucose (FPG) ≥ 7mmol/L and/or glycated haemoglobin (HbA1c) ≥ 6.5%, In addition, the diagnosis of T2DM should always be confirmed by repeating the same test on another day and should be based on formal laboratory test results (not point of care tests) before the initiation of any therapy (SEMDSA, 2017, SADoH, 2014).

2.2 PATHOPHYSIOLOGY OF T2DM

Refer to the figure below for an overview of the pathophysiology of T2DM as will be discussed in the sections below.

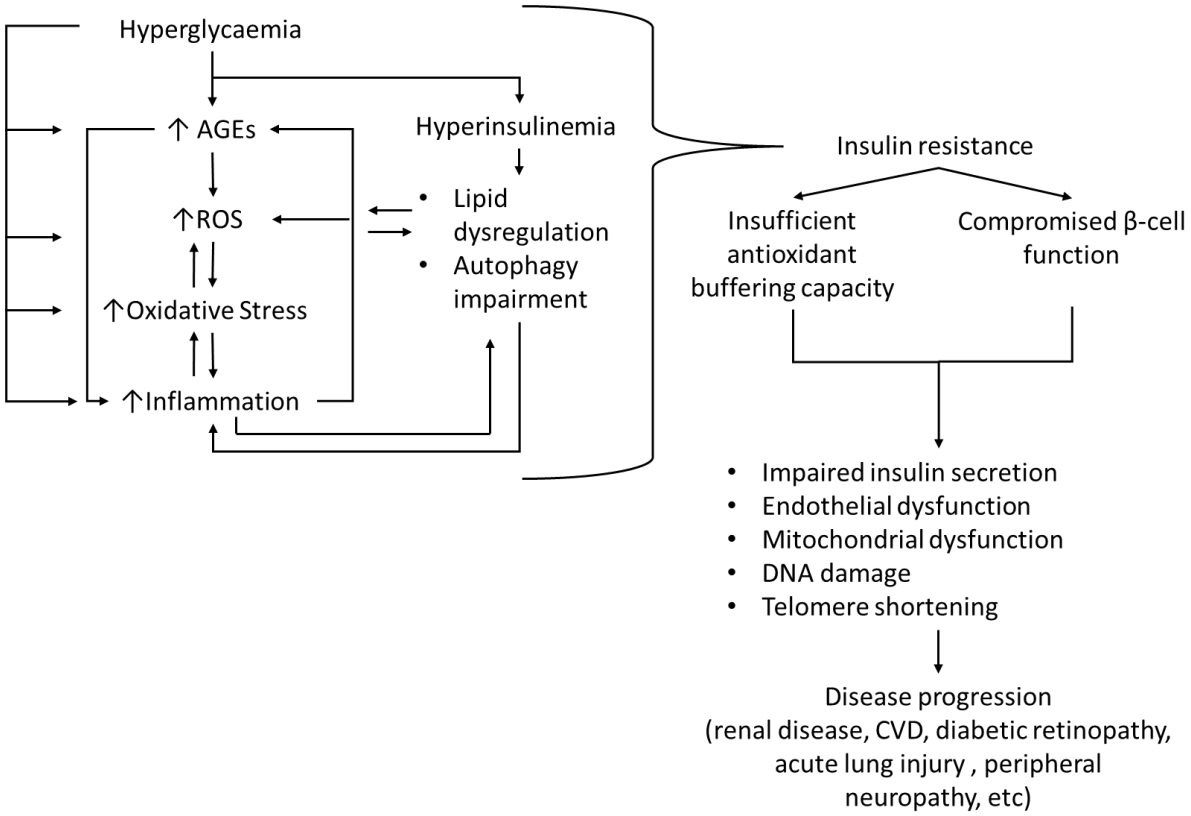


Figure 2.2: Overview of pathophysiology of T2DM: Changes in microenvironmental factors brought about by hyperglycaemia can cause DNA damage, promote telomere shortening, impaired insulin secretion, endothelial dysfunction and cause an excessive release of pro-inflammatory cytokines that persistently promote T2DM disease progression. **Abbr:** AGEs, advanced glycation end products; CVD, cardiovascular diseases; ROS, reactive oxygen species.

2.2.1 T2DM, insulin resistance and hyperglycaemia

Insulin release and action are essential processes for glucose homeostasis. The molecular mechanisms involved in the synthesis and release of insulin, as well as in its binding are therefore closely regulated (Galicia-Garcia et al., 2020). In pancreatic β-cells, under normal physiological conditions when there is an increasing in circulating glucose, glucose uptake by glucose transporters (GLUT) increase accordingly. This in turn initiates a series of reactions that cause the depolarization of these pancreatic β-cells and open calcium channels. Calcium

then enters the cells and signals the release of insulin into plasma via exocytosis (Boland et al., 2017, Rorsman and Ashcroft, 2018, Seino et al., 2011). The main function of insulin is to promote cellular uptake, use, and storage of absorbed nutrients, mainly glucose. It promotes uptake of glucose from the blood into insulin-sensitive peripheral tissues, such as the heart, liver, skeletal muscle and adipose tissue. It does this through recruitment of plasma membrane carriers, GLUT (Wondmkun, 2020).

Once GLUT facilitates entry of glucose into a cell it is immediately enzymatically phosphorylated to glucose-6-phosphate (G6P) by hexokinase and has no means out of the cell, unlike glucose. Two biochemical processes are activated to convert G6P, namely glycolysis and glycogen synthesis. Glycolysis is a biochemical process taking place in the cell's cytosol that breaks down glucose into pyruvate molecules that contribute to the body's energy production in the forms of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Whereas glycogen synthesis is the conversion of glucose into glycogen for the purpose of storing excess, unused glucose (Komoda and Matsunaga, 2015). These processes work synergistically and thus reduces the blood glucose concentration (Sherwood, 2016). Defects in any of the mechanisms involved in the processes mentioned namely, defective insulin secretion by pancreatic β-cells and inadequate insulin response of insulin-sensitive tissues, cause T2DM (Galicia-Garcia et al., 2020).

Insulin resistance (IR) is defined as a sub-optimal response of tissues such as skeletal muscle, adipose tissue and the liver, to insulin resulting in deficient glucose uptake from blood (Czech, 2017). Insulin resistance precedes apparent T2DM and is a strong and early detectable risk factor to predict T2DM. Obesity due to high fat diet and lack of physical activity leads to IR. Insulin resistance is present in numerous tissue types but has the greatest effect on skeletal muscle, adipose tissue and the liver as they are responsible for the majority of glucose uptake following food intake (Galicia-Garcia et al., 2020). Insulin resistance which precedes T2DM has mainly been linked to the phosphatidylinositol 3-kinase (PI3K) pathway which promotes glucose transporter type 4 (GLUT4) translocation to the plasma membrane, resulting in glucose uptake into skeletal muscle. Increased serine phosphorylation of insulin receptor substrates (IRS) proteins in this pathway, inhibits tyrosine phosphorylation, leading to insulin resistance. In addition, serine phosphorylation increases IRS degradation, further contributing to the insulin resistance (Abdul-Ghani and DeFronzo, 2010). Serine phosphorylation is caused by many factors, including ectopic lipid accumulation, mitochondrial dysfunction, inflammation, oxidative stress and endoplasmic reticulum stress. Insulin resistance, together with a multitude of factors such as ageing, genetic abnormalities, incretin hormone resistance and/or deficiency, lipotoxicity, glucotoxicity, hypersecretion of islet amyloid polypeptide (IAPP),

oxidative stress and inflammation contribute to β-cell failure and a steady decline in insulin secretion (DeFronzo et al., 2015).

T2DM develops in stages. Initially normal glucose tolerance is maintained although insulin resistance is already present. This is due to increasing insulin release via pancreatic β-cells that compensate for the insulin resistance, to maintain normal physiological blood glucose levels (Ferrannini and Mari, 2014, Kahn et al., 2014). As the disease progresses, glucose metabolism becomes impaired and this manifests as impaired glucose tolerance (random plasma glucose ≥ 11mmol/L, fasting plasma glucose (FPG) ≥ 7mmol/L and glycated haemoglobin (HbA1c) ≥ 6.5%). This phase is characterized by hyperglycaemia and hyperinsulinemia as β-cell compensation still takes place, however tissue sensitivity to insulin has declined to a point where insulin cannot exert its metabolic effects (ADA, 2017, Galicia-Garcia et al., 2020). In the final stage of apparent T2DM, chronic hyperglycaemia is accompanied by low insulin levels, chronic inflammation, oxidative stress and hyperlipidaemia which progressively impede blood glucose homeostasis and result in the development of micro- and macrovascular complications. The decreased insulin levels are a result of β-cell dysfunction brought about by several mechanisms that cause damage to pancreatic β-cells (DeFronzo et al., 2015). T2DM has no cure, therefore the clinical treatment goal for managing T2DM patients is to prevent acute metabolic and long-term complications by focusing on glycaemic control management to relieve symptoms, reduce premature morbidity and mortality, improve quality of life and productivity of patients and reduce the economic burden on individual, family, and community (SADoH, 2014).

2.2.2 T2DM and oxidative stress

One of the major contributors of diabetes complications as well as insulin resistance development, is oxidative stress (Yaribeygi et al., 2020). Cells consume oxygen and glucose to generate energy in the form of ATP and in the process, produce free radicals that are predominantly ROS such as hydroxyl (OH), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydrochlorous acid (HOCl) as well as RNS such as nitric oxide (NO), nitrogen dioxide (NO_2), and the non-radical peroxynitrite ($ONOO^-$) (Dal and Sigrist, 2016, Tangvarasittichai, 2015). The mitochondria are the main source of ROS production but there are also non-mitochondrial sources of ROS, such as nicotinamide adenine nucleotide phosphate oxidase (NOX), xanthine oxidase (XO), cyclooxygenases (COXs), monoamine oxidases, hemeoxygenases (HO) and cytochrome P450 reductase (CYPOR) (Zhang et al., 2020b).

At low concentrations, ROS and RNS have beneficial effects on cellular responses and immune function, while at high concentrations, they generate oxidative stress. Oxidative stress is defined as an imbalance between production and accumulation of ROS in cells and tissues

and the ability of a biological system to detoxify these reactive products. Oxidative stress plays a significant role in the development of chronic and degenerative diseases (Pham-Huy et al., 2008, Pizzino et al., 2017). Oxidative stress leads to disease progression by further reducing peripheral insulin sensitivity, impairs glucose uptake in muscle and fat cells and induces β-cell dysfunction which ultimately contributes to the development of co-morbidities via several molecular mechanisms (Yaribeygi et al., 2020).

Persistent high glucose levels associated with T2DM cause the formation of AGEs. AGEs form through the non-enzymatic reaction of glycation, between reducing sugars and protein molecules, amino phospholipids, or nucleic acids (Kuzan, 2021). A Schiff base is formed, through a Maillard reaction where the carbonyl group of a glucose molecule reacts with an amino group of a protein, lipid, or nucleic acid. Schiff bases may rearrange to form Amadori products which are unstable and further undergo rearrangement and oxidation, which in turn increases ROS production by mitochondria through the electron transport chain, and increases the release of pro-inflammatory cytokines, which all perpetuates oxidative stress and in turn secondary tissue damage (Burgos-Moron et al., 2019, Kuzan, 2021, Nowotny et al., 2015).

Common characteristics of T2DM such as hyperglycaemia, hyperlipidaemia, and inflammation have all been shown to promote oxidative stress and by doing so induces β-cell damage. Excessive oxidative stress is detrimental to pancreatic β-cell health and survival as these cells display high levels of endogenous ROS production and have a lower antioxidative capacity (Eguchi et al., 2021). Excessive ROS overproduction under high glucose conditions not only cause β-cell dysfunction but is also associated with widespread cellular/tissue dysfunction (DNA damage, epigenetic alterations) which exacerbates the disease condition (increased inflammation, insulin resistance) and the subsequent development of complications (nephropathy, cataract, neuropathy, retinopathy, cardiovascular disease) (Burgos-Moron et al., 2019, He et al., 2017, van de Vyver, 2023). Refer to table 2.1 for the intracellular signalling pathways involved in ROS production, the effect they have on the pathological micro-environment and T2DM complications. ROS causes dysfunction so by regulating important intracellular signalling pathways, such as p38 Mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and Nuclear factor-κB (NF-kB), as well as several other mitochondrial pathways such as protein kinase C (PKC) activation, increased intracellular AGEs formation and hexosamine and polyol pathways activation (Eguchi et al., 2021, Zhang et al., 2020b).

Various cell types (neural stem cells, leukocytes, fibroblasts, mesenchymal stem cells, glomerular endothelial cells, Müller cells, hepatocytes, cardiac progenitor cells, vascular endothelial cells, endothelial progenitor cells) are affected by the pathogenesis of T2DM. If the

function of these affected cells is not restored, it impairs endogenous regenerative mechanisms and prevents tissue repair and restoration. The disruption of the regular functioning of these cells and thus tissue homeostasis cause various complications and comorbidities (Burgos-Moron et al., 2019, Maartens et al., 2021). Vascular endothelial cells are especially sensitive to glucose toxicity and one of the main targets of hyperglycaemic damage (Burgos-Moron et al., 2019). The endothelium, which is the thin, inner, single-celled layer of epithelial cells lining the blood vessels of the entire circulatory system, plays an important role in tissue maintenance and repair. Thus, endothelial dysfunction brought on by hyperglycaemia-induced oxidative stress and inflammation play an early and critical role in the functional decline of various organs and micro- and macro-angiopathies in T2DM (Dal and Sigrist, 2016, Sherwood, 2016). Endothelial dysfunction amplifies the inflammatory responses by triggering leukocyte migration/ adhesion and platelet aggregation. It is characterized by impaired vasodilatation and vascular wall remodelling leading to tissue ischaemia and secondary damage (Burgos-Moron et al., 2019). Various cell types are affected by the pathogenesis of T2DM which leads to cellular dysfunction in these different cell types. If these affected cells are not treated, it may cause irreversible damage that prevents tissue repair and restoration. Thus, regular functioning of these cells is disrupted and homeostasis is no longer maintained resulting in various complications and comorbidities . Oxidative stress and T2DM have complex interactions and they both intensify and aggravate each other (Luc et al., 2019, Yaribeygi et al., 2020).

Table 2.2: The role of intracellular signalling pathways involved in ROS production on T2DM complications.

Pathway	Effect	Associated T2DM complications	Ref
Polyol	In response to hyperglycaemia, a metabolic shift causes the excess glucose to enter the polyol pathway in which glucose is reduced to the intermediate product, sorbitol, by aldose reductase in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction. Overload of polyol pathway: • NADPH depletion – redox imbalance due to glutathione deficiency • Accumulation of sorbitol – cause glycation of respiratory chain proteins leading to intracellular damage.	Vascular dysfunction and neuro-glial degeneration. Retinal, nerve and kidney-related microvascular complications.	Garg and Gupta (2022), Hosseini and Abdollahi (2013), Ighodaro (2018)
Hexosamine	Under hyperglycaemic cellular conditions, Fructose-6-phosphate derived from glycolysis is increased and metabolized to glucosamine-6-phosphate which is then converted to UDP-GlcNAc. The accumulation of UDP-GlcNAc activates the hyperactivity of Oglucosamine-N-Acetyl transferase, which is associated with toxic and pro-oxidative roles in T2DM. Hyperactivity of hexosamine pathway: • Increased expression of inflammation proteins (TGF-α, TGF-ß, PAI-1) which are responsible for the toxic and pro-oxidative status.	Diabetic nephropathy	Hosseini and Abdollahi (2013), Ighodaro (2018)
P38 MAPK	High glucose and lipid excess cause increased production of oxidants by mitochondria which is associated with the upregulation of p38 MAPK. p38 MAPK is involved in the regulation of numerous physiological and pathological processes such as apoptosis, differentiation, survival, proliferation, inflammation and other stress responses Hyperactivity of P38 MAPK pathway: • Dephosphorylated deactivation of pathway that increases ROS generation, inflammation and leads to insulin resistance.	Cardiac hypertrophy and diabetic cardiac myopathy, nephropathy and retinopathy	Ighodaro (2018), Wang et al. (2016), Zhang and Liu (2002)
JNK	The JNK pathway is involved in the regulation of various physiological processes such as inflammatory responses, cell differentiation, cell proliferation, cell death, cell survival and expression of proteins. Under diabetic conditions, increased endoplasmic reticulum stress activates the JNK pathway in various tissues, which is involved in both insulin resistance and β-cell dysfunction. Overload of JNK pathway: • Induction of apoptosis in various cells. • Stress induced reduction of insulin gene expression which interferes with insulin action and reduces insulin biosynthesis. • Results in insulin resistance and glucose intolerance	Diabetic nephropathy	Kaneto et al. (2005), Kumar et al. (2015)

NF-kB	Hyperglycaemia activates NF-kB, which is a nuclear transcription factor found in all cell types and is involved in the immune and inflammatory responses. NF-kB activation is a key event early in the pathophysiology of T2DM. NF-kB activation and induced-oxidative stress and inflammation in hyperglycaemia • Dysregulation of lipid and glucose homeostasis that plays a primary role in insulin resistance. • Modulates the expression of several proinflammatory factors (cytokines, tumour necrosis factor, nitric oxide synthase) that result in increased ROS production and inflammation.	Diabetic nephropathy, neuropathy, retinopathy, cardiomyopathy	Liu et al. (2017), Patel and Santani (2009)
PKC	Chronic activation by hyperglycaemia stimulates the PKC pathway. PKC is a is a key enzyme in cellular signalling pathways involving DAG, phosphactidyl serine and calcium. Increased cellular level of DAG which induces oxidative stress through up regulating the PKC pathway and its isoforms PKC activation and induced-oxidative stress in hyperglycaemia • Dephosphorylated deactivation of pathway that increase ROS generating enzymes (NADPH-oxidases and lipooxygenses) which exacerbate cellular oxidative environment and decrease insulin action.	Diabetic neuropathy and vascular abnormalities in retinal, renal and cardiovascular tissues	Ighodaro (2018), Nandipati et al. (2017)

Footnote: The table above describes the intracellular signalling pathways (MAPK, JNK and NF-kB) and several other mitochondrial pathways (PKC, hexosamine and polyol) that ROS regulates to bring about dysfunction in T2DM. **Abbr:** DAG, diacylyglycerol; NADPH, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C; ROS, reactive oxygen species; NF-kB, Nuclear factor-κB ; T2DM, type 2 diabetes; JNK, c-Jun N-terminal kinase; P38 MAPK, P38 Mitogen-activated protein kinase; UDP-GlcNAc , UDP-N-Acetyl glucosamine; TGF-α, transforming growth factor alpha; TGF-ß, transforming growth factor beta; PAI-1, plasminogen activator inhibitor-1.

2.2.3 T2DM and inflammation

Inflammation is an important pathophysiological factor in obesity associated T2DM, it is both a cause and consequence of metabolic dysfunction (van de Vyver, 2023). Acute inflammation is defined as an innate series of highly interrelated immune events (involving neutrophils and macrophages) that are activated in response to either pathogen invasion and/or tissue damage (Sherwood, 2016). Although acute inflammation is a necessary immune process that is required for phagocytosis of cellular and tissue debris prior to the commencement of regeneration, it can also cause secondary tissue damage and become self-perpetuating. Under pathological conditions such as those associated with T2DM, there is simultaneous crosstalk between immune regulation and abnormal metabolism resulting in sterile metabolic inflammation (van de Vyver, 2023). It is defined as chronic low-grade systemic inflammation fuelled by metabolic disturbances in the absence of any infection (Furman et al., 2019). This chronic metabolic inflammation originates from adipose tissue hypertrophy, which leads to insulin resistance giving rise to hyperglycaemia and subsequent glucose toxicity and oxidative stress-induced cellular damage. This in turn perpetuates the inflammatory response through a positive feedback loop. Simultaneously, glucose toxicity negatively affects the ability of immune cells to perform their innate functions through epigenetic changes and by altering the activation status (metabolic changes favouring glycolysis) of these cells. Together this persistent low grade metabolic inflammatory state gives rise to numerous harmful macro- and microvascular complications (van de Vyver, 2023).

Chronic inflammation is brought about by numerous pathways that originate from various factors such as insulin resistance, increased circulating free fatty acids (FFA), hyperglycaemia, hyperinsulinemia and oxidative stress. These factors all participate in a vicious cycle that persistently promote T2DM disease progression and causes an impaired response to insulin stimulation in white adipose tissue. This impaired insulin response in known as adipose-IR. Adipose-IR causes adipose tissue to abnormally increase in mass and size. These now hypertrophied white adipocytes as well as adipose tissue-resident immune cells (macrophages and lymphocytes) stimulate the release of adipokines (resistin, TNF-α and RBP4) and chemokines (TNF, IL-1β, IL-6, IL-17 and IL-23) that contribute to increased circulating levels of proinflammatory cytokines and overall low-grade systemic inflammation. Additionally, adipose-IR is associated with glucose intolerance and elevated release of FFA into plasma that accumulates in muscle or liver. The accumulation of FFA activate adenine nucleotide translocase 2 (ANT2), an inner mitochondrial protein that leads to adipose tissue dysfunction and inflammation through adipocyte hypoxia via the transcription factor hypoxia-inducible factor-1α (HIF-1α), (Galicia-Garcia, 2020) (Koufakis, 2020).

2.2.4 Skeletal muscle cell dysfunction in type 2 diabetes mellitus

Skeletal muscles play a major role in the maintenance of glycaemic control as they are the main tissue responsible for glucose uptake and thus a target of insulin (Sehrawat et al., 2023). Under regular physiological conditions, insulin stimulates muscle glycogen synthesis by increasing glucose uptake from plasma. Glycogen synthase, hexokinase and the glucose transporter GLUT4 are three primary rate-limiting factors associated with glucose uptake and glycogen synthesis. In muscle cells, when insulin binds to insulin receptor (INSR), it activates the INSR tyrosine kinase activity which is essential for the action of insulin on glucose metabolism. Insulin binding to the α-subunit of the INSR causes phosphorylation of the β-subunit on multiple tyrosine residues and allows insulin-mediated signalling. This then activates the translocation of GLUT4 from intracellular compartments to the plasma membrane. This process allows glucose to be taken up by the muscle cells and reduces circulating glucose plasma levels (Galicia-Garcia et al., 2020).

Several defects in this process do however occur in T2DM, these include: a) mutations that reduce the expression of insulin receptor and/or GLUT4 translocation, b) dysregulation of key proteins of either upstream or downstream signalling pathways (IRS1/PI3K/ Akt pathway); c) mutations in some of the main phosphorylation sites (IRS1 and IRS2). Thus, impairing insulin action on skeletal muscle and reducing glucose uptake into muscle cells resulting in a hyperglycaemic state (Galicia-Garcia et al., 2020, Han et al., 2020, Nolan et al., 2011). As mentioned before the pathophysiology of T2DM involves insulin resistance, which severely alters glucose, lipid, and protein metabolism. A decline in the secretion of certain organokines such as adiponectin and increased concentrations of inflammatory cytokines and non-esterified fatty acids (which accumulates in muscle tissue) aggravate insulin resistance in muscle which in turn negatively affects skeletal muscle quality through protein degradation and mitochondrial dysfunction, (Han et al., 2020, Nolan et al., 2011). T2DM alters many biochemical and morphological characteristics of skeletal muscle, such as, reduction in fibre integrity and impairments in the regenerative response to muscle damage (Burton et al., 2022).

Organokines are small, secreted proteins produced by target organ/tissues (liver, adipose, muscle) that act in an endocrine, paracrine, and autocrine fashion. Their actions regulate several metabolic pathways and the dysregulation of organokines (specifically in muscle and adipose tissue) is a prominent feature of T2DM. Adipokines (adipose derived organokines) such as adiponectin contribute to insulin sensitivity in the liver and skeletal muscles through the activation of adenosine monophosphate kinase (AMPK). It also suppresses gluconeogenesis and increases the phosphorylation of acetyl-CoA carboxylase (ACC),

glucose uptake, and fatty acid oxidation. Myokines (muscle derived organokines) such as IL-6, IL-15 and METRNL play an important role in the endocrine response in T2DM. IL-6, usually associated with inflammatory functions, is the best characterized myokine. Treatment with IL-6 in a murine model suggested that insulin signalling through Akt was improved and reduced expression of gluconeogenic genes was observed as well. In addition, IL-6 was associated with enhanced lipolysis and fat oxidation via AMPK activation in adipose tissue (Peppler et al., 2019). IL-15 contributes to reduced visceral adipose tissue and enhanced insulin action while, METRNL has been reported to contribute to energy expenditure through glucose and FFA oxidation (Gholamrezayi et al., 2020, Oh et al., 2016). Taken together these studies suggest that skeletal muscles have a central role in T2DM induced oxidative stress and inflammation and its overall pathogenesis. Thus, for a better understanding of T2DM, interpretation of the roles of organokines in the regulation of metabolic homeostasis should be further explored (Sanches et al., 2023).

del Aguila et al. (1999) showed that increases in TNF-α concentrations in cultured myotubes promote IR in skeletal muscle. A possible mechanism through which this is achieved is by inhibiting IRS-1- and IRS-2-mediated PI 3-kinase activation as well as p42(MAPK) and p44(MAPK) tyrosine phosphorylation. Thus, leading to impaired insulin-stimulated glucose uptake, confirming the role of inflammatory cytokines in disease progression. Apart from defective intracellular signalling that impair insulin action on skeletal muscle and reduce glucose uptake into muscle cells, environmental factors (lack of physical activity and diet) may also play an important role in glucose uptake by muscle. Lack of regular physical activity decreases blood flow into skeletal muscle cells and in so doing decreases glucose utilization (Galicia-Garcia et al., 2020).

Several studies have however shown the therapeutic potential of various agents to improve the utilization and uptake of glucose within skeletal muscle. Miranda-Nunez et al. (2021) conducted a study to investigate the possible antioxidant effect of the fractions and 5,7,4´-trihydroxy-3,6,3´,5´-tetramethoxyflavone (Flav1) on GLUT4. They used C2C12 cells (mouse myoblasts) and revealed that fractions of *Tillandsia usneoides* (C2F9-12, C2F38-44) and Flav1 increased GLUT4 translocation in these cells. This effect has been observed in other studies using different flavonoids such as tangeretina (Kim et al., 2012), kaempferitrin, quercetin, rutin (Cazarolli et al., 2013, Jiang et al., 2019, Kappel et al., 2013) and (Hossain et al., 2014) as treatment, in muscle cells.

2.3 PHARMACEUTICAL INTERVENTION: EXISTING THERAPIES

SADoH management objectives in the treatment of T2DM is to relieve symptoms, prevent acute metabolic and long-term complications with reduction in premature morbidity and

mortality, improve quality of life and productivity of patients as well as to reduce the economic burden on individual, family, and community. In the majority of patients, management should aim to achieve and maintain a HbA1c ≤ 7% (target fasting plasma glucose of 4.0 – 7.0mmol/L and target post-prandial glucose of 5.0 – 10.0mmol/L) (SEMDSA, 2017, SADoH, 2014). To achieve effective glycaemic management in T2DM, both non-pharmacological and pharmacological interventions are prescribed. Non-pharmacological treatment includes diabetes awareness and education as well as lifestyle modification (appropriate eating and weight loss plans).

Diabetes education and awareness consists of both, people with T2DM, their families and communities being provided with the correct information and support (counselling and motivational interviewing) for self-management of the disease. This allows for regular self-monitoring for the effective management of the disease in their homes. Diabetes education has been associated with better glycaemic control and is one of the best predictors of disease progression and development of diabetes related complications (SADoH, 2014). Lifestyle modification include behaviour change (reducing tobacco and alcohol use), increasing physical activity (moderate-intensity aerobic and resistance exercises) and making healthy nutritional choices (reducing high fat and sugar foods intake). These modifications can lead to modest weight loss, reduce HbA1c by up to 2% and improve outcomes in overweight and obese individuals with T2DM and prediabetes, (SEMDSA, 2017, SADoH, 2014).

