Antioxidant supplementation as protective strategy against stem cell impairment in Type 2 Diabetes

by
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Abstract

Type 2 Diabetes Mellitus (T2DM) is a global epidemic. It is a complex disorder that leads to cellular dysfunction and the development of co-morbidities. The underlying pathologic microenvironment in T2DM, associated with hyperglycaemia, include the accumulation of advanced glycation end products, excessive oxidative stress, and chronic inflammation. Bone marrow mesenchymal stem cells (MSC) are especially susceptible to this damaging microenvironment and as consequence the endogenous repair mechanisms within the body fail giving rise to secondary complications such as non-healing wounds, retinopathy, and neuropathy.

There is thus a need for a holistic approach when it comes to disease management in which anti-diabetic drugs focussed on glucose control is complemented by supplementary treatment aimed at restoring homeostasis. Natural and synthetic antioxidants such as Ascorbic acid-2-phosphate (AAP) and N-acetyl-l-cysteine (NAC) are known to protect cells against oxidative stress-induced damage *in vitro* and have been shown to reduce inflammation in various models. The efficacy of these antioxidants to restore homeostasis and prevent cellular dysfunction in T2DM is however still unknown.

The aim of this study was to investigate the protective effects of combined NAC and AAP supplementation against MSCs impairment using an animal model of obese diabetic (B6.C-Lep^{ob}/J) (ob/ob) (n=14) and wild type control (C57BL6/J) (n=20) mice. All mice received jelly cubes containing either antioxidants (NAC7.5mM+AAP0.6mM) or placebo (vehicle control) for a period of 6-weeks. Metabolic parameters (weight and blood glucose) were assessed on a weekly basis and the overall antioxidant status of animals assessed at the end of the 6-week period by analysing the total antioxidant capacity (TAC) and Malondialdehyde (MDA) levels in serum. Bone marrow MSCs were isolated from mice in each of the respective treatment groups and their *ex vivo* growth rate, viability, multi-lineage differentiation capacity and paracrine responsiveness upon stimulation with wound fluid assessed.

Compared to the wild type (C) mice, excessive weight gain (weight: C 28.7±1.6 g; DM 44.3±3.5 g) ($p<0.05$) and hyperglycaemia (blood glucose: C 10.1±1.3 mmol/L; DM 23.6±5.7 mmol/L) ($p<0.05$) was evident in the DM animals validating the animal model as representative of T2DM. The antioxidant supplementation did not affect metabolic parameters indicating that differences observed between supplement and placebo treated mice were not due to weight loss or changes in glucose metabolism. NAC/AAP significantly ($p<0.05$) reduced lipid peroxidation in DM animals (DM:P 39.0±14.7 nmol/L; DM:S 21.5±11.6 nmol/L) to a level comparable to that of controls (C:P 22.9±11.4 nmol/L; C:S 30.5±3.3 nmol/L) and increased the overall TAC (C:P 3.7±1.3 U/mL; C:S 4.0±0.7 U/mL; DM:P 5.2±0.9 U/mL; DM:S 5.8±1.5 U/mL). The *ex vivo* growth rate of cells derived from DM animals were impaired (Time to confluence: C 8 days; DM 12 days). NAC/AAP supplementation did however improve the growth rate and viability of MSCs derived from both animal models. NAC/AAP

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furthermore reduced the adipogenic differentiation capacity of MSCs but could not restore osteogenesis in DM MSCs. Upon stimulation with wound fluid, slightly increased IL6 (DM:S+WF 1583.3±481.9 pg/mL) and IL10 (DM:S+WF 27.1±9.8 pg/mL) release was evident in MSCs derived from NAC/AAP supplemented animals. In conclusion, NAC/AAP supplementation was able to reduce oxidative stress in animals and improved MSCs viability.

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Opsomming

Tipe 2 Diabetes Mellitus (T2DM) is ‘n wêreldwye epidemie. Dit is ‘n ingewikkelde siekte wat tot selwanfunksionering en die ontwikkeling van komorbiditeite lei. Die onderliggende patologie van T2DM word met hiperglukemie geassosieer en sluit die vermeerdering van gevorderde glykasie-eindprodukte, buitengewone oksidatiewe-spanning en chroniese inflammasie in. Beenmurg-mesenchimale-stamselle (MSS) is veral vatbaar tot hierdie skadelike mikro-omgewing. Gevolglik word die liggaam se inwendige herstelmeganismes beskadig wat tot die ontstaan van sekondêre komplikasies soos ongeneesbare wonde, retinopatie en neuropatie lei.

Dus word daar ‘n holistiese-aanvlug ten opsigte van behandeling benodig. Diabeetmedisyne, gefokus op die beheer van glukosevlakke, word met aanvullende behandeling wat beoog om homeostase reg te stel, gepaar. Natuurlike en sintetiese anti-oksidante soos askorbiensuur-2-fosfaat (AAP) en N-asetiel-I-sisteïen (NAC) is daartoe in staat om selle *in vitro* teen oksidatiewe-spanning-geïnduseerde skade te beskerm. Dit is met behulp van verskeie modelle bewys dat dié anti-oksidante ook inflammasie verminder. Daar is egter nog onsekerheid met betrekking tot hierdie anti-oksidante se vermoë om homeostase te herstel en selwanfunksionering te voorkom.

Die doel van hierdie studie is om aanvullings NAC en AAP se beskermende effek op diabeet-aangetaste MSS deur middel van ‘n diermodel wat uit vetsugtige diabeet-B6.C-Lep^{ob}/J-muise (ob/ob) (n=14) en wilde-tipe-C57BL6/J-muise (n=20) bestaan, te ondersoek. Al die muise was vir ‘n tydperk van 6 weke jellieblokkies wat óf die aanvulling (NAC7.5 mM+AAP0.6 mM) óf die plasebo (kontrole) bevat het, gevoer. Die metaboliese parameters (gewig en bloedglukosevlakke) was weekliks geëvalueer. Die diere se universele anti-oksidant-status was teen die einde van die 6-week-periode in terme van totale anti-oksidant-kapasiteit (TAC) en Malondialdehiedvlak (MDA) in serum geëvalueer. Die muise se geïsoleerde beenmurg-MSS se *ex vivo* groeitempo, lewensvatbaarheid, multi-kapasiteit differensiasievermoë en parakrien-reaksie op wondvog-stimulasie was geassesseer.

In vergelyking met die wilde-tipe-muise (WT), het die ob/ob-muise buitensporig gewig opgetel (gewig: WT 28.7±1.6 g; ob/ob 44.3±3.5 g) ($p<0.05$) en hiperglukemie (bloedglukose: WT 10.1±1.3 mmol/L; ob/ob 23.6±5.7 mmol/L) ($p<0.05$) ontwikkel, wat bekragtig dat die diermodel ‘n geldige verteenwoordiging van T2DM is. Die anti-oksidantaanvulling het nie die metaboliese parameters geaffekteer nie wat aandui dat die verskille tussen die aanvulling- en plasebo-behandelde muise nie weens gewigsverlies of veranderinge in hul glukosemetabolisme is nie. Die NAC/AAP-aanvulling het lipiedperoksidasie in ob/ob-diere (plasebo: 39.0±14.7 nmol/L; behandeling: 21.5±11.6 nmol/L) aansienlik ($p<0.05$) verlaag en is vergelykbaar met dié van WT-diere (plasebo: 22.9±11.4 nmol/L; behandeling: 30.5±3.3 nmol/L). Dit het die totale TAC (WT: plasebo: 3.7±1.3 U/mL; behandeling: 4.0±0.7 U/mL; ob/ob: plasebo: 5.2±0.9 U/mL; behandeling: 5.8±1.5 U/mL) verhoog. Die *ex vivo* groeitempo van die ob/ob-dierselle was vertraag (tyd tot samevloeiing: WT 8 dae; ob/ob 12 dae).

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Die NAC/AAP-aanvulling het egter die groeivermoë en lewensvatbaarheid van MSS van beide die ob/ob- en WT-diermodel verhoog. Bowendien het die NAC/AAP-aanvulling die adipogenese-differensiasiekapasiteit van MSS verlaag. Ongelukkig kon dit nie die osteogenese-differensiasie in ob/ob-MSS herstel nie. Daar was ‘n effense toename in die vrystelling van IL6 (ob/ob-MSS: 1583.3±481.9 pg/mL) en IL10 (ob/ob-MSS: 27.1±9.8 pg/mL) in MSS van NAC/AAP-aangevulde diere na wondvog-stimulasie. Die gevolgtrekking is dat die NAC/AAP-aanvulling in staat is om oksidatiewe-spanning in diere te verminder, asook MSS se lewensvatbaarheid te verbeter.

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Abbreviations

4PL	4 parameter logistic regression
AAP	Ascorbic acid-2-phosphate
AED	Animal Equivalent Dosage
AGEs	Advanced Glycation End Products
AM	Adipogenic induction media
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BM	Bone Marrow
BM-MSCs	Bone Marrow residing Mesenchymal Stem Cells
BrdU	Bromodeoxyuridine
°C	Degrees Celsius
C	Control
CAT	Catalase activity
CB-MSCs	Compact Bone Mesenchymal Stem Cells
CD90	Cluster of Differentiation 90
CD105	Cluster of Differentiation 105
CD73	Cluster of Differentiation 73
CD34	Cluster of Differentiation 34
CD45-	Cluster of Differentiation 45 negative
C/EBP β	CCAAT/enhancer binding protein beta
CF	Cystic Fibrosis
CNS	Central Nervous System
CO_2	Carbon Dioxide
C:P	Control Placebo
COPD	Chronic Obstructive Pulmonary Disease
CREB	cAMP response element-binding protein
C:S	Control supplement
CXCL9	C-X-C motif chemokine ligand 9

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CXCL10	C-X-C motif chemokine ligand 10
Cys	Cysteine
DAMPs	Danger-associated molecular patterns
DFS	Diabetic foot syndrome
ddH2O	Double distilled water
DHA	Dehydroascorbic acid
DM	Diabetic
DM:P	Diabetic placebo
DM:S	Diabetic supplement
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ERK	Extracellular-signal-regulated kinase
ESL	Endothelial surface layer
EtOH	Ethanol
FBS	Foetal bovine serum
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
FGF-2	Fibroblast growth factor 2
Fig	Figure
FMHS	Faculty of Medicine and Health Science
g	G-force
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
HbA1c	Glycated haemoglobin

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HED	Human equivalent dosage
HGF	Hepatocyte growth factor
HIFα	Hypoxia inducible factor alpha
HO	Hydrogen oxide
HO(x)	Hydrogen oxide radicals
HRP	Horseradish peroxidase
GATA-4	GATA binding protein 4
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GLUT4	Glucose transporter 4
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GSK3	Glycogen synthase kinase 3
HGF	Hepatocyte growth factor
HDL	High density lipoprotein
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IGF-1	Insulin-like growth factor 1
IL1	Interleukin 1
IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IRS1/2	Insulin receptor substrate one and two
JAK	Janus activated kinases
JNK	c-Jun N-terminal kinase
K^+	Potassium ion
Kg/m^2	Kilogram per meter squared

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K_m	Michaelis constant
M	Molar
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
mg	Milligram
mg/dL	Milligram/ decilitre
min	Minutes
MIP1α/β	Macrophage inflammatory protein 1 alpha/ beta
MIP2	Macrophage inflammatory protein 2
mL	Millilitre
mm^2	Millimetre squared
mM	Millimolar
mmol/L	Milli mol per Litre
MSCs	Mesenchymal stem cells
NAC	N-acetyl-l-cysteine
nmol/mL	nanomole/ millilitre
nm	Nanometre
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuroD1	Neuronal differentiation 1
NF-κB	Nuclear factor kappa B
Nkx 2.5	Homeobox protein Nkx 2.5
NO	Nitric Oxide
NO_2	Nitric dioxide
Nox4	NADPH oxidase 4
O_2	Singlet oxygen
O_2^-	Superoxide
ob/ob	Obese

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OD	Optical density
OGTT	Oral glucose tolerance test
OM	Osteogenic induction media
$ONOO^-$	Peroxynitrite
Ox-LDL	Oxidized low-density lipoprotein
%	Percentage
p70S6K	Phosphoprotein 70 ribosomal protein S6 kinase 1
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate Buffered Saline
PDK1	3-phosphoinositide-dependent protein kinase-1
Pen/Strep	Penicillin-Streptomycin
pg	Picogram
PGE2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKB	Protein Kinase B (Akt)
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor
qPCR	Quantitative Polymerase Chain Reaction
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RPM	Revolutions per minute
Sca-1	Stem cells antigen 1
SD	Standard deviation
SDF-1	Stromal cell derived factor 1
sec (s)	Seconds
SGLT2	Sodium-glucose transport protein 2
SGM	Standard growth media

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SOD	Superoxide dismutase
STAT	Signal transducer and activation of transcription
SU	Stellenbosch University
SVCT1	Sodium dependent co-transport 1
SVCT2	Sodium dependent co-transport 2
T2DM	Type 2 Diabetes Mellitus
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TLR	Tol-like receptor
TNFα	Tumour Necrosis Factor alpha
TNFγ	Tumour necrosis factor gamma
TGFβ	Transforming growth factor beta
TTG	Total thiol groups
µL	Microliter
µg	Microgram
U/mL	Units per millilitre
USA	United States of America
VEGF	Vascular endothelial growth factor
WF	Wound Fluid
WT	Wild type

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Chapter 1

Introduction

Anti-diabetic drugs alone are not enough to treat the pathogenesis of Type 2 Diabetes Mellitus (T2DM). Mainly anti-diabetic drugs target hyperglycaemia by treating glucose uptake and insulin sensitivity, however due to non-compliance of patients, hyperglycaemia is often not well controlled and as result the underlying oxidative stress and inflammation threatens. These two underlying factors along with the accumulation of hyperglycaemia induced advanced glycation end products (AGEs) cause cellular dysfunction leading to disease progression and the development of comorbidities (Oguntibeju, 2019). There is thus a need for supplementary interventions to compliment anti-diabetic drugs in combating the whole disorder.

In 2019, diabetes mellitus was the 9th leading cause of death globally and the leading cause of kidney failure, blindness and lower limb amputations (Centres for Disease Control and Prevention, 2020; World Health Organization, 2020). The multifactorial nature of T2DM causes end-organ failure and in addition to the well-known complications several other systems in the body such as the bone marrow is also negatively affected by the pathologic microenvironment. Bone marrow remodelling is prominent in T2DM (Picke *et al.*, 2019) and together with the microenvironmental changes lead to the dysfunction and impairment of bone marrow resident mesenchymal stem cells (MSCs). Dysfunctional MSCs have impaired viability, proliferation and differentiation, and as a result become senescent losing their ability to promote regeneration (Kornicka, Houston and Marycz, 2018). These cells are particularly important for connective tissue formation, wound healing, immunomodulation and the overall maintenance/repair of tissues upon mobilization from the bone marrow (Andrzejewska, Lukomska and Janowski, 2019; Ayala-Cuellar *et al.*, 2019). In T2DM, these MSCs become unharmonized and contribute to the pathologic microenvironment through their altered paracrine signalling that amplifies inflammation instead of restoring homeostasis (Vinci *et al.*, 2020). Thus, protecting these MSCs against dysfunction using therapeutic intervention could prevent disease progression and the development of secondary complications, since the body will be able to heal itself.

In this thesis, the literature review (Chapter 2) will give an overview of T2DM pathogeneses and the development of comorbidities as a result of cellular dysfunction. Furthermore, the importance of MSCs and their vulnerability to impairment as a consequence of T2DM will be discussed. Thereafter, the therapeutic potential of antioxidant intervention to combat low-grade inflammation and oxidative stress will be discussed. It is hypothesized that combined supplementation of obese T2DM animals with two antioxidants namely, N-acetyl-l-cysteine (NAC) and Ascorbic acid-2-phosphate (AAP) will protect bone marrow MSCs against the pathologic microenvironment.

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The aims and objectives of this study is described in Chapter 3 followed by a detailed description of the study design and methodology in Chapter 4. The study consisted of two phases namely: phase 1 (*in vivo*) wherein the animals received oral supplementation of either the combined antioxidants or placebo for a period of 6 weeks and phase 2 (*ex vivo*) during which animals were euthanized, and bone marrow MSCs isolated for *in vitro* assessment of their functional and molecular capacity. The results of the study are presented in Chapter 5 and is followed by a detailed discussion (Chapter 6) and conclusion (Chapter 7) which includes the limitations of the study as well as future perspectives.

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Chapter 2

Literature review

2.1. Type 2 Diabetes Mellitus

2.1.1. Epidemiology

T2DM is a global epidemic with 463 million people currently affected, leading to approximately 1.6 million deaths annually (World Health Organisation, 2020; International Diabetes Federation, 2019). T2DM is in essence a metabolic disorder (dysregulated glucose metabolism) that gives rise to secondary complications such as non-healing wounds, retinopathy, neuropathy, and nephropathy which in turn affect the patient's quality of life and in severe cases can be the main cause of death. In the United States of America (USA) 90-95% of reported diabetes cases are of adult onset T2DM with early mortality predicted to be as high as 60% (Centre for Disease Control, 2020). The high prevalence of T2DM in developed countries can be attributed to modernized human lifestyle wherein risk factors such as unhealthy dietary choices (energy-dense food) and a sedentary lifestyle is prominent. Similarly, the prevalence in developing Sub-Saharan Africa countries is increasing at an alarming rate due to urbanization, with the looming estimate of 47 million people being affected by 2045 (International Diabetes Federation, 2020). An estimated 60% of people in developing countries are however unaware of their T2DM status with deaths attributed to T2DM-related complications in these countries occurring more frequently due to late diagnosis (Pastakia *et al.*, 2017, International Diabetes Federation, 2019). In a cross-sectional study, Mutyambizi Id *et al.* (2019) indicated that the proportion of diagnosed vs. undiagnosed diabetes cases in urban and rural communities is 0.78 and 0.22, respectively. Suggesting that the social process of communities evolving to cities accelerates disease development. This is further exacerbated by socio-economic status since employment, income and education contribute to lifestyle outcome.

Lifestyle choice be it through socio-economic terms, cultural norms, or own will, influences the health status of a person. In Sub-Saharan African countries, cultural norms such as being overweight or obese are seen as a sign of wealth and desirability (Agyemang *et al.*, 2015). Overweight (body mass index > 25kg/m^2) and obesity (body mass index > 30kg/m^2) are however major risk factors for developing T2DM as excessive body weight (fat accumulation) contributes to insulin resistance and glucose intolerance leading to hyperglycaemia (Burhans *et al.*, 2019). Body weight is influenced by diet and physical activity, both of which are equally important to maintain a person's health. In general, moderate physical activity is known to be greatly beneficial and helps reduce the progression of various diseases. On the contrary, a sedentary lifestyle together with a unhealthy diet contributes to the development of metabolic syndrome (increased cholesterol levels, elevated

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triglycerides, lower high-density lipoprotein (HDL) levels and hypertension) that ultimately leads to T2DM and significantly increases the risk for cardiovascular disease and stroke (Pitsavos *et al.*, 2006; Tune *et al.*, 2017).

Other non-modifiable factors that contribute towards the risk of developing T2DM are age and genetic inheritance/ family history. The risk of developing T2DM increases with age and is mainly diagnosed in adults after 45 years of age (American Diabetes Association, 2020). Although adult onset is far more frequent, the disorder is not uncommon in children and adolescents. An increase in the incidence of T2DM amongst the youth have been observed with gender, ethnicity, family history and body weight identified as potential mediators (Candler *et al.*, 2018). Family history and genetic vulnerabilities are known to predispose individuals and increase the risk for the development of T2DM at a younger age. A study done by Scott *et al*. (2013) in individuals of European origin indicated a 2.5 fold increased risk if a person has an immediate family member with T2DM, a 5.1 fold increased risk with biparental history and a 4.7 fold increased risk with T2DM history on maternity side of the family (Scott *et al.*, 2013). This observation was supported by Noh *et al.* (2018) demonstrating that the mean age of T2DM onset is higher in individuals without family history (51 years) than with family history (46 years), confirming family history as mediator for the age of disease onset.

In South Africa health care associated with T2DM management alone costs the country approximately R2.7 billion annually (Erzse *et al.*, 2019), with the majority of affected individuals being of working age. This is a major concern since T2DM takes a toll on a person's health and wellbeing and therefore impacts their ability to work. This disorder is a growing issue and in addition to opposing further development, interventions are needed to prevent the onset of secondary complications in individuals already affected by the disorder.

2.2. Pathophysiology of Type 2 Diabetes Mellitus

The body is comprised of intricate systems that keep the internal environment at stable equilibrium (homeostasis). Unfortunately, homeostasis can be disrupted by disease through altering the internal microenvironment causing it to become pathologic. In T2DM, glucose metabolism is dysregulated causing hyperglycaemia and in turn the accumulation of AGEs, the development of inflammation and excessive oxidative stress.

2.2.1. Insulin signalling, glucose metabolism and hyperglycaemia

Glucose, an important source of energy, is found in abundance in certain types of food and is utilized as fuel after metabolism to power various systems in the body. When food is consumed and digested, glucose is absorbed into the circulation through concentration gradient sodium channels by the brush

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border (microvilli) of the small intestine (jejunum). The increase in blood glucose concentrations is sensed by the β-cells located in the pancreatic islets and stimulates the release of insulin. Insulin is a peptide hormone responsible for the regulation/ absorption of glucose in the liver, adipocytes and skeletal muscle cells (Tokarz, MacDonald and Klip, 2018) and plays a key role in the metabolic signalling pathway.

Insulin binds to the extracellular alpha subunits of transmembrane insulin receptors located on the membrane surface of insulin sensitive cells. Upon ligand binding, the insulin receptor activates its transmembrane/intracellular beta subunits through auto phosphorylation. Next, insulin receptor substrate one and two (IRS1/2) becomes activated by the beta subunits of the insulin receptor through phosphorylation. Phosphoinositide 3-kinase (PI3K) is then summoned from within the cytosol to IRS1/2 and binds to the protein through a p85 subunit (Luo *et al.*, 2005). PI3K in turn phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol-3, 4, 5-triphosphate (PIP3), this process continues until high concentrations of PIP3 recruit 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Protein Kinase B (PKB, also known as AKT), to the plasma membrane (Świderska *et al.*, 2020). PIP3 activates PDK1 which in turn phosphorylates AKT. The activation of AKT facilitates the translocation and embedding of intracellular vesicles containing glucose transporter 4 (GLUT4), a transmembrane protein comprised of 12 alpha subunits, to the plasma membrane (Vargas and Carrillo Sepulveda, 2019). This occurs because AKT deactivates protein AS160 through phosphorylation and by doing so prevents it from inhibiting the translocation of GLUT4. AKT is furthermore also responsible for initiating glycogen synthesis by activating glycogen synthase through inhibition of its inhibitor, glycogen synthase kinase 3 (GSK3) (Lizcano and Alessi, 2002). Upon GLUT4 translocation to the plasma membrane, glucose can enter the cell via a concentration gradient and thereafter be broken down through the biochemical process of glycolysis to produce energy in the form of pyruvate, Adenosine triphosphate (ATP) and Nicotinamide adenine dinucleotide (NADH) (Chaudhry and Varacallo, 2018). The energy produced is used in glycogen synthesis, lipogenesis, protein synthesis and gene expression modification (Świderska *et al.*, 2020).

