

Effect of an organic *Cannabis sativa* extract exposure on glucose
metabolism in obese and lean Wistar rats

Submitted in fulfillment of the requirement for the degree of
Doctor of Philosophy in the Faculty of Health Sciences at the
University of the Witwatersrand, Johannesburg

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I, Ruby-Ann Levendal, herewith declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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31 March 2015

Date

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
2-AGE	2-Arachidonyl glyceryl ether/noladin ether
AC	Adenylate cyclase
ACC	Acetyl co-enzyme A carboxylase
ACEA	Arachidonyl-2-chloroethylamide
Acrp30/AdipoQ	Adiponectin
ACPA	Arachidonylcyclopropylamide
ACTH	Adrenocorticotrophic hormone
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AEA	N-arachidonoyl ethanolamine (anandamide)
AGEs	Advanced glycation end products
AIP	Atherogenic index of plasma
AIDS	Autoimmune deficiency syndrome
Akt	Protein kinase B
AM251/A1	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-morpholinyl-1 <i>H</i> -pyrazole-3-carboxamide
AM630/A2	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1 <i>H</i> -indol-yl](4-methoxyphenyl) methanone
AMPK	Adenosine monophosphate kinase
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
AUC _g	Area under the curve for glucose
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C	0.0 ng/mL THC/untreated control
cAMP	Cyclic adenosine monophosphate
Ca ²⁺	Calcium ion

[Ca ²⁺] _i	cytosolic calcium concentration
CB1/CB1R	Cannabinoid receptor type 1
CB1A	CB1, isoform A
CB1 ^{-/-}	CB1 receptor knockout
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CBN	Cannabinol
cDNA	copy DNA
c-fos	FBJ murine osteosarcoma viral oncogene homolog
c-Jun	Proto-oncogene Jun
c-MYC	Myelocytomatosis oncogene
CNS	Central nervous system
Cq	Threshold cycle
CT RIA	Coated tubes radioimmunoassay
CVD	Cardiovascular disease
Cyclo A	Cyclophilin A
D1	24 hours
D4	96 hours
DAGs	Diacylglycerols
DIO	Diet-induced obesity
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPPIV	Dipeptidyl peptidase IV
dUTP	Deoxyuridine triphosphate
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAAH	Fatty acid amide hydrolase

FAO	Food and Agriculture Organization of the United Nations
Fas	CD95 cell surface receptor
FBPase	Fructose 1,6-bisphosphatase
FDA	Food and Drug Administration
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
FLICE	Fas-associated death-domain-like IL-1 β -converting enzyme
FLIP	Fas-associated death-domain-like IL-1 β -converting enzyme (FLICE)-like inhibitory protein
GIP	Glucose-dependent insulintropic polypeptide
GIP-R	GIP G-protein-coupled receptor (GIP-R)
GK	Glucokinase
GLP-1	Glucagon-like peptide-1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GOI	Genes of interest
GPCRs	G protein-coupled receptors
GPO	Glycerol-3-phosphate oxidase
GPR55	G-protein coupled receptor 55
GRO/KC	Growth-related oncogene/cytokine-induced neutrophil chemoattractant
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine triphosphate
H	Hyperglycemic
HA1	Hyperglycemic untreated islets with CB1 antagonist
HA1T1	Hyperglycemic with CB1 antagonist and cannabis-treated islets
HA2	Hyperglycemic untreated islets with CB2 antagonist
HA2T1	Hyperglycemic with CB2 antagonist and cannabis-treated islets
HbA1c	Glycated hemoglobin
HBSS	Hanks balanced salt solution

HC	Hyperglycemic untreated islets
HD1C	24-hours hyperglycemic untreated
HD1T1	24-hours hyperglycemic with extract containing 2.5 ng/mL THC
HD1T2	24-hours hyperglycemic with extract containing 5.0 ng/mL THC
HD4C	96-hours hyperglycemic untreated
HD4T1	96-hours hyperglycemic with extract containing 2.5 ng/mL THC
HD4T2	96-hours hyperglycemic with extract containing 5.0 ng/mL THC
HDL	High density lipoprotein
HEK293	Human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF	hepatocyte nuclear factor-1 α /1 β /4 α
HT1	Hyperglycemic cannabis-treated islets
IDF	International Diabetes Federation
IDV	Integrated Density Value
IFN- γ	Interferon- γ
IL	Interleukin-1 α /-1 β /-4/-6/-8/-10/-12
IL-1Ra	Interleukin-1 receptor antagonist
IL-1Ra ^{-/-}	IL-1 receptor antagonist deficient
IP ₃	Inositol 1,4,5-trisphosphate
IP ₃ R	IP ₃ receptor
IPGTT	Intra-peritoneal glucose tolerance test
IRS	Insulin receptor substrate-1/-2
JNK	c-Jun N-terminal kinase
K _{ATP}	ATP-sensitive potassium channel
K _i	Dissociation constant
Kir	Inwardly rectifying potassium channel
KRB	Krebs ringer buffer

Krox-24	Early growth response 1
LDL	Low density lipoprotein
LNC	Lean control
LNE	Lean experimental
MAGL	Monoacylglyceride lipase
MAPK	Mitogen-activated protein kinase
MCC	Medicines Control Council
MCH	Melanin-concentrating hormone
MCT	Monocarboxylate transporter
MetS	Metabolic syndrome
MIP	Macrophage inflammatory protein -1 α /-1 β /-2
MODY	Maturity onset diabetes of the young-1/-2/-3/-4/-5/-6
mRNA	Messenger ribonucleic acid
N	Normoglycemic
NADA	<i>N</i> -Arachidonoyl-dopamine
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NAPE	<i>N</i> -Arachidonoyl-phosphatidylethanolamine
NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NA1	Normoglycemic untreated islets with CB1 antagonist
NA1T1	Normoglycemic with CB1 antagonist and cannabis-treated islets
NA2	Normoglycemic untreated islets with CB2 antagonist
NA2T1	Normoglycemic with CB2 antagonist and cannabis-treated islets
NC	Normoglycemic untreated islets
ND1C	24-Hours normoglycemic untreated
ND	Not determined
ND1T1	24-Hours normoglycemic with extract containing 2.5 ng/mL THC

ND1T2	24-Hours normoglycemic with extract containing 5.0 ng/mL THC
ND4C	96-Hours normoglycemic untreated
ND4T1	96-Hours normoglycemic with extract containing 2.5 ng/mL THC
ND4T2	96-Hours normoglycemic with extract containing 5.0 ng/mL THC
NeuroD1/BETA-2	Neuronal differentiation 1
NK	Natural killer cells
Non-SU	Non-sulfonylurea
NT1	Normoglycemic cannabis-treated islets
2-OG	2-Oxoglutarate
OAD	Oral antidiabetic drug
OBC	Obese control
OBE	Obese experimental
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
Pax4	Paired box gene 4
PBS	Phosphate buffered saline
PC-1	Plasma cell membrane glycoprotein-1
PDX-1	Pancreatic duodenal homeobox-1
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphatidyl bisphosphate
PI-PLC	Phosphoinositol phospholipase C
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC- γ	Phospholipase C- γ
PPAR- γ	Peroxisome proliferator-activated receptor- γ

PTP1B	Protein tyrosine phosphatase 1B
qPCR	Quantitative real-time polymerase chain reaction
RAGE	Receptors for advanced glycation end products
RANTES	Regulated on activation normal T cell expressed and secreted
Ras	Rat sarcoma
REST	Relative Expression Software Tool
RIA	Radioimmunoassay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high pressure liquid chromatography
RPMI	Roswell's Memorial Park Institute
Rt	Retention time
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGLT-2	Sodium-dependent glucose transporter-2
SR141716	5-(4-Chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide hydrochloride / Rimonabant
SREBP-1c	Sterol regulatory element binding factors-1c
STZ	Streptozotocin
SU	Sulfonylurea
SUR1/2	Sulfonylurea receptor 1 or 2
T1	2.5 ng/mL THC
T2	5.0 ng/mL THC
T1D	Type 1 diabetes
T2D	Type 2 diabetes

TCA	Tricarboxylic acid
THC	Tetrahydrocannabinol
Δ^8 -THC	Δ^8 -Tetrahydrocannabinol
Δ^9 -THC	Δ^9 -Tetrahydrocannabinol
THCA	Δ^1 -Tetrahydrocannabinolic acid
THCV	Δ^9 -Tetrahydrocannabivarin
TM1	Transmembrane region 1
TNF- α	Tumor necrosis factor- α
TRPV1	Transient receptor potential vanilloid type 1
TRIS-HCl	2-Amino-2(hydroxymethyl)-1,3-propanediol-hydrochloride
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
TZDs	Thiazolidinediones
U	Uniporter for Ca ²⁺
UCP2	Uncoupling protein 2
V	ATP synthase
VGCC	voltage-gated Ca ²⁺ channel
VR1	Vanilloid type 1 receptor
WHO	World Health Organization
WIN55212-2	R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo [1,2,3-de]-1,4-oxazinyl]-(1-naphthalenyl)-methanone-mesylate
ZDF	Zucker diabetic fatty rats

ABSTRACT

Introduction: Renewed interest in cannabinoid compounds arose since the discovery of the endocannabinoid system in the early 1990's and its role in mediating the body's energy balance. The aim of this study was to investigate the effect of an organic *Cannabis sativa* (hereafter referred to as *C. sativa*) extract on β -cell secretory function using an *in vivo* diet-induced obese rat model and an *in vitro* isolated rat pancreatic islet model and to determine the associated molecular changes within the pancreatic tissue.

Materials and methods: Diet-induced obese Wistar rats and rats fed on standard pellets were subcutaneously injected, over a 28 day period, with an organic *C. sativa* extract or the vehicle (1% Tween 80[®] in saline). The effect of diet and treatment was evaluated using the intraperitoneal glucose tolerance tests (IPGTTs) and quantitative polymerase chain reaction (qPCR) analysis on rat pancreata. *In vitro* studies were conducted using isolated rat islets exposed to 11.1 (representative of normoglycemic conditions) and 33.3 mM glucose levels (representative of hyperglycemic conditions) over a 24-(D1; acute) and 96-hour (D4; chronic) period, and treated with *C. sativa* extract containing an equivalent of 2.5 (T1) and 5 ng/mL (T2) tetrahydrocannabinol (THC). Glucose-stimulated insulin secretion (GSIS), immunohistochemistry for apoptosis and proliferation detection and western blotting for detection of cannabinoid receptor type 1 (CB1), CB2 receptors and specific transduction factors were undertaken. Antagonist studies were conducted using AM251 (A1) and AM630 (A2) to block CB1 and CB2, respectively, to determine the role of cannabinoid receptors in insulin secretion.

Results: The overall increase in body weight in the experimental groups occurred at a significantly slower rate than the control groups ($P < 0.01$), irrespective of diet. In the lean group, the area under the curve for glucose (AUC_g) was significantly higher compared to the diet-induced obese group ($P < 0.001$), while *C. sativa* treatment significantly improved the AUC_g in the lean rats ($P < 0.05$). The cafeteria diet did not induce hyperglycemia and insulin resistance in the obese rats and *C. sativa* treatment maintained a plasma glycemic profile similar to the obese control rats. The lower AUC_g values in the obese group may, in part, be due to the inclusion of milk products (shown to be beneficial in reducing diabetes) in the cafeteria diet. qPCR analysis showed that the cafeteria diet induced down-regulation of the following genes in the obese control group,

relative to lean controls: UCP2 ($P < 0.01$), c-MYC ($P < 0.05$) and FLIP ($P < 0.05$), and upregulation of CB1 ($P < 0.01$), GLUT2 ($P < 0.001$), UCP2 ($P < 0.001$) and PKB ($P < 0.05$), relative to the obese control group, while c-MYC levels were down-regulated ($P < 0.05$), relative to the lean control group.

In the *in vitro* study, results showed *C. sativa* treatment decreased chronic insulin secretion in islets cultured under normoglycemic condition for D1 ($P < 0.05$), but not for D4. In islets cultured under hyperglycemic conditions, *C. sativa* treatment for the D4 period showed a significant increase in their chronic insulin secretion (HD4T1, $P = 0.07$; HD4T2, $P < 0.001$), increase in basal insulin secretion (HD4T1, $P < 0.001$; HD4T2, $P < 0.001$), increase in GSIS (HD4T1, $P < 0.05$; HD4T2, $P < 0.001$), reduction in glucose-stimulated:basal insulin production (HD4T1, $P < 0.05$; HD4T2, $P < 0.05$), reduction in insulin content (HD4T1, $P < 0.001$), increase in the percentage basal : content ratio (HD4T1, $P < 0.001$; HD4T2, $P < 0.01$) and increase in the percentage GSIS : content ratio (HD4T1, $P < 0.001$; HD4T2, $P < 0.05$), relative to ND4C islets.

In antagonist studies, A2 preconditioning did not affect suppress the stimulatory effect of *C. sativa* treatment on chronic insulin secretion under normo- and hyperglycemic conditions, relative to the NC and HC islets, respectively. qPCR studies showed that *C. sativa* exposure induced a 2.2-fold increase in CB1 gene expression, relative to normoglycemic control islets ($P < 0.05$), while c-MYC and FLIP expression was significantly reduced by 12% (ND4T1, $P < 0.05$) and 37% (HD4T1, $P < 0.05$), respectively. *C. sativa* treatment also induced increased secretion of anti-inflammatory cytokines/chemokines under hyperglycemic conditions.

Conclusion: These results suggest that *C. sativa* protects pancreatic islets against the negative effects of obesity (*in vivo* studies) and hyperglycemia (*in vitro* studies). In light of these findings, further investigation into the potential of *C. sativa* as a complementary therapeutic agent in the treatment of the deleterious effects of hyperglycemia in diabetic patients is warranted. In addition, the significant effect of *C. sativa* treatment on adipose tissue in experimental rats needs further investigation to determine how the cannabinoids affect the mechanisms of adipogenesis and lipolysis in diet-induced obesity. **Keywords:** Diet-Induced Obesity, Cannabinoids, *C. sativa*, THC, β -cell, AM251, AM630.

ACKNOWLEDGEMENTS

“Education is the most powerful weapon which you can use to change the world.” –

Nelson Mandela

Lord God, my heavenly father, thank you for your many blessings you bestow on me on a daily basis. Without you I am nothing. Praise, glory and honour to you forever, Amen. To my children – Remi, Reece and Andrea, my sisters – Cheryl, Gail and Desireé, and Mommy and Daddy - you have each contributed in your various ways – thank you for your love, encouragement and generous support provided throughout my studies. I love you all very much. Des, a special word of appreciation and thanks to you for all you have done to assist me - too numerous to mention. Distance is a reality, but the bond of love is strong. A very special word of thanks and gratitude to Carminita Frost– you were and still are there for me through good and bad times. No words can express how much I appreciate the encouragement, support and comfort you provided and continue to provide. Thank you for your friendship – I feel truly blessed to know someone like you. Thank you to Nigel Crowther for agreeing to supervise my project and for your inputs. Thank you to Marc Donath for his generous support with all the *in vitro* studies conducted in his laboratory in Switzerland, and to Kathrin Maedler and Garetha Siegfried-Kellerberger for their technical assistance. Thanks to Coos Bothma for assisting with the statistical modeling. Thanks to the academic and technical staff, past and present, who assisted me during my time in the Department of Biochemistry and Microbiology. Last but not least, I would like to acknowledge and thank the Nelson Mandela Metropolitan University and National Research Foundation for the financial support provided for this project.

CHAPTER 1: LITERATURE REVIEW

1. OVERVIEW OF CHAPTER

This chapter provides a brief introduction of the prevalence of diabetes and obesity at a global and South African level to contextualize the need for alternative treatments to enable effective health management in light of the ever-increasing burden placed on the resources of countries generally, especially third-world, developing and emerging economies. In addition, the introduction briefly introduces *C. sativa* as a medicinal plant its various biological effects it elicits upon stimulation of the endocannabinoid system.

The specific diagnostic parameters for diabetes mellitus (DM), as provided by the World Health Organization (WHO), in collaboration with the International Diabetes Federation (IDF) and the American Diabetes Association (ADA), will be discussed, differentiating between the different types of diabetes. Thereafter, the different types of anti-diabetic treatments that are currently available to diabetics are discussed, as well as other possible treatments being investigated at clinical trial level.

Since most products are developed from natural products, a section will follow that deals with hypoglycemic medicinal plants, after which *Cannabis sativa* (*C. sativa*) is introduced as a medicinal plant. *C. sativa* contains several phytocannabinoids, including tetrahydrocannabinol (THC), which will be dealt with in more detail in terms of its chemical structure and biosynthesis. There are many therapeutic properties associated with *C. sativa* as well as specific cannabinoids.

The discovery of specific cannabinoid receptors within the body has led to the discovery of the endocannabinoid system, which will be elaborated on in terms of biosynthesis of key endocannabinoids found in the body. In addition, the cannabinoid receptors, as constituents of the endocannabinoid system, will be dealt with in detail, with regards to their distribution and associated signal transduction pathways triggered upon stimulation or inhibition and the subsequent physiological responses elicited. Specific focus will be placed on the role of endocannabinoid system in pancreatic tissue. Having provided a

detailed background to the study, the aim and research objectives of the study will be set outlined.

1.1 INTRODUCTION

In 2014, the global prevalence of diabetes was estimated to be 9% among adults aged 18 years and above (WHO, 2015). The WHO estimates that more than 347 million people have diabetes globally, with obesity affecting more than 180 million people worldwide, and that this number will double by 2030 (WHO, 2013). The International Diabetes Federation (IDF) Atlas estimated that 14.7 million people have diabetes in sub-Saharan Africa in 2011, and predicts that this number would increase to 28.0 million by 2030 (IDF, 2012). Research has shown that rural-urban migration in sub-Saharan Africa is a major contributor to the increase in non-communicable diseases including obesity and type 2 diabetes (T2D) (Gill et al., 2009; Godfrey and Julien, 2005). In South Africa, the first National Demographic and Health Survey conducted in 1998 found that 19.2% of urban adult South Africans were obese (Puoane et al., 2002). In 2000, the estimated mortality due to diabetes for sub-Saharan Africa was 2.2% of all male deaths and 2.5% of all female deaths, while this percentage was estimated at 4.3% of all deaths in South Africa (Levitt, 2008). The IDF estimates that 63 061 diabetes-related deaths occurred in South Africa in 2012 (IDF, 2012).

The prevalence of diet-induced obesity (DIO) continues to increase, resulting in increasing demands on healthcare resources to treat associated life-threatening diseases such as hypertension, cardiovascular disease (CVD) and diabetes. The progressive development of diabetes is characterized by changes in metabolic parameters, and loss in β -cell mass and function resulting in an increase in blood glucose levels (Kargar and Ktorza, 2008; Maedler and Donath, 2004). Diabetes is one of the features associated with the metabolic syndrome (MetS). Abdominal obesity, insulin resistance, dyslipidemia, hypertension, hyperfibrinogenemia, hyperuricemia, microalbuminuria and elevated circulating proinflammatory and/or prothrombotic markers including C-reactive protein, tumor necrosis factor- α (TNF- α), interleukin (IL)-6 and plasminogen activator inhibitor-1 (PAI-1) or reduced levels of tissue plasminogen activator and anti-inflammatory

molecules like adiponectin (also referred to as AdipoQ/Acrp30) are all factors associated with MetS (Alberti et al., 2006; Alberti et al., 2005; Eckel et al., 2005). Research has shown that exercise and weight loss improve these associated conditions (Balducci et al., 2010; Després and Lemieux, 2006). In addition to weight loss, pharmaceutical drugs are being used to manage and minimize the metabolic sequelae of diabetes. The withdrawal of rosiglitazone from the global market due to increased risk of cardiovascular disorders (Psaty and Furberg, 2007), have contributed to a reduction in the diversity of therapies available for the treatment of diabetes, thus increasing the need for alternative methods of therapy. Subsequent to the withdrawal of rosiglitazone, the Food and Drug Administration (FDA) released a ruling on 25 November 2013 to remove certain restrictions on prescribing and use of rosiglitazone based on the findings from the Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes clinical trial that showed no elevated risk of heart attack or death in patients being treated with rosiglitazone relative to standard T2D medicines, viz. metformin and sulfonylurea (SU)

(<http://www.fda.gov/downloads/Drugs/DrugSafety/UCM381108.pdf>).

The associated healthcare costs and unavailability of resources in many rural communities in developing countries provides impetus and urgency to finding affordable pharmacotherapeutic approaches to the diabetes pandemic.

The increasing demand on limited resources within the healthcare sector has fueled a global upsurge in research on the use of medicinal plants over the past 20 years, in an attempt to adequately manage this disorder (Ojewole, 2002). *C. sativa* (Cannabaceae) has been in use as a medicinal plant for thousands of years, with the earliest formal record of its use having appeared in a Chinese pharmacopoeia dated 2737 BC. Approximately 70 terpenophenolic constituents called phytocannabinoids are found in varying quantities in the plant material, with the primary psychoactive phytocannabinoids being tetrahydrocannabinol (THC) (Russo, 2011; ElSohly and Slade, 2005; Gaoni and Mechoulam, 1964). Used as a weak infusion in indigenous medicine, it serves as a remedy for asthma, bronchitis, headache, migraine, pain, pain associated with childbirth,

hypertension and diabetes (Van Wyk and Gericke, 2000). Crude *C. sativa* extract has been shown to have potent anti-inflammatory properties, being 80 times more potent than aspirin and twice as potent as hydrocortisone (Sofia et al., 1974). *C. sativa* modulates the anti-inflammatory effect by suppressing cytokine and chemokine secretion, increasing apoptosis in activated immune cells and reducing cell proliferation in T-regulatory cells (Nagarkatti et al., 2009). THC is also associated with neuroprotective properties in rat cortical-neuron cultures via a mechanism independent of cannabinoid receptors (Hampson et al., 1998; Shen and Thayer, 1998). *In vivo* studies conducted on Wistar rats and mice showed that intra-tumoral administration of THC induced significant regression of malignant gliomas (Sánchez et al., 2001). In A549 cells, a pulmonary transformed cell line, exposure to THC caused disruption of mitochondrial and cellular energetics by rapid depletion of adenosine triphosphate (ATP) stores and diminished mitochondrial function (Sarafian et al., 2003). In astrocytes and lymphocytes, THC enhances glucose utilization mediated via cannabinoid receptor type 1 (CB1) and mitogen-activated protein kinase (MAPK) activation (Guzmán and Sánchez, 1999). In animals, including rats, THC mediates its physiological effects via CB1 and cannabinoid receptor type 2 (CB2) binding, activating intracellular G-proteins which provide signals to a variety of effectors such as ion channels, the MAPK cascade and the induction of myelocytomatosis oncogene (c-MYC) expression (Compton et al., 1992; Howlett et al., 2002).

In the early 1960's, THC was isolated and characterized by Gaoni and Mechoulam (1964). This discovery together with the cloning of the two distinct cannabinoid receptor subtypes in the early 1990's prompted a renewed interest in cannabinoid-based medicines (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993). A large body of evidence is emerging around the role of the CB1 receptor and its endogenous ligands in the regulation of food intake and peripheral lipogenic mechanisms. Recent advances have been made in identifying the therapeutic potential of cannabinoids, with therapies specifically aimed at enhancing appetite in eating disorders such as anorexia, cancer- and autoimmune deficiency syndrome (AIDS)-associated cachexia. A CB1 knockout mouse model exhibited hypophagia, with subsequent reduction in total fat mass and reduced body weight (Trillou et al., 2004; Cota et al., 2003b; Di Marzo et al., 2001). In humans,

C. sativa and THC are known to increase appetite approximately three hours after use (a predictable phenomenon known colloquially as ‘the munchies’) (Berry and Mechoulam, 2002). In disorders such as obesity and MetS, the CB1 antagonist, Rimonabant, was widely prescribed for its anorexic effects (Greineisen and Turner, 2010), and induced hypophagia and weight loss in adult, non-obese Wistar rats (Thornton-Jones et al., 2006; Colombo et al., 1998). In the Rimonabant in Obesity-Lipids study conducted on obese patients, it was found that Rimonabant significantly improved cardio metabolic parameters (reducing body weight, waist circumference, plasma triglyceride levels, and improving plasma glucose-insulin homeostasis, insulin resistance and high-density lipoproteins (HDL) cholesterol levels), which indirectly reduce the risk of developing obesity-induced diabetes (Després et al., 2005). However, in 2008 clinical trials associated with Rimonabant and other CB1 antagonists were halted due to the psychiatric side effects (sleep disorders, headache, suicidal ideation, depressed mood and anxiety) associated with the use of this drug (Butler and Korbonits, 2009). Studies have however shown that Rimonabant has therapeutic potential to counter drug dependency, specifically tobacco and ethanol dependency (Maldonado et al., 2006).

To better understand the rationale for the development of a CB1 antagonist as both an anti-diabetic and anti-obesity drug, a more detailed definition of DM will be outlined below.

1.2 DIABETES MELLITUS

1.2.1 Definition

Normal glucose homeostasis is maintained primarily through the action of insulin, when a balance exists between glucose production and glucose utilization. The ADA (2013) has defined DM as a group of metabolic diseases caused by defects in insulin secretion and/or insulin action, resulting in chronic hyperglycemia. These defects may be inherited and/or acquired (WHO, 2011). Symptoms of chronic hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, blurred vision, susceptibility to infections and impaired healing (ADA, 2013).

1.2.2 Primary forms of DM

1.2.2.1 Type 1 Diabetes (T1D)

Type 1 diabetes (T1D) comprises 5% to 10% of all diabetic cases. T1D was previously known as insulin-dependent DM or juvenile onset DM, with its prevalence being primarily amongst children and adolescents. However the incidence of T1D is increasing amongst adults (WHO, 2011). The etiology of T1D primarily involves gene-environment interactions, with viral infection being the major environmental factor identified (Graves and Eisenbarth, 1999). T1D is etiologically classified into autoimmune (Type 1A) which makes up 85 to 90% of all T1D cases, and the rest constitutes the idiopathic (Type 1B) diabetes. Type 1B has been found to be associated with patients of African or Asian descent, with a strong hereditary link (ADA, 2013). The pathogenesis of T1D involves a complicated interplay between genetic predisposition, the immune system and environmental factors (Hober and Sauter, 2010). The autoimmune pathogenesis of T1D is associated with a number of autoantibodies, including islet cell autoantibodies, and autoantibodies directed at glutamate decarboxylase 65, islet cell antigen-2, insulin and zinc transporter 8 (ZnT8) (Thrower and Bingley, 2010). It is well documented that the human lymphocyte antigen region of chromosome 6 influences the susceptibility and resistance of an individual to T1D (Davies et al., 1994). The human lymphocyte antigen region is a cluster of more than 150 genes, a number of which code for transmembrane proteins that are responsible for the presentation of antigens to receptors on CD8⁺ (cytotoxicity) and CD4⁺ (helper) T cells.

The major signs and symptoms associated with T1D are directly attributable to insulin deficiency in the body. The characteristic symptoms include hyperglycemia, glucosuria, polyuria, polydipsia, weight loss, fatigue, and ketoacidosis (WHO, 2013, Nowak and Handford, 2004).

1.2.2.2 Type 2 Diabetes (T2D)

Type 2 diabetes (T2D), previously known as non-insulin-dependent DM or adult onset DM, accounts for approximately 90% to 95% of all diabetes cases and is characterized

by β -cell dysfunction and insulin resistance at the level of the liver, skeletal muscle and adipose tissue (WHO, 2013; ADA, 2013).

The ADA, in consultation with the IDF and European Association for the Study of Diabetes (EASD), has developed a set of standards of medical care in diabetes that provides criteria for the screening and diagnosis of T1D, T2D, gestational diabetes and other specific types of diabetes resulting from any other underlying conditions (such as cystic fibrosis, HIV and AIDS) as well as guidelines for the treatment of diabetes complications. The person is diagnosed with diabetes if any one of the following criteria is fulfilled: glycated hemoglobin (HbA1c) of $\geq 6.5\%$, or fasting plasma glucose level (no caloric intake at least 8 hours prior to testing) is ≥ 7.0 mM (126 mg/dL), or displays a plasma glucose level of ≥ 11.1 mM (200 mg/dL) 2 hours after a 75 g oral glucose load is consumed during an oral glucose tolerance test (OGTT), or a random plasma glucose level (anytime during the day regardless of time of last meal) of ≥ 11.1 mM (200 mg/dL) and displays symptoms of hyperglycemia or hyperglycemic crisis; or in the absence of indisputable hyperglycemia, the testing should be repeated to confirm diagnosis (ADA, 2013).

Hyperglycemia that develops as a consequence of defective insulin action and/or secretion, progressively leads to the production of glycated end products that induce micro- and macro-angiopathies and peripheral neuropathies, resulting in damage to various organs, including kidneys, retina, feet and the heart.

1.2.2.3 Other specific types of Diabetes

Gestational DM (GDM) is defined as any degree of glucose intolerance associated with the onset or progression of pregnancy (WHO, 2013). Long-term studies have shown that pregnant women with GDM have an increased risk of developing T2D subsequent to their pregnancy (Buchanan and Xiang, 2005). In a study conducted by Di Cianni et al., (2003), 3 950 women were assessed by universal screening for GDM during the period of 1st June 1995 to 31st December 2001. The prevalence of GDM was significantly higher in women with a history of diabetes, increasing age, previous pregnancies, maternal obesity and

short stature. Regression analysis showed that GDM diagnosis was significantly correlated with age, high pre-pregnancy body mass index, weight gain and family history of diabetes (Di Cianni et al., 2003).

Another type of diabetes is maturity onset diabetes of the young (MODY) which involves genetic defects in hepatic and β -cell genes whilst mitochondrial deoxyribonucleic acid (DNA) mutations, drug, chemical or disease-induced pancreatic damage, can also lead to diabetes. Essentially MODY is a monogenic form of T2D and characterized by early age of onset (generally before 25 years of age) and autosomal dominant transmission. Mutations in genes coding for transcription factors which regulate the expression of key genes modulating β -cell function, including hepatocyte nuclear factor (HNF)-1 α (associated with MODY-3), HNF-4 α (MODY-1), insulin promoter factor (IPF)-1/pancreatic duodenal homeobox (PDX)-1 (MODY-4), HNF-1 β (MODY-5) and neuronal differentiation 1 (NeuroD1, also referred to as BETA-2) (MODY-6). Mutations in glucokinase (GK), the enzyme responsible for the phosphorylation of glucose to glucose-6-phosphate is associated with MODY-2 (Shao et al., 2009; Malecki, 2005; Fajans et al., 2001; Habener et al., 1998).

Both environmental and genetic factors may contribute to the pathogenesis of T2D, resulting in impaired insulin secretion and diminished insulin sensitivity. The next section will provide an overview of the molecular mechanisms associated with insulin secretion by the β -cell and associated dysfunction in T2D.

1.3 β -CELL AND INSULIN SECRETION

Insulin secretion is modulated by several factors other than glucose. The β -cell has the capability of detecting changes in circulating nutrients such as glucose, amino acids, fatty acids, oxygen consumption, ATP or the ATP/adenosine diphosphate (ADP) ratio, the mitochondrial inner membrane potential, lactate release and nicotinamide adenine dinucleotide phosphate (NAD(P)H) levels, and therefore adjusts insulin secretion according to the physiological needs of the organism. Other factors such as glucagon, gastric inhibitory peptide and glucagon-like peptide-1 (GLP-1), incretins, other nutrients,

vitamins, systemic growth factors, nerve innervations and intra-islet autocrine and paracrine factors which interact with regulatory elements associated with the insulin gene further modulate changes in insulin gene expression, resulting in changes in insulin secretion (Leibiger et al., 2010; Wiederkehr and Wollheim, 2008; Bertram et al., 2007; Berggren and Leibiger, 2006). The abovementioned factors trigger β -cell signal transduction via membrane receptors which are mostly linked to either G-proteins or tyrosine kinases (Leibiger et al., 2010; Berggren and Leibiger, 2006). Once activated, secondary messenger systems are induced, leading to the initiation of insulin secretion (figure 1.1).

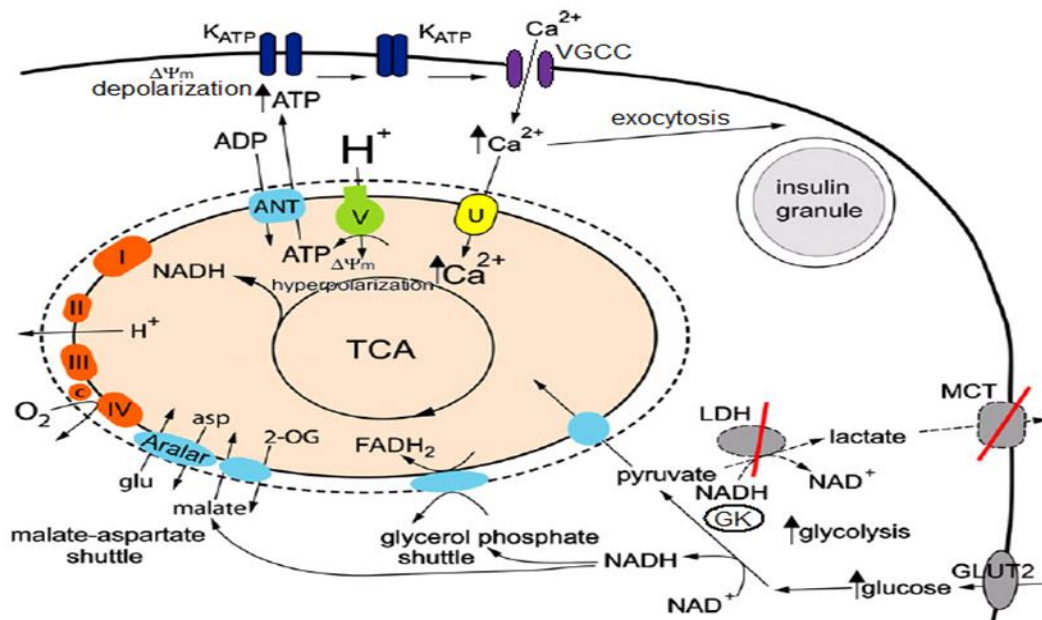


Figure 1.1 Schematic representation of the coupling of glucose metabolism to insulin secretion in pancreatic β -cells. 2-OG, 2-oxoglutarate; ANT, adenine nucleotide translocase; asp, aspartate; GK, glucokinase; GLUT2, glucose transporter type 2; glu, glutamate; KATP, ATP-sensitive potassium channel; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; TCA, tricarboxylic acid cycle; VGCC, voltage-gated Ca^{2+} channel; U, uniporter for Ca^{2+} ; V, ATP synthase (adapted from Wiederkehr and Wollheim, 2008; Fujimoto et al., 2007).

The β -cell responds to minute increases in circulating blood glucose levels by rapid release of insulin. Insulin concentrations oscillate at a frequency of 5 to 15 minutes per oscillation (Pørksen et al., 2002). Oscillations in insulin secretion have been measured in two phases: a fast first phase which lasts approximately 15 seconds, and a slower second phase which lasts between 4 to 6 minutes (Bertram et al., 2007; Pørksen et al., 2002).

Bertram et al. (2007) proposes that the fast oscillations in insulin secretion result from electrical mechanisms, primarily emanating from the effect of cytosolic calcium on plasma membrane ion channels, while the slow oscillations result from metabolic changes such as glucose levels.