Pharmacological treatment includes three main types of hypoglycaemic agents (biguanides, sulphonylureas, and insulin) available for use. Guidelines for pharmacotherapy for the management of stable patients (non-pregnant adults without metabolic decompensation or cardiovascular disease) with T2DM with suboptimal glycaemic control, who are being managed at primary care facilities, are shown in Table 3. In South Africa, the biguanide, metformin (3-(diaminomethylidene)-1,1-dimethylguanidine), is available and is the first line oral medication used for the treatment of T2DM (SEMDSA, 2017, SADoH, 2014).

Table 2.3: Guidelines for pharmacotherapy for the management of stable T2DM patients with suboptimal glycaemic control, who are being managed at primary care facilities

Type of therapy	Treatment	Alternative options without motivation*	Not recommended if glycaemic target is attainable with other agents
Monotherapy	Metformin	DPP4i Gliclazide MR Pioglitazone	GLP1a Insulin
Dual therapy	Metformin + DPP4i or Gliclazide (max dosage: 320mg daily, in two divided doses) or Glibenclamide (max dosage: 15 mg daily, in two divided doses)	Pioglitazone SGLT2i	GLP1a Insulin
Complex therapy	Metformin + Insulin • Intermediate- (NPH) or long-acting (Maintain all oral medications) • Biphasic (Discontinue gliclazide and glibenclamide)	Oral therapy + basal insulin+GLP1a	

Footnote: The table above describes the current guidelines used in South African primary care facilities for pharmacotherapy for the management of stable patients with T2DM with suboptimal glycaemic control. **Abbr:** DPP4i: dipeptidyl peptidase-4 inhibitor; SGLT2i: sodium glucose cotransporter-2 inhibitor; GLP-1a, glucagon-like peptide-1 receptor agonist. *These alternatives do not require motivation to funders as they offer similar benefits and are selected for the individual circumstances based on clinical judgement. Adapted from SEMDSA 2017 Guidelines and updated SADoH Management of Type 2 Diabetes in Adults at primary Care level 2014.

2.3.1 Metformin

2.3.1.1 Indication

Metformin was developed from galegine, a guanidine derivative found in French lilac (Galega officinalis). Metformin's clinical use to treat diabetes was first reported by French physician Jean Sterne, in 1957 (Bailey, 2017). It belongs to the drug class of biguanides and is currently the major oral anti-diabetic agent in the management of T2DM. Metformin is prescribed at a max dosage of 2550mg daily, divided into 2 or 3 doses (SADoH, 2014). Metformin has been shown to reduce diabetes mortality and complications by 30% compared to insulin and sulphonylureas (glibenclamide and chlorpropamide) (Nasri and Rafieian-Kopaei, 2014). When used as monotherapy, metformin can reduce HbA1c by 1 – 2 % and maximum glucose lowering is typically evident within six months. When in combination with other anti-diabetic drugs, it is equally effective at reducing blood glucose with approximate reductions of 0.8–1.2%. in HbA1c levels (SEMDSA, 2017, SADoH, 2014). Besides maintaining glycaemic targets, benefits of metformin include minimal side effects, affordability and positive cardiovascular benefit (O'Brien and Travis, 2022). Another beneficial effect is that metformin is associated with less weight gain compared with insulin and sulfonylureas, helping improve T2DM outcome (Kaneto et al., 2021, Nasri and Rafieian-Kopaei, 2014). It is not just used as

an anti-diabetic drug but there is evidence supporting the use of metformin in the management of polycystic ovarian syndrome, cancer, prediabetes and gestational diabetes (Flory and Lipska, 2019, O'Brien and Travis, 2022).

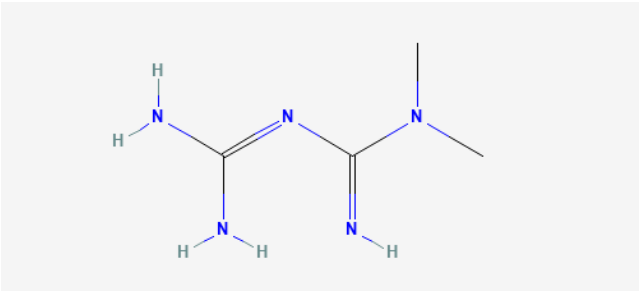


Figure 2.3: Metformin chemical structure. Metformin, chemically known as 1, 1-Dimethyl biguanide hydrochloride with a molecular formula of C4H12Cl N5 is a biguanide and major oral anti-diabetic agent in the management of T2DM

2.3.1.2 Pharmacokinetics

Metformin is taken orally and does not bind to plasma proteins, thus causing its rapid distribution throughout the body. The peak plasma concentration after receiving a single dose of 0.5 g metformin orally ranges between 7.74–12.39 µM after 3 hours. Due to its hydrophilic nature, passive absorption through the plasma membrane is limited and thus it requires active transport (Di Magno et al., 2022, Rena et al., 2017). The oral absorption, hepatic uptake and renal excretion of metformin are mediated chiefly by organic cation transporters (OCTs) such as organic cation transporter 1 (OCT1) and organic cation transporter 2 (OCT2) and multidrug and toxin extrusion (MATE) transporters such as multidrug and toxin extrusion 1 transporter (MATE1) and multidrug and toxin extrusion 2 transporter (MATE2) (Di Magno et al., 2022, Feng et al., 2022). The activity of these transporters directly determines the tissue concentration of metformin and thus its clinical efficacy (Feng et al., 2022). The fractional oral bioavailability of metformin is 55 ± 16% and is predominately absorbed from the small intestine. Metformin is excreted unchanged in urine. The elimination half-life of metformin during multiple dosages in patients with good renal function is approximately 5 hours (Di Magno et al., 2022, Bailey, 2017).

Metformin has been used clinically for over sixty years and trial data has yielded virtually no safety concerns (Flory and Lipska, 2019). Metformin has minimal significant side effects. Approximately 30% of users report gastrointestinal intolerance (bloating, abdominal discomfort and diarrhoea). This side effect can be minimised by titrating the dose steadily over 1-2 months or by temporarily halting the use of the drug before re-introducing it (Flory and Lipska, 2019, O'Brien and Travis, 2022) (SADoH, 2014). Lactic acidosis (increased production of lactic acid produced via physiologically normal processes that becomes comorbid with

decreased clearance) is a rare and usually occurs due to drug overdose or in various contraindicated conditions. Although rare, it causes serious adverse effects such as dizziness, severe drowsiness, muscle pain, tiredness, chills, cold skin, difficult breathing, irregular heartbeat, stomach pain with diarrhoea, nausea or vomiting (Nasri and Rafieian-Kopaei, 2014, Foucher and Tubben, 2023). Metformin very rarely causes hypoglycaemia (low blood glucose) and may occur if the drug is used with other anti-diabetic drugs, with excessive exercise, consuming large quantities of alcohol and insufficient caloric food intake (Nasri and Rafieian-Kopaei, 2014).

2.3.1.3 Mechanism of action

Metformin reduces serum glucose level by several different non-pancreatic mechanisms, meaning it does so without increasing insulin secretion but increases the effects of insulin. Metformin suppresses the endogenous glucose production by the liver, through activation of the enzyme adenosine monophosphate kinase (AMPK) resulting in the reduction of fatty acid synthesis and gluconeogenesis and has a minimal effect on glycogenolysis. Additionally, metformin increases the peripheral glucose absorption into skeletal muscle, through increased AMPK activation and translocation of glucose transporter 4 (GLUT4) to the cell membrane. Metformin reduces autophagy failure observed in pancreatic β-cells under diabetic conditions. Furthermore, it alters the gut microbiome and facilitates the transport of glucose from circulation into excrement (Nasri and Rafieian-Kopaei, 2014, Rena et al., 2017). The full understanding of metformin's mechanism of action remains unknown and its effects are likely pleiotropic (Flory and Lipska, 2019). Refer to Figure 4 below for an overview of the mechanisms by which metformin improves glucose homeostasis.

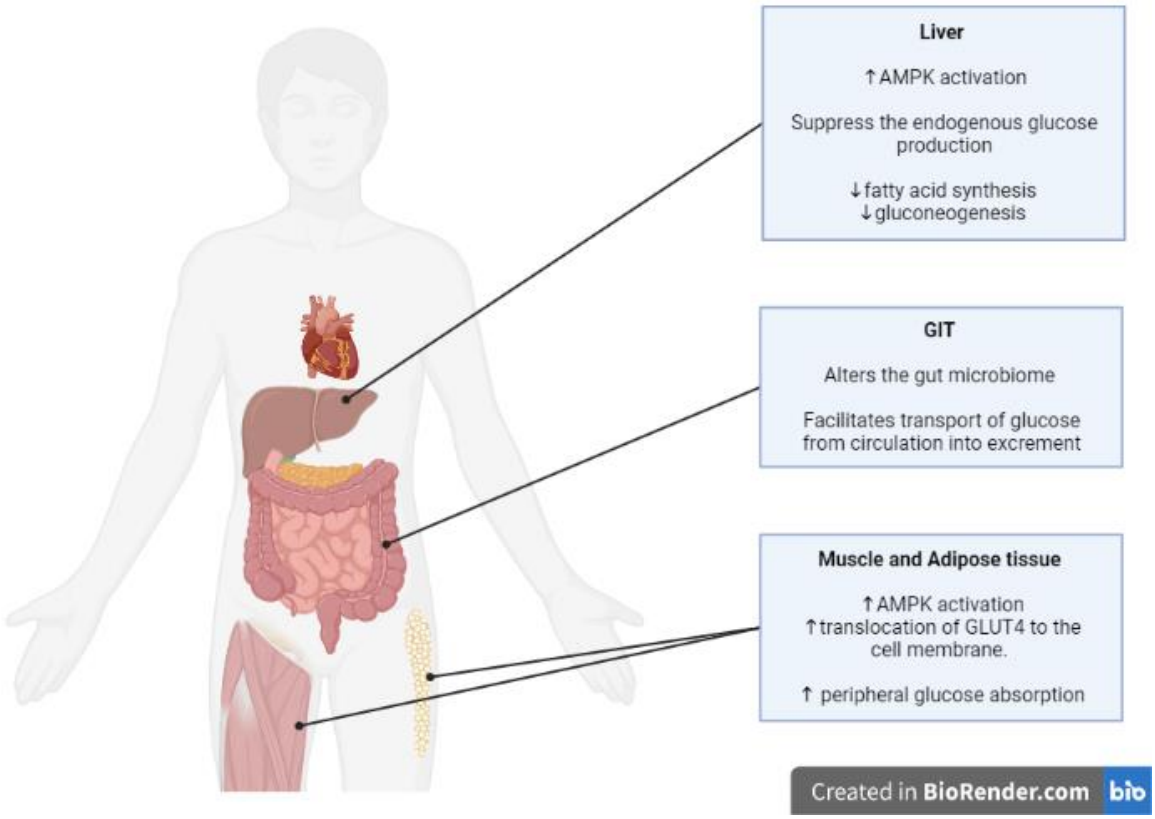


Figure 2.4: The mechanisms of action of metformin. In the liver, metformin downregulates gluconeogenesis by activation increasing AMPK. Additionally, it inhibits fatty acid synthesis and upregulates fatty acid oxidation. In the gastrointestinal tract (GIT), it alters the gut microbiome and decreases glucose absorption from the intestine by facilitating transport of glucose from circulation into excrement. In the skeletal muscle and adipose tissues, it increases glucose uptake through increased translocation of GLUT4 to the cell membrane and thus increases peripheral glucose absorbance. **Abbr:** AMPK, Adenosine monophosphate kinase ;GLUT4, Glucose transporter type 4.

Metformin is thought to possess anti-inflammatory properties and might inhibit NFkB activation in macrophages, leading to a reduction in the proinflammatory cytokines, interleukin 1β and 6 (IL-1β, IL-6) and Tumour Necrosis Factor alpha (TNF-α). It may exhibit its anti-inflammatory effects through action via activation of AMPK that inhibits NFκB via phosphoinositide-3-kinase-protein kinase B (PI3K-Akt) pathway in human vascular smooth muscle cells as well as via inhibition of NFκB activation in macrophages. In addition, it regulates inflammation through SIRT1/LKB1/AMPK pathway and suppression of ROS/Poly ADP-ribose polymerase (PARP) signalling (Kothari, 2022) (Kothari et al., 2016). Adeshara et al. (2020) showed that a 3-month treatment regime of metformin in T2DM patients had showed a maximum percent declined from baseline in biomarker levels of plasma protein glycation and oxidative stress markers (fructosamine, b-amyloid, sRAGE) and inflammatory cytokines (IL-6, TNF-α). Additionally, Saxena et al. (2020) performed an in vitro analysis to investigate the role of antidiabetic drugs (metformin, pioglitazone and insulin) on TNF-α in subcutaneous adipocytes derived from mesenchymal stem that were cultured in high glucose (5–20 mM) conditions. They concluded that the TNF-α gene expression level was decreased significantly when the adipocytes were

treated with metformin (p=0.015) and pioglitazone (p=0.020). They also observed that a combination of these drugs showed that the expression of TNF-α was nearly the identical for metformin alone. However, insulin up-regulated the TNF-α gene expression in mild or severe glucose load. Table 2.4 further details the effects of metformin on inflammation in *in-vivo* and *in-vitro* models.

Table 2.4: The effects of metformin on inflammation

Model	Treatment	Main Outcome	Reference
In-vivo	3-month treatment regime of metformin (500 mg tablets taken two times daily) treatment as prescribed by the doctor. No dose adjustment was made during the follow up 3-month period.	The regime of metformin in T2DM patients had showed a maximum percent declined from baseline in biomarker levels of plasma protein glycation and oxidative stress markers and inflammatory cytokines.	Adeshara et al. (2020)
	4-week treatment in newly diagnosed T2DM patients to receive either metformin or pioglitazone. The metformin group received 1000 mg metformin daily in two equal doses. The pioglitazone group received 30 mg pioglitazone daily in a single dose.	Highlighted that metformin and pioglitazone both significantly improved oxidative stress as reflected by reduction in malondialdehyde (oxidative stress marker) but antioxidant effect (increase in SOD) was seen with metformin only.	Singh et al. (2016)
	12-week treatment in newly diagnosed T2DM patients to receive either metformin or gliclazide. The metformin group was started at a dose of 850 mg/day. The gliclazide was started at a dose of 80 mg/day. Dosages of the drugs were increased as needed up to a maximum of 2550 mg/day for metformin and of 240 mg/day for gliclazide.	The data suggested that metformin could improve oxidative stress and preserve antioxidant function.	Formoso et al. (2008)
In-vitro	Subcutaneous adipocytes derived from mesenchymal stem cells were were exposed to variable concentrations metformin (50–200 mg/mL) and pioglitazone(5–20 mg/mL)	TNF-α gene expression level was decreased significantly when the adipocytes were treated with metformin and pioglitazone. They also found that a combination of these drugs showed that the expression of TNF-α was nearly the identical for metformin alone	Saxena et al. (2020)
	Human HepG2 cells were exposed to ethanol to simulate alcohol-induced liver injury associated inflammation and oxidative stress. They were then treated with Visbiome® probiotic or metformin treatment (1mM) alone and in combination	Visbiome® probiotic and metformin in combination prevented ethanol-induced cellular injury, oxidative stress, and regulated lipid metabolism as well as inflammatory response in HepG2 cells	Patel et al. (2021)
	Human retinal vascular endothelial cells were exposed to varying doses of metformin with final concentrations of 0~200mM.	Metformin reduced several inflammatory molecules including NFκB p65, ICAM1, MCP-1 and IL-8	Han et al. (2018)

Footnote: The table above describes the effects of metformin on inflammation in in-vivo and in-vitro models. **Abbr:** TNF-α, tumour necrosis factor alpha; NFκB p65, nuclear factor kappa B p65; ICAM1, intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; IL-8, interleukin-8.

Many studies have proven that metformin reduces inflammatory biomarkers while several other studies have shown it does not (Luc et al., 2019). One such study, conducted by Pradhan et al. (2009), investigated whether insulin alone or combined with metformin lowers levels of

inflammatory biomarkers such as high-sensitivity C-reactive protein (hsCRP), IL-6 and soluble tumour necrosis factor receptor 2 (sTNFr2) in patients with recent-onset T2DM. In their study they concluded that in patients with recent-onset T2DM, treatment with insulin or metformin compared with placebo did not reduce inflammatory biomarker levels even though it improved glucose control.

Although metformin possess these anti-inflammatory effects, it remains unclear if this may be an indirect effect mediated through the improvement of insulin sensitivity, weight gain, lipid profile and hyperglycaemia (Kothari et al., 2016). Metformin alone cannot control micro- and macrovascular complications caused by sterile metabolic inflammation and oxidative stress that is associated with apparent T2DM, as its primary objective is to regulate the impeded blood glucose homeostasis (chronic hyperglycaemia and low insulin levels). Despite improvements in the regulation of blood glucose that protect against hyperglycaemia-associated complications, even minor fluctuations in blood glucose levels are sufficient to increase free radical species (ROS and NOS), which through accelerated generation of oxidative stress reduce the existing low antioxidant levels characteristic of T2DM (Dludla et al., 2018). As result various cell types are still exposed to the pathologic T2DM microenvironment and are susceptible to secondary tissue damage. Thus, T2DM patients are still at risk of developing associated comorbidities. Therefore, the treatment of T2DM requires interlinked, simple, inexpensive and broad-based adjuvant therapies to limit T2DM micro- and macrovascular complications and therefore justifies the potential clinical use of exogenous antioxidant supplementation as a synergistic treatment in T2DM to not only ameliorate oxidative stress related damage but holistically treat the disease.

2.4 ANTIOXIDANTS

An antioxidant is a chemical compound obtained from plant and animal extracts, consisting of large molecules like proteins, peptides and polysaccharides, as well as smaller molecules such as peptides, phenols and flavonoids (Pham-Huy et al., 2008). These compounds play a vital role in regulating the redox state of the body by reducing or slowing down the oxidation of ROS and RNS to limit cellular damage (Zhang et al., 2020a). Antioxidants are categorised into two groups namely, enzymatic, and non-enzymatic. Enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, break down free radicals converting the end product (hydrogen peroxide) into water (Haida and Hakiman, 2019, Jeeva et al., 2015). Non-enzymatic antioxidants such as glutathione (GSH), vitamins A, C and E, polyphenols, flavonoids, flavanols and carotenoids, disrupt free radical chain reactions to prevent the formation of free radicals (Nimse and Pal, 2015, Mirończuk-Chodakowska et al., 2018). Synthetic antioxidants such as NAC and AAP which are artificially synthesised in

laboratories by combining chemical compounds, may perform both enzymatic and non-enzymatic antioxidant functions as they can supplement enzyme activity in catalysing radical formation as well as disrupt radical chain reactions (Thomas Amarachukwu, 2022, Ziad et al., 2019). Synthetic antioxidants also display higher stability and performance, are low cost and widely available (Lourenço et al., 2019).

In a diabetic condition, persistent hyperglycaemia cause increased production of ROS via both auto-oxidation of glucose and non-enzymatic protein glycation. The production of ROS is controlled by different antioxidant enzymes which is diminished in a diabetic condition (Adeshara et al., 2020). However, there is increasing evidence from animal studies confirming oxidative stress-induced insulin resistance and the improvement in insulin signal transduction and glucose homeostasis by using antioxidative agents (Yaribeygi et al., 2020). Hininger-Favier et al. (2009) evaluated the antioxidative effects of green tea on insulin sensitivity and found that it markedly improved insulin resistance in diabetic rats. Bagul et al. (2012) reported that antioxidative properties of resveratrol reversed oxidative stress-dependent insulin resistance in diabetic rats.

Subsequently, existing experimental studies and clinical trials have demonstrated the effectiveness of antioxidants in the treatment of T2DM and preventing its associated complication. Particularly through counteracting oxidative stress and oxidative stress related inflammation through increasing endogenous antioxidant defences (Dal and Sigrist, 2016, Johansen et al., 2005, Rajendiran et al., 2018). The mechanisms responsible for the anti-inflammatory properties of carotenoids (β-carotene and lycopene) which are tetraterpene pigments that are found in photosynthetic bacteria, fungi, algae and plants that act as antioxidants, have been found to reduce the generation of ROS mediated by TNFα, while simultaneously increasing the availability of NO at the endothelial level. This then leads to a decrease in oxidative stress and inflammation and has an overall positive and beneficial impact on vascular health (Dal and Sigrist, 2016, Maoka, 2020). Additionally, antioxidant supplementation with vitamins C and E, coenzyme Q_{10}, alpha-lipoic and acid L-arginine, may potentially improve endothelial dysfunction in T2DM. This is done via the re-coupling endothelial NO synthase (eNOS) and mitochondrial function, in addition to decreasing vascular NAD(P)H oxidase activity. This antioxidant supplementation not only improves endothelial health and vascular homeostasis whose importance has been mentioned above but also better controls blood pressure, dyslipidaemia and glucose homeostasis. Thus, preventing the development of diabetic associated complications such as diabetic retinopathy, nephropathy, neuropathy and cardiomyopathy (Bajaj and Khan, 2012, Dal and Sigrist, 2016). Polyphenols such as flavonoids, which are naturally occurring compounds found mainly in fruits, vegetables and cereals that possess antioxidant functions, have shown to have a

protective effect in vascular tissue due to the direct antioxidant activity of polyphenols on endothelial function (Dal and Sigrist, 2016). Furthermore, Various preclinical studies from our lab and others, have demonstrated the protective effect of antioxidants, such as NAC against diabetes induced hepatopathy, nephropathy, retinopathy, myocardial injury, and stem/progenitor cell impairment (Genc and Peker, 2021, Liu et al., 2021, Maartens et al., 2021, Mehrbani Azar et al., 2018, Wang and Kang, 2020, Yalçın and Gürel, 2021).

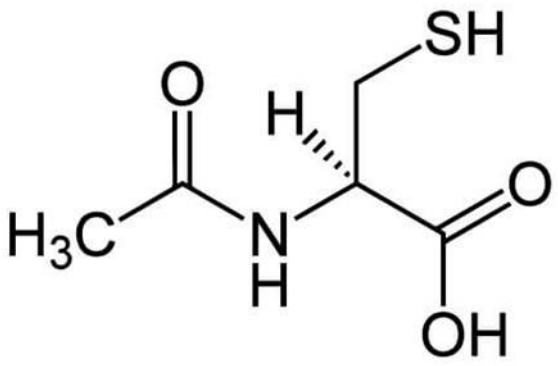
Literature provides evidence that suggests that antioxidants are beneficial to human health and may be used as a potential complementary therapeutic strategy to combat diseases associated with oxidative stress and inflammation (Arulselvan et al., 2016, Hussain et al., 2016). Thus, using natural and/or synthetic antioxidant and/or anti-inflammatory therapeutic agents to target oxidative damage and inflammation could potentially prevent the development of secondary complications in T2DM patients and in doing so improve their quality of life.

2.4.1 NAC

2.4.1.1 Indication

N-acetylcysteine is thiol compound that is the acetylated form of L-cysteine and a precursor of reduced glutathione (Figure 2.5). It has been used clinically for more than 40 years (Lasram et al., 2015). NAC is indicated for the treatment of various disorders. It is used as the standard antidote for acetaminophen (paracetamol) overdose and as an adjuvant treatment for patients with abnormal and viscous mucous secretions associated with acute and chronic bronchopulmonary disorders (pneumonia, bronchitis, emphysema, tuberculosis). Additionally, it is used during anaesthesia and in the preparation of patients for diagnostic bronchial studies (bronchograms, bronchospirometry, bronchial wedge catheterization), (Sahasrabudhe et al., 2023, Pedre et al., 2021). NAC is also a known thiol-containing antioxidant that may act as a ROS scavenger to treat oxidative stress and its associated diseases (diabetes, cancer, cardiovascular disease, etc) (Pedre et al., 2021).

NAC is generally well tolerated and mild adverse effects. Common side effects from oral administration include stomatitis, nausea, vomiting, rhinorrhoea, drowsiness, clamminess and rash with or without fever. Intravenous (IV) administration side effects include rash, urticaria and pruritus, (hypersensitivity reactions), wheezing, dyspnoea and hypotension. Adverse reactions to intravenous NAC are due to complications associated with the treatment for acetaminophen poisoning and its associated high adult doses (loading dose of 150 mg/kg, administered as soon as possible followed up with two maintenance doses of 50 mg/kg and 100 mg/kg) (McEvoy and American Society of Health-System, 2005, Sahasrabudhe et al., 2023, Yarema et al., 2018).



N-ACETYLCYSTEINE

Figure 2.5: NAC chemical structure. It is a thiol compound and a prodrug of the endogenous amino acid L-cysteine which is a precursor of the antioxidant GSH. It has a chemical formula of C5H9NO3S and molecular weight of 163.2 g/mol.

2.4.1.2 Pharmacokinetics

NAC is administered orally or via IV infusion (McEvoy and American Society of Health-System, 2005). NAC is more efficiently absorbed in the acetylated form and complete absorption provides a vast amount of NAC for cellular uptake, deacetylation of cysteine (Cys) and synthesis of glutathione (GSH). NAC and its metabolites are rapidly and almost completely absorbed at the intestinal wall after oral administration. Further, it is primarily metabolized by the liver and undergoes extensive first-pass metabolism. Due to this, the bioavailability of free NAC is only 10%. After 600 mg oral or IV administration, NAC has a maximum concentration in of plasma 15 nmol/mL and 300 nmol/mL via oral and Intravenous administration, respectively. It has a half-life estimated at around 2 hours. 13-38% of NAC is excreted in the urine, while 3% is excreted in the faeces within the first 24 hours. The major metabolites excreted are cystine and cysteine with small amounts of taurine and sulphates excreted in urine (Lasram et al., 2015).

2.4.1.3 Mechanism of action

NAC exerts its mechanism in a number of ways (Pedre et al., 2021). NAC reduces the viscosity of pulmonary secretions and facilitates their removal by mechanical means (coughing) and postural drainage. This mucolytic effect (Figure 2.6) is achieved through NAC's free sulfhydryl groups hydrolysing disulphide bonds within mucoproteins, breaking down the oligomers, and making pulmonary secretions less viscous (Sahasrabudhe et al., 2023).

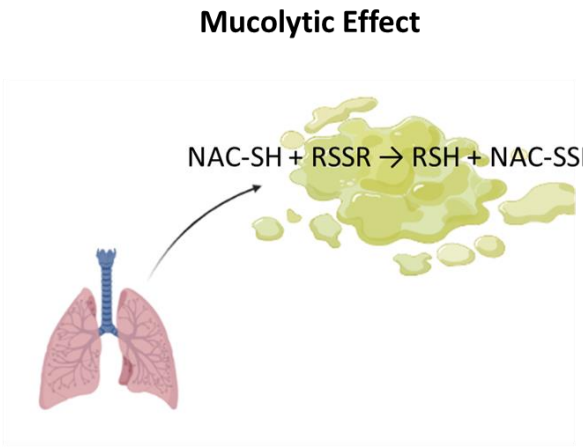


Figure 2.6: Mucolytic effect of NAC. The free-sulfhydryl (-SH) group in NAC hydrolyses mucus disulphide bonds, in so doing causes the viscosity of mucus to become significantly reduced. NAC is thus indicated in chronic broncho-pulmonary diseases (chronic emphysema, chronic emphysema with bronchitis, chronic asthmatic bronchitis, TB, bronchiectasis and pulmonary amyloidosis of the lung) and acute bronchopulmonary diseases (pneumonia, bronchitis, and tracheobronchitis) as these diseases produce abnormal, extremely viscous and thick mucus secretions. **Abbr:** NAC-SH, N-acetylcysteine with free-sulfhydryl; RSSR, mucoproteins with disulphide bonds; RSH, hydrolysed mucoprotein; NAC-SSR, N-acetylcysteine bonded to sulphide from mucoprotein.