Negative feedback of the insulin mediated pathway is regulated by the duration insulin is bound to the insulin receptor. It is controlled by the glucose concentration in the blood as well as through phosphoprotein 70 ribosomal protein S6 kinase 1 (p70S6K), an intracellular protein which phosphorylates and inhibits IRS1/2 (Zhang *et al.*, 2008). Leading to the retraction of GLUT4 transmembrane proteins from the plasma membrane back into the cytosol. Another intrinsic mechanism to stop insulin signalling from allowing glucose to enter the cell is by PTEN inhibition of PI3K which in turn inhibits the activation of PKB (Hemmings and Restuccia, 2012).

The insulin signalling pathway becomes disrupted in T2DM as result of excessive glucose accumulation in circulation leading to glucose toxicity. The glucose toxicity theory propose that an

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elevated postprandial glucose level over a prolonged period leads to pancreatic β-cell functional and structural impairment (Robertson *et al.*, 2003). Resulting in reduced β-cell insulin secretion and therefore insulin resistance as consequence of reduced insulin action. Robertson *et al.* (2003) furthermore suggests that glucose toxicity alters insulin gene expression resulting in irreversible damage to insulin production, secretion, and action.

This dysregulation of glucose metabolism therefore results in a further increase in blood glucose levels, known as hyperglycaemia. T2DM-associated hyperglycaemia develops when insulin sensitive cells become desensitized to insulin (insulin resistance) causing the pancreas to over produce insulin (hyperinsulinemia) in attempt to compensate for the increased blood glucose levels (Cerf, 2013). This results in pancreatic β-cell hyperplasia, hypertrophy and eventual dysfunction and/or cell death (Kawahito, Kitahata and Oshita, 2009; Cerf, 2013). Given that the insulin sensitive cells are starved from glucose, important processes such as glycolysis and glycogen synthesis cannot occur to convert glucose into a suitable energy form. To compensate, gluconeogenesis takes place and the hepatic release of glucose into circulation occurs, worsening the already existing hyperglycaemia (Rines *et al.*, 2016). When hyperglycaemia is left untreated, glucose toxicity contributes to the development of serious co-morbidities due to cellular dysfunction caused by the accumulation of AGEs, persistent inflammation and increased oxidative stress (Campos, 2012).

2.2.2. AGEs, systemic inflammation, and oxidative stress

The formation of AGEs are a normal process in the human body but excessive AGEs accumulate during prolonged hyperglycaemia and/or ageing (Omsland *et al.*, 2006) and disrupts homeostasis. Lipids, proteins and deoxyribonucleic acid (DNA) are especially vulnerable to undergo glycation (sugar binding) (Ott *et al.*, 2014). Under T2DM conditions AGEs accumulate throughout the body causing cellular damage in various tissues and organs. AGEs bind to the receptor for advanced glycation end products (RAGE) and by doing so activates various downstream signalling cascades. These trigger the formation of reactive oxygen species (ROS) and promote proinflammatory cytokine production through activation of the nuclear transcription factor kappa B (NF-κB) signalling pathway (Piperi *et al.*, 2015). The upregulation of RAGE is influenced by the concentration of the accumulating AGEs. Upon AGE/RAGE activation, the transmembrane receptor activates protein kinase C (PKC), a protein family that signals various targets including extracellular signal regulated kinase (ERK), PKB, p38 mitogen-activated protein kinase (MAPK), NADPH-oxidase and NF-κB (Geraldes and King, 2010). The activation of these downstream pathways together with AGE accumulation result in sustained RAGE activation leading to increased formation of free radicals and development of oxidative stress.

Oxidative stress is the imbalance between free radicals and free radical scavenging molecules, in favour of the former. Free radicals such as ROS are generated in the mitochondria via oxidative

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metabolism or by enzymatic generation through NADPH-oxidase in complex I and III (Valko *et al.*, 2007). It can also be generated by AGEs glycating proteins of the respiratory chain causing dysregulation. Oxidative stress is a core factor in the development of intracellular damage to proteins, lipids and DNA that in turn cause organelle damage resulting in cell death (Ott *et al.*, 2014). Excessive ROS in the cytosol attack the lipid bilayer of the cell membrane leading to membrane permeability and can also cause mitochondrial fragmentation.

In T2DM sustained RAGE activation together with oxidative stress, therefore, amplifies the destructive signalling cascade leading to DNA damage and programmed cell death (apoptosis) (Liu *et al.*, 2020). Cellular damage and apoptosis ultimately lead to tissue dysfunction and the development of secondary complications. In addition to the RAGE-associated activation of proinflammatory NF-κB signalling, cellular death also promotes inflammation through the release of danger associated molecular patterns (DAMPs) (Feldman, Rotter-Maskowitz and Okun, 2015). These molecules are in turn recognized by innate immune cells through toll-like receptors (TLRs) located on the membranes of these cells. Over expression of specific TLRs have been reported in diabetic patients (Tanti *et al.*, 2013). Reacting to the DAMPs released, immune cells such as macrophages, mast cells, eosinophils, neutrophils, natural killer cells and T-helper cells secrete inflammatory cytokines such as Interleukin 1 (IL1), Interleukin 6 (IL6) and Tumour Necrosis Factor alpha (TNFα) (Chen *et al.*, 2018). Under normal/healthy circumstances inflammation will clear and repair of tissue will take place, however in T2DM the inflammation promoting pathways are not switched off resulting in a persistent response and secondary damage. The inflammation promoting pathways involved include NF-κB, c-Jun N-terminal kinase (JNK), JAK/STAT, and MAPK (Castro, Macedo-de la Concha and Pantoja-Meléndez, 2017). Together, these pathways regulate DNA transcription, cell survival, cytokine production, proliferation, apoptosis, autophagy, viability, migration, and play a role in the innate immune response (Oeckinghaus and Ghosh, 2009; Liu *et al.*, 2017).

2.3. Diabetic complications and co-morbidities

The pathologic microenvironment as discussed above (Section 2.2) is the main cause of cellular dysfunction with endothelial damage being prominent in secondary complications. The endothelium is important in homeostasis, tissue maintenance and repair. A prolonged pathologic state induced by the altered microenvironment affects micro- and macrovascular health resulting in angiopathy, hypoperfusion, hypoxia and neuropathy, (throughout the body, but especially within bone marrow) which in turn causes tissue damage and prevents the mobilization of stem/progenitor cells (mobilopathy) leading to the development of various comorbidities (Table 2.1).

Pathologies that arise as result of microvascular complications include nephropathy, peripheral neuropathy, retinopathy, non-healing wounds and bone marrow remodelling. Cardiovascular

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disease such as stroke, cardiomyopathy and atherosclerosis are associated with macrovascular complications. Table 2.1 summarizes the various cell types affected by T2DM pathogenesis and the associated comorbidity. If not treated, dysfunction of these different cell types is irreversible and together with mobilopathy, tissues cannot be repaired to restore normal function and maintain homeostasis. Vascular health is dependent on the inner lining (endothelium layer) of the vessels that control vasodilation, vasoconstriction and vessel elasticity (Sandoo *et al.*, 2010). When the endothelium layer is compromised by hyperglycaemia and repair does not occur due to the lack of stem/progenitor cell mobilization and dysfunction, it allows for the development of vascular disease (Hu *et al.*, 2018). Furthermore, the broken endothelial barrier causes the development of edema that in turn stimulates the immune system and activates an inflammatory response which consequently leads to further tissue damage.

The quality of life is severely affected in T2DM patients who develop co-morbidities. The Australian Institute of Health and Welfare reported that 11% of Australians living with diabetes are disabled and 59% of Australians receiving treatment for diabetic co-morbidities experience restricted daily activity (Australian Institute of Health and Welfare, 2013). Common disabilities caused by diabetes include vision loss which is the leading cause of blindness worldwide (International Diabetes Federation, 2020) and mobility limitations due to lower limb amputations (Yusof *et al.*, 2019). T2DM patients who develop diabetic foot ulcers or non-healing wounds are most likely to face reoccurring infections, hospitalisation and amputation (Beyaz, Güler and Bağır, 2017; Wensley, Kerry and Rayman, 2018). In a study by Prompers *et al.* (2007), 49% of diabetic patients were reported to have developed non healing wounds and 58% of reported wounds were infected. Ingelfinger *et al.* (2017) estimates the reoccurrence of diabetic foot ulcers after a year of remission to be approximately 30% and increases to 65% within 5 years. The life expectancy of an amputee due to untreatable foot ulcers is less than 3 years (Beyaz, Güler and Bağır, 2017). Although, infections of non-healing wounds are a major cause of death among patients with T2DM, the leading cause of death related to diabetes is cardiovascular disease. According to the International Diabetes Federation, a third to a half of deaths related to diabetes are due to cardiovascular disease (International Diabetes Federation, 2020).

Targeting oxidative damage and inflammation using antioxidant and/or anti-inflammatory agents of natural and/or synthetic origins could thus potentially prevent the development of secondary complications in T2DM patients and by doing so might improve quality of life.

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Table 2.1 Cellular dysfunction and co-morbidity development in T2DM pathogenesis.

Cell type(s) and function	Cellular dysfunction	Co-morbidity	References
Microvascular complications			
Dorsal root ganglion sensory neurons **Convey sensory information from internal and external stimuli to CNS.**	• Neuronal atrophy • Altered gene expression	Peripheral neuropathy	(Hidaka *et al.*, 2013; Fujimaki *et al.*, 2015; Kobayashi and Zochodne, 2018; Chen *et al.*, 2019)
Neural stem cells **Generate neuronal and glial cells**	• Inhibition of NeuroD1 • Decreased neurogenesis • Increased ROS • Decreased migration • Decreased proliferation		

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Macrophages **Phagocytic cell responsible for detecting and eliminating harmful organisms.**	• Increased proinflammatory cytokine secretion (TNFα, IL6) • TNF-γ activated M1 macrophage • Impaired efferocytosis	Non-healing wounds	(Wall *et al.*, 2008; Khanna *et al.*, 2010; Shin and Peterson, 2012)
Fibroblasts **Synthesize extracellular matrix components.**	• Decreased proliferation • Premature senescence • Decreased chemokine expression		
Mesenchymal stem cells **Generate connective tissue, modulate immune system.**	• Impaired regenerative capacity • Decreased proliferation • Reduced viability • Increased apoptosis • Increased proinflammatory cytokine secretion • Altered transcriptional profile		

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Müller cells (glia of retina) **Regulate and maintain vascular and neural function in retina.**	• Decreased glutamate transport and glutamine synthesis • Decreased K^+ uptake Hypertrophic (macular edema) • Increased AGEs formation • Increased proinflammatory cytokine secretion (IL1β, IL6, TNFα) • Pyroptosis	Retinopathy	(Coughlin, Feenstra and Mohr, 2017)
Glomerular endothelial cells **Regulates blood filtration, leukocytes trafficking and secretes glycocalyx.**	• Decreased ESL • Albuminuria	Nephropathy	(Fu *et al.*, 2015)
Hepatocytes **Synthesize and store protein, breakdown fat and store energy,**	• Increased TNFα secretion • Decreased adiponectin secretion • Lipid peroxidation • Protein cross-linkage • Cell death	Non-alcoholic fatty liver disease	(Tolman *et al.*, 2007)

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Macrovascular complications			
Cardiac progenitor cells **Regenerate cardiac tissue.**	• ROS induced apoptosis • Premature myocyte senescence and death • Aging and failure	Cardiomyopathy	(Rota *et al.*, 2006)
Vascular endothelial cells **Vasomotor control and maintain structural and functional integrity of vessels.**	• Decreased NO secretion • Increased fatty acid production • Atherosclerosis • Thrombosis	Stroke	(Chen, Ovbiagele and Feng, 2016; Hu *et al.*, 2018)
Endothelial progenitor cells **Repair endothelium via angiogenesis and secrete VEGF, HGF, IL8, eNOS.**	• Reduced EPCs • Decreased NO production Supressed VEGF, SDF-1 secretion • Decreased cell adhesion • Decreased cell migration		

Footnote: The table indicates various cellular dysfunctions from underlying hypoxia, hyperglycaemia and neuropathy associated with diabetic co-morbidities. **Abbreviations:** CNS, Central nervous system; K^+, Potassium ion; TNF-α, Tumour necrosis factor alpha; IL, Interleukin; TNF-γ, Tumour necrosis factor gamma; AGEs, Advanced Glycation End products; ESL, Endothelial surface layer; NO, Nitric Oxide; EPCs, Endothelial progenitor cells; VEGF, Vascular endothelial growth factor; HGF, Hepatocyte growth factor; SDF-1, stromal cell-derived factor 1

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2.4. Murine models used for T2DM research

Murine and rodent models are valuable and widely used to study diabetes and its related research as these animals are genetically similar to humans. The most abundantly used mouse strains in T2DM research are ob/ob, db/db, KKAy and Tsumura Suzuki Obese Diabetes (TSOD) due to the disorder development in these animals (King, 2012; Yazdi, Clee and Meyre, 2015). The ob/ob and db/db strains (monogenic) share the same phenotypical attributes including obesity, hyperinsulinemia, and hyperglycaemia however their hyperglycaemia differs in severity (Fang *et al.*, 2019). Both strains are used in pharmaceutical studies where the aim is to target β-cell dysfunction as well as diabetic comorbidity research including neuropathy, non-healing wounds and retinopathy (Katsuda *et al.*, 2013; Wang, Chandrasekera and Pippin, 2014). Polygenic mouse strains (KKAy and TSOD) are considered resourceful in diabetic complication studies as their phenotypes cover diabetic characteristics including multi-organ failure. The KKAy mouse strain for example is used in diabetic nephropathy research due to histologically observed renal failure (Tomino, 2012). The TSOD mouse strain is considered an excellent model for peripheral neuropathy research. A study by Iizuka *et al.*(2005) reported histological abnormalities in the pancreatic islands, kidney and sciatic nerve of TSOD mice compared to non-diabetic control mice.

2.5. Mesenchymal stem cell dysfunction in Type 2 Diabetes Mellitus

2.5.1. Mesenchymal stem cell physiology

Mesenchymal stem cells (MSCs) originate from mesenchyme tissue that form part of the mesoderm; a germ layer that is responsible for the formation of organs during early development. The mesenchyme on the other hand, is responsible for the formation of connective tissue, bone and cartilage (Panawala, 2018). Most commonly MSCs reside in the bone marrow of long bones (femur) however, various studies have discovered MSCs in adipose tissue, skin, umbilical cord tissue, amniotic fluid, peripheral blood, cord blood, placenta, dental pulp, foetal liver and lung tissue (Mafi *et al.*, 2011; Stanko *et al.*, 2014; Rajabzadeh, Fathi and Farahzadi, 2019; Ahmed Al-Anazi, K. Al-Anazi and M. Al-Jasser, 2020). These cells are characterised as having fibroblast like morphology, express cluster of differentiation (CD) surface markers CD90, CD105, CD73, CD34, CD45-, stem cells antigen (Sca-1) and when cultured in standard cell culture conditions are plastic adherent (Rilo *et al.*, 2017; Mehrbani Azar *et al.*, 2020). By possessing multipotency, MSCs have regenerate capacity to promote cellular growth through paracrine signalling, become specialized through differentiation, proliferate to increase cell number, modulate the immune system and maintain tissue homeostasis (Spees, Lee and Gregory, 2016; Moreira, Kahlenberg and Hornsby, 2017; Ayala-Cuellar *et al.*, 2019). It should be noted that MSCs specific function is dependent on their tissue of

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origin (Caplan, 2016; Mastrolia *et al.*, 2019). Bone marrow resident (BM) MSCs are highly reactive to their environment thus, any change to the chemical (DAMPs) or structural (tissue injury) composition of their niche will activate and mobilize dormant MSCs. To maintain tissue homeostasis at the damaged site, MSC secrete various bioactive molecules to suppress an aggressive inflammatory response and simultaneously induce a pro-regenerative environment (Caplan, 2016).

In the bone marrow MSCs (stromal) reside in a niche situated within bone alveolar matrices. These cells modulate this environment through their interaction with surrounding factors such as extracellular matrix (ECM), other cell types, signalling factors and nutrient supply (Pennings, Liu and Qian, 2018). The niche keeps MSCs passive and at low metabolic state until their function is required at a specific location in the body (Andrzejewska, Lukomska and Janowski, 2019). Cellular support comes from the ECM which provide collagen and fibronectin fibrous proteins along with adhesion molecules to provide structure and anchor cells within their niche (Kusindarta and Wihadmadyatami, 2018). The ECM plays a role in MSC proliferation and differentiation (Park *et al.*, 2011). Bone marrow remodelling as result of T2DM therefore also affects MSC function.

As previously mentioned, the MSCs share the niche environment with other cells. These include haematopoietic stem cells that are responsible for blood cell production. What makes these populations important is the ability of MSCs to modulate haematopoietic stem cells through their immunomodulatory function. Interaction of MSCs with various immune cells are mostly through paracrine signalling via cell to cell communication by supressing, activating or inhibiting the functions of the immune cells (Pennings, Liu and Qian, 2018). Signal dependent activation of MSCs drive them into MSC1 (proinflammatory) or MSC2 (anti-inflammatory) phenotype (Waterman *et al.*, 2010). Proinflammatory phenotype MSC is activated in the presence of DAMPs secretion post tissue injury. In this state MSCs secrete various proinflammatory chemokines that ultimately signal immune cells to migrate to the site of injury. It has been reported that proinflammatory chemokines secreted by the MSC1 phenotype in the form of extracellular vesicles or exosomes are macrophage inflammatory protein (MIP1) and chemokine motif ligands 9 and 10 (CXCL 9 and CXCL10) (Bernardo and Fibbe, 2013).

Through immune surveillance MSCs react to a proinflammatory microenvironment by changing their phenotype to MSC2. In this state it has been reported that MSCs promote regeneration, signal macrophages to change phenotype from phagocytic to pro-regenerative and supress the overly active immune system to avoid additional damage. To accomplish this MSCs secrete transforming growth factor beta (TGF-β), NO, prostaglandin E2 (PGE2), indolamine 2,3 dioxygenase (IDO) and IL6 (Duffy *et al.*, 2011; Bernardo and Fibbe, 2013; Ayala-Cuellar *et al.*, 2019). Furthermore, the anti-inflammatory MSC phenotype encourages the release of anti-inflammatory IL10. However, in T2DM dysfunctional MSCs have been shown to favour inflammation by releasing additional

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proinflammatory cytokines instead of promoting regeneration (Rodrigues *et al.*, 2015; van de Vyver *et al.*, 2016; Fijany *et al.*, 2019).

The pathophysiology of T2DM affects all tissue types throughout the body however some systems are more susceptible to damage than others. Bone marrow remodelling and MSC impairment is prominent in T2DM patients (Kume *et al.*, 2005; Kornicka, Houston and Marycz, 2018). Under T2DM conditions MSCs immunomodulatory function is skewed to promote inflammation instead of signalling to relieve it. In this regard MSC phenotype will remain as MSC1 promoting phagocytosis instead of tissue repair. The dysfunctional MSCs further compromises bone architecture due to their impaired osteogenic differentiation and increased adipogenic differentiation capacity in T2DM. Bone loss as a result of T2DM is attributed to increased fat deposition, osteoblast apoptosis and increased bone resorption by osteoclasts (Catalfamo *et al.*, 2013; Sanches, Daher Vianna and De Carvalho Barreto, 2017). The altered bone marrow microenvironment retains cells from migrating into the circulation (mobilopathy) to where their functions are needed most. Subsequently, their dysfunction is worsened by the constant exposure to the pathologic microenvironment within their remodelled bone marrow niche. Therefore, restoring MSC function and/or protecting them against the degrading T2DM environment is an important step in maintaining tissue homeostasis and should be taken into consideration when treating T2DM patients.

2.5.2. Mesenchymal stem cell dysfunction

There is a great deal of evidence showing that T2DM affects the multi-functional potency of MSCs. A study by Al-Qarakhli *et al.* (2019) investigated the effects of hyperglycaemia (25mM) and normoglycaemia (5.5mM) on MSCs isolated from endosteal niche lining compact bone (CB-MSCs) in male Wistar rats *in vitro*. The authors observed that mainly osteogenic and adipogenic differentiation capabilities of CB-MSCs were affected by hyperglycaemia and that it impairs the bone repair capabilities of CB-MSCs (Al-Qarakhli *et al.*, 2019). Furthermore, MSC functional potency is affected by inflammation and premature aging as observed by Kizilay Mancini *et al.* (2015) in donor MSCs from patients who have atherosclerosis and T2DM. The study observed that MSCs ability to suppress T-cells was impaired in these patients compared to age-matched controls (Kizilay Mancini *et al.*, 2015). The pathogenesis of T2DM also affects MSC number. In a human bone marrow study MSCs were assessed for cell number and phenotype characteristics. The study indicated that MSCs from T2DM donors had a decreased MSC number compared to age matched healthy controls (Cassidy *et al.*, 2020). This is supported by a study done by Shin and Peterson (2012) indicating that MSCs harvested from bone marrow in a diabetic animal model lost cellular number when cultured *in vitro*.

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Similarly, MSCs isolated from Streptozotocin-induced type 1 diabetic rats had impaired growth rate upon expansion in culture and expressed reduced angiogenic factors (Kim *et al.*, 2015). Another streptozotocin-induced study by Khan *et al.* (2011) studied the effects of insulin-like growth factor (IGF-1) and fibroblast growth factor (FGF-2) on MSCs. The results showed upregulation of IGF-1, FGF-2, Akt, GATA binding protein 4 (GATA-4), and homeobox protein Nkx 2.5 which in untreated MSCs were downregulated.

Recent studies performed by our laboratory have shown that oxidative stress and inflammation in T2DM change the phenotype of MSCs by targeting the genetic machinery inside cells. Dysfunctional MSCs take on a more proinflammatory profile and are known to produce and secrete copious amounts of proinflammatory cytokines consequently worsening the existing highly inflamed environment (Mehrbani Azar *et al.*, 2018). The intracellular mechanism responsible for skewing immunomodulatory property of MSCs is the IL6/STAT3 pathway (van de Vyver *et al.*, 2016). Resulting in transcription and secretion of proinflammatory cytokines such as IL6 and TNFα (Mehrbani Azar *et al.*, 2020).

Other than skewing the immunomodulatory properties of MSCs, oxidative stress and inflammation contribute to impaired migration, proliferation, differentiation, and viability. Causing multipotent MSCs to lose the ability to self-regenerate and becoming senescent. Taken together, these studies confirm that diabetic MSCs are dysfunctional and remain impaired even if introduced to healthy cell culture environment.