Insulin synthesis (via increased insulin gene transcription), occurs within ten minutes of elevation of blood glucose levels, starting with the movement of glucose into the β -cells via glucose transporter type 2 (GLUT2). The glucose undergoes phosphorylation by GK to form G-6-P, which proceeds to undergo glycolysis. The metabolic by-product, pyruvate, gets co-transported with a proton across the inner mitochondrial membrane to enter the tricarboxylic acid (TCA) cycle in the mitochondria, producing nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide in the mitochondrial matrix (see figure 1.1). These co-factors enter the respiratory chain and are re-oxidised by the respiratory chain. This process is coupled to the transport of protons from the mitochondrial matrix into the cytosol, thereby establishing a hyperpolarizing membrane potential across the inner mitochondrial membrane. Glucose oxidation therefore serves as the driving force for maintaining a hyperpolarizing electrochemical gradient across the inner mitochondrial membrane, which in turn serves as a stimulus for increased ATP-synthase (V) activity. As activity increases, adenine nucleotide translocase exchanges matrix ATP for ADP to provide ATP for energy-consuming processes in the cytosol. The exchange gives rise to an increase in the ATP/ADP ratio in the cytosol of the β -cell, leading to the activation of sulfonylurea receptor 1 (SUR1) which results in the closure of the ATP-sensitive potassium channels (K_{ATP}). This closure brings about depolarization of the plasma membrane and opening of the voltage-gated Ca^{2+} channel (VGCC), resulting in an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cell. The glucose-induced $[Ca^{2+}]_i$ signal stimulates pulsatile insulin secretions, via exocytosis of insulin-containing secretory vesicles, based on the oscillatory pattern of the $[Ca^{2+}]_i$. The presence of sufficient ATP or a high ATP/ADP ratio is crucial for insulin secretion. ATP provides the phosphate group which phosphorylates protein kinase A (PKA), which in turn phosphorylate various proteins that modulate the process of exocytosis (Wiederkehr and Wollheim, 2008; Fujimoto et al., 2007; Doyle and Egan, 2003; Henquin, 2000).

Pancreatic duodenal homeobox-1 (PDX-1) plays a crucial role in pancreatic development, β -cell differentiation and regulating β -cell neogenesis (Shao et al., 2009; Jonsson et al., 1994). PDX-1 is a mediator of glucose-regulated insulin gene expression, and is a key transactivator of the insulin gene, and other islet-specific genes, including GLUT2, GK, islet amyloid polypeptide and somatostatin (Melloul et al., 2002). PDX-1 knock-out mice showed significant β -cell dysfunction resulting in a severe diabetic phenotype (Ganno et al., 2008). Ahlgren et al. (1998) reported that chronic hyperglycemia reduced β -cell function by inducing oxidative stress and reducing PDX-1 activities, while Hagman et al. (2008) found that prolonged exposure of rat islets to palmitate impairs nuclear localization of PDX-1 resulting in inhibition of insulin gene transcription.

1.4 β -CELL DYSFUNCTION AND DM

β -Cell mass plasticity has been implicated in the pathogenesis of T2D. β -Cell mass is dependent on the interplay between four mechanisms: (1) proliferation, (2) neogenesis (3) hyperplasia/hypertrophy and (4) apoptosis. The net β -cell growth rate results from the difference between the total sum of the first three factors and the last factor (Sachdeva and Stoffers, 2009; Rhodes, 2005).

The progression of diabetes with time is characterized by a concomitant change in metabolic parameters, loss in β -cell mass as well as β -cell function, thus resulting in subtle changes in glycemia. Weir and Bonner-Weir (2004) proposed that there are five stages in the progression of diabetes in relation to β -cell function and/or plasticity. Stage one involves compensation, where insulin secretion increases to maintain normal glucose levels despite insulin resistance brought about by obesity, sedentary lifestyle and genetic predisposition. Stage two involves a stable state of β -cell adaptation, where normal glucose levels can no longer be maintained, and fasting levels range between 5.0 and 7.3 mM. Stage three is referred to as the transient stage of decompensation due to a critical reduction in β -cell mass and/or an increase in insulin resistance resulting from glucotoxicity and lipotoxicity. The deleterious effect of hyperglycemia on β -cell efficiency exacerbates the glycemic problem, eventually resulting in glucose levels

ranging between 16 and 20 mM. Stage four, stable decompensation, lasts a lifetime in most T2D patients. Typically, there is a reduction in the number of β -cells, however, the numbers are sufficient to secrete enough insulin to maintain glucose levels in the range specified in stage three, rather than progress to ketoacidosis. In stage five, severe decompensation, is marked by a severe loss in β -cell mass such that the diabetic becomes ketotic and dependent on insulin for survival, with glucose levels typically ranging above 22 mM (Weir and Bonner-Weir, 2004).

The transition from obesity-related insulin-resistance to T2D is β -cell failure resulting from a partial loss in β -cell mass and reduced β -cell function (Muio and Newgard, 2008). In response to prolonged elevated nutrient levels such as glucose, as in the case of obesity-induced insulin resistance, β -cells usually compensate by increasing insulin secretion to maintain glucose within normal physiological levels. This compensatory mode may last for long periods of time, thus protecting the individual from diabetes, before the β -cells give way to β -cell failure. Research has shown that β -cell mass increased four-fold in non-diabetic Zucker *fa/fa* rats relative to lean control rats (Pick et al., 1998), which show a similar trend to findings of increased β -cell mass found in non-diabetic obese humans (Prentki and Nolan, 2006; Lingohr et al., 2002).

The gerbil *Psammomys obesus* (*P. obesus*) develops nutrition-dependent diabetes and two lines of *P. obesus* animals have been established by selected breeding, viz. diabetes-prone (which develops diabetes in response to high-energy feeding) and diabetes resistant (which maintains normoglycemia in response to high-energy feeding) (Kalman et al., 1993; Kalderon et al., 1986). Neshet et al. (1999) investigated the relative contribution of diet and predisposition to β -cell dysfunction in islets isolated from both diabetes-prone and diabetes resistant *P. obesus* animals. They found amongst others that despite a four-fold increase in glucose phosphorylation in islets from the diabetes-prone *P. obesus* animals, there were no differences in islet GK and hexokinase K_m activity. However, the maximum reaction rate (V_{max}) of GK was 3.7- to 4.6-fold higher in diabetes-prone islets, while the V_{max} of HK was augmented 3.7-fold in diabetes-prone islets isolated from *P. obesus* fed on a high energy diet. They therefore concluded that both genetic

predisposition and β -cell maladaptation to nutritional load appear to contribute to the progression to overt diabetes.

Kaiser et al. (2005) investigated the contribution of β -cell dysfunction and reduced β -cell mass to the initiation and progression of T2D. Diabetes-prone *P. obesus* animals were used in their study since this species is characterized by inherent insulin resistance. The insulin resistance is due to the lack of complete suppression of hepatic glucose production and reduced total body glucose disposal during a hyperinsulinemic-euglycemic clamp (Kaiser et al., 2005). These changes were observed in both lines of the *P. obesus* animals irrespective of their glycemic condition. Animals were subjected to a change from its natural low-calorie diet to a calorie-rich laboratory food, which resulted in the development of moderate obesity associated with postprandial hyperglycemia. Prolonged dietary load together with its innate insulin resistance resulted in inappropriate production of insulin, depletion of pancreatic insulin stores, and increased proportions of insulin precursor molecules in the pancreas and the blood. A major contributor to the progression of diabetes was found to be the inadequate response of the pre-proinsulin gene to the increased insulin demands due to the prolonged dietary load, rather than the changes in β -cell mass. Their results showed that in *P. obesus* animals, changes in β -cell mass did not correlate with pancreatic insulin stores and therefore was unlikely to play a role in the initiation and progression of diabetes.

There are various proposed mechanisms by which β -cell function is affected. Early β -cell death with the onset of T2D can be induced via toxic metabolic by-products, dysfunctional triglyceride/free fatty acid (FFA) cycling, mitochondrial dysfunction, oxidative stress, endoplasmic reticulum (ER) stress and inter-organ communication mediated via hormones and cytokines. In response to a diabetic milieu, including gluco- and lipotoxicity, β -cell death is accelerated by processes such as islet inflammation and increased apoptosis (Poitout et al., 2010; Poitout and Robertson, 2008; Muoio and Newgard, 2008; Prentki and Nolan, 2006).

1.4.1 Glucotoxicity

Plasma insulin levels diminish as hyperglycemia persists, a hallmark associated with T2D. Both the rapid and slow phases of the insulin secretion are impaired in response to hyperglycemia in T2D patients (Van Haefen, 2002).

Despite convincing evidence that hyperglycemia contributes to β -cell apoptosis, there are differences in the islet's susceptibility to glucose-induced apoptosis. Rodent islets survive best at 11 mM glucose, while islets from humans and *P. obesus* that have been exposed to glucose levels ranging from 5.5 to 33 mM show a higher incidence of β -cell apoptosis (Donath et al., 2005). In studies conducted on islets from Sprague-Dawley rats and *P. obesus*, a rodent that is naturally prone to diet-induced type 2-like diabetes, the initial transition from normoglycemia to hyperglycemia was accompanied by an initial increase in β -cell proliferation, followed by a prolonged increase in β -cell apoptosis, resulting in a significant reduction in β -cell mass (Donath et al., 1999). Contrasting the responses observed in rat and *P. obesus* islets showed that elevated glucose concentrations stimulated β -cell proliferation in both species, however, this response was brief and diminished in *P. obesus* islets, relative to the prolonged effect in rat islets. Prolonged exposure to elevated glucose levels inhibited β -cell proliferation. Similar results were observed by Leibowitz et al. (2001a) in β -cells exposed to high glucose concentrations (20.0 mM). The changes observed in these islets were similar to those observed in diabetic animals, such as deficient insulin secretion, depleted insulin content, increased levels of insulin precursor molecules, increased DNA fragmentation and a brief proliferative response. The β -cells were unable to sustain insulin synthesis due to increased demands resulting from the hyperglycemic conditions. It was found that the expression of PDX-1, a key insulin gene regulator, was deficient in these β -cells (Leibowitz et al., 2001b).

PDX-1 is also considered the most important transcription factor in β -cells due to its key role in normal β -cell function, viability, and compensation capacity (Ackermann and Gannon, 2007). Evidence from studies conducted by Gupta et al. (2013; 2010; 2008) shows that PPAR- γ plays a central role in β -cell compensation and/or failure. They found that PPAR- γ regulates two key genes in pancreatic β -cells, viz. PDX-1 and glucose-dependent

insulinotropic polypeptide (GIP) G-protein-coupled receptor (GIP-R), via binding to the PPAR response element that is found within the PDX-1 and GIP-R promoters, respectively. GIP plays a role in normal mealtime glucose tolerance and insulin secretion (Tseng et al., 1996). Hyperglycemic rats have shown impaired PPAR- γ expression which resulted in lowered GIP-R expression (Gupta et al., 2010). Gupta et al. (2013) has shown that the PPAR- γ /FoxO1 network mediates PDX-1 expression and other key β -cell genes. FoxO1 is highly expressed in β -cells, and is a key integrator of metabolic stimuli, inhibits PPAR- γ expression and also targets PDX-1 (Kitamura et al., 2002). Gupta et al. (2013) found that the PPAR γ /FoxO1-mediated network acts as a core component of β -cell adaptation to metabolic stress. In non-diabetic insulin resistant rats they found that PPAR- γ expression in the islets and its target genes were increased, while in diabetic rats these were markedly reduced. In addition, strong nuclear localization of FoxO1 was observed in the β -cells of diabetic rats, thus maintaining its inhibitory effect on PDX-1. These findings suggest a central role of PPAR- γ in the FoxO1-regulation of PDX-1, thus providing a novel mechanism for β -cell failure (Gupta et al., 2013)

Depending on duration of exposure to hyperglycemic conditions and the genetic make-up of islets, glucose may induce or impair β -cell proliferation and have pro- or anti-apoptotic effects with extensive overlap existing between apoptotic and proliferative pathways. Under normoglycemic conditions CD95 cell surface receptor (Fas) would trigger β -cell proliferation, in the presence of sufficient levels of Fas-associated death-domain-like IL-1 β -converting enzyme (FLICE) inhibitory protein (FLIP). Hyperglycemia induces β -cell proliferation in the short-term, but apoptosis in the long-term (Maedler et al., 2001b). In human β -cells hyperglycemia triggers apoptosis by increasing IL-1 β production which induces upregulation of Fas expression. IL-1 β mediates the interaction between the upregulated Fas receptors and Fas ligand present on neighbouring β -cells (Maedler et al., 2002a). The formation of Fas-Fas ligand complexes, triggers the activation of Caspase-8, which in turn promotes Caspase-3 activation, which results in DNA fragmentation (Maedler et al., 2001b). In islets from T2D patients that were exposed to chronic hyperglycemic conditions, there is a reduction in FLIP expression, increased production and release of IL-1 β , nuclear factor-kappa B (NF κ B)

activation, Fas upregulation, DNA fragmentation, and impaired β -cell function (Elouil et al., 2005; Maedler, 2002a&b; Maedler, 2001b). Therefore Fas, in the presence of FLIP, induce β -cell proliferation (Tschopp et al., 1998).

Other critical genetic regulators of β -cell proliferation and/or differentiation include the paired box gene 4 (Pax4) and c-MYC. Pax4, is crucial for the proliferation and survival of the β -cells, and is activated via the phosphoinositide 3-kinase (PI3K) pathway. Pax4 mediates β -cell replication via PI3K and two of its downstream targets, p38 mitogen-activated protein kinase and protein kinase C (PKC) (Brun et al., 2004). In addition to PKC, protein kinase B (Akt/PKB) has also been closely linked to β -cell proliferation with over-expression resulting in increased β -cell size and total islet mass, improved glucose tolerance and complete resistance to experimental diabetes in mice (Tuttle et al., 2001).

Pax4 increases c-MYC and the anti-apoptosis regulatory protein, B-cell lymphoma-extra-large gene expression, thus protecting it from apoptosis (Brun et al., 2004). In a normal adult pancreas, c-MYC levels are low (Jonas et al., 1999), however, in a diabetic rat model, hyperglycemia induces over-expression of c-MYC, which resulted in a reduction in glucose-stimulated insulin secretion (GSIS) and suppression of insulin gene transcription (Kaneto et al., 2002, Jonas et al., 2001). c-MYC is a stress-induced gene that serves as an early marker of alterations induced by hyperglycemia in β -cell gene expression (Jonas et al., 1999) Hyperglycemic conditions also activate MAPK and extracellular signal-regulated kinase (ERK) 1/2 which are important regulators of glucose-dependent nuclear activities within the β -cells (Brun et al., 2004).

Several clinical studies have shown that T2D patients are subjected to chronic oxidative stress (Shin, 2001; Sakuaba, 2002) and the deteriorating glycemic control results in increased levels of oxidative stress markers in β -cells (Ihara et al., 1999).

1.4.2 Chronic oxidative stress

Pancreatic islets have the lowest intrinsic antioxidant capacity compared to other metabolic tissues such as fat, skeletal muscle and the liver (Tiedge, 1998). Therefore, due

to their low antioxidant capacity, the cellular stress induced by hyperglycemia does not induce higher activities of the antioxidant enzymes (Tiedge, 1998). Chronically elevated glucose and free fatty acids, as evident in obesity and T2D, increase mitochondrial superoxide production and uncoupling protein 2 (UCP2) expression in pancreatic β -cells (Rabuazzo et al., 2003; Krauss et al., 2003). Mitochondrial superoxide induces UCP2-mediated proton leak which results in the lowering of ATP levels and impairs GSIS. Elevated superoxide production causes increased exposure of the β -cell to reactive oxygen species (ROS). The generation of ROS is known to occur via autoxidation of glucose, where glyceraldehydes, in the presence of heavy metals, form a radical anion, which in the presence of oxygen, form superoxide, which is changed to hydrogen peroxide via the action of superoxide dismutase. The increase production of UCP2 can be seen as a compensatory mechanism to dissipate elevated mitochondrial membrane potential and promote the uncoupling of oxidative phosphorylation, resulting in a reduction in ROS production - a price for its survival in the presence of excess nutrients (Wiederkehr and Wollheim, 2012 and 2008; Prentki and Nolan, 2006; Krauss et al., 2003).

Evans et al. (2003) proposed that the ROS and reactive nitrogen species (RNS) formed due to chronic hyperglycemia, together with fatty acids, directly induce damage to DNA, proteins and lipids (figure 1.2). The ROS and RNS indirectly induce insulin resistance and β -cell dysfunction by activating cellular stress-activated signaling pathways, including nuclear factor $\kappa\beta$ (NF κ B), p38 MAPK, proto-oncogene Jun (c-Jun) N-terminal kinases (JNK)/stress activated protein kinases (SAPK), and hexosamines. Increased mitochondrial ROS and RNS, resulting from elevated glucose and fatty acid levels, trigger the activation of pathways involved in the development of diabetes sequelae. Thus, stress-activated signaling pathways result in increased levels of products such as advanced glycation end products (AGEs), receptors for AGE (RAGE), and PKC, that cause cellular damage and the development of diabetes complications (Evans et al., 2003).

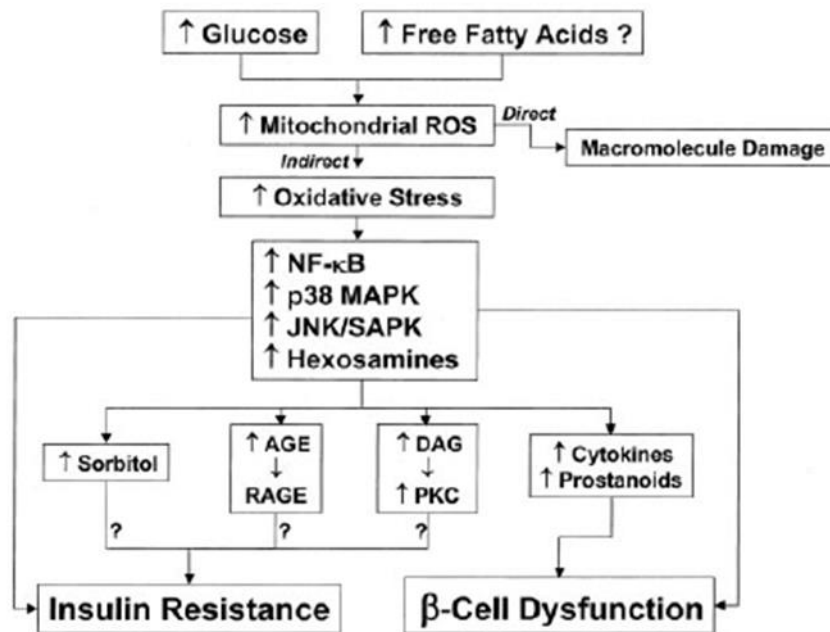


Figure 1.2 Proposed mechanism of the pathophysiology of T2D. Taken from Evans et al. (2003).

AGE production, RAGE activation and impaired prostaglandin metabolism contribute to the pathogenesis of diabetic complications, particularly atherosclerosis (Niedowicz and Daleke, 2005). Activated PKC modulates mechanisms that promote atherosclerosis via increased *de novo* synthesis of diacylglycerols (DAGs). PKC and RAGE increase NFκB activation, which is a transcription factor that regulates expression of various chemokines and cytokines such as TNF- α , tissue factor, vascular endothelial growth factor, cell adhesion molecules, and chemotactic proteins (Niedowicz and Daleke, 2005).

Poitout and Robertson (2002) showed that chronic exposure to glucotoxicity and lipotoxicity resulted in deterioration of β -cell function, while chronic hyperglycemia exposure, independent of lipotoxicity, is toxic to β -cells. In T2D patients, lipotoxicity only occurs in the context of pre-existing chronic hyperglycemia, since most individuals with hyperlipidemia have normal β -cell function (Robertson et al., 2004).

1.4.3 Lipotoxicity

The primary role of insulin is to lower blood glucose levels by facilitating uptake into skeletal muscle while inhibiting hepatic gluconeogenesis and promoting the storage of energy (mainly in the form of glycogen and fat). Poitout and Robertson (2002) have shown that lipid dysregulation only occurs once glucotoxicity is present. They also found that once plasma glucose is normalized in Zucker diabetic rats, the accumulation of plasma triglycerides is prevented. In obese and diabetic patients, insulin-induced glucose uptake is impaired and lipogenesis in the liver and adipose tissues remain unchanged or elevated resulting in changes in their plasma lipid profile occur which affects glucose metabolism at multiple levels, including the deleterious effects on β -cells. These deleterious effects of lipid overload on the different organs and systems are referred to as lipotoxicity (Chavez and Summers, 2010; Unger et al., 2010).

As indicated above, the development of diabetes is underpinned by abnormalities in lipid metabolism. Under normal physiological conditions, FFAs provide an alternative fuel source to glucose during prolonged fasting and stimulate insulin secretion in nondiabetic individuals (Boden, 1997). Hyperphagia/overnutrition results in hyperinsulinemia which promotes lipogenesis in non-adipocytes. Ingestion of high fat diets contributes to the absorption of excess FFAs thus resulting to increased plasma FFAs (Yang and Li, 2012). In a fed state, the antilipolytic effect of insulin suppresses FFA levels by inhibiting lipolysis and promoting glucose utilization. However in obese subjects FFA levels are characteristically elevated due to the insulin insensitivity displayed as a result of the increased adipocyte mass. Insulin resistance/insensitivity results in increased lipolysis in the adipose tissue, which in turn causes increased plasma FFAs. The increased availability of plasma FFAs results in the production of increased fatty acyl Co-enzyme A and citrate content in the skeletal muscle tissue of obese subjects. Fatty acyl Co-enzyme A inhibits pyruvate dehydrogenase which results in reduced glucose oxidation, while citrate inhibits phosphofructokinase 1, thus directly inhibiting glycolysis (Boden, 1997; Unger, 1995). Besides affecting glucose oxidation and glycolysis, FFAs inhibit insulin-induced glucose uptake via inhibition of glucose transport or phosphorylation and decreased muscle glycogen synthase activity (Boden, 1997).

FFAs serve as a substrate for non-oxidative metabolism, such as lipid peroxidation and ceramide-mediated apoptosis (Unger and Orci, 2001). Depending on the type of FFA, it has been shown to protect the β -cells against apoptosis, where exposure to monounsaturated fatty acids protects the β -cells against lipo- and glucotoxicity, while prolonged exposure to saturated fatty acids is deleterious to β -cell function and turnover (Maedler et al., 2001b; Maedler et al., 2003). Besides the effect of excess FFAs on glucose metabolism, lipotoxicity induces damage in pancreatic β -cells via a number of mechanisms which will be discussed below.

The increase in fat deposits in obese subjects results in a concomitant increase in leptin, an adipocyte-derived satiety factor and an anorexigenic hormone (Jo et al., 2005). Leptin inhibits glucose-stimulated insulin secretion by reducing cyclic adenosine monophosphate (cAMP) levels, which prevents activation of PI3-K (Kieffer and Haberner, 2000) and induces β -cell apoptosis by increasing IL-1 β secretion and decreasing levels of the IL-1 receptor antagonist (IL-1Ra) in human islets (Maedler et al., 2004). Leptin activates adenosine monophosphate kinase (AMPK), which in turn inactivates acetyl co-enzyme A carboxylase (ACC) and blocks malonyl co-enzyme A synthesis which effectively augments FFA oxidation and reduce FFA synthesis (Unger et al., 2010). Unlike leptin, glucose negatively regulates AMPK resulting in increased malonyl co-enzyme A levels, reduced fatty acid oxidation and increased lipogenesis (Lim et al., 2010; Bandyopadhyay et al., 2006; Xue and Kahn, 2006; Poitout, 2004). AMPK is a key molecular regulator in energy balance, mediating the metabolic effects of hormones such as leptin, ghrelin, Adipoq, glucocorticoids, insulin as well as cannabinoids (Lim et al., 2010). Besides caloric restriction and exercise that activate AMPK activity, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside and metformin, AMPK activators, reduce lipogenesis (especially the ectopic lipid overload in non-adipocytes), increase fatty acid oxidation and insulin secretion, thus protecting the β -cell from lipotoxicity (Diraison et al., 2004; Yamashita et al., 2004).

β -Cell functionality is negatively regulated by sterol regulatory element binding protein-1c (SREBP-1c) which is a key mediator of insulin-induced glycolytic and lipogenic gene transcription in mouse and human adipose tissue as well as in muscles in rats and humans, and inhibit hepatic gluconeogenic gene expression (Foufelle and Ferré, 2002). Over-expression of SREBP-1c in rat β -cells and INS-1 cells resulted in increased UCP2 levels, reduced intracellular ATP/ADP ratio, increased lipogenesis and reduced insulin secretion. Islets exposed to fatty acids showed increased peroxisome proliferator-activated receptor- γ (PPAR- γ) and UCP2 levels, while treatment with PPAR- γ antagonists reduced UCP2 levels, thus restoring insulin secretion (Patanè et al., 2002). UCP2-knockout mice were protected from lipotoxicity when fed on a high-fat diet and showed increased insulin secretory capacity (Joseph et al., 2002).

PPAR- γ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors that serves as a key regulator of adipocyte differentiation and adipogenesis (Bell and Polonsky, 2001; Murphy and Holder, 2000). As stated above, PPAR- γ is expressed in the pancreatic β -cells (Dubois et al., 2000). Welters et al. (2004) found that PPAR- γ does not mediate protective responses within the pancreatic β -cells exposed to lipo- and glucotoxic conditions. FoxO1 is an integrator of metabolic stimuli and inhibits PPAR γ expression in β -cells. Gupta et al. (2013, 2010) have shown that FoxO1/PPAR γ signaling regulates the transcription of key β -cell genes including PDX1, glucose-dependent insulintropic polypeptide receptor, and pyruvate carboxylase – all important regulators of β -cell function, survival, and compensation.

The known mechanisms associated with glucotoxicity, lipotoxicity and chronic oxidative stress in the various organs provide the basis for the development of anti-diabetic treatments, which will be discussed next.

1.5 ANTI-DIABETIC DRUG TREATMENTS

Dietary modifications, weight loss and exercise are all recommended for T2D patients in its prevention and management, since it enhances insulin sensitivity and glucose utilization, whilst improving lipid and lipoprotein levels (WHO, 2013). The Diabetes

Prevention Program Research Group found that changes in lifestyle which translates into a 7% weight loss and 150 min of physical activity per week resulted in a 58% reduction in the incidence of diabetes (Knowler et al., 2002). Besides introducing lifestyle modifications in T2D patients where β -cell functionality is impaired, the improvement of glycemic control can be achieved by introducing pharmacological therapy that is tailored according to the patient's needs, while also considering the safety profiles, tolerability, ease of use, and cost of these pharmacological agents (Inzucchi et al., 2012; Nathan et al., 2009).

In T2D patients, different types of oral hypoglycemic agents are prescribed to enhance glycemic control. Figure 1.3 provides an overview of the types of anti-diabetic treatments which are targeting different organs and tissues implicated in the pathogenesis of T2D. There are various categories of OADs on the market, with ongoing development of newer ones as the underlying etiology of T2D is unraveled. Currently, there are three broad categories of oral anti-diabetic drugs (OADs): those that reduce insulin resistance, those that increase insulin secretion and those that reduce glucose absorption from the gut. Insulin is often used as a rescue therapy after the first line OADs have failed. The goal of these strategies is to eventually normalize glucose levels and maintain glycemic levels within the non-diabetic range thus reducing the prevalence of long-term complications.

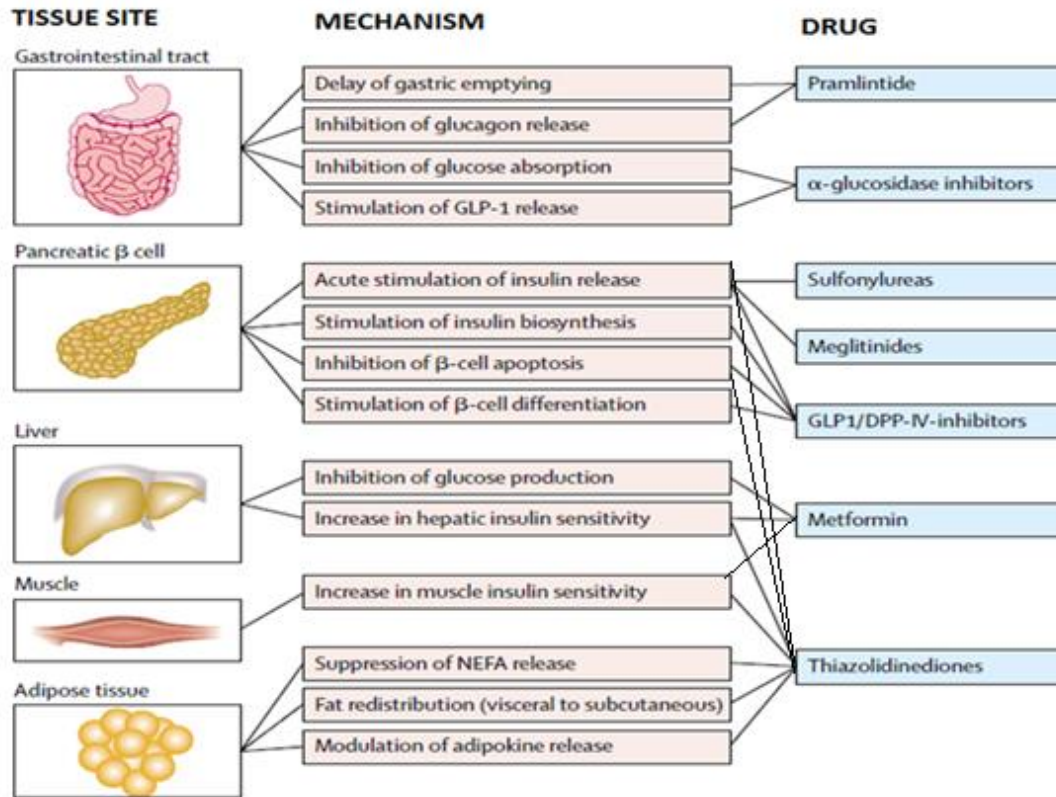


Figure 1.3 Hypoglycemic treatments for diabetes and their primary sites of action. Taken from Stumvoll et al. (2005).

1.5.1 Insulin sensitizers

1.5.1.1 Biguanides

These compounds reduce blood glucose level without causing overt hypoglycemia or stimulating insulin secretion. This is achieved by increasing peripheral insulin sensitivity and glucose utilization, while reducing hepatic gluconeogenesis and glycogenolysis. Metformin, derived from extracts of the French Lilac (*Galega officinalis*) (Schäfer, 1983), is the most widely used OAD for T2D because of its efficacy, low cost, lack of weight gain, no increased risk of hypoglycemia, and its proven long-term efficacy on enhancing glycemic control (Holman et al., 2008). Metformin improves hepatic and muscle insulin sensitivity by indirectly stimulating AMPK via inhibition of complex I of the respiratory chain, resulting in an increase in the AMP:ATP ratio (Kahn et al., 2005). Activated AMPK gauges cellular energy levels and regulates metabolic pathways involved in glucose and fatty acid metabolism and protein synthesis, switching on catabolic processes

that produce ATP and switches off ATP consuming processes (Lim et al., 2010; Xue and Kahn, 2006; Zheng et al., 2001; Zhou et al., 2001; Owen et al., 2000). AMPK mediates food intake, body weight and hepatic glucose production via the AMPK–ACC–malonyl CoA pathway in the hypothalamus and also mediates the effects of adipocyte-derived and gut-derived hormones and peptides on fatty acid oxidation and glucose uptake in peripheral tissues (Lim et al., 2010; Xue and Kahn, 2006). Therefore, the overall effect of metformin is to enhance glucose uptake into the liver and muscles, induce hepatic fatty acid oxidation and inhibits hepatic glucose production and expression of lipogenic enzymes thereby reducing the hypertriglyceridemia associated with diabetes (Lim et al., 2010).

The major side effect is gastrointestinal discomfort, most notably diarrhea, reported by approximately 15 % of patients. There is a growing recognition for the increased risk of lactic acidosis and its consequences, especially in the elderly and those with renal dysfunction (Lipska et al., 2011; Ross, 2004). Renal dysfunction in patients with T2D is typically progressive. Diabetic patients using metformin that have mild to moderate renal dysfunction are at risk of developing lactic acidosis due to their impaired ability to eliminate metformin via the kidneys (Lipska et al., 2011). Examples include metformin, phenformin and buformin. Only metformin remains on the market due to the high risk of lactic acidosis associated with phenformin and buformin.

1.5.1.2 Thiazolidinediones

Thiazolidinediones (TZDs) act as high-affinity ligands to PPAR- γ which modulates genes involved in carbohydrate and lipid metabolism. The overall effect of TZD treatment is increased sensitivity to endogenous and exogenous insulin by muscle, liver and fat tissues (Nathan et al., 2009; Inzucchi, 2002). Stimulation of PPAR- γ by TZDs increases glucose uptake due to increased glucose transporter type 4 (GLUT4) expression and concurrently enhances insulin signaling through insulin receptor substrate (IRS)-1 expression and activation as well as inhibition of the MAPK pathways (DeFronzo, 2009). TZDs improve glucose utilization and enhanced peripheral insulin sensitivity by increasing glucose disposal in skeletal muscle and decreasing hepatic gluconeogenesis, increase glycogen

synthase activity and glucose metabolism in skeletal muscle and adipocytes (Cheng and Fantus, 2005). The TZDs also induce adipocyte differentiation through the activation of PPAR- γ , which is the master regulator of adipocyte differentiation, and redirect fatty acids away from circulating blood, muscle and liver tissues to adipose tissue and inhibit leptin and TNF- α gene expression while increasing AdipoQ levels (Nathan et al., 2009; Cheng and Fantus, 2005; Inzucchi, 2002). TZDs exert anti-atherogenic effects by increasing AdipoQ levels, reducing plasma TNF- α levels and infiltration of macrophages into fat tissue and endothelial cells, and improving and preserving β -cell function (DeFronzo, 2009; Nathan et al., 2009; Cheng and Fantus, 2005; Di Gregorio et al., 2005; Maeda et al., 2001). TZDs are paradoxically also potent inhibitors of lipolysis (DeFronzo, 2009). Like metformin, TZDs do not induce insulin secretion or hypoglycemia (Ross, 2004). The most common adverse effects associated with TZDs are weight gain, bone fractures, anemia, fluid retention, peripheral and pulmonary edema and a two-fold increased risk for congestive heart failure, with an increase in subcutaneous adiposity (Nathan et al., 2009; Inzucchi, 2002). TZDs are the most costly of the OADs. Examples include pioglitazone and rosiglitazone.

1.5.2 Insulin Secretagogues

1.5.2.1 Sulfonylurea (SU)

These are sulfonamide drugs that contain a SU moiety. They serve as insulin secretagogues, enhancing the release of preformed insulin by increasing the β -cell's sensitivity to glucose, thereby mimicking the acute phase of the normal response to elevated glucose levels. They elicit their effect by blocking the K_{ATP} on the β -cell by binding to the SUR1, which is a subunit of the K_{ATP} channel. Blocking of the channel causes depolarization of the membrane, and subsequent opening of voltage-gated Ca^{2+} channels (VGCC) (see figure 1.1). The Ca^{2+} that flows into the cell binds to calmodulin, which triggers exocytosis of insulin. Hypoglycemia and weight gain are common side-effects. Examples include tolbutamide, glibenclamide, gliclazide, glimepiride and glipizide (Akkati et al., 2011; Ross, 2004).