During acetaminophen over dosage (Figure 2.7), NAC acts as an alternate substrate for conjugation and detoxification of N-acetyl-p-benzoquinoneimine (NAPQI) which is a toxic intermediate metabolite of acetaminophen. In addition, the antioxidant properties of NAC play a role in reversing the liver toxicity associated with acetaminophen over dosage (Mazaleuskaya et al., 2015).

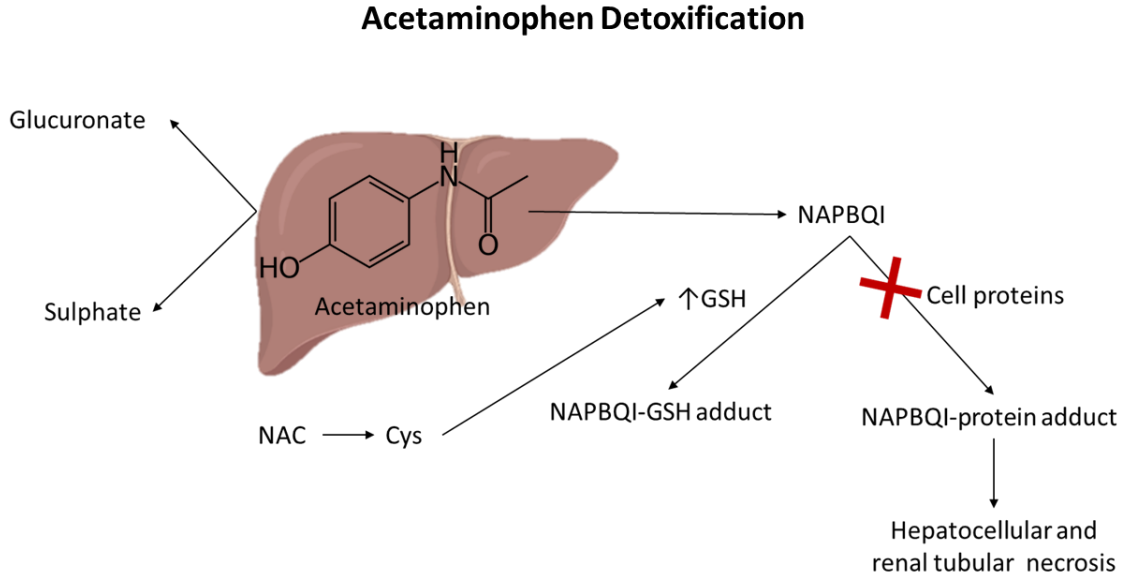


Figure 2.7: Acetaminophen detoxification effect of NAC. Abnormally high doses of acetaminophen saturate the regular detoxification of the drug into glucuronate and sulphate and consequently produce a toxic metabolite, NAPBQI. NAPBQI may be inactivated through conjugation with GSH. However, the abnormally high toxic doses deplete GSH reserves and NAPBQI is then free to react with cell proteins which causes hepatic injury, potential hepatic necrosis as well as renal tubular necrosis (rarely). **Abbr:** NAPBQI, N-acetyl-p-benzoquinone imine; GSH, glutathione; Cys, cysteine.

NAC is a known antioxidant due to its chemical structure containing Cys. NAC displays both direct and indirect antioxidant properties (Figure 2.8). It has a direct effect in acting as a ROS scavenger to decrease oxidative stress and inflammation. This direct effect is accomplished via the thiol group present in NAC that serves as an electron donor to prevent the oxidation of ROS (hydrogen peroxide and hypochlorous acid). Its indirect effect is as a precursor in the production of GSH (an antioxidant that contains Cys, glycine and glutamic acid which counter ROS) (Demirkol and Ercal, 2012, Lasram et al., 2015, Santus et al., 2014). The antioxidant properties of NAC are both significant and valuable in the context of the pathological T2DM microenvironment as it may be used as a complimentary therapeutic agent to target hyperglycaemic induced oxidative stress and sterile metabolic inflammation to potentially prevent the development of secondary tissue damage. This concept will be further elaborated below (section 2.4.1.4) bringing into light the connection between NAC, T2DM and oxidative stress.

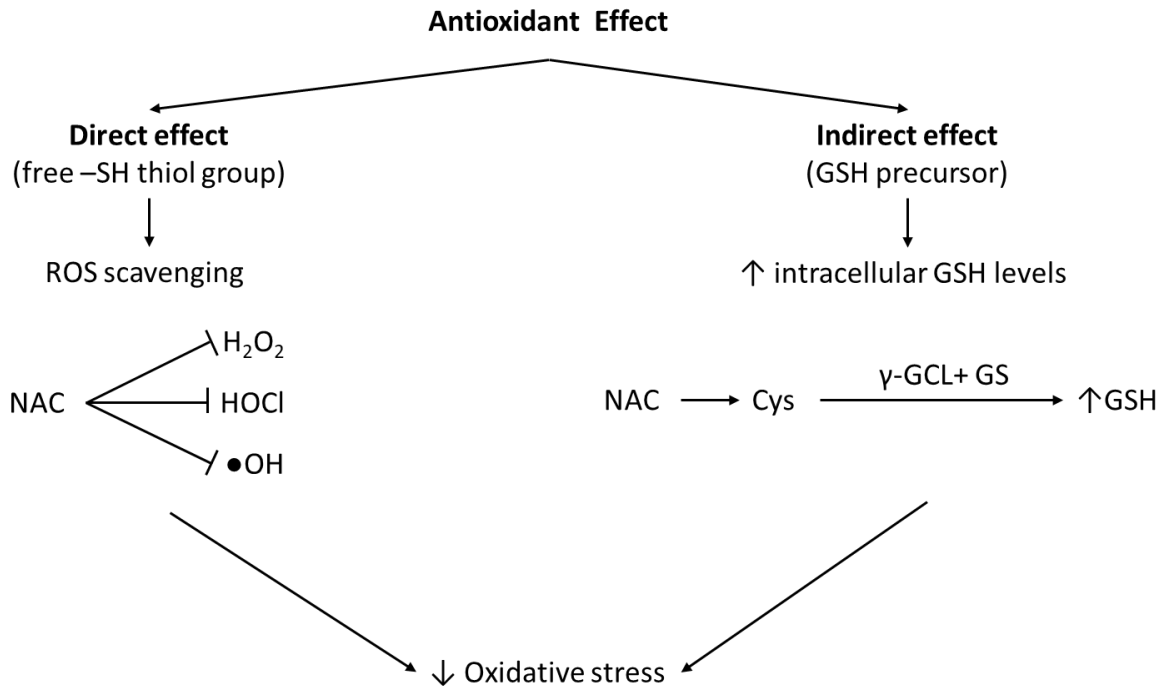


Figure 2.8: Antioxidant effect of NAC. NAC displays both direct and indirect antioxidant properties. Its direct effect as a ROS scavenger is accomplished via the free thiol (-SH) that acts as an electron donor to prevent the oxidation of ROS (hydrogen peroxide and hypochlorous acid). Its indirect effect is as a precursor of GSH and thus increases the synthesis of GSH to further counter ROS and oxidative stress. **Abbr:** ROS, reactive oxygen species; GSH, glutathione; GS, glutathione synthase; γ-GCL, γ-glutamylcysteine; Cys, cysteine; H2O2, hydrogen peroxide; HOCl, hydrochlorous acid; -OH, Hydroxyl

2.4.1.4 NAC, diabetes and oxidative stress

A number of clinical and experimental studies have reported the potential effects of NAC as a therapeutic agent against T2DM and its complications (Lasram et al., 2015). Table 2.5 below depicts preclinical and clinical studies that have been shown to improve the diabetic outcome

via NAC supplementation. NAC has been reported to reduce inflammation, inhibit lipid accumulation and improve insulin sensitivity (Dludla et al., 2019, Falach-Malik et al., 2016). There is increasing evidence from animal studies confirming oxidative stress-induced insulin resistance and the improvement in insulin signal transduction and glucose homeostasis by using NAC (Yaribeygi et al., 2020). Additionally, studies have reported NAC to be protective against oxidative stress induced by hyperglycaemia via the generation of GSH (Dludla et al., 2019, Johnson et al., 2019, Kamboj et al., 2010). GSH is the most abundantly present non-protein thiol in all mammalian tissues and is the most important intracellular antioxidant. GSH performs several vital functions including defence against oxidative stress, modulating redox signalling, detoxification of xenobiotics (chemical substances that are foreign to an organism's regular metabolism), and regulating cell proliferation, apoptosis, immune function and fibrogenesis (Lasram et al., 2015, Lu, 2013, Patterson et al., 2010). NAC has the ability to enhance endogenous levels of GSH, thus indicating its importance as an antioxidant to protect against oxidative stress and prevent the development and/or progression various metabolic conditions such as diabetes mellitus, musculoskeletal disorders, CVD and cancer (Dludla et al., 2018).

The acetylated form of NAC is almost completely absorbed and after being metabolised primarily by the liver it provides a large amount of NAC for cellular uptake. NAC is then converted to cysteine via a deacetylation reaction which is then utilised in GSH biosynthesis to increase levels of GSH and thus antioxidant activity (Lasram et al., 2015). GSH biosynthesis (Figure 2.9) occurs in the cytosol and is tightly regulated via the availability of the Cys and activity of glutamate cysteine ligase (GCL) which is a rate-limiting enzyme (Lu, 2013). The biosynthesis of GSH occurs in a negative feedback loop via two enzymatic steps that require ATP. The first step (catalysed by GCL) conjugates Cys with glutamate, producing γ-glutamylcysteine. The second step (catalysed by GSH synthase) joins glycine to γ-glutamylcysteine to form GSH. GSH performs its antioxidant function chiefly through GPx-catalysed reactions. These reactions reduce ROS (hydrogen peroxide and lipid peroxide) while GSH is oxidized to disulfide oxidized GSH (GSSG). GSSG is then reduced back to GSH by GSSG reductase through the consumption of NADPH, forming a redox cycle and preventing the excessive accumulation of ROS, thus preventing oxidative stress (Lu, 2013).

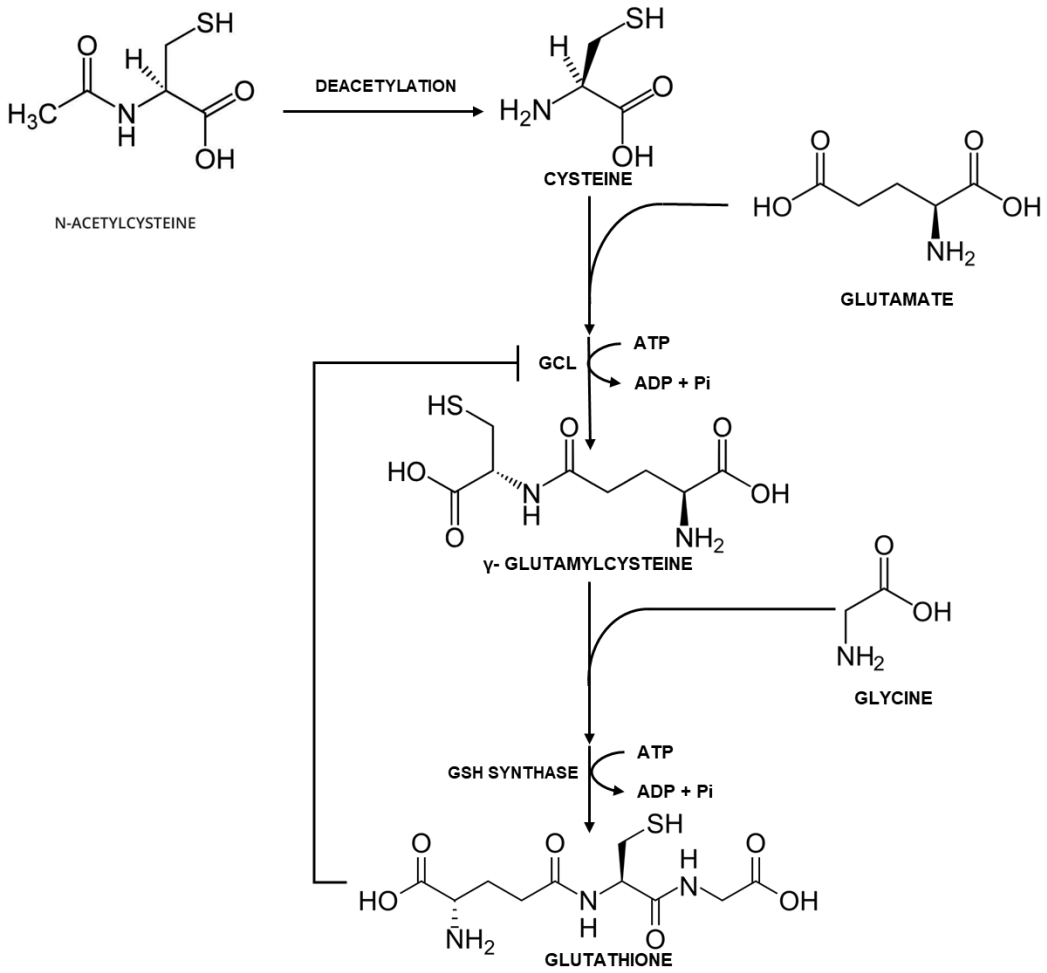


Figure 2.9: GSH biosynthesis via NAC supplementation. NAC is taken up by cells and is then converted to cysteine via a deacetylation reaction. Cysteine is then utilised in GSH biosynthesis which occurs in a negative feedback loop via two enzymatic steps that require ATP. The first step (catalysed by GCL) conjugates Cys with glutamate, producing γ-glutamylcysteine. The second step (catalysed by GSH synthase) joins glycine to γ-glutamylcysteine to form GS. GCL is then inhibited by GSH via a negative feedback mechanism. **Abbr:** ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate; GSH, glutathione; GCL, glutamate cysteine ligase.

As previously mentioned, numerous preclinical studies from our lab and others, have demonstrated the protective effect of NAC against diabetes induced hepatopathy, nephropathy, retinopathy, myocardial injury, and stem/progenitor cell impairment (Genc and Peker, 2021, Liu et al., 2021, Maartens et al., 2021, Mehrbani Azar et al., 2018, Wang and Kang, 2020, Yalçın and Gürel, 2021). Shen et al. (2018) investigated whether the effect of NAC and its time point of intervention can affect insulin resistance in a diet-induced obesity mouse model. Obese mice that received the 6-month NAC treatment showed improved intraperitoneal glucose and insulin tolerances throughout the study. In addition, these mice showed that NAC significantly increased motor activity and improved the high-fat high-sucrose diet induced mitochondrial and intracellular ROS expression, DNA and protein oxidative damage and adipose tissue inflammation.

Table 2.5: Preclinical and clinical studies (cell culture, animal and human models) that have been shown to improve the diabetic outcome through NAC supplementation.

Model	Study Context	Treatment	Study Outcomes	Key Findings	Reference
Animal Model	Male Wistar rats	2 mg/L NAC for 30 days	Hyperglycaemia, dyslipidaemia and oxidative stress were assessed.	NAC reduced the oxidative stress, enhancing glutathione-peroxidase activity, and normalizing lipid hydroperoxide.	Diniz et al. (2006)
Animal Model	Male Sprague-Dawley rats	NAC infusion of 45.9 ± 3.5 mg/kg/min	Insulin-stimulated glucose uptake and oxidative stress markers were measured.	Prevented a significant decrease in insulin-stimulated glucose uptake and blocked the increase in soleus muscle protein carbonyl content (a marker of oxidative stress)	Haber et al. (2003)
Animal Model	Diabetic adult male Wistar rats	NAC administered at 75 mg/kg	Oxidative stress biomarkers were quantified.	Significant decrease in hepatic and renal lipid peroxidation and an increase in δ-aminolevulinate dehydratase activity, especially in the renal tissue, were observed.	Ribeiro et al. (2011)
Cell culture Model	Human vascular endothelial cell (cell line ECV304)	NAC at 30 mmol/L or 1 mmol/L, respectively, for 24 h	TNF-α-induced apoptosis, NO production and lipid peroxidation were assessed.	Restored eNOS expression. Increased superoxide dismutase activity and glutathione peroxidase production Reduced malondialdehyde levels	Xia et al. (2006)

Animal and Cell Culture Model	Zucker diabetic fatty rats HIT-T15 cells	6 through 12 weeks of age	Blood oxidative stress markers, hyperglycaemia and glucose intolerance were measured.	Prevented a rise in blood oxidative stress markers and partially prevented hyperglycaemia, glucose intolerance, defective insulin secretion as well as declines in β-cell insulin content.	Tanaka et al. (1999)
Animal Model	Male Wistar rats fed a high-sucrose (HS) diet	5.8 or 20g NAC per kilogram of food given for 6 weeks	Oxidative stress and glucose homeostasis were assessed.	Prevented HS-induced oxidative stress. Prevented HS-induced glucose intolerance, impaired postprandial glycaemic control, and decrease in muscle and liver insulin-induced activation of insulin receptor substrate 1 and Akt	Blouet et al. (2007)
Animal Model	Male Wistar rats fed Glucose-	NAC administer at 1 g/kg/day for 5 weeks	Hypertension, plasma glucose and insulin concentrations, insulin resistance and kinin B1 receptor expression were assessed.	High systolic blood pressure, hyperinsulinemia, insulin resistance and kinin B1 receptor expression were normalized or attenuated in glucose-fed rats.	Ismael et al. (2008)
Animal Model	Diabetic KK-Ay mice	NAC at 200-1800 and 60-600 mg/kg/day, respectively	Glucose and insulin tolerance tests were performed and plasma insulin and lipid peroxidation were measured	Improved glucose tolerance Increased insulin sensitivity Glucose tolerance was improved in Lower weight gain and hepatic TG	Falach-Malik et al. (2016)

Animal model	Streptozotocin-induced diabetic rats	NAC (25 mg/kg/day) was administrated by gavage, for 37 days mg/day,	lipid profile and the atherogenic index was assessed.	NAC decreased glycemia, energy intake, lipid profile, AI as well as carbohydrate and protein consumption in diabetic rats	Kaga et al. (2018)
Human Model	Patients with metabolic syndrome. Randomized, double-blind, placebo-controlled trial	NAC at 1800 mg/day	Metabolic parameters and serum adiponectin levels were measured.	NAC significantly reduced fasting plasma glucose, fasting serum insulin, insulin resistance index and serum C-reactive protein and increased serum HDL-cholesterol and plasma total glutathione	Panahi et al. (2022)

Footnote: The table above describes preclinical and clinical studies that have been shown to improve the diabetic outcome both oxidative stress and glucose homeostasis related via NAC supplementation. **Abbr:** eNOS, endothelial NO synthase; HDL, high-density lipoprotein; NAC, N-acetylcysteine; AI, atherogenic index; TG, triglyceride; Akt, protein kinase B; HS, high sucrose.

The literature mentioned in the table above demonstrates NAC's protective effect in T2DM induced oxidative stress as well as restoration and regulation of glucose homeostasis. Thus, this highlights the potential use of NAC as an adjuvant therapy in the treatment of T2DM. Additionally, this warrants additional research, both preclinical and clinical, on whether NAC alters glucose uptake, the requirement for metformin treatment as well as how it may improve the pathogenic diabetic microenvironment.

2.4.2 AAP

2.4.2.1 Indication

The ascorbic acid (vitamin C) derivative ascorbic acid-2-phosphate is a precursor for ascorbate, an important molecule used by the body in a variety of ways. It is an essential nutrient and plays a vital role in immune defence by supporting various cellular functions in both the innate and adaptive immune systems (Carr and Maggini, 2017, Shi et al., 2020). It specifically important in redox reactions such as maintaining the balance between antioxidants and oxidants by acting as ROS scavenging agents inside cells (Blaszczak et al., 2019).

The daily dietary requirements for Vitamin C range between 100–200 mg. This dosage is estimated to cover general requirements for the reduction of chronic disease risk (Carr and Maggini, 2017). In the United States and Canada, the recommended dietary allowance for vitamin C is 75 mg for women and 90 mg for men according to the Food and Nutrition Board (Padayatty and Levine, 2016). Vitamin C is indicated in prevention and treatment of scurvy but may also be indicated for respiratory and systemic infections (asthma pneumonia, sinusitis, rheumatic fever). Additionally it may be indicated in chronic illnesses such as T2DM as well as haemovascular disorders, burns and delayed wound healing (Abdullah et al., 2022).

Oral Vitamin C supplements taken at appropriate doses are generally considered safe, (Food and Nutrition Board, 2000b; Levine et al, 1999a). Doses above 2g per day are associated with side effects, such as nausea, vomiting, bloating and osmotic diarrhoea heartburn, skin flushing, fatigue and drowsiness (Padayatty and Levine, 2016). Vitamin C supplementation may increase uric acid and oxalate accumulation causing kidney stones in those with impaired renal function (Canavese et al., 2005, Padayatty and Levine, 2016).

2.4.2.2 Pharmacokinetics

The pharmacokinetics of vitamin C are complex. Vitamin C is primarily administered via oral ingestion in the form food or supplements (Lykkesfeldt and Tveden-Nyborg, 2019). Majority of vitamin C is completely absorbed in the small intestine via enterocytes facilitated by secondary active transporters, namely sodium-dependent Vitamin C transporters 1 and 2 (SVCT1 and

SVCT2) (Lykkesfeldt and Tveden-Nyborg, 2019, Wilson, 2005). Vitamin C is then ionised into its two main forms, namely, its reduced and oxidized which are ascorbate (ASC) and dehydro-ascorbate (DHA), respectively. DHA is more predominant than ASC (Lykkesfeldt and Tveden-Nyborg, 2019). These forms exit the enterocytes and enters systemic circulation (May and Harrison, 2013). Vitamin C plasma concentrations are dependent on dosage and rates of disposition. However, it typically has a plasma concentration of 40-60 µM (May and Harrison, 2013, Padayatty and Levine, 2016). Vitamin C is easily filtered and excreted through the kidneys. However, it is reabsorbed in the renal proximal tubule by renal epithelial cells which assists to conserve ascorbate in the blood for uptake into the tissues (Lykkesfeldt and Tveden-Nyborg, 2019, May and Harrison, 2013).

2.4.2.3 Mechanism of action

Vitamin C functions as a cofactor, chelating agent, enzyme complement and co-substrate in a number of reactions and metabolic processes (Abdullah et al., 2022). Through chelate complex formation, it increases iron solubility and is an important coenzyme collagen, noradrenaline, carnitine and peptide hormones such ads vasopressin, calcitonin, oxytocin (Pazirandeh and Burns, 2015). In particular, it plays an important role in immune system processes. Vitamin C enhances, antibody levels, phagocytosis, chemotaxis, microbial killing as well as enhances differentiation and proliferation of B and T lymphocytes. Furthermore, modulates cytokine production, decreases histamine levels and necrosis as well as facilitates apoptosis and clearance (Abdullah et al., 2022, Carr and Maggini, 2017). Vitamin C is a reducing agent, that acts as a donor of single reducing equivalents (H or H+ +e−) cycling between its two forms thus making it a good free radical scavenger and powerful antioxidant, (Njus et al., 2020). The antioxidant and free-radical scavenging actions of ascorbate are due to its ability to non-enzymatically reduce free radicals such superoxide, hydroxyl, alkoxyl, peroxyl (ROO•) and other radicals. These free radicals remove a single H atom from ascorbic acid, oxidizing it to dehydroascorbic acid (DHA) (Njus et al., 2020). Therefore, neutralising these free radicals, attenuating the inflammatory response and protecting important biomolecules (proteins, lipids, carbohydrates, and nucleic acids) from ROS damage (Abdullah et al., 2022, Carr and Maggini, 2017). The antioxidant properties of AAP are both significant and valuable in the context of the pathological T2DM microenvironment as it may be used as a complimentary therapeutic agent to scavenge the excess free radicals produced via hyperglycaemic induced oxidative stress and sterile metabolic inflammation.

Thus, it may prove useful to potentially prevent the development of secondary tissue damage. This concept will be further elaborated (Section 2.4.2.4) below bringing into light the connection between AAP, T2DM and oxidative stress.

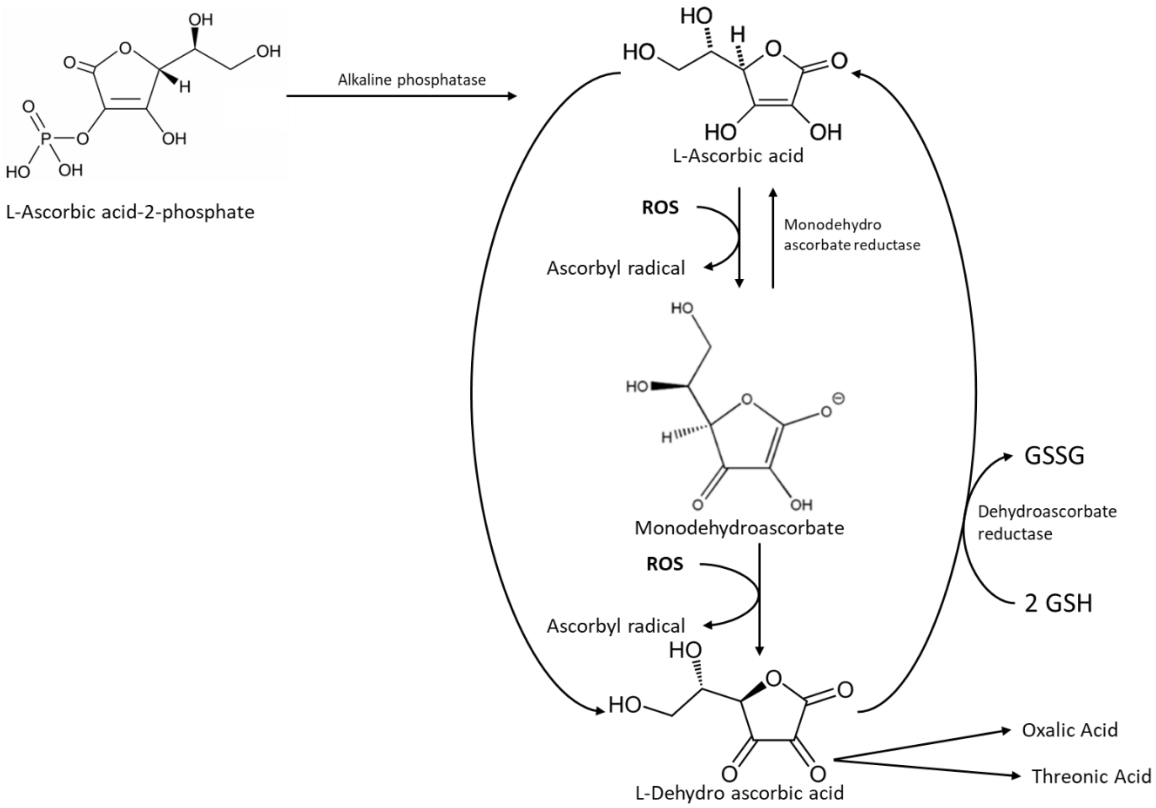


Figure 2.10: Metabolism and Antioxidant effect of AAP. Ascorbic acid-2-phosphate (AAP), via the action of alkaline phosphatase on the plasma membrane of various kinds of cells is hydrolysed into L-Ascorbic acid which is then transported into the cells by either simple diffusion or active transport. L-Ascorbic acid is then oxidised via single electron reduction by substrates such as free radicals (superoxide, hydroxyl, alkoxyl, peroxyl) and forms monodehydroascorbate and an ascorbyl radical which is poorly reactive. Monodehydroascorbate is then further oxidised again via single electron reduction by substrates such as free radicals and forms L-Dehydro ascorbic acid and a poorly reactive ascorbyl radical. Thus, ascorbate accomplishes its antioxidant effect through its ability to donate electrons from both the second and third carbon. L-Dehydro ascorbic acid is mainly reduced back into L-ascorbic acid via dehydroascorbate reductase using GSH as a source of reducing power. Monodehydroascorbate reductase can also reduce monodehydroascorbate back to L-Ascorbic acid. The enzymatic reduction of monodehydroascorbate and L-Dehydro ascorbic acid help replenish and preserve L-Ascorbic acid stores for continuous antioxidant activity. Small amounts of L-Dehydro ascorbic are irreversibly hydrolysed to form diketogulonic acid and further breakdown products such as oxalate and threonate. **Abbr:** ROS, reactive oxygen species; GSH, glutathione; GSSG, reduced glutathione.