2.6. Pharmaceutical intervention

2.6.1. Existing therapies for Type 2 Diabetes Mellitus treatment

Anti-diabetic drugs are introduced when lifestyle intervention and modification is not enough to counteract the progression of T2DM. Administration of anti-diabetic drugs are dependent on the severity of the disorder, administration method, cost, age of patient and comorbidities (Chaudhury *et al.*, 2017). These drugs are divided into first-, second- and third-line administration and depending on the severity of the disorder dual or multi therapy is possible. Biguanides (metformin) are used as first line drug and second- and third-line drugs are insulin replacement therapy, insulin secretagogues, alpha-glucosidase inhibitors, thiazolidinediones, dipeptidyl/peptidase-4 inhibitors, sulfonylureas, sodium glucose transport protein (SGLT2)-inhibitors and meglitinides to counteract disease progression. The main function of these drugs is to reduce hyperglycaemia and their specific mechanisms of action either target glucose by controlling glucose uptake, digestion, and excretion, or by targeting insulin sensitivity, production, and secretion. These drugs primary objective is to regulate glucose metabolism but has the unfortunate disadvantage of not directly targeting oxidative

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stress and inflammation, which play a central role in T2DM pathology, leaving the patient with the possibility of comorbidity development.

The change in microenvironment brought by anti-diabetic drugs does not sufficiently counteract inflammation and oxidative stress as well as in reducing hyperglycaemia to control glucose metabolism. As result the endogenous MSCs are still exposed to the pathologic microenvironment within their bone marrow niche and become dysfunctional. A supplementary treatment is thus necessary to work in synergy with anti-diabetic drugs to holistically treat T2DM. Most antioxidants used in treating diabetes are used to manage the disorder. Investigation into medicinal plants have been an ongoing research area. Medicinal plants (*Spilanthes Africana, Portulaca oleracea* and *Sida rhombifolia*) from the West Cameroon region have been used to manage diabetes as these plants have hypoglycaemic, antioxidant, and hypolipidemic properties (Moukette *et al.*, 2017). In South Africa, a variety of different indigenous plant species have shown medicinal properties with some familiar species used for their antidiabetic attribute. These include silver cluster-leaf (*Terminalia sericea), holy thistle (Centaurea benedicta),* and Cape periwinkle (*Catharanthus roseus)* (Afolayan and Sunmonu, 2010; van Wyk, van Oudtshoorn and Gericke, 2017). The inherent polyphenolic compounds that naturally occur in plants have been extracted from the plant such as resveratrol. A compound used in the treatment of diabetic foot syndrome (DFS) (Bashmakov, Assaad-Khalil and Petyaev, 2011). Since medicinal plants have antioxidant potential which is beneficial for disease management this leaves the floor open for antioxidants to be used in their pure or synthetic form because much like medicinal plants antioxidants have also shown therapeutic potential to treat diabetes (Bajaj and Khan, 2012; Rajendiran, Packirisamy and Gunasekaran, 2018). Especially through counteracting oxidative stress and oxidative stress related inflammation (Johansen *et al.*, 2005). However, before antioxidants can be used factors such as toxicity, physiological effects and pharmacological parameters of antioxidants must first be investigated before these treatments can be used to treat diabetes.

2.6.2. Antioxidants

Antioxidants are compounds of natural and synthetic origin that inhibit or counteract oxidation. Naturally occurring antioxidants can be produced endogenously as part of various signalling pathways in the body or consumed as part of our diet from exogenous plant sources (Stokes, Belay and Ko, 2020). Evidence suggest that antioxidants are beneficial for human health and are increasingly seen in the literature as a potential complementary therapeutic strategy to combat diseases associated with oxidative stress and inflammation (Arulselvan *et al.*, 2016; Hussain *et al.*, 2016).

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Categorically antioxidants are divided into two groups namely, enzymatic, and non-enzymatic. Enzymatic antioxidants role in the body is to break down free radicals turning the end product (hydrogen peroxide) into water (Zainol Haida, 2019). Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase are examples of enzymatic antioxidants (Jeeva *et al.*, 2015). On the other hand, non-enzymatic antioxidants include glutathione (GSH), vitamins A, C and E, polyphenols, flavonoids, flavanols and carotenoids (National Center for Complementary and Integrative Health, 2013), are responsible for disrupting free radical chain reactions to halt the formation of free radicals (Nimse and Pal, 2015). Synthetic antioxidant compounds play a role in both enzymatic and non-enzymatic antioxidant categories as it can augment enzyme activity in catalysing radical formation as well as interrupt radical chain reactions (Moussa, Judeh and Ahmed, 2019). For the purpose of this review the synthetic antioxidants NAC and AAP will be discussed further.

2.6.2.1. NAC significance and mechanism of action

The synthetic compound NAC is derived from the amino acid cysteine (Cys) and is available "over-the-counter" without requiring a prescription. NAC have various direct and indirect functions which can be attributed to its chemical structure (Fig. 2.1). In addition to its antioxidant capacity, the versatile nature of NAC allows it to be used for a variety of applications the most common of which include a countermeasure to paracetamol overdose and a mucolytic agent (disulphide breaking) in cold/flu, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (Waring, 2012; Rushworth and Megson, 2013; Aldini *et al.*, 2018). NAC is commonly administered intravenously, given as oral supplement or inhaled in tolerable dosages of up to 1200mg or lower without the appearance of adverse effects (Millea, 2009).

Noted in NACs chemical structure is a thiol group responsible for NACs direct antioxidant effect in serving as a ROS scavenger molecule to reduce oxidative stress and inflammation (Santus *et al.*, 2014). NAC is a potent antioxidant as it can directly scavenge oxidants especially oxygen free radical species such as singlet oxygen. However there have been controversies in the literature whether NAC functions better as direct or indirect antioxidant (Aldini *et al.*, 2018; Ezeriņa *et al.*, 2018). Ezeriņa *et al.* (2018) demonstrated NAC to be a weak antioxidant when used alone *in vitro* and suggest that NAC's use as indirect antioxidant is much more efficient in seizing oxidants in cells. In support of this, a review article by Aldini *et al.* (2018) investigated NAC's reaction rate to reduce various oxidants and observed a weak effect in reducing hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), superoxide (O_2^-) and hydroxyl radical (OH^-) whereas halohydrin (HO(x)) and nitrogen dioxide (NO_2) was successfully reduced. The indirect properties of NAC are attributed to its chemical structure containing Cys, an important amino acid that serves as precursor to replenish GSH levels. GSH is a powerful antioxidant consisting of Cys, glycine and glutamic acid and responsible for keeping

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reactive oxygen species at bay that contribute to cellular component destruction (Demirkol and Ercal, 2012). GSH is synthesized by reducing oxidised glutathione (GSSG) through the enzyme glutathione reductase (Santus *et al.*, 2014). Reduced GSH is an alarming status and could mean serious disorder in the body. Reduced GSH levels in T2DM patients have been reported (Lutchmansingh *et al.*, 2018).

NACs ability to enhance intracellular antioxidants improves mitochondria function in disease. Mitochondrial function is important for ATP production and β-Cell insulin secretion and regulation (Kwak *et al.*, 2010). Mitochondria dysfunction plays a role in insulin resistance, decreased oxidative phosphorylation, pancreatic β-Cell dysfunction, diabetes pathology and risk factors have a toll on mitochondrial dysfunction. By increasing intracellular antioxidants NAC indirectly increases the production of oxidant reducing enzymes SOD and glutathione reductase (GR) (Dludla, Orlando, *et al.*, 2019). Oxidative stress markers in serum of patients with diabetic neuropathy taking NAC as supplement showed decreased total antioxidant capacity (TAC), total thiol groups (TTG), catalase activity (CAT), lipid peroxidation (MDA), NO and increased SOD and GPx markers compared to baseline measurements (Heidari *et al.*, 2019). Conflicting literature exists on the ability of NAC to influence CAT activity. CAT is an antioxidant and should be increased by NAC administration. A study by Kamboj, Vasishta and Sandhir (2010) demonstrated NAC to increase CAT activity in diabetes induced (Streptozotocin) Wistar rats with neuropathy.

The functions of NAC allow it to be explored in different settings such as diabetes and the associated secondary complications of the disorder. Investigations of NAC treatment in diabetes thus far have explored the compound to inhibit lipid accumulation, improve insulin sensitivity and target and diminish inflammatory markers involved in the inflammatory response (Falach-Malik *et al.*, 2016; Dludla, Mazibuko-Mbeje, *et al.*, 2019). Thus, NAC interacts with peroxisome proliferator activated receptor gamma (PPARγ), CCAAt/enhancer binding protein beta (C/EBP β), modulates IRS-1 in the PI3K/PKB pathway and suppress PAI-1, TNFα, IL8, NF-kB and MCP-1. A study by Ma, Gao & Liu (2016) observed NAC to reduce hyperglycaemia and hyperinsulinemia as a result of enhanced adiponectin and decreased macrophage infiltration in a high-fat died induced obese model. The capacity of NAC expands to the effect of modulating hormones such as adiponectin and leptin responsible for glucose uptake and fatty acid breakdown and controlling energy expenditure by inhibiting hunger, respectively (Raffaele *et al.*, 2018). Furthermore, NAC supplementation in an animal neuropathy model defended nerve deterioration by suppressing apoptotic factors cytochrome C and active caspase 3, and also attenuated oxidative stress by reducing lipid peroxidation and thiol groups (Kamboj, Vasishta and Sandhir, 2010).

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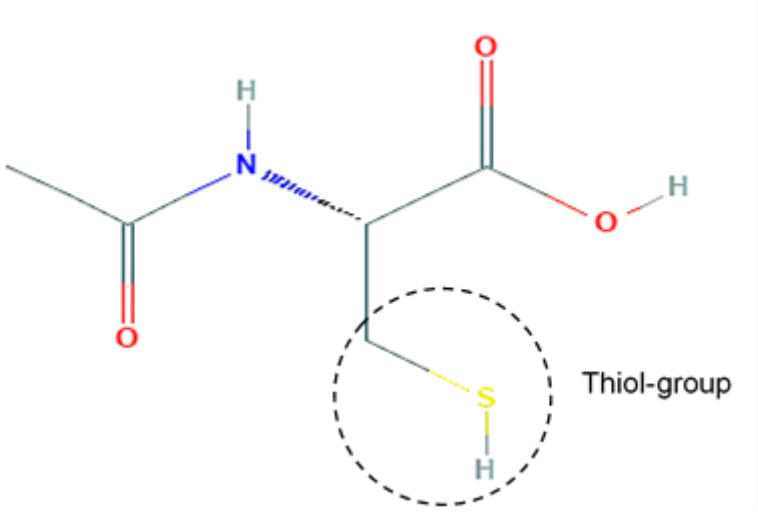

Figure 2.1 N-acetyl-l-cysteine chemical structure. Encircled in the image is the thiol group that benefits the direct antioxidant effect of N-acetyl-l-cysteine. (Image obtained from PubChem, ID: 12035).

2.6.2.2. AAP significance and mechanism of action

The ascorbic acid (vitamin C) derivative AAP is a precursor for ascorbate, an important molecule used by the body in a variety of ways. Refer to figure 2.2 for a representation of AAP used in this study. It is also long-acting and oxidation-resistant (Li, Sun and Pang, 2015). The compound is water soluble which facilitate the transport of AAP molecules to the surrounding tissue. Furthermore, AAP is extremely important to the human body not only for its versatile function but also as an essential nutrient. The human body cannot synthesize AAP due to a lack of l-gluconolactone oxidase and thus it becomes a dietary requirement (Drouin, Godin and Page, 2011). Food's rich in ascorbic acid are mostly fruits and vegetables however AAP can also be ingested as a supplement in various concentrations.

After ascorbic acid is ingested it is absorbed by enterocytes in the intestine through secondary active transport by ascorbate specific sodium dependent co-transporters 1 and 2 (SVCT1 and SVCT2) located on the basolateral membrane (Wilson, 2005). As ascorbic acid moves across the basolateral membrane through SVCT1/2 it is oxidized to ascorbate ions which in turn moves out of enterocytes into the circulation by anion channels. Inside the circulation ascorbate molecules either move freely or are bound to albumin for transport. From the circulation ascorbate is transported by simple diffusion or active transport to various tissues where it is utilized (May and Harrison, 2013). Most cells in the human body absorb ascorbate by SVCT2 transporters from the extracellular fluid with only endothelial cells to absorb ascorbate through SVCT1 transporters (Wilson, 2005). Inside the cell, ascorbate is stored in the cytosol and reversely reduced to dehydroascorbic acid (DHA) for use in cellular processes. DHA molecules are also absorbed by enterocytes through GLUT1/3 and released into the circulation. Red blood cells, neutrophils and lymphocytes are of the few types of

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cells that only absorb and utilize DHA. Thus, ascorbic acid in the reduced form of DHA is important to maintain healthy immune cells and modulate the immune system (Carr and Maggini, 2017). DHA is absorbed more rapidly than ascorbate and is converted back to ascorbate in tissues. Further catabolism of DHA results in oxalic acid, threosone and erythrulose end products. Thus, the two active molecules after AAP metabolism are ascorbate and DHA.

Ascorbic acid has many biological functions including serving as cofactor in collagen formation by facilitating the merging of proline and lysine by hydroxylation to form procollagen (Pinnell, 1985; Santosh and David, 2017). Collagen is an abundant protein component of the body providing structure to the skin, bones, tendons, ligaments, blood vessels and teeth (Christie-David, Girgis and Gunton, 2015; Jennings, 2020). Other than forming part of the process synthesizing collagen, ascorbic acid is also involved in the maturation, secretion and degradation of the protein in the body's wound healing phases hemostasis, inflammatory, proliferation and maturation (Moores, 2013; Bikker *et al.*, 2016).

Various metabolic pathways involve ascorbic acid as facilitator (cofactor/ chelator) in providing metabolic outcome (product). Tyrosine oxidation by ascorbic acid in the metabolism of this amino acid is important for neurotransmitter formation (La Du and Zannoni, 1961). In carbohydrate metabolism, ascorbic acid can restore the fundamental energy supply to the body in ascorbic deficient environment (Banerjee, 1943). Another common and important involvement of ascorbic acid is its role as chelator in iron metabolism with the goal to maintain homeostasis between iron species (Fe^{3+} to Fe^{2+}) by preventing iron toxicity in the body (Lane and Richardson, 2014).

Ascorbate ions and DHA are specifically important in redox reactions such as maintaining the balance between antioxidants and oxidants by acting as ROS scavenging agents inside cells. Most commonly ascorbic acid reduces mitochondrial generated singlet oxygen species (O_2), O_2^- and OH^- (Aniello *et al.*, 2017). And thus, ascorbic acid can reduce oxidative stress. Furthermore, ascorbic acid has the ability to regenerate other antioxidants such as α- tocopherol a form of vitamin E responsible for inhibiting lipid peroxidation of lipid bilayers (Traber and Stevens, 2011). Osteoblasts benefits from ascorbic acid as it promotes gene expression of bone matrix formation by osteoblasts to maintain bone structure (Aghajanian *et al.*, 2015). As mentioned before lymphocyte function is augmented by ascorbic acid that help rid the body of viruses, bacteria and toxins (Carr and Maggini, 2017).

Dispersion of ascorbic acid into the various tissues after absorption results in different thresholds of the molecule throughout the body. The circulation stores the least amount and the tissue the most. Evidence in the literature suggests that AAP is best absorbed in small amounts as AAP absorption largely by the gastrointestinal tract and to a lesser extent by the inner mouth lining and renal system

quickly reach saturation levels (Wilson, 2005; Santosh and David, 2017). AAP is recycled by the body to maintain optimum concentrations by reabsorption by renal tubules however when systemic thresholds are saturated AAP is excreted from the body through urine.

Ascorbic acid deficiency most commonly leads to scurvy, delayed wound healing as result of connective tissue degradation and capillary fragility (Devaki and Raveendran, 2017). The structure of AAP is similar to glucose and thus it comes to no surprise that competitive affinity for these two molecules will mediate to benefit transport of the one or the other. This is especially observed in diabetes where a surge in glucose molecules flood the extracellular environment of the cells keeping AAP from entering the cell. Vitamin C deficiency have been reported in patients suffering from T2DM (Christie-David, Girgis and Gunton, 2015). Both AAP and glucose are transported through the cell membrane by SVCT2. Large amounts of systemic vitamin C in diabetes could possibly reduce glycation of proteins and ROS (Santosh and David, 2017).

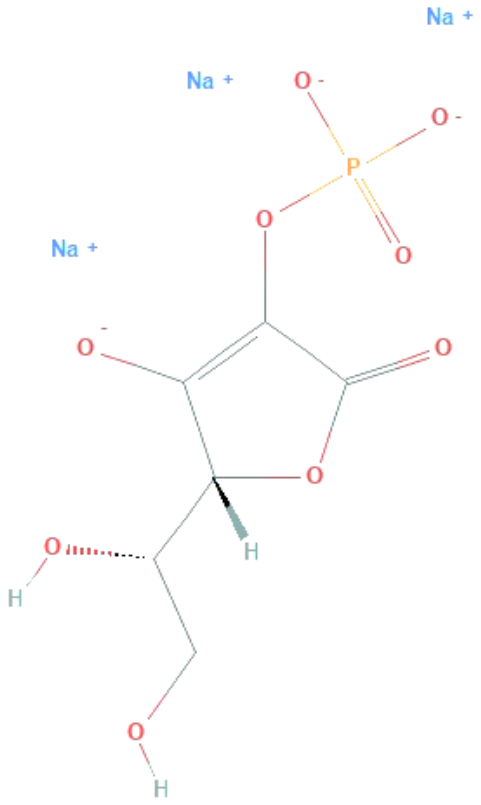

Figure 2.2 L-Ascorbic acid-2-phosphate trisodium salt chemical structure. The image depicts the synthetic compound L-Ascorbic acid-2-phosphate trisodium salt which is oxidant resistant and long-acting (Image obtained from PubChem, ID: 10990876).

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Chapter 3

Aims and objectives

We hypothesize that a synergistic approach targeting the underlying oxidative stress and inflammation in T2DM should include antioxidant supplementation to protect patients against cellular dysfunction. This hypothesis was tested by 1) feeding obese diabetic and control animals an antioxidant supplement and 2) assessing the functional capacity of MSC through various techniques.

3.1. Aim

Investigate the efficacy of *in vivo* antioxidant (NAC and AAP) supplementation as preventative measure against mesenchymal stem cell impairment using obese diabetic (B6.C-Lep^{ob}/J) (ob/ob) and wild type control (C57BL6/J) mouse models.

3.2. Objectives

3.2.1. Nutritional supplementation of wild type control and ob/ob mice with either placebo or antioxidants (NAC/AAP) for a period of 6 weeks with weekly assessment of metabolic profile (weight and blood glucose).

3.2.2. Determine the overall oxidative stress in placebo vs. antioxidant supplemented groups by assessing total antioxidant capacity and lipid peroxidation in serum.

3.2.3. Isolation of bone marrow derived MSCs (BM-MSCs) from the femurs of mice within each treatment (placebo vs antioxidant) group and determine their phenotype, responsiveness, and functionality.

3.2.3.1. Function – Assess ex vivo proliferation (BrdU ELISA and image analysis), viability (Crystal Violet) and multi-lineage differentiation (adipogenesis and osteogenesis) capacity of MSCs.

3.2.3.2. Responsiveness – Assess the paracrine function of MSCs at baseline (without stimulation) and following exposure to chronic wound fluid on protein (ELISA) level.

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Chapter 4

Materials and Methods

4.1. Ethical approval

This research study was conducted in accordance with the South African National Standard: The care and use of animals for scientific purposes (SANS10386:2008). Ethical approval (ACU-2019-3857) was granted by Stellenbosch University (SU) Research Ethics Committee: Animal Care and Use (REC:ACU) (Appendix A). The experimental procedures of this study were performed in either the Animal research facility (phase 1) or in the Stephen Hough Research Laboratories (phase 2), Department of Medicine, Faculty of Medicine and Health Sciences (FMHS), SU.

4.2. Overview of study design

This study consisted of 2 phases: ***Phase one:*** *In vivo* antioxidant (NAC and AAP) supplementation of healthy control (wild-type C57BL6/J) and obese diabetic (B6.C-Lep^{ob}/J) (ob/ob) mice for a period of 6 weeks. ***Phase two:*** Isolation of BM-MSCs from the femurs of these mice at the end of the 6-week supplementation period with *ex vivo* assessment of cellular function.

Mice were obtained from the animal research facility at FMHS at the age of 4-5 weeks old. These mouse strains were chosen because of their phenotype. The ob/ob strain has a genetic mutation on the gene that codes to produce the protein, leptin. Leptin is a hormone mainly produced by adipocytes and regulates appetite and fat storage (Klok *et al*., 2006; Society for Endocrinology, 2018). This mutation supresses the production of leptin and thus causes the mouse to spontaneously become obese and pre-diabetic at approximately 3 weeks of age. No special diet was therefore required for the ob/ob animals to develop obesity-associated type 2 diabetes. These animals are very well characterized and is known to also have hyperphagia, hyperglycaemia, glucose intolerance, hyperinsulinemia, subfertility, impaired wound healing, and an increase in hormone production from both pituitary and adrenal glands (Ewart-Toland *et al*., 1999; Katsuda *et al*., 2013; Kennedy *et al*., 2010; The Jackson Laboratory, 2020). The wild type control mice have the same genetic background as the ob/ob mice with the only exception being the leptin mutation.

Refer to figure 4.1 below for an overview of the study design.