1.5.2.2 Sulfonylurea derivatives

These compounds are referred to as non-sulfonylurea (non-SU) insulin secretagogues since they are structurally distinct from the traditional SUs, but have some chemical similarity (Fuhendorff et al., 1998). They have a shorter circulating half-life than the SUs and are administered more frequently (Nathan et al., 2009). Meglitinide analogues include mitiglinide, nateglinide and repaglinide, where mitiglinide and repaglinide are benzoic acid derivatives, and nateglinide is a phenylalanine derivative (Cheng and Fantus, 2005; Ross, 2004). They enhance insulin release by closing the K_{ATP} channels in β -cells resulting in calcium-dependent insulin secretion, thus having a similar mechanism of action to SUs (Cheng and Fantus, 2005). K_{ATP} channels are comprised of Kir (Kir6.1 or Kir6.2) subunits that form the K^+ -selective inwardly rectifying potassium ion pore and subunits that are SUR1 or 2 (Nagashima et al., 2004). In pancreatic β -cells the K_{ATP} channels are comprised of the Kir6.2 subunit that forms the K^+ -selective ion channel pore and the SUR1 subunit. Both nateglinide and mitiglinide (which are structurally unrelated to SU) inhibit pancreatic β -cell K_{ATP} channels by binding to a tolbutamide-binding site on the SUR1 subunit. Repaglinide binds to the benzamide-binding site on the SUR1 subunit (Nagashima et al., 2004).

Adverse effects associated with meglitinide analogues include hypoglycemia and weight gain, but to a lesser extent than that induced by SUs. These compounds are contraindicated in patients with severe liver dysfunction and in patients with severe kidney dysfunction, where the dosage should be reduced (Cheng and Fantus, 2005). These compounds may have an adverse cardiovascular effect, specifically with respect to ischemic preconditioning, as they bind to K_{ATP} channels associated with the sarcolemma and mitochondrial membranes of cardiomyocytes (Ross, 2004).

1.5.2.3 Incretins: Incretin Mimetics and Incretin Enhancers

Ingestion of glucose results in the stimulation of insulin secretion by gut-derived peptides called incretins (Doyle and Egan, 2007; Drucker, 2006). Incretins are known to have hypoglycemic properties by inducing GSIS and have also been associated with β -cell regeneration by promoting proliferation and neogenesis and reducing apoptosis (Nauck

et al., 2009; Drucker, 2006; Bonner-Weir and Weir, 2005). The primary incretin hormone is GLP-1, which acts on the β -cells via the GLP-1 receptor, activating adenylate cyclase (AC) resulting in increased levels of cAMP, leading to cAMP-dependent activation of second messenger pathways (Doyle and Egan, 2007). GLP-1 is secreted by neuroendocrine L cells in the ileum and colon, and triggers glucose-stimulated insulin release, increased proinsulin gene expression and translation, inhibits glucagon release, slows gastric emptying and nutrient absorption, increases satiety thus reducing food intake (Nauck et al., 2009; Drucker, 2006). The half-life of GLP-1 is approximately two minutes, as it is rapidly inactivated by dipeptidyl peptidase IV (DPPIV), thus providing a narrow therapeutic window within which to elicit a desirable effect (Drucker, 2003). To extend the therapeutic window of GLP-1, two approaches were embarked on: (1) the development of GLP-1 analogues (incretin mimetics) geared at reducing GLP-1 plasma clearance, and (2) identifying and/or developing DPPIV inhibitors to prevent DPPIV cleaving GLP-1 (incretin enhancers). Various GLP-1 analogues have been developed and form part of the current therapeutic interventions, including exenatide, liraglutide, lixisenatide and taspoglutide, while DPPIV inhibitors (also referred to as gliptins) include vildagliptin, sitagliptin and saxagliptin (Akkati et al., 2011). The oral DPPIV inhibitor, linagliptin, attenuated postprandial glucose and significantly reduced HbA1c after only 4 weeks of treatment (Forst et al., 2011). As indicated above, oral DPPIV inhibitors are available, however, the lack of orally bioavailable dosage forms of GLP-1 analogues is a significant limitation, besides being expensive, as these drugs are administered by injection either intravenously or subcutaneously. In addition, the stability of GLP-1 analogues in solution is limited by time, temperature and pH (Nathan et al., 2009; Arulmozhi and Portha, 2006).

Adverse effects associated with sustained GLP-1 and DPPIV administration include nausea and vomiting due to the slowing down of gastric emptying, which may in part contribute to the weight loss of patients using GLP-1 analogues (Nathan et al., 2009). Further concerns were raised by the research of Butler et al. (2013) which linked incretin use to cancerous lesions found in the pancreata of T2D volunteers. According to Butler et al. (2013) incretin therapy resulted in a marked increase in exocrine and endocrine

pancreatic cells. The exocrine proliferation was accompanied by dysplasia, while in the islets, α -cell hyperplasia showed the potential of developing into neuroendocrine tumors. Based on a meta-analysis of research published on incretin treatment, including GLP-analogues and DPPIV inhibitors, Labuzek et al. (2013) reported that in animal and human studies there could be a connection between incretin-based therapies and pancreatitis, pancreatic cancer, thyroid cancer and other neoplasms, and recommends that use of this therapy should proceed with caution, more especially if there is a familial tendency for cancer. However, the benefits of incretin therapy far outweigh the potential risks associated with its use. Thus, Nauck (2013) emphasises the improved cardiovascular risk profile and states that insufficient data is available to directly link incretin therapy to chronic pancreatitis and pancreatic cancer, with thyroid cancer mainly evident in rodents with very few cases observed in humans.

1.5.3 Drugs acting on carbohydrate/glucose absorption

1.5.3.1 α -Glucosidase inhibitors

α -Glucosidase is a brush border enzyme in the proximal small intestine that is responsible for the final catabolic step in disaccharides and polysaccharide digestion (Inzucchi, 2002). α -Glucosidase inhibitors (e.g. acarbose and miglitol) retard the absorption of carbohydrates in the intestine and thus reduce fluctuations in postprandial insulin and glucose levels. Adverse effects associated with the use of AGIs occur as a result of the delay in carbohydrate absorption with subsequent increased delivery of carbohydrates to the colon which brings about abdominal discomfort, increased flatulence and diarrhea. The unabsorbed carbohydrates increase the osmotic pressure in the colon, resulting in increased fluid retention in the lumen and gaseous waste products being formed from the sugars being metabolized by the intestinal flora (Nathan et al., 2009; Cheng and Fantus, 2005; Ross, 2004). AGIs are contraindicated in patients with irritable bowel syndrome, severe kidney or liver dysfunction (Cheng and Fantus, 2005).

1.5.4 Insulin

Exogenous insulin therapy is usually initiated in T2D patients to support OADs which enhance insulin sensitivity such as metformin, when it becomes ineffective in the presence

of inadequate levels of endogenous insulin. Insulin can be used in combination with other OADs (table 1.1), particularly insulin sensitizing agents, with the aim of improving glycemic control and hemoglobin A_{1c} concentrations in the diabetic patient.

Table 1.1 Potential Combinations of OADs for the Treatment of Type 2 Diabetes. (Taken from Cheng and Fantus, 2005).

<p>Metformin plus</p> <ul style="list-style-type: none"> · SU · Non-SU insulin secretagogues · Thiazolidinedione · α-Glucosidase inhibitor · Insulin <p>α-Glucosidase inhibitor plus</p> <ul style="list-style-type: none"> · Metformin · Thiazolidinedione · SU · Non-SU insulin secretagogues · Insulin 	<p>Thiazolidinedione plus</p> <ul style="list-style-type: none"> · Metformin · SU · Non-SU insulin secretagogues · α-Glucosidase inhibitor <p>Do not combine:</p> <ul style="list-style-type: none"> · SU + non-SU insulin secretagogue · Insulin secretagogues + preprandial insulin · Thiazolidinedione + insulin
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Combinations of submaximal doses of different classes of orally administered antihyperglycemic agents may be equally effective as or more effective than maximum dose of monotherapy in improving glucose control with fewer adverse effects.

There are various formulations of insulin, varying from ultra-fast acting to slow acting. The major disadvantages associated with insulin therapy are hypoglycemia, weight gain, high costs of insulin analogues, and the mode of administration i.e. subcutaneous injection (Nathan et al., 2009).

Non-invasive products being developed include Afrezza[®] which is an inhaled pulmonary drug delivery system awaiting US FDA approval, the Transgene[®] oral delivery system by Biotek, which is being tested in human trials, and involves biodegradable nanoparticles containing insulin; In-105[®] by Biocon, is an oral tablet containing conjugated insulin, which is at a phase-III clinical trial level, an oral capsule, ORMD-0801[®] by Oramed Pharmaceuticals which is at the phase-IIb stage of development, ORMD-0802[®] which is a rectal insulin suppository at Phase-1a stage of development and Oral-Lyn[®] (manufactured

by Generex Biotechnology Corporation), which is a liquid formulation containing insulin that is sprayed into the mouth and is at various phases of clinical development (Akhter and Nijhu, 2012; Soares et al., 2012; Akkati et al., 2011; Hollander et al., 2004).

1.5.5 Other forms of therapy

Ongoing research has resulted in the development of various new drugs which vary in their sites of action and mode of action. Amylin is a peptide co-secreted with insulin by the β -cells, which lowers glucagon release, slows gastric emptying and reduces food intake (Akkati et al., 2011; Chapman et al., 2005). Amylin analogues include Pramlintide and Symlin (Akkati et al., 2011). Inhibitors of protein tyrosine phosphatase 1B (PTP1B), the enzyme required for shutting off the insulin receptor after activation, have been developed. Bis(maltolato)oxovanadium(IV), a nonspecific PTP1B inhibitor, improved glucose tolerance and prevented diabetes in a study conducted on Zucker diabetic fatty rats (Winter et al., 2005). AdipoQ is a potent insulin sensitizer, and clinical trials are currently underway to investigate its potential as a new treatment modality.

Another class of FDA-approved oral anti-diabetic drugs are the sodium-dependent glucose transporter (SGLT)-2 inhibitors. SGLT-2 transporters are primarily located in the cortex of the kidney specifically in the renal proximal tubule, and are the principal glucose transporters which are highly selective for glucose over galactose (Abdul-Ghani et al., 2011). SGLT-2 transporters are also expressed at low levels in the brain and liver (Turk et al., 1994). SGLT-2 inhibitors suppress reabsorption of renal glucose, thereby reducing the amount of glucose being re-introduced into the blood circulation. Some SGLT-2 inhibitors include canagliflozin and dapagliflozin. No long-term data is available regarding the side-effects associated with SGLT-2 inhibitor treatment. However, up to 48-week studies have been conducted, where treatment resulted in a slight reduction in blood pressure, a slight increase in urinary tract infections. If used in combination with insulin sensitizing agents such as SU, the dosages of these should be reduced to prevent the occurrence of hypoglycemia. No other effects have been observed in the liver or brain where SGLT-2 transporters are also located (Nigro et al., 2013; Abdul-Ghani et al., 2011; Akkati et al., 2011).

The gluconeogenesis pathway in the liver is elevated in T2D patients, and fructose 1,6-bisphosphatase (FBPase) has been identified as a rate-limiting enzyme in this pathway (Dang et al., 2009). AMP is a potent inhibitor of FBPase (Dang et al., 2009; Sarabu and Tilley, 2005). The hypoglycemic capabilities of AMP-mimetics were shown in rat hepatocytes and *in vivo* in Sprague-Dawley rats, where purine analogues bound to the AMP site on FBPase, inhibiting FBPase, resulting in the suppression of the gluconeogenesis pathway, thereby reducing hepatic glucose release, blood glucose levels and HbA1c levels (Akkati et al., 2011; Dang et al., 2009).

T2D involves both insulin resistance and β -cell dysfunction. Therefore, the ideal therapy would be one that improves both these aspects of metabolic dysfunction. Current treatment regimes for T2D can only provide such improvements by combination therapy. Furthermore, each therapy has its own drawbacks that include hypoglycemia, intestinal side effects, lactic acidosis, weight gain and primary or secondary failure. Thus, there is a need for improved therapeutics that act on both the metabolic defects that characterize T2D and that lack the side effects of current therapies. It is for these reasons that attention is now being focused on the development of new anti-diabetic therapies derived from plants.

1.6 HYPOGLYCEMIC MEDICINAL PLANTS

Many medicinal plants are used as dietary supplements and in the treatment of numerous diseases without proper scientific or medical scrutiny, with a large number of these plants possessing some degree of toxicity. Despite this, plants have always been an exceptional source of drugs and many of the drugs that are currently available as therapeutic agents are derived either directly or indirectly from them. These chemical compounds include alkaloids, glycosides, galactomannan, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions (Grover et al., 2002). A classical example is the class of biguanides to which the hypoglycemic drug, metformin, belongs. It is derived from the French lilac (*Galega officinalis*), which was in use for several centuries for its anti-diabetic properties.

In recent reviews, the families of plants with the most potent hypoglycemic effects include: Leguminosae (11 species), Lamiaceae (8 species), Liliaceae (8 species), Cucurbitaceae (7 species), Asteraceae (6 species), Moraceae (6 species), Rosaceae (6 species), Euphorbiaceae (5 species) and Araliaceae (5 species) (Patel et al., 2012; Bnouham et al., 2006). The most active plants are *Allium sativum*, *Gymnema sylvestre*, *Citrullus colosynthis*, *Trigonella foenum greacum*, *Momordica charantia* and *Ficus bengalensis*, with the hypoglycemic effects of these plants attributed to chemicals such as amino acids, alkaloids, alkyl disulphides, coumarins, flavonoids, glycoproteins, guanidines, inorganic ions, iridoids, lipids, peptides, phenolics, polysaccharides, steroids, terpenoids and xanthone (Patel et al., 2012; Jarald et al., 2008).

1.6.1 *Cannabis sativa* (*C. sativa*)

C. sativa, is also referred to as dagga (Afrikaans), marijuana (English), umya (Xhosa), matokwane (Sotho), and nsangu (Zulu), and is not indigenous to southern Africa. It is proposed that Arab traders introduced it into this region via Mozambique, many centuries ago, during pre-colonial times. It was subsequently used as an intoxicant and medicinal plant by the Khoikhoi, by either chewing it or drinking it as a weak infusion. Smoking of dagga, derived from the Khoikhoi word Dachab, only began after the European colonialists introduced the smoking pipes (Perkel, 2005). *C. sativa* is the illicit drug of choice in South Africa (Perkel, 2005). It is extensively used as a recreational drug by millions of people globally, and is the third most commonly used drug in Britain, after tobacco and alcohol (Baker et al., 2003).

1.6.1.1 Cannabinoid biosynthesis

The *C. sativa* plant contains over 400 compounds including at least 66 terpenophenolic compounds, i.e. volatile organic compounds generally associated with plant fragrances, referred to as the classical cannabinoids. The plant content of tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol (also referred to as Δ^9 -Tetrahydrocannabinol or Δ^9 -THC), the primary psychoactive agent in *C. sativa* varies depending on the age of the plant (increasing with age), the geographical area where it is cultivated, the strain of plant

from which the material originated and the type of preparation (Hillig and Mahlberg, 2004). Three discrete chemotypes (I, II and III) of *C. sativa* have been identified, based on the THC:Cannabidiol (CBD) ratio in the plant material (De Backer et al., 2009; Hillig and Mahlberg, 2004). There is, however, great intra-species variability in *C. sativa* due to strains specifically being cultivated for their narcotic properties, fibre and oilseed (Small et al., 2004; Guzmán and Sánchez, 1999). However, not all cannabinoids are psychoactive.

Other classical cannabinoids found in *C. sativa* include cannabiniol (CBN), CBD, Δ^8 -Tetrahydrocannabinol (Δ^8 -THC) and Δ^9 -tetrahydrocannabivarin (THCV) (figure 1.4) (Baker et al., 2003).

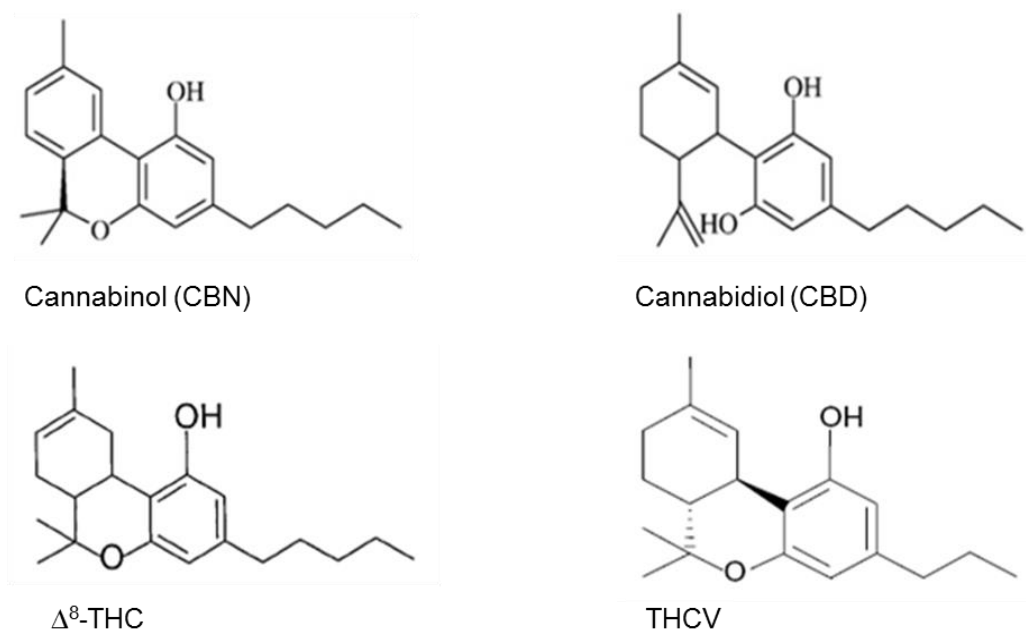


Figure 1.4 Chemical structure of classical cannabinoids found in *C. sativa*. Taken from Baker et al. (2003).

C. sativa is the unique source of at least 66 cannabinoids: 9 Δ^9 -THC-types, 2 Δ^8 -THC-types, 7 CBD-types, 6 cannabigerol-types, 5 cannabichromene-types, 3 cannibicyclol-types, 5 cannabielsoin-types, 9 cannabitriol-types, and 11 other miscellaneous types, with CBN and cannabiodiol-types (which are air-oxidation artefacts) (Pertwee, 2006).

Cannabinoids have been shown to interact, to varying degrees, with cannabinoid receptors distributed throughout the body (Pertwee, 2008; Pertwee, 2006).

Research has shown that non-cannabinoid compounds derived from plant material are able to interact with cannabinoid receptors. Due to these findings, phytocannabinoids have been defined as any plant-derived natural compounds which are capable of interacting directly with that cannabinoid receptor, and/or which are chemically similar to cannabinoids (Gertsch et al., 2010). There are three other classes of cannabinoids: the non-classical cannabinoids (consisting of bicyclic and tricyclic analogues of Δ^9 -THC, that lack a pyran ring, e.g. $1\alpha,2$ -(R)-5-(1,1-dimethylheptyl)-1-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-phenol (also known as CP 55940); aminoalkylindoles (these compounds are structurally different from classical cannabinoids, possess cannabimimetic properties, and are analogs of pravadoline, e.g. R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo [1,2,3-de]-1,4-oxazinyl]-(1-naphthalenyl)-methanone-mesylate (also referred to as WIN55212-2) and the eicosanoids (arachidonic acid derivatives which include most endocannabinoids, e.g. anandamide (AEA)) (figure 1.5) (Howlett et al., 2002).

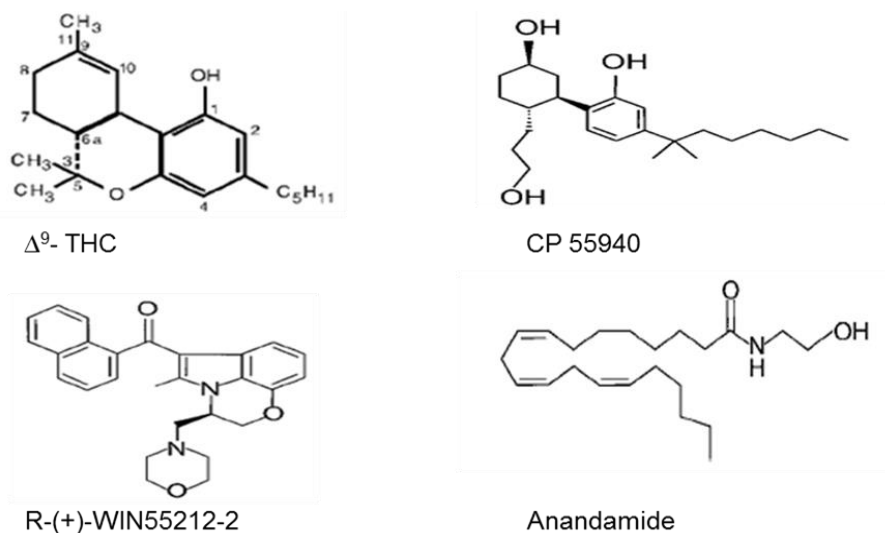


Figure 1.5 Examples of the different classes of cannabinoids. Taken from Howlett et al. (2002).

The biosynthesis of the classical cannabinoids occur in all *C. sativa* plant tissues however, their expression varies between tissue types, and is most abundant in flowers, especially the glands (trichomes). The biosynthesis of THC is depicted in figure 1.6.

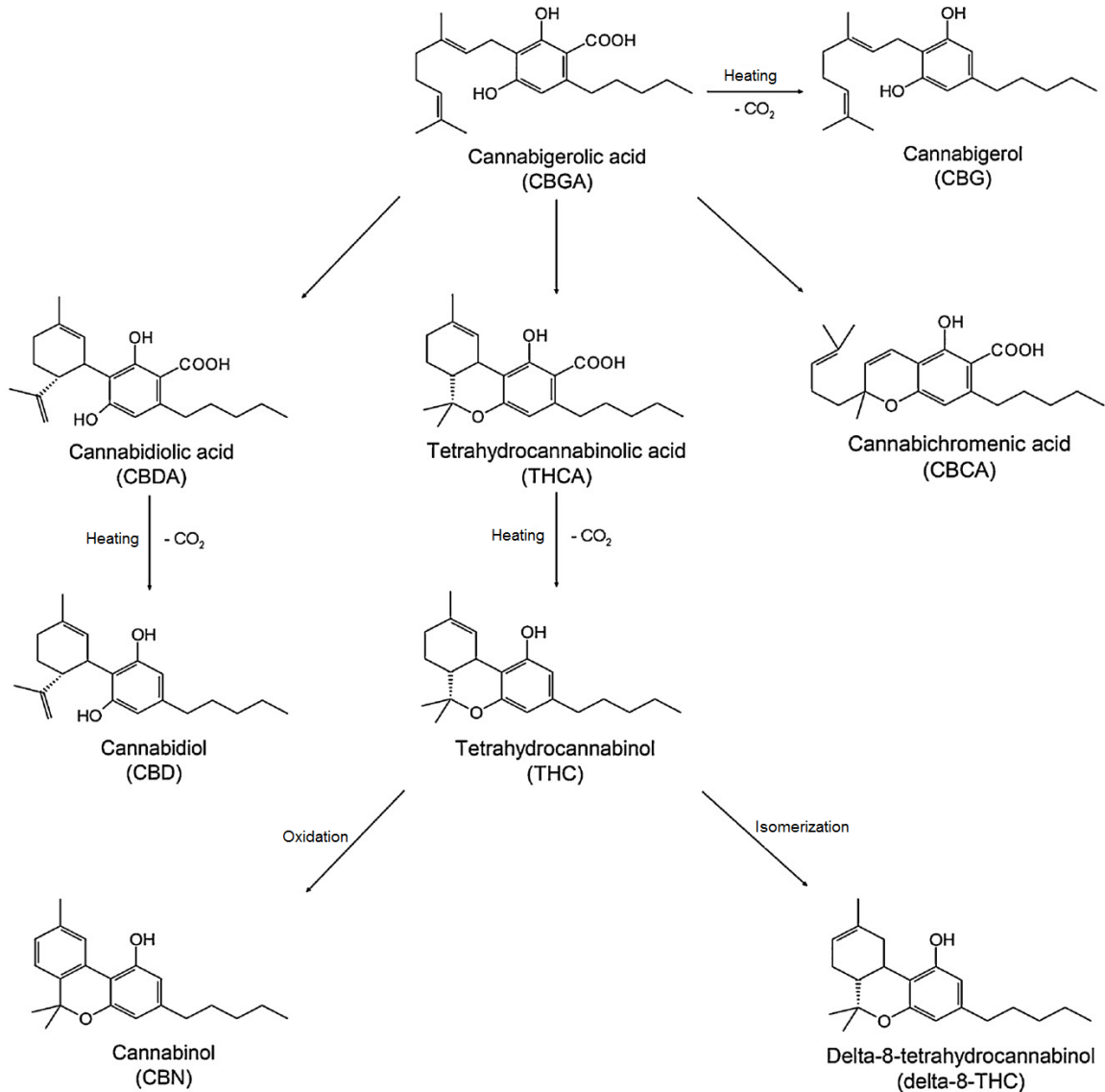


Figure 1.6 Schematic representation showing THC biosynthesis and by-products. Taken from De Backer (2009).

Cannabigerolic acid is oxidatively cyclized by Δ^1 -tetrahydrocannabinolic acid synthase to form Δ^1 -tetrahydrocannabinolic acid (THCA). THC is a derivative of THCA via non-enzymatic decarboxylation. Cannabinoids are highly lipophilic and some have been shown to exert their effect in humans via the endogenous cannabinoid receptors, CB1

and/or CB2, identified in 1988 and 1992, respectively (Sirikantaramas et al., 2004). CBN is the oxidation by-product of THC, with the ratio of CBN:THC increasing the longer the plant material is stored (Ross and Elsohly, 1997).

1.6.1.2 Therapeutic properties of *C. sativa* and cannabinoids

Despite the psychotropic properties associated with *C. sativa* due to the THC component, the therapeutic capacity of cannabinoids is immense. Extensive research is ongoing for the use of cannabinoids in the treatment of various neurological disorders, including multiple sclerosis, Parkinson's disease, and Huntington's disease. MS is characterized by increased spasticity, pain, tremor, ataxia (irregularity of movement), and incontinence. In a study conducted using 7.5 mg THC, patients reported an improvement in tremor and ataxia, and reduced spasticity, as well as relief in chronic pain (Baker et al., 2003). Nabilone, a synthetic THC analogue, was found to improve muscle spasm at a dosage of 1 mg every alternate day. Cannabinoids have both anticonvulsant and proconvulsant effects. CBD was found to be a potent anticonvulsant in a study conducted on fifteen poorly controlled epileptic patients over a period of 4.5 months (Robson, 2001).

Both natural and synthetic cannabinoids, such as nabilone (4-8 mg per day) and dronabinol are commonly prescribed to cancer patients, who prove resistant to the standard anti-emetic drugs. These prescriptions are restricted to the treatment of nausea and vomiting, which is usually a side-effect associated with the cytotoxic drugs used in chemotherapy. Despite producing unwanted side-effects, e.g. drowsiness, dizziness and lethargy, patients prefer to use them. In addition, cancer patients are prone to depression, and studies have shown that low doses of THC, 0.1 mg/kg, have anti-depressant properties (De Petrocellis et al., 2004). Studies have shown that *C. sativa* and cannabinoids improved appetite and slowed down weight loss in AIDS-related illnesses and cancer patients (Baker et al., 2003).

Cannabinoids have the ability to control cell survival or cell death by inducing proliferation, growth arrest or apoptosis, depending on drug concentration, timing of drug

delivery, and the type of cell used in the experimentation (Guzmán et al., 2002; Guzmán et al., 2001). Neuroprotection is provided in the central nervous system (CNS) against the toxic effects of factors such as ischemia, hypoglycemia and oxidative damage (Liu et al., 2006; Heck et al., 1999). However, cannabinoids induce apoptosis of glioma cells (*in vitro*) and regression of malignant gliomas (*in vivo*) (Carracedo et al., 2006; Gómez del Pulgar et al., 2002; Sánchez et al., 2001), as well as breast and prostate cancer cells (Sarfaraz et al., 2008; Sarfaraz et al., 2006; Ligresti et al., 2006). In the immune system, cannabinoids enhance cell proliferation at low doses, while growth arrest or apoptosis is induced at high doses (Guzmán et al., 2001). Sarafian et al. (2001) found that THC inhibited Fas-induced caspase-3 activation, but did not prevent necrotic cell death.

Natural and synthetic cannabinoids have been found to affect hormonal pituitary secretions in rodent models. THC administration to rats resulted in adrenal depletion of cholesterol ester and ascorbic acid, with concomitant increases in corticosterone and unesterified fatty acid levels in the plasma. This was due to the stimulation of the release of adrenocorticotrophic hormone (ACTH) (Pagotto et al., 2001). It is known that corticosterone stimulates lipolysis, and this implies that cannabinoids induce lipolysis via ACTH secretion. Gunasekaran et al. (2009) found that ACTH was a very potent lipolytic agent since there was a significant positive correlation between the level of THC and glycerol released from THC pre-treated adipocytes into the cell media upon exposure to ACTH. These findings were supported in the *in vivo* rat model (treated for 10 consecutive days with 10 mg/kg body weight) which also showed increased plasma levels of THC and its metabolite, THC-COOH, upon administration of ACTH. Contrary to these findings, *In vitro* studies conducted by Cota et al. (2003b) showed that cannabinoids exerted little or no lipolytic activity and rather promoted lipid storage in adipocytes. Interestingly, AEA, an endogenous cannabinoid, was found to inhibit lipogenic pathways associated with rat hepatocytes, by inhibiting ACC activity, resulting in suppression of fatty acid, cholesterol and glycerolipid synthesis (Guzmán and Sánchez, 1999). However, this effect on rat hepatocytes does not occur via the cannabinoid receptors as THC treatment did not bring about similar results as those observed with AEA (Guzmán and Sánchez, 1999).

Cannabinoid treatment appears to be beneficial in metabolic disorders such as insulin resistance and diabetes. Insulin resistant 3T3-L1 cells exposed to an organic *C. sativa* extract enhanced IRS-1, IRS-2 and GLUT4 gene expression levels and reduced fat content (Gallant et al., 2009). In a streptozotocin (STZ)-induced diabetes rat model, *C. sativa* exposure was found to increase energy utilization by reducing hepatic and skeletal muscle glycogen stores, while increasing the plasma glucose, total cholesterol, HDL, and triglyceride levels (Levendal and Frost, 2006). CBD treatment inhibited islet cell destruction and pancreatic tissue infiltration by mononuclear cells in non-obese T1D mice (Izzo et al., 2009; Weiss et al., 2008). In a study conducted on isolated rat pancreatic islets, it was found that THC stimulated basal release of insulin and also potentiated the secretory response to glucose (Laychock et al., 1986). In addition, THC also increased arachidonic acid hydrolysis from phospholipids, a process which was inhibited by a cyclooxygenase inhibitor, resulting in increased insulin release. The metabolism of arachidonic acid in pancreatic cell membranes has been linked to insulin secretion (Laychock et al., 1986).

Cannabinoid effects on energy consumption and metabolism in animal models, with results being inconsistent due to the varying responses associated with the animal species and experimental protocol used. Abel (1975) reported that only three out of twenty five studies showed an increase in food intake – the majority showed increased intake at low cannabinoid doses, while high doses decreased food intake. Furthermore, in studies conducted on various species exposed to acute or chronic cannabinoid administration, significant reductions in body and liver weight, hepatic glycogen content and reduced plasma glucose, were observed (Sprague et al., 1973; Sanz et al., 1985; Thompson et al., 1975). The cannabinoids affect energy metabolism in a dose dependent manner by stimulating glucose utilization in tissues that express the cannabinoid receptors, including hepatocytes, C6 glioma cells, primary astrocytes and lymphocytes (Guzmán and Sánchez, 1999).

Cannabinoids are involved in immune modulation by decreasing T- and B-cell proliferation, and suppressing cytotoxic T lymphocyte activity. Proinflammatory and anti-inflammatory effects are exerted by THC. THC suppresses production and action of interferon γ (IFN γ), IL-12, IL-6, TNF- α , and Granulocyte/Macrophage Colony Stimulating Factors (Klein et al., 2000a). Other ILs such as IL-1, IL-4, IL-10 and IL-6 were found to increase with THC exposure, as well as chemokines such as IL-8, Macrophage Inflammatory Protein (MIP)-1 α and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (Klein et al., 2000b). Srivastava et al., (1998) investigated the *in vitro* effect of THC and CBD on cytokine production by human leukemic T, B, eosinophilic and CD8q natural killer (NK) cell lines. THC reduced the normal production of IL-8, MIP-1 α , MIP-1 β , and RANTES as well as the phorbol ester-stimulated production of TNF- α , Granulocyte/Macrophage Colony Stimulating Factors and Interferon- γ (IFN- γ) by NK cells. It inhibited MIP-1 β in human T-cell lymphotropic virus (HTLV-1)-positive B-cells, but tripled IL-8, MIP-1 α and MIP-1 β in B-cells and MIP-1 β in eosinophil cells, while doubling IL-8. Both THC and CBD inhibited IL-10 production by human T-cell lymphoma T-cells.

Other therapeutic applications associated with THC and/or its analogues include ocular and systemic hypotensive agents in patients with glaucoma and hypertension, respectively, bronchodilator in asthmatic patients, and antimicrobial and antineoplastic activity (van Wyk and Gericke, 2000). Coetzee et al. (2007) found that THC and an organic *C. sativa* extract exhibited anticoagulant activity by inhibiting thrombin-induced coagulation. Several studies have shown that cannabinoids exert antinociceptive effects, comparable to codeine, with its effect peaking about five hours after administration (De Petrocellis et al., 2004).

The discovery of an endogenous endocannabinoid system has resulted in extensive research being conducted into its role in relation to maintaining health and contributing to disease states. From the section discussing the effects of THC within the body, it is apparent that the endogenous cannabinoid system affects various organ systems. The role

of endogenous cannabinoid receptors and their ligands will now be explored in relation to their distribution, mechanism of action and effect on energy metabolism.

1.7 ENDOCANNABINOID SYSTEM

The discovery of the cannabinoid receptors paved the way to the discovery of an entire endogenous signaling system associated with these receptors and their ligands. The endocannabinoid system is comprised of the cannabinoid receptors, their endogenous ligands (the endocannabinoids), the proteins involved with their synthesis and degradation, and all the other molecular targets affected by the endocannabinoids (De Petrocellis et al., 2004). The wide distribution of the cannabinoid receptors in various mammalian organs and tissues is indicative of the numerous physiological roles associated with the endocannabinoid system.

In 1992 the first endocannabinoid, AEA was isolated from porcine brain (Figure 1.7).