2.4.2.4 AAP, diabetes and oxidative stress

As mentioned before the consumption of antioxidants nutrients such as vitamin C and E have demonstrated the protective effect of antioxidants against T2DM associated complications such as oxidative injure associated with hyperglycaemia and pancreatic β cell function (Rafighi et al., 2013). Table 2.6 below depicts preclinical and clinical studies that have been shown to improve the diabetic outcome via ascorbic acid supplementation. Additionally, people living with T2DM show evidence of lower circulating vitamin C and thus Vitamin C deficiency (Christie-David et al., 2015, Sun et al., 2022). This may be the result of poor food choices leading to decreased consumption of vitamin C rich foods, AAP being inhibited due to it having a similar structure to glucose and competing for binding sites on GLUT transporters (Rumsey

et al., 1997) and the increased oxidative stress associated with T2DM disease progression (Sun et al., 2022).

Rafighi et al. (2013) conducted a study to evaluate the outcome of vitamin C and E supplementation on T2DM patients. Treatment groups were treated for 3 months and received a daily dosage of 266.7 mg vitamin C alone or in combination with vitamin E (300 IU). Parameters such as HbA1c, glucose, SOD and glutathione peroxides were evaluated confirmed that patients with T2DM that were treated with vitamins C and E demonstrated significantly low levels of hypertension, decreased levels of blood glucose and increased SOD and GSH enzyme activity that may reduce insulin resistance by greater lowering oxidative stress parameters. Vitamin C intakes, serum vitamin C, fasting plasma glucose, and HbA1c levels of 6807 T2DM and 428 T1DM from the NHANES database between 1999 and 2018 were analysed to investigate whether low vitamin C intake and serum vitamin C level may be an associated health risk for US adults with diabetes (Sun et al., 2022). These analyses revealed that, vitamin C intake and serum vitamin C levels were inversely associated with markers of pre-diabetes and T2DM, namely, fasting plasma glucose and HbA1c levels and that the risks of T2DM increased in adults with vitamin C intake below the estimated average requirement as well as with no vitamin C supplement.

Furthermore, Ellulu et al. (2015) investigated the effect of vitamin C on reducing the levels of inflammatory markers in hypertensive and/or diabetic obese adults. Obese patients living with hypertension and/or T2DM and having high levels of inflammatory markers were randomized into two groups and the experimental group was treated with 500 mg vitamin C twice daily for 8 weeks. In the experimental group, vitamin C had shown to significantly reduce the levels of hsCRP, IL-6, fasting blood glucose and triglyceride. Thus, suggesting that Vitamin C (500 mg twice daily) has the potential effect of improving the inflammatory status associated obese patients living with hypertension and/or T2DM. A study conducted to investigate the effect of vitamin C on polymorphonuclear (PMN) cell (mediators of the innate immune response to invading microorganisms) primary functions in T2DM patients with poor glycaemic control found that a 6-week vitamin C (1000 mg daily) increased PMN functions namely PMN phagocytosis and oxidative burst in treated T2DM patients (Chuangchot et al., 2020)

Table 2.6: Preclinical and clinical studies (cell culture, animal and human models) that have been shown to improve the diabetic outcome with AAP supplementation.

Model	Study Context	Treatment	Study Outcomes	Key Findings	Reference
Cell culture model	Bone marrow stem cells derived from wild-type mice and diabetic obese mice	Ex vivo antioxidant treatment of NAC (7.5mM) and AAP (0.6mM)	Paracrine responsiveness on both the molecular and protein level were assessed	Significantly dampened the excessive TNFa response observed in obese mice and improved the secretion of IL10.	Mehrbani Azar et al. (2018)
	Bone marrow stem cells derived from wild-type mice (SCWT) and ob/ob mice (SCob)	Antioxidant preconditioning of NAC (7.5mM) and AAP (0.6mM)	Growth rate, migration ability and viability of impaired MSCs were assed.	Improved ex vivo MSC viability and protected MSCs in the presence of Diabetic wound fluid. Could not rescue the reduced proliferation and migration capacity of impaired diabetic MSCs.	Mehrbani Azar et al. (2020)
	Wild-type mice (male, n = 24) and obese diabetic mice (male, n = 24)	Antioxidant NAC (7.5mM) and AAP (0.6mM) supplementation for a period of 6 weeks	Metabolic parameters (weight and blood glucose) and the oxidative status (serum total serum antioxidant capacity, malondialdehyde) of animals were assessed Bone marrow MSCs were isolated and their functionality (growth rate, viability, adipogenesis, and osteogenesis) assessed ex vivo.	No change in the metabolic profile Improved antioxidant status of diabetic animals and reduced lipid peroxidation, Improved population doubling time of MSCs and significantly downregulated the expression of two genes (Nox1 and Rag2) associated with oxidative stress.	Maartens et al. (2021)
	Male Wistar rats	Vitamin C and E at the doses of 100 mg/kg and 100 mg/kg body weight, respectively	Total antioxidant capacity, nitric oxide, malondialdehyde, calcium, magnesium levels were assessed.	Provided better ameliorative benefits than a combination of Vitamin C, E, calcium and magnesium in diabetics	Bassey et al. (2022)
	Human adipose tissue-derived mesenchymal stem cells (hADMSCs)	Various concentrations of NAC and/or AAP for 20 h	Major signal transduction pathways that were responsible for the survival of hMSCs were assessed.	Protected mitochondria from H2O2-induced oxidative stress and rescues hMSCs from mitoptosis, necroptosis and apoptosis.	Li et al. (2015)
Animal and Cell Culture Model	Diabetic male albino mice HepG2cells	2 concentrations 60 and 120 mg/kg) ascorbic acid were given orally to mice for 21 days. Low:	Molecular interactions and cytotoxicity determination	Significant reduction in body weight, blood glucose level and SFRP4 expression.	Bukhari et al. (2022)

Animal model	Diabetic male albino rats	Orally administered with ascorbic acid (25 mg/kg), or combination of metformin (25 mg/kg) and ascorbic acid (25 mg/kg) for 11 consecutive days.	Potency and efficacy of ascorbic acid against immobility period, hypercorticosteronemia, adrenal hyperplasia, hyperglycemia, hypoinsulinemia, oxidative stress, and inflammatory response were assessed.	Significantly reduced immobility period, glucose and corticosterone levels Increased the levels of insulin and monoamines Significant reductions in oxidative stress and proinflammatory cytokines.	Shivavedi et al. (2017)
Human Study	Individuals with type 1 diabetes mellitus	Complex 1 had 0.08, 0.04, 0.0008 µM of ascorbic acid, β-carotene and α-tocopherol respectively. Complex 2 had 1.6, 0.8, 0.0016 µM of ascorbic acid, β-carotene and α-tocopherol respectively	Production of ROS, reduction capacity, expression of NADPH oxidase subunits, superoxide dismutase and catalase and levels of cytokines were assessed.	Reduced ROS production, expression of NADPH oxidase subunits and pro-inflammatory cytokines Raised the expression of antioxidant enzymes and reducing pro-inflammatory cytokines	de Oliveira et al. (2013)
Human Study randomized cross-over study	Individuals with type 2 diabetes	500mg oral ascorbic acid twice daily for 4 months	Ascorbic acid concentration and oxidative stress markers that included basal measures and insulin-stimulated measures were measured.	Improved skeletal muscle oxidative stress improved insulin-mediated glucose disposal	Mason et al. (2016)
Human Study randomized cross-over study	Individuals with type 2 diabetes	500mg oral ascorbic acid twice daily for 4 months	Postprandial glucose incremental areas under the curve, duration of day in hyper- and hypo-glycaemia status, average 24-hour and daily postprandial glucose concentrations, HbA1c, insulin, blood pressure and oxidative stress were measured.	Improved postprandial and 24-hour glycaemia Decreased blood pressure after 4 months of supplementation	Mason et al. (2019)
Human Study prospective, randomized,	Individuals with type 2 diabetes	Group A: 60 mg Rutin in combination with 160 mg vitamin C three times daily in addition to usual oral	Fasting Blood Glucose (FBG), HbA1c, fasting insulin, Malondialdehyde, Superoxide dismutase, Lipid profile and patients' quality of life using were assessed	Significantly reduced the % change of FBG No effect on HbA1c, overall FBG, total cholesterol, fasting insulin, Homeostatic Model Assessment for Insulin Resistance or oxidative stress in T2DM patients	Ragheb et al. (2020)

controlled study		antidiabetic treatment for 8 weeks Group B: 500 mg vitamin C once daily in addition to usual oral antidiabetic treatment for 8 weeks			
Human Study Crossover trial	Individuals with type 2 diabetes	1000 mg vitamin C for 6 weeks	blood pressure, oxidative stress and nitric oxide release were measured.	Decreased pre-exercise and post-exercise blood pressures, possibly due to improved oxidative stress and nitric oxide release.	Boonthongkaew et al. (2021)

Footnote: The table above describes preclinical and clinical studies that have been shown to improve the diabetic outcome related to both oxidative stress and glucose homeostasis via AAP supplementation. **Abbr:** NAC, N-acetylcysteine; MSC, mesenchymal stem cells; AAP, ascorbic acid-2 phosphate; IL10, interleukin-10; TNF-a, tumour necrosis factor alpha; H2O2, Hydrogen peroxide; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; FBG, fasting blood glucose; T2DM, type 2 diabetes; SFRP4, secreted frizzled-related protein 4; HbA1c, glycated haemoglobin.

The sources mentioned in the table above exhibits AAP's protective effect in T2DM induced oxidative stress as well as restoration and regulation of glucose homeostasis. AAP, at appropriate doses as well as being a precursor for ascorbate may facilitate, support and restore various important cellular functions in the pathogenic diabetic microenvironment. Specifically, through maintaining the balance between antioxidants and oxidants by acting as a ROS scavenging agent inside cells. Thus, it emphasises the potential use of AAP as a complementary therapy in the treatment of T2DM. Additionally, this merits additional research, both preclinical and clinical, on whether AAP alters glucose uptake, the dose requirement for metformin treatment as well as how it may improve the pathogenic diabetic microenvironment and ultimately the diabetic prognosis.

2.5 THE FUTURE OF T2DM AND OXIDATIVE STRESS

Metformin alone cannot control micro- and macrovascular complications caused by sterile metabolic inflammation and oxidative stress that is associated with apparent T2DM, as its primary objective is to regulate the impeded blood glucose homeostasis (chronic hyperglycaemia and low insulin levels). As result various cell types are still exposed to the pathologic T2DM microenvironment and are susceptible to secondary tissue damage. Thus, T2DM patients are still at risk of developing associated comorbidities. Therefore, the treatment of T2DM requires interlinked, simple, inexpensive and broad-based adjuvant therapies to limit T2DM micro- and macrovascular complications and therefore justifies the potential clinical use of exogenous antioxidant supplementation as a synergistic treatment in T2DM to not only ameliorate oxidative stress related damage but holistically treat the disease.

Literature provides evidence that suggests that antioxidants are beneficial to human health and may be used as a potential complementary therapeutic strategy to combat diseases associated with oxidative stress and inflammation (Arulselvan et al., 2016, Hussain et al., 2016). Thus, using natural and/or synthetic antioxidant and/or anti-inflammatory therapeutic agents to target oxidative damage and inflammation could potentially prevent the development of secondary complications in T2DM patients and in doing so improve their quality of life. Specifically, the use of NAC and AAP have exhibited its protective effects in T2DM induced oxidative stress as well as restoration and regulation of glucose homeostasis.

NAC not only acts as a ROS scavenger but increases the synthesis of GSH to further counter ROS and oxidative stress. Therefore, indicating its importance as an antioxidant to protect against oxidative stress and prevent the development and/or progression various metabolic conditions such as T2DM and its associated complication. AAP, being a precursor for ascorbate maintains the balance between antioxidants and oxidants by acting as a ROS scavenging agent inside cells and may facilitate, support and restore various important cellular

functions in the pathogenic diabetic microenvironment. Therefore, it emphasises the potential use of AAP as a complementary therapy in the treatment of T2DM. Additionally, the use of GSH as a source of reducing power in the enzymatic reduction of L-Dehydro ascorbic to replenish and preserve L-Ascorbic acid stores for continuous antioxidant activity, reasons the use of NAC and AAP in conjunction.

Taken together, this merits additional research, both preclinical and clinical, on whether the synthetic antioxidants NAC and AAP alters glucose uptake, the dose requirement for metformin treatment as well as how it may improve the pathogenic diabetic microenvironment and ultimately the diabetic prognosis.

3 AIMS AND OBJECTIVES

Although the beneficial effects of the antioxidants, NAC and AAP is well established. It is unclear if the antioxidants NAC and AAP will interfere with the insulin sensitizing function of metformin or reduce the required daily dose. It is hypothesised that there will be beneficial synergistic drug interactions between metformin, NAC and AAP when used in combination with each other, with regards to glucose uptake and oxidative stress reduction.

Aims:

The main aim of this study was to investigate if there is any drug interaction and synergistic effects between metformin and the antioxidants, NAC and AAP. This was assessed in an in-vitro model, using a skeletal muscle myoblasts cell line (mouse C2C12, ATCC). Skeletal muscle is known to be the major tissue in the body for glucose uptake and plays a crucial role in glucose homeostasis.

Objectives:

1. Assess the cellular growth and viability under low and high glucose culture conditions over a period of 6 days.
2. Determine the optimal and non-toxic concentration of NAC and AAP by performing a dose response experiment over a period of 24h, 48h, 96h and 6 days by exposing cells to 5 different concentrations of the specific antioxidants and quantify cell viability using a crystal violet assay. Calculate how the optimal concentration relates to the recommended human equivalent doses.
3. Optimise and establish a standard operating procedure for measuring the glucose uptake using the glucose analogue, (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose) 2-NBDG, to assess the glucose lowering and insulin sensitizing effect of metformin before and after the pretreatment of NAC and AAP at optimal and non-toxic doses.
4. Assess the beneficial effect(s) of single dose and combination therapy of NAC, AAP and metformin by determining the total capacity and ROS levels within the cell culture supernatant.

4 METHODS

4.1 ETHICAL APPROVAL

This study was performed using a C2C12 skeletal muscle myoblast - immortalized cell line (ATCC) and is thus exempt from requiring either human or animal ethics approval. Thus, to assess the health & safety risk and approve the standard operating procedures for this project, the project was granted approval (BEE-2022-24398) by the Biosafety and Environmental Ethics committee (REC:BES via committee review procedures). The operating procedures form part of the standard practices within our laboratory and facilities which are registered as both a Genetically Modified Organisms as well as a BSL2 facility.

4.2 OVERVIEW OF PROCEDURES

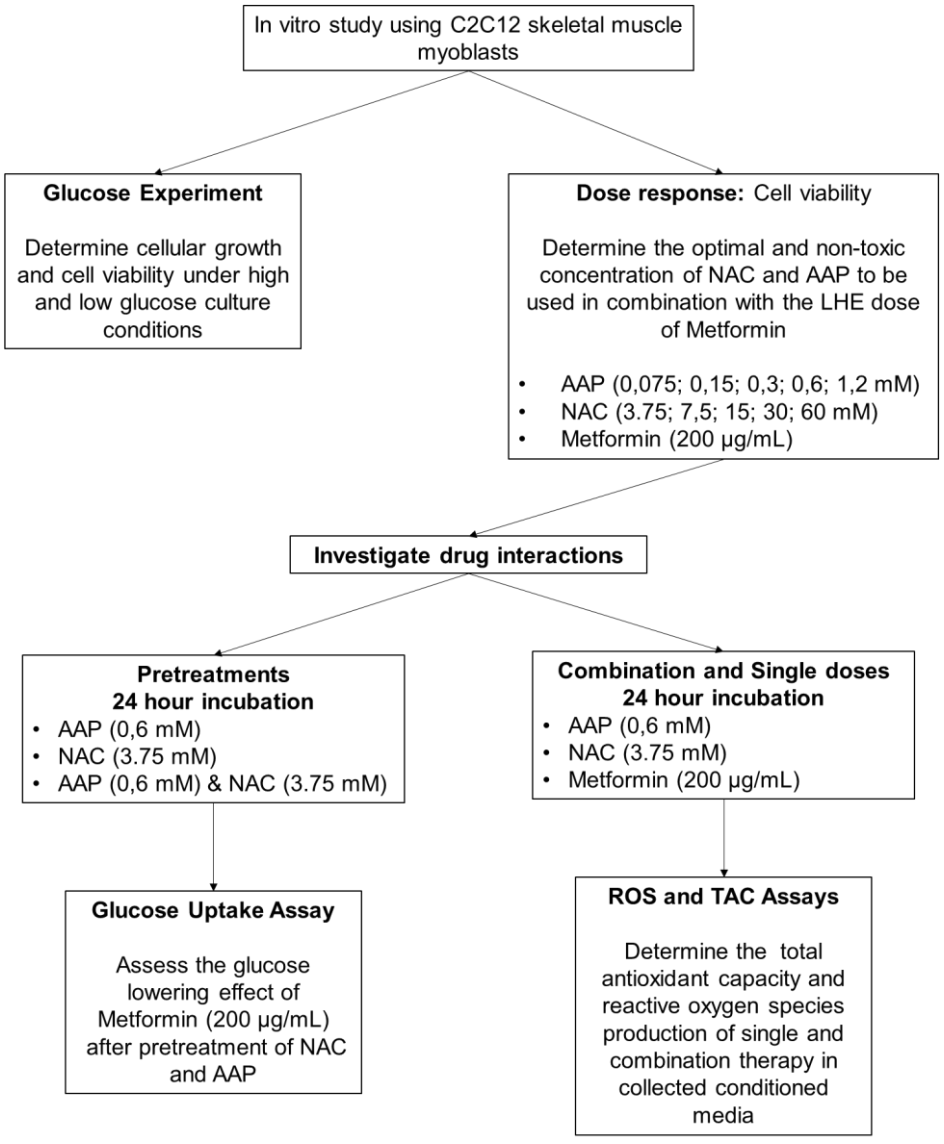


Figure 4.1: Study design and method overview. Abbr: NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate; LHE, lowest human equivalent

4.3 CELL CULTURE

C2C12 skeletal myoblast cell stocks (ATCC® CCL-1772™), preserved in liquid nitrogen, were thawed in a 37ºC water bath for one minute. Cells were seeded (5000 cells/cm^2) into 10cm culture plates (Nest, SoCal BioMed, LLC, US) containing 10mL standard growth medium (SGM) and incubated at 37ºC, in 90% humidified air with 5% CO_2. SGM consisted of high-glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) with ultra-glutamine 2mM (BioWittaker, Lonza, Basel, Switzerland), 1% penicillin (100 U/ml) /streptomycin (100 µg/ml) (BioWittaker, Lonza, Basel, Switzerland) and 10% foetal bovine serum (FBS) (Biochrom, Berlin, Germany). Media was changed every 3 days until cells reached 70-80% confluence. All experiments were performed on cells between passages 7-16.

Once 70% confluence was reached, old media was aspirated, and the cells rinsed with prewarmed phosphate buffer solution (PBS). Prewarmed trypsin/EDTA (#BE17-161E, BioWhittaker; Lonza, Switzerland) was added for 3 minutes to detach the cells from the plate. Once detached, the trypsin enzymatic activity was deactivated using double the volume of SGM. The cell suspension was transferred to a 15 mL falcon tube (NEST, China) and centrifuged at 400 x g (1500 rpm) for 5 minutes (Eppendorf 5804; Sigma-Aldrich, Germany) before resuspending the cell pellet in pre-warmed SGM. 10 µL of cell suspension and 10 µL of trypan blue (0.4%, Invitrogen™, Life technologies corporation, USA) were mixed in a microfuge tube (508-GRD-Q, QSP, US) and the trypan blue-cell suspension pipetted onto a haemocytometer grid with a coverslip. Under a light microscope (Olympus CKX41, CachN 10x/0.25 PhP objective) the number of viable cells in 4 outermost corner squares of a haemocytometer (Fuchs-Rosenthal, Marienfield, Germany) were counted. The total number of viable cells per mL was calculated and the required volume of cell suspension needed along with pre-warmed SGM for each well was added. Plates were placed in the incubator (8000 WJ series, Thermo Electron Corporation, Waltham, MA, USA) and the media refreshed every third day.

For differentiation, C2C12 cells were cultured as described above and seeded at a seeding density of 5000 cells/cm^2 in either a 24-well plate or 8-well chamber dish depending on the experiment performed. Upon reaching 80-90% confluence, SGM was removed and wells washed twice with pre-warmed PBS. Pre-warmed differentiation media which consisted of high-glucose DMEM, 2% horse serum, 1% ultra-glutamine and 1% Pen-strep, was added to differentiate cells into skeletal muscle myotubes. Differentiation media was changed every 24 hours for 3-5 days until cells reached 70% differentiation which was determined by qualitative visual measurement. Once 70% differentiated, differentiation media was removed, and subsequent experiments conducted as described below.

4.4 CELLULAR GROWTH UNDER LOW AND HIGH GLUCOSE CULTURE CONDITIONS

C2C12 cells at passage 7-16, stored in 90% FBS were thawed and plated in 10 mL of SGM on a 10cm^2 petri dish. Cells were then incubated at 37 °C and 40-60 % humidity with 5 % CO_2. After 24 hours, cell attachment was confirmed with a light microscope. Cells were maintained in SGM until 70-80% confluency was reached and then sub-cultured into 6-well plates at 5000 cells/cm^2. Cells (triplicate wells) were then exposed to either low glucose (1.0 g/L) or high glucose (4.5 g/L) media and allowed to adhere for 5 hours. Five random images per well were then taken at 10x magnification at the 0, 6, 24- and 48-hour time points post adherence. Image analysis using Image-J software (Version 1.52, NIH, MD, United States; nih.gov) was conducted to determine in vitro cellular growth rate and generate growth curves.

4.5 DOSE RESPONSE

A dose response within our study context refers to the relationship between the applied dose/concentration (the amount of a drug administered, purposely to cultured cells) and the effect that is observed (confluency and cellular survival rate) (Vandenberg, 2022).

Stock solutions of AAP (Sigma-Aldrich®, 49752-10G, St. Louis MO, USA) at a concentration of 2.4mM and NAC (Sigma-Aldrich®, A9165-100G, St. Louis MO, USA) at a concentration of 120mM were prepared in SGM and PBS respectively. However, both AAP and NAC stock solutions may be prepared in PBS as they are water soluble. Stock solutions were further diluted in SGM to create working concentrations for the dose response experiment. A stock solution of metformin (Sigma-Aldrich®, BCCC7544, UK) at a concentration of 2mg/mL was prepared in PBS and later diluted in SGM to the working concentration of 200ug/mL. All stock solutions were aliquoted into 2mL microfuge tubes and stored at -20°C. A fresh tube was thawed when conducting experiments to minimise the freeze-thawing.

The chosen concentrations and time intervals of this study are in line with existing literature as many toxicology studies employ a dose response to examine the safety and efficacy of a chosen drug on a representative cell line (Maartens et al., 2021, Mehrbani Azar et al., 2018). The *in vitro* lowest human equivalent (LHE) dose of metformin (200ug/mL) was calculated according using equations adapted from various literature (Levy, 2020, Nair et al., 2018, Nair and Jacob, 2016, Saadh et al., 2020). These equations consider the dietary human recommended dose intake (2550mg daily), lowest drug bioavailability (39%), human weight (60kg) and human blood volume (5L) to produce an *in vitro* equivalent dose.

C2C12 cells were cultured according to the subculturing methods in Section 4.3 and seeded at a seeding density of 5000 cells/cm^2. Cells were seeded into 36 wells of four 96-well plates

(Nest, SoCal BioMed, LLC, US) (one for each time point) in SGM and incubated overnight to adhere to the bottom of the wells. The following day, media was removed from all plates and the cells treated in triplicate with varying concentrations of AAP (0,075; 0,15; 0,3; 0,6; 1,2 mM), NAC (3.75; 7,5; 15; 30; 60 mM) and the human equivalent dose of metformin (200 µg/mL). SGM in triplicate was used as the positive control. Treatment media was changed on day 3 for both day 4 and 6 plates. The varying concentrations of AAP and NAC were treated alone and not in combination. This was done as we aimed to observe cellular confluency and survival rate in the single treatments under the assumption these cells should correspondingly survive in combination of the two optimised doses.

At each of the respective end points, the crystal violet (hexamethyl pararosaniline chloride) stain was used to assess cell viability. This assay is based on the principle that all cells attached to the plate are viable and all dead cells are detached and washed away and thus did not stain. Crystal violet is a triarylmethane dye that binds to ribose type molecules such as DNA in nuclei. Thus, the amount of Crystal violet staining in the assay is directly proportional to the cell biomass that is attached to the plate. This cell biomass was then used to infer levels of cell viability. For this experiment the number of viable cells per mm^2 was quantified for each of the time points.

At days 1, 2, 4 and 6 post treatment, media was removed from each well, cells washed with PBS and fixed with 150µL ice cold methanol (Sigma Aldrich, Merck chemicals (Pty) Ltd., RSAUS) for 30 seconds. C2C12 cells were then stained with Crystal violet working solution (0.01% v/v in distilled H_2O) (Sigma-Aldrich, Germany) for 5 min and washed twice with 150µL PBS. Five random images were taken of each well using a light microscope at 10x magnification. Images were analysed using Image J software according to the protocol described by Busschots et al. (2015). This method analyses images for confluence using Image J software resulting in an Area Fraction output which is a measure of confluence.

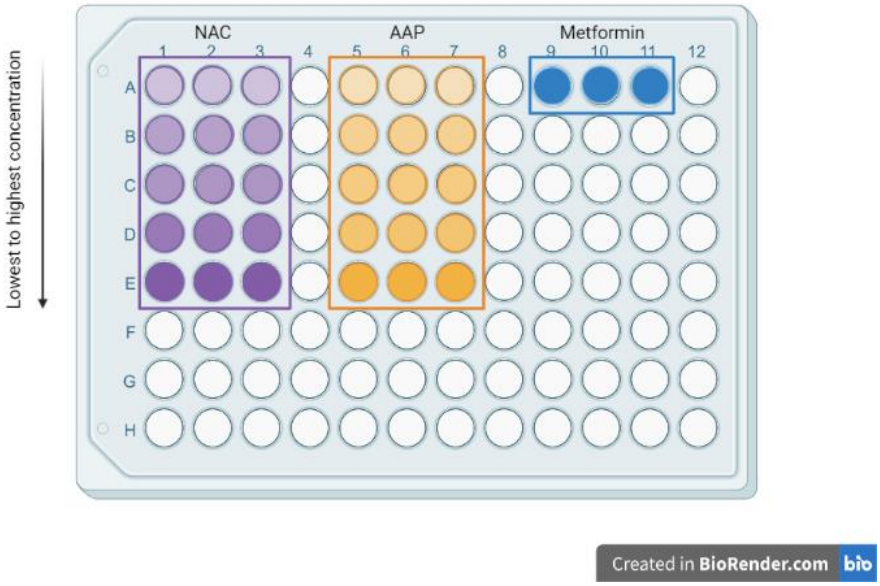


Figure 4.2: Plate layout of dose response experiment. Cells were treated in triplicate with varying concentrations of AAP (0,075; 0,15; 0,3; 0,6; 1,2 mM), NAC (3.75; 7,5; 15; 30; 60 mM) and the human equivalent dose of metformin (200 µg/mL). **Abbr:** NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate

4.6 DETERMINATION OF OXIDATIVE STRESS AND ANTIOXIDANT CAPACITY OF CELLS

C2C12 cells were cultured according to the above subculturing methods and seeded in 24-well plates at a seeding density of 5000 cells/cm^2. After desired confluency was achieved, cells were differentiated according to the above differentiation procedure described above. Once 70% differentiated, differentiation media was removed, the antioxidant pretreatments (described in table 4.1) were added to the cells and incubated at 37°C for 24 hours (time period commonly used in literature). Conditioned media (the cell culture supernatant) containing the secretome of the cells (consisting of proteins containing various enzymes, cytokines, growth factors and other soluble factors), was collected and stored at -80°C. The conditioned media was subsequently analysed to determine the levels of free radicals (ROS) and the total antioxidant capacity (TAC) of cells within the various treatment groups.