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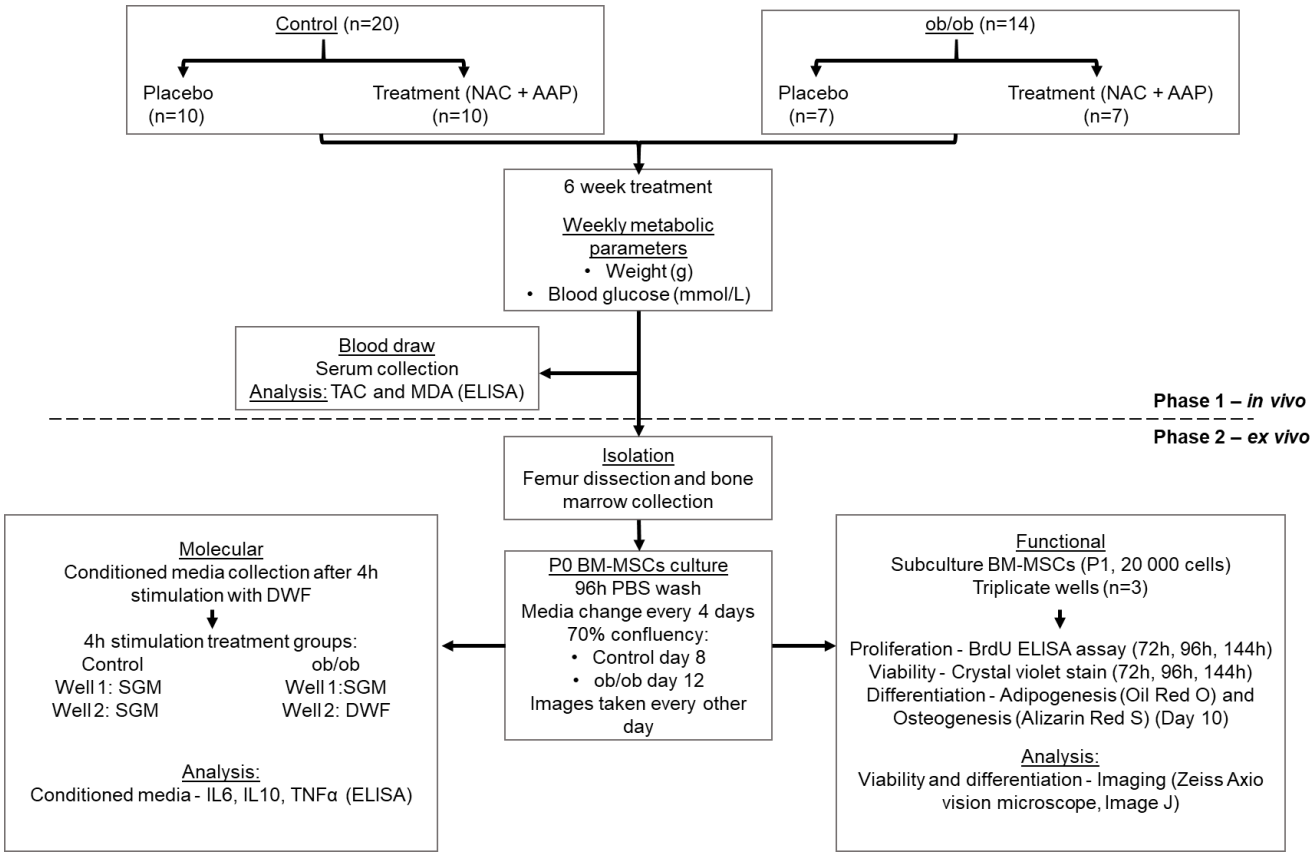

Figure 4.1 Study design. The mice were divided into four treatment groups, namely control placebo (n=10), control supplement (n=10), obese placebo (n=7) and obese supplement (n=7). All treatment groups underwent a 6-week treatment regime with weekly assessment of metabolic parameters. Post euthenization, blood was drawn for serum assessment and mesenchymal stem cells isolated from bone marrow, cultured, and assessed for functional and molecular capabilities. **Abbreviations:** Ob/Ob – Obese diabetic; TAC – Total Antioxidant Capacity; MDA – Malondialdehyde (Lipid peroxidation); PBS – Phosphate Buffered Saline; SGM – Standard growth media; DWF – Diabetic wound fluid; IL6 – Interleukin 6; IL10 – Interleukin 10; TNFα – Tumour Necrosis Factor alpha; ELISA - enzyme-linked immunosorbent assay; BM-MSCs - bone marrow-derived mesenchymal stem cells; BrdU - Bromodeoxyuridine.

4.3. PHASE 1: 6-WEEKS OF *IN VIVO* ANTIOXIDANT SUPPLEMENTATION

4.3.1. Animal housing and husbandry

Healthy wild type control (C57BL/6J) (male, n=20) and obese prediabetic (B6.C-Lep^{ob}/J) (ob/ob) (male, n=14) mice were housed at a constant temperature of 21°C with a 12-hour light/dark cycle and ad libitum access to chow (Rodent breeder, Labchef, SA) and drinking water, unless otherwise stated.

At the start of the study, mice aged 4-5 weeks were familiarized with the experimental setup and trained to eat jelly cubes (placebo) for a period of 1 week. During the training stage, animals were housed individually. After the training was completed, animals were housed in groups of 2-3 animals

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per cage for a period of 6-weeks whilst receiving nutritional supplementation. At the end of the supplementation period, prior to euthanasia, animals were thus 11-12 weeks old (Adult).

4.3.2. Nutritional supplementation

4.3.2.1. Optimisation and training of animals

All animals underwent a training week, during which they were accustomed to being handled by the researcher and were trained to consume a placebo jelly cube daily. This was necessary to ensure consistency in the uptake of the oral supplement by all animals. Although the gavage technique allows for more precise dose delivery, it is stressful for the animals as well as the handler and incorrect technique can have detrimental effects. The use of jelly cubes as delivery method was thus a safer alternative, it did however require optimisation. At first a whole jelly cube ($1cm^3$) weighing 1g was given to each mouse, but the cube was too large for a mouse to consume, and resizing was necessary. According to Jensen *et al*. (2013) a mouse eats approximately 5g of food per day and the company Labchef (Stellenbosch, SA) recommends a daily intake of 5-8g of chow. The jelly cube was therefore resized to 0.5g (10% of recommended daily food).

The type of jelly also played a role in the consumption of the cube by the mice. At first a sugar free jelly (Live well jelly strawberry flavour, Pick n Pay Retailers (Pty) Ltd., SA) was used to make the cubes. Only the control mice consumed these cubes, however, mice from the ob/ob strain did not seem interested. It was substituted with jelly containing 50% less sugar (than standard jelly) (Tower, Pioneer Foods (Pty) Ltd, SA) and both strains consumed this jelly with ease.

During the training week the mice were individually housed. A plastic weighing boat was cut in half and placed in each cage. Thereafter, once daily a cube of placebo jelly was placed in each cage on the plastic weighing boat. The cube was left in the cage until the next day and if not consumed replaced with a fresh cube. Some of the animals showed interest and others ignore the cube. By day three if an animal has not yet shown interest to consume the cube, small pieces would be broken off the cube with sterile tweezers and held in front of the animal's nose or placed in front of the animal until it tries to eat it. Usually by the fourth day of training the animals would have gotten used to the cube of jelly and consumed it with ease. With the start of nutritional supplementation, all animals received 1 cube of jelly (containing either placebo or active antioxidants) 5 days per week for a period of 6-weeks.

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4.3.2.2. Jelly cube preparation

The supplement administered consisted of a combination of NAC (7.5mM) and AAP (0.6mM) dissolved in phosphate buffered saline (PBS) and double distilled (ddH_2O), whereas the placebo treatment consisted only of the vehicle controls (PBS, ddH_2O).

The jelly cubes were prepared by dissolving 20g flavoured gelatine (50% less sugar strawberry flavoured) (Tower, Pioneer Foods (Pty) Ltd, SA) and 10g unflavoured gelatine (Sheridans, SA) in 112.5mL boiling water. Thereafter, 112.5mL cold water was added and mixed into the gelatine mixture and set aside to completely cool down. Next, NAC (3.6M) and AAP (4.5M) stock solutions were prepared by dissolving 0.73g NAC in 4mL PBS and 1.15g AAP in 2mL ddH_2O, respectively. In two separate falcon tubes labelled placebo and supplement, 200uL of the NAC stock solution and 10µL of the AAP stock solution was added to the supplement tube. In the tube labelled placebo, 200µL PBS and 10µL ddH_2O was added. The cooled gelatine mixture was then added to fill each falcon tube up to a final volume of 30mL (Refer to appendix B for concentration calculations). The supplement and placebo jelly were then pipetted into separate moulds in 1mL aliquots and placed at 4°C to set. After the cubes had set it was removed from the moulds, cut in half and weighed to ensure they were of the correct size. Each jelly cube therefore contained 0.6083mg (608µg) NAC and 0.0958mg (95.83µg) AAP. The cubes were stored in separate containers at 4°C and prepared fresh on a weekly basis.

Refer to Table 4.1 for the content, volumes and final concentrations of supplement and placebo jelly.

Table 4.1 Supplement and placebo jelly content.

		Supplement			
Component	**Composition (%)**	**30mL jelly (µL)**	**Final [mM]**	**Per cube (µL)**	**Final [mM]**
Jelly stock solution (20g flavoured gelatine; 10g unflavoured gelatine dissolved in 225mL dH_20)	99.3	2979		496.5	
NAC stock solution (3.6M)	0.56	200	7.5	3.33	7.5
AAP stock solution (4.5M)	0.14	10	0.6	0.17	0.6
		Placebo			
Component	**Composition (%)**	**30mL jelly (µL)**	**Final [mM]**	**Per cube (µL)**	**Final [mM]**
Jelly stock solution (20g flavoured gelatine; 10g	99.3	2979		496.5	

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unflavoured gelatine dissolved in 225mL dH_20)					
PBS	0.56	200	0	2.8	0
ddH_2O	0.14	10	0	0.7	0

Footnote: This table indicates the concentration and volumes of antioxidants and vehicle controls inside the jelly cubes that was administered to mice daily. **Abbreviations:** ddH_2O, double distilled water; PBS, Phosphate Buffered Saline; NAC, N-Acetylcysteine; AAP, Ascorbic acid-2-Phosphate; mM, milimolar.

4.3.3. Metabolic parameters: Weight and blood glucose

Each animal's weight (g) was measured at baseline and on a weekly basis thereafter for the duration of the study. Fasting blood glucose levels (tail vein prick using a lancet, Microlet™, Ascensia Diabetes Care Holdings AG, Poland) were determined using the Contour plus glucometer system (Bayer, Leverkusen, Germany). The detectable glucose range for the glucometer is 0.6 to 33.3mmol/L. In cases where the blood glucose measurement exceeded the measuring range of the glucometer system (Bayer, Leverkusen, Germany) (>33mmol/L), the highest possible measurement (33.3mmol/L) was recorded. Note: All animals were fasted for a period of 4h prior to taking blood glucose measurements.

4.3.4. Animal wellness: Appearance and behaviour

Animal wellness was monitored for each animal daily throughout training and 6-week treatment period. Wellness was determined by criteria score on appearance and behaviour (Refer to Appendix C for the animal well-being monitoring sheet template and score parameters). Appearance parameters included: 1 - well groomed; bright facial expression; no discharges; no lesions; normal posture/ breathing, 2 - coat slightly rough; subtle discharges/ lesions; dull facial expression; mild hunching; abnormal breathing, 3 – staring coat; obvious discharges/ lesions, facial bulge/ squinting; hunched posture; breathing difficulty. Behaviour parameters included: 1 - Alert; curious; active; interactive with cage mates; normal gait, 2 – less active; isolated from cage mates; less responsive; abnormal gait, 3 – inactive; poorly responsive.

4.3.5. Euthanasia and serum collection

Prior to euthanasia (cervical dislocation), the animals were placed under general anaesthesia using Isoflurane (Safeline pharmaceuticals, SA) gas via vaporizer (Penlon Ltd, UK) for 3-5 min. Immediately, following cervical dislocation, blood was drawn from each animal via cardiac puncture using a 25-gauge needle (Avacare™, Lasec, SA) and serum separating tubes (BD Microtainer® SST™, USA). Collected blood samples were left at room temperature for 15 min to coagulate. After coagulation, the tubes were placed on ice and centrifuged (Prism™, Labnet Inc., USA) at 4000 RPM

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for 5 min. Serum was aliquoted (approximately 200-400µL per animal) and stored at -80°C until subsequent analysis (Refer to section 4.3.6).

4.3.6. Serum analysis

4.3.6.1. Total antioxidant capacity

The total antioxidant capacity of the animals within each treatment group were determined using a colorimetric assay (E-BC-K136-100 EL, Elabscience®, US). Due to the small volume of serum available per animal, the protocol of the kit was amended to accommodate the amount of serum available to test. The serum volumes used were half of what the original protocol suggested (50µL per test instead of 100µL), this did not affect the sensitivity and detection was within range for all samples. The assay was performed according to the manufacturer's instructions. All reagents used were supplied in the kit.

Serum samples from both control and ob/ob mice were placed on ice to thaw. Microcentrifuge tubes (508-GRD-Q, QSP, US) were labelled with the sample labels and a control tube was labelled for each sample tube. Buffer solution was added to all the tubes. Next, serum samples were added to the respective sample tubes. Thereafter, Chromogenic agent was added to both sample and control tubes following ferric salt solution. The tubes were vortexed and placed in a 37°C heating block (D1100, Labnet International, Inc., USA) for 30 min. After the samples were heated, stop solution was added to all the tubes. Lastly, serum samples were added to the respective control tubes. The tubes were vortexed and allowed to stand at room temperature (21°C) for 10 min. Prior to measuring the absorbance of the samples at 520nm (Multiskan™ FC, Thermo Fischer ™, US), samples were pipetted into a 96 well plate (Nest, SoCal BioMed, LLC, US) in duplicate and a blank of ddH_2O was also added in duplicate.

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

$$\frac{ODsample - ODcontrol}{0.01} \div 30(min) \times \frac{Total\ volume\ of\ reaction\ system\ (mL)}{The\ volume\ of\ sample\ (mL)}$$

Principle of the test: This kit gives a quantitative measure of the total antioxidant capacity within a specific sample by measuring the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}). Several antioxidants (enzymes and/or macromolecules) can reduce Fe^{3+} to Fe^{2+} and Fe^{2+} can form stable complexes with phenanthroline substances which can be measured at an absorbency of 520nm. The kit has a sensitivity of 0.62 U/mL and an intra-assay variability of 2.7% with an average recovery rate of 105%.

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4.3.6.2. Lipid peroxidation

Lipid peroxidation was measured by using a Malondialdehyde (MDA) assay kit (E-BC-K025 EL, Elabscience®, US) to indirectly reveal the level of cellular damage caused by free radicals. The assay was performed according to the manufacturer's instructions and all reagents used were supplied in the kit.

Serum samples from both control and ob/ob mice were thawed on ice. Falcon tubes (Cellstar®, Greiner, Germany) were labelled with sample labels and control tubes were added for haemolysed samples. A blank and a single standard tube was also labelled. Serum samples were added (100µL) to each respective sample tube as well as their control (if the sample was haemolysed). Clarification solution was added to all the tubes followed by acid reagent. Thereafter, 50% glacial acetic acid was added to only the control tubes which is necessary to control for haemolysis that alters the detection of MDA by giving a false result. Next, absolute ethanol was added to the blank tube and the standard was added to the tube labelled standard. All the tubes were vortexed and placed in a water bath to incubate for 40min at 95°C. Following the incubation period, the tubes were cooled by placing them under cold running water and immediately centrifuged at 3100xg for 10min. Prior to measuring the absorbance of the samples at 532nm (Multiskan™ FC, Thermo Fischer ™, US). The supernatant was pipetted into a 96 well plate in duplicate for each sample, their respective control and the standard. ddH_2O was also added in duplicate to serve as a background control (blank).

The following formula was used to calculate the MDA content (nmol/mL) of the samples:

$$\frac{ODsample - ODcontrol}{ODstandard - ODblank} \times Concentration\ of\ standard\ (10nmol/L)$$

Principle of the test: This kit gives a direct quantitative measurement of lipid peroxidation in a sample by detecting MDA content. Oxygen free radicals produced by (non-) enzyme systems in the body break down unsaturated fatty acids resulting in cellular damage through lipid peroxidation. MDA in the presence of lipid peroxide catabolites reacts with Thiobarbituric acid (TBA) to produce colour which can be measured at an absorbance of 530nm. The kit has a sensitivity of 0.38nmol/mL with an inter-assay variability of 8% and recovery rate of 101%.

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4.4. PHASE 2: *EX VIVO* ASSESSMENT OF THE REGENERATIVE CAPACITY OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS

4.4.1. Isolation of bone marrow derived MSCs

Bone marrow-MSCs were isolated from the femurs of the experimental animals using an established method (Mehrbani Azar *et al.*, 2020). After euthanasia (cervical dislocation), the hind legs of each animal were dissected as follow: The skin was separated from the muscle tissue until the hip and knee joints were visible. Muscle tissue and ligaments were excised to expose the femurs. Each femur was harvested by dislocating the femur head from the pelvis and cutting through the ligaments at both the hip and knee joints to ensure that an intact femur was removed. The excised femurs (left and right) were rinsed in 70% EtOH and placed in separate Falcon tubes (Nest Biotechnology, US) containing pre-warmed transport media. Transport media consisted of Dulbecco's Modified Eagle Medium (DMEM) (L-glutamine Lonza, Switzerland) and 1% Penicillin/ Streptomycin (Pen/Strep) (Lonza, Switzerland). Under sterile conditions in the cell culture laboratory, the femurs were cleaned by scraping the bone with a scalpel blade to remove any excess soft tissue. The proximal and distal ends were cut open with a sterile bone cutter and the bone marrow flushed from the shaft using a 25-gauge needle and syringe containing 1mL prewarmed isolation media. Isolation media consisted of DMEM with ultra-glutamine (4.5g/L high glucose Lonza, Switzerland), 20% Foetal bovine serum (FBS) (Biochrom Superior S0615, Germany) and 1% Pen/Strep (Lonza, Switzerland). The bone marrow derived from each femur were flushed into separate wells of a six well plate (1 well = 1 femur) already containing 1mL isolation media (total volume per well = 3mL). Bone marrow aspirates were incubated for 96h at 37°C and 5% CO_2 to allow for the plastic adherence of MSCs.

After 96h the adherend cells (MSCs) were washed x3 times with 2mL PBS to remove all debris. The media was replaced with standard growth media (SGM) consisting of FBS (Biochrom Superior S0615, Germany), DMEM with ultraglutamine (4.5 g/L high glucose, BioWittaker, Lonza, Basel, Switzerland) containing 1% penicillin/streptomycin (BioWittaker, Lonza, Basel, Switzerland) and maintained in a humidified incubator at 5% CO_2 and 37°C. Media was changed every four days for both control and ob/ob MSCs until the cells reached 70% confluence.

Cell confluency over time post isolation was determined using the image analysis method previously described by Busschots *et al*. (2015). Four images were taken per well at 20x magnification with a camera (Canon EOS 600D, Canon Inc., Taiwan; attachment: SN:0569, Martin microscope company-BX, US) attached to an inverted light microscope (Olympus CKX41SF, Olympus Corporation, Phillipenes). Images were taken every other day starting from the 96h PBS wash. The isolated MSCs (all treatment groups) were allowed to expand in culture for a period of 8 days (control) or 12 days (ob/ob), respectively.

NOTE: For the assessment of the molecular responsiveness of MSCs derived from animals within each treatment group, cells (passage 0; 70% confluent) were stimulated with diabetic wound fluid for a period of 4h prior to collecting conditioned media (refer to section 4.4.4). For assessment of functional measures (viability, proliferation rate, multilineage differentiation capacity) the MSCs were subcultured (passage 1) prior to performing functional assays (refer to section 4.4.3).

4.4.2. Cell counting and Subculture (Passaging).

Subculture involved dissociating adherent cells from the plate by adding cell dissociating reagent (StemPro® Accutase®, Gibco, Life technologies corporation, US) to each well and incubating the plate for 5min. After 5min, the enzymatic activity was quenched by adding standard growth media (SGM) into each well. The cells derived from both femurs for each animal (two wells of each 6-well-plate) were pooled together into a single falcon tube (CellStar®, Greiner, Germany) and the number of viable cells determined using a haemocytometer (Fuchs-Rosenthal, Marienfield, Germany).

For cell counting purposes, one to one ratio of homogenous cell suspension and Trypan blue (0.4%, Invitrogen™, Life technologies corporation, US) was prepared and the mixture loaded onto the haemocytometer counting grid (Fuchs-Rosenthal, Marienfield, Germany). Under the microscope (Olympus CKX41SF, Olympus Corporation, Phillipenes) the number of viable cells in the four outer quadrants (squares) were counted (Refer to figure 4.2). Cell density (cells/mL) was calculated using the following formula:

$$Number\ of\ cells\ counted \times Cell\ suspension\ dilution\ factor\ (mL) \times \frac{Trypan\ blue\ dilution\ factor\ (mL)}{Squares\ counted} \times 10\,000$$

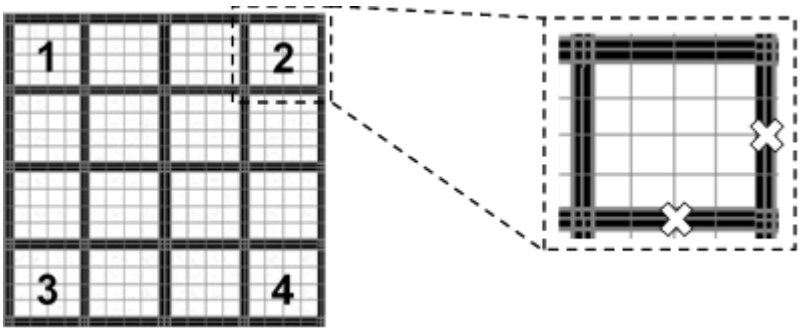

Figure 4.2 Haemocytometer cell counting principle. All cells were counted in the outer four quadrants of the haemocytometer grid. Each square has three lines on each side. Cells touching the middle line of the three bottom and right lines were excluded from the count.

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4.4.3. Functionality assessment of bone marrow derived MSCs

4.4.3.1. Viability

Following subculture, MSCs were seeded into three 96 well plates at a seeding density of 2×10^4 cells/well. Once the cells reached approximately 60% confluence in passage 1, cell viability was assessed over a period of 72h, 96h and 144h using crystal violet staining. Crystal violet stains the nuclei (DNA) of viable cells and is used to detect maintained adherence of cells (Feoktistova *et al.*, 2016), thus only viable cells are stained and quantified. At each of the respective end points (72h, 96h, 144h), the media was removed from each well, the MSCs washed with PBS (x3) and fixed with 150µL ice cold methanol (SAAR4164060LP, Merck chemicals (Pty) Ltd., RSA) for 30s. Afterward methanol was discarded and the wells containing cells washed once with PBS. Following the wash step, MSCs were stained with Crystal violet stain (0.01% Crystal violet working solution, made up by dissolving 1% crystal violet stock solution with dH_2O) for a period of 5 min. Subsequently crystal violet stain was removed, and the cells washed twice with PBS. Thereafter, PBS was added to each well and the plates were imaged by using light microscopy (Zeis Axio Observer 7 inverted microscope Axiocam 305 color, Germany) at 10x magnification. Images were analysed using the method previously described by Venter & Niesler (2019). The program Image J (version 1.52, NIH) was used to quantify the number of adherent cells stained with Crystal violet. All experiments were performed in triplicate wells for at least n=3 biological repeats per treatment group.

4.4.3.2. Bromodeoxyuridine (BrdU) Proliferation ELISA

Following subculture, MSCs were seeded into three 96 well-plates at a seeding density of 2×10^4 cells/well. Once the cells reached approximately 60% confluence in passage 1, proliferation rate of the cells was assessed over a period of 72h, 96h and 144h using a colorimetric BrdU-cell proliferation assay (11647229001, Roche, Switzerland). The assay was performed according to the manufacturer's instructions. BrdU (pyrimidine analogue) is incorporated into the DNA of proliferating cells by replacing thymidine. Resulting in partially denatured DNA which Anti-BrdU can bind to with ease. The substrate reaction develops colour and absorbance can be measured at 370nm. The absorbance values and DNA synthesis are directly correlated and thus an indication of the number of proliferating cells.