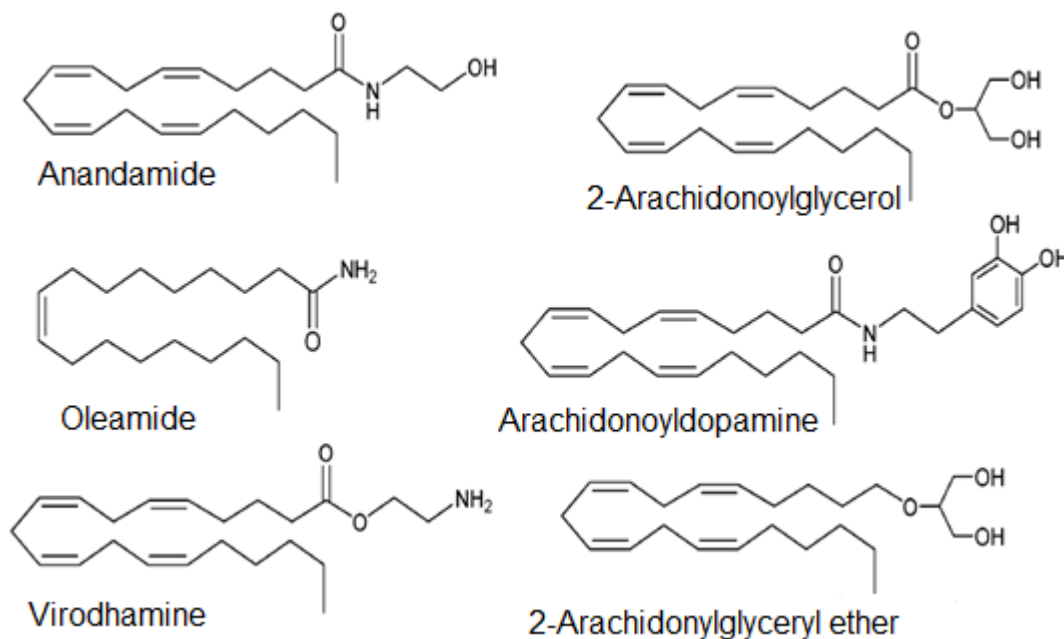


Figure 1.7 Structures of six endocannabinoids. Taken from Lambert and Fowler (2005).

AEA is based on the Sanscrit word “ananda” meaning “bliss”. It is an amide of arachidonic acid, also referred to as arachidonoyl ethanolamide and belongs to the class

of lipids referred to as N-acylethanolamines (Martin et al., 1999). Figure 1.7 shows the other endocannabinoids including 2-arachidonoyl-glycerol (2-AG), the ester of arachidonic acid and ethanolamine, *O*-arachidonoyl-ethanolamine (virodhamine, where the Sanskrit “virodha” meaning opposed), an analogue of AEA, 2-arachidonoylglyceryl ether (noladin ether, 2-AGE), an analogue of 2-AG, *N*-arachidonoyldopamine (NADA) and oleamide (Lambert and Fowler, 2005).

1.7.1 Endocannabinoid Biosynthesis and Receptor Binding

The metabolic pathways involved in the biosynthesis of these compounds occur via a phospholipid-dependent pathway in the various tissues where the cannabinoid receptors are located. Two biosynthetic pathways have been suggested for the synthesis of AEA and 2-AG. Biosynthesis of N-acylethanolamines, e.g. AEA, occurs via a phospholipid-dependent pathway. *N*-arachidonoyl-phosphatidylethanolamine (NAPE), the precursor of AEA, is formed upon the transfer of an acyl group from a phosphoglyceride to a phosphatidylethanolamine. This is catalyzed by a Ca^{2+} -dependent *trans*-acylase. Enzymatic hydrolysis of the NAPE by a Ca^{2+} -dependent phospholipase D that is specific for NAPEs, results in the formation of AEA (figure 1.8) (De Petrocellis et al., 2004; Pertwee and Ross, 2002).

The biosynthesis of 2-AG occurs via a different route, involving the hydrolysis of 2-arachidonate-containing DAGs, catalyzed by a DAG lipase. DAGs are the precursors of 2-AG and can be produced by two different mechanisms: the hydrolysis of 2-arachidonate-containing phosphatidic acid by phosphohydrolase or hydrolysis of phosphoinositides by a phosphoinositide-selective phospholipase (De Petrocellis et al., 2004).

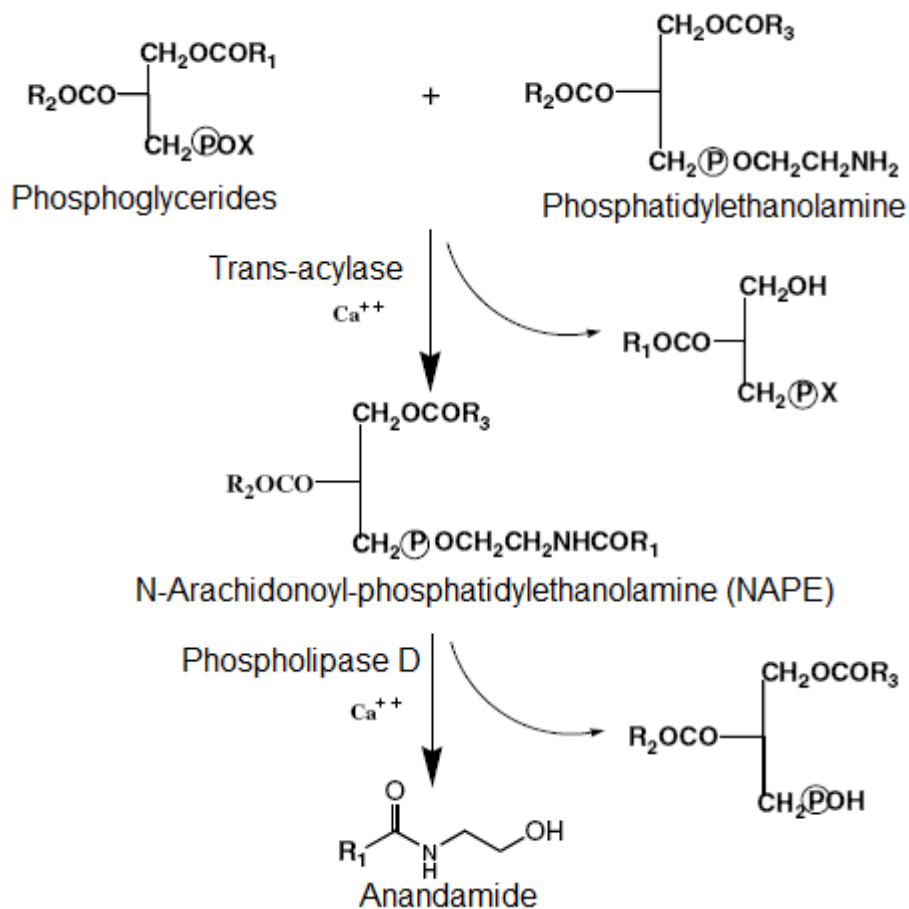


Figure 1.8 Proposed biosynthesis pathway for AEA. Taken from De Petrocellis et al. (2004).

The endocannabinoids have a half-life of less than five minutes. Endocannabinoid signaling is terminated in a two-step process, involving the uptake into cells via selective, saturable, temperature-dependent and Na⁺-independent membrane transporters i.e. the AEA membrane transporters, followed by hydrolysis by two specific enzymatic systems, fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL) to form arachidonic acid and ethanolamine or glycerol, for AEA and 2-AG, respectively (figure 1.9) (Di Marzo et al., 2009a; Di Marzo et al., 1994).

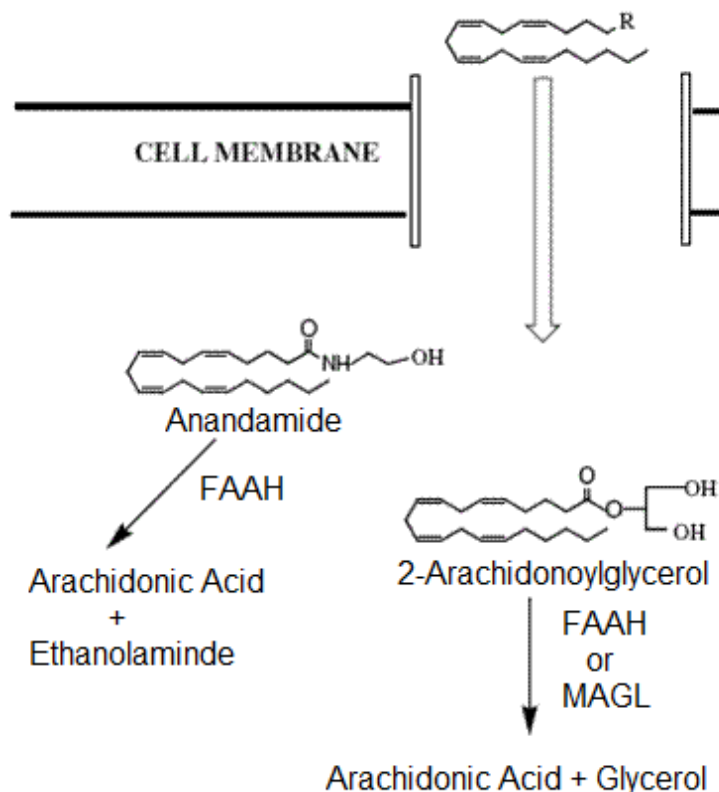


Figure 1.9 Endocannabinoid inactivation. Modified from illustration of De Petrocellis et al. (2004).

1.7.2 Cannabinoid Receptors

CB1 and CB2 consist of 361 and 360 amino acid residues, respectively. The basis of distinction between CB1 and CB2 includes the difference in their amino acid sequence (CB1 and CB2 receptors display 44% similarity in amino acid sequence), their signal transduction mechanisms, localization and their highly selective affinity for certain agonists and antagonists (Howlett et al., 2004). The CB1 receptor displays a 97% to 99% amino acid similarity across species, with the rat CB1 receptor showing a 97.3% similarity to the human CB1 receptor (Howlett et al., 2002). Two isoforms of the CB1 receptor have been identified, CB1 and CB1A (CB1, isoform A) (figure 1.10). Alternative splicing in the regions flanking the CB1 coding region results in the formation of two fragments – one containing the entire CB1 coding region and the other lacking a 167-base pair intron within the sequence encoding the amino-terminal tail of the receptor. The CB1A isoform is shorter than CB1 by sixty one amino acids and also differs from the

CB1 in the first twenty eight amino acids associated with the truncated receptor sequence (Shire et al., 1995).

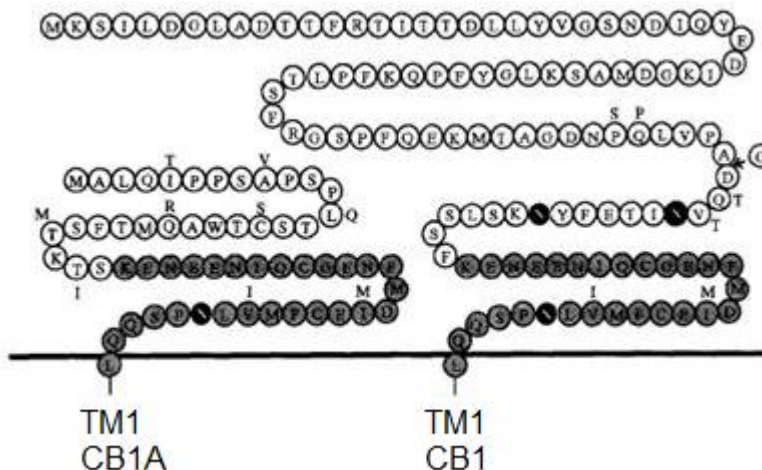


Figure 1.10 Illustration of the NH₂-terminal extremities of CB1 and CB1A of the human and rat cannabinoids receptors. The human sequences are inside the circles, while differences found in the rat receptor are shown at the relevant positions outside the circles. The first transmembrane (TM1) region is below the membrane surface. Taken from Shire et al. (1995).

Cannabinoid receptors are members of the rhodopsin-like G-protein-coupled receptors (GPCRs), consisting of seven transmembrane (TM) helices (characteristic of this superfamily of G_{i/o} proteins), connected by three intracellular loops and three extracellular loops which inhibit AC activity via G_{i/o} (Howlett et al., 2002; Pertwee and Ross, 2002; Spiegel et al., 1992; Matsuda et al., 1990). However, some cannabinoid receptor ligands stimulate AC activity, depending on their interaction with various AC isozymes and the cellular environment within which the receptors occur (Rhee et al., 1998; Bonhaus et al., 1998; Spiegel et al., 1992).

Each GPCR is a heterotrimer consisting of α , β and γ subunits. Cannabinoid agonists activate multiple G_{i/o} transmembrane region 1 (TM1) subunits, leading to the activation of G α subunit due to guanosine triphosphate (GTP) binding. Receptor-mediated activation of the GPCR result in the dissociation of the GTP-coupled α subunit from the G $\beta\gamma$ -dimer complex. Both the G $\beta\gamma$ -dimer complex and the G α subunit have been shown to interact with and either stimulate or inhibit various AC isozymes, thus affecting

specific physiological events in the target cells (Rhee et al., 1998; Spiegel et al., 1992). Due to the intrinsic GTPase activity of the $G\alpha$ subunit, GTP is hydrolysed to guanosine diphosphate. As a result of the hydrolysis of GTP, the affinity between the $G\alpha$ subunit and $G\beta\gamma$ -dimer complex increase, and the binding of these results in the re-assembly of the receptor (Rhee et al., 1998; Shire et al., 1995; Spiegel et al., 1992).

Toxins covalently modify the $G\alpha$ -subunit thereby resulting in altered signal transduction. Cholera toxin activates the α -subunit resulting in cAMP formation independent of ligand binding (Spiegel et al., 1992). Figure 1.11 shows the primary signaling pathways of CB1 and CB2 receptors activated by ligands such as THC or AEA. Pertussis toxin blocks signal transduction by uncoupling the G-proteins from their receptors. Mutations associated with the α -subunit block signal transduction by uncoupling it from the receptors, or preventing GTP activation (Spiegel et al., 1992). Amplification associated with receptor activation is low, with each cannabinoid receptor (specifically CB1) only activating three G proteins, compared to at least 20 in opioid receptors. This low amplification could be due to the high number of cannabinoid receptors present in the brain, thus reducing the necessity for a high level of amplification (Pertwee, 2008; Pertwee, 1997). However, findings have shown that psychoactive cannabinoids (THC, and to a lesser extent CBN) are able to produce changes in the physical properties of artificial membranes containing only cholesterol and phospholipids. It has therefore been suggested that the psychoactive cannabinoid effects result from their structure-dependent ability to disorder membrane lipids, which relies on their 'awkwardness of fit' into asymmetric components of the membrane matrix, rather than on their 'goodness of fit' into specific receptors (Pertwee, 1988).

CB1 receptor activation (by a CB1 receptor agonist/ligand/THC/AEA) results in a reduction in the excitability of the membrane potential due to the opening of inwardly rectifying potassium channels (Kir), resulting in inhibition of VGCC and inhibition of Ca^{2+} influx (Howlett, 2005; Howlett et al., 2002; Mackie et al., 1995) (figure 1.11, A). CB1 and CB2 receptor-evoked $G_{i/o}$ activation inhibits AC and subsequent reductions in cAMP (figure 1.11, B).

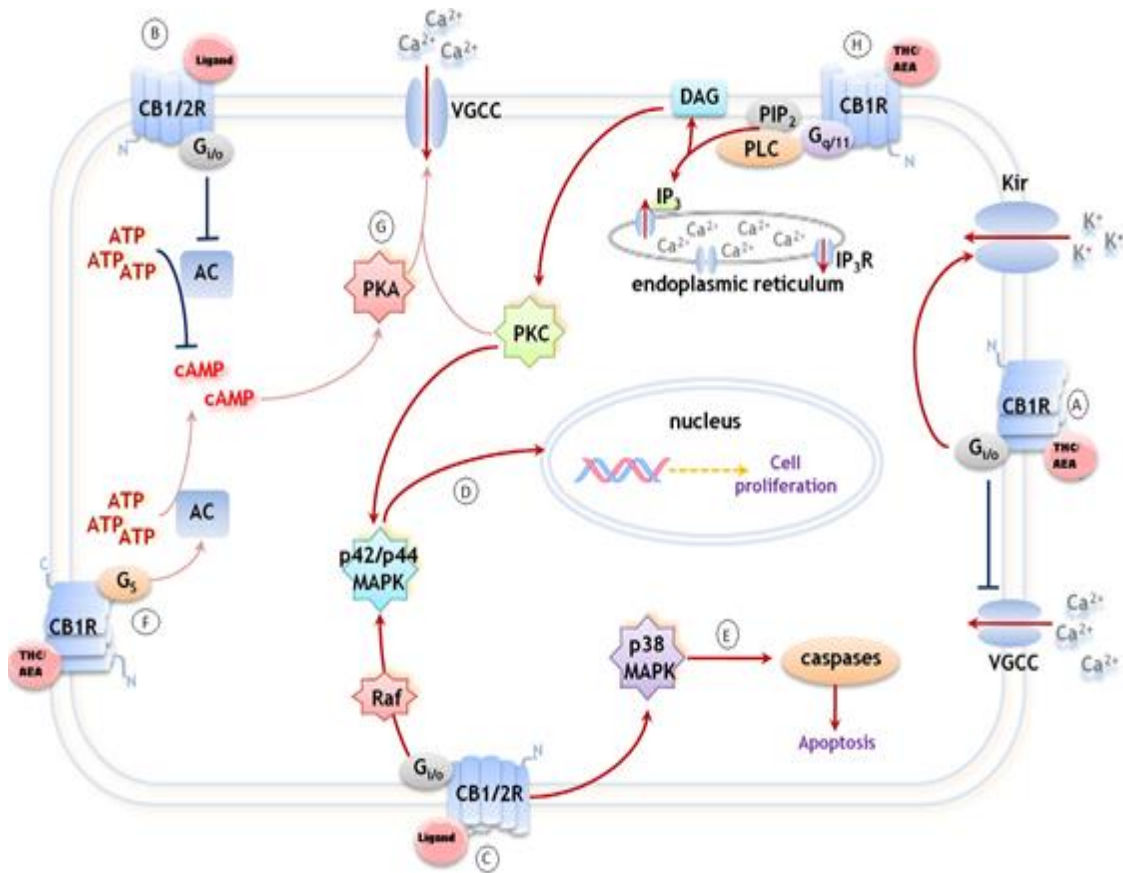


Figure 1.11 Schematic diagram showing the signaling pathways downstream of cannabinoid receptor activation. AC, adenylate cyclase; cAMP, cyclic AMP; CB1R, CB1 receptor; CB1/2R, CB1 or CB2 receptor; DAG, diacylglycerol; Kir, inwardly rectifying potassium channel; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; MAPKs, p38 and p42/p44 mitogen activated protein kinases; PIP₂, phosphatidyl bisphosphate; PLC, phospholipase C; PKC, protein kinase C; VGCC, voltage-gated calcium channels. Adapted from Li et al. (2011).

Rhee et al. (1998) found that expression of CB1 or CB2 cannabinoid receptors in host cells together with AC isoforms 1, 3, 5, 6, or 8 resulted in inhibition of cAMP accumulation, while co-expression with AC isoforms 2, 4, or 7 resulted in stimulation of cAMP accumulation. CB1 and CB2 receptor activation also induce activation of MAPKs (Melck et al., 1999) (figure 1.11, C). Once activated, MAPKs translocate to the nucleus (figure 1.11, D), phosphorylate and regulate nuclear proteins such as c-MYC which ultimately regulate the expression of genes essential for proliferation, such as FBJ murine osteosarcoma viral oncogene homolog (c-fos), early growth response 1 (Krox-24) and c-Jun. MAPK isoforms p42/p44 stimulates cellular proliferation, while p38 MAPK

activates caspases (figure 1.11, E) to induce apoptosis (Pearson et al., 2001; Rueda et al., 2000; Bouaboula et al., 1997). Research has shown that under certain conditions CB1 receptors may stimulate AC via G_s , resulting in increased cAMP, which then activates PKA (figure 1.11, F). This in turn may phosphorylate VGCCs which result in Ca^{2+} influx (figure 1.11, G). CB1 receptor activation of PLC leads to the formation of inositol 1,4,5-trisphosphate (IP_3) and DAG through phosphatidyl bisphosphate (PIP_2) hydrolysis (figure 1.11, H). In rat insulinoma RIN m5F cells CB1 receptor activation was found to be coupled to phosphoinositol phospholipase C (PI-PLC)-mediated mobilization of $[Ca^{2+}]_i$ (De Petrocellis et al., 2007). IP_3 in turn mobilizes Ca^{2+} from the ER following binding to IP_3 receptors (IP_3R) and DAG activates PKC, which phosphorylate VGCCs thus allowing the influx of Ca^{2+} . The activation of PKC results in the activation of p42/44MAPKs which induces cell proliferation (Li et al., 2011b).

Most of the endocannabinoids which have been identified serve as agonists and bind to CB1 and CB2 receptors with varying efficiency, with AEA, NADA and 2-AGE being functionally more selective for CB1, while 2-AG shows equal selectivity for both receptor types. THC has a high affinity for the CB1 receptor with a dissociation constant (K_i) of 41.0 ± 2.0 nM, while K_i for CB2 is 36.0 ± 10.0 nM (Huffman et al., 1996). Virodhamine however has been shown to behave as a CB1 receptor antagonist/inverse agonist (Pertwee, 2005a). Endocannabinoids act via the G_i/G_o proteins which then give rise to several intracellular signaling events. These include inhibition of AC (with subsequent reduction in cAMP/protein kinase A-mediated effects), stimulation of MAPK signaling, for both receptor types, while activation of CB1 results in inhibition of voltage-gated P, Q and N-type Ca^{2+} channels, stimulation of inwardly rectifying G-protein-coupled K^+ channels, stimulation of PI3K, and intracellular mobilization of Ca^{2+} via activation of phospholipase C- γ (PLC- γ) (Guzmán et al., 2002; Pertwee, 2006; Pertwee, 1997). However, several cannabinoid agonists have been found to reduce intracellular Ca^{2+} in cells expressing CB1 receptors by inhibiting voltage-activated inward Ca^{2+} currents in a dose-dependent manner (Vilches-Flores et al., 2013; Li et al., 2010a&b; Bermúdez-Silva et al., 2008). This inhibitory effect on Ca^{2+} conductance was found to occur via N-type and P/Q type Ca^{2+} channels (Howlett et al., 2004; Twitchell et al., 1997). In pancreatic

β -cells, the inhibition of Ca^{2+} together with the reduction in cAMP resulted in a reduction in insulin exocytosis (Li et al., 2011b; Juan-Picó et al., 2006; Howlett et al., 2002; Prentki and Matschinsky, 1987).

1.7.2.1 CB1 Receptor Distribution

The distribution of CB1 receptors is similar across species, though subtle differences do occur. CB1 receptors are associated with the CNS, being most densely distributed in the areas associated with cognition and short-term memory (cerebral cortex and hippocampus), and with areas involved in motor function and movement (basal ganglia, and cerebellum) (Pertwee, 2006). Low levels of expression are found in the hypothalamus, spinal cord and respiratory centres of the brainstem. In these areas, CB1 receptors are found either on the pre- or post-synaptic neural terminals where they mediate inhibition of neurotransmitter release (Howlett et al., 2004). Lower levels of CB1 messenger ribonucleic acid (mRNA) has been detected in several peripheral tissues including the rat spleen, testes, vas deferens, urinary bladder, human prostate, ovary, uterus, bone marrow, thymus, tonsils, pituitary gland, adrenal glands, heart, lungs, stomach, colon, bile duct, leukocytes, skeletal muscle, liver, kidney, pancreas and placenta (Li et al., 2011b; Rueda et al., 2000).

1.7.2.2 CB2 Receptor Distribution

CB2 receptors are associated with tissues involved in immune cell production and regulation, including the spleen, thymus and tonsils (having the highest concentration of CB2 mRNA). CB2 mRNA has been isolated from primary and cultured immune cells including B and T lymphocytes, natural killer cells, monocytes, macrophages, microglial and mast cells (Li et al., 2011b; Pertwee, 2010; Pertwee, 2006; Howlett et al., 2002). It has also been associated with Peyer's patches (i.e. aggregated lymphoid nodules) in the rat (Howlett et al., 2002). In human tissues, CB2 mRNA has been detected in the thymus gland, bone marrow, adrenal gland, heart, lung, prostate gland, uterus, pancreas, ovary and testes (Pertwee, 2006; Lambert and Fowler, 2005; Howlett et al., 2002). Interestingly, CB2 mRNA greatly exceeded CB1 mRNA levels in human tonsils, spleen, thymus, pancreas and leukocytes (Guzmán et al., 2001).

1.7.3 Non-Cannabinoid Receptors

Receptors other than CB1 and CB2 have been associated with cannabinoid ligands, both exogenous and endogenous. Di Marzo et al. (2000) found that THC (10 mg/kg) reduced spontaneous activity in CB knockout (CB^{-/-}) mice, a typical CB1 receptor mediated response, despite the absence of CB1 receptors, while AEA failed to evoke spontaneous activity, increased immobility *in vivo*, and increased *in vitro* regulation of G-proteins. The CB1 antagonist, 5-(4-Chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide hydrochloride (also referred to as Rimonabant or SR141716), did not reverse these effects (Di Marzo et al., 2000; Breivogel et al., 2001). These data raised the possibility that cannabinoids were able to interact with targets other than cannabinoid receptors. Ryberg et al. (2007) found that the human orphan G-protein coupled receptor 55, GPR55, transfected into human embryonic kidney (HEK293) cells, was specifically bound and activated by THC, AEA, palmitoyl-ethanolamide and (6aR,10aR)-9-(Hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo[c]chromen-1-ol and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-morpholinyl-1H-pyrazole-3-carboxamide (AM251), with activation of downstream signaling pathways. Oka et al. (2007) found that GPR55 was able to bind cannabinoids, but categorized it as an ‘atypical’ cannabinoid receptor, due to its ability to bind both agonists and antagonists of CB1 and CB2 receptors. Research findings to date has clearly established lysophosphatidylinositol is an agonist to GPR55, while WIN55212-2 is unable to activate GPR55 (Sharir and Abood, 2010).

In addition, evidence suggests that the transient receptor potential vanilloid type 1 (TRPV1) receptor can bind cannabinoids (Li et al., 2011b). Interestingly, CB1 and CB2 receptors are often co-expressed with TRPV1 receptors, either on the same or neighbouring cells (De Petrocellis and Di Marzo, 2009). Evidence has emerged that TRPV1 is expressed on brain neurons (Ross, 2003), and non-neural cells (epithelial, endothelial, glial, smooth muscle, mast and dendritic cells, lymphocytes, keratinocytes, osteoblasts, hepatocytes, myotubes, fibroblasts and pancreatic β -cells) (Idris et al., 2010; De Petrocellis and Di Marzo, 2009; Szallasi and Gunthorpe, 2008; Cavuoto et al., 2007b;

Akiba et al., 2004) . Human and rodent skeletal muscle express CB1, CB2, and TRPV1 receptors as well as FAAH (Cavuoto et al., 2007b). TRPV1 receptors have been expressed in Sprague-Dawley rat islet β -cells, radiation-induced rat insulinoma β -cell lines (RIN and INS-1), as well as in the neurons in rat pancreata (Akiba et al., 2004). TRPV1 acts as a non-selective cation (especially Ca^{2+}) channel, and activation of TRPV1 receptors stimulates Ca^{2+} signaling which leads to increased insulin secretion (Akiba et al., 2004). In rat insulinoma RIN m5F cells, CB1, CB2 and TRPV1 receptors are co-expressed and there is evidence of CB1-TRPV1 cross-talk (De Petrocellis and Di Marzo, 2009). Stimulation of CB1 and TRPV1 receptors increase $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} (De Petrocellis et al., 2007).

1.7.4 Signal transduction mechanisms

Cannabinoid activation of G-proteins influences multiple effector systems. Activation of both CB1 and CB2 receptors inhibit AC via pertussis toxin-sensitive G_i proteins, resulting in a reduction in cAMP production. Pertussis toxin induces ADP-ribosylation of G-proteins, thereby preventing the dissociation of the α and $\beta\gamma$ -dimer subunits, thus blocking G-protein-mediated inhibition of AC (Rueda et al., 2000). However, regional differences in the brain do exist, as there is evidence that cannabinoids stimulate cAMP accumulation in rat globus pallidus slices and striatal neurons, possibly via G_o protein activation or alternative mechanisms associated with the regulation of this transduction process (Sánchez et al., 1998).

Conductance of K^+ via inwardly rectifying currents is enhanced by cannabinoid agonists. The effect on calcium channels and G-protein-coupled inwardly rectifying K^+ channels are due to direct coupling to G-proteins and independent of cAMP (Sánchez et al., 1998). Activation of pre-synaptic CB1 receptors can mediate the suppression of release of excitatory or inhibitory neurotransmitters in the brain and peripheral nervous system through changes in conductance of Ca^{2+} and K^+ currents (Sánchez et al., 1998). Cannabinoid-induced neuroprotection from glutamate excitotoxicity is believed to be based on the modulation of Ca^{2+} fluxes that prevents excessive glutamate release (Robson, 2001). Cannabinoid agonists have been found to induce effects independent of

cannabinoid receptors, such as stimulating the release of arachidonic acid and inhibiting the re-acylation of arachidonic acid (Felder and Glass, 1998).

Studies have shown that cannabinoid receptors regulate different members of the MAPK family, such as ERK, (Howlett et al., 2002), JNK and p38 (Baker et al., 2003). MAPK pathways are involved in cellular regulation of proliferation and differentiation. The relationship between the activation of these pathways and cell fate depends on the duration of stimulation. In neural cells, long-term exposure to THC induces peak ERK activation. THC exposure to glial cells resulted in ceramide production via CB1 receptor activation. Increased ceramide levels lead to Raf-1 mediated sustained activation of the ERK cascade (Robson, 2001). The CB1 receptor is coupled to the ERK and JNK cascades via a common upstream mechanism involving the G $\beta\gamma$ -dimer complex, class I_B PI3K and Rat Sarcoma (Ras) (a small G protein lying upstream of JNK) (Baker et al., 2003).

1.7.5 Physiological Effects of Endocannabinoids

Endocannabinoids are released from the postsynaptic neuron upon stimulation, and diffuse back to the presynaptic neurons where they act on CB1 receptors, resulting in reduced neurotransmitter release (Pertwee, 2006). In a CB1 knockout mouse model, AEA has been shown to elicit antinociceptive effects and a decrease in locomotor activity (Howlett et al., 2002). One of the receptors to which AEA binds is vanilloid type 1 receptors (VR1). It has been found that blockage of CB1 receptors unmasks the VR1-mediated effects of AEA. CB1 and VR1 may therefore be considered ionotropic and metabotropic receptors for the same class of endogenous fatty acid amides, including AEA and NADA (Di Marzo et al., 2002). Some of the physiological effects resulting from the neuromodulatory functions associated with endocannabinoids include inhibition of central pain perception (Hanuš et al., 2001), stimulating dopamine release thereby affecting motor behaviour (Chen et al., 1990), impairing learning and memory consolidation, and playing a role in sleep regulation by increasing slow-wave and rapid eye movement sleep in rats at the expense of wakefulness (Murillo-Rodriguez et al., 1998), playing a regulatory role in the prefrontal cortex (which houses integrative cognitive and emotional functions) in stress and cognition (Fried et al., 1998) and immunomodulation via effects on cytokine production (low AEA

levels decrease IL-6 and IL-8 release whilst high AEA levels increase TNF- α , IFN γ and IL-4 release) (Klein et al., 2000a). Two critical reproduction-related functions are associated with endocannabinoids: embryonal implantation and newborn milk intake. Deficiency of endocannabinoids can be fatal due to failure of these reproductive processes (Fride, 2002).

Extensive research has been conducted on the role of endocannabinoids in energy metabolism and it has become apparent that the endocannabinoid system plays a pivotal role in regulating energy metabolism at central and peripheral levels (Quarta et al., 2010; Kunos et al., 2009; Pagotto et al., 2006). Stimulation by endocannabinoids of CB1 receptors, situated outside of the hypothalamus, increase appetite for palatable food leading to dietary-induced obesity in rats (Di Marzo et al., 2001). In addition to having central mechanisms for regulating food intake (Gómez et al., 2002), CB1 receptors are present on the nerve terminals innervating the digestive tract, and mediate gut-derived satiety signals. Activation of CB1 receptors results in reduced ACh release from the enteric nerves, which induce inhibition of intestinal motility and secretion, inhibition of gastric motility and acid secretion as well as relaxation of the lower esophageal sphincter, while endocannabinoids stimulate intestinal motility via VR1 located on the primary sensory neurons (Izzo and Croutts, 2005).

Cannabinoids strongly stimulate feeding via the CB1 receptors (Di Marzo et al., 2001). It has been proposed that when AEA levels in the gut are high, following food deprivation, this serve as a hunger signal to promote feeding, and inhibit gastric emptying and intestinal peristalsis. It is therefore proposed that the endogenous cannabinoid system modulates energy balance via the central pathways governing appetite, due to the co-expression of CB1 mRNA with neuropeptides such as corticotrophin-releasing hormone, cocaine-amphetamine-regulated transcript, and melanin-concentrating hormone (MCH) and prepro-orexin (Cota et al., 2003b).

Endocannabinoids have been implicated in the development of obesity (Nisoli, 2011). Epididymal fat and pancreatic tissue from DIO mice were found to contain higher levels

of endocannabinoid compared to lean mice (Matias et al., 2006). Similarly, patients with T2D caused by obesity or hyperglycemia also showed higher levels of endocannabinoids in their visceral fat or serum, relative to non-diabetic lean controls (Quarta et al., 2010; Matias et al., 2006). The tissue levels of endocannabinoids, cannabinoid density and/or binding efficacy is enhanced in obesity (see table 1.2) (Pertwee, 2005b).

Table 1.2 Changes in endocannabinoid levels in various *in vivo* rodent experimental models relating to obesity. Taken from Pertwee (2005b).

Experimental Model	Effect Observed in Experimental Model				
	Endocannabinoid Concentration		Protein Expression, mRNA or Binding site density		Activity or Protein Expression
	AEA	2-AG	CB ₁	CB ₂	FAAH
Obesity model (Zucker <i>fa/fa</i> rat hypothalamus)	0	+	ND	ND	ND
Obesity model (<i>db/db</i> mouse hypothalamus)	+	+	ND	ND	ND
Obesity model (<i>ob/ob</i> mouse hypothalamus)	0	+	ND	ND	ND
Obesity model (<i>ob/ob</i> mouse uterus)	+	+	0	-	-
Obesity model (Obese <i>fa/fa</i> rat adipose tissue and mouse cultured differentiated 3T3-F442A adipose cells)	ND	ND	+ (mRNA or expression)	ND	ND

ND = not determined; (0) = none found; (-) = decreased levels; (+) = increased levels

In obese individuals AdipoQ levels are low (Xydakis et al., 2004). Intravenous injection of AdipoQ increased non-esterified fatty acid oxidation and reduced body weight in obese mice (Fruebis et al., 2001). Administration of SR141716 increases AdipoQ mRNA expression in adipose tissue (Bensaid et al., 2003). The synthetic CB1 receptor antagonist, Rimonabant has been used as a weight-reducing agent for the treatment of obesity. In a study conducted on overweight patients with dyslipidemia, rimonabant produced a moderate weight loss and improvement in HDL and triglyceride levels, whilst increasing plasma AdipoQ levels (Després et al., 2005). In a different study rimonabant inhibited preadipocyte cell proliferation and induced differentiation without lipid droplet accumulation (Gary-Bobo et al., 2007 & 2006). The rimonabant phase III clinical trial was terminated due to the psychological side-effects observed in participants receiving the treatment. Evidence suggests that the cannabinoid system plays roles in both feeding and reward pathways. Endocannabinoids increase c-fos expression in the hypothalamus, a chemical that plays an important role in the regulation of energy balance. The precise

function of c-fos is not understood, however it is found in the nucleoprotein complex involved in the regulation of expression of the adipocyte gene 2 (Tratner et al., 1990). Leptin, an adipokine, is a key signal to the hypothalamus on the nutritional state of the body. Research has shown that endocannabinoid levels decrease in response to increased leptin levels in obese subjects, while endocannabinoid levels increase in animals with defective leptin signaling (Gary-Bobo et al., 2006). Abnormalities in endocannabinoid levels have also been found in pancreatic tissue of mice fed with a high-fat diet (Starowicz et al., 2008).