Table 4.1: Treatment conditions prepared to treat cells prior to ROS and TAC assay using conditioned media.

Single pretreatments	Combination pretreatments
AAP (0,6 mM)	AAP (0,6 mM) + NAC (3.75 mM)
NAC (3.75 mM)	AAP (0,6 mM) + metformin (200 µg/mL)
Metformin (200 µg/mL)	NAC (3.75 mM) + metformin (200 µg/mL)
	AAP (0,6 mM) + NAC (3.75 mM) + metformin (200 µg/mL)

Footnote: Differentiated were pretreated with antioxidant and metformin combinations described in the table above and incubated at 37°C overnight. The conditioned media was collected and stored at -80°C. The conditioned media

was subsequently used to determine the levels of free radicals (ROS) and the total antioxidant capacity (TAC) of cells. **Abbr:** NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate.

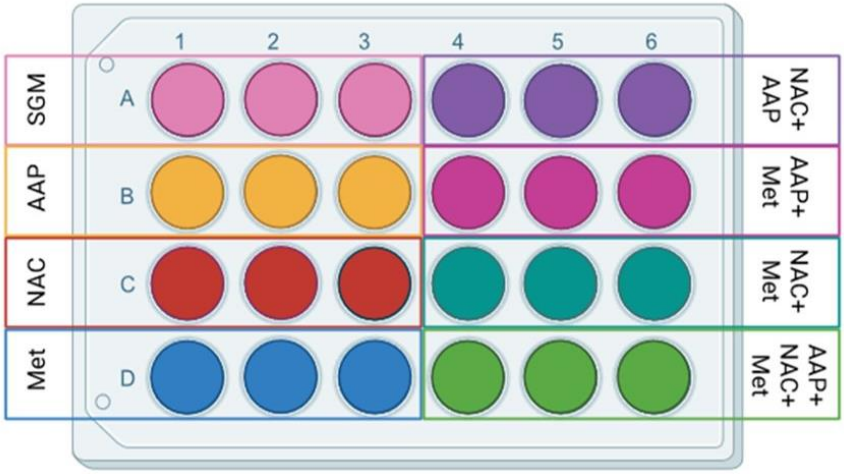


Figure 4.3: Plate layout of treatment conditions prepared to treat cells prior to ROS and TAC assay using conditioned media. Abbr: NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate; Met, metformin. Image created in BioRender.com

4.6.1 ROS assay

The total level of intracellular reactive oxygen species of the collected conditioned media within each treatment group was determined using a fluorometric assay (E-BC-K138-F, Elabscience®, US). The assay was performed according to the manufacturer's instructions adapting the protocol to be applied to conditioned media. All reagents used were supplied in the kit. 2,7-dichlorofuorescin diacetate (DCFH-DA) is a fluorescent probe that can freely cross the cell membrane. Once in the cell, DCHF-DA is hydrolysed by intracellular esterase to form dichlorofluorescin (DCFH). In the presence of ROS, DCFH is oxidized to dichlorofluorescein (DCF) which fluoresces a strong green and cannot penetrate the cell membrane, therefore DCF intensity is proportional to the level of intracellular reactive oxygen species.

Conditioned media samples from all treatment groups were placed on ice to thaw. 50µL of SGM and 50µL of 10mmol/L DCFH-DA working solution was added in duplicate to a 96-well plate. 50µL of SGM and 50µL of 10mM tert-Butyl hydroperoxide (TBHP) working solution was added in duplicate to the plate. SGM containing no added reagents or treatments was added in duplicate to the plate. Finally, 50µL 10mmol/L DCFH-DA working solution and 50µL of treated collected conditioned media (final concentration 20µL) was added to the plate. The plate was then incubated at 37°C for 30-60 minutes. The plate was removed from the incubator and absorbance values were read using a colourimeter plate reader (Multiskan™ FC, Thermo Fischer ™, US) at a wavelength of 525 nm.

ROS production of conditioned media was calculated in MFI (x fold change) using the average absorbance value of SGM as the standard to compare the other treatment conditions to, using the following ratio:

$$\frac{Absorbance\ value\ of\ treatment}{Avergae\ absorbance\ value\ of\ SGM}$$

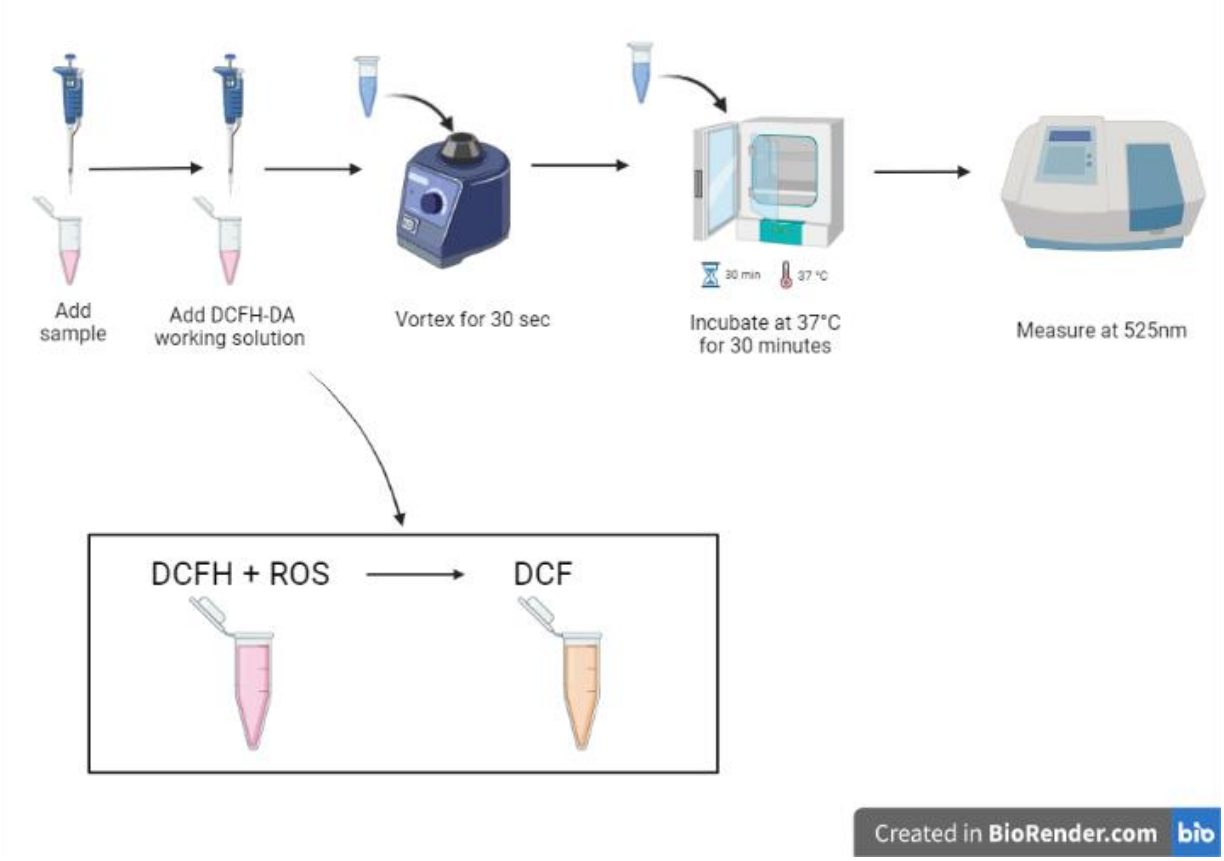


Figure 4.4: ROS assay study design of procedures followed. The beneficial effect(s) of combination therapy was determined by assessing reactive oxygen species (ROS) (fluorometric assay) within the culture supernatants with and without NAC/AAP treatment. **Abbr:** DCFH-DA, 2,7-dichlorofuorescin diacetate; DCFH, dichlorofluorescin; DCF, dichlorofluorescein; ROS, reactive oxygen species

4.6.2 TAC Assay

The total antioxidant capacity of the collected conditioned media within each treatment group was determined using a colorimetric assay (E-BC-K136-M, Elabscience®, US). The assay was performed according to the manufacturer's instructions. All reagents used were supplied in the kit.

A variety of antioxidant macromolecules, antioxidant molecules and enzymes in a system can eliminate all kinds of reactive oxygen species and prevent oxidative stress induced by reactive oxygen species. The total level reflects the total antioxidant capacity in the system. Many antioxidants in the body can reduce Fe^{3+} to Fe^{2+} and Fe^{2+} can form stable complexes with phenanthroline substance. The antioxidant capacity (T-AOC) can be calculated by measuring the absorbance at 520 nm.

Conditioned media samples from all pretreatment groups were placed on ice to thaw. Microcentrifuge tubes were labelled with the treatment names and a control tube was labelled for each treatment group. Buffer solution was added to all the tubes. Conditioned media samples were added to the respective sample tubes. Thereafter, chromogenic agent and ferric salt solution was added to both sample and control tubes. Samples were vortexed and placed in an incubator at 37°C for 30 min. After samples were incubated, stop solution was added. Finally, supernatant samples were added to the respective control tubes. The samples were vortexed and allowed to stand at room temperature (21°C) for 10 min. Before measuring the absorbance of the samples at 520nm , samples were pipetted into a 96 well plate in duplicate.

The Total Antioxidant capacity activity (U/mL) was determined by the following formula:

$$\frac{\text{Optical density of sample} - \text{Optical density of control}}{0.01} \div 30 \text{ min} \times \frac{\text{Total volume of reaction system (mL)}}{\text{The volume of sample (mL)}}$$

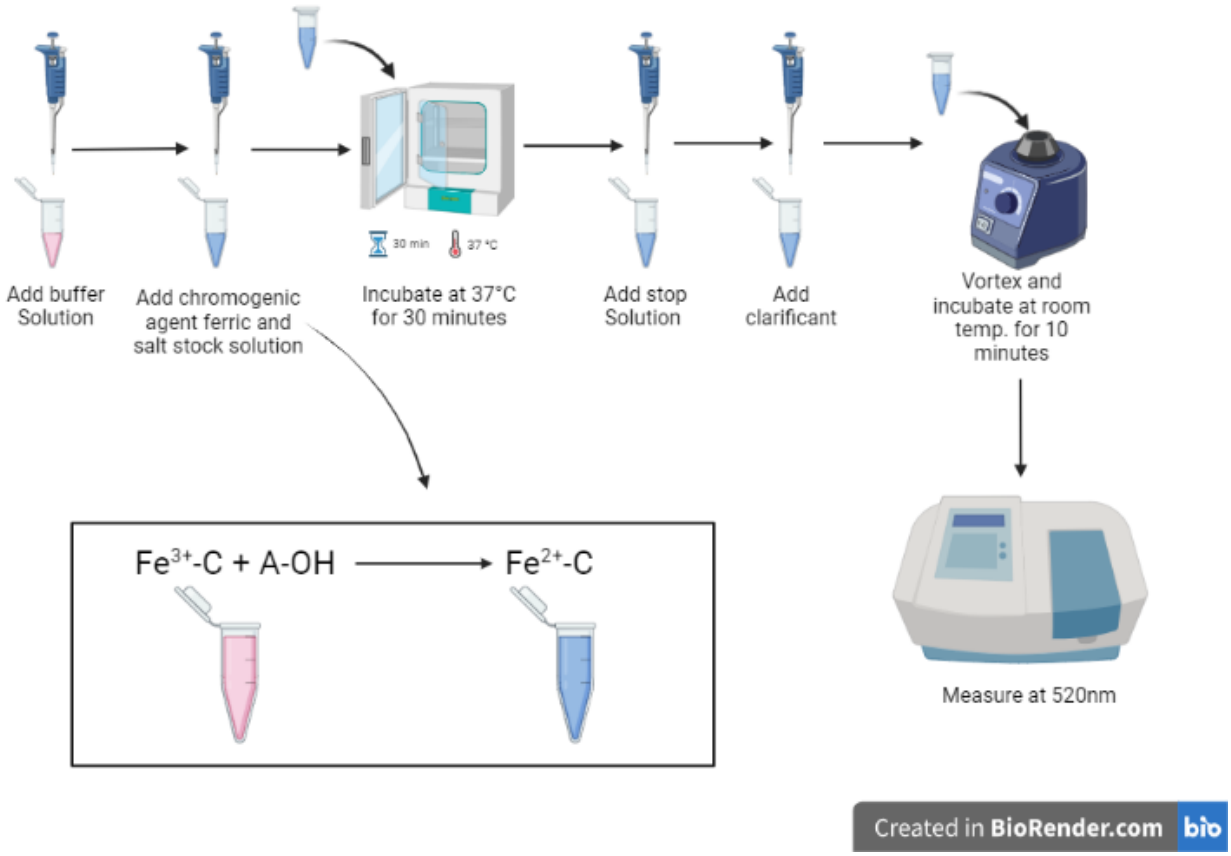


Figure 4.5: TAC assay study design of procedures followed. The beneficial effect(s) of combination therapy was also determined by assessing the Total Antioxidant Capacity (colorimetric assay within the culture supernatants with and without NAC/AAP treatment.

4.7 GLUCOSE UPTAKE ASSAY OPTIMISATION

The 2-[N-[7-Nitrobenz-2-oxa-1,3-diazol-4-yl] Amino]-2-Deoxyglucose (2-NBDG) based assay is a widely used approach in various cells including skeletal muscle to assess glucose uptake. 2-NBDG which is a glucose analogue, is transported into the cells and phosphorylated by hexokinase to 2-NBDG 6-phosphate (2-NBDG6P). 2-NBDG6P cannot be further metabolized and accumulates in cells. This accumulation is then directly proportional to the glucose uptake by cells. In this assay, 2-NBDG uptake is determined by a coupled enzymatic assay in which 2-NBDG6P is oxidized, resulting in the generation of NADPH, which reacts with the probe to generate a fluorometric product, proportional to the 2-NBDG taken up by the cell at a wavelength of 535-587 nm. Additionally, 2-NBDG is light sensitive thus, all laboratory work using 2-NBDG such as adding 2-NBDG into 8-well chamber was conducted in low light and additionally covered immediately with foil.

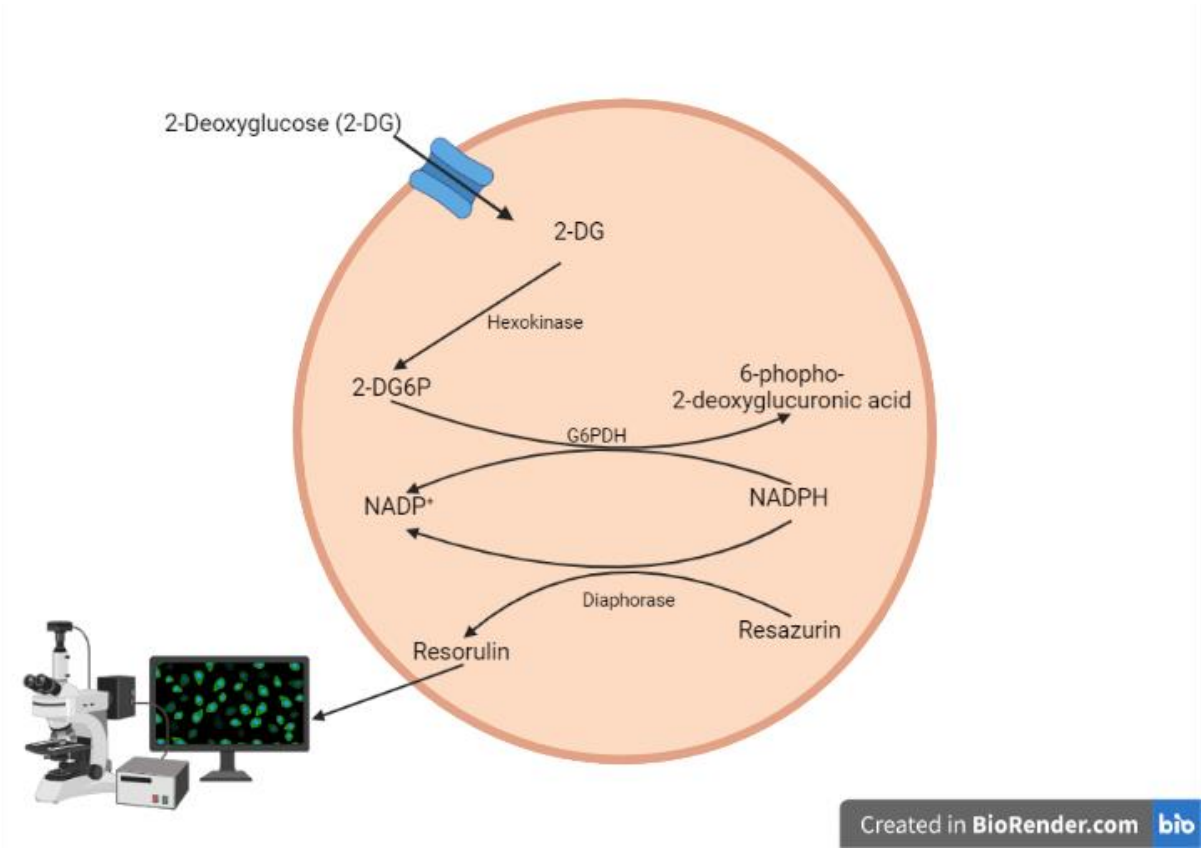


Figure 4.6: Glucose uptake assay mechanism of action. 2-NBDG, which is taken up by cells and phosphorylated by hexokinase to 2-NBDG6P. 2-NBDG6P cannot be further metabolized and accumulates in cells. This is then directly proportional to the glucose uptake by cells. **Abbr:** G6PDH, glucose-6-phosphate dehydrogenase; NADPH/NADP, nicotinamide adenine dinucleotide phosphate.

However, literature provides various protocols that possess large variability in the study design which impacts the overall data reproducibility. Additionally, glucose starvation of cells using glucose/serum free media, which is one of the common prerequisite conditions for glucose uptake assay, induces stress during longer preincubation periods and alters muscle cell

metabolism and morphology (Bala et al., 2021). Therefore, using C2C12 skeletal myoblasts, we investigated the conditions such as concentration of 2-NBGG, pre-incubation time period, insulin pre-incubation time period and concentration of glucose while maintaining the cultured myoblasts in a morphologically healthy state. The outcome of these optimisation experiments is described below.

4.7.1 Determination of optimal 2-NBDG concentration for glucose uptake measurement

Zou et al. (2005) showed that fast 2-NBDG uptake by L6 rat skeletal muscle cells at concentration as low as 5μM generated a significant signal-to-noise ratio that could be measured. In order to optimize 2-NBDG (Thermo Fisher Scientific, USA) concentration for glucose uptake measurement in the current study, undifferentiated cells were incubated with 10μM and 50μM of 2-NBDG for 2 hours, respectively.

Glucose uptake was assessed (mean fluorescent intensity (MFI)) using fluorescent microscopy under various conditions using the 2-NBDG assay with or without metformin treatment (200μg/mL). Figure 4.4 shows the results of the different concentrations (10μM and 50μM) of 2-NBDG tested. Cells treated with metformin using a concentration of 50μM 2-NBDG (MFI= 6.471 ± 1.037) had significantly higher levels of glucose uptake compared to cells without metformin with the same concentration of *2*-NBDG (MFI= 2.834 ± 0.739) ($p<0.01$). Based on our optimisation results, 50μM yielded a brighter signal. Thus, the concentration of 2-NBDG at 50μM was chosen for all subsequent experiments. Additionally, based on this the cells treated with metformin in the presence of 50uM 2-NBDG significantly increased glucose uptake.

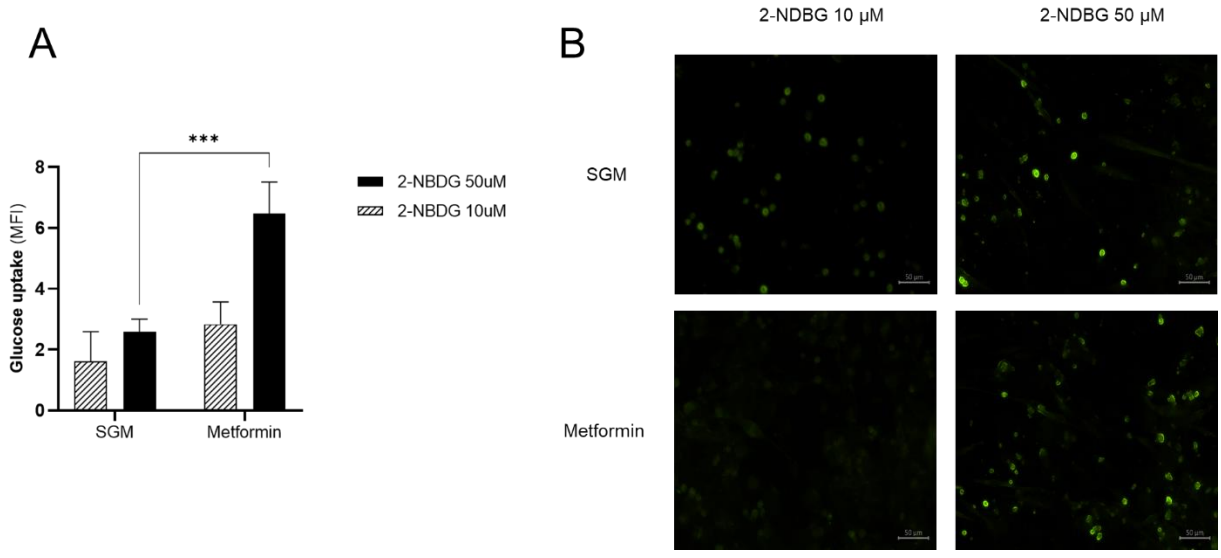


Figure 4.7: Determination of optimal 2-NBDG concentration for glucose uptake measurement A: Cells that received metformin with 50μM 2-NBDG showed a significant increase in glucose uptake when compared to cells

that received without metformin with 50µM 2-NBGG. **B:** Representative images indicating the fluorescent intensity differences between cells cultured in the different concentrations of 2-NBDG. **Scale bar,** 50µm **Statistical analysis:** two-way ANOVA with Šídák's post hoc test. ***p<0.01 was considered significant.

4.7.2 Determination of optimal media conditions for glucose starvation prior to 2-NBDG uptake

Serum and glucose starvation, a common prerequisite condition for the glucose uptake assay could be the main reason for inducing extreme alterations in the muscle cell physiology (Kawai et al., 2015, Mirza et al., 2014). Therefore, the study design for the 2-NBDG glucose uptake assay was optimised to maintaining the cultured myoblasts in a morphologically healthy state. The differentiated cells were divided into two groups and glucose staved for 2-5 hours in different media conditions (labelled as media I and media II). Group I cells were glucose starved in media I which consisted of live cell imaging solution (1X) (Thermo Fisher Scientific, USA) which is both FBS and glucose free. Group II cells were glucose starved in media II which consisted of FBS-free and low glucose media (1g/L Glucose DMEM). After glucose starvation, each group was treated with a combination of insulin (100 nM) and 2-NBDG (50 µM) for 2 hours and the uptake of glucose assessed using fluorescent microscopy. Figure 4.5 shows the study design followed and the representative images of the different media conditions tested for glucose starvation. Since this was part of optimisation, the experiment was only performed once (n=1) and thus not quantified. Based on morphology, cells appeared unhealthy (rounded and flat) after complete glucose starvation in FBS and glucose free media (Group I) compared to cells which were glucose starved in low glucose media (Group II). Thus, the FBS-free and low glucose media (1g/L Glucose DMEM) was chosen to be used in all further experimentation.

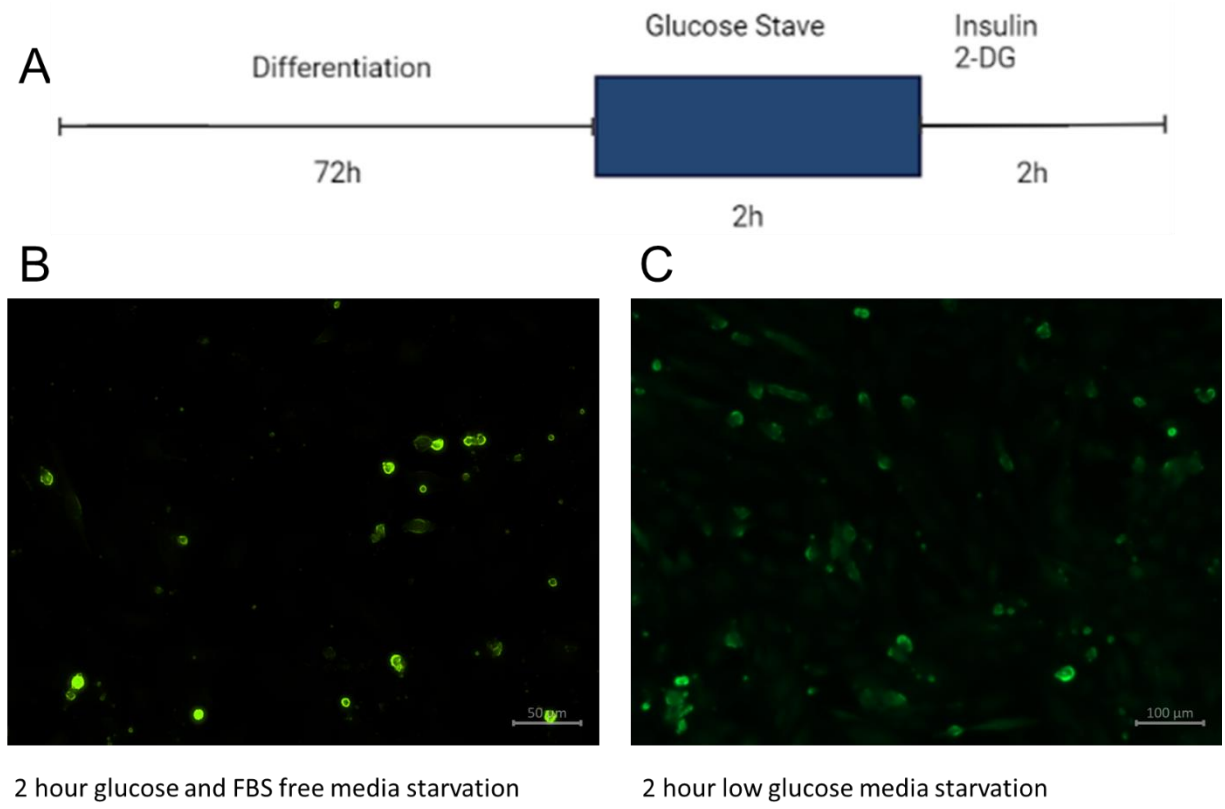


Figure 4.8: Determination of optimal media conditions for glucose starvation prior to 2-NBDG uptake. A: Study design followed to optimize 2-NBDG uptake for glucose uptake measurement while maintaining the cultured myoblasts in a morphologically healthy state **B & C:** Representative images indicating the morphological and fluorescent intensity differences between cells cultured in FBS-free and glucose media (B) and FBS-free and low glucose media (C). (B) morphologically cells appeared unhealthy (rounded and flat) after glucose starved with FBS and glucose free media; (C) morphologically cells appeared healthy after glucose starved low glucose media. **Scale bar**, (B) 50µm; (C) 100µm

4.7.3 Determination of optimal insulin pre-incubation for the 2-NBDG uptake assay

Various different approaches have been used in literature. Zou et al. (2005) exposed L6 rat skeletal muscle cells to 2-DG (10 µM) together with Insulin (unknown concentration), for 1 hour whereas Bala et al. (2021), treated C2C12 myotubes with insulin (100 nM) for 30 minutes and then added 2-DG (60µM) for 1 hour. To optimize 2-NBDG uptake in this study, healthy differentiated cells that were glucose starved in FBS-free and low glucose media were divided into two groups (group I and II). Following glucose starvation, Group I cells were exposed to a combination of 2-NBDG (50µM) and insulin (100nM) for 2 hours prior to assessing glucose uptake based on fluorescence intensity. Group II on the other hand, were pretreated with insulin (100nM) for 30 minutes before addition of the 2-NBDG (50µM) and subsequently incubated for 2 hours. Higher glucose uptake (fluorescence intensity) was observed in cells that received insulin pretreatment for 30 minutes prior to 2-NBDG incubation compared to cells that were treated with a combination of insulin and 2-NBDG immediately post glucose starvation. Refer to Figure 4.6 below for an overview of the study design and representative

microscopy images. Thus, for all further experiments, if treatments required insulin, it was incubated for 30 minutes before the addition of either drug treatments and 2-NBDG.