The media was removed from each well 4-hours before the respective end points (68h, 92h and 140h), washed with PBS (x1) and BrdU labelling solution (0.01% BrdU in SGM) added to each well before incubation at 37°C for 4-hours. Following the 4-hour labelling period, the cells were fixed with 70% ethanol for 30min. Thereafter, the fixative was discarded, and the cells treated with Anti-BrdU-POD consisting of 0,01% Anti-BrdU and Antibody diluent for 90 min at room temperature (21°C).

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Prior to adding the substrate, the cells were washed with PBS three times. The plate was left to stand at room temperature for 5 min before absorbance was measured at 370nm with a microplate photometer (Multiskan™ FC, Thermo Fischer™, US).

All experiments were performed in triplicate wells for at least n=3 biological repeats per treatment group.

4.4.3.3. Multi-lineage differentiation capacity

Following subculture, MSCs were seeded into three 96 well-plates at a seeding density of 2×10^4cells/well. 48h after seeding, the MSCs were 100% confluent and SGM replaced with either adipogenic induction media (AM) or osteogenic induction media (OM) for a period of 10 days (Mehrbani Azar *et al*., 2020; van de Vyver *et al*., 2014). AM consists of SGM supplemented with insulin (10µM) (human, I9278-5mL, Sigma-Aldrich, US), 3-Isobutyl-1-methylxanthine (0.5mM) (I5879-1G, Sigma-Aldrich, US), Dexamethasone (1µM) (D4902-100MG, Sigma-Aldrich, US) and Indomethacin (56µM) (I7378-5G, Sigma-Aldrich, US). For adipogenic differentiation, AM was changed three times a week for the duration of the induction period. OM consist of SGM supplemented with Ascorbic acid (50µM) (A4544-25G, Sigma-Aldrich, US), Dexamethasone (10nM) (D4902-100MG, Sigma-Aldrich, US) and β-glycerophosphate (10nM) (G5422-100MG, Sigma-Aldrich, US). For osteogenic differentiation, OM was changed twice a week for the duration of the induction period.

Following the 10-day induction period, the extent of lipid accumulation (adipogenesis) and mineralization (osteogenesis) were determined using Oil Red O and Alizarin Red S staining, respectively. MSCs induced with OM were washed with PBS and fixed with 70% ethanol for 5 min. Subsequently the PBS was discarded, and the wells washed with dH_2O once. Alizarin Red S stain was added to each well and incubated overnight with agitation. After the staining period, the stain was removed followed by washing of wells with dH_2O three times. Thereafter, the wells were rinsed once with PBS and filled with PBS before imaging. MSCs induced with AM for the duration of adipogenesis differentiation, were stained with Oil Red O (dissolving 1% Oil Red O stock in isopropanol and thereafter diluting the stock in dH_2O to make 70% Oil Red O working solution) for 30min. Following the 30min period, the stain was removed, and the wells washed with dH_2O three times. Subsequently dH_2O was added to the wells before imaging.

For both adipogenesis and osteogenesis, the entire well was imaged using light microscopy (Zeiss Axio Observer 7 inverted microscope Axiocam 305 colour, Germany) in tile scan formation (4x4 tiles and 10x magnification). All images were analysed using the program Image J (version 1.52, NIH) to determine the percentage surface area positively stained. The images were opened, and the scale parameters set to 637.60 pixels, known distance to 1, the unit of length to mm and lastly, the scale

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was set to global (all images will be analysed with the same scale). The images were then converted to RGB stack and set to green channel. Next, the threshold was adjusted for Oil Red O images (±80) and Alizarin Red S images (±50) to highlight the fat droplets or mineral pockets, respectively. Prior to the analysis of the threshold image, the measurement parameters were set (area, area fraction, limit to threshold and display label).

4.4.4. Assessment of Molecular responsiveness

4.4.4.1. Diabetic wound fluid stimulation and sample collection

MSCs (passage 0; 70% confluent) derived from obese prediabetic animals that received either placebo (n=4) or supplementation (n=4), were stimulated with either diabetic wound fluid (DWF) (MSCs derived from femur 2) or SGM (MSCs derived from femur 1) for a period of 4h. For the stimulation with DWF, SGM was supplemented with 30% DWF (300µL DWF + 700µL SGM). This was previously determined in our laboratory to be the optimal concentration for stimulation (Mehrbani Azar *et al.*, 2018) based on total protein content. After the 4-hour stimulation, supernatant (conditioned media) was collected, centrifuged at 5000xg for 10min to remove cellular debris and stored at -80°C for subsequent cytokine analyses (refer to section 4.4.4.2).

4.4.4.2. Conditioned media analysis: IL6, IL10 and TNFα ELISA

The cytokines of interest (IL6, IL10 and TNFα) were detected individually using ELISA kits (E-EL-M0044, E-EL-M0046, E-EL-M0049, Elabsciences®, US) as per the manufacturer's instructions. All reagents used were supplied within the respective kits.

Conditioned media was thawed on ice and centrifuged at 1000xg at 4°C for 20 min. On each 96-well ELISA plate, the known standards, conditioned media samples, an SGM control and DWF were added in duplicate wells and incubated for 90min at 37°C. Immediately after the incubation period, the liquid was discarded and the appropriate Biotinylated Detection Antibody working solution added to each well. Each plate was then incubated for 1 hour at 37°C. Following the second incubation period, the liquid was once again discarded, and the plate washed using wash buffer by soaking the wells for 1min prior to removing the buffer. This process was repeated three times to ensure excess antibody was removed from the wells. Next, Avidin-Horseradish Peroxidase (HRP) Conjugate working solution was added to the wells and incubated for 30min at 37°C followed by another three washing steps as previously described. The appropriate substrate was then added to each well and the plate incubated for approximately 15min or until sufficient colour has developed to measure the optical density (OD) at 450nm with a microplate reader (Multiskan™ FC, Thermo Fischer ™, US). For quantification of the raw data, the cytokine concentration within each sample was interpolated from the four-parameter logistic curve (4PL) standard curves using GraphPad Prism (version 5.01,

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GraphPad software, California, US) (Refer to Fig. 4.4). For each sample, the dilution factor and background absorbance were considered when determining the actual cytokine concentration.

Principle of the test: All kits (IL6, IL10, TNFα) follow the Sandwich-ELISA principle. Each micro-ELISA plate is pre-coated with a specific capture antibody to recognize the antigen of interest within sample (IL6/ IL10/ TNFα). After antigen recognition occurred the addition of the detection antibody binds to the antigen (biotinylated detection antibody). A secondary antibody conjugate (HRP) binds to the detection antibody and lastly, an enzymatic reaction between the secondary antibody conjugate and substrate gives off a colour that is spectrophotometrically measurable. The kits have a sensitivity of 18.75pg/mL (IL6), 9.38pg/mL (IL10) and 18.75pg/mL (TNFα).

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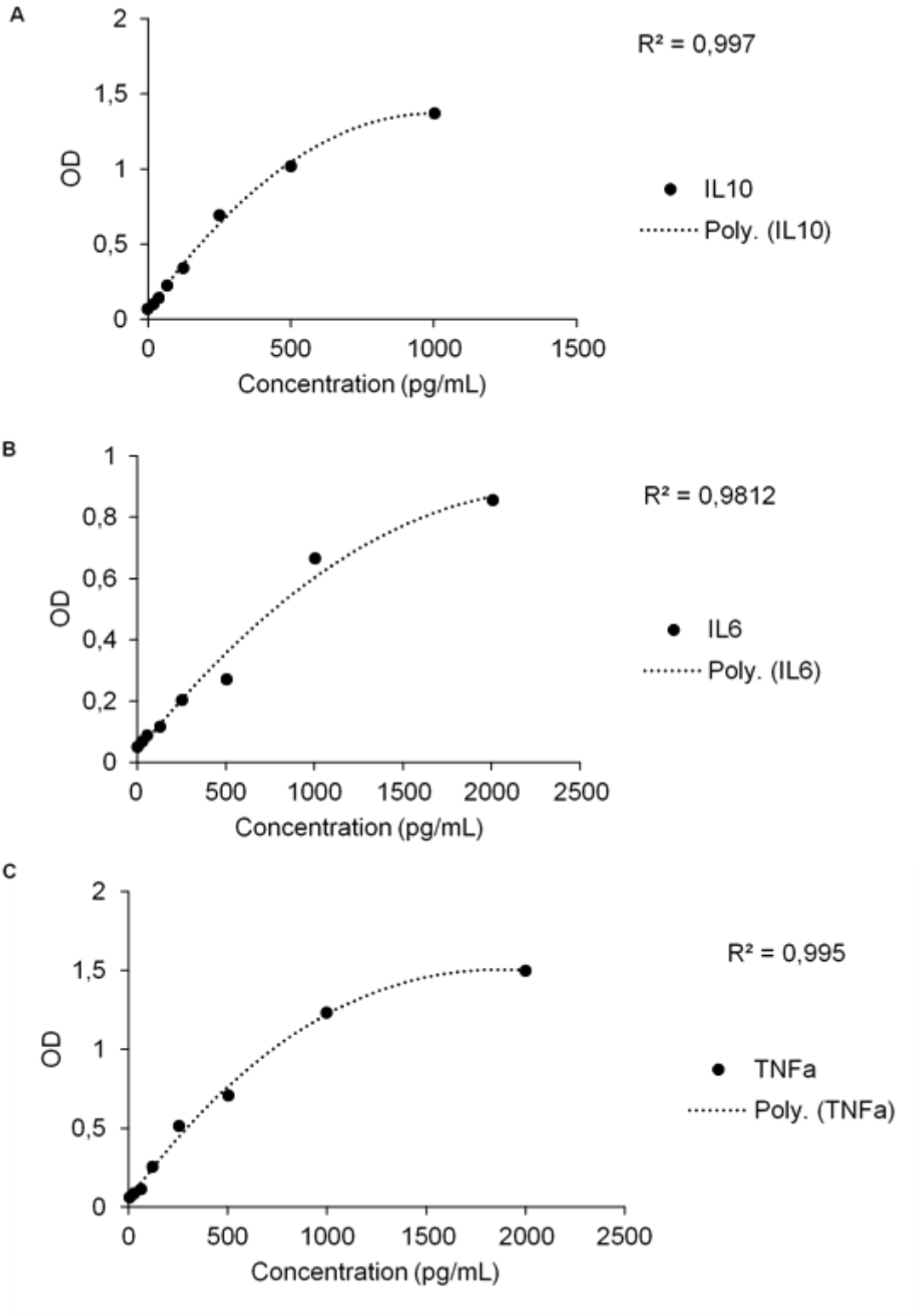

Figure 4.3 Cytokine standard curves. Standard curves were obtained from serial dilutions with known concentrations A) IL10 with R^2=0.997; B) IL6 with R^2=0.9812; C) TNFα with R^2=0.995. **Abbreviations:** OD, optical density; IL, Interleukin; TNFα, Tumour necrosis factor alpha; pg/mL, picogram/millilitre.

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4.5. Statistical analysis

The data is presented as mean ± standard deviation (mean±SD). Statistical analysis was performed using Statistica software (Version 13, StatSoft). Factorial ANOVA with Tukey post hoc test was used to compare time to treatment (placebo and supplement) to group (control and obese). One-way ANOVA with Tukey post hoc test used to compare treatment (placebo and supplement) to groups (control and obese). Statistical significance was accepted at $p<0.05$.

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Chapter 5

Results

5.1. Animal well-being and metabolic profile

Animal weight was assessed on a weekly basis from baseline (age 3-4 weeks) to the end of the supplementation period (age 11-12 weeks). A normal growth curve was observed in the wild-type control animals (C) (C57BL/6J WT) with body weight increasing significantly over time from 20.8±1.7 g at baseline to 28.7±1.6 g after 6 weeks of supplementation ($p<0.05$) (Fig. 5.1A). Excessive weight gain was evident in the obese diabetic (DM) (B6.Cg-Lep^{ob}/J, ob/ob) animals, with body weight increasing from 25.6±1.6 g at baseline to 44.3±3.5 g at the end of the 6 weeks supplementation period ($p<0.001$) (Fig. 5.1A). In the DM animals significant weight gain was observed from week 1 (DM:P 33.7±1.9 g, DM:S 33.3±2.0 g) onwards compared to baseline measurement (DM:P 26.1±2.4 g, DM:S 25.1±2.3 g) ($p<0.05$). No difference in body weight between NAC/AAP antioxidant supplementation and placebo treatment groups were observed (Fig. 5.1A).

Fasting blood glucose levels at baseline (C:P 11.3±0.5 mmol/L, C:S 10.0±0.7 mmol/L) and throughout the 6-week treatment period remained consistent in control animals (Fig. 5.1B). Whereas DM animals had significantly ($p<0.01$) increased blood glucose levels from week 3 (DM:P 17.0±1.6 mmol/L, DM:S 17.7±2.1 mmol/L) until week 6 (DM:P 23.5±2.7 mmol/L, DM:S 23.7±1.7 mmol/L) compared to baseline (DM:P 10.9±1.0 mmol/L, DM:S 9.5±0.7 mmol/L). A significant difference was observed in the blood glucose levels between control and DM animals ($p<0.01$), with hyperglycaemia indicative of diabetes (fasting blood glucose >16.7 mmol/L; >300 mg/dL) evident from week 3 onwards in the DM animals. The NAC/AAP antioxidant supplementation had no effect on the blood glucose levels of control or DM animals (Fig. 5.1B).

Animal wellness was assessed using a criterion score of appearance and behaviour at baseline and over the 6-week treatment period. The healthy control and DM animals had a consistent score of 1 for both appearance and behaviour (Fig. 5.1C-D), indicating that no signs of distress were observed in any of the animals. No mortalities occurred and animals had a 100% survival rate.

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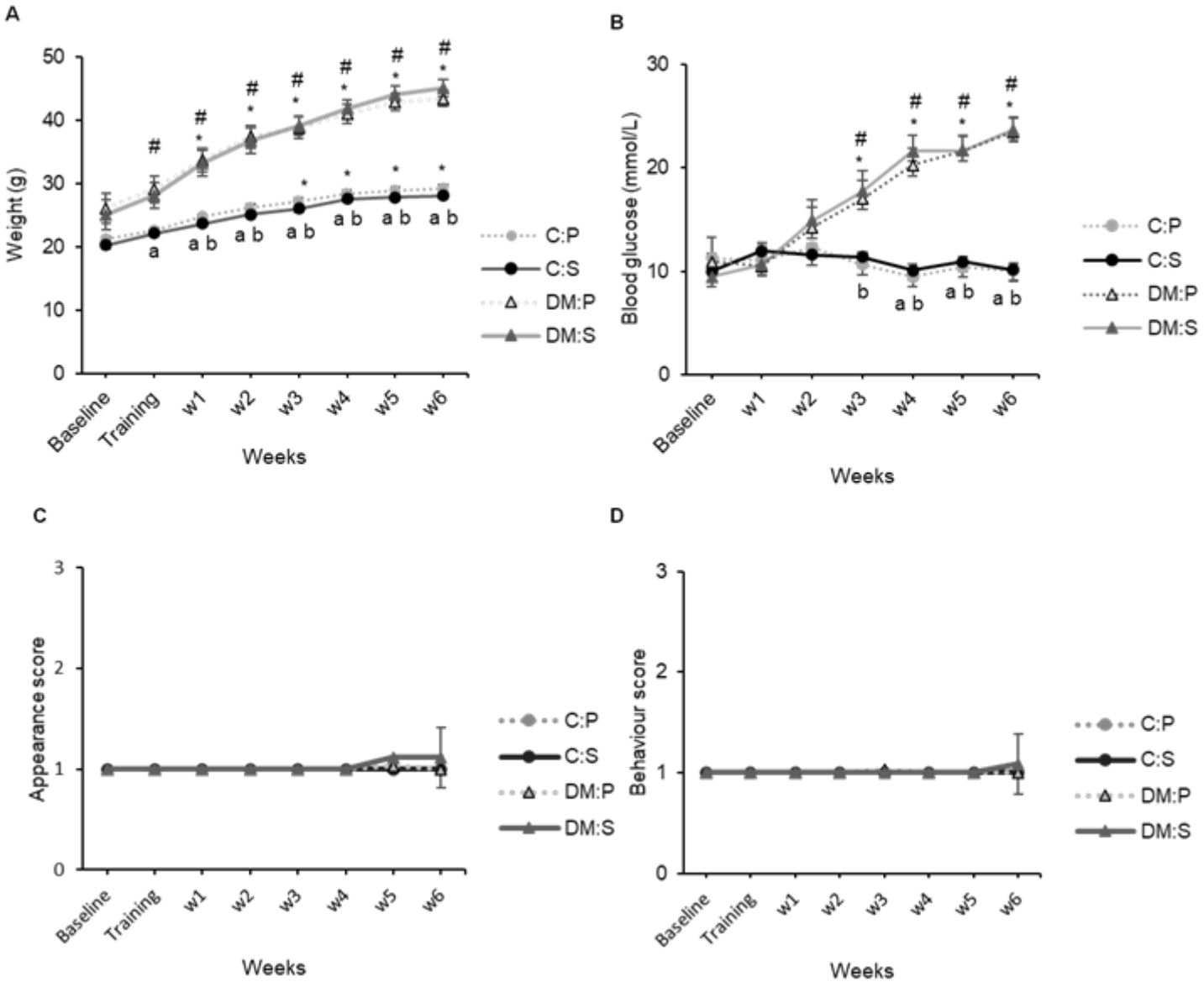

Figure 5.1 Animal wellbeing and metabolic profile. Animal wellbeing was measured at baseline, training and 6-week treatment period by assessing A) Weight (g), B) Fasting blood glucose (mmol/L), C) Appearance and D) Behaviour. Data is presented as mean±SD. The score indication for C) Appearance: 1 - well groomed; bright facial expression; no discharges; no lesions; normal posture/ breathing, 2 - coat slightly rough; subtle discharges/ lesions; dull facial expression; mild hunching; abnormal breathing, 3 – staring coat; obvious discharges/ lesions, facial bulge/ squinting; hunched posture; breathing difficulty, and D) Behaviour: 1 - Alert ; curious; active; interactive with cage mates; normal gait, 2 – less active; isolated from cage mates; less responsive; abnormal gait, 3 – inactive; poorly responsive. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** Three-way ANOVA with Tukey's post hoc test, * indicates significance from baseline, # indicates significance over time, a indicates significance between control placebo and obese diabetic groups, b indicates significance between control supplement and obese diabetic groups at the same time point. Animals per group C:P n=10; C:S n=10; DM:P n=7; DM:S n=7

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5.2. Antioxidant status of animals

At the end of the supplementation period, the DM animals tended to have an overall higher total serum antioxidant capacity (TAC) (DM:S 5.9±1.5 U/mL; DM:P 5.2±0.9 U/mL) than healthy control animals (C:P 3.8±1.3 U/mL; C:S 4.1±0.8 U/mL) (Fig. 5.2A). A significant difference was evident between the TAC in DM:S and control (C:P and C:S) animals ($p<0.05$) (Fig. 5.2B). Consistent with the TAC data, supplementation with NAC/AAP was able to reduce serum MDA levels in DM animals (DM:P 39.0±14.7 nmol/mL; DM:S 21.5±11.6 nmol/mL) ($p<0.01$) to levels comparable to that of healthy control animals (C:P 22.9±11.4 nmol/mL) (Fig. 5.2B).

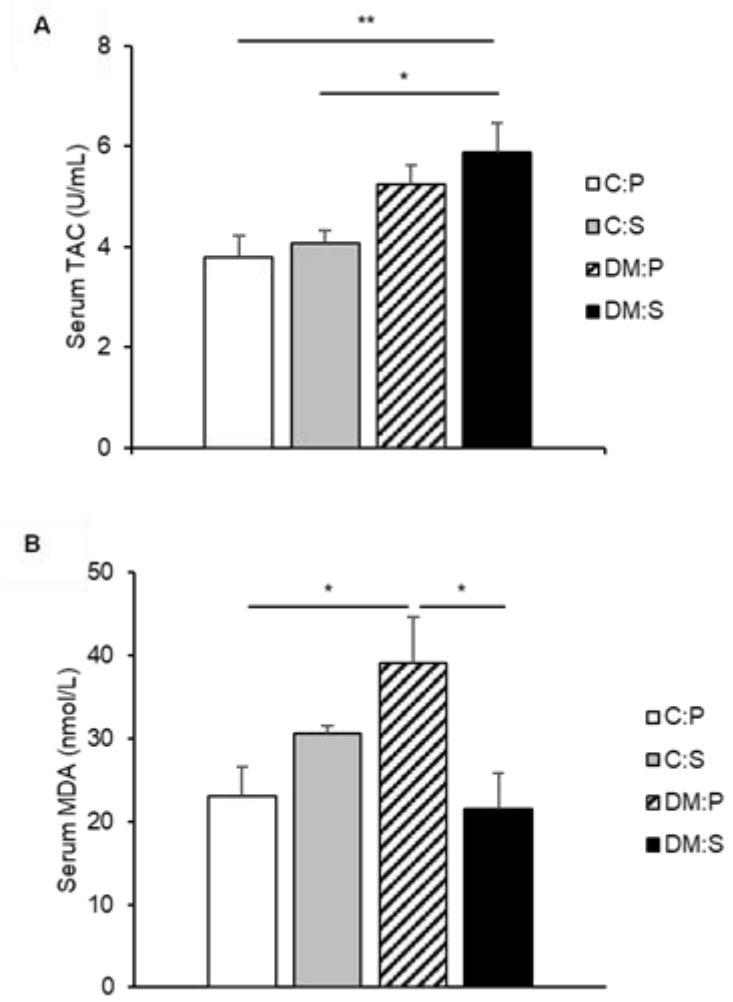

Figure 5.2 Oxidative status of animals. Serum was collected and oxidative stress status measured by assessing A) Total serum antioxidant capacity (TAC) (U/mL) and B) Lipid peroxidation (serum malondialdehyde, MDA) (nmol/L) after 6-week treatment period. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** One Way ANOVA with Tukey's post hoc test, *$p<0.05$, **$p<0.01$. Outliers and values with more than 2 SD away from the mean were excluded from MDA data set (C:P, n=10; C:S, n=9; DM:P, n=6; DM:S, n=7 remained).