Insulin is the primary hormone that regulates glucose metabolism (Saltiel and Kahn, 2001). Results from various studies have been inconsistent with regard to the effect of endocannabinoids and cannabinoid agonists on insulin secretion in isolated islets (mice, rats, humans) and β -cell lines (RIN-m5F, MIN6), showing either inhibition or stimulation of insulin secretion (Vilches-Flores et al., 2013, 2010a&b; Anderson et al., 2013; Li et al., 2010a&b; Bermúdez-Silva et al., 2008; Nakata and Yada, 2008; Juan-Pico et al., 2006; Matias et al., 2006). CB1 ligands were also found to potentiate insulin secretion in rat, mouse and human β -cells (Vilches-Flores et al., 2013, 2010a&b; Li et al., 2010a; Bermúdez-Silva et al., 2008). In isolated islets from fasted rats, AEA increased basal insulin secretion and insulin content but not GSIS (Vilches-Flores et al., 2010a). Exposure to HU-210 [3-(1,1-dimethylheptyl)-(-)-11-hydroxy-delta8-tetrahydrocannabinol], a CB1 agonist, increased insulin secretion in RIN-m5F β -cells (Matias et al., 2006). In mouse and rat β -cells, CB1 agonists (AEA, arachidonylcyclopropylamide [ACPA]) inhibited insulin secretion (Anderson et al., 2013; Nakata and Yada, 2008; Juan-Pico et al., 2006). Differences in experimental methodology and changes in the signaling and/or metabolic pathways of endocannabinoids in islet/ β -cell have been advanced as possible factors contributing to these inconsistencies (Anderson et al., 2013).

Over the past few decades, subsequent to the discovery of the endocannabinoid system, scientific research has been geared toward a greater understanding of the physiological activity of cannabinoids and their derivatives in maintaining homeostasis as well as in the etiology of disease conditions, with the aim of developing cannabinoid-related

compounds that can be used as therapeutics in their treatment. A large number of South Africans incorporate the use of traditional medicine, including medicinal plants, in the treatment regime for diseases. With the increase in the incidence of obesity and diabetes, as specified in the introduction, the need to establish alternative and/or complementary therapeutic strategies has increased.

1.8 AIM OF THE STUDY

Obesity has been linked to insulin resistance and T2D. The aim of this project was therefore to determine how administration of an organic *C. sativa* extract would affect glucose regulation in an obese rat model, specifically focusing on β -cell function. Various metabolic parameters were also investigated, as outlined below.

The research objectives include the following:

- To obtain an organic extract of *C. sativa* from plant material, and quantify the THC content using reverse-phase high performance liquid chromatography (RP-HPLC).
- To determine how *C. sativa* treatment affects the following parameters in lean and obese rats:
 - Body weight, food intake, mass of epididymal fat, skeletal muscle mass (soleus and thigh), liver, pancreas, and adrenal glands.
 - Blood parameters: glucose, triglycerides, total cholesterol, HDL-cholesterol, low-density lipoprotein (LDL)-cholesterol, very low-density lipoprotein (VLDL)-cholesterol, atherogenic indices, insulin and leptin.
 - Quantitative polymerase chain reaction (qPCR) was used to determine how *C. sativa* treatment affected insulin and CB1 gene expression in the pancreatic tissue.
- To determine how *C. sativa* treatment affected the *in vitro* physiological functioning of isolated rat islets, the following aspects were investigated:
 - Insulin secretion in response to normoglycemic and hyperglycemic conditions, at various *C. sativa* concentrations (0; 2.5 and 5.0 ng/mL) over a 24-hour period.
 - Insulin secretion in response to normoglycemic and hyperglycemic conditions, at various *C. sativa* concentrations (2.5 ng/mL) over a 96-hour period.
 - Insulin secretion in response to normoglycemic and hyperglycemic conditions, in the presence and absence of CB1 (AM251) and CB2 (6-iodo-2-methyl-1-[2-(4-

morpholinyl)ethyl]-1*H*-indol-yl](4-methoxyphenyl) methanone) (AM630) receptor antagonists.

- Cell proliferation using immunohistochemical detection with Ki-67 as a specific indicator.
- Cellular apoptosis using immunohistochemical detection with terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP nick end labeling (TUNEL) technology.
- Determine effect of *C. sativa* on cytokine/chemokine secretion by isolated islets, after 96 hours exposure period, under normo- and hyperglycemic conditions.
- Determine effect of *C. sativa* on cytokine/chemokine secretion by isolated islets, in the presence and absence of CB1 and CB2 inverse agonists/antagonists, after 48 hours exposure period.
- Signal transduction via ERK1/2 and JNK1 pathways using western blotting to establish these pathways are activated in isolated islets.
- Detection of CB1 and CB2 receptors using western blotting to determine the presence of cannabinoid receptors in isolated islets;
- qPCR was used to determine how *C. sativa* treatment affected expression levels of genes linked to insulin secretion and cell differentiation (insulin, CB1, GLUT2, c-MYC, PKB, PDX-1, FLIP, and UCP2).

The experimental design and methodology associated with the abovementioned investigations will be discussed in subsequent chapters.

CHAPTER 2: *IN VIVO* STUDY ON THE EFFECTS OF AN ORGANIC *C. sativa* EXTRACT ON GLUCOSE METABOLISM IN LEAN AND DIET-INDUCED OBESE WISTAR RATS

2 INTRODUCTION

The prevalence of DIO continues to increase, resulting in associated life style diseases such as hypertension, cardiovascular disease and diabetes (WHO, 2008). The IDF estimated that 63 061 diabetes-related deaths occurred in South Africa in 2012 (IDF, 2012). According to the WHO, the global occurrence of obesity has nearly doubled since 1980 (WHO, 2015). This steep increase in the trajectory of obesity has brought into stronger focus the need to develop effective therapeutic interventions to reduce obesity, so as to reduce obesity-related disorders such as diabetes.

Most therapeutic agents are derived directly or indirectly from plant material. The medicinal use of *C. sativa* goes back thousands of years, with it being used for conditions including inflammation, bronchitis, pain and diabetes (van Wyk and Gericke 2000). Worldwide, *C. sativa* usage has increased for both medicinal and recreational purposes. Research into the functions of the phytocannabinoids increased when in the 1960s, THC (the best characterized phytocannabinoid), was isolated and characterized, followed by the cloning of two distinct cannabinoid receptor subtypes (viz. CB1 and CB2) in the early 1990s. Renewed interest in cannabinoid research was further prompted by the detection of a lipid-signaling system (i.e. the endocannabinoid system) that was found to modulate physiological activities including inflammation, tissue injury, energy metabolism, and oxidative stress (Horváth et al., 2012; Munro et al. 1993). Understanding of phytocannabinoid effects will therefore contribute to unravelling the role of the endogenous cannabionoids.

CB1 mRNA has been detected in several peripheral tissues including the rat adrenal glands, skeletal muscle, adipose tissue, liver and pancreas (Howlett et al., 2004; Rueda et al., 2000). THC has been shown to exert a central orexigenic effect on various peripheral tissues, regulating their energy metabolism (Kola et al., 2005). In STZ-induced diabetic

rats treated with *C. sativa* extract, increased liver weights and reduced hepatic and muscle glycogen content have been reported (Levendal and Frost, 2006). The lipid profiles of the various experimental groups were investigated since Coetzee et al. (2007) found that *C. sativa* treatment in an obese rat model reduced the atherogenic index of plasma (AIP) by 1.5-fold, relative to untreated rats.

Diet-induced animal models are used as a polygenic model of obesity in the quest to develop novel and effective interventions and/or treatments to stem the scourge of obesity and obesity-related disorders. Therefore an *in vivo* study was conducted utilizing lean and diet-induced obese male Wistar rats to establish the effects of treatment with an organic *C. sativa* extract over a 28-day period on various metabolic parameters, including food consumption, changes in body weight and body tissue mass, glucose tolerance and blood biochemistry. At a molecular level, various genes of interest (GOI) that play a key role in β -cell function and insulin secretion were investigated, viz. FLIP, PDX-1, c-MYC, GLUT2 and UCP2 (Kim and Kim, 2012; Zini et al., 2010). In addition, the detection of CB1 and CB2 receptors within pancreatic tissue was investigated to confirm their presence in insulin-secreting β -cells.

2.1 MATERIALS AND METHODS

2.1.1 Plant material

Plant material was obtained from various South African Police Services offices within the Nelson Mandela Metropolitan Municipal area. The plant material was confirmed by a botanist, Kirsten Ellis as *C. sativa*, and a sample has been placed in the botany herbarium, voucher no. 20084. Authorization for the use of a schedule 8 narcotic substance for scientific research purposes was obtained for the duration of the study. Annexure A provides a copy of one of the permits provided by the Medicines Control Council (MCC), permit UPE 36/2004/2005.

2.1.1.1 THC extraction protocol

Plant material included short stalks, leaves, flowers, and seeds. Several chloroform extractions was prepared during the duration of treatment according to a modified

protocol as described by Turner and Mahlberg (1984) and standardized against the THC content using RP-HPLC. Briefly, twenty grams of *C. sativa* plant material were ground with pestle and mortar and immersed in 50 mL analytical grade chloroform. The plant material was left in the dark for 60 minutes to extract at room temperature. The supernatant was removed and fresh chloroform was added and left for 60 minutes. This process was repeated once more, after which the three fractions were combined and filtered using Watman No.1 filter paper to remove any plant debris from the fractions. Thereafter the filtrate was filtered using a 0.45 µm Millex Millipore filtration unit to remove any other plant debris from the extract. The chloroform was evaporated under a gentle stream of nitrogen at 4°C. The remaining resin was redissolved in 5 to 10 mL of pure methanol, flushed with nitrogen, and stored under vacuum at 4°C in a dark container to protect it from light. Prior to injecting the experimental rats, the methanol was removed by evaporation using a speedvac (Savant SC100) and the remaining resin pellet was resuspended in Tween 80® (Polysorbate 80) (Sigma) which was made up to a 1% solution in saline (Levendal and Frost, 2006). Tween 80® is a hydrophilic, nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid, commonly used in *in vivo* studies (Zhang et al., 2003). The saline solution was filtered with a 0.22 µm Millex Millipore filtration unit to remove any particulate matter before injecting the rats. In total eight organic extractions were performed during the 28 days treatment period, and the THC content of each organic extraction quantified as outlined below. The volume of 1% Tween 80® saline solution (containing the *C. sativa* extract) that was injected into experimental rats was dependent on two factors, viz. firstly, the THC content in each of the organic extractions, and secondly, the weight of each rat. Annexure B provides an example of the table used to calculate the volume of 1% Tween 80® saline solution to be injected into the rats. Similarly, control rats were injected with an equivalent volume of 1% Tween 80® saline solution (without the *C. sativa* extract) as their experimental counterparts, using the same variables as stated above. Previous *in vivo* studies have shown that the use of Tween 80, ranging from 1% to 10%, had no effect on the parameters being investigated (Levendal and Frost, 2006; Bermúdez-Silva et al., 2006; Jafri et al., 2000).

2.1.1.2 THC quantification of chloroform extract using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

A C18 10 μm (0.46 cm x 25 cm) Grace Vydac Reverse Phase Column was connected to the Beckman Coulter HPLC System. Methanol was evaporated from the plant extract solution and the commercial THC standard using the speed vacuum dryer (Savant SC100). The resin was then resuspended in a mixture of acetonitrile and phosphate buffer at a ratio of 70:30, respectively. The standard or extract compounds were eluted based on their hydrophobic character, with more polar compounds being eluted first, using a hydrophobic stationary phase and a polar mobile phase. The solvent used was acetonitrile:0.01 M phosphate buffer, pH 7.4, (70:30) at a flow rate of 1 mL/min. Absorbance was read at 241 nm and the Beckman System Gold software was used to analyze the data. A THC standard (Industrial Analytical, SA) was used to construct a standard curve (figure 2.1), which reflected the peak area associated with various known THC concentrations (0 to 200 $\mu\text{g/mL}$), and which was used to quantify the unknown THC content in the extracts. Retention time (R_t) was used to identify the THC content in the extracts.

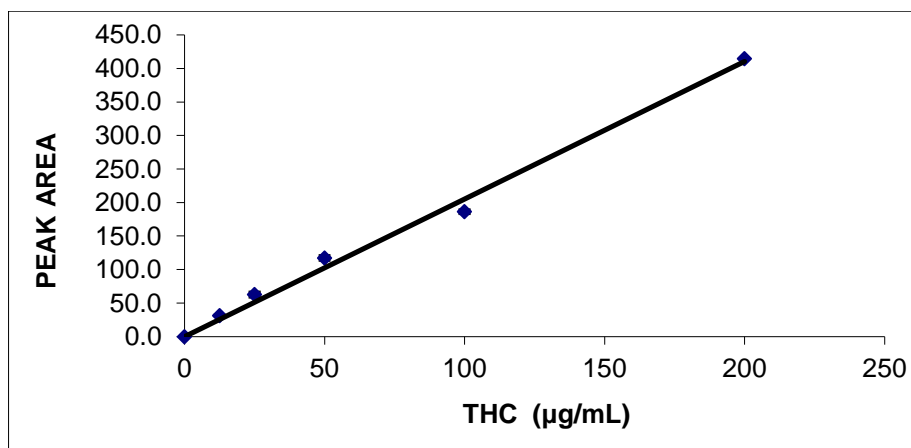


Figure 2.1 THC standard curve generated using THC standard concentrations ranging between 12.5 - 200 $\mu\text{g/mL}$. Error bars represent the standard deviation of triplicate samples ($n = 1$). $R^2 = 0.994$.

2.1.2 Animals

Approval for the use of animals for this study was obtained from the University of Port Elizabeth Animal Ethics Committee (Annexure C). Forty-eight male Wistar rats (*Ratus norvegicus*), weighing approximately 100 grams were purchased from the University of Cape Town Animal Unit. Sexual dimorphism occurs in adult Wistar rats with regard to body weight, where male rats are 20% heavier than females (Vital et al., 2006). The animals were randomly assigned to four groups, lean control (LNC) consisting of 10 rats, lean experimental (LNE) consisting of 11 rats, obese control (OBC) consisting of 13 rats and obese experimental (OBE) consisting of 14 rats. Each experimental group had rats located in 2-3 separate cages, thus collectively housing all the rats in 8 cages. These cages were located in an air-conditioned room ($22 \pm 3^{\circ}\text{C}$) under a 12 hour light/dark cycle, and allowed to acclimatize for 7 days.

The lean rats were maintained on standard mouse pellets (Epol), while the obese rats were fed for 8 weeks on a palatable, high energy cafeteria diet to induce obesity prior to the start of the experiment, and were maintained on this diet throughout the experiment. The composition (g/100 g wet matter) of the standard mouse pellets included 9.77 g moisture, 7.72 g fibre, 6.30 g ash, 16.82 g protein, 57.17 g carbohydrates, 2.22 g fat (MacKenzie et al., 2012). Moisture, fibre, calcium and phosphorus were non-caloric constituents of the pellet composition. The caloric composition of the standard mouse pellets were derived from 72.4% (1 884.1 kcal/kg) carbohydrates, 21.3% (554.3 kcal/kg) protein and 6.3% (164.6 kcal/kg) fat. According to the manufacturer's specifications, the total theoretical metabolisable energy of the mouse pellets was equivalent to 2 603 kCal/kg. The caloric composition of the diets were determined according to the food energy conversion factors set out in the publication on Food and Nutrition by the Food and Agriculture Organization (FAO) of the United Nations (2003). The cafeteria diet utilized in this study was a modified version of that used by Brandt et al. (2010), where the standard Muracon-G pellets (Carfil Quality, Belgium) were substituted with standard mouse pellets (Epol, South Africa). The cafeteria diet contained 330 g/kg ground standard mouse pellets (Epol), 330 g/kg Nestlé full-fat sweetened condensed milk, 70 g/kg sugar and 270 g/kg water. The caloric composition of the cafeteria diet was 73.1% (2 266.0 kcal/kg)

carbohydrates, 12.7% (395.2 kcal/kg) protein and 14.2% (440.6 kcal/kg) fat. The metabolisable energy associated with this diet was equivalent to 3 101.8 kCal/kg. This constituted 19.2% more calories in the cafeteria diet compared to an equivalent mass of standard mouse pellets.

All animals had access to food and tap water, *ad libitum*. Food consumption was monitored on a daily basis by calculating the difference between the weight of feed given and the left-over feed. Wastage was not taken into account but appeared consistent across all experimental groups. References to pre-experimental data relate to data collected prior to the rats being treated with *C. sativa* extract (dissolved in saline containing 1% Tween 80[®]) or vehicle (1% Tween 80[®] in saline), while references to post-experimental data relate to data collected 24 or 48 hours after termination of treatment with *C. sativa* extract or vehicle. The reason for the differences in timeframes was to reduce stress on the animals after conducting the intra-peritoneal glucose tolerance tests (IPGTTs) and harvesting their organs.

2.1.2.1 Experimental procedure

Numerous chronic studies have been conducted on animals, ranging from a few days to several months (Shen et al., 2011; Lamarque et al., 2001; Breivogel et al., 1999). An experimental duration of 28 days was selected, based on previous research protocols (Levendal and Frost, 2006; Watson, 1989; Abel, 1980), with treatment commencing on day 1. Figure 2.2 provides an overview of the experimental design and timeframes associated with measurement of blood glucose (fasting and post-prandial), body weight and food consumption for the duration of the experiment.

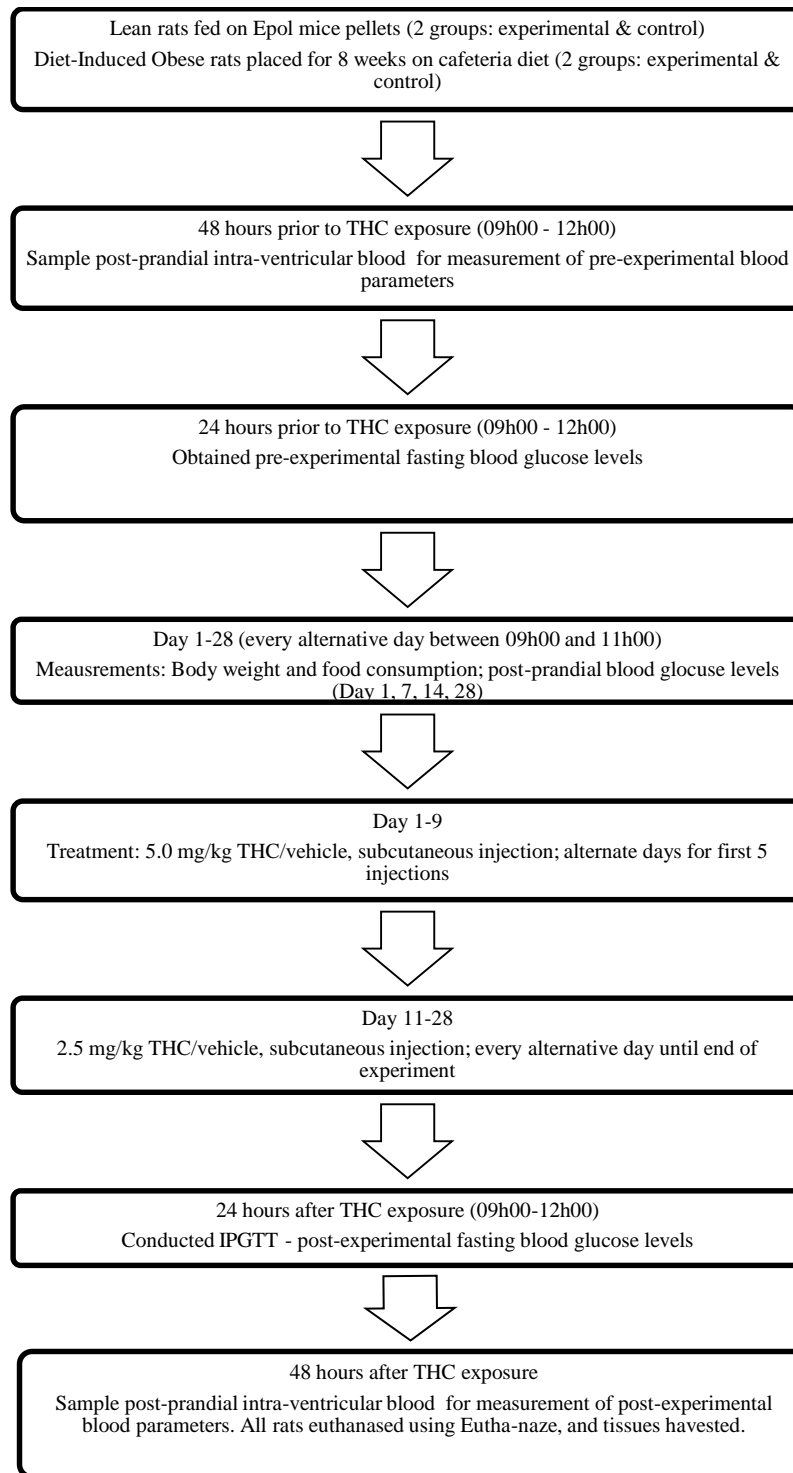


Figure 2.2 Overview of experimental design and data collection timeline.

Control animals were vehicle-treated, i.e. 1% Tween 80[®] in saline, while experimental animals were treated with the organic (chloroform) *C. sativa* extract dissolved in 1%

Tween 80[®] in saline. Experimental rats were treated every alternate day with an equivalent of 5.0 mg THC per kg body weight via subcutaneous injection, for the first 5 injections, after which the dosage was reduced to 2.5 mg THC per kg body weight for the duration of the experiment. This reduction in dosage was due to cutaneous lesions developing after the first two weeks. The THC dosage used corresponds with previous chronic studies conducted (Shen et al., 2011; Quinn et al., 2008; Cha et al., 2006; Levendal and Frost, 2006). Despite the development of the cutaneous lesions (see figure 2.3), no visceral abnormalities were observed once the rats were sacrificed and various tissues harvested at the end of the experimental period. Control rats were treated similarly, except that their injections contained the vehicle only. No lesions were observed in the control rats.



Figure 2.3 Lesion on skin of rat after two weeks of administration of *C. sativa* extract.

2.1.2.2 Intraperitoneal glucose tolerance test

IPGTTs are used to determine the body's ability to regulate its haemostatic control of glucose, through insulin secretion. The sensitivity of the rats to glucose loading and their responses were conducted 24 hours after completion of THC treatment. Animals were fasted overnight for 15-16 hours. IPGTT involved the intraperitoneal injection with a glucose bolus of 2 g/kg body weight, using a 40% glucose stock solution (Deushi et al., 2007). Blood glucose levels were monitored at time intervals of 0, 30, 60, 120 and 150

minutes, after bolus injection, using the Accutrend Glucometer GC (Roche). Blood samples (approximately 20 – 40 μL) were obtained from the tail vein and deposited directly on to the Accu-Chek test strips (Roche Diagnostics). These test strips have glucose dehydrogenase inside them, which converts any glucose present in the blood sample to gluconolactone. Oxidation of glucose results in the transfer of electrons to a mediator molecule, viz. hexacyanoferrate III which is then reduction of hexacyanoferrate II. Application of electrical voltage, reoxidize the hexacyanoferrate II which creates a small current that is recorded by the glucometer. AUC_g (fasted state) was calculated using the following formula: $\text{AUC}_g = (\text{concentration}_0 + \text{concentration}_1)/2 \times \text{Time}_{1-0}$ (Le Floch et al., 1990; Chiou, 1978).

2.1.2.3 Blood sampling

Approximately 2 mL of blood was collected from the fed animals 48 hours prior to the first THC exposure, via intraventricular puncture (see figure 2.4), in a syringe containing 0.105 M sodium citrate aqueous solution, at a ratio of 0.1 : 0.9 citrate solution to blood, in order to prevent coagulation.



Figure 2.4 Picture depicting pre-experimental blood collection via intraventricular puncture.

The blood was then placed in a centrifuge tube and centrifuged using a benchtop Eppendorf (5804R) centrifuge at 3000 rpm for 15 minutes. The plasma collected was stored at -20°C until used for the various assays described below. After sedation, the same procedure was followed 48 hours after the last THC treatment. Blood glucose levels were monitored on a weekly basis throughout the experiment using post-prandial blood samples obtained from the tail vein. Post-prandial blood samples were used because it would have been too stressful on the animals to fast them for 15 hours every alternate day, therefore fasting blood glucose levels were only measured pre- and post-experimentally.

2.1.2.4 Blood glucose quantification

To determine the effect of *C. sativa* administration on blood glucose levels, post-prandial blood samples were obtained by puncture of the tail vein on day 1, 7, 14 and 28 and quantified using the Accutrend Glucometer GC (Roche). These readings were consistently taken between 09h00 and 11h00 on the days specified above. Pre- and post-experimental fasting blood glucose levels were obtained 24 hours before and after the last THC exposure, after a period of 15-16 hours of fasting.

2.1.2.5 Plasma insulin determination using radioimmunoassay (RIA)

The RIA is based on the competitive binding of labeled tracer and unlabeled antigen for a limited and constant number of antibody binding sites. The quantity of tracer bound to the antibody will decrease as the quantity of unlabeled antigen increases. The Linco rat insulin RIA (Linco Research, USA) utilizes ^{125}I -labeled insulin and rat insulin antiserum according to the protocol outlined in table 2.1.

Table 2.1 Protocol of rat insulin RIA (Linco). Taken from the Linco Rat Insulin RIA handbook.

DAY 1						DAY 2		
SET UP	STEP 1	STEP 2&3	STEP 4	STEP 5	STEP 6	STEP 7	STEP 8	STEP 9-11
Tube No.	Add Assay buffer	Add standards, QC, samples	Add ¹²⁵ I Insulin Tracer	Add Rat Insulin Antibody	Vortex, cover and incubate for 20 – 24 hours at 4°C	Add precipitating reagent	Vortex and incubate for 20 minutes at 4°C	Centrifuge for 20 minutes at 4°C, decant and count
1, 2	-	-	100 µL	-		-		
3, 4	200 µL	-	100 µL	-		1.0 mL		
5, 6	100 µL	-	100 µL	100 µL		1.0 mL		
7, 8	-	100 µL of 0.1 ng/mL	100 µL	100 µL		1.0 mL		
9, 10	-	100 µL of 0.2 ng/mL	100 µL	100 µL		1.0 mL		
11, 12	-	100 µL of 0.5 ng/mL	100 µL	100 µL		1.0 mL		
13, 14	-	100 µL of 1.0 ng/mL	100 µL	100 µL		1.0 mL		
15, 16	-	100 µL of 2.0 ng/mL	100 µL	100 µL		1.0 mL		
17, 18	-	100 µL of 5.0 ng/mL	100 µL	100 µL		1.0 mL		
19, 20	-	100 µL of 10.0 ng/mL	100 µL	100 µL		1.0 mL		
21, 22	-	100 µL of QC1	100 µL	100 µL		1.0 mL		
23, 24	-	100 µL of QC2	100 µL	100 µL		1.0 mL		
25, n	-	100 µL of unknown	100 µL	100 µL		1.0 mL		

The assays were performed using the post-prandial plasma samples of individual rats according to the manufacturer's specifications, and quantified using the respective standard curves for insulin (figure 2.5) using the Packard Tri-Carb 2300TR liquid scintillation analyzer (Life and Analytical Science (Pty) Ltd, RSA).

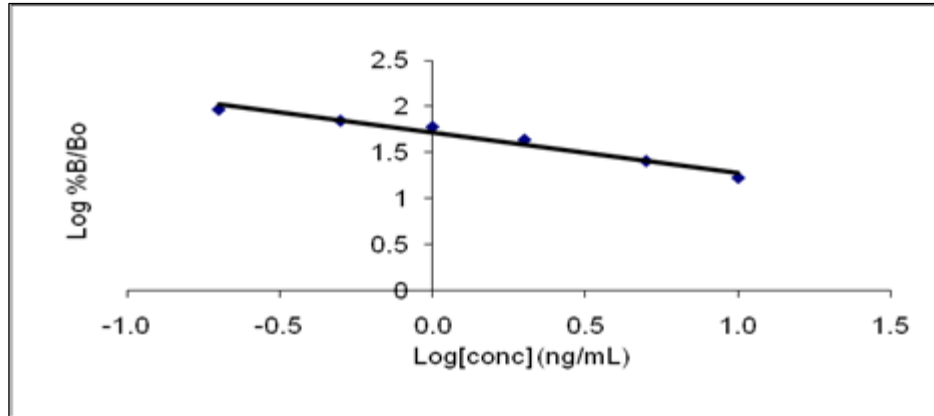


Figure 2.5 Standard curve for rat insulin radioimmunoassay (Linco), 33% tracer binding efficiency, $R^2 = 0.97$. Error bars represent the standard error of the mean (SEM) of duplicate standards.

2.1.2.6 Multiplex plasma endocrine detection

Plasma generated from intraventricular blood was used for endocrine detection. *Lincoplex* kits (Linco Research, USA) are based on laser detection of fluorescently labeled microsphere antibody-immobilized beads with specific dimensions associated with the analytes being quantified, using the Luminex¹⁰⁰ system (Luminex Corp., USA). Table 2.2 summarizes the protocol followed according to the manufacturer's specifications.

Only seventy-six samples could be processed in each 96-well assay plate provided, therefore eight individual pre- and post-experimental plasma samples were randomly selected from the OBC and LNC groups, while nine individual pre- and post-experimental plasma samples were selected from the OBE and LNE groups. The rat endocrine *Lincoplex* kit contained the leptin analyte which was used to quantify the unknown levels present in the plasma.

Table 2.2 Assay protocol for the rat endocrine Lincoplex kit as illustrated in the RENDO-85K protocol handbook.

Well #	Well ID	Assay Buffer	Assay Buffer	Standard/Control/Sample	Serum/Diluent/Test Media	Mixed Beads	Detection Antibody	SA-PE	Sheath Fluid
1A, 1B	0 pM standard (background)	200 µL	10 µL	-	10 µL	25 µL	50 µL	50 µL	100 µL
1C, 1D	6.2 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
1E, 1F	18.5 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
1G, 1H	55.6 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
2A, 2B	166.7 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
2C, 2D	500 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
2E, 2F	1500 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
2G, 2H	4500 pM standard	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
3A, 3B	Control I	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
3C, 3D	Control II	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
3E, 3F	Sample	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
3G, 3H	Sample	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
4A, 4B	Sample	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL
4C, 4D	Sample	200 µL	-	10 µL	10 µL	25 µL	50 µL	50 µL	100 µL

Seal, agitate, incubate for 10 minutes at RT. Remove assay buffer by vacuum.

Seal, agitate, incubate overnight at 4°C. Wash 3X with 200 µL wash buffer

Seal, agitate, incubate for 60 minutes at RT.

Seal, agitate, incubate for 30 minutes at RT. Wash 3X with 200 µL wash buffer.

2.1.2.7 Determination of plasma lipid profile and atherogenic indices

Total cholesterol, HDL-cholesterol and total triglycerides were determined using pooled plasma samples from all the rats as set out in table 2.3. No further pooling of plasma occurred since pooling diminishes the ability to detect differences between experimental groups.

Table 2.3 Summary of rat plasma samples that were pooled within each experimental group

Experimental Group	Rats #	Plasma volume (µL) per rat
LNC	C1/3; C1/4; C1/5; C2/1; C2/2; C2/5 ^a	60
LNE	E1/1; E1/2; E1/3; E1/6; E2/1; E2/3; E2/4 ^b	60
OBC	C1/2; C1/3; C1/6; C1/7; C2/1; C2/3; C2/5; C2/6 ^c	60
OBE	E1/2; E1/5; E1/6; E1/7; E2/1; E2/2; E2/3; E2/4 ^d	60

^a Insufficient plasma in the following rats: C1/1; C1/2; C2/3; C2/4

^b Insufficient plasma in the following rats: E1/4; E1/5; E2/2; E2/5

^c Insufficient plasma in the following rats: C1/1; C1/4; C1/5; C2/2; C2/4

^d Insufficient plasma in the following rats: E1/1; E1/3; E1/4; E2/5; E2/6; E2/7

Relevant assays modified to a microtitre plate format according to the methods stipulated by the various manufacturers. Briefly, triglyceride levels were determined in triplicate using 200 µL of reagent from the Triglyceride Glycerol-3-Phosphate Oxidase (GPO)-PAP kit (Roche, SA) mixed with 10 µL plasma, incubated for 5 minutes at 37°C and thereafter the colorimetric change were measured spectrophotometrically at 540 nm, and quantified against a standard curve set up using known glycerol concentrations (figure 2.6)

The reaction principle involves triglycerides being hydrolyzed by lipoprotein lipase to form glycerol and fatty acids. Glycerol kinase then converts the glycerol to glycerol-3-phosphate which gets oxidized by GPO to form hydrogen peroxide. The oxidative coupling of 4-aminophenazone and 4-chlorophenol, in the presence of hydrogen peroxide, is catalyzed by peroxidase (POD), to form a red dye. The absorbance measured

is proportional to the concentration of triglycerides and free glycerol in the sample (Stein and Myers, 1995; Henkel and Stoltz, 1982).

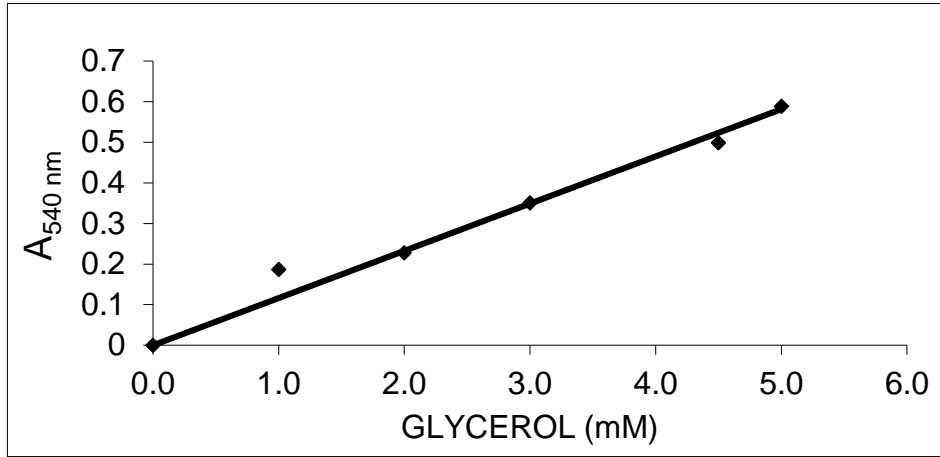


Figure 2.6 Triglyceride standard curve using glycerol, $R^2 = 0.9757$. Error bars represent the standard error of the mean (SEM) of triplicate samples.