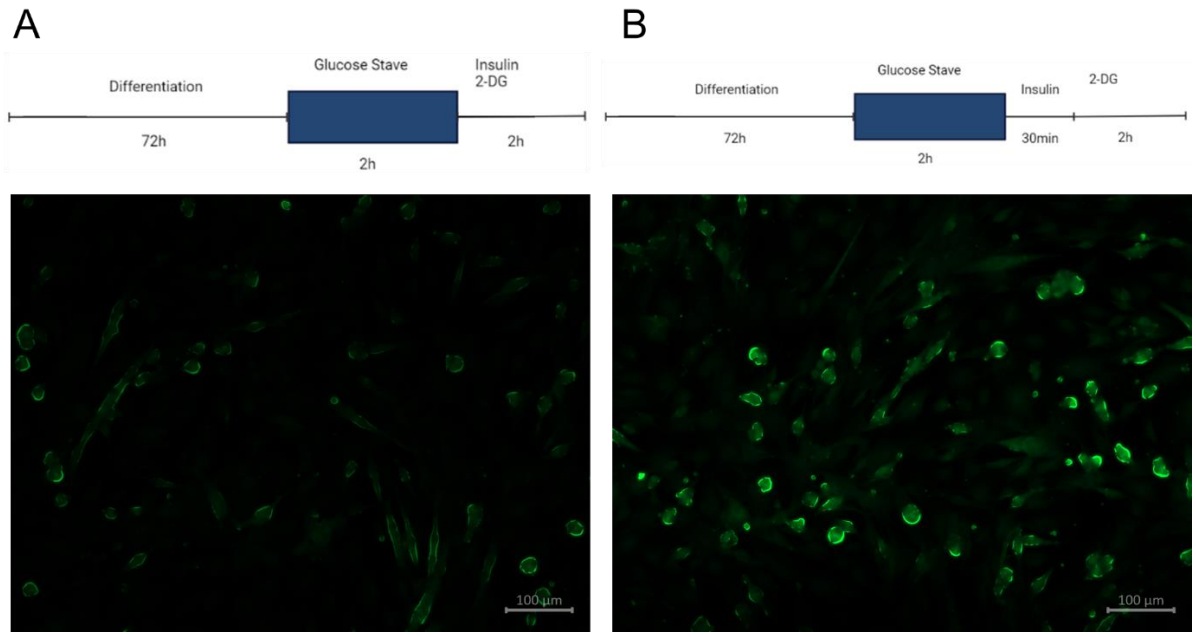


Figure 4.9: Determination of optimal insulin pre-incubation for the 2- NBDG uptake assay. A: study design and representative image of Group I cells. **B:** study design and representative image of Group II cells. It was observed that fluorescence intensity increased in cells that received insulin treatment for 30 minutes compared to cells that were treated directly with 2-NBDG. **Scale bar**, 100µm

4.7.4 Determination of optimal incubation period for 2-NBDG uptake

In literature, various different time frames for incubation with 2-NBDG have also been used. Zou et al. (2005), determined optimal staining time in L6 rat skeletal muscle cells by adding 10µM 2-NBDG and incubating it for 0, 15, 30, 60, 120 or 180 minutes, with longer durations showing increased glucose uptake. Thus, in order to optimize the time frame for the 2-NBDG glucose uptake assay in this study, healthy differentiated cells were divided into two groups (group I and II). Both groups were glucose starved in FBS-free and low glucose media and pretreated with insulin (100nM) for 30 minutes prior to the addition of 2-NBDG. Group I cells were incubated with 2-NBDG (50µM) for a period of 2 hours and group II cells were incubated with 2-NBDG (50µM) overnight (8-15 hours) prior to assessing fluorescent intensity. Based on our experimental results, glucose uptake was similar with both pre-incubation periods. Thus, the 2-hour pre-incubation period was chosen for all further experimentation (data not shown).

4.7.5 Optimised Glucose Uptake Assay: Antioxidant treatments

To test our hypothesis, 2-NBDG uptake was performed to assess changes in glucose uptake in the various treatment groups using the optimised conditions.

C2C12 cells were cultured according to the above subculturing methods and seeded into an 8-well chamber at a seeding density of 5000 cells/cm^2. Once desired confluence was achieved, cells were differentiated to 70% (frequently adopted in literature to simulate a human biological system), differentiation media was removed and the cells pre-treated with either NAC and/or AAP (concentrations determined by dose response assay). Following pre-treatment media was removed and cells maintained in FBS-free and low glucose media (1g/L Glucose DMEM) for 2 hours. This step enhances glucose uptake pattern by reducing the influence of some growth factors and insulinomimetic action in FBS. There after cells the 2-NBDG glucose uptake assay was performed under various conditions (Insulin, metformin) as described below.

After incubation was completed, media was removed and cells fixed with fluorescent mounting media (Sigma-Aldrich, Germany) in a dark room. Five random images were taken immediately using a florescent microscope (Zeiss Axio Observer 7 Inverted Microscope, Zeiss, Germany) at 10x magnification at 465/540nm. Exposure and microscope settings were kept consistent throughout the study. Images were then analysed using ZEN blue software (version 3.6, Carl Zeiss Microscopy, Germany) and Image J software.

The treatment conditions were as follows:

- **Control I (only glucose starvation):** Once glucose starvation was complete, media was removed and 2-NBDG (50µM) prepared in SGM was added and incubated for an additional 2 hours. Once incubation was completed, media was removed, cells fixed with fluorescent mounting media in a dark room and images taken immediately in florescent microscope using conditions mentioned above.
- **Control II (Insulin pretreatment only):** Once glucose starvation was complete media was removed and insulin (100nM) was added to cells in SGM and allowed to incubate for 30 minutes. 2-NBDG (50µM) prepared in SGM was then added and incubated for an additional 2 hours. Once incubation was completed , media was removed, cells fixed with fluorescent mounting media in a dark room and Images were taken immediately in florescent microscope using conditions mentioned above.
- **Antioxidant Pretreatments:** Pretreatments prepared in SGM were added at the optimal concentration of AAP (0,6mM) and NAC (3.75mM) both as single doses and a combination doses. Cells were then incubated at 37°C for 24 hours. Media was removed and cells maintained in FBS-free and low glucose media for 2 hours. If treatments required insulin, insulin (100nM) was added and incubated for 30 minutes before 2-NBDG and metformin were added. This media was then removed and 2-NBDG (50µM) and metformin (200 µg/mL) prepared in SGM were added. The plate was covered in foil and incubated at 37°C for 2 hours. Cells were then retrieved from the incubator, media removed and cells fixed using mounting media. Images were taken immediately in florescent microscope using conditions mentioned above.

Refer to Figures 4.7 and 4.8 below for an overview of the study design and the various pretreatment conditions used.

Pretreatments	
AAP (0,6 mM)	
NAC (3.75 mM)	
AAP (0,6 mM) + NAC (3.75 mM)	

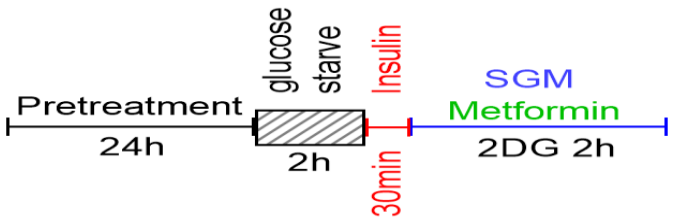


Figure 4.10: Study design and various pretreatment conditions. Abbr: NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate; 2-DG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose)

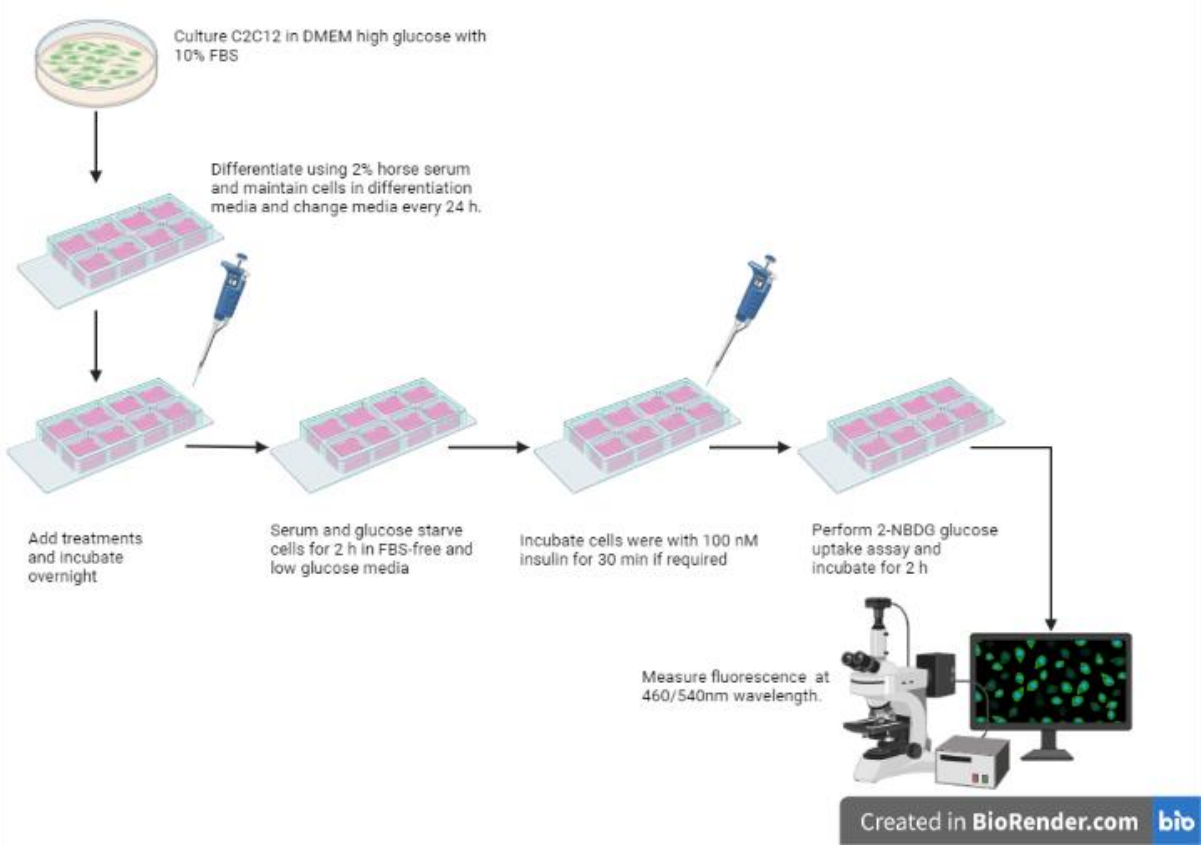


Figure 4.11: Glucose uptake assay study design of procedures followed. Glucose uptake was assessed using fluorescent microscopy and the 2-NBDG assay under various conditions (Insulin, Metformin) following pretreatment with either NAC and/or AAP. **Abbr:** DMEM, Dulbecco's modified Eagle medium; FBS, foetal bovine serum; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose).

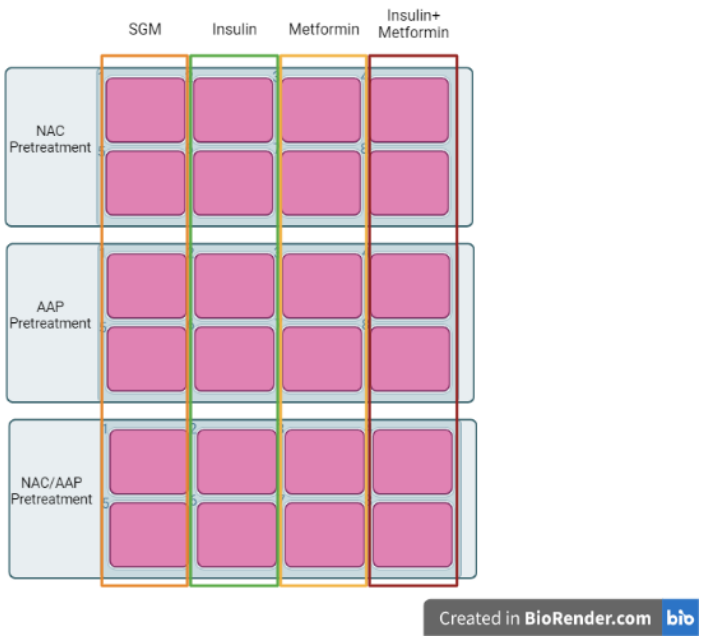


Figure 4.12: Plate layout of glucose uptake assay with pretreatment groups. Pretreatments were added at optimal concentration (AAP (0,6 mM); NAC (3.75 mM)) prepared in SGM and incubated at 37°C for 24 hours. **Abbr:** NAC, N-acetylcysteine; AAP, ascorbic acid-2 phosphate.

4.8 STATISTICAL ANALYSIS

All experiments were performed using three biological repeats (n=3) with two or three internal technical repeats as specified in above methodology. The data is presented as mean ± standard deviation (mean±SD). Statistical analysis was performed using Prism-Graph Pad software (Version 9, Domantics). A one-way ANOVA with Tukey post hoc test or a two-way ANOVA with Šídák's post hoc test was used to compare treatment groups. Repeated measures ANOVA with Tukey post hoc test was used to determine the effect of treatment, time and treatment x time. Statistical significance was accepted at $p<0.05$.

5 RESULTS

5.1 CELLULAR GROWTH UNDER LOW AND HIGH GLUCOSE CULTURE CONDITIONS

C2C12 skeletal myoblasts were treated in triplicate with low glucose (LG) (1.0 g/L) and high glucose (HG) (4.5 g/L) media. In vitro cellular growth rate and characterisation of cell morphology was determined. Images of cells, taken at 10x magnification using a light microscope were morphologically assessed using ImageJ software. Healthy C2C12 cells were characterised as mononucleated having radial branching morphology consisting of long fibres extending in many directions unhealthy (potential apoptotic and/or non-viable) were characterised by their flattened and rounded structure (Fig. 5.1). Cells cultured in high glucose conditions showed normal morphology, whereas cells cultured in low glucose conditions exhibited shrinking and lifting from the plate indicative of cellular death. The low glucose conditions showed less connections between cells and a decreased number of viable cells. Refer to Figure 5.1 for representative light microscopy images illustrating cell morphology over time.

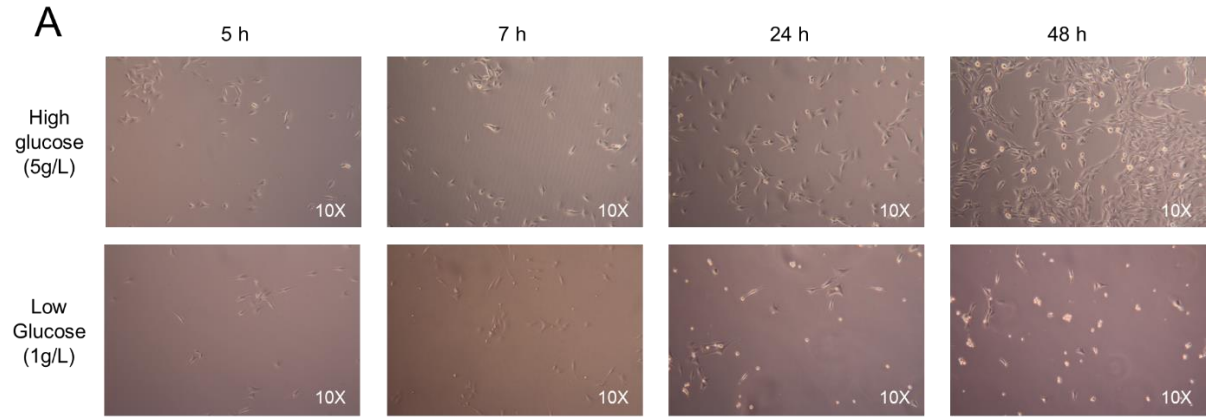


Figure 5.1: Cell morphology in high and low glucose conditions over time. Cells cultured in high glucose conditions showed a higher cell number of healthy cells compared to cells cultured in low glucose conditions. C2C12 cells cultured in low glucose conditions was markedly inhibited. Cells exhibited increased shrinking and lifting from the plate

Although not significant, quantification of cell number indicated a normal growth curve under high glucose conditions with cell number increasing 4 fold over the 48 hour period (Fig. 5.2). Under low glucose conditions, there was a significant decrease ($p<0.5$) in cell number between the 7h (4.65 ± 0.92 cells/10mm^2) and 48h period (2.49 ± 0.71 cells/10mm^2). Over the 48- hour period, cells cultured in high glucose conditions displayed a significantly ($p<0,0001$) higher cell number per 10mm^2 plate surface area when compared to cells cultured in low glucose conditions (48h time point: HG=18.88 ± 4.66 cells/10mm^2; LG=2.49 ± 0.71 cells/10mm^2).

Suggesting that low glucose levels do not support cellular growth over time and that high glucose is thus a requirement for standard growth media conditions.

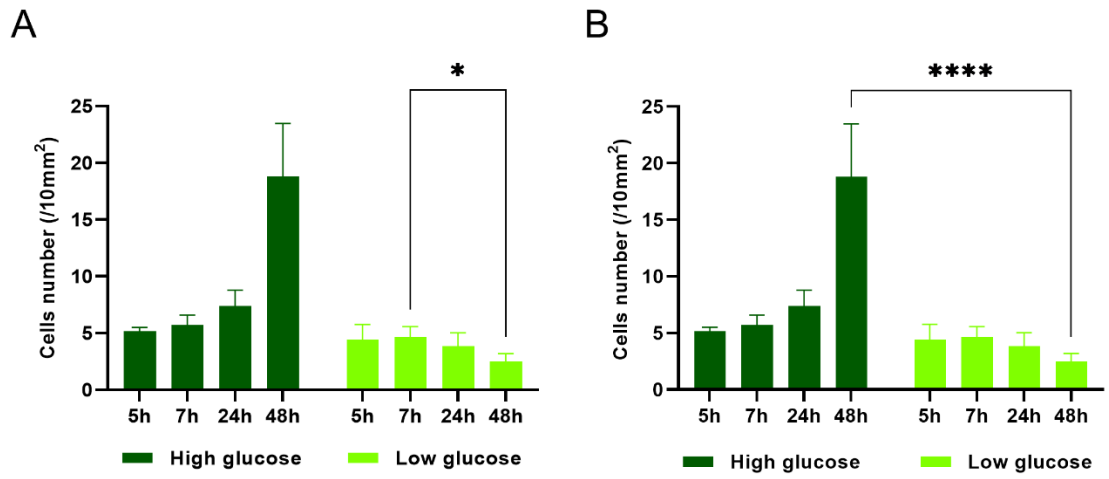


Figure 5.2: The effect of high and low glucose conditions on cell number. A: Cell number increased by a factor of 4 over the 48-hour period in high glucose conditions. Under low glucose conditions, there was a significant decrease in cell number between the 7 hour and 48-hour period. **B:** Over the 48-hour period, cells cultured in high glucose conditions displayed a significantly higher cell number per 10mm2 plate surface area when compared to cells cultured in low glucose conditions. **Statistical analysis:** two-way ANOVA with Šídák's post hoc test. *p<0.05 **** p< 0.0001 was considered significant.

5.2 OPTIMAL AND NON-TOXIC DOSE RESPONSE CONDITIONS

Prior to the assessment of drug interaction, optimal and non-toxic concentrations of antioxidants, NAC and AAP that support cell viability was determine via a dose response experiment. Confluency and survival rates were calculated to determine cell viability. Confluency refers to the percentage of the adhesive surface of the culture vessel that is covered with cultured cells. This is an indication of cell viability and proliferation as they continue to cover the plate it indicates that they healthy and functioning and not being inhibited by the drug concentration. Survival rate (% Survival) refers to the percentage of cells that have survived when compared to SGM (represented by the dotted line in bar graphs).

Compared to control (SGM) of all the concentrations tested, the high concentration of 0.6mM AAP had the most significant effect ($p<0.5$) on the proliferation rate of C2C12s over a period of 6 days (100%< confluency and 99.7 ± 13.4% survival rate) (Fig. 5.3D). This evident in Figure 5.4, where cells can be seen clumped together as a result of high cell yield. Although all concentrations of AAP supported the growth and proliferation of cells, based on these results, the optimal non-toxic concentration that promoted cell proliferation and survival was 0.6mM AAP and was used for subsequent experimentation. In contrast, compared to control group, the low concentration of 3.75mM NAC had the most significant effect on cell survival and growth (100%< confluency and 68.0 ± 28.7% survival rate) (Fig. 5.4A). Cell growth steadily

increased, at NAC concentrations of 3.75 and 7.5 mM before media was refreshed on day 3. Following media change, cellular proliferation began to change on day 4 for 7.5mM and 15mM while in control, cell growth continued to increase. Concentrations of 30 and 60mM NAC did not support cell growth as cell number continued to significantly decline at day 2 onwards. Whereas 3.75mM NAC, even at day 4 (100% confluency and 66.2 ± 17.5% survival rate), cells increased in number and followed a similar confluency trend as the control. Morphology of cells started to change as NAC concentration increase, from the standard spindle shape (3.75mM) to pointed jagged ends (7.5 – 15Mm) until cells could no longer survive and lifted off the plate (60Mm) (Fig. 5.6). Cellular survival rate was significantly lower ($p<0.5$) on day 6 in NAC concentrations 7.5, 15, 30 mM when compared to control. Overall, the optimal and non-toxic concentration that supported cell growth was determined to be 3.75mM NAC and was used for subsequent experimentation. The lowest human equivalent dose of metformin (200µg/mL) was observed to support the growth and proliferation of cells and at this concentration cells increased in number and followed a similar confluency trend as the control over the 6-day period (day 1: 67.40% confluency; day 6: 100%< confluency) (Figure 5.7). At this concentration survival rate of cells did not significantly decrease over the 6 day period (day 1: 100% survival rate; day 6: 78.9 ± 20.8% survival rate) and thus it was sufficient enough to continue experimentation. Figure 5.2.7 displays the confluency trends of chosen optimal concentrations of NAC (3.75mM) and AAP (0.6mM) as well as lowest human equivalent dose of metformin (200µg/mL) compared to standard conditions (SGM).

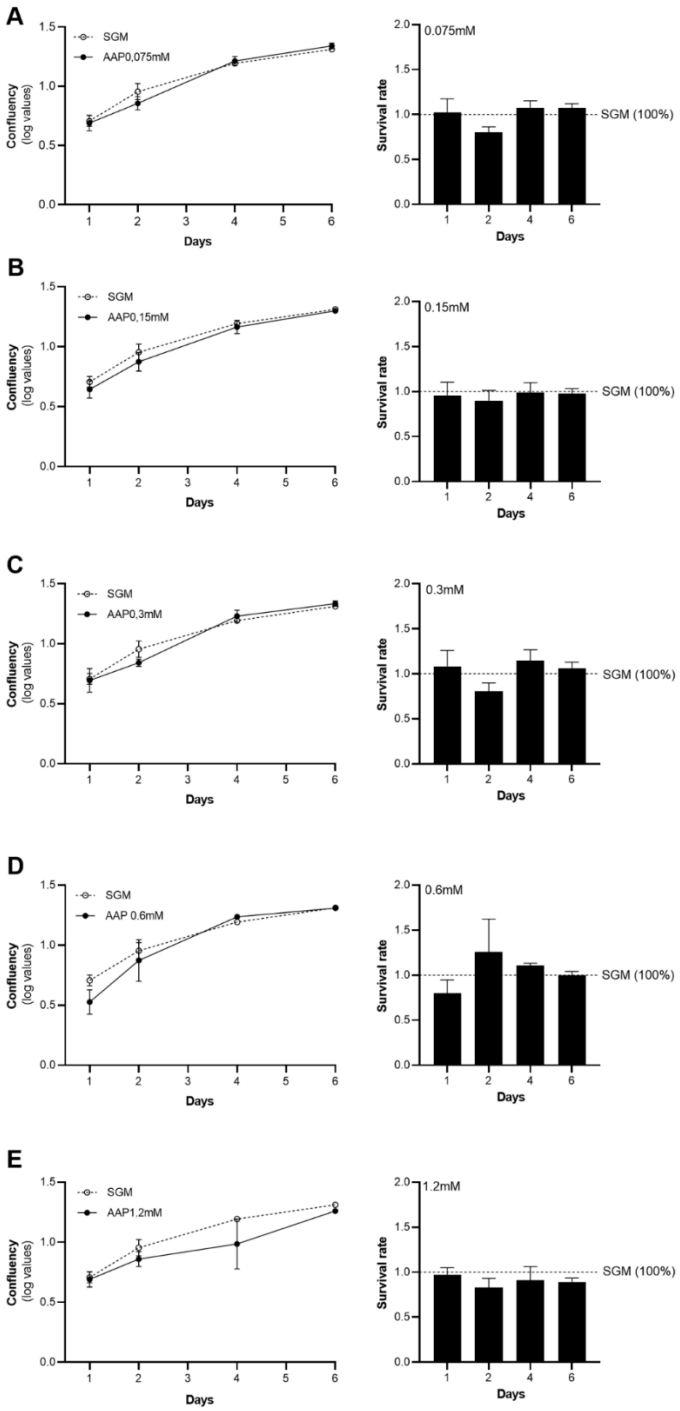


Figure 5.3: Confluency and survival rates of C2C12 cells under different concentrations of AAP. A: 0.075mM AAP confluency and survival rate over 6-day dose response period. Confluency and cellular survival rate followed a similar trend as the control over the 6-day period. **B:** 0.15mM AAP confluency and survival rate over 6-day dose response period. Confluency and cellular survival rate followed a similar trend as the control over the 6-day period. **C: 0**.3mM AAP confluency and survival rate over 6-day dose response period Confluency and cellular survival rate followed a similar trend as the control over the 6-day period. **D:** 0.6mM AAP confluency and survival rate over 6-day dose response period. Of all the concentrations tested, 0.6mM AAP had the most significant effect on the proliferation rate of C2C12s over a period of 6 days confluency and survival rate. **E:** 1.2mM AAP confluency and survival rate over 6-day dose response period. Confluency and cellular survival rate followed a similar trend as the control over the 6-day period. **Statistical analysis:** two-way ANOVA with Šídák's post hoc test.