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5.3. NAC/AAP supplementation improved the growth rate of MSCs post isolation (Passage 0)

The growth rate of MSCs derived from DM animals were slower with cells only reaching 70% confluency on day 12 post isolation, whereas MSCs derived from healthy control animals reached a similar confluency level on day 8 post isolation (Fig. 5.3). Refer to Fig. 5.3 A-B for representative microscopy images over time. Quantification of cellular growth using image analysis was possible from day 4 post isolation onwards. The images demonstrated that the cell number/field of view (0.3mm²) increased significantly ($p<0.001$) over time in both control and DM groups with NAC/AAP supplementation, whereas this effect of time was less pronounced in the placebo groups (Fig. 5.4 A-B). A significant difference ($p<0.05$) was evident on days 4 (C:P 7978 ± 358, C:S 9417 ± 996) and 6 (C:P 8760 ± 826, C:S 10573 ± 853) between the placebo and supplementation groups in control animals and on days 6 (DM:P 8145±882, DM:S 9343±751), 8 (DM:P 7437±546, DM:S 9336±1081) and 10 (DM:P 8501±562, DM:S 9744±1012) in the DM animal groups (Fig. 5.4 A-B). Cellular senescence had little impact on MSC growth rate as the percentage of cells with a senescent morphology was less than 1% in each group (Fig. 5.5 A-C).

As validation of the image analysis data, absolute quantification of viable cell number (cell yield at end of passage 0) was performed using a haemocytometer. The data indicated that isolations from healthy control animals (C:P 1 908 895±778 134 cells/1.2mL; C:S 2 569 000 ± 695 963 cells/1.2mL) produced a 2-fold higher viable MSC yield than isolations from obese diabetic animals (DM:P 901 000±139 309 cells/1.2mL; DM:S 1 282 000± 358 463 cells/1.2mL) prior to subculture (Fig. 5.6). In both control and DM animals, NAC/AAP supplementation tended to increase the yield of viable MSCs by 1.5-fold (Fig. 5.6), due to the small sample size (n=3 per group) and variability this observation was however not significant.

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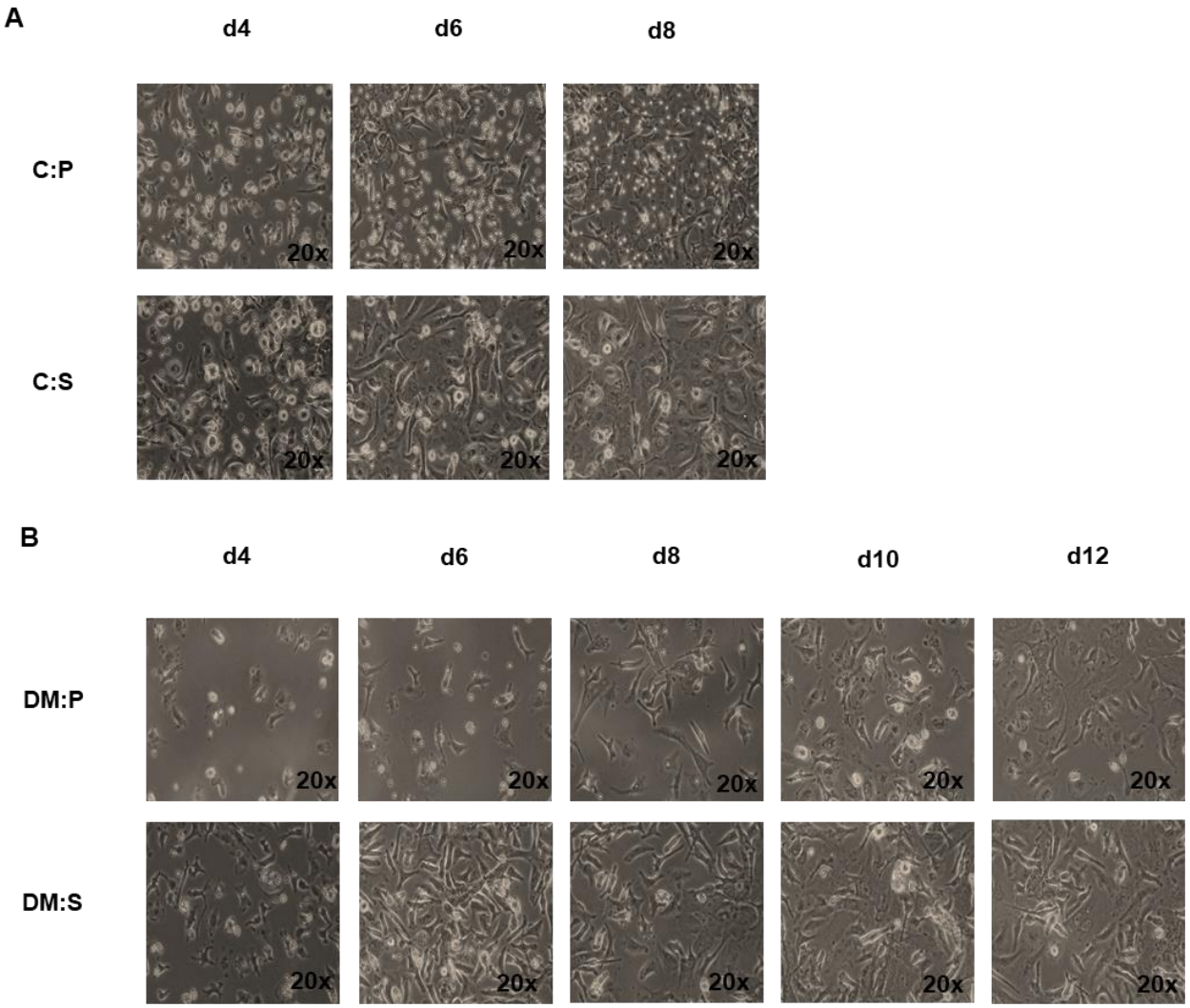

Figure 5.3 Representative images of MSC confluency post isolation. Images of cultured MSCs post isolation were taken every other day of A) Control MSCs from day 4 post isolation to day 8 and of B) Diabetic MSCs from day 4 to day 12 to demonstrate change in growth rate over time. Magnification x20. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement.

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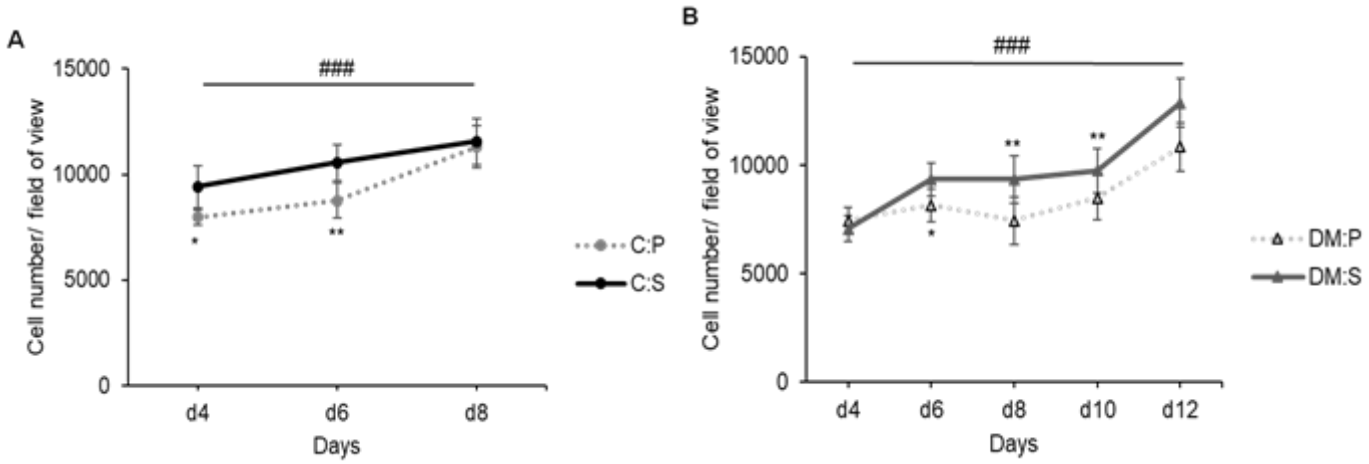

Figure 5.4 MSC growth rate post isolation (passage 0). Growth rate post isolation over time was determined by image quantification of A) Control MSCs from day 4 to 8, and B) Diabetic MSCs from day 4 to 12. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** Two-way ANOVA with Tukey's post hoc test, *p<0.05 indicates significance between groups, **p,0.01 indicates significance between groups, ###p<0.001 indicates significance over time.

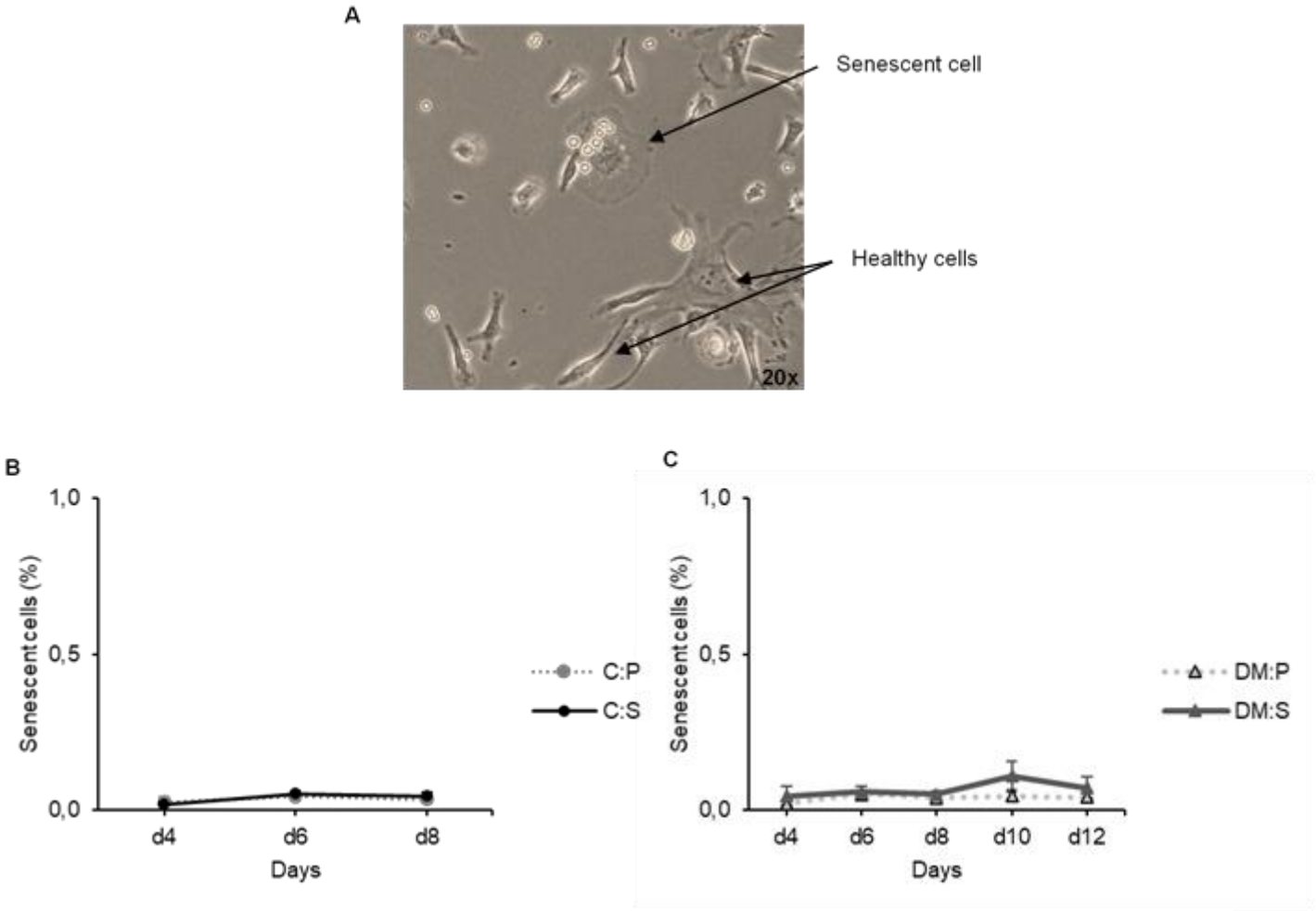

Figure 5.5. MSC cellular senescence. Senescence was quantified by morphology over the culturing period post isolation. A) Representative image of senescent and healthy cells, B) Control MSCs senescence at day 4 to 8, C) Diabetic MSCs senescence at day 4 to 12. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** Two-way ANOVA with Tukey's post hoc test. No significant difference was evident.

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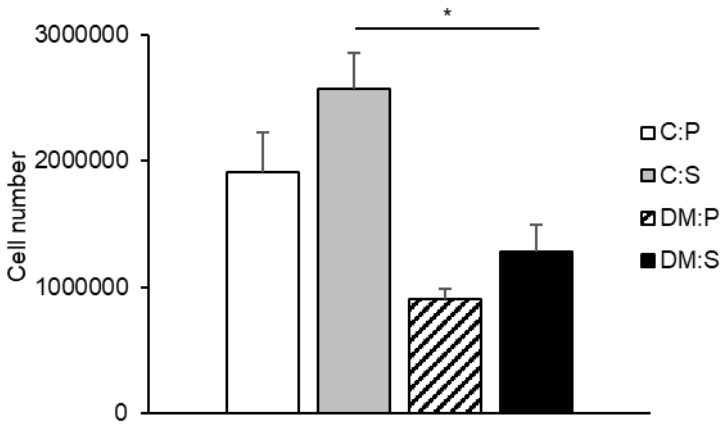

Figure 5.6 Cell number at the end of passage 0, prior to subculture. Cell number was determined by haemocytometer cell counting principle prior to subculture for all treatment groups. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** One-way ANOVA with Tukey's post hoc test, *p<0.05 indicates significance between groups.

5.4. MSC viability following subculture was slightly improved with NAC/AAP supplementation (Passage 1)

Viability of MSCs was assessed over a period of 72h, 96h and 144h post seeding in passage 1 using Crystal Violet staining. Refer to Fig. 5.7 for representative images of Crystal Violet staining. Despite seeding the same number of cells per well (666 cells/mm^2) for all groups, MSC viability was higher in the healthy control compared to DM groups (p=0.008) (Fig. 5.8). In the C:P group, MSC number increased to 1351±1105 cells/mm^2 (2-fold) on day 3 (72h) post seeding and to 2409±927 cells/mm^2 (3.6-fold) (p<0.001) on day 4 (96h). MSC viability was however not maintained in the C:P group after 6 days (144h) (1393±803 cells/mm^2) (2.1-fold). In contrast the C:S group showed a slower but steady increase in MSCs over time, with cell number increasing to 1338±846 cells/mm^2 on day 3 (72h) (2-fold), 1850±1286 cells/mm^2 on day 4 (96h) (2.78-fold) (p=0.02) and 2530±1851 cell/mm^2 on day 6 (144h) (3.79-fold) (p<0.001) post seeding. A significant difference in MSC number was evident between the C:P and C:S groups on day 6 (144h) (p=0.03) (Fig. 5.8).

In the DM:P group MSC number did not increase over time, instead some cell death was evident with MSC number remaining below seeding density at 459±444 cells/mm^2 (0.69-fold) throughout the 6 days. NAC/AAP supplementation in the DM:S group slightly improved MSC survival with cell number increasing from 369±188 cells/mm^2 on day 3 (72h) (0.55-fold) to 852±739 cells/mm^2 on day 4 (96h) (1.27-fold) and 718±421 cells/mm^2 (1.08-fold) on day 6 (144h) post seeding (Fig. 5.8).

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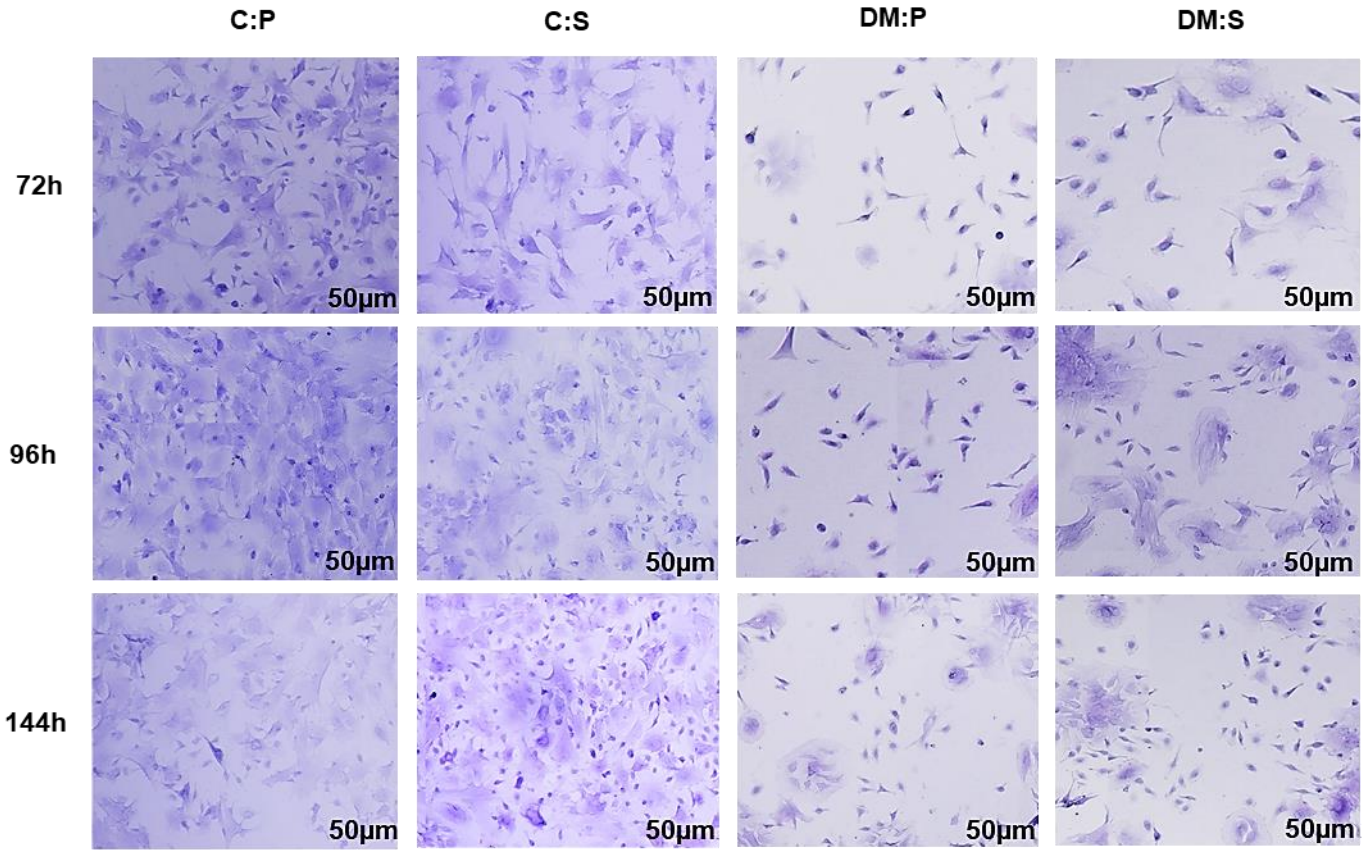

Figure 5.7 Representative images of crystal violet stained control and diabetic derived MSCs. MSCs in passage 1 of all the treatment groups were stained with crystal violet stain at 72h, 96h and 144h to determine viability. The images here are each a tile scan of the whole images which was taken in 10x/200µm. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement.

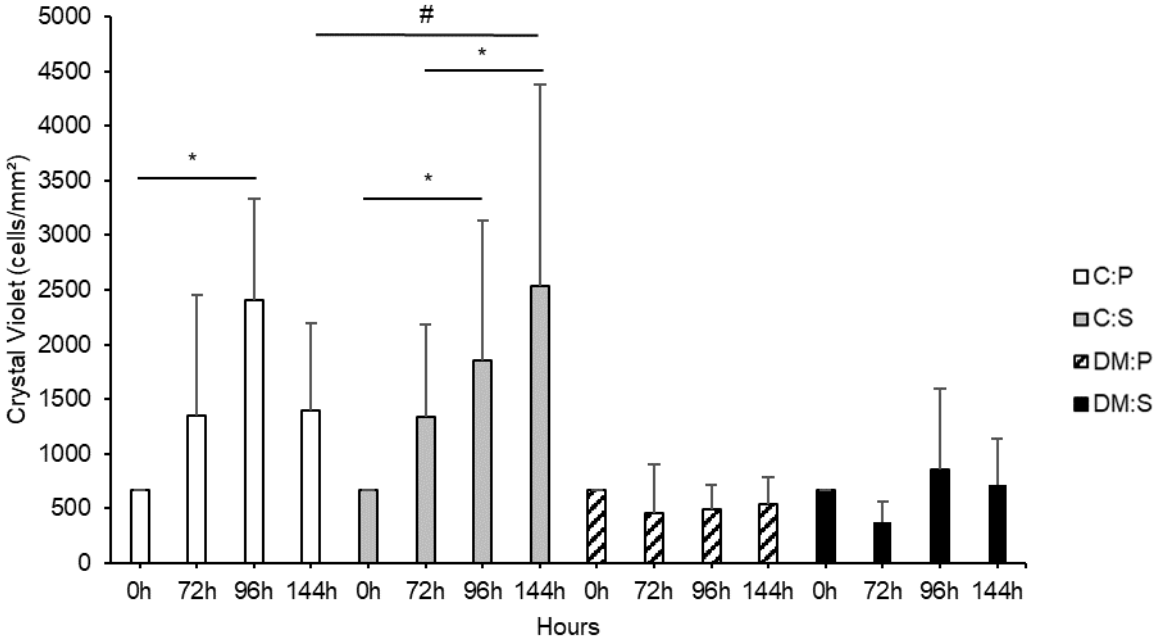

Figure 5.8 Crystal violet staining indicating viable MSCs at 72h, 96h and 144h post seeding. MSCs of all treatment groups were assessed for viability by image quantification of crystal violet stained MSCs at 72h, 96h and 144h. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** Three-way ANOVA with Tukey's post hoc test, *p<0.05. * indicates significance within groups, # indicates significant treatment effect at the same time.

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5.5. NAC/AAP supplementation and the proliferation rate of MSCs

Active cellular proliferation was determined in healthy control and DM derived MSCs using BrdU absorbance at 72h, 96h and 144h post seeding in passage 1. No difference was evident in the proliferation rate between treatment groups at the respective time points (Fig. 5.9 A-B). Cellular proliferation peaked on day 4 (96h) in all groups, followed by a decline towards day 6 (144h). This decline in proliferation coincided with an increase in cell confluency as was evident in the Crystal Violet data. C:P had steady proliferation from day 3 (72h) (0.42±0.04 OD) to day 4 (96h) (0.44±0.07 OD) however proliferation declined at day 6 (144h) (0. 28±0.05 OD) (Fig. 5.9A). A similar proliferation curve can be observed in C:S (day 3 0.34±0.02 OD, day 4 0.39±0.10 OD; day 6 0.24±0.03 OD). Proliferation rate in DM:P had a strong incline from day 3 (0.35±0.09 OD) to day 4 (0.63±0.12 OD) however proliferation declined until day 6 (0.46±0.06 OD) (Fig.5.9B). The same trend was observed for DM:S however at a steadier rate. An obvious decrease in proliferation rate at day 6 compared to day 3 was noticed though not significant. Overall, both control and diabetic supplement groups had a slightly lower incorporation of BrdU at the specific time points compared to control and diabetic placebo groups. It should be noted that there was variability in the background control sample, so these results should ideally be confirmed.