The cholesterol CHOD-PAP kit (Roche, SA) was used for total cholesterol determination. Total cholesterol levels were quantified against a standard curve set up using cholesterol (figure 2.7). Briefly, 200 μ L pooled plasma was added to 200 μ L cholesterol reagent, incubated for 5 min at 37°C and read at 540nm (NIH, 1990).

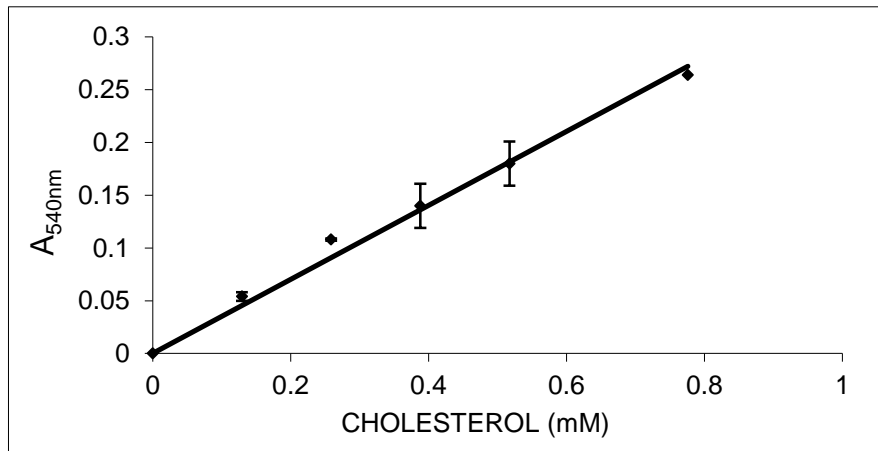


Figure 2.7 Cholesterol standard curve, $R^2 = 0.9817$. Error bars represent the standard error of the mean of triplicate samples.

Determination of HDL-cholesterol was conducted using the HDL-cholesterol precipitant (Roche, SA) on the same plasma samples as those used for the total cholesterol determination. HDL-cholesterol was measured in duplicate using 200 μ L plasma which was added to 500 μ L HDL reagent, mixed and left at room temperature for 10 minutes. Thereafter the precipitate was compacted by centrifugation, using an Eppendorf Minispin Plus Microcentrifuge at 4000 rpm for 10 minutes. 20 μ L of supernatant was added to 200 μ L of cholesterol reagent to determine the remaining cholesterol, measured at 540 nm. The HDL-cholesterol levels were calculated as the difference in absorbance between total cholesterol and post HDL-cholesterol precipitation readings (Lopes-Virella et al., 1977).

LDL-cholesterol levels were determined using the total cholesterol, HDL-cholesterol and total TG levels, and not via ultra-centrifugation. The LDL-cholesterol levels were calculated according to the following formula:

$$\text{LDL-cholesterol (mM)} = (\text{TC}/1.19) + (\text{TG}/0.81) - (\text{HDL}/1.1) - 0.98$$
 (Ahmadi et al., 2008), where TC = total cholesterol; TG = total triglycerides; HDL= HDL-cholesterol. VLDL-cholesterol levels were calculated according to the formula set out by Friedewald et al. (1972):
$$\text{VLDL-cholesterol (mM)} = (\text{TG}/2.17).$$

AIP is used as a predictor of MetS, T2D, hypertension and coronary heart disease in humans (Onat et al., 2010). In rats, a decrease in the AIP is considered to be a beneficial effect since HDL level is inversely correlated to coronary heart disease (Hussein, 2011), since increased HDL-cholesterol levels are associated with reduced cardiovascular risk (Assmann and Gotto, 2004). The AIP was calculated according to the following formula:
$$\text{AIP} = \log([\text{Triglycerides}] / [\text{HDL cholesterol}])$$
 (Dobiášová and Frohlich, 2001).

2.1.3 Quantitative real-time polymerase chain reaction (qPCR)

qPCR is a highly sensitive techniques used to quantify small changes of the level of gene expression within tissues. In order to detect these minute amounts of product formed, a fluorescent dye, in this case SYBR[®] Green, binds to all double-stranded DNA after excitation at 494nm, and emission of a fluorescent signal at 521nm (Pfaffl, 2001). The number of cycles required for the fluorescent signal to cross the threshold, i.e. to exceed

the background signal, is referred to as the quantification cycle (C_q). C_q levels are inversely proportional to the amount of complementary nucleic acids present in the sample that binds to the specific primer sequences. Therefore, the lower the C_q level, the greater the amount of complementary nucleic acids in the sample. The quantification of expression could be absolute expression or relative expression. For this study, relative expression was used, where the expression level of the GOI was normalized relative to the expression levels of two reference genes.

Numerous reference genes reported in the literature were evaluated with the aid of geNorm 3.5 software, (Vandesompele et al., 2002), and the two reference genes that showed expression stability values of less than 1.5 and least variation across all treatment conditions, were selected. Cyclophilin A, α -tubulin and β -tubulin are well known reference genes for pancreatic islets (Tschen et al., 2011; Laybutt et al., 2002a&b; Federici et al., 2001), and for this study β -tubulin and cyclophilin A (cyclo A) were selected with expression stability values of 1.441.

The following GOI were investigated in this study and are expressed exclusively or predominantly in pancreatic β -cells, relative to other endocrine and exocrine cells, in relation to obesity, diabetes and/or hyperglycemia: Akt/PKB (Cai et al., 2008; Elghazi et al., 2006); CB1 (Vilches-Flores et al., 2010b; Nakata and Yada, 2008); c-MYC (Elouil et al., 2005; Kaneto et al., 2002; Jonas et al., 2001); FLIP (Brun et al., 2008; Mohanty et al., 2005; Maedler et al., 2002a&b); GLUT2 (Arava et al., 1999; Bonny et al., 1997; Thorens et al., 1992); insulin (Arava et al., 1999; Niwa et al., 1997; Neophytou et al., 1996); PDX-1 (McKinnon and Docherty, 2001; Arava et al., 1999) and UCP2 (Anello et al., 2005; Laybutt et al., 2002b; Chan et al., 2004; Chan et al., 2001; Chan et al., 1999).

In order to evaluate amplification efficiencies of each primer set, standard curves were generated, using copy DNA (cDNA) from rat tissues that showed high levels of expression of the relevant GOI. The amplification efficiency provides information about the efficiency of the reaction, and is directly related to the slope of the standard curve, where:

Efficiency = $[10^{(-1/\text{slope})}] - 1$. At 100% efficiency, i.e. a slope of -3.3, the template doubles after each cycle during exponential amplification.

cDNA samples were run in duplicate, and the average Cq value was used for calculating the relative gene expression ratios using the Relative Expression Software Tool (REST) 2009 V2.0.13 (QIAGEN). This software uses pair-wise fixed reallocation randomisation testing to calculate the relative expression ratios based on qPCR efficiencies and the difference between mean Cq of the control and experimental samples. The randomisation (permutation) testing is used to improve the confidence interval and probability associated with the qPCR data (Pfaffl et al., 2002; Pfaffl, 2001). The randomisation test involves repeated and random reshuffling/re-ordering of the original observed data associated with the control and experimental groups, and notes the expression ratio with each permutation. In this study, 10 000 random reallocations of the respective control and experimental groups were calculated. The mathematical model calculates the random expression ratios generated relative to the mean of the original observed data of the control and experimental groups to determine whether there is a significant difference between these or not, i.e. the P-value. The mathematical model sets a 95% confidence interval based on the ratios generated using the equation above and provides a ratio range, i.e. a minimum and maximum ratio, that has been calculated based on an α value of 0.05. If the proportion of the random expression ratios is small, then there is evidence that the observed effect as reflected in the original observed data is not simply the result of random allocation but (Pfaffl, 2004; Pfaffl et al., 2002; Pfaffl, 2001).

The expression of the GOIs was normalized to the expression of two reference genes, viz. β -tubulin and cyclo A. The relative gene expression ratios were calculated according to the following equation:

$$\text{Ratio} = \frac{(E_{\text{GOI}})^{\Delta Cq_{\text{GOI}}(\text{Mean Control} - \text{Mean Experimental})}}{(E_{\text{REF}})^{\Delta Cq_{\text{REF}}(\text{Mean Control} - \text{Mean Experimental})}}$$

where E = efficiency of relevant GOI and reference (housekeeping gene); ΔCq refers to the difference in gene expression between control and experimental samples which is assessed in group means (Pfaffl et al., 2002). LNC relative expression ratios were compared to LNE, OBC and OBE rats, while OBE relative expression ratios were compared to OBC ratios. Data will be expressed as the mean and standard error interval for above comparisons between experimental groups.

2.1.3.1 RNA isolation

RNeasy mini kits (Qiagen) were used to extract total RNA from the pancreatic tissue, as per manufacturer's instructions (figure 2.8). RNA was extracted from pooled pancreatic tissue samples (see table 2.4), as routinely conducted for qPCR studies (Fereidouni et al., 2012; Chen et al., 2008; Medhurst et al., 2000). Despite tissue samples being pooled, the number of pooled samples per experimental group did not compromise the ability to assess variability within and between experimental groups. RNA quality was verified spectrophotometrically with an Ultraspec 2100 pro, using the A_{260}/A_{280} ratio (Imbeaud et al., 2005) (see table 2.4). Quartz cuvettes were used to quantify total RNA, with 1 unit corresponding to 40 μg of RNA per mL.

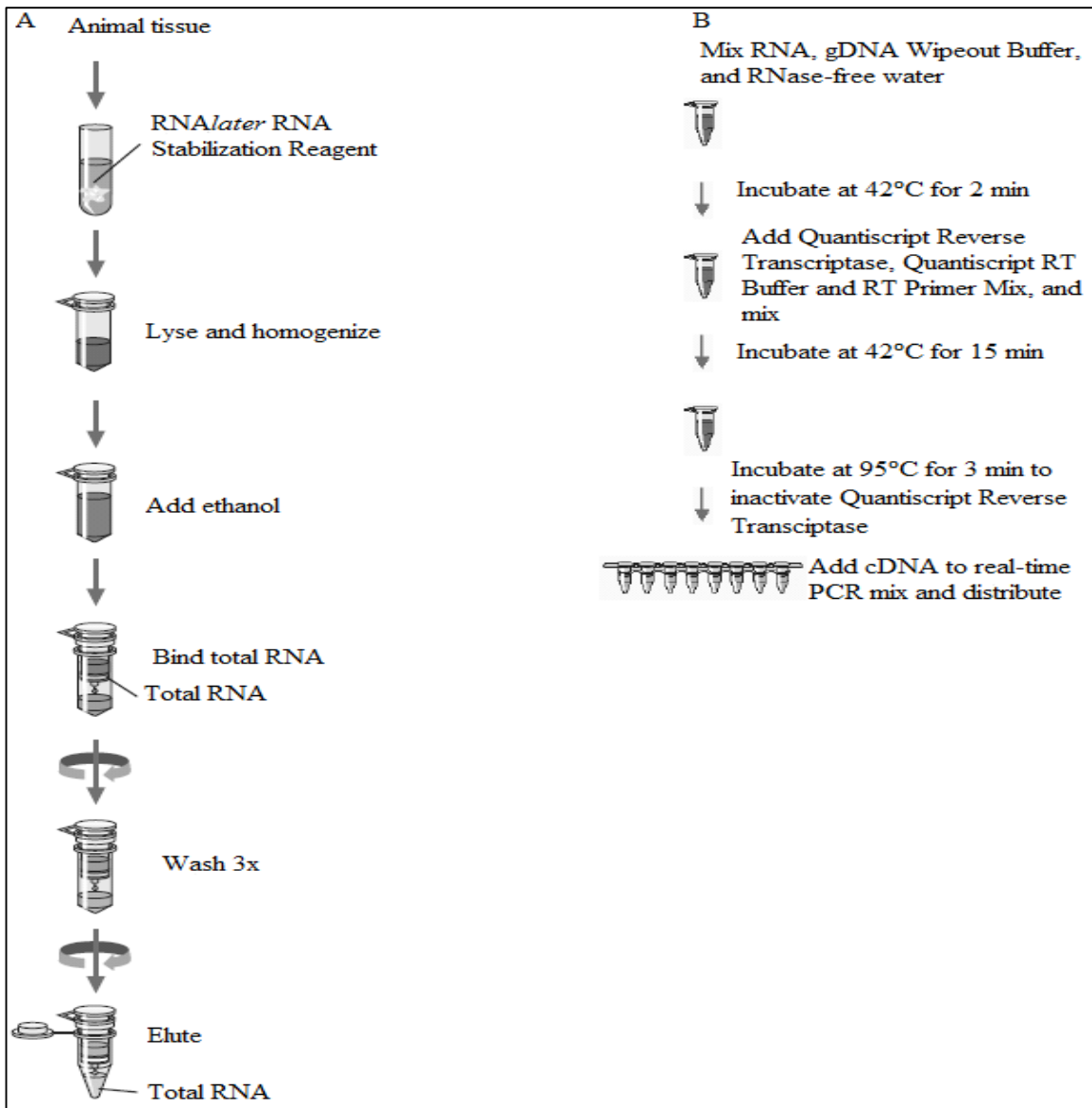


Figure 2.8 (A) RNA extraction protocol used for rat pancreatic tissue, (B) QuantiTect reverse transcription protocol (as illustrated in the RNeasy Mini Handbook 04/2006, and QuantiTect Reverse Transcription Handbook 04/2005).

Table 2.4 Total RNA extract from pooled rat samples.

Experimental Group	Tube #	Rat #	260/280	[RNA] $\mu\text{g}/\mu\text{L}$
LNC	1	C1/2;C1/5	2.12	0.705
	2	C1/3;C1/1	2.18	0.226
	3	C2/2;C2/3	2.92	0.066

	4	C2/4;C2/5	2.55	0.071
	5	C2/1	2.56	0.084
LNE	6	E1/1;E1/6	2.01	0.342
	7	E1/2;E1/5	2.25	0.148
	8	E2/4;E2/1	2.45	0.111
	9	E2/2	2.08	0.188
	10	E2/5	1.89	0.275
OBC	11	C1/3;C1/5	2.14	1.321
	12	C1/4;C1/6	2.14	1.514
	13	C1/7;C1/2	2.14	1.540
	14	C1/1;C2/3	2.08	1.526
	15	C2/2;C2/1	2.12	0.861
	16	C2/5;C2/6;C2/4	2.10	0.245
OBE	17	E1/5;E1/3	2.18	0.839
	18	E1/7;E1/2	2.14	1.204
	19	E1/1;E1/6	2.16	0.996
	20	E1/4;E2/6	2.48	0.119
	21	E2/7	1.90	0.368
	22	E2/5;E2/3	2.58	0.048
	23	E2/2;E2/4	2.19	0.232

QuantiTect Reverse Transcription kits (Qiagen), which include an integrated genomic DNA contamination removal process, were used for cDNA synthesis in accordance to the manufacturer's specifications (figure 2.8 and table 2.5).

Table 2.5 Genomic DNA elimination reaction components (www.qiagen.co.za)

Reagent	Volume (1 reaction)	Volume (2 reactions)
DNA Wipeout buffer, 7x (µL)	2	4
RNA Template	±1 µg	±2 µg
RNase-free water	Variable	Variable

Total volume (μL)	14	28
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The reagent mixture from the DNA elimination step was added to the RT mastermix (see table 2.6) according to the protocol outlined in figure 2.8.

Table 2.6 Components of Reverse Transcription Mastermix (www.qiagen.co.za)

Component	Volume (μL)
Quantiscript Reverse Transcriptase	2
Quantiscript RT Buffer, 5x	8
RT Primer Mix	2
Entire genomic DNA elimination reaction	28
Total Volume	40

All primers were based on the rat genome, and designed using the Beacon Designer Software (version 3.0) (Premier Biosoft International), synthesized by Inqaba Biotech (SA) and Microsynth (CH), reconstituted in sterile water to a 100 μM solution, which was further diluted with sterile water to a 10 μM working stock. The sequences of the various primers (Table 2.7) were tested for species cross-reactivity using the basic local alignment search tool (BLAST) (Altschul et al., 1990). Except for CB1 (rat brain) and UCP2 (rat liver), all other primer standard curves were set up using rat pancreatic tissue.

Table 2.7 Primers of reference genes and GOI with their associated annealing temperatures, PCR efficiencies and correlation coefficients used in qPCR protocols.

GENE	ACCESSION NO.	Forward Primer (5' – 3')	Reverse Primer (3' – 5')	Annealing T°C	PCR efficiency (%)	R ² (%)
Akt/PKB	NM_017093	CAACTGGCAGGATGTGGTAC	AGGCTGTCATATCGGTCTGG	58	105.1	96.1
CB1	NM_012784	CTGGTTCTGATCCTGGTGGT	TGTCTCAGGTCCTTGCTCCT	60	101.7	99.7
c-MYC	AY294970	AAGGAAGGACTATCCAGC	CCTCTTGTCGTTTTCTC	53	105.0	96.4
CycloA	XM341363	ACGTGGTCAAGACTGAGTGG	CGTGCTTCCCACCAGACC	58	104.8	99.1
FLIP	AF244366	GGGACCTCCTGGATTGTTTAAAG	ACGGCTGCTTTATCTGTCTTC	55	102.5	96.0
GLUT2	NM_012879	ATGACATCAATGGCACAGACAC	GGACACAGACAGAGACCAGAG	60	100	97.2
Insulin	NM_019129	CTGCCAGGCTTTTGTCAA	TCCACTTCACGACGGGACTT	60	95.6	100.0
PDX-1	NM_022852	CTGCCTCTCGTGCCATGTGAAC	GGCTGTTATGGGACCGCTCAAG	61	104.8	98.0
β-Tubulin	NM_173102	AACCCAGCCCAGTTCTAAG	GCGATTCCCGTGTCTAAATG	58	103.3	98.8
UCP2	NM_019354	TGGCGGTGGTCGGAGATAC	GGGCAACATTGGGAGAGGTC	59	99.4	95.0

2.1.3.2 qPCR protocol

All qPCR reactions were conducted on the iCycler iQ Multicolour Real-Time Detection System (BIO-RAD), V3.1. The iQ SYBR Green Supermix (Biorad) which was used in the qPCR reactions contained the following components: 50 mM KCl; 20 mM 2-Amino-2(hydroxymethyl)-1,3-propanediol-hydrochloride (Tris-HCl), pH 8.4; 0.2 mM each deoxynucleotide triphosphate, 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate and 2'-deoxythymidine 5'-triphosphate; 25 U/mL iTaq DNA polymerase (25 units/mL); 3 mM MgCl₂; SYBR Green 1; 10 nM fluorescein and stabilizers. For each primer pair, the optimal concentration was determined prior to evaluating the effect on the different treatments. The qPCR reaction mix with a primer concentration of 500 nM for per well contained the following: 10 µL iQ SYBR Green Supermix (Biorad), 1 µL each of the forward and reverse primers of a specific GOI, 6 µL sterile water and 2 µL cDNA template. In all qPCR runs DNA contamination and primer dimer formation were monitored by including blank samples, where the cDNA templates were replaced with water.

The standard format of the qPCR protocols used consisted of 5 cycles: Cycle 1 was a hotstart at 95°C for 3 minutes. Cycle 2 included a forty times repeat of the following three steps: step 1 at 95°C for thirty seconds; step 2 at specified temperature (refer to table 2.7) for 30 seconds; and step 3 at 72°C for 30 seconds. Cycle 3 includes 1 step at 95°C for 30 seconds. The first step of cycle 4 runs for 10 seconds at the temperature specified in step 2 of cycle 2. Thereafter, the temperature was adjusted by 0.5°C for 10 seconds for each repeat step, until the temperature reached 95°C to allow for product denaturation, thus enabling the generation of amplicon specific melt curves. Cycle 5 includes an infinite holding step at 22°C. Product specificity was examined by melting curve analysis of SYBR-Green double-stranded DNA amplicons and gel electrophoresis of qPCR products. Furthermore, Western blots were used to confirm the protein expression in the pancreatic tissues.

2.2 STATISTICAL ANALYSIS

All data are expressed as means ± standard error of the mean (SEM), unless otherwise indicated, and differences between the various experimental groups were statistically analyzed using the single factor analysis of variance (ANOVA) test, as well as the Student's *t*-test. A P-value of < 0.05 was interpreted as being statistically significant. Outliers were

identified using the Grubb's Test, and excluded from the parameter being analyzed. The Grubb's Test statistic can be calculated as follows: $Z = (\bar{Y} - y_i)/s$, where \bar{Y} is the sample group mean; y_i is the individual sample value and s is the standard deviation of the sample group. The Grubb's critical value was determined at $P < 0.05$. Any value being considered that is equal to or above the critical value is considered to be an outlier. Taking into account the total number of rats within each experimental group, the Z values for the respective experimental groups were as follows: LNC = 2.18; LNE = 2.23; OBC = 2.33 and OBE = 2.37. This critical value may vary depending on the parameter being analysed and the number of rats being analysed.

2.3 RESULTS

2.3.1 THC quantification

Figure 2.9 and 2.10 are chromatograms of a THC standard and a chloroform extract, respectively. The profile of the THC standard (see figure 2.9) shows negligible degradation of the standard. As can be seen in the chromatogram of the extract (figure 2.10), other components formed part of the extraction. As previously stated there are numerous cannabinoids present in the *C. sativa* extract. Consumption of the plant material, whether smoking or by infusion, does not distinguish between the different cannabinoids, therefore THC was used in this study as a measure for standardizing the dosage administered to the animals, relative to other components of the extract. The same plant material was used to perform various extractions, with an average THC concentration of 4.13 ± 0.27 mg/mL. Figure 2.10 shows a *C. sativa* extract profile where CBD levels (R_t 6.01 minutes) were found to be lower than THC levels (R_t 8.52), while CBN levels (R_t 7.51 minutes) were found to be higher than THC levels. The high CBN content was not due to oxidative degradation of THC after organic extraction occurred since the extract was used within 3 to 4 days after extraction and quantification. The plant material that were obtained from the SAPS may have been aged and/or stored for a prolonged period of time, which may account for the high CBN content in the extract. The ratio of THC:CBN:CBD across all extractions equated to approximately 1:10:1.

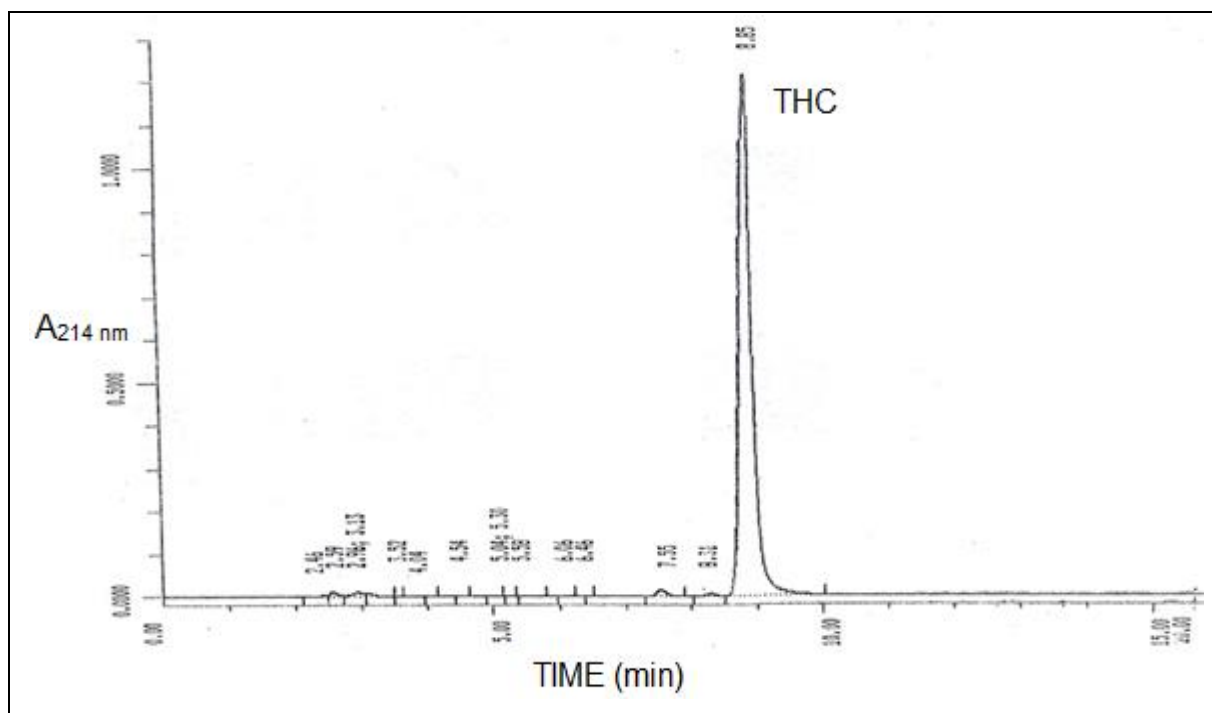


Figure 2.9 Chromatogram of 150 µg/mL THC standard with a major peak detected at 214 nm, R_t 8.85 minutes, with a C18 10µm (0.46cm x 25cm) Grace Vydac Reverse Phase Column, using a solvent mix of acetonitrile:0.01 M phosphate buffer, pH 7.4, (70:30) at a flow rate of 1 mL min⁻¹.

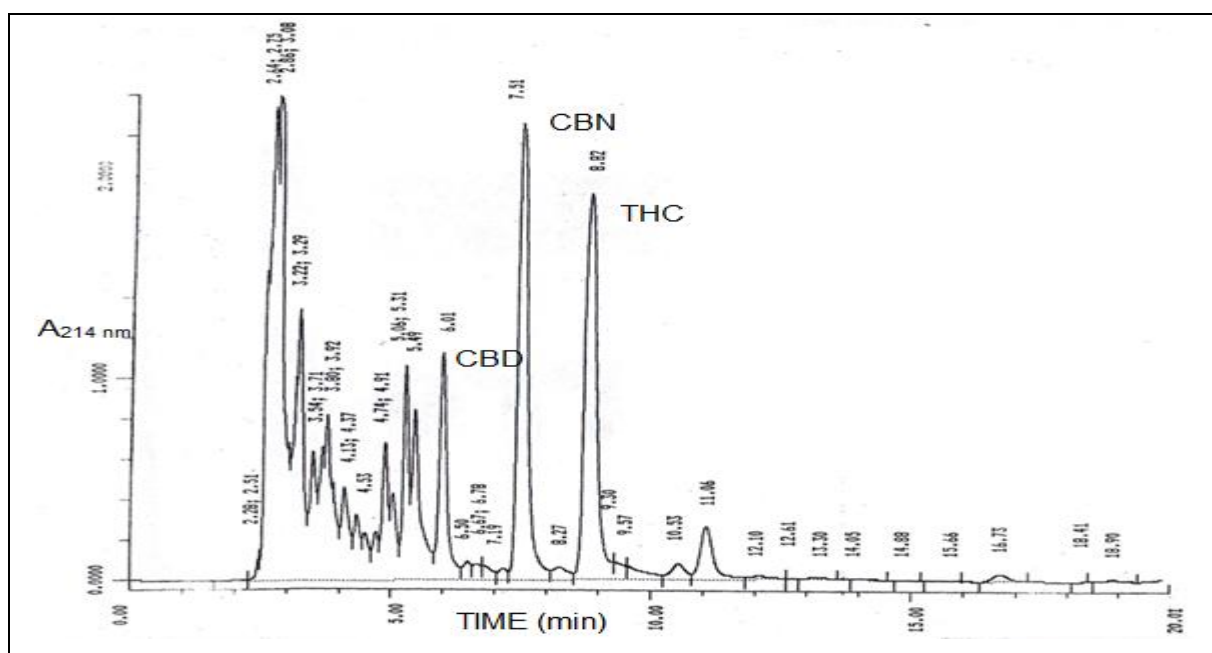


Figure 2.10 Chromatogram of chloroform *C. sativa* extract with THC detected at 214 nm, R_t 8.82 minutes, with a C18 10µm (0.46cm x 25cm) Grace Vydac Reverse Phase Column, using a solvent mix of acetonitrile:0.01 M phosphate buffer, pH 7.4, (70:30) at a flow rate of 1 mL min⁻¹.

2.3.2 Effect of *C. sativa* treatment on body weight and food intake

Table 2.8 provides a summary of the average body weights of the various experimental groups on the pre-experimental (day 1) and post-experimental (day 28) time points, as well as their respective weight gain. The average starting body weight in the obese group (425.41 ± 6.94 g) was significantly higher ($P < 0.01$) than that of the lean group (398.61 ± 6.63 g), and indication that the cafeteria diet induced a higher degree of weight gain in the obese rats, relative to the standard chow diet in the lean rats. Both control groups showed a significant increase in weight over the 28 day period, with the OBC group increasing by 61.20 ± 4.72 g ($P < 0.001$), and the LNC group gaining 49.23 ± 7.93 g ($P < 0.01$). The effect of the cafeteria diet was maintained throughout the duration of the study, where the body weights of LNC and OBC rats remained significantly different ($P < 0.05$).

Table 2.8 Effect of diet and *C. sativa* extract on body weight of lean and obese Wistar rats.

Groups	Body Weight (g)		Food intake per day (g)	% Weight Gain	
	Pre	Post		Intra-group	Relative to Controls
LNC	395.85±10.01	445.08±14.93 ^b	45.37±0.12	12.35±1.88%	
LNE	401.12±9.18	424.51±8.63	50.03±0.54 ^c	5.93±1.05% ^b	47.51%
OBC	427.05±9.21 ^a	488.24±11.68 ^{ac}	64.61±1.18 ^c	14.32±0.06% ^c	
OBE	423.90±10.62	439.83±9.21 ^b	57.42±0.44 ^c	3.94±0.92% ^c	26.03%

Body Weight:

^a $P < 0.05$, pre-LNC vs pre-OBC; post-LNC vs post-OBC

^b $P < 0.01$, pre-LNC vs post-LNC ; post-OBC vs post-OBE

^c $P < 0.001$, pre-OBC vs post-OBC; post-LNE vs post-OBC

Food intake:

^c $P < 0.001$, LNC vs LNE, OBC, OBE; LNE vs OBC, OBE; OBC vs OBE;

Weight Gain:

^b $P < 0.01$, LNC vs LNE

^c $P < 0.001$, LNC vs OBE; LNE vs OBC

C. sativa treatment in both the lean and obese groups resulted in a significantly lower percentage weight gain of 5.83% ($P < 0.01$) and 3.76% ($P < 0.001$), compared to the respective controls. The average food consumption of 57.42 ± 0.44 g per rat per day in the OBE group was significantly less when compared to the respective control group, $64.61 \pm$

1.18 g ($P < 0.001$). This was not observed in the lean group, where experimental rats consumed significantly more, 50.03 ± 0.54 g, than the respective control group, 45.37 ± 0.12 g ($P < 0.001$).

The change in body weight over the 28 days, as observed on a weekly basis in lean and obese rats is graphically represented in figure 2.11.

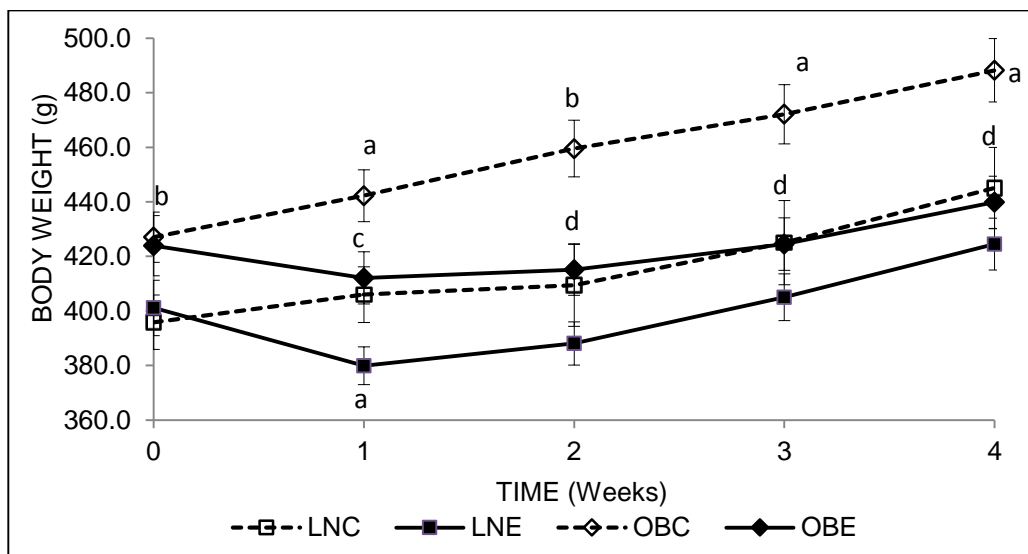


Figure 2.11 Effect of diet and *C. sativa* treatment on body weight in lean and obese Wistar rats over a 4-week period.

^a $P < 0.05$, relative to LNC; ^b $P < 0.01$, relative to LNC; ^c $P < 0.05$, relative to OBC; ^d $P < 0.01$, relative to OBC

A significant difference existed at the start of the 4-week experiment in the average body weight of obese rats relative to the average body weight of lean rats ($P < 0.01$). The body weights of LNC and LNE rats were significantly different in the first two weeks of treatment, with a higher dosage of *C. sativa* extract being administered ($P < 0.05$). However, with a lowering of the dosage, the body weights of the lean rats were found not to be statistically different in week 2 to 4. The significant weight gain induced by the cafeteria diet was maintained over the 28 day period, with OBC rats having a significantly higher body weight than LNC rats at all time-points ($P < 0.05$). *C. sativa* treatment reduced the difference in weight between the OBE and LNC rats, whereby the initial difference induced by the cafeteria diet was obliterated once *C. sativa* treatment commenced. *C. sativa* treatment induced a significant reduction in weight in the OBE rats, relative to the OBC rats, within a week of commencing treatment, and maintained the significant difference in weight for the duration of the treatment period ($P < 0.01$).

Based on the experimental data (figure 2.11), a validated predictive polynomial model for body weight was developed (figure 2.12), within a 95% confidence interval, taking into account the effect of time, treatment, diet and the interaction between these factors:

Predicted body weight (\hat{Y}) = $\beta_0 + \beta_1\text{Time} + \beta_2\text{Time}^2 + \beta_3\text{Treatment} + \beta_4\text{Diet} + \beta_5\text{Time}$.
 Treatment ($r^2 = 0.31$), where $\beta_0 = 400.09$ ($P < 0.0001$); $\beta_1 = -6.71$ ($P = 0.42$); $\beta_2 = 8.21$ ($P = 0.001$) and $\beta_3 = -7.16$ ($P = 0.40$); $\beta_4 = 31.91$ ($P < 0.0001$); $\beta_5 = -11.63$ ($P < 0.01$).