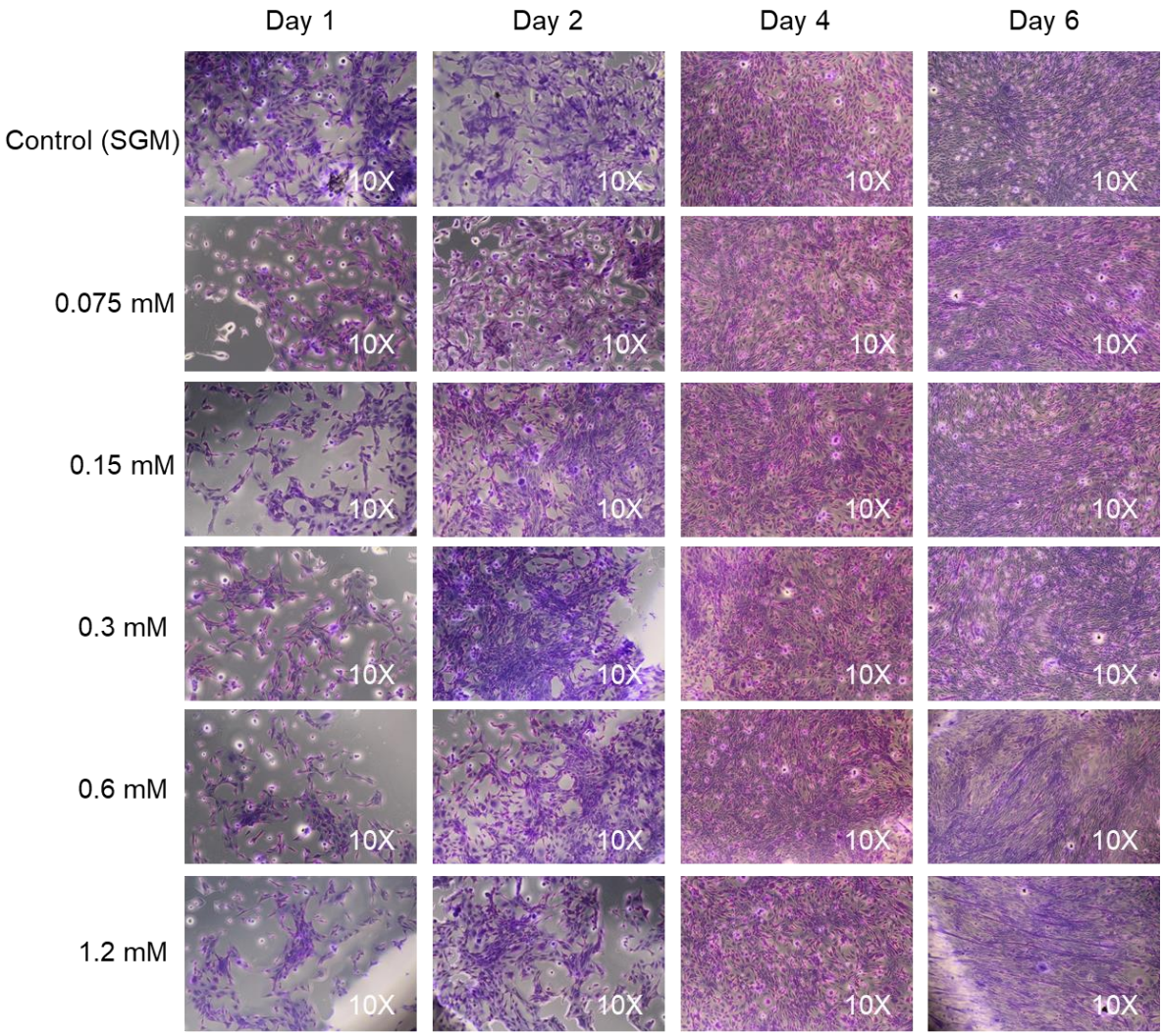


Figure 5.4: Cellular growth and proliferation of C2C12 cells under different concentrations of AAP. All concentrations of AAP supported the growth and proliferation of cells. This is evident as cells can been seen clumped together as a result of high cell yield.

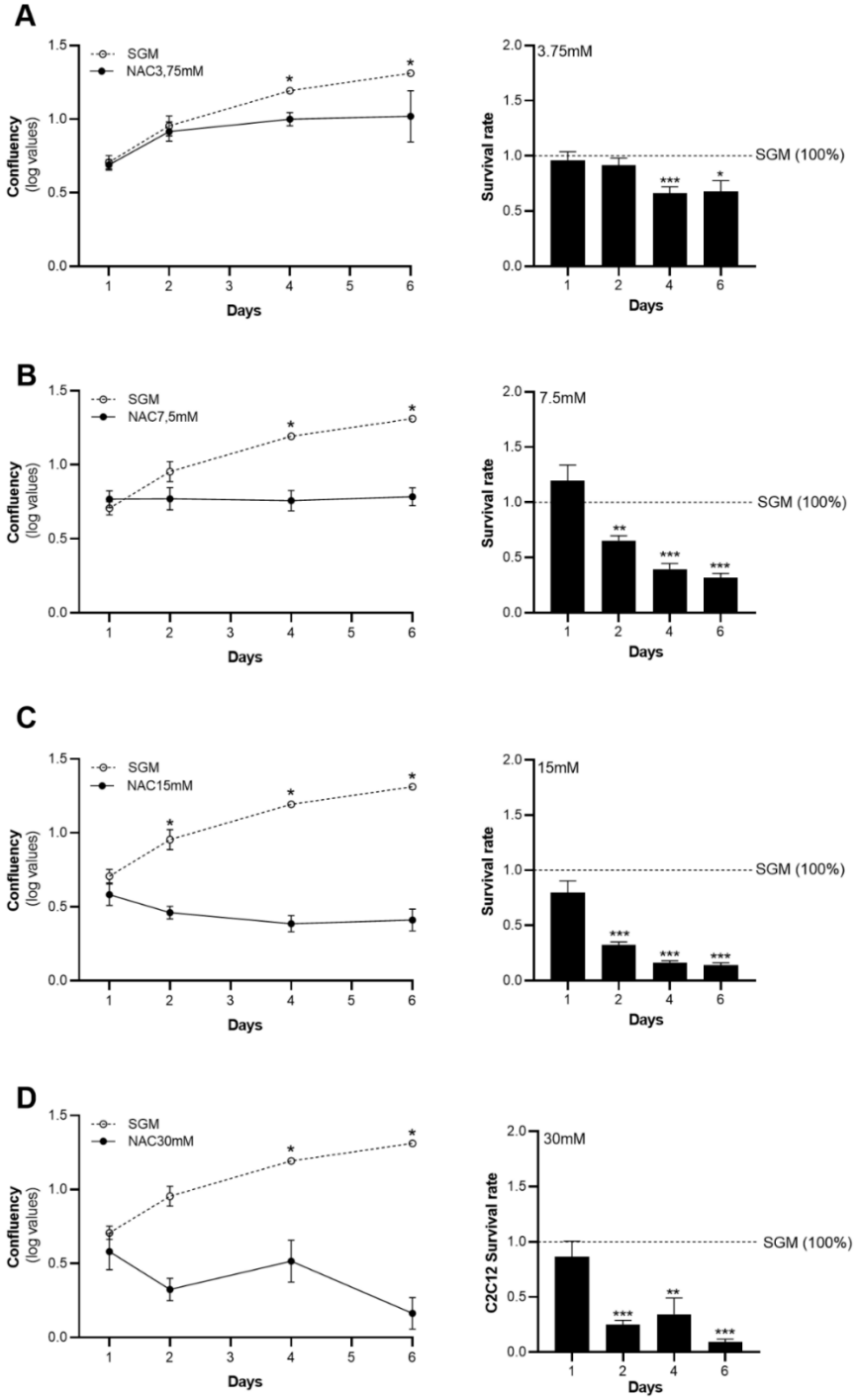


Figure 5.5: Confluency and survival rates of C2C12 cells under different concentrations of NAC. A: 3.75mM NAC confluency and survival rate over 6-day dose response period. At day 4 (cells increased in number and followed a similar confluency trend as the control over the 6-day period. **B:** 7.5mM NAC confluency and survival rate over 6-day dose response period. Following media change, cellular proliferation began to change on day 4 while in control, cell growth continued to increase. Confluency and cellular survival rate were significantly lower on day 6 when compared to control. **C:** 15mM NAC confluency and survival rate over 6-day dose response period. Following media change, cellular proliferation began to change on day 4 while in control, cell growth continued to increase. Confluency and cellular survival rate were significantly lower on day 6 when compared to control. **D:** 30mM NAC confluency and survival rate over 6-day dose response period. 30mM NAC did not support cell growth as cell number continued to significantly decline. Confluency and cellular survival rate were significantly lower on day 6 when compared to control. **Statistical analysis:** two-way ANOVA with Šídák's post hoc test. *p<0.05; **p<0.01; ***p< 0.0001 was considered significant.

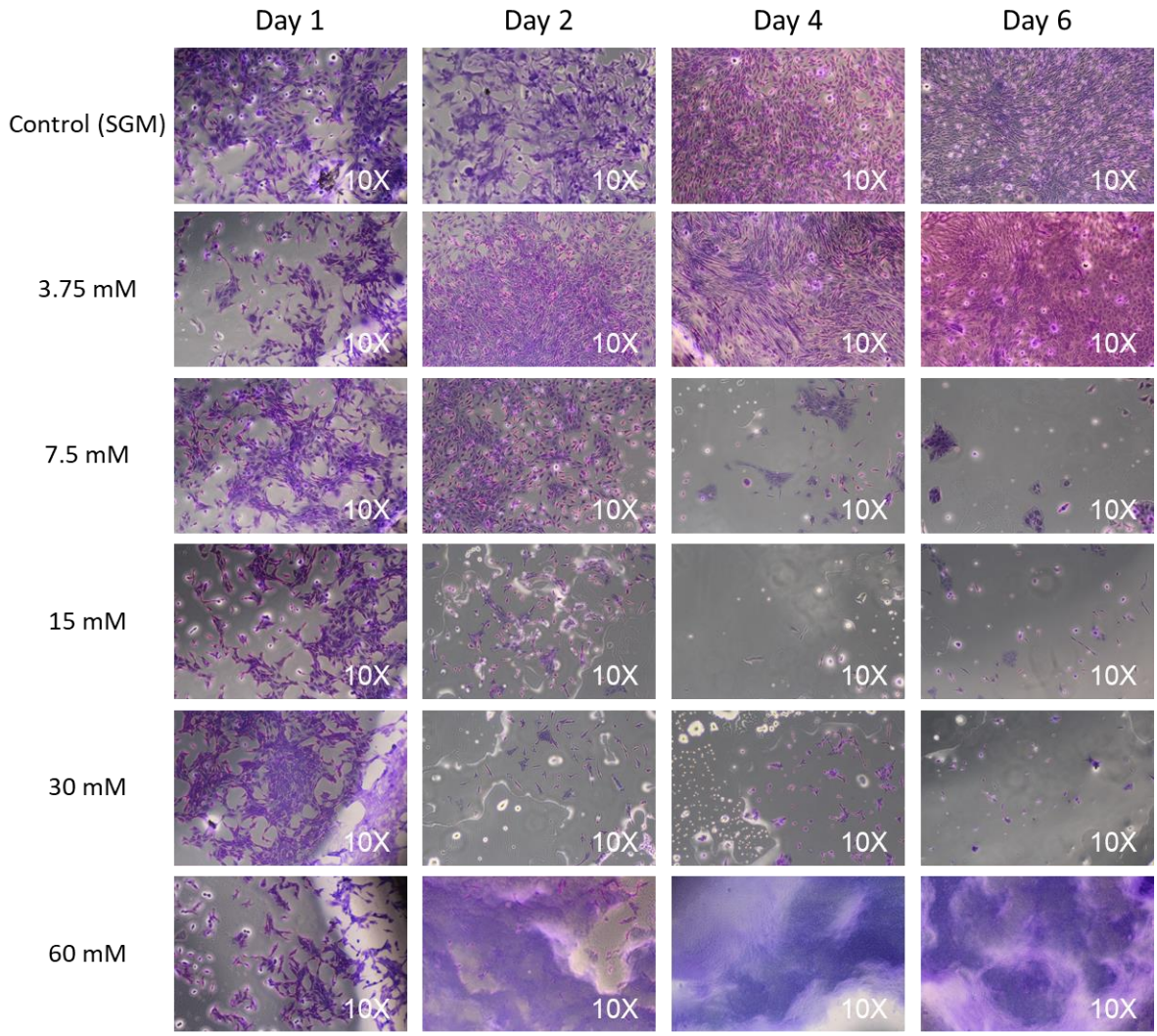


Figure 5.6: Cellular growth and proliferation of C2C12 cells under different concentrations of NAC. Morphology of cells started to change as NAC concentration increase, from the standard spindle shape (3.75mM) to pointed jagged ends (7.5 – 15Mm) until cells could no longer survive and lifted off the plate (60Mm).

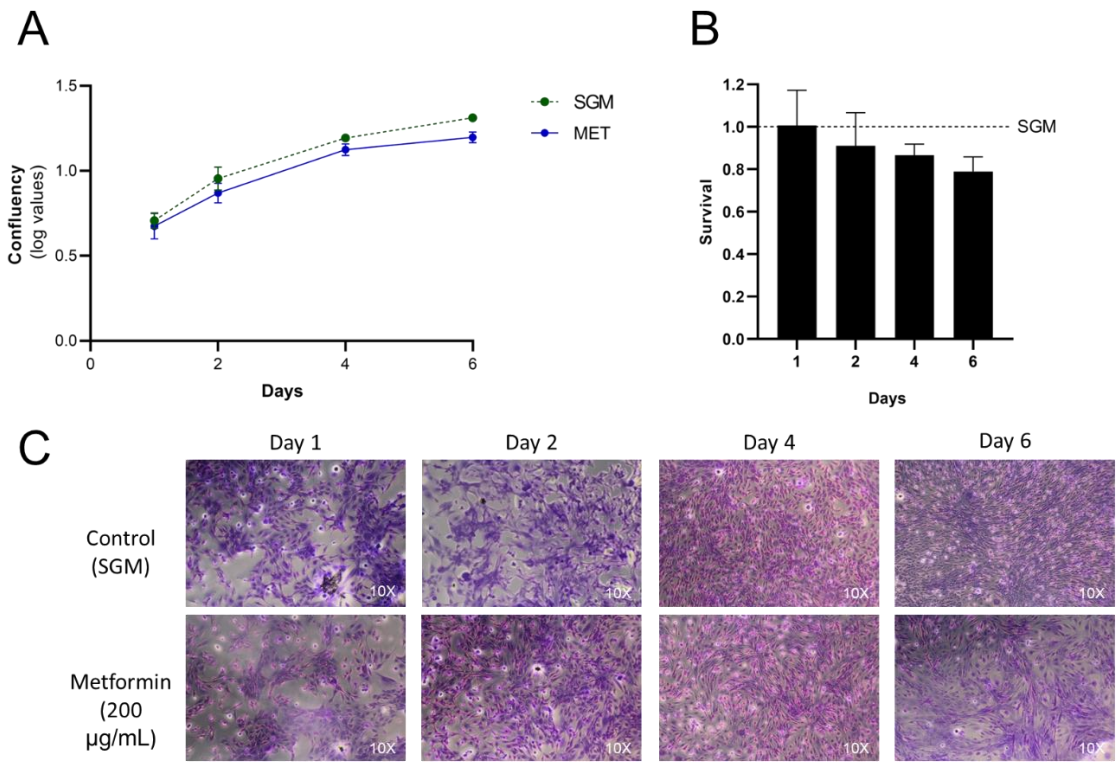


Figure 5.7: Cellular growth and proliferation of C2C12 cells under lowest human equivalent of metformin. A: Confluency over 6-day dose response period. At this concentration cells increased in number and followed a similar confluency trend as the control over the 6-day period**. B:** Survival rate over 6-day dose response period. At this concentration cells increased in number and did not significantly decrease in survival over the 6-day period; thus, it was sufficient enough to continue experimentation**. C:** Representative images displaying cellular growth and proliferation of C2C12 cells under 200µg/mL concentration of metformin. Lowest human equivalent dose of metformin was observed to support the growth and proliferation of cells. **Statistical analysis:** two-way ANOVA with Šídák's post hoc test.

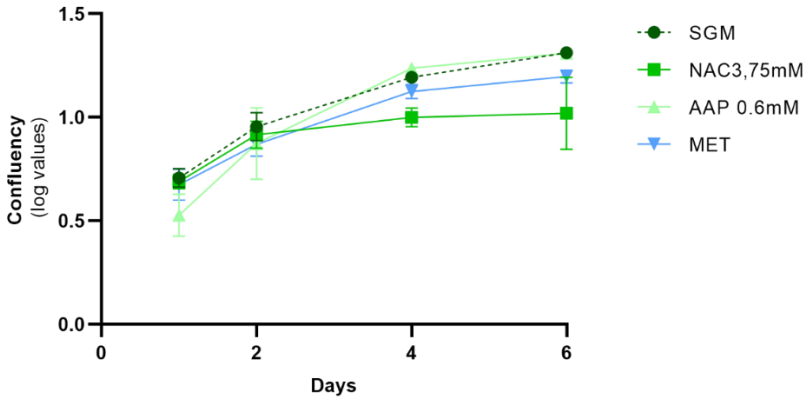


Figure 5.8: Cellular confluence over time. Confluency trends of chosen optimal concentrations of NAC (3.75mM) and AAP (0.6mM) as well as lowest human equivalent dose of metformin (200µg/mL) compared to standard conditions (SGM).

5.3 REDOX STATUS OF C2C12 CELLS

After combination therapy, the total level of intracellular reactive oxygen species and total antioxidant capacity of the collected conditioned media within each treatment group was determined using a Total Antioxidant Capacity (T-AOC) colorimetric assay and ROS fluorometric assay. Please refer to Figure 5.3.1 that displays overall ROS production and Total Antioxidant Capacity. At the end of the 24-hour supplementation period, the combination of AAP, NAC and metformin significantly decreased ROS levels (2-fold, $p<0.05$) and increased the total antioxidant capacity ($p<0.01$) (11.57 ± 5.66U/mL) when compared to metformin treatment on its own (0.81±2.48 U/mL). The combination of AAP, NAC and metformin had a significant increase in supernatant antioxidant capacity (11.57 ± 5.66 U/mL) when compared to the combination of AAP and metformin treatment ($p<0.01$) (0.72 ± 2.35 U/mL). Although no significant difference was evident between the ROS intracellular levels of single treatments AAP and NAC as well as the combination of NAC and AAP when compared to standard conditions (SGM), we did observe an overall decrease trend. In addition, single NAC treatment (29.15 ± 41.98 U/mL) had a significant increase ($p<0.5$) supernatant antioxidant capacity when compared to single AAP treatment (-5.65 ± 1.18 U/mL).

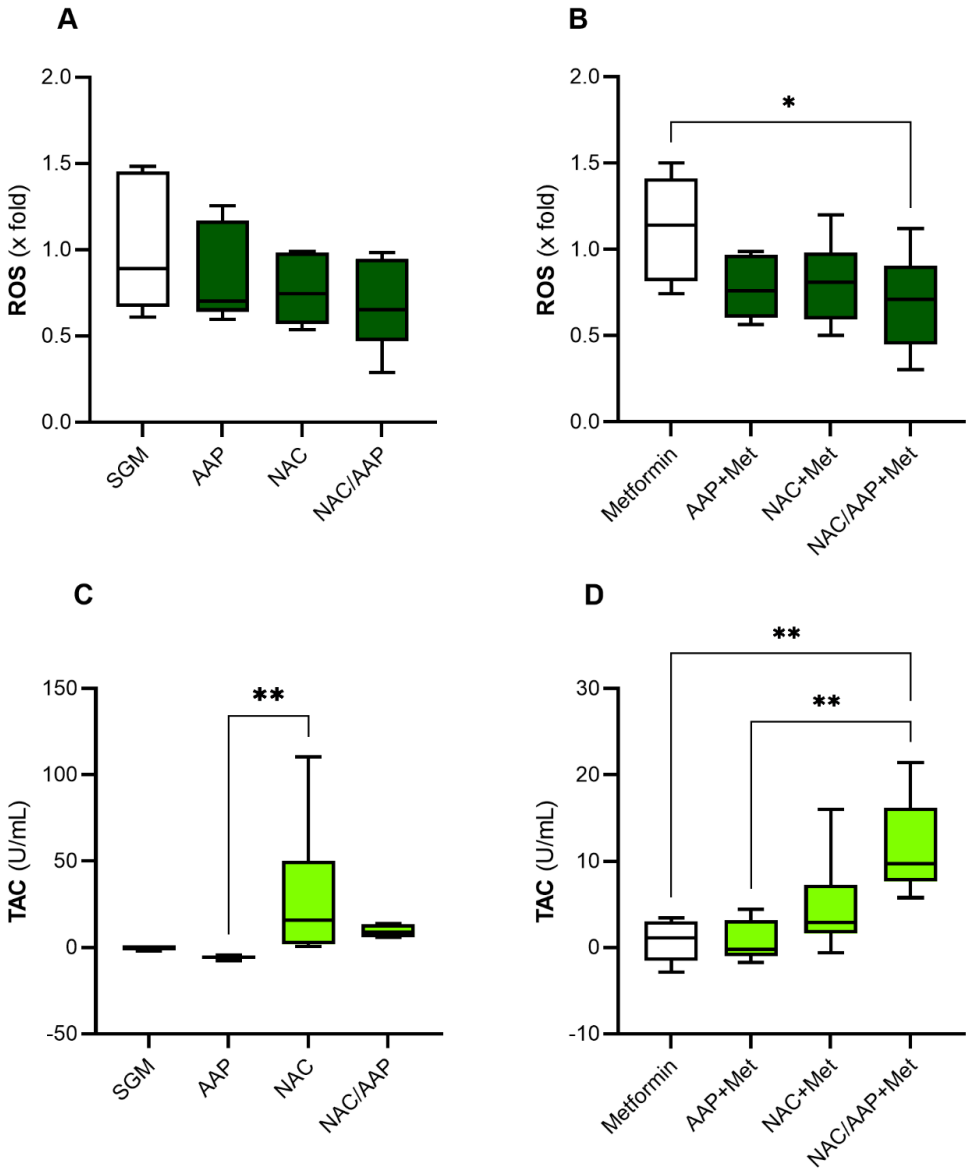


Figure 5.9: ROS fluorometric and TAC colorimetric assay results. A and B: ROS production of conditioned media containing antioxidants NAC (3.5 mM), AAP (0.6 mM) and metformin (200µg/mL) in various treatment combinations. At the end of the 24-hour supplementation period, the combination of AAP, NAC and metformin significantly decreased ROS levels. No significant difference was evident between the ROS intracellular levels of single treatments AAP and NAC as well as the combination of NAC and AAP when compared to standard conditions (SGM). We did however observe an overall decrease trend. **C and D:** Total Antioxidant Capacity in U/mL of conditioned media containing antioxidants NAC, AAP and metformin in various treatment combinations. At the end of the 24-hour supplementation period, the combination of AAP, NAC and metformin had a significant increase in supernatant antioxidant capacity when compared to the single metformin treatment. The combination of AAP, NAC and metformin had a significant increase in supernatant antioxidant capacity when compared to the combination of AAP and metformin treatment. In addition, single NAC treatment had a significant increase supernatant antioxidant capacity when compared to single AAP treatment. two-way ANOVA with Šídák's post hoc test. *p<0.05; **p<0.01 was considered significant.

5.4 GLUCOSE LOWERING EFFECT OF METFORMIN IN THE PRESENCE OF NAC AND AAP

Cells were divided into 4 pre-treatment groups 1) control (no pre-treatment); 2) AAP pre-treatment; 3) NAC pre-treatment; 4) AAP/NAC pre-treatment and the glucose uptake measured using the mean fluorescent intensity (MFI) value (Fig. 5.11). In the control (no pre-treatment) group, cells that received a 30 minute insulin incubation (MFI=2.33 ± 1.13) showed a significant increase in glucose uptake when compared to cells that received metformin alone ($p<0.5$) (MFI=1.24 ± 0.62) and combination insulin and metformin ($p<0.01$) (MFI=1.00 ± 0.60) incubation. In the AAP pre-treatment group, cells that received a combination insulin and metformin incubation (MFI=1.82 ± 1.48) showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin ($p<0.01$) (MFI=1.03 ± 6.21), 30 minute insulin ($p<0.05$) (MFI=1.20 ± 0.79) and metformin ($p<0.05$) (MFI=1.24 ± 1.00) alone incubation. In the NAC pre-treatment group, cells that received metformin alone incubation (MFI=1.94 ± 1.27) showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin ($p<0.01$) (MFI=1.22 ± 0.83) and a combination insulin and metformin incubation (MFI=1.26 ± 0.81). In the AAP/NAC pre-treatment group, cells that received a combination insulin and metformin incubation (MFI=3.26 ± 2.43) showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin ($p<0.001$) (MFI=1.37 ± 0.69), 30 minute insulin ($p<0.001$) (MFI=1.31 ± 1.10) and metformin ($p<0.001$) (MFI=1.36 ± 1.06) alone incubation. Representative images displayed in Figure 5.12indicates the fluorescent intensity under various conditions (Insulin, metformin) following pretreatment with either NAC and/or AAP. The 2-NBDG fluorescent probe lost its intensity as we conducted subsequent experiments and thus these groups could not be compared to each other using a two-way analysis of variance.

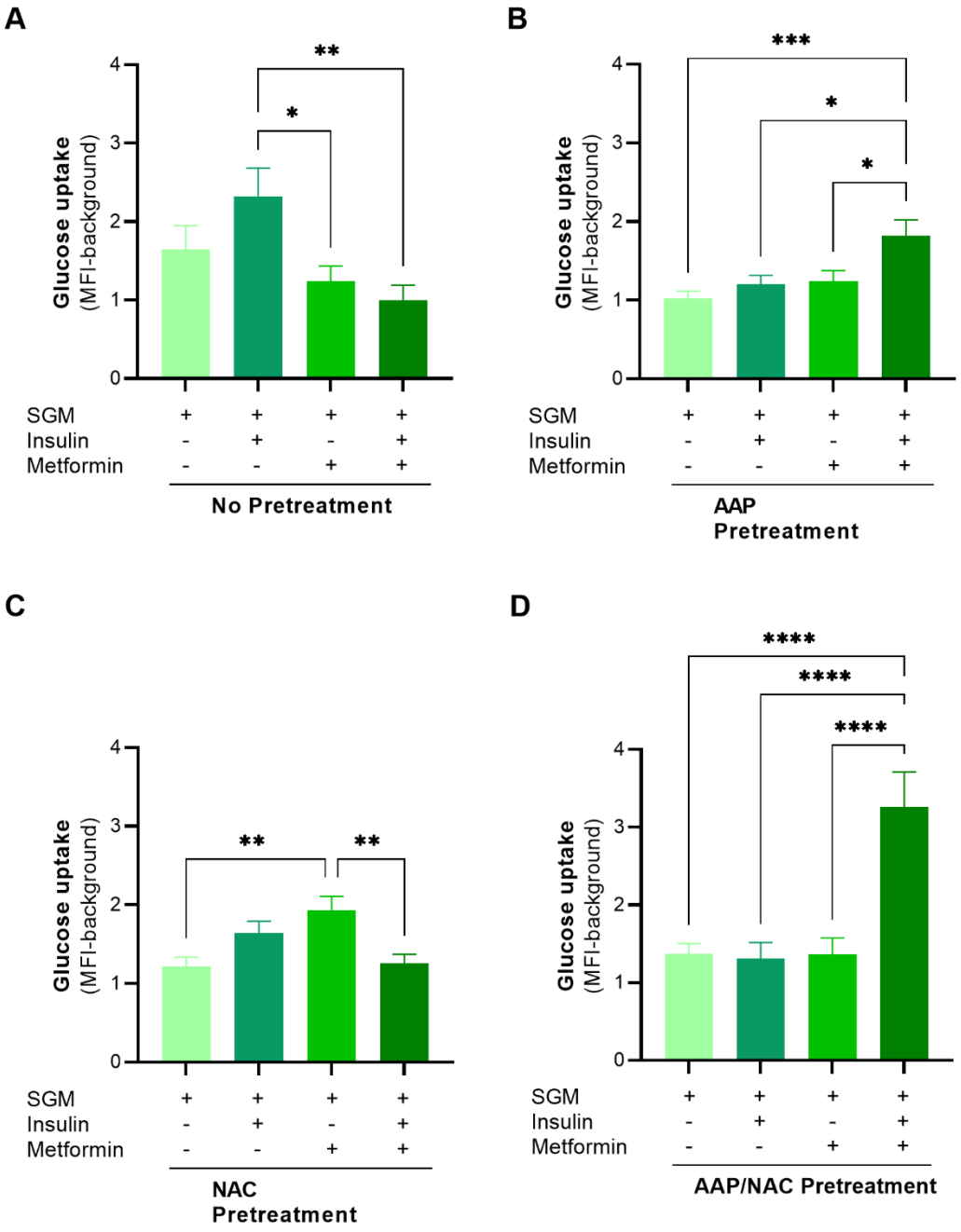


Figure 5.10: Glucose uptake profiles under various conditions (Insulin, Metformin) following pretreatment with either NAC and/or AAP. A: Control (no pre-treatment) group: cells that received a 30 minute insulin incubation showed a significant increase in glucose uptake when compared to cells that received metformin alone and combination insulin and metformin incubation. **B:** AAP pre-treatment group: cells that received a combination insulin and metformin showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin, 30-minute insulin and metformin alone incubation. **C:** NAC pre-treatment group: cells that received metformin alone incubation showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin and a combination insulin and metformin incubation. **D:** NAC/AAP Pretreatment (n=2): cells that received a combination insulin and metformin incubation showed a significant increase in glucose uptake when compared to cells that received no insulin and metformin, 30-minute insulin and metformin alone incubation. **Statistical analysis:** one-way ANOVA with Tukey post hoc test. *p<0.05; **p<0.005; ***p< 0.01; ****p< 0.0001 was considered significant.