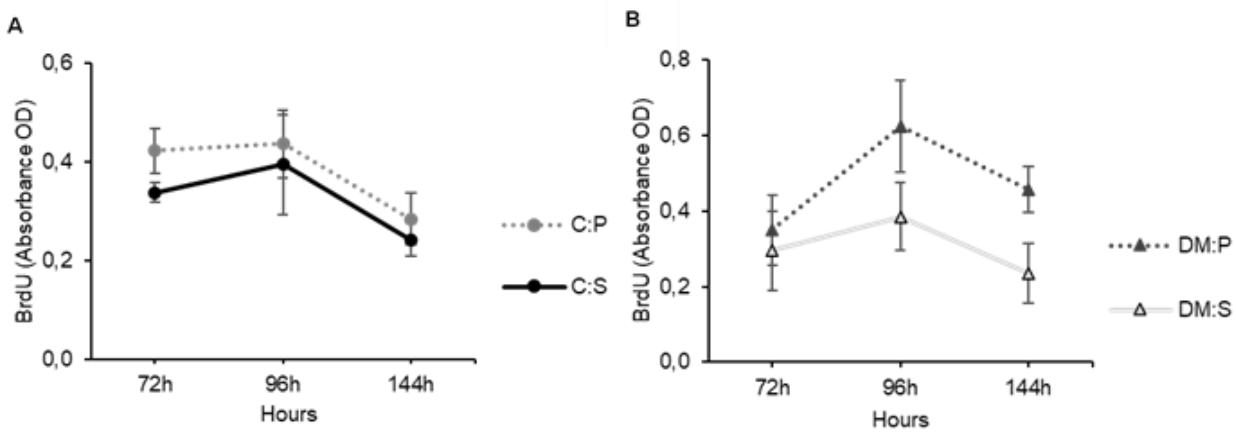

Figure 5.9 Proliferation assay. Proliferation of MSCs in passage 1 was assessed by BrdU ELISA assay at 72h, 96h and 144h. A) Healthy control MSCs B) Obese diabetic MSCs. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement.; BrdU, Bromodeoxyuridine; OD, Optical Density.

5.6. NAC/AAP supplementation reduced adipogenesis in diabetic derived MSCs however could not aid osteogenic differentiation.

The osteogenic and adipogenic differentiation capacity of MSCs was determined over a period of 10 days. Extensive mineralization (>60% of surface area) was evident in the MSCs derived from healthy control animals, whereas osteogenic differentiation was significantly impaired in the MSCs derived from DM animals (<10% surface area) ($p<0.001$) (Fig. 5.10A). Although NAC/AAP supplementation did show some signs of improved osteogenesis in the DM:S group (6±3% surface area) compared

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to placebo treatment (DM:P 2±1% surface area), it was not significant (Fig. 5.10B). Similarly, lipid accumulation during adipogenesis was impaired in the MSCs derived from DM animals compared to controls ($p<0.01$), with NAC/AAP supplementation showing a tendency to suppress adipogenesis even further though not significant (Fig. 5.10C, D).

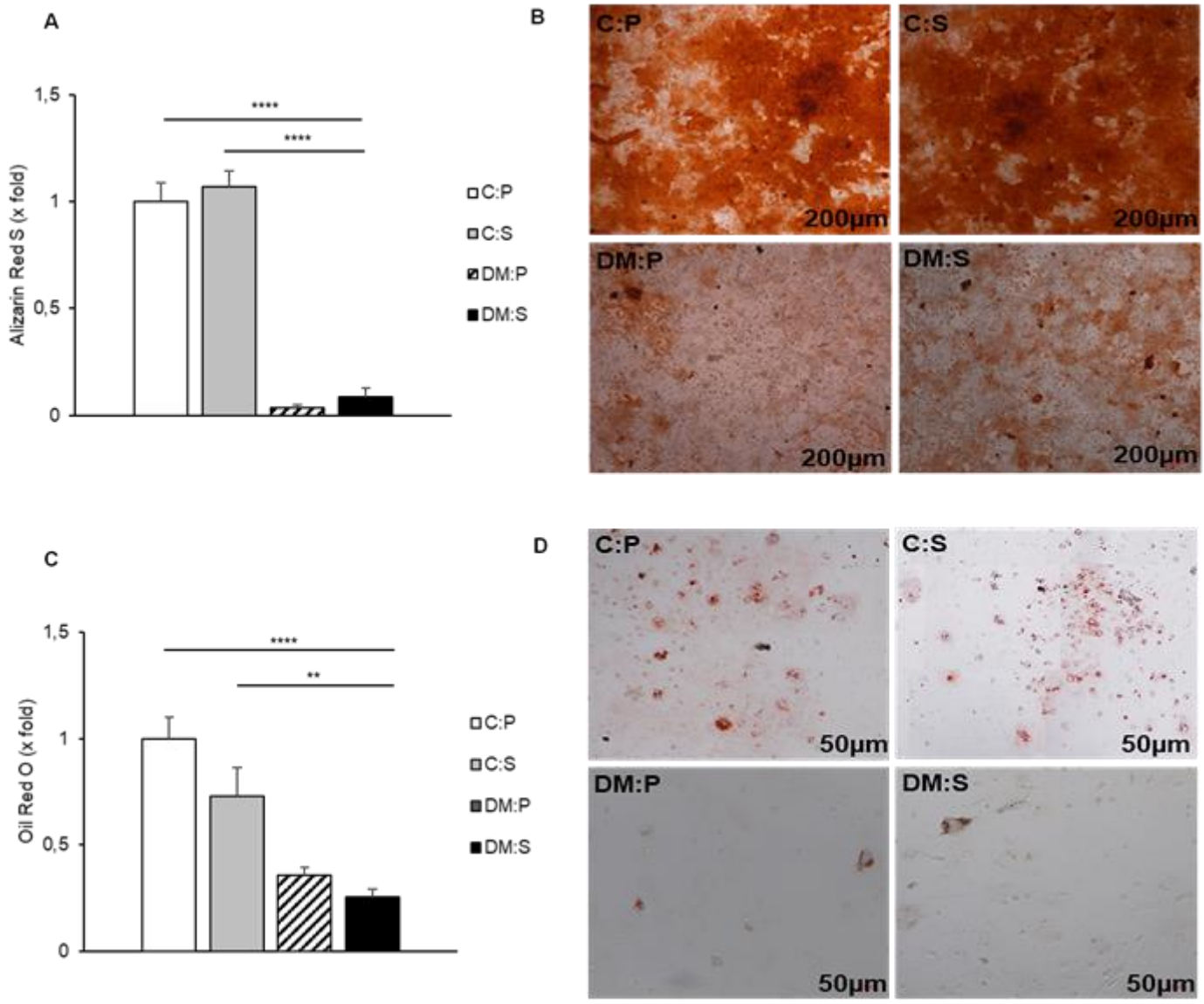

Figure 5.10 MSC osteogenic and adipogenic differentiation. 10 Days after induction media (AM and OM) was introduced to MSC in culture, differentiation was assessed by A) Alizarin Red S staining for osteogenic differentiation, indicative of mineralization (fold change) between treatment groups, B) Representative images of osteogenic differentiated MSCs, and C) Oil Red O staining for adipogenic differentiation, indicative of lipid droplet formation (fold change) between treatment groups, D) Representative images of adipogenic differentiated MSCs. The images here are each a tile scan of the whole images which was taken in 10x/200µm. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement. **Statistical analysis:** One-way ANOVA with Tukey's post hoc test, **$p<0.01$, ****$p<0.0001$ indicates significance between groups.

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5.7. Cytokine expression in conditioned media from wound fluid stimulated diabetic MSCs

The secretome of MSCs was assessed by quantifying cytokine levels within conditioned media at baseline (WT control vs. DM) and following stimulation with diabetic wound fluid (WF) (DM:P vs. DM:S). The cytokine expression levels of IL6, IL10 and TNFα in stimulated (diabetic wound fluid, WF) and unstimulated (SGM) conditioned media were overall near the detection limit of the assays.

Under standard culture conditions large variability was evident with no significant difference in IL6 (pro- and anti-inflammatory) (DM:P 53.56±23.01 pg/mL, DM:S 45.74±14.11 pg/mL) and IL10 (anti-inflammatory) (DM:P 6.8±5.45 pg/mL, DM:S 11.99±8.87 pg/mL,) levels in the DM groups compared to their healthy control counterparts (IL6: C:P 36.29±7.15 pg/mL, C:S 34.81±7.75 pg/mL; IL10: C:P 10.7±5.51 pg/mL, C:S 6.2±3.91 pg/mL) (Fig. 5.11 A-B). TNFα (proinflammatory) levels was significantly higher ($p<0.01$) in the DM group that received supplementation (DM:S 12.16±1.08 pg/mL) compared to the healthy control groups (C:S 8.56±0.72 pg/mL) (Fig. 5.11 C).

The paracrine responsiveness of DM MSCs was assessed in the presence of WF (Figure 5.11 D-F). A significant ($p<0.001$) increase in IL6 levels (DM:P+WF 1351.36±442.65 pg/mL, DM:S+WF 1583.30±481.96 pg/mL) was observed in response to WF stimulation compared to standard culture conditions (DM:P 53.56±23.01 pg/mL, DM:S 45.74±14.11 pg/mL) (Fig. 5.11 D). Similarly, increased IL10 expression was evident in stimulated DM MSCs, with DM:S+WF showing the greatest response (27.11±9.80 pg/mL) ($p<0.05$) compared to DM:P (6.80±2.73 pg/mL) (Fig. 5.11 E). Proinflammatory TNFα expression was slightly increased in DM:S+WF (16.67±4.35 pg/mL) ($p<0.05$) compared to DM:P under standard culture conditions (10.55±0.74 pg/mL) (Fig. 5.11 F). Due to the limited volume of WF available, control MSCs were not stimulated with WF and comparisons with DM could thus not be performed.

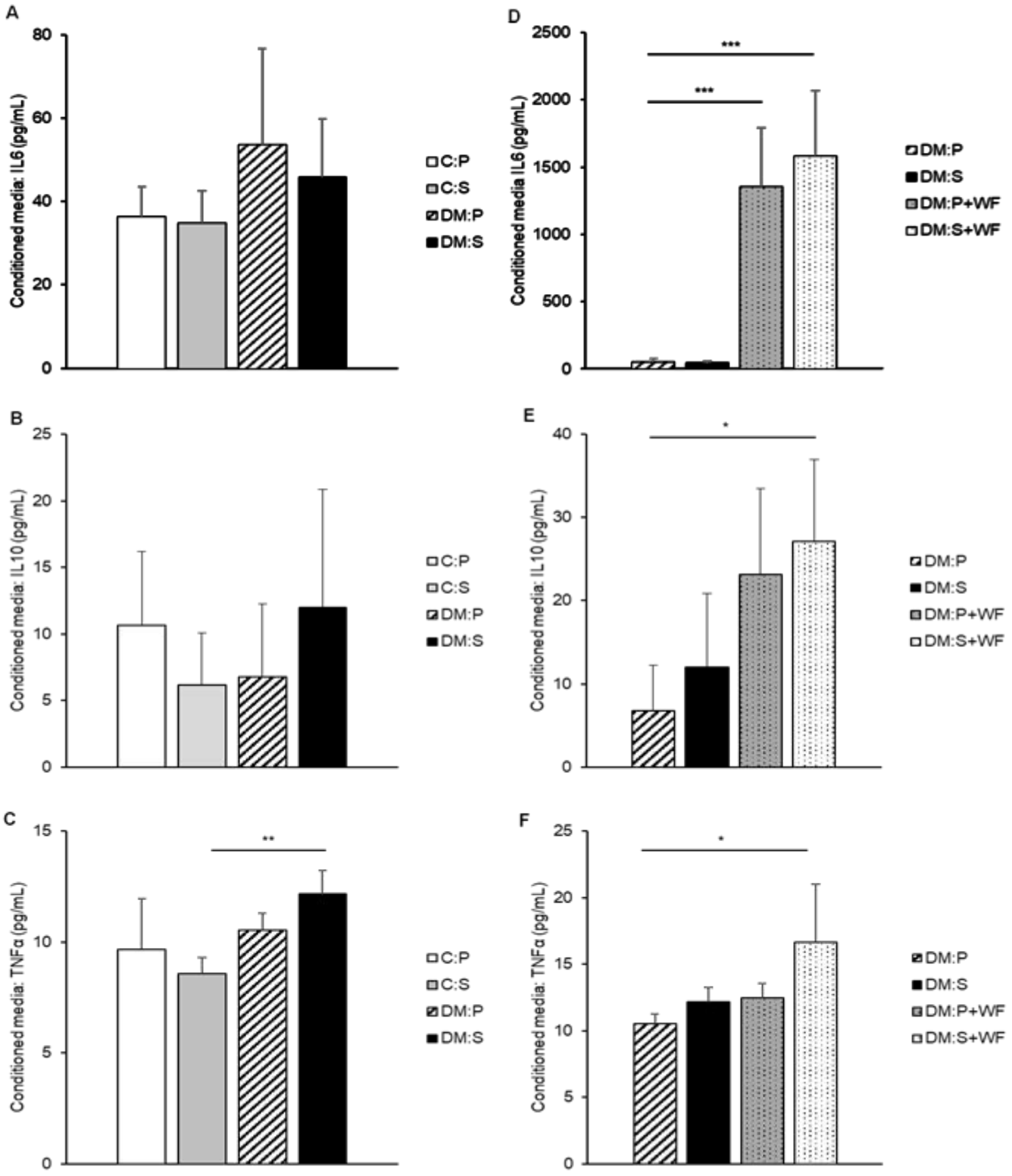

Figure 5.11 IL6, IL10 and TNFα cytokine levels in conditioned media from SGM and wound fluid stimulated MSCs. Conditioned media collected from MSCs stimulated with standard growth media and diabetic wound fluid for 4 hours was assessed for inflammatory cytokine protein content by ELISA. A-C) IL6, IL10 and TNFα (pg/mL) cytokine response in conditioned media under standard growth media conditions. D-F) IL6, IL10 and TNFα (pg/mL) cytokine response in conditioned media from wound fluid stimulated diabetic MSCs. **Abbreviations:** C:P, Control: Placebo; C:S, Control: Supplement; DM:P, Diabetic: Placebo; DM:S, Diabetic: Supplement; IL, Interleukin, TNFα, Tumour Necrosis Factor alpha; WF, wound fluid. **Statistical analysis:** One-way ANOVA with Tukey's post hoc test, *p<0.05, **p<0.01, ***p<0.001. * indicates significance between groups.

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Chapter 6

Discussion

MSC dysfunction is a serious and often overlooked complication of T2DM (Kornicka, Houston and Marycz, 2018). The pathologic microenvironment associated with T2DM occur as result of prolonged hyperglycaemia leading to AGE formation and build up, which in turn triggers persistent inflammation and oxidative stress. Combined, this toxic environment (also known as glucose toxicity) causes irreversible cellular damage to various systems inside the body instigating the development of secondary complications such as retinopathy and non-healing wounds. In healthy individuals, bone marrow resident MSCs play an important role in the body's innate repair system and maintaining tissue homeostasis (Kalinina *et al.*, 2011). These MSCs are however vulnerable to the pathologic microenvironment in T2DM and as result cannot perform their vital immune surveillance and pro-regenerative functions. Consequently, excessive tissue damage and the failure of endogenous repair mechanisms in T2DM patients is a serious concern.

The current study demonstrated that combined *in vivo* antioxidant supplementation consisting of NAC and AAP, was able to reduce lipid peroxidation and slightly improve the overall antioxidant capacity of obese diabetic mice. These protective effects related to the anti-inflammatory and antioxidant nature of NAC/AAP was also evident on cellular level upon isolation of bone marrow MSCs and assessment of their function *ex vivo*. This study showed that MSC growth rate and viability post isolation improved following *in vivo* supplementation. Furthermore, the combined antioxidant supplementation reduced the adipogenic differentiation of MSCs which is advantageous since unwanted ectopic lipid accumulation within bone marrow (bone marrow adiposity) has been shown in T2DM patients (Ferland-Mccollough *et al.*, 2018). These key findings demonstrate that NAC/AAP as supplementary treatment has the potential to limit the development of secondary complications by protecting T2DM patients against glucose toxicity and warrants further investigation.

6.1. Model validation

During metabolic profile assessment of control (C57BL6/J) and diabetic (B6.Cg-Lep^{ob}/J, ob/ob) animals, control animals showed appropriate weight gain and constant blood glucose levels within the normal range throughout the treatment period. These results are consistent with the Jackson laboratory's observations on the C57BL6/J (control) mouse strain (The Jackson Laboratory, 2020). Excessive weight gain was however observed in diabetic animals from the first week. Furthermore, diabetic animals had significantly increased blood glucose levels indicative of diabetes (>16.7mmol/L). The observation that 16.7mmol/L is considered diabetic is supported by Alipio *et al.* (2010) who observed 300mg/dL (16.7mmol/L) blood glucose level in their db/db T2DM model at 3-4 weeks of age. Blood glucose levels of control animals were approximately 9.0 mmol/L throughout

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the treatment period and is considered to be high when comparing it to normal human blood glucose values (4.0-5.4mmol/L). However, normal physiological blood glucose levels of C57BL6/J (control) mouse strain at 8 weeks of age is 17.09 mmol/L (The Jackson Laboratory, 2007) which is much higher than what was observed in this study. Thus, our control animals blood glucose levels were in normal healthy range. The ob/ob strain has a particular phenotype characterised by obesity and prediabetes which develop due to leptin deficient hyperphagia (Kennedy *et al.*, 2010). Glycated haemoglobin (HbA1c) and oral glucose tolerance test (OGTT) was not performed on the animals to confirm the disease state. This limited our full understanding of the diabetic state of the animals. Controversy on the use of glucometer readings instead of biochemical assays on blood glucose measurements exists however, the resources that were available for this study were utilised in a consistent matter to achieve results. Also performing an OGTT test requires oral gavage of a glucose bolus to assess glucose metabolism. This method was excluded from the study as oral gavage is difficult and dangerous to perform on mice aged 4-5 weeks. Taken together, significant weight gain and increased blood glucose levels in diabetic animals over the treatment period highlighted obesity and hyperglycaemia. With the appropriate background control and dysregulated metabolic factors, the ob/ob animals in this study represent insulin resistance (phase 3 of diabetes). Thus, validating our model as being representative of T2DM including its associated factors. The NAC/AAP supplementation furthermore did not affect either animal weight or blood glucose levels indicating that the observed beneficial effects were not due to improved metabolic parameters.

6.2. Antioxidant status of animals

In vivo supplementation of NAC and AAP did not affect the animals negatively as behaviour, appearance, blood glucose and weight did not differ from placebo treated animals. A major concern when using phytochemicals is the toxicity that may develop due to the misconception that natural compounds are not harmful. The affectless observation of NAC and AAP in the current study demonstrate that the concentration antioxidants are low to not cause any harm. To compare concentration used and that of human equivalent, the mice were administered with NAC 0,608mg and AAP 0.095mg compared to NAC 188mg and AAP 30mg (adult human male equivalent). Refer to appendix B for animal and human equivalent dosages. For perspective the recommended daily dose of these antioxidants for an adult male human is 600mg and 90mg, respectively (Goodson, 2018; Snyder, 2019).

Oxidative stress is prominent in T2DM pathogenesis. A study by Ganjifrockwala, Joseph & George (2017), reported that T2DM patients from a South African cohort had increased MDA, fasting plasma glucose (FPG), HbA1c, triglycerides and oxidized low-density lipoprotein (ox-LDL) with a decreased total antioxidant level. The authors concluded that hyperglycaemia and dyslipidaemia lead to the increased lipid peroxidation and oxidative stress observed in patients with T2DM compared to healthy individuals (Ganjifrockwala, Joseph and George, 2017). Serum analysis by the current study

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indicated the same trend, with increased MDA and decreased TAC levels from obese diabetic animals without supplementation compared to healthy control animals. MDA is a polyunsaturated fatty acid peroxidation product in cells and is excessively produced in the presence of increased free radicals (Gaweł *et al.*, 2004). The altered microenvironment in diabetic animals as a result of hyperglycaemia and obesity increase generation of free radicals leading to oxidative stress (Oguntibeju, 2019). With the addition of NAC/AAP antioxidant supplementation an increased TAC level in obese diabetic animals was evident while lipid peroxidation levels were reduced. Increased oxidant scavenging agents in the circulation exerted a diminishing action on oxidative stress related damage. The cysteine derivative NAC is a powerful antioxidant and functions by direct ROS scavenging or indirect function by replenishing GSH to reduce oxidative stress (Lasram *et al.*, 2014). NAC supplementation in a study by Zukowski *et al.* (2018) reduced oxidative stress in a high fat fed rat model.

6.3. Bone marrow MSC function

In a previous study done by our group, standard growth rate of MSCs *in vitro* was restored with a combination of 7.5mM NAC and 0.6mM AAP (Mehrbani Azar *et al.*, 2018). Furthermore, the study demonstrated MSC viability lasted 6 days in culture due to the addition of AAP. Thus, the research question shifted toward whether these antioxidants in combination and specific concentration will enhance MSC functionality *in vivo*.

Coherent with literature, MSCs derived from healthy control animals had consistent growth rate whereas diabetic derived MSCs were impaired upon *ex vivo* culture (Kim *et al.*, 2015; Mehrbani Azar, Niesler and van de Vyver, 2020). Decreased cellular growth is attributed to excessive cell death, senescence, decreased stemness and impaired function (Li *et al.*, 2007; Liu *et al.*, 2020). Furthermore, impaired growth rate could reflect DNA damage, mitochondrial fragmentation and defected membrane repair (van de Vyver, 2017; Seo, Yoon and Do, 2018). The difference in growth rate between control and diabetic groups point to impairment in diabetic groups as is expected from the diabetic microenvironment these cells resided in. Improved growth rate was however evident in MSC derived from supplement treated mice (both control and diabetic). Suggesting the antioxidant combination have protective effects on MSCs by increasing cell number. The implication of improved growth rate not only increases MSCs intrinsic health, however, also indicate the body's ability to repair damaged tissue through cell division. Growth rate is the looking glass into cell fate as it can indicate cells intrinsic health status. In the current study no excessive cell death was observed in either of the groups and senescence was not a factor (<1%) indicating that proapoptotic proteins are down regulated. This observation could however not be justified because senescence was only assessed by morphology and not by using β-galactosidase, limiting total cellular senescence quantification.

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Antioxidant supplementation slightly improved viability post subculture in both control and diabetic MSCs. The slight increase in viability could be as result of decreased protectiveness by antioxidants as supplementation did not continue throughout cell culture – a future perspective for increasing MSC viability post isolation for longer period. *Ex vivo* MSC supplementation with NAC and AAP combination have improved MSC viability post isolation for up to 6 days (Mehrbani Azar, Niesler and van de Vyver, 2020). Various studies have observed AAP (vitamin C) to be more prominent in enhancing viability of MSCs (Li *et al.*, 2016; Fujisawa *et al.*, 2018).