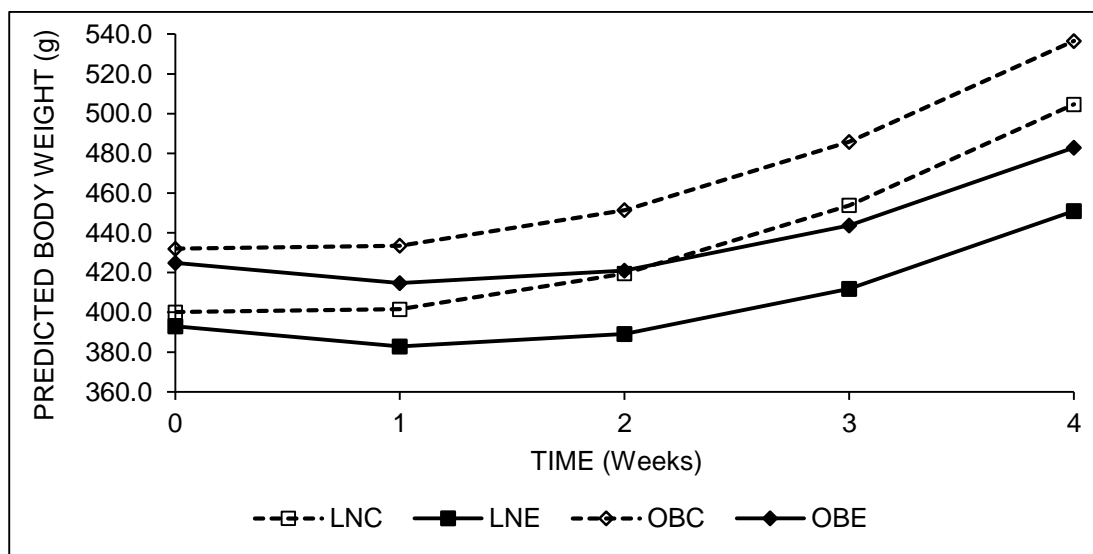


Figure 2.12 Effect of diet, time and *C. sativa* treatment on predicted body weight in lean and obese Wistar rats over a 4-week period.

Regression analysis shows that body weight increased by an average of 8.21 g over the experimental period ($P < 0.0001$), irrespective of diet or treatment, while the cafeteria diet induced an average weight difference of 31.9 g ($P < 0.0001$) in obese rats, between lean and obese rats. For the same period, *C. sativa* exposure on its own did not have a significant influence on body weight, contributing an average decrease in body weight of 7.16 g ($P = 0.40$), while the combined effect of time and *C. sativa* exposure significantly reduced body weight by an average of 11.63 g ($P < 0.01$) over the experimental period.

2.3.3 Effect of *C. sativa* treatment on body organ weights

The effect of diet and *C. sativa* treatment on the weights of the various body organs is summarized in figure 2.13 and table 2.9 provides an overview of the ratios of the various organs in relation to the post-experimental body weights across all experimental groups.

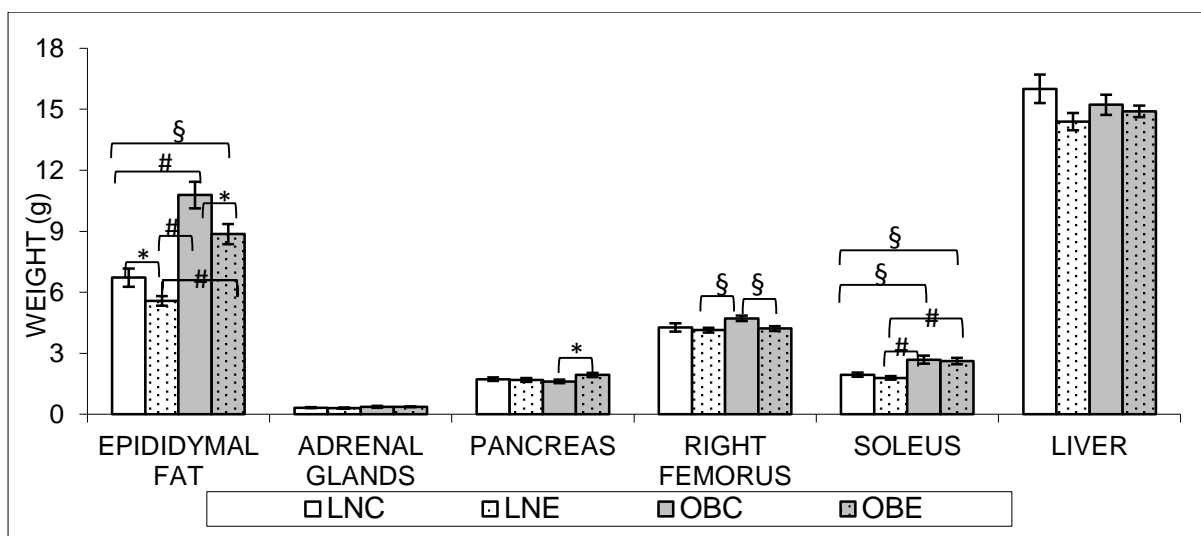


Figure 2.13 Effect of *C. sativa* treatment on various body tissues in lean and obese rats.

*P < 0.05, Epididymal fat - LNC vs LNE, OBC vs OBE;

Pancreas - OBC relative to: OBE

§P < 0.01, Epididymal fat - LNC relative to: OBE

Right femorus - LNE relative to: OBC; OBC relative to: OBE

Soleus - LNC relative to: OBC, OBE

#P < 0.001, Epididymal fat - LNC relative to: OBC; LNE relative to: OBC, OBE

Soleus - LNE relative to: OBC, OBE

The epididymal fat mass was significantly reduced in both the experimental groups. Treatment with *C. sativa* extract significantly decreased the average weight of epididymal fat to 5.58 ± 0.24 g in the LNE group, relative to 6.72 ± 0.45 g in the LNC group ($P < 0.05$). Similarly, treatment significantly reduced epididymal fat in OBE, 8.86 ± 0.49 g, relative to 10.7 ± 0.65 g, in the OBC group ($P < 0.05$). Both the obese groups had a significantly higher amount of epididymal fat, relative to the lean groups, resulting in a higher fat:body weight percentage. The OBC ($2.19 \pm 0.10\%$) and OBE ($2.00 \pm 0.08\%$) rats had a significantly higher fat percentage than LNC ($1.50 \pm 0.07\%$; $P < 0.001$) and LNE ($1.32 \pm 0.06\%$; $P < 0.001$) rats, respectively.

The pancreata of the OBE group (1.94 ± 0.10 g) weighed significantly more, relative to the OBC group (1.62 ± 0.09 g) ($P < 0.05$). No difference in pancreas weight was found within the lean group and between lean and obese rats. When evaluating the pancreas/body weight percentages, significant differences were found between the OBC ($0.33 \pm 0.01\%$) and OBE ($0.44 \pm 0.02\%$; $P < 0.001$) rats. The OBC group also differed significantly from the LNC group ($0.40 \pm 0.03\%$; $P < 0.05$) and LNE ($0.40 \pm 0.02\%$; $P < 0.01$) rats.

Table 2.9 Summary of organ weights expressed as a percentage of body weight.

	Epididymal Fat (%)	Adrenal Glands (%)	Pancreas (%)	Right Femorus (%)	Soleus (%)	Liver (%)
LNC	1.50±0.07	0.07±0.01	0.40±0.03	0.96±0.03	0.44±0.02	3.62±0.19
LNE	1.32±0.06 ^c	0.07±0.01	0.40±0.02 ^b	0.97±0.02 ^b	0.42±0.02	3.39±0.07 ^a
OBC	2.19±0.10 ^c	0.07±0.01	0.33±0.01 ^a	0.97±0.02	0.55±0.04 ^a	3.12±0.07
OBE	2.00±0.08 ^c	0.09±0.01	0.44±0.02 ^c	0.96±0.0 ^b	0.60±0.04 ^c	3.39±0.06 ^b

^aP < 0.05, Pancreas – LNC vs OBC
Soleus - LNC vs OBC
Liver – OBC vs LNE

^bP < 0.01, Pancreas – OBC vs LNE
Soleus – LNC vs OBE; OBC vs LNE
Liver – OBC vs OBE

^cP < 0.001, Epididymal fat – LNC vs OBC; LNC vs OBE; OBC vs LNE; LNE vs OBE
Pancreas – OBC vs OBE
Soleus – LNE vs OBE

C. sativa treatment significantly reduced the mass of the left *rectus femorus* muscles of OBE rats (4.22 ± 0.12 g) ($P < 0.01$), relative to the OBC rats (4.72 ± 0.14 g). This reduction was not observed in the lean group. The soleus muscle was not found to be affected by *C. sativa* treatment. However, the cafeteria diet did induce a highly significant increase in the mass of the soleus muscles of the obese group (2.81 ± 0.17 g and 2.62 ± 0.15 g, OBC and OBE, respectively) compared to the lean groups (1.95 ± 0.10 g and 1.79 ± 0.09 g, for LNC and LNE, respectively) ($P < 0.001$). Analysis of the soleus:body weight ratios showed that the OBC ($0.55 \pm 0.04\%$) and OBE ($0.60 \pm 0.04\%$) rats had significantly higher percentages than the LNC ($0.44 \pm 0.02\%$) ($P < 0.05$, OBC; $P < 0.01$, OBE) and LNE rats ($0.60 \pm 0.04\%$) ($P < 0.01$, OBC; $P < 0.001$, OBE).

No difference was observed in the weights of the adrenal glands across all experimental groups. No difference was found in the organ/body weight ratios associated with adrenal glands.

The effect of a treatment on the liver is optimally evaluated using organ:body weight ratio (Bailey et al., 2004). No differences were found in the liver weights across all experimental groups, with the mean liver weight of the LNE group (14.89 ± 0.28 g) being lower than that of the LNC group (15.22 ± 0.50 g) ($P=0.058$). Similar to the trend observed in the

pancreas/body weight percentages, a significant difference in the liver/body weight percentage was observed with the OBC group ($3.12 \pm 0.07\%$) being significantly lower than the OBE ($3.39 \pm 0.06\%$; $P < 0.01$), LNC ($3.62 \pm 0.19\%$; $P < 0.01$) and LNE ($3.39 \pm 0.07\%$; $P < 0.05$) rats. *C. sativa* treatment did not affect liver:body weight percentage levels in lean rats and restored the ratio of OBE rats to levels similar to lean rats.

2.3.4 Effect of *C. sativa* treatment on blood profile

2.3.4.1 Fasting plasma glucose levels

There was no significant difference in fasting glucose levels between the obese and the lean rats before treatment with *C. sativa* started (figure 2.14). Over the treatment period there was a reduction in the fasting blood glucose in obese rats, resulting in an average fasting blood glucose level of 3.84 ± 0.18 mM in obese rats at the end of the treatment period, relative to lean rats, 5.48 ± 0.28 mM ($P < 0.001$). There was no significant change in fasting blood glucose levels in LNC and LNE rats over time or due to *C. sativa* treatment.

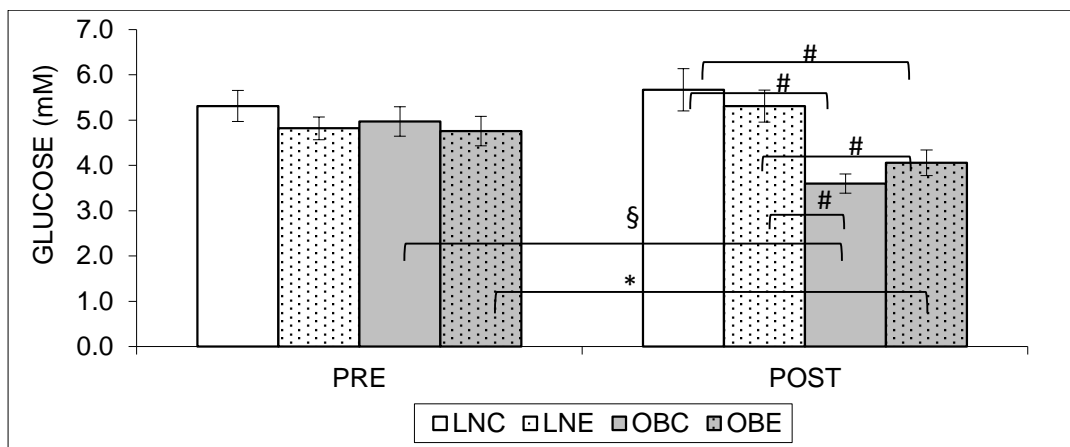


Figure 2.14 Effect of *C. sativa* treatment on fasting blood glucose levels in lean and obese rats.

* $P < 0.05$, pre-OBE relative to: post-OBE

§ $P < 0.01$, pre-OBC relative to: post-OBC

$P < 0.001$, post-LNC relative to: post-OBC, post-OBE; post-LNE relative to: post-OBC, post-OBE

2.3.4.2 Postprandial blood glucose levels

Postprandial blood glucose was monitored on a weekly basis for the period over which *C. sativa* extract was administered to the rats (table 2.10).

Table 2.10 Overview of postprandial blood glucose levels across all experimental groups for the duration of the experiment.

Experimental Groups	Blood Glucose (mM)			
	Day 1	Day 7	Day 14	Day 28
LNC	6.23 ± 0.28	5.80 ± 0.26	4.15 ± 0.19 ^b	7.65 ± 0.38 ^{ac}
LNE	6.80 ± 0.49 ^{bc}	5.39 ± 0.41 ^a	3.85 ± 0.26 ^{ab}	6.81 ± 0.49 ^c
OBC	4.90 ± 0.24 ^b	4.95 ± 0.19 ^a	5.26 ± 0.52	6.27 ± 0.29 ^{bc}
OBE	4.49 ± 0.40 ^b	5.84 ± 0.30 ^{ab}	4.74 ± 0.28 ^a	6.04 ± 0.29 ^b

Within groups:

Day 1 vs Day 7: LNE (^aP < 0.05); OBE (^bP < 0.01)

Day 7 vs Day 14: LNC (^bP < 0.01); LNE (^bP < 0.01); OBE (^aP < 0.05)

Day 14 vs Day 28: LNC (^cP < 0.001); LNE (^cP < 0.001); OBE (^bP < 0.01)

Within groups - pre- and post-experimental:

^aP < 0.05 - LNC

^bP < 0.01 - OBE

^cP < 0.001 - OBC

Between groups:

^aP < 0.05, relative to LNC: OBC (day 7)

^aP < 0.05, relative to OBC: OBE (day 7); LNE (day 14)

^aP < 0.05, relative to OBE: LNE (day 14)

^bP < 0.01, relative to LNC: OBC (day 1, 28); OBE (day 1, 28)

^bP < 0.01, relative to OBC: LNE (day 1)

^cP < 0.001, relative to OBE: LNE (day 1)

Blood glucose levels comparisons within each experimental group showed significant changes in lean rats were significantly different from the obese rats on day 1 of the study, while LNC rats maintained this significant difference on day 28 as well, but not LNE rats (see table 2.10). Significant differences were observed between the pre- and post-experimental blood glucose levels in all experimental groups LNC (P < 0.05), OBC (P < 0.001) and OBE (P < 0.01), except LNE. Inter-group comparisons showed that no significant differences in blood glucose levels were observed between the LNC and LNE rats for the duration of the study. Blood glucose levels between the lean and obese rats were significantly different on day 1, while LNC rats maintained a significantly higher level of blood glucose compared to both obese groups. No significant differences in blood glucose levels were observed between OBC and OBE rats, except on day 7 where OBE rats had significantly higher levels at 5.84 ± 0.30 mM (P < 0.05) compared to OBC levels at 4.95 ± 0.19 mM. Diet induced a significantly lower blood glucose level in OBC rats, with *C. sativa* extract inducing a further reduction in blood glucose levels in OBE rats, relative to LNC rats.

2.3.4.3 IPGTT

Figure 2.15 provides a graphic representation of the pre-experimental IPGTT of lean and obese rats which showed no statistical differences between their response and recovery profiles. The AUC_g of the lean and obese rats were 755.57 ± 27.52 and 788.89 ± 20.37 , respectively.

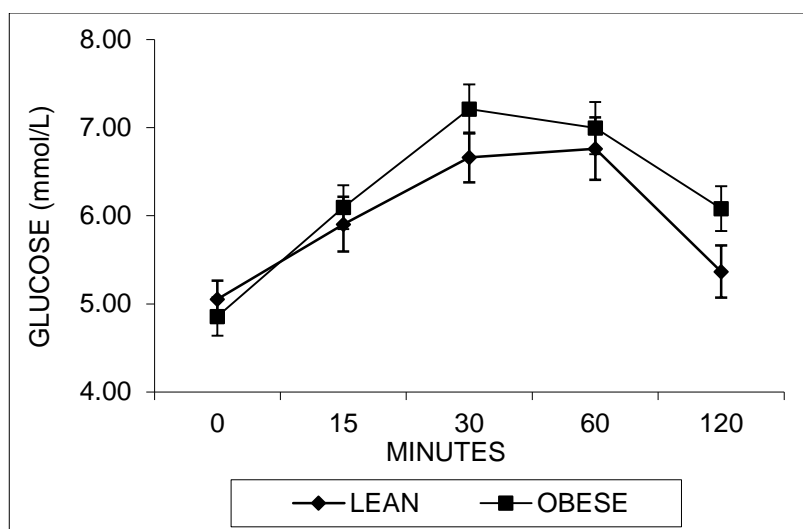


Figure 2.15 Pre-experimental IPGTT of lean (n=21) and obese (n=28) rats.

Figure 2.16 provides a graphic representation of the post-experimental IPGTT of all experimental groups. Plasma glucose in the LNE group (6.32 ± 0.21 mM) differed significantly from that of the LNC group (7.31 ± 0.27 mM) during the initial response period (30 minutes) to the glucose bolus ($P < 0.01$), but no significant differences were observed during the recovery period (post 30 minutes). *C. sativa* extract induced a significant improvement in glycaemia over the two hour period, resulting in an 8% reduction in the AUC_g of LNE (707.45 ± 21.87 , $P < 0.05$) rats, relative to LNC (769.20 ± 14.36) rats (table 2.10).

Significant differences were observed in the initial response and recovery blood glucose levels of the OBC and OBE rats, relative to the LNC and LNE rats ($P < 0.001$ for all time points). No significant differences were induced by *C. sativa* treatment in the OBE rats during the response and recovery phases, relative to OBC rats.

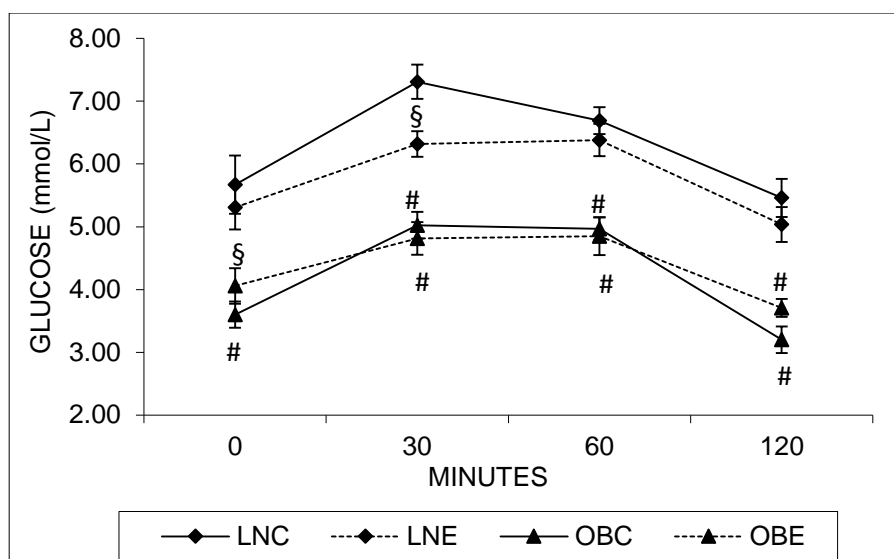


Figure 2.16 IPGTT of obese rats treated with *C. sativa* for 28 days compared to untreated control rats.

Inter-group analysis of AUC_g confirms significant differences between lean and obese rats ($P < 0.001$ for all comparisons) (table 2.10). No significant difference was observed between the pre- and post-experimental AUC_g associated with LNC rats. *C. sativa* treatment significantly lowered (8%) the AUC_g in the LNE rats relative to the LNC rats ($P < 0.05$), thus improving responsiveness to glucose stimulation. The cafeteria diet induced a significant improvement in glucose uptake in OBC rats, relative to LNC rats ($P < 0.001$). *C. sativa* treatment maintained the significant difference in AUC_g between OBE and LNC rats ($P < 0.001$) as well as LNE rats ($P < 0.001$). However, relative to OBC rats, *C. sativa* treatment showed no significant effect on the AUC_g of OBE rats.

Table 2.11 Total area under the curve for IPGTT (AUC_g)

Experimental Group	AUC_g (IPGTT) mM * minute
LNC	769.20 ± 14.36
LNE	707.45 ± 21.87^a
OBC	524.31 ± 13.22^b
OBE	534.75 ± 20.83^{bc}

^a $P < 0.05$, relative to LNC; ^b $P < 0.001$, relative to LNC; ^c $P < 0.001$, relative to LNE

2.3.4.4 Plasma insulin levels

All the data pertaining to insulin and leptin levels relate to rats in a fed state.

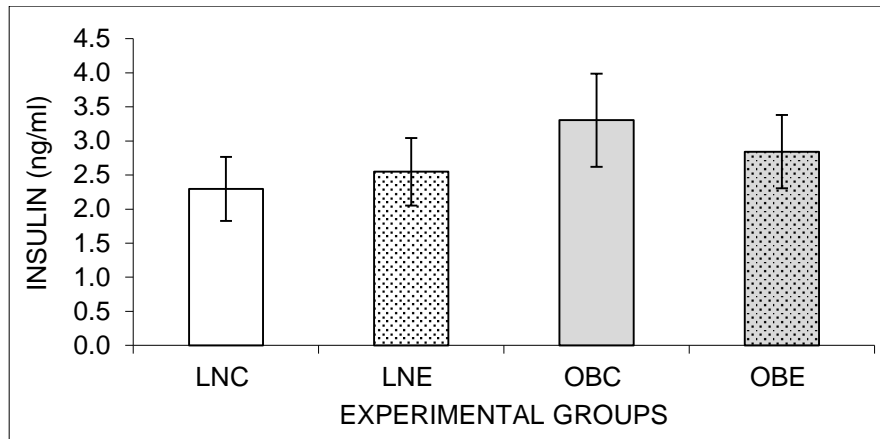


Figure 2.17 Plasma insulin levels in fed rats treated with *C. sativa* extract as determined using the Linco Rat Insulin RIA. LNC (n=10); LNE (n=11); OBC (n=13); OBE (n=13).

A comparison of the insulin levels measured with the RIA showed no significant differences between the various experimental groups (figure 2.17). One of the values (13.91 ng/mL) was statistically excluded from the OBE group. The average insulin measured in OBC rats (3.30 ± 0.68 ng/mL) increased by 1.44-fold, relative to the LNC rats (2.30 ± 0.47 ng/mL), but this increase was not statistically significant. The average level of insulin secreted in OBE rats (2.84 ± 0.54 ng/mL) was 0.86-fold the level found in OBC rats. OBE insulin levels showed a 1.24-fold increase, relative to LNC rats.

2.3.4.5 Plasma leptin levels

The average leptin level in LNE rats (234.84 ± 32.71 pmol/L) showed a significant (0.56-fold) reduction, relative to LNC rat leptin levels (419.58 ± 70.76 pmol/L; $P < 0.05$) (figure 2.18). Leptin levels associated with the obese rats showed a 1.43- and 1.26-fold increase in OBC (598.87 ± 94.90 pmol/L) and OBE (528.88 ± 38.50 pmol/L) rats, relative to LNC rats. These changes were not statistically different for the LNC rats, however, the levels in the LNE rats were significantly lower than levels secreted in the OBC ($P < 0.01$) and OBE rats ($P < 0.001$).

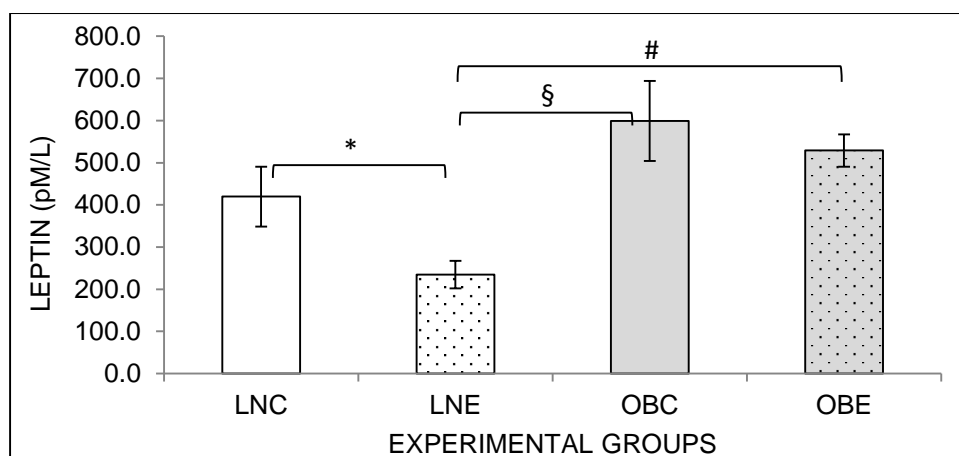


Figure 2.18 Effect of *C. sativa* treatment on plasma leptin levels in fed lean and obese rats, using the Lincoplex Rat Endocrine immunoassay kit. LNC (n=6); LNE (n=8); OBC (n=7); OBE (n=6).

*P < 0.05, LNC relative to: LNE

§P < 0.01, LNE relative to: OBC

#P < 0.001, LNE relative to: OBE

2.3.4.6 Plasma lipid profile and atherogenic indices

Table 2.12 summarizes the lipid profile of across the respective experimental groups where data is expressed as the average \pm standard deviation, where the standard deviation represents triplicate values). *C. sativa* treatment reduced the lipid profile in LNE rats, relative to LNC rats (not statistically significant) as follows: TG (0.75-fold), TC (0.83-fold, P = 0.09), HDL (0.52-fold, P = 0.10), LDL (0.41-fold) and VLDL (0.75-fold).

Table 2.12 Effect of *C. sativa* treatment on triglycerides (TG), total cholesterol (TC), HDL, LDL and VLDL of pooled rat plasma samples. Data is presented as the average \pm SD.

	Concentration (mM)				
	TG	TC	HDL	LDL	VLDL
LNC	1.02 \pm 0.21	0.49 \pm 0.07	0.15 \pm 0.07	0.55 \pm 0.26	0.47 \pm 0.10
LNE	0.76 \pm 0.14	0.40 \pm 0.04	0.08 \pm 0.04	0.23 \pm 0.17	0.35 \pm 0.06
OBC	1.10 \pm 0.03 ^c	0.78 \pm 0.01 ^{be}	0.35 \pm 0.01 ^{ad}	0.72 \pm 0.04 ^c	0.51 \pm 0.01 ^c
OBE	1.08 \pm 0.02 ^c	0.74 \pm 0.01 ^{bef}	0.33 \pm 0.02 ^{ae}	0.67 \pm 0.02 ^c	0.50 \pm 0.01 ^c

^aP < 0.05, relative to LNC; ^bP < 0.01, relative to LNC; ^cP < 0.05, relative to LNE; ^dP < 0.01, relative to LNE; ^eP < 0.001, relative to LNE; ^fP < 0.001, relative to OBC

The cafeteria diet increased the overall lipid profile of the OBC rats, relative to the LNC rats in the following manner: TG (1.08-fold), TC (1.60-fold, P < 0.01), HDL (2.27-fold, P < 0.05), LDL (1.31-fold) and VLDL (1.09-fold). The lipid profile of the OBC rats was significantly higher than that of the LNE rats across all the lipid categories (see Table 2.12) Similar to the

LNE rats, *C. sativa* treatment reduced the lipid profile of OBE resulting in the following lipid profile, relative to LNC rats: TG (1.06-fold), TC (1.51-fold, $P < 0.01$), HDL (2.20-fold, $P < 0.05$), LDL (1.22-fold) and VLDL (1.06-fold). The lipid profile of OBE was significantly higher than the LNE rats as well (see Table 2.12). *C. sativa* treatment significantly reduced the TC levels in OBE rats to 0.95-fold ($P < 0.001$), relative of OBC rats, while all other lipid categories in OBE rats showed a reduction, despite the reduction not being statistically significant.

Changes in lipid profiles affect the AIP of these rats and Table 2.13 summarises the effects of the cafeteria diet and *C. sativa* treatment on the AIP across the various experimental groups.

Table 2.13 Atherogenic indices of rat plasma samples. Data is presented as the average \pm SD

GROUPS	AIP
LNC	0.83 \pm 0.20
LNE	0.99 \pm 0.17
OBC	0.50 \pm 0.02 ^a
OBE	0.52 \pm 0.01 ^a

^a $P < 0.05$, relative to LNE

C. sativa treatment increased the AIP in LNE relative to LNC rats: card to 1.19-fold, relative to LNC rats. Surprisingly, the cafeteria diet reduced the AIP in OBC rats to 0.60- and 0.51-fold ($P < 0.05$), relative to LNC and LNE rats, respectively. Similarly, *C. sativa* treatment in OBE rat sustained the reduction in AIP to 0.63- and 0.53-fold, relative to LNC and LNE rats, respectively. In both experimental groups where *C. sativa* treatment was used, the AIP increased, however, this was not significantly different to the respective untreated rats.

2.3.5 qPCR

Figure 2.19 illustrates a typical standard curve for quantifying mRNA levels. The example used is for CB1 receptor mRNA quantification. The unknowns used were isolated control islets and untreated pancreatic tissue, to test expression levels relative to the known standards.

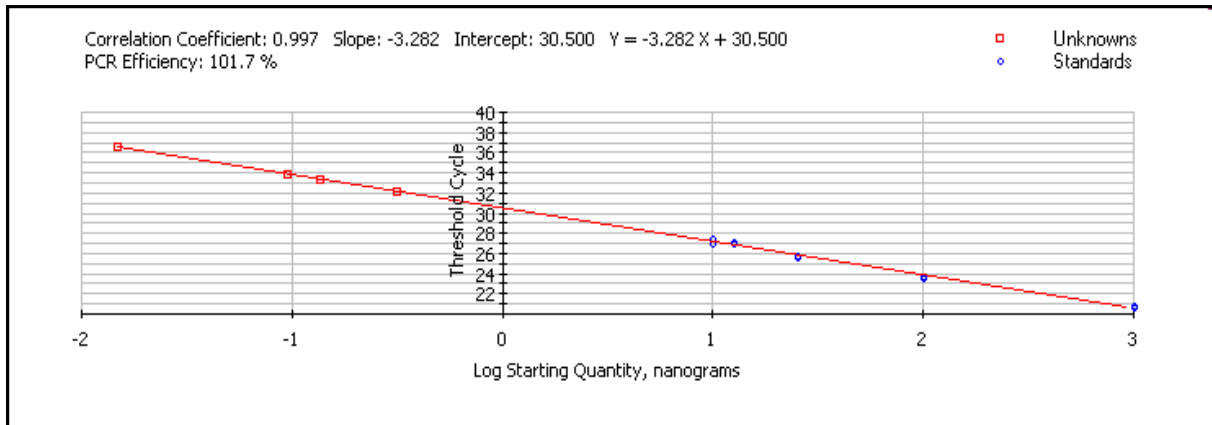


Figure 2.19 A typical qPCR standard curve generated using CB1 receptor primers and untreated rat brain cDNA ranging from 5 to 1000 ng, showing a PCR amplification efficiency = 101.7%, and $R^2 = 0.997$. All samples were prepared in triplicate.

2.3.5.1 GIO analysed using REST

Analysis was conducted on the pancreatic tissues from pooled pancreatic samples as reflected in table 2.14. Annexure D provides tables containing more details regarding the statistical analysis.

Table 2.14 Summary of the relative gene expression ratios of selected genes in pancreatic tissue across the various experimental groups.

Gene	Fold change (relative to LNC)			Fold change (relative to OBC)
	LNE	OBC	OBE	OBE
CB1	1.229	0.333	1.033	<u>2.923^d</u>
c-MYC	1.055	<u>0.009^a</u>	<u>0.025^a</u>	2.676
FLIP	0.432	<u>0.177^a</u>	0.386	2.053
GLUT2	1.997	0.568	1.667	<u>2.765^c</u>
INSULIN	0.201	0.441	0.541	1.156
PDX-1	1.462	1.479	1.123	0.716
PKB	1.041	0.495	1.345	<u>2.562^c</u>
UCP2	0.401	<u>0.137^b</u>	0.466	<u>3.199^e</u>

^aP < 0.05, relative to LNC: OBC (c-MYC); OBC (FLIP); OBE (c-MYC)

^bP < 0.01, relative to LNC: OBC (UCP2)

^cP < 0.05, relative to OBC: OBE (PKB)

^dP < 0.01, relative to OBC: OBE (CB1)

^eP < 0.001, relative to OBC: OBE (GLUT2; UCP2)

LNE rats showed no significant differences in the gene expression ratios of selected GOI subsequent to *C. sativa* treatment, relative to LNC rats. Despite GLUT2 showing a 2.0-fold increase in expression levels, it was not found to be statistically significant. PDX-1 showed an increase to 1.5-fold, while FLIP (0.43-fold), UCP2 (0.40-fold) and insulin (0.20-fold) showed a reduction, relative to LNC rats.

OBC rats showed a significant down-regulation in the expression ratios of c-MYC (P = 0.032), FLIP (P = 0.038) and UCP2 (P = 0.004) levels, while PDX-1 levels showed a 1.5-fold increase (albeit not statistically significant), relative to LNC rats.

All other GOI showed down-regulation in expression, but these changes were not statistically significant. OBE rats showed a significant reduction in c-MYC levels (P=0.016) to 0.03-fold, relative to LNC rats. All other GOI were found not to be significantly different to expression levels of LNC rats.

In OBE rats, *C. sativa* treatment induced an increase in most of the genes investigated, except PDX-1, where expression was reduced to 0.72-fold of that observed in OBC rats. The following genes were significantly up-regulated: CB1 (2.92-fold, P = 0.006), GLUT2 (2.77-fold, P < 0.0001), PKB (2.56-fold, P = 0.03) and UCP2 (3.20 fold, P < 0.0001), relative to OBC rats.

The results observed in this study will now be discussed in the context of previous research and highlighting key findings.

2.4 DISCUSSION AND CONCLUSIONS

Rodents are commonly used in diabetic studies, and most anti-obesity and anti-diabetic agents are taken into clinical trial phase on the basis of their efficacy in rodent models. Studies are conducted in which obesity, T2D, insulin resistance, or environmentally-induced factors (such as dietary treatment) are used to determine the mechanisms of the biological events associated with or responsible for the pathogenesis of the disease. Most of these rodent models, despite the development of certain aspects of the diabetic phenotype, e.g. hyperinsulinemia and hyperglycemia, do not develop all the complexities associated with diabetes, thus allowing research to be conducted into the pathogenesis of the disease. The onset of T2D in humans and non-human primates occur over many years, while in rodents,

the phenotypic changes occur within the first twelve months of the rodent's life (Ross et al., 2004). The choice of the DIO rat model was to enable the researcher to investigate the effect of DIO and *C. sativa* treatment on the responsiveness of β -cell functionality. It should however be noted that despite achieving a significant increase in body weight, the DIO model used in the current study did not show hyperglycemia or glucose intolerance. The lack of manifestation of impaired glucose tolerance and/or insulin resistance within this DIO model was a major limitation of this study, since the researcher could not determine whether *C. sativa* treatment affected obesity-induced glucose intolerance and/or insulin resistance. No conclusions could therefore be made in relation to the effect of *C. sativa* treatment on modulating insulin resistance in this DIO rat model.