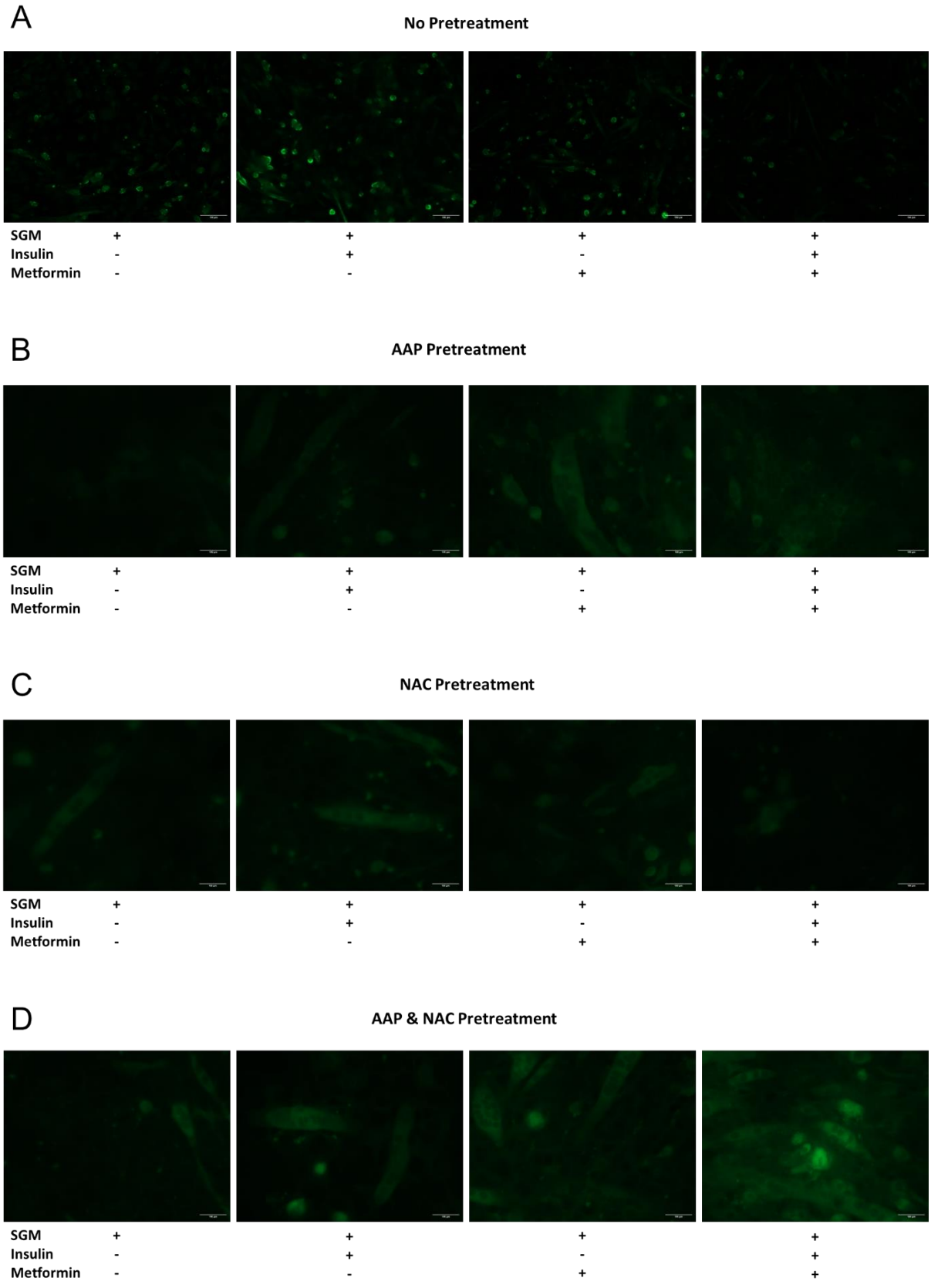


Figure 5.11: Representative images of glucose uptake profiles under various conditions (Insulin, Metformin) following pretreatment with either NAC and/or AAP A: Glucose uptake of No Pretreatment group. **B:** Glucose uptake of AAP Pretreatment group. **C:** Glucose uptake of NAC Pretreatment group. **D:** Glucose uptake of AAP/NAC Pretreatment group. **Scale bar**, 100µm.

6 DISCUSSION

The pathologic T2DM microenvironment exposes tissue, specifically skeletal muscle tissue, to continuous oxidative stress and inflammation that is rooted in uncontrolled hyperglycaemia (Galicia-Garcia et al., 2020, Han et al., 2020, Nolan et al., 2011). As a result, T2DM patients are still at risk of developing associated comorbidities. Therefore, the treatment of T2DM requires connected, simple, inexpensive and comprehensive adjuvant therapies to limit these micro- and macrovascular complications (Atun et al., 2017). Literature provides evidence that suggests that antioxidants such as NAC and AAP may be used as a potential complementary therapeutic strategy to combat diseases associated with oxidative stress and inflammation (Arulselvan et al., 2016, Hussain et al., 2016). Metformin's primary treatment goal is to achieve effective glycaemic management. Thus, it alone cannot control the micro- and macrovascular complications caused by sterile metabolic inflammation and oxidative stress that is associated with apparent T2DM. This necessitates the use of exogenous NAC and AAP antioxidant supplementation as a synergistic treatment with metformin in T2DM to not only ameliorate oxidative stress related damage but restore regular blood glucose homeostasis and holistically treat the disease.

Our study had a number of main findings. Firstly, we observed that over the 48-hour period, cells cultured in high glucose conditions had a significantly ($p<0,0001$) higher cell number per $10mm^2$ plate surface area when compared to cells cultured in low glucose conditions. The optimal concentrations of NAC and AAP was determined to be 3.75mM and 0.6mM, respectively. Additionally, the combination of AAP, NAC and metformin treatment significantly decreased ROS levels (2-fold, $p<0.05$) and increased the total antioxidant capacity ($p<0.01$) (11.57±5.66 U/mL) when compared to metformin treatment on its own (0.81±2.48 U/mL). Lastly, the pretreatment (24h) of cells with a combination of AAP and NAC prior to glucose starvation (2h) and exposure to either insulin (30min) and/or metformin (2h) significantly increased glucose uptake compared to cells without pre-treatment.

Highly proliferating mammalian cells such as mouse C2C12 and L6 cells consume large amounts glucose as a major energy source (Furuichi et al., 2021). Additionally, it has been calculated that growing mammalian cells require 30% more energy than non-growing or quiescent cells (Mulukutla et al., 2010). Therefore, proliferating cells have the preference for high glucose conditions in tissue culture (Furuichi et al., 2021). High glucose conditions support their rapid proliferation and has widely been used for culturing muscle cells (Pasut et al., 2013, Shefer and Yablonka-Reuveni, 2005). Our study showed that C2C12 cells cultured in high glucose (4.5 g/L) conditions over a 48-hour period showed a higher cell number of

healthy cells per 10mm^2 plate surface area compared to cells cultured in low glucose (1 g/L) conditions which exhibited increased shrinking and lifting from the plate.

The optimal concentrations of NAC and AAP was determined to be 3.75mM and 0.6mM, respectively using crystal violet staining. These concentrations are considered to be relatively low even when translated into human equivalent doses. Crystal violet staining is a quick and versatile assay for screening cell viability under diverse stimulation conditions especially compounds that may impact cell survival. This assay is directly proportional to the cell biomass that is attached to the plate. The cell biomass can be used to infer levels of cell viability or cytotoxicity. However, it is insensitive to changes in cell metabolic activity and only gives an absolute value based on cell biomass (Aslantürk, 2018). An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay could have also been employed to not only measure cell viability, proliferation and cytotoxicity but gain additional insight on cell metabolic and mitochondrial activity of cells and possible changes in cell physiology (Grela et al., 2018).

Antioxidants may act as prooxidants under certain condition such as high pH, being in the presence of transition metal ions (Cu^{2+} or Fe^{3+}) and especially at high concentration (Rajashekar, 2023). Additionally, the prooxidant properties of Vitamin C can be exhibited at high concentration (Pal and Jana, 2022). Therefore, these concentrations were chosen not only because we observed higher confluency and survival rates but also to ensure minimal oxidative stress and cell toxicity. Chosen concentrations and time intervals of this study are in line with existing literature as many toxicology studies employ a dose response to examine the safety and efficacy of a chosen drug on a representative cell line (Maartens et al., 2021, Mehrbani Azar et al., 2018).

This study demonstrated in an in-vitro model, using a skeletal muscle myoblasts cell line that the combination of AAP, NAC and metformin treatment significantly decreased ROS levels and increased the total antioxidant capacity when compared to singular metformin treatment. Additionally, with regards to total antioxidant capacity, NAC alone was observed to outperform the combination treatment of AAP, NAC and metformin. This is consistent with literature that both AAP and NAC are potent antioxidants that reduce oxidative stress and that metformin is thought to possess anti-inflammatory properties that suppresses ROS. Ascorbic acid is known to be unstable in cell culture and has an estimated half-life of less than 1 hour in tissue culture media, (Farhat and Fry, 2023). Additionally, it is limited by its rapid oxidation and potential H_2O_2-induced cytotoxicity. Thus, AAP being a more stable derivative of ascorbic acid that also promotes the growth of various cell types, is commonly adopted as an alternative for culturing various cell types (Takamizawa et al., 2004, Wu et al., 2020). However, it is easily degraded into ascorbic acid in the presence of phosphatase from living tissues (Tsutsumi et al., 2012).

Furthermore, AAP and NAC accomplish their antioxidant effects via different mechanisms of action. AAP is a precursor for ascorbate, which is a good free radical scavenger and powerful antioxidant that displays direct antioxidant properties. It non-enzymatically reduces free radicals cycling between its two forms (Njus et al., 2020). Therefore, it neutralises free radicals, attenuates the inflammatory response and protects important biomolecules (proteins, lipids, carbohydrates, and nucleic acids) from ROS damage (Abdullah et al., 2022, Carr and Maggini, 2017). NAC displays both direct and indirect antioxidant properties. Its direct antioxidant effect is accomplished via the thiol group present in NAC that serves as a ROS scavenger to decrease oxidative stress and inflammation. Its indirect effect is accomplished due to it being a precursor in the production of GSH, which is the most abundant and important non-protein thiol antioxidant in all mammalian tissues (Demirkol and Ercal, 2012, Lasram et al., 2015, Santus et al., 2014). Healthy C2C12 cells were used in experiments and oxidative stress was not intentionally induced or simulated. Any oxidative stress induced was due to the standard cell culture techniques used which may or may not have required the powerful antioxidant properties of both AAP and NAC. However, taken together, this may explain the significant increase in supernatant antioxidant capacity of single NAC treatment when compared to single AAP treatment as well as the significant difference between the ROS intracellular levels of single treatments AAP and NAC and the combination of NAC and AAP when compared to standard conditions. We did however observe an overall decrease trend in ROS from these single treatments which again displays that both AAP and NAC are important antioxidants that protect against oxidative stress. Our results show that there seems that the combination of metformin, NAC and AAP may diminish the effectiveness of NAC with respect to Total Antioxidant Capacity and thus the combination of metformin, NAC and AAP had a comparable effect in increasing TAC levels and reducing ROS levels.

Cell culture is universally used as laboratory technique to examine metabolic pathways, disease pathogenesis and to reveal mechanisms involved in signal transduction, regulation of gene expression, cell proliferation and cell death. However, cell culture imposes a state of oxidative stress on cells through numerous ways (type of cell culture media, change in pH, drop in temperature) throughout the process (Halliwell, 2003). The use of NAC and AAP as an antioxidant pretreatment protects cells from oxidative stress induced through the culturing process and allows cells to adequately uptake glucose. Although no studies have shown this specifically in using a 2-NDBG assay, it is similar to many studies that have shown NAC and AAP to improve glucose homeostasis (Falach-Malik et al., 2016, Mason et al., 2016, Mason et al., 2019, Panahi et al., 2022, Ragheb et al., 2020)

Glucose uptake and disposal accounts for more than 80% in skeletal muscles under regular physiological conditions after a meal is digested and thus makes this tissue a primary site of

glucose utilization in the human body. Thus, it plays a significant role in the regulation of glucose homeostasis (Hansen et al., 1994, Kim et al., 2021, Park et al., 2014). Glucose uptake occurs by two apparent cellular mechanisms in skeletal muscles that set off a series of reactions that enhance GLUT-4 translocation and consequently glucose uptake. The insulin signalling pathway which regulates glucose uptake and glycogen synthesis via stimulation of phosphoinositide 3 kinase (PI3K) and subsequently Protein kinase B (PKB/Akt) activation and the insulin-independent AMP-activated protein kinase (AMPK) pathway (Kamga-Simo III et al., 2021, Park et al., 2014). AMPK is considered a 'master' sensor as it plays an important role in the regulation of cellular and whole-body energy homeostasis. Essentially all cultured cells regardless of tissue origin express AMPK (Mulukutla et al., 2010). It is activated by various pharmacological, pathological and metabolic triggers (Ong et al., 2012). AMPK mediates the cellular adaptation to nutritional and environmental variations that deplete intracellular ATP levels (e.g., heat shock, hypoxia, starvation or prolonged exercise) (Viollet et al., 2009). Through phosphorylation of transcription factors and co-activators, AMPK is able to regulate various physiological processes (Mihaylova and Shaw, 2011). AMPK regulates glucose homeostasis by upregulating the glucose and fatty acids metabolism. Additionally, it brings about a decrease in blood glucose by increasing peripheral glucose uptake through the translocation of GLUT-4 from intracellular membranes to plasma membranes and suppresses glucose production (Kim et al., 2021, Mihaylova and Shaw, 2011, Ong et al., 2012). T2DM has been shown to be associated with reduced AMPK activity in skeletal muscles (Guan et al., 2016). Therefore, AMPK has been investigated as a potential factor to control muscle regeneration and mass in people with metabolic disorders (Furuichi et al., 2021).

The factors within our study context such as high glucose conditions, reduced oxidative stress due to NAC and AAP pretreatment as well as insulin and metformin incubation may have activated the AMPK pathway and reason why the pre-treatment (24h) of cells with AAP, NAC and a combination of AAP and NAC prior to glucose starvation (2h) and exposure to either insulin (30min) and/or metformin (2h) increased glucose uptake compared to cells without pre-treatment. It is widely accepted that AMPK is activated by antidiabetic drugs such as metformin (Kim et al., 2021). Furthermore, insulin's main function is to promote cellular uptake, use, and storage of absorbed nutrients, mainly glucose. Thus, numerous studies show that cells are commonly treated with insulin to be used as a positive control for the measurement of glucose uptake, while the untreated cells are commonly used as negative control (Bala et al., 2021, Kamga-Simo III et al., 2021, van de Venter et al., 2008, Zhang et al., 2018). It is important to note that the direct phosphorylation of PKB/Akt by insulin inhibits AMPK (Jeon, 2016) thus dual activation of these pathways would not contribute to more GLUT-4 being translocated.

However, the data implies that the pretreatment with AAP and NAC may increase GLUT-4 expression and consequently glucose uptake.

Glucose uptake is a fundamental necessity to investigate T2DM, its associated insulin resistance, comorbidities such as skeletal muscle defects and possible therapeutic strategies. There are various approaches such as western blotting, enzyme-linked immunosorbent assay (ELISA), scintillation counting, flow cytometry, fluorometry and spectrophotometry (Bala et al., 2021). However, 2-NBDG which is a fluorescent-tagged glucose analogue as well as bioprobe, is the most common approach and has been used in numerous studies conducted in living cells for cell viability, glucose absorption and metabolic activity in (Bala et al., 2021, Louzao et al., 2008). The use of 2-NBDG as a fluorescent indicator has many benefits such as being inexpensive, is not restricted to screening insulin-mimetic compounds and can be used in both primary cells or cell lines to provide insights into the mechanisms of glucose accumulation and metabolism (Zou et al., 2005). Additionally, 2-NBDG provides a more sensitive measurement of glucose uptake in real time in single, living cells and allows for simultaneous measurement of other cellular activities by different methods (intracellular calcium concentration, pH, or membrane potential) (Yamada et al., 2007). In muscle types that exhibit high rates of stimulated glucose transport activity, the use of 2-NBDG allows for the accurate determination of transport activity (Hansen et al., 1994).

2-NBDG is transported into skeletal muscle mainly via facilitated diffusion by glucose transporters GLUT1 and GLUT4 and partially through active transport by sodium glucose cotransporters (SGLT) (Pajak et al., 2019, Ueyama et al., 2000). After uptake, 2-NBDG is phosphorylated by hexokinase, to produce 2-NBDG 6-phosphate which is a fluorescent derivative. 2-NBDG 6-phosphate is then trapped inside the cell as it is unable to undergo isomerization to fructose-6-P, resulting in intracellular accumulation of 2-NBDG 6-phosphate and the cessation of glycolysis and glucose metabolism. However, 2-NBDG 6-phosphate may also be converted back into 2-NBDG by the enzyme glucose 6-phosphatase. After this conversion, 2-NBDG enters the glycolytic pathway and is rapidly degraded to non-fluorescent products (Kim et al., 2012, Pajak et al., 2019) or may accumulate as glycogen (Zhang et al., 2018). Additionally, evidence shows that fluorescent tagged glucose bioprobes (2-NBDG and 6-NBDG) permeate the cell 50–100 times slower than glucose (Kim et al., 2012). Understanding the transport of 2-NBDG into skeletal muscle may provide insight into the limitations of the 2-NBDG based assay as well as the altered glucose uptake profiles between pretreatment groups. Additionally, the high glucose incubation conditions used to preserve cellular health may have competed with fluorescent-tagged 2-NBDG for binding as they both utilise the same glucose transporters to enter muscle cells.

Although, the 2-NBDG based assay is the most commonly used approach in numerous cell types including skeletal muscle, all available protocols have large variability which impacts the overall data reproducibility (Bala et al., 2021). The glucose uptake results demonstrated in this study shows great variability due to the modified approach in the study design we used. Park et al. (2014) examined the effect of two essential fatty acids (linoleic acid and α -linolenic acid) on glucose uptake of C2C12 skeletal muscle cells. In their study, glucose uptake activity was determined by cellular uptake of 2-NBDG intensity per cell viability in each well and displayed as a percentage of untreated control cells. The assay method was modelled after Zou et al. (2005) with slight modifications. Cells were incubated with insulin (100 nM) in glucose-free DMEM for 10 min before the addition of 2-NBDG (60 µM) that was then left to incubate for 1 hour. Additionally, Gorelick et al. (2015) evaluated the anti-diabetic activity of W. somnifera (Asghawhanda) that is traditionally used to treat many medical problems including diabetes. The study assessed anti-diabetic activity via glucose uptake using fluorescent glucose 2-NBDG in L6 myotubes and 3T3 adipocytes. Differentiated L6 myotubes and adipocytes were incubated in low glucose and serum free media overnight, treated with increasing concentrations of W. somnifera extract or insulin for 4 hours and finally 2-NBDG (200 uM) was added for 1 hour. These studies and other investigations using specific cell lines that are derived from tissues known to play a crucial role in insulin resistance and glucose homeostasis, all make use of modified approaches to the 2-NBDG based assay.

Glucose starvation which is the use of glucose and serum free medium, is one of the prerequisite conditions for glucose uptake assay. It allows for sufficient uptake of 2-NBDG that can be quantified but may induce stress and alter muscle cell metabolism and morphology, (Bala et al., 2021). Our study prioritised preventing severe alterations in muscle cell physiology and as such chose to glucose starve our cells in low glucose (1 g/mL) media rather than glucose-free media. Additionally, pre-treatments, insulin, metformin and 2-NBDG incubation were all completed in SGM as to preserve cell integrity. Although there are several limitations associated with this study, it did show altered glucose uptake profiles between groups depending on pretreatment. Suggesting that despite these limitations that the decisions made to modify the approach to the 2-NBDG based assay resulted in the combination of metformin, NAC and AAP showing improved glucose uptake in C2C12 mouse myoblasts in vitro. Future studies are however required to determine the exact mechanism responsible for the observed effects in this study.

7 CONCLUSION, LIMITATIONS AND FUTURE IMPLICATIONS

With the prevalence of T2DM increasing rapidly in sub-Saharan Africa (Atun et al., 2017) coupled with the major negative impacts on the well-being of patients, the economy and global health systems (Atun et al., 2017, Saeedi et al., 2019). The treatment of T2DM demands simple, inexpensive, and broad-based alternative preventative strategies to limit T2DM progression and delay the onset of acute metabolic and long-term complications (Atun et al., 2017). One of the major contributors of diabetes complications as well as insulin resistance development, is oxidative stress (Yaribeygi et al., 2020). Oxidative stress and T2DM interact by complex means and both intensify and aggravate the other (Luc et al., 2019, Yaribeygi et al., 2020). Taken together with the supporting literature, warrants the potential clinical use of antioxidants as an adjuvant therapy in the treatment of metabolic disorders such as T2DM. Although the beneficial effects of the antioxidants, NAC and AAP are well established, it is unclear if the antioxidants NAC and AAP will interfere with the insulin sensitizing function of metformin or reduce the required daily dose. Thus, our study aimed to investigate using an in-vitro skeletal muscle myoblasts cell line (mouse C2C12, ATCC) model, if there is any drug interaction and synergistic effects between metformin and the antioxidants, NAC and AAP.

Our study demonstrated that the combination of AAP, NAC and metformin treatment significantly decreased ROS levels and increased the total antioxidant capacity when compared to metformin treatment on its own. Additionally, we observed that the pre-treatment (24h) of cells with a combination of AAP/NAC prior to glucose starvation (2h) and exposure to either insulin (30min) and/or metformin (2h) significantly increased glucose uptake compared to cells without pre-treatment. Therefore, in conclusion, there is a comparable effect between metformin, NAC and AAP when used in combination with each other, which reduces oxidative stress in vitro. Additionally, the combination of metformin, NAC and AAP improves glucose uptake in C2C12 mouse myoblasts in vitro that resulted in altered glucose profiles thus patients taking adjuvant antioxidants may require glucose monitoring and changes in metformin requirements. This study proves that NAC and AAP may serve as a possible adjuvant therapeutic agent to target oxidative damage and inflammation in the T2DM pathogenesis to potentially prevent the development of secondary complications in T2DM patients and in doing so improve quality of life of patients with T2DM.

However, our study was limited by numerous means. Chiefly due to the variability in our glucose uptake assay study design. Glucose uptake results observed in this study are restricted to physiologically healthy, differentiated mouse C2C12 skeletal myoblasts in which IR was not induced and cells being incubated with insulin and/or metformin in SGM containing a 4.5 g/L glucose concentration, which could differ from cells being incubated with insulin

and/or metformin prepared with other lower glucose concentration solvents. Additionally, a realistic, human biochemical muscle glucose uptake system could not be completely mimicked in differentiated C2C12 myoblasts and thus, our study be could not elucidate, the responsible underlying mechanisms and possible interaction between drugs that brought upon the synergistic effect between metformin, NAC and AAP that improved glucose uptake and reduced oxidative stress in vitro. Finally, our study was limited in other study approaches that may have been used to better analyse and quantify whether NAC and AAP interfere with the insulin sensitizing function of metformin or reduce the required daily dose. Further testing such as, western blotting to determine the activity of pathways (PKB/Akt and AMPK pathways) involved glucose uptake, flow cytometry using other glucose analogues (6-NBDG) to assess glucose uptake, assessing calcium (Ca^{2+}) flux in live cells as Ca^{2+} plays a central role in the context of metabolic stress and T2DM as well as using fluorescent cationic dyes (rhodamine-123, DiOC6, JC-1) to evaluate mitochondrial viability and function with regards to oxidative stress, should be performed to establish valid conclusions. Additionally, in-vitro studies using adipose tissue derived stromal cells (ADSCs) and HepG2 liver cells as these cell lines are derived from tissues known to play a crucial role in insulin resistance and glucose homeostasis.

Future implications of this study include using its results to warrant further screening of not only the above-mentioned drugs in this study but also screen the efficacy of other anti-diabetic agents and antioxidants that target both glucose homeostasis and oxidative stress within a diabetic microenvironment as well as its associated comorbidities. Subsequently, more in depth studies are needed to research and elucidate the mechanism(s) of action and drug-drug interactions involved in cell culture, animal and human biological systems when metformin, NAC and AAP are used in combination with each other. Additionally, the application of 2-NBDG as a fluorescent indicator to quantify and clarify the mechanisms underlying dynamic glucose uptake-function in living cells, provides a novel yet simple approach to not only develop anti-diabetic drugs but also to further vital research on T2DM. Overall, this study contributes towards the development for further opportunities and newer approaches to modern medicine in combatting T2DM.

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9 APPENDICES

9.1 APPENDIX A: ETHICS APPROVAL LETTER

REC: Biological and Environmental Safety

Feedback Letter: *Approved*

22 August 2022

PI: Dr M Van de Vyver

REC: BES Reference #: BEE-2022-24398

Title: Drug interaction between metformin and antioxidants

Dear Dr M Van de Vyver

Your New Application, with BEE-2022-24398 was reviewed by the Research Ethics Committee: Biological and Environmental Safety **(REC: BES)** via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Approval Date: **22 August 2022 - 21 August 2023**

Please remember to use your REC: BES reference number: # BEE-2022-24398 on any documents or correspondence with the REC: BES concerning your research protocol.

If you have any questions or need further help, please contact the REC: BES office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: BES policy and procedures.

Sincerely,

Mr. Winston BeukesCoordinator:
Research Ethics (Biosafety) E:
applyethics@sun.ac.za
(Ethics Help-Desk)