DNA replication is important for cell growth and tissue repair. Though can be dysregulated by ROS which cause DNA damage by blocking DNA replication resulting in impaired proliferation ability. Incorporation of BrdU (proliferation assessment) in both control and diabetic supplement groups was slightly lower compared to control and diabetic placebo groups at various time points. In a study by Fujisawa *et al.* (2018), human MSCs isolated from bone marrow treated with ascorbic acid had increased proliferation when expanded in culture. Furthermore, the authors reported on an increased hydroxylase of hypoxia inducible factor alpha (HIFα) which promoted mitochondria activation (Fujisawa *et al.*, 2018). In support of this study Li, Sun & Pang (2015) demonstrated that a combination treatment of NAC and AAP on human adipose derived MSCs maintained mitochondrial function protecting the organelle against mitoptosis (programmed destruction of mitochondria) and the cells from subsequent programmed cell death brought by ROS production. Vitamin C can promote proliferation through telomerase activity (Zheng *et al.*, 2015). In the current study these effects were not observed but could be explained by overly confluent MSCs due to the increased growth rate observed in supplement groups. Contact inhibition theory states that cells will signal the inactivation of proliferation when encountering one another (Pavel *et al.*, 2018). Therefore, future research should consider controlling proliferation of MSCs by reducing *ex vivo* expansion time before quantifying proliferation.

In healthy non-diabetes environment ROS is required by MSCs to commit to a specific lineage in their ability to differentiate. However, excessive ROS production as result of oxidative stress can skew the differentiation potential of MSCs by inducing adipogenic differentiation (Kanda *et al.*, 2011). Skewed adipogenic differentiation results in excessive adipocyte formation that reduce osteogenic differentiation needed for healthy bone architecture. Kanda *et al.* (2011) studied the effect of NAC on multipotent mesenchymal stem cell line (10T1/2 cells) and observed that NAC successfully blocked NAD(P)H oxidase 4 (Nox 4) excessive ROS production by blocking cAMP response element-binding protein (CREB) through transcriptional activation which reduced adipogenic differentiation. This observation is supported in our study as MSCs derived from supplemented treated mice (control and diabetic) had reduced adipogenic differentiation. Osteogenic differentiation of MSCs from diabetic derived animals was however not rescued by antioxidant protection. Impaired osteogenic differentiation has direct relations to altered bone architecture as result of decreased bone density due to development of increased fat deposition, inflammation and oxidative stress in

T2DM pathology (van de Vyver, 2017; Kornicka, Houston and Marycz, 2018). This is important as MSCs reside in bone marrow and require their niche environment to fulfil homeostasis. Bone remodelling is defected in T2DM due to fat accumulation in bone marrow that create pathologic environment for the cells that reside there. Although the opposite was observed for adipogenic differentiation a longer treatment of NAC/AAP supplementation could potentially rescue osteogenic differentiation.

The current study shows novelty as the first to protect MSCs *in vivo* by NAC/AAP supplementation whereas the studies discussed above only focussed on preconditioning stem cells *in vitro* to obtain protective effect.

6.4. Paracrine responsiveness of MSCs

MSCs phenotype and response to change in their environment is an important indicator of their ability to maintain tissue homeostasis. When exposed to prolonged pathologic environment such as T2DM MSCs become dysfunctional. Dysfunctional MSCs cannot perform their vital function (immune surveillance) when exposed to a highly inflamed environment, rather these cells will exert proinflammatory cytokines to add to instead of aid the pathologic environment (van de Vyver *et al.*, 2016; Mehrbani Azar *et al.*, 2018; Fijany *et al.*, 2019; Vinci *et al.*, 2020). Thus, MSCs (depending on state) exposed to a chronic proinflammatory environment (wound fluid) will either counteract or exacerbate it. Antioxidants exert a beneficial protective factor on MSCs, and it is claimed/ alleged that response to wound fluid should retain an anti-inflammatory profile.

Upon tissue damage caused by wounds, the body produces exudate during the inflammatory phase of wound healing to moisten the wound area and prepare it for tissue repair (Adderley, 2010). The wound exudate is a clear liquid sourced from capillary leakage, lymphocytes and surrounding cells secretome that uniquely represents the various factors involved in the wound microenvironment (Adderley, 2010). Persistent inflammation in chronic wounds favouring a proinflammatory profile has been associated with factors detected in diabetic wound fluid (exudate) namely TNFα, IL6, IFNγ and IL1 (Lasram *et al.*, 2014). Diabetic wound fluid used in this study was obtained from a previous diabetic wound model study done by our group (Research by Azar, 2019). Following wound fluid collection 21 days post wounding, analysis was performed on protein content by Bio-Plex assay and resulted in the detection of 23 cytokines. The wound fluid used represents a chronic proinflammatory profile with overexpression of IL1, IL12 and TNFα associated markers. In the current study MSCs response to the induced proinflammatory microenvironment differed between placebo derived and supplement derived MSCs. The increased expression of proinflammatory markers IL6 and TNFα observed in diabetic placebo group supports proinflammatory activation. The proinflammatory profile follows the pathway of NF-kB signalling which leads to the inactivation of IRS-1 and ultimately insulin resistance (Chen *et al.*, 2015). Chronic inflammation not only dysregulates cells extrinsic environment however also intrinsically by skewing diabetic MSCs immunomodulatory function to

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overexpress proinflammatory mediators MIP1α, MIP1β, MIP2, TNFα and IL6 (Bernardo and Fibbe, 2013; van de Vyver *et al.*, 2016). Whereas diabetic animals with supplementation had decreased expression of proinflammatory markers and slight increase in IL10 (anti-inflammatory). NAC *in vitro* diminishes abnormal inflammatory state by targeting and reducing JNK, ERK1/2, PPARγ, NF-κB and MCP1 levels (Lasram *et al.*, 2014). IL6 and IL10 both share anti-inflammatory properties and are secreted by MSCs in response to inflammation. IL6 regulate inflammation, immune response, cell fate and proliferation (Kyurkchiev *et al.*, 2014). TNFα expression also slightly increased in response to wound fluid. This observation could possibly be attributed to the 12-day culture which decreased protectiveness provided by antioxidants. Overall, the antioxidants could potentially drive the inflammatory profile of diabetic groups to favour anti-inflammatory modulating properties in the presence of WF. The genetic expression of the expressed cytokines was not assessed with qPCR. Thus, the data is inconclusive and further investigation into the expressed genes of these cytokines are required.

Comorbidities in T2DM develop due to the favourable environment created by the disorder leading to cellular dysfunction. From the current study the overall trend observed was that MSCs functionality improved due to the antioxidant supplementation and will thus protect MSCs from contributing to the development of comorbidities. The protection in some instances only lasted for a short while *in vitro*. A consensus on the main pathway responsible for secondary complication development in T2DM is the AGE/RAGE pathway which is prominent in oxidative stress and inflammation development (Goh and Cooper, 2008; Singh Jaggi *et al.*, 2014). RAGE activation has been associated with the acceleration of atherosclerosis, neuropathy, nephropathy and reduced angiogenesis (Toth *et al.*, 2008; Giacco and Brownlee, 2010; Manigrasso *et al.*, 2014). A RAGE knockout study performed on mice induced with Streptozotocin (type 1 diabetes) by Soro-Paavonen *et al.* (2008) showed decreased atherosclerosis development compared to controls. Supporting the above-mentioned study, another RAGE knockout study with the focus on neuropathy demonstrated that the absence of RAGE reduced neuropathy characteristics in mice (Toth *et al.*, 2008). To combat the underlying oxidative stress and inflammation antioxidants have been proposed and used in diabetic patients to attenuate development of secondary complications. Antioxidants such as NAC have the ability to increase physiological antioxidants such as GSH while reducing inflammatory cytokines and apoptotic factors (Tun *et al.*, 2020). NAC has been observed to reduce lipid accumulation in the liver by suppressing PPARγ and C/EBP β and enhance the genes responsible to breakdown lipids (Ma, Gao and Liu, 2016). The current study supports this observation as NAC/AAP supplementation successfully reduced adipogenic differentiation in diabetic MSCs. AAP has been associated with lowered blood glucose and decreased blood pressure in individuals with diabetes which is promising since hyperglycaemia is associated with the development of comorbidities such as vascular disease (Dakhale, Chaudhari and Shrivastava, 2011; Mason, Rasmussen and Wadley, 2018). Many studies also reported on the use of AAP with Metformin to manage diabetic complications (Gillani *et al.*, 2017). Thus, the NAC/AAP supplementation used in the current study could potentially limit

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secondary complication development in T2DM through their antioxidant and anti-inflammatory functions to attenuate hyperglycaemia, oxidative stress, and inflammation.

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Chapter 7

Conclusion, future perspectives, and limitations

T2DM is a multifactorial disorder with hyperglycaemia, AGEs, oxidative stress and inflammation affecting tissue and organ systems such as bone marrow resulting in bone marrow MSC dysfunction (Kornicka, Houston and Marycz, 2018). MSC dysfunction leads to impaired tissue regeneration and impaired immunomodulation, important functions of MSCs as these cells maintain tissue and regulate the immune response (Duffy *et al.*, 2011; Kalinina *et al.*, 2011; Picke *et al.*, 2019). The progression of the disorder therefore leads to cellular dysfunction and ultimately the development of comorbidities. Thus, affecting the quality of life of patients suffering from T2DM. Poorly controlled hyperglycaemia as result of non-compliance of patients to their anti-diabetic drug regime warrants investigation into a complementary supplement treatment to use with prescribed anti-diabetic drugs.

In our study, we had a validated T2DM model as the ob/ob animals represented obesity and hyperglycemia with their significant weight gain and significantly increased blood glucose levels compared to control animals. The NAC/AAP supplementation successfully decreased lipid peroxidation and increased TAC of ob/ob animals. Our study did not assess the bioavailability of the antioxidants used *in vivo*. Bioavailability would have indicated the fraction of each antioxidant in the circulation which would have also revealed absorption of antioxidants inside the animals. Thus, future studies should measure bioavailability of antioxidants to determine antioxidant absorption. Another limitation occurred during serum analysis. Some samples had haemolysis (pink/red serum colour) due to red blood cell rupturing during blood collection. This occurred especially in ob/ob animals because of their huge amount of abdominal fat that made it difficult to accurately perform cardiac puncture. The haemolysis of some samples was controlled for during serum analysis however due to insufficient volumes not all could be controlled for. It is advisable to use kits that require smaller volumes of serum to determine oxidant content for future testing. In addition, the chest cavity of the animals should be opened to expose the heart of the animals to accurately perform cardiac puncture.

This study did not have separate treatment groups of NAC and AAP to justify if the combination or antioxidants alone were the leading cause of change in diabetic animals and MSCs. This limitation was addressed with literature to possibly justify the synergistic effect of NAC and AAP in combination. The number of animals also played a role as they were already limited in the current study.

Furthermore, the effects of 6-week NAC/AAP supplementation *in vivo* provided limited protection for the MSCs. Although *ex vivo* growth rate improved, viability increased and adipogeneses reduced, antioxidant protection diminished in culture as osteogenesis was not fully restored. For future research, the decline in protection offered by antioxidants post isolation, suggest that MSCs be

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treated with antioxidants *ex vivo* as well to prevent protection from declining. Variability in the data as a result of contamination of some cell cultures decreased sample size leading to skewing of the results. For future studies care should be taken to precisely follow cell culture protocols to avoid contamination. In addition, it is advisable to increase the internal sample size of each biological sample when culturing cells.

Lastly, our study assessed MSC responsiveness after *in vivo* supplementation and wound fluid stimulation. Evidence that supplemented diabetic MSCs are in favour of activating the anti-inflammatory response is weak, as both pro- and anti-inflammatory cytokines were expressed. Again, antioxidant protection decreased and *ex vivo* supplementation is required to prevent protection from diminishing. Molecular responsiveness by real-time polymerase chain reaction (qPCR) was not assessed due to time constraints however would have given insight into the expression of anti- and proinflammatory cytokines observed in our study. Wound fluid stimulation in control MSCs for inflammatory marker assessment in MSC responsiveness was not tested due to small volume of wound fluid available. The pro-inflammation activation through protein and pathway NF-kB can be assessed by western blot analysis of liver in mice to determine changes in inflammatory response with and without antioxidant treatment. This will determine if NF-κB is down regulated in antioxidant treated animals.

Non-compliance of T2DM patients with their prescribed medications makes it inevitable to trust that an antioxidant regime would be followed. However as seen from the current study control MSCs benefitted from the combined antioxidant supplementation as well. Thus, arguably the assumption can be made that the antioxidants can be used in well controlled and under controlled T2DM patients.

In conclusion, our study is novel as it is the first to demonstrate NAC/AAP supplementation in an *in vivo* diabetic mouse model. The NAC/AAP supplementation offered limited protection to dysfunctional MSCs *in vivo* from glucose toxicity in the diabetic microenvironment and could possibly limit the development of comorbidities. Further investigation is required to prove NAC/AAP supplementation can limit comorbidity development and improve the quality of life for patients suffering from T2DM.

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Chapter 8

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Chapter 9

Appendices

Appendix A: Ethics approval letters

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Protocol Approval

Date: 06 February 2019

PI Name: Dr. Mari Van de Vyver

Protocol #: ACU-2019-3857

Title: Development of a chronic wound model using B6.Cg-Lepob/J obese diabetic and C57BL/6J wild-type control mice: Characterizing cellular changes that impair regenerative potential of wound-derived fibroblasts and endogenous mesenchymal stem cells.

Dear Mari Van de Vyver ,

Your request for an amendment, was reviewed on 05 February 2019 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved

Amendment Approval Date: 05 February 2019

Amendment - In vivo nutritional supplementation with anti-oxidants (NAC/AAP) for a period of 6 weeks prior to euthanasia.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 3857 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

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UNIVERSITEIT
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UNIVERSITY

Amendment Approval

Date: 30 April 2020

PI Name: Dr. Mari Van de Vyver

Protocol #: ACU-2020-3857

Title: Development of a chronic wound model using B6.Cg-Lepob/J obese diabetic and C57BL/6J wild-type control mice: Characterizing cellular changes that impair regenerative potential of wound-derived fibroblasts and endogenous mesenchymal stem cells.

Dear Mari Van de Vyver ,

Your request for amendments, was reviewed on 29 April 2020 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved.

Approval Date: 29 April

2020 **Amendment (s)**

Details:

1. The use of wound fluid collected from chronic wounds on days 2 and 7 post wounding to stimulate an immortalized monocyte/macrophage cell line (J774.1A) in vitro.

2. (a) Assessment of blood glucose levels (weekly - every Friday) during the 6 weeks of nutritional supplementation (tail prick); (b) Following euthanasia, the collection of whole blood via cardiac puncture for serum collection.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 3857 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

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Appendix B: Administered antioxidants concentration calculations and animal and human equivalency dosages

AAP concentration calculations

AAP: 0.6mM in 30 mL jelly

0.6mM x 30 = 18mM [working]

18mM/1000 = 0.018M (0.018mol per 1L)

AAP molecular weight = 322.05g per 1mol x 0.018mol = 5.7969g per 1000mL (1L)

0.0057969g per 1mL (Value too small to measure off)

0.018M x 100 = 1.8M (1.8 mol per 1L)

Gram per 1L = 1.8mol x 322.05 = 579.69g per 1L

0.5769g per 1mL

Prepare 1.8M (0.57969g + 1mL ddH_2O)

10µL AAP of 1.8M + 990µL jelly.

Amendment to calculations. AAP does not dissolve in 1mL ddH_2O however it is soluble in 2mL ddH_2O. Calculations adjusted to accommodate solubility of AAP. Weighed off 1.15g AAP and dissolved in 2 mL ddH_2O. 10µL of new AAP stock added to 990µL of jelly to make up 1ml. Added 1ml AAP to 28mL jelly. Volume and concentration increased thus the ratio stayed the same.

AAP quantity per jelly cube

1.15g NAC in 2mL ddH_2O (stock solution)

10µL stock in 30mL jelly

Cube size: 1mL/cube (1g) (÷2)

Cube size used: 500µL/cube (0.5g)

1.15g = 1150mg in 2mL (÷ 2)

= 575mg/mL (x 0.010)

= 5.75mg in 10µL stock

5.75mg in 30mL jelly (÷ 30)

= 0.1916mg/mL (÷2)

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= 0.0958mg in 500µL cube

= 95.83µg/cube

NAC concentration calculations

NAC: 7.5mM in 30mL jelly

7.5mM x 30 = 225mM [working]

225mM/1000 = 0.225M (0.225mol per 1L)

NAC molecular weight = 163.19g per 1mol x 0.225mol = 36.71775g per 1000mL (1L)

0.03671775g per 1mL (Value too small to measure off)

0.225M x 100 = 2.25M (2.25mol per 1L)

Gram per 1L = 2.25mol x 163.19g= 367.1775 g per 1L

0.3671775g per 1mL

Prepare 2.25M (0.3671775g + 1mL ddH_2O)

100µL NAC of 2.25M + 900µL jelly.

Amendment to calculations. NAC does not dissolve in 1mL PBS however it is soluble in 4mL PBS. The calculations were adjusted to accommodate the solubility of NAC. Weighed off 0.73g NAC dissolved in 4mL PBS. Added 200µL of new NAC stock to 800µL jelly to make up 1mL. Added 1mL to 28mL jelly.

NAC quantity per jelly cube

0.73g NAC in 4mL PBS (stock solution)

200µL stock in 30mL jelly

Cube size: 1mL/cube (1g) (÷2)

Cube size used: 500µL/cube (0.5g)

0.73g = 730mg in 4mL (÷ 4)

= 182.5mg/mL (x 0.200)

= 36.5mg in 200µL stock

36.5mg in 30mL jelly (÷ 30)

= 1.2166mg/mL (÷2)

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= 0.6083mg in 500µL cube

= 608 µg/cube

Animal and human equivalent dosage

Table 9.1 Animal and human equivalent dosage.

	Concentration	**AED** (mg/kg)	**HED** (mg/kg)	**Daily dose** (mg) **for human weighing 80kg**
NAC	7.5mM (608µg/cube)	28.94	2.34	188 mg
AAP	0.6mM (95.83µg/cube)	4.56	0.369	30 mg

Footnote: Animal dose (AED) (mg/kg) was based on the body weight of the animals at baseline before the onset of supplementation. The human equivalent (HED) dose was calculated using the following formula: HED (mg/kg) = (AED) x (Mouse Km/ Human Km). Km human = 37; Km mouse = 3. Km ratio = 3/37 = 0.081 (Nair and Jacob, 2016).

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Appendix C: Animal wellness monitoring sheet and example

FMHS animal Research Facility **Health and Welfare Monitoring Sheet**

Cage ID:	**Group description:**	
PI:	**Email:**	**Tel:**
Researcher:	**Email:**	**Tel:**
Protocol#:		

Date	Time	Appearance	Behaviour	Cage	Comments	Initials

Appearance	**1: Well-groomed; bright facial expression; no discharges; no lesions; normal posture/breathing** **2: Coat slightly rough; subtle discharges/lesions; dull facial expression; mild hunching; abnormal breathing** **3: Staring coat; obvious discharges/lesions; facial bulge/squinting; hunched posture; breathing difficulty**
Behaviour	**1: Alert; curious; active; interactive with cage mates; normal gait** **2: Less active; isolated from cage mates; less responsive; abnormal gait** **3: Inactive; poorly responsive**
Cage	**Cage is clean and dry; faecal pellets are normal; water and food available**

Monitoring must be performed twice a day if a cage is scored "2"

Veterinary staff must be alerted if a cage is scored "3"

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Born : 18/12/2019

2020

FMHS Animal Research Facility — **Health and Welfare Monitoring Sheet**

Cage ID: mmobSS //	**Group description:**	
PI: Dr M vd Vyver	**email:**	**tel:**
Researcher: M Maartens	**email:**	**tel:**
Protocol #: ACU - 2019 - 3857		

Date	Time	Appearance	Behaviour	Cage	Comments	Initials
20/01/20	09:45	1	1		Received animal, inserted weighing boat + jelly cube removed food	
					H_2O ad libitum	
	10:00	1	1		Showing interest in the cube, sniffing + sitting next to it very active	
			:		Returning in 2 hrs	
	12:15	1	1		Not eaten food given	
21/01/20	09:10	1	1		Ate cube, removed food gave fresh cube, H_2O ad libitum	
	09:20	1	1		Nibbling ~~food~~ jelly after realizing there's no food	
	12:15	1	1		Jelly consumed, food given	
22/01	09:30	1	1		Removed food, gave fresh cube, H_2O ad libitum	
					~~Ate~~ Nibbled as soon as it was observed.	

Appearance	1: well-groomed; bright facial expresion; no discharges; no lesions; normal posture/breathing
	2: coat slightly rough; subtle discharges/lesions; dull facial expression; mild hunching; abnormal breathing
	3: staring coat; obvious discharges/lesions; facial bulge/squinting; hunched posture; breathing difficulty
Behaviour	1. Alert; curious; active; interactive with cage mates; normal gait
	2: less active; isolated from cage mates; less responsive; abnormal gait
	3: inactive; poorly responsive;
Cage	Cage is clean and dry; faecal pellets are normal; water and food available

Monitoring must be performed twice a day if a cage is scored "2"

Veterinary staff must be alerted if a cage is scored "3"

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Date	Time	Appearance	Behaviour	Cage	Comments	Initials
	09:35	1	1		Jelly consumed: food supplied	
	12:55	1	1		Happily sleeping	
23/01/20	08:55	1	1		Fe food removed fresh cube added; H2O ad libitum	
	09:03	1	1		Ate immediately	
			:		Jelly consumed: food supplied	
24/01/20	09:30	1	1		Removed food; gave jelly	
					H2O ad libitum	
	09:35	1	1		Immediately ate jelly	
27/01/20	08:~~55~~	1	1		Finished; food given	
					Starved by removing food	
	11:~~15~~25	1	1		H2O ad libitum	
					weighed: 37,12 g	
					put under for 3min @ 2,5% Isoflurane, tail marked with red marker; two stripes, tail point pricked for blood glucose measure = 16,5	
		1	2 jumpy		Placed back in cage, gave supplement cube	
	11:47	1	1		Started eating	
		1	1		Finished; placed in one cage	
28/01/20	07:15	1	1		~~Placed~~ food given Starved	
	11:15	1	: 2 jumpy		Placed in separate cage,	
					Supplement jelly given	
	11:25	1	1		finished; placed in one cage with mates	
					food given	
29/01/20	07:45	1	1		Starved	
	11:~~05~~10	1	1		Placed in separate cage	
					Supplement jelly given	
					Ate immediately	
	11:20	1	1		Finished, placed in one cage, food supplied	
					Starved	
30/01/20	07:45	1	1			