DIO has become a global health problem, with its associated increased risk of life-threatening pathologies such as hypertension, CVD and diabetes. Weight loss has been reported to ameliorate these associated conditions (Trillou et al., 2003). DIO rat models have shown a strong correlation to human genetic and dietary influences associated with obesity and the MetS (Sampey et al., 2011; Sclafani and Springer, 1976). The cafeteria diet is considered a robust model of the human metabolic syndrome with regards to liver and adipose tissue inflammation (Sampey et al., 2011), and has been shown to be the most effective induction model of obesity in rats, specifically for the use of investigating the anti-obesity potential of novel drugs (Reuter, 2007). Cafeteria diets have been associated with greater hyperphagia (Kennedy et al., 2010; Morris et al., 2008; Kretschmer et al., 2005; Rodríguez et al., 2004). These studies support the observation of hyperphagia in the DIO rats of the current study.

The model of cafeteria diet used in this study contained 330 g/kg standard pellets, 330 g/kg Nestlé full-fat sweetened condensed milk, 70 g/kg sugar and 270 g/kg water. Brandt et al. (2010) used the above palatable diet with an energy composition of 69% carbohydrates, 15% protein and 16% fat and exposed male Wistar rats for 12 weeks. Results from their study showed that the cafeteria-style diet induced a 10.1% increase in weight where the obese rats weighed 522 ± 9 g (a total gain of 305 g), relative to the control rats that weighed 474 ± 16 g (a total gain of 256 g) after being fed the standard Muracon-G pellets (63% carbohydrates, 26% protein and 11% fat). The blood profile of the control rats was 3.3 ± 0.15 mmol/L for fasting glucose and 0.9 ± 0.14 ng/mL for fasting insulin levels, while the obese rats measured 3.66 ± 0.16 mmol/L for fasting glucose and 2.52 ± 0.25 ng/mL for fasting insulin, with only

the insulin being significantly higher than the control rats. In a study conducted by Naderali et al. (2001), male Wistar rats were exposed for 12 weeks to a cafeteria diet with an energy composition of 68% carbohydrates, 16% protein and 13% fat. Results from their study showed that the cafeteria-style diet induced a 12.4% increase in weight where the obese rats weighed 523 ± 11.1 g (a total gain of 271.5 g), relative to the control rats that weighed 465.6 ± 8.6 g (a total gain of 221.0 g) after being fed the standard laboratory pellets supplied by CRM Boisure (Cambridge, UK) that consisted of an energy composition of 76.8% carbohydrates, 19.2% protein and 4.3% fat. Their control rats had plasma glucose levels at 5.48 ± 0.38 mmol/L and plasma insulin levels at 7.30 ± 0.88 ng/mL. This was significantly different from levels in their obese rats, with plasma glucose and insulin levels at 6.07 ± 0.44 mmol/L and 8.55 ± 1.75 ng/mL, respectively.

In the current study, the lean rats were fed standard Epol pellets, with an energy composition of 72.4% carbohydrates; 21.3% protein and 6.3% fat, while the cafeteria diet had an energy composition of 73.1% carbohydrates, 12.7% protein and 14.2% fat. This translated into the following energy being generated per gram standard Epol pellets: carbohydrates (1.9 kcal/g), protein (5.5 kcal/g) and fat (1.7 kcal/g), while the energy was generated per gram cafeteria diet was as follows: carbohydrates (2.3 kcal/g), protein (4.0 kcal/g) and fat (3.1 kcal/g). The body weight of the lean rats in the current study was comparable to the control rats of both abovementioned studies. The cafeteria diet induced a significant weight gain of 9.7% ($P < 0.05$), relative to lean rats, with OBC rats at 488.24 ± 11.68 g and the LNC rats at 445.08 ± 14.93 g. Despite the significant weight gain induced by the cafeteria diet, the weight gain was substantially lower compared to that reported by Brandt et al. (2010) and Naderali et al. (2001). The plasma glucose of the control rats was 7.65 ± 0.38 mmol/L (5.67 ± 0.46 mmol/L for fasting glucose) and 2.30 ± 0.47 ng/mL for plasma insulin levels, while plasma glucose for the obese rats measured 6.27 ± 0.29 mmol/L (3.60 ± 0.21 mmol/L for fasting glucose) and 3.30 ± 0.68 ng/mL for plasma insulin levels. The blood glucose and insulin levels of the obese rats associated with both Brandt et al. (2010) and Naderali et al. (2001) supported that observed in the current study, while the blood glucose associated with the control rats were significantly lower than the levels observed in the LNC rats. However, both blood glucose and blood insulin of the LNC rats were significantly higher in the current study compared to that observed by both Brandt et al. (2010) and Naderali et al. (2010), despite their body weights being comparable. This may have contributed to the higher AUC_g profile in LNC

rats, relative to the OBC rats. The dairy component of the cafeteria diet (full cream milk and whey) may have contributed to improved insulin sensitivity in the OBC and OBE rats. Dairy enhances lipid and glucose metabolism in the liver and skeletal muscle of diet-induced obese rats. The significant increase in skeletal muscle mass together with the increase in insulin levels (albeit not statistically significant), may also have contributed to the reduction in the AUC_g observed in the OBC rats.

There was no statistical difference between the pre-experimental fasting glucose levels across all four groups. However, analysis of post-prandial glucose levels showed significant differences between the pre-experimental lean and obese groups. These significant differences were also reflected in post-experimental post-prandial blood glucose levels of LNC and OBC rats. In addition, comparative analysis between pre- and post-experimental fasting glucose levels showed that only the OBC rats had significantly lower fasting glucose levels ($P < 0.01$), while OBE rats also showed lower fasting glucose levels, but it was not statistically significant ($P = 0.086$), relative to LNC rats. These differences in pre- and post-experimental post-prandial blood glucose levels may have been indicative of changes in the glucose regulation induced by the respective diets. Differences in responsiveness to glucose metabolism were confirmed by the significant differences observed in the AUC_g of the LNC (769.20 ± 14.36) and OBC (524.31 ± 13.22) rats ($P < 0.001$) when the IPGTTs were conducted. When pre-experimental IPGTTs were conducted with the lean and obese rats, the AUC_g for the lean rats (755.57 ± 27.52) was not significantly different from that of the obese group (788.89 ± 20.37), thus confirming that the cafeteria diet did not induce glucose intolerance. A factor which may have contributed to the improved glycemic profiles in the OBC rats could be one of the dietary components of the cafeteria diet. Despite being fed high-carbohydrate low-fat diets, the rats responded differently when their glucose tolerance was assessed. The lean (non-obese) rats showed significantly impaired glucose tolerance relative to the obese rats ($P < 0.001$). One of the factors which may have in part contributed to this unusual observation could be the use of condensed milk in the modified cafeteria diet. A growing body of evidence have been published that shows the benefits of milk and dairy consumption which enhance insulin sensitivity, inflammation, oxidative stress, reduce the risk of diabetes and CVD in humans and rodents (Crichton and Alkerwi, 2014; Tong et al., 2014; Aune et al., 2013; Eller et al., 2013; Crichton et al., 2011; Eller and Reimer, 2010; Elwood et al., 2008). According to the CODEX standard for condensed milks issued by FAO

(2011), milk, milk powder, cream, cream powders and milkfat products form part of its composition, with the product having to contain at least 8% m/m milkfat, 28% m/m milk solids and 34% m/m milk protein in milk solids-not-fat. Supplementation with whey has been shown to improve insulin resistance in non-obese male Wistar rats (Tong et al., 2014), while Eller et al. (2013) found that whole-body insulin sensitivity was improved, independent of adiposity, in DIO Sprague–Dawley rats treated for 8-weeks on a leucine-supplemented diet composed of 42% carbohydrates, 10% protein. They found that diets containing complete dairy and leucine modulated fatty acid metabolism and glucose homeostasis in both liver and muscle tissue. These beneficial effects associated with dairy consumption in DIO and insulin resistant rats support the findings in the current study, where obese rats showed a better glycemic response after consumption of the cafeteria diet (containing milk products), relative to lean rats fed on a high-carbohydrate low-fat diet. In addition, it is well established that skeletal muscle plays a crucial role in glucose homeostasis by disposing the bulk of whole-body insulin-mediated glucose. The hyperphagic consumption of the cafeteria diet together with the significant increase in skeletal muscle mass in the obese rats (see figure 2.13), may thus have contributed to the overall reduction in the AUC_g associated with their IPGTTs, relative to the LNC rats, as well as the lowering of the post-experimental fasting blood glucose levels in the obese rats, relative to their pre-experimental blood glucose levels.

A significant body of evidence exists that associate *C. sativa* use with reduced body mass index, lower blood glucose levels despite increased appetite and caloric intake. Data collected from the National Health and Nutrition Examination Survey III showed that *C. sativa* use was associated with a lower body mass index, significantly lower blood glucose levels and decreased prevalence of diabetes (Rajavashisth et al., 2012). In another study conducted by Penner et al. (2013) it was found that frequent *C. sativa* users had significantly lower blood glucose than non-*C. sativa* smokers. This data supports the findings from the present study, where the AUC_g of LNE rats was significantly lower than the LNC rats ($P < 0.05$) (see table 2.11). Interestingly, *C. sativa* treatment significantly increased the initial insulin response, relative to LNC rats, but no difference was observed during the recovery phase. This significantly reduced the AUC_g in LNE rats, relative to LNC rats. The LNE group consumed significantly more food (50.03 ± 0.50 g per rat per day), relative to the LNC group (45.37 ± 0.12 g per rat per day) ($P < 0.001$). Despite the higher food intake in LNE rats, the LNE rats showed a significant reduction in weight gain as well as a significant improvement in glucose

tolerance, relative to LNC rats. These observations is supported by results from previous studies (Sansone and Sansone, 2014; Penner et al., 2013; Rajavashisth et al., 2012). Food consumption in the obese group was significantly higher than that of the lean rats. *C. sativa* treatment induced a significant decrease in food intake in OBE rats (57.42 ± 0.44 g per rat per day, $P < 0.001$), relative to OBC rats (64.61 ± 1.18 g per rat per day), which may have contributed, in part, to the significant reduction in body weight in the OBE rats ($P < 0.01$). No conclusions can be made regarding the effect of *C. sativa* treatment on weight loss in the OBE rats, since the reduction in food consumption may have contributed to the weight loss observed in the OBE rats. Furthermore, no conclusions can be made regarding the effect of *C. sativa* treatment on DIO-related insulin resistance and glucose intolerance, since the modified cafeteria model used in this study did not result in glucose intolerance and/or insulin resistance. *C. sativa* treatment induced weight loss in the lean rats which may have contributed to the significant improvement in glucose tolerance observed in these rats.

The majority of animal studies conducted on the effect of *C. sativa* and/or various cannabinoid treatments on food intake, have either failed to find an effect on eating behaviour, or found that cannabinoids induced an anorectic or hyperphagic effect (Pagotto et al., 2006; Kirkham and Williams, 2001a&b). Drewnowski and Grinker (1978) found that 15 days of THC treatment resulted in a reduction of food and water intake with simultaneous reduced activity levels. Rahminiwati and Nishimura (1999) found that in male *ddY* strain mice treated for 7 days with THC (4 mg/kg), hyperphagia was induced and significant weight loss observed. Research findings suggest that THC, as the active agent in *C. sativa*, exerts a biphasic dose response, with appetite stimulation at low doses and inhibition at high doses (Cota et al., 2003a; Berry and Mechoulam, 2002; Evans et al., 1976). In this study, *C. sativa* exposure showed a varied effect on the feeding behaviour of LNE and OBE rats. *C. sativa* administration over the 28 day period had both a hyperphagic (lean rats) and an anorectic (obese rats) effect (see table 2.8). Food consumption in the OBE rats was 15% higher than in LNE rats ($P < 0.001$), which supports the findings of Koch (2001) where a higher volume of high-fat and sweetened high-fat diet was consumed, relative to chow-fed rats. It has been reported in Indian literature that *C. sativa* was used as an appetite suppressant to overcome the sensation of hunger, therefore the dual role of *C. sativa* could possibly be due to the different potencies of the *C. sativa* preparation, where weak preparations ('bhang') acts as a stimulant, and more potent ones ('ganja', 'chara') as an appetite suppressant (Cota et al.,

2003a). Other factors have been proposed to contribute to the biphasic effect of THC on food intake, including the differential involvement of the G_s and G_i proteins, which are activated at high and low doses, respectively, activation of presynaptic cannabinoid receptors by low doses of cannabinoid, and storage of unused cannabinoids in fat deposits (Hall et al., 2001; Sulcova et al., 1998). In a study conducted by Kreuz and Axelrod (1973), radiolabeled THC was injected subcutaneously into rats for 26 days. They found that fat tissue, relative to brain tissue, contained 21-fold higher levels of THC and its metabolites after seven days of consecutive exposure, and 64-fold higher levels after 27 days. It is important to note that THC and its metabolites are highly lipid-soluble, and accumulate in the fatty tissues of the body and are slowly released into other body compartments. Interestingly, unmetabolized THC can be stored in the fatty tissues for more than 28 days (Hall et al., 2001). The half-life of THC in fatty tissues is approximately 8 days (Ashton, 2001), with complete elimination of a single dose taking up to 30 days (Maykut, 1985).

Cannabinoids are metabolized in the liver, after which they are excreted in urine (25%), and mainly into the gut (65%), from where they are reabsorbed, further prolonging their actions (Ashton, 2001). The body weights of the lean group were significantly lower ($P < 0.05$) than the obese group (see table 2.8), and the lower level of fat tissue present in the lean rats (relative to the obese rats), may have contributed to a reduced storage capability of THC (and possibly other cannabinoids) in these rats. The presence of increased adipose tissue in the OBE rats may have contributed to increased accumulation of THC and its metabolites in the fatty tissues, resulting in higher levels of THC being released within these rats during the experimental period, thus leading to the reduction in food intake. It is therefore proposed that the retention of THC and its metabolites in the fat depots partially contributed, in part, to the different responses to food consumption in the LNE and OBE rats. Further to this, research has shown that endocannabinoids, orexigenic agents which play a regulatory role in food intake via activation of CB1 receptors in the hypothalamus, are negatively modulated by leptin, an anorexigenic hormone released by adipocytes (Di Marzo et al., 2001). Di Marzo et al. (2001) found that in obese *ob/ob* and *db/db* mice as well as Zucker rats with defective leptin signaling, both 2-AG and AEA levels were elevated in the hypothalamus, whilst a single injection of leptin into normal rats and *ob/ob* mice reduced levels of 2-AG and AEA in the hypothalamus (Di Marzo et al., 2001). Jo et al. (2005) subsequently conducted experiments on lateral hypothalamic neurons that are essential for the control of food intake.

They found that these neurons were subject to CB1-mediated depolarization-induced suppression of inhibition, and that leptin exposure blocked the voltage-gated Ca^{2+} channels of these neurons. In these neurons, the synthesis of endocannabinoids in response to direct depolarization is strongly dependent on increased calcium influx (Di Marzo et al., 1998). Reduced Ca^{2+} influx therefore diminished endocannabinoid release which in turn affected the excitability of these appetite-regulating neurons. The lower leptin levels in LNE rats may therefore have reduced the leptin-induced inhibitory effect on endocannabinoid release, which may in part also have contributed to the increase in food intake, relative to the LNC rats. In addition, with a lower endocannabinoid tone in lean subjects, *C. sativa*/THC treatment may have increased central CB1 receptor stimulation, thus increasing food intake in LNE rats. However, Matias et al. (2006) showed peripheral endocannabinoid overactivity in rodent and human obesity. This may explain, in part, the hyperphagia associated with the obese rats in this study, as is generally found in diet-induced obesity. Despite the hyperphagia, *C. sativa* treatment induced a decrease in food intake in the OBE rats. This may, in part, be due to THC acting as an antagonist to CB1 receptors in situations of increased endocannabinoid tone, such as obesity (Di Marzo and Matias, 2005), thus resulting in reduced food intake, which was observed in OBE rats, relative to OBC rats. The biphasic effect of THC as reported in previous studies (Cota et al., 2003a; Berry and Mechoulam, 2002; Evans et al., 1976) therefore support the findings in this study.

Adiposity directly influences leptin levels. Leptin is one of the key adipokines released by adipose tissue, which is viewed as an adipostat, monitoring the balance between sufficient and insufficient energy stores, and providing a signal to the brain when the balance is disturbed (Frederich et al., 1995). During times of low energy intake (eg. fasting or starvation), leptin levels decrease which signals the brain to promote increased energy intake, reduced energy expenditure, and increased fat energy storage (Flier, 1998; Frederich et al., 1995). DIO is normally characterized by high plasma leptin levels in humans and rodents, which are in direct proportion to the amount of adipose tissue (Levin and Dunn-Meynell, 2002). Chronic use of *C. sativa* has been associated with reduced body weights as well as reduced weight gain (Le Foll et al., 2013). In a study conducted by Klein et al. (2011), chronic (i.e. 21 days) exposure to injections of THC or a 1:1 THC and CBD combination resulted in reduction in body weight in both treatment groups, relative to untreated rats. Interestingly they also found that, relative to untreated rats, the rats on THC/CBD combination treatment

showed a significantly lower body weight on Day 10, while rats only treated with THC showed a significant lower body weight on Day 14. They proposed that CBD potentiated an inhibition of body weight gain, most likely by delaying the metabolism and elimination of THC. Weight loss in rats treated with THC has been associated with changes in the number and morphology of adipocytes and macrophage infiltration (Teixeira et al., 2010), which in turn contribute to the overall reduction in body weight and adiposity. The reduction in body weight and adiposity induced by chronic *C. sativa*/THC treatment supports the findings in the current study, where body weight and epididymal fat levels were significantly lower in *C. sativa*-treated rats, relative to the respective controls. The lower fat levels in both experimental groups when compared to their respective controls may explain the parallel trend for lower leptin levels in the former groups. However, the leptin levels were only statistically significantly lower in the LNE compared to the LNC animals whilst the difference was not significant in the obese animals. The reason for this is not known however, only epididymal fat mass was measured and therefore differences in total body fat mass, which would be more influential on systemic leptin levels than just the epididymal depot, are not known.

Obesity places increased demand on β -cells, resulting in increased β -cell mass, with a decrease in numbers leading to diabetes (Butler et al., 2003; Bonner-Weir, 2000). Studies have revealed that there is a relationship between β -cell mass and body mass, where a linear correlation exists between β -cell mass and body weight throughout the lifespan of Lewis rats, due to β -cell hyperplasia and hypertrophy (Montanya et al., 2000). The β -cell mass is governed by cell differentiation, neogenesis, proliferation, increased or decreased cell size, apoptosis and necrosis. With obesity and the associated insulin resistance, the physiological compensatory response at the level of the β -cell is to increase β -cell mass via replication/neogenesis and hypertrophy (Lee and Nielsen, 2009; Liu et al., 2005; Pick et al., 1998). β -Cell numbers decrease with increasing age, and animals became glucose intolerant when the β -cell number decreased below ~10 million cells per kilogram body weight (Bouwens and Rooman, 2005). Research has shown that β -cell mass increases in non-diabetic obese humans (Prentki and Nolan, 2006; Kloppel et al., 1985), while non-diabetic obese Zucker Diabetic Fatty (ZDF) *fa-fa* rats showed a four-fold increase in β -cells, relative to the lean controls (Pick et al., 1998). In the present study, no difference was found in the pancreatic tissue mass of the OBC group in relation to the LNC group. However, the pancreas:

body weight percentage of OBC rats ($0.33 \pm 0.01\%$) was significantly lower than LNC rats ($0.40 \pm 0.03\%$) ($P < 0.05$) (see table 2.8). *C. sativa* treatment did not affect the pancreas:body weight percentage of LNE rats ($0.40 \pm 0.02\%$), while in the OBE rats the pancreatic tissue mass was significantly increased by 20%, relative to the OBC rats, resulting in a significant increase in the pancreas:body weight percentage ($0.44 \pm 0.02\%$, $P < 0.001$). The pancreatic tissue was not further analyzed to determine the proportion of α , β , δ and pancreatic polypeptide cells to assess whether the increase in pancreatic tissue mass can be attributed to increased β -cell hyperplasia or hypertrophy.

With blood glucose homeostasis being regulated by the endocrine cells of the pancreas, changes in the composition of the pancreatic tissue affect hormone production and consequently glucose regulation, as is evident in DM patients. Various studies using *in vivo* models to determine the effect of *C. sativa* and/or THC on insulin secretion have generated conflicting results. Matias et al. (2006) conducted a study in which DIO mice showed higher levels of AEA and 2-AG as well as increased insulin levels, relative to lean control mice (Matias et al., 2006). De Pasquale et al. (1978) however found that injecting dogs with *C. sativa* resin containing THC ranging from 2.5 to 10.0 mg/kg body weight, reduced plasma insulin levels in a dose-dependent manner, resulting in increased plasma glucose and ammonia levels. The increased levels of ammonia corresponded with the inhibition of insulin release since ammonia obstructs the entry of calcium into the β -cells, thus preventing the activation of the cytoskeletal system, and consequently reducing the release of insulin. In a US survey conducted on a nationally representative population, Penner et al. (2013) found that current *C. sativa* users had lower levels of fasting insulin, lower insulin resistance (according to the homeostasis model assessment of insulin resistance) and smaller waist circumference. In yet another study conducted on normal and STZ-induced diabetic Sprague-Dawley rats, 7 days of *C. sativa* exposure (3 mg THC/kg body weight) resulted in a significant increase in insulin secretion in the diabetic rats, but had no effect on the insulin levels in the normal rats (Coskun and Bolkent, 2013). In a study conducted on HIV-positive male patients that received chronic *C. sativa* treatment (1% – 8% THC content per cigarette smoked), no change in insulin levels were observed (Riggs et al., 2012). These latter findings support the observations in the present study, where *in vivo C. sativa* extract treatment showed no significant changes to plasma insulin levels in lean or obese rodents when compared against the appropriate control animals (see figure 2.17). Similarly, at the insulin gene level, no significant differences were observed in *C.*

sativa-treated rats, relative to the relevant control animals (see table 2.14). It should be noted that El-Souroy et al. (1966) reported that intramuscular administration of CBN increased blood glucose levels by reducing hepatic glycogen levels. However, the researcher could not find further publications to support these findings.

Insulin is the primary hormone that regulates blood glucose homeostasis, eliciting physiological responses within peripheral tissues that effectively maintain blood glucose levels within physiological parameters. The skeletal muscle is a major site of energy expenditure, with much research being conducted towards the development of agents that will enhance insulin sensitivity to improve glycemic control in insulin resistant individuals with obesity and DM. CB1 receptors are expressed in mice, rat and human skeletal muscle (Pagotto et al., 2006; Liu et al., 2004; Cavuoto et al., 2007a). CB1 receptor levels in the soleus muscle isolated from obese mice are higher, relative to the lean controls (Pagotto et al., 2006), and Liu et al. (2004) found that SR141716 exposure induced increased glucose uptake and oxygen consumption in isolated soleus muscles. In the present study, *C. sativa* treatment had no significant effect on the mass of the soleus muscles of LNE and OBE rats, relative to their respective controls (see figure 2.13). However, when comparing both obese groups to the respective lean groups, statistically significant increases were observed between LNC ($P < 0.01$) and LNE ($P < 0.05$) with regards to the soleus muscle mass and the soleus: body weight percentage level (see table 2.9). These differences in skeletal muscle mass may therefore account, in part, for the changes in body composition induced by the cafeteria diet.

The liver was another peripheral organ that showed a higher liver to body weight ratio in *C. sativa*-treated obese rats, in comparison to non-treated obese rats. In a study conducted on STZ-induced diabetic rats that were subjected to *C. sativa* extract treatments, the liver weights were significantly increased with reduced hepatic and muscle glycogen content (Levendal and Frost, 2006). Yassa et al. (2010) found that chronic (90 days) treatment of Wistar rats with *C. sativa* extract via a gastric tube resulted in areas of necrosis, with congestion of blood vessels, vacuolation or rarified/hyalinized cytoplasm of hepatocytes with their nuclei being dense and irregular. Human studies have shown that in chronic *C. sativa* users, 57.7% have hepatomegaly. However, the hepatomegaly was not due to parenchymal lesion but due to cell hypertrophy and hyperplasia, especially Kupffer components and showed no relationship with the prevalence of

liver enzyme alterations (Borini et al., 2004). No further morphological analysis was conducted on the liver samples of the OBE rats.

Insulin also enhances the storage of triglycerides primarily in white adipose tissue during excess energy consumption, while glucocorticoids modulate fatty acid release (from lipolysis) occur during excess energy expenditure. CB1 receptors and endocannabinoid levels increase during adipocyte differentiation, with CB1 stimulation leading to faster adipocyte differentiation (Matias et al., 2006). In primary cultured adipocytes isolated from obese ZDF rats, CB1 expression increased, relative to their lean controls (Bensaid et al., 2003). In addition, studies conducted on obesity and *C. sativa* use by Le Strat and Le Foll, (2011) and Le Foll et al. (2013) found that participants who did not smoke *C. sativa* showed a 22.0% and 25.3% prevalence of obesity, relative to 14.3% and 17.2% in participants reporting the use of *C. sativa* for 3 or more days a week. These findings support the observations in the present study, where rats exposed to *C. sativa* extract showed a significant reduction in epididymal fat mass, in both lean and obese groups (see figure 2.13).

DIO is associated with impaired glucose tolerance, hyperinsulinemia, dyslipidemia (increased VLDL, small dense LDL, TG, and decreased HDL), a pro-inflammatory and pro-atherogenic state (Macedo et al., 2012). Research has shown that low-fat diets, i.e. less than 30% of total caloric intake, increase circulating VLDL levels, reduce HDL, and thus adversely affects atherogenic dyslipidemia (Siri-Tarino et al., 2010). Consumption of high-carbohydrate diets is associated with increased plasma TG, which was evident in the LNC rats. In the current study, the rats on the cafeteria diet had higher cholesterol and HDL levels compared to the rats on the standard rat chow. Generally, studies conducted using cafeteria diets show an increase in plasma TG, TC, LDL- and VLDL-cholesterol levels, with a reduction in HDL-cholesterol levels, compared to normal chow diets (MacKenzie et al., 2012; Hussein et al., 2011; Naderali et al., 2001). In a study conducted by Hussein et al. (2011), where the TG levels were found to be higher than the levels in the LNC rats in the current study, TG levels in the control rats were 1.31 mmol/l and 2.21 mmol/L in the DIO rats, an increase of 1.69-fold, relative to control rats. Cafeteria diets induce increases in plasma TG ranging from 30 – 90%, however in the current study, the cafeteria diet induced an increase of 7.8% in plasma TG levels in OBC rats, relative to LNC rats. LDL levels were relatively similar to that found in the current study. In MacKenzie et al. (2012) the TG levels were at

0.59 mmol/L in the control rats, and 0.86 mmol/L in the DIO rats, an increase of 1.46-fold, relative to control rats. The profile of other plasma lipids shows that total cholesterol, HDL- and VLDL-cholesterol levels were lower than that observed in lean control rats. The effect of the *C. sativa* treatment in LNE rats showed a significant reduction across all lipid categories including HDL-cholesterol (reduced by 47%), relative to LNC rats. The drastic reduction in HDL-cholesterol resulted in a 1.66-fold increase in cardiac risk ratio (1.66-fold, $P < 0.01$), the atherogenic coefficient (1.93-fold, $P < 0.01$) and the AIP (1.19, $P < 0.05$) of LNE rats (see table 2.12). In Neraldi et al., (2001), the TG levels were at 0.44 mmol/L in the control rats, and 0.79 mmol/L in the DIO rats, an increase of 1.80-fold, relative to control rats. The major difference between the current study and other studies is that the cafeteria diet increased HDL levels, which may explain the more favourable AIP readings in both OBC and OBE groups compared to the lean groups. The reason for the increase in HDL-cholesterol is not known. Each 1 mg/dL (0.0259 mM) increase in HDL is associated with a 6% reduction in CVD risk (Ashen and Blumenthal, 2005). Interestingly, the animals treated with *C. sativa* had lower levels of all 3 lipid molecules when compared to the respective control groups. The reduction in HDL levels (although the difference was not significant) led to higher AIP levels in the *C. sativa*-treated rats, relative to their respective control rats. However, the lowering of HDL levels by *C. sativa* is a cause for concern and requires further investigation. Other studies have also shown lipid lowering effects of *C. sativa* in rodents. In a study conducted by Coetzee et al. (2007) where Wistar rats were also fed a palatable cafeteria diet and standard pellets, the cafeteria diet altered TG levels by 1.11-fold, total cholesterol by 1.30-fold ($P < 0.01$), HDL-cholesterol by 1.10-fold ($P < 0.05$), and LDL-cholesterol by 2.95-fold ($P < 0.01$). Exposure to 28 days of *C. sativa* treatment improved the lipid profile and atherogenic index of lean rats to 0.74-fold for TG, 0.83-fold for TC, 0.83-fold for HDL and 0.78-fold for LDL. This resulted in an AIP of 0.110, relative to 0.120 in the control rats, an improvement of 8%. In the DIO rats, *C. sativa* treatment enhanced the lipid profile and atherogenic index of the obese rats to 0.95-fold of TG, 1.16-fold of TC, 1.27-fold of HDL and 0.83-fold of LDL. This resulted in an AIP of 0.205, relative to 0.312 in the DIO control rats, an improvement of 35%. In another study conducted on STZ-induced diabetic rats, *C. sativa* treatment improved the lipid profile of both lean and diabetic rats (Levendal and Frost, 2006). These results support the observations of the current study where *C. sativa*-treated rats show improved AIPs.

In obesity, Matias et al. (2006) suggest that dysregulation of the endocannabinoid system in the adipocytes and β -cells may contribute to hyperlipidemia, hypoadiponectinemia, and hyperinsulinemia in obesity. Di Marzo et al. (2009a&b) found that insulin infusions into obese and non-obese individuals reduced their plasma endocannabinoid (anadamide and 2-AG) levels in a manner that is inversely related to metabolic predictors of insulin resistance and dyslipidemia. To determine the molecular effect of *C. sativa* on pancreatic tissue, specific GOI that play key roles in β -cell function, proliferation and/or differentiation were investigated, viz. FLIP, PDX-1, c-MYC, GLUT2 and UCP2. These genes and hormones all have a direct and/or indirect effect on β -cell functionality, which in turn affects insulin secretion.

UCP2 has been established as the main determinant of the insulin secretory response to glucose (Krempler et al., 2002). Pi et al. (2009) observed increased oxidative stress and impaired insulin secretion in UCP2 knockout mice, while Produit-Zengaffinen et al. (2007) found that increased UCP2 expression in β -cells did not alter insulin secretion, but enhanced the cellular defence by reducing ROS production. Insulin secretion is highly dependent on the calcium and ATP levels in the β -cell and Dalgaard (2012) found that a reduction in β -cell UCP2 expression is associated with increased islet ATP levels and insulin secretion. Increased UCP2 expression impairs impaired GSIS through impaired glucose sensing and insulin secretion in murine β -cells, both *in vivo* and *in vitro* (Iwasaki et al., 2009). Normal glucose uptake and subsequent metabolic signalling for GSIS cannot be achieved without GLUT2, since β -Cell-specific knockout of GLUT2 results in infant death because of severe hyperglycemia (Thorens et al., 2000). Ohneda et al. (1995) found in pre-diabetic male Zucker diabetic fatty rats that their beta cells exhibited high basal insulin secretion, absent insulin response to glucose and loss of GLUT2 glucose transporters. In addition their β -cell volume had increased at the onset of diabetes, but declined thereafter. β -Cell-specific knockout of GLUT2 results in infant death because of severe hyperglycemia, which was confirmed in diabetic subjects, where GLUT2 expression decreased before the loss of GSIS. (Ohneda et al., 1995).

Therefore according to above, a reduction in UCP2 and increase in GLUT2 expression should improve insulin secretion and GSIS. These changes were observed in the LNE rats, where UCP2 and GLUT2 gene expression were changed to 0.40- and 2.0-fold, respectively, These

changes may have contributed, in part, to the overall 11% increase in insulin release as well as the improved glucose uptake ($AUC_g = 0.92$ -fold) observed, relative to LNC rats. In the OBC rats, a further reduction in UCP2 expression may have contributed to the 44% increase in insulin released by the β -cells, which may also have modulated more effective glucose uptake ($AUC_g = 0.68$ -fold, relative to LNC) with significantly increased pancreatic and skeletal muscle mass. The 2.8-fold increase in GLUT2 expression in OBE rats did not translate into an improved IPGTT, since the UCP2 gene expression increased to 3.2-fold in OBE rats, which may have contributed, in part, to the 14% reduction in insulin secretion and impaired GSIS, relative to OBC rats. Despite the reduction in insulin secretion, their glycemic profile and control was significantly better than that of LNC rats. As in the case of the OBC rats, the improved glucose uptake may have been due, in part, to the increased skeletal muscle mass in these rats, which increased demand for energy, since no hyperglycemia was detected. These observations are supported by previous research, as quoted above, conducted on genes modulating insulin secretion.

β -Cell function, proliferation and/or differentiation are affected by c-MYC, FLIP and PKB gene expression (Laybutt et al., 2002c; Maedler et al., 2002a; Tuttle et al., 2001). c-MYC is expressed ubiquitously during embryogenesis and can induce both proliferation and apoptosis in a variety of cell types, sensitizing the cells to apoptosis (Pelengaris and Khan, 2003). In c-MYC deficient mice it was found that their pancreata contained fewer differentiated acinar cells and severe acinar hypoplasia (Nakhai et al., 2008). Pelengaris et al. (2002) found that activation of switchable c-MYC expression in the pancreatic β -cells of transgenic mice resulted in initial β -cell proliferation, which was rapidly outpaced by β -cell apoptosis. The β -cell apoptosis and loss of islet differentiation resulted in a reduction in insulin secretion, giving rise to hyperglycemia. Inactivation of c-MYC was accompanied by concurrent β -cell and pancreatic islet regeneration, with sufficient β -cell proliferation resulting in increased insulin secretion to restore normoglycemia. In OBC pancreatic tissue, significant changes were also observed in the gene expression of c-MYC and FLIP, relative to LNC rats (see table 2.14). The significantly lower c-MYC expression in OBE rats, relative to LNC rats, may have contributed, in part, to the changes in pancreatic tissue mass and organ/body weight percentage observed in these rats. It is however unclear whether the increase in pancreatic tissue mass was due to β -cell proliferation, as no further analysis was conducted on the pancreatic tissues.

In β -cells, the presence of FLIP induces the Fas signalling switch from apoptosis to cell replication (Maedler et al., 2002a; Tschopp et al., 1998). A significant reduction in FLIP expression was observed in OBC rats, relative to LNC rats, which may, in part, have contributed to the significant reduction in pancreas weight and pancreas/body weight percentage in OBC rats, relative to LNC rats. In OBE rats, *C. sativa* treatment, albeit not statistically significant, resulted in increased FLIP expression (2.1-fold) in OBE rats, relative to OBC rats. The increased FLIP expression together with the significant increase in PKB expression in OBE rats, relative to OBC rats, may have contributed to the significantly higher pancreatic weight and pancreas/body weight percentage observed. PKB provides a survival signal that protects cells from apoptosis induced by various stresses, and also mediates a number of metabolic effects of insulin (Downward, 1998). Gómez del Pulgar et al. (2000) have shown that THC induced activation of PKB via CB1 receptors, not CB2 receptors, stimulates glycolysis and anti-apoptotic cellular activities. The significant increase in CB1 receptor expression found in OBE rats, relative to OBC rats, may also have facilitated the effect of PKB activation, resulting in increased pancreatic weight. Literature over the past decade has shown that the endocannabinoid signaling system responds to stimuli and maintains the homeostatic balance of some neurotransmitters, mediators or hormones, including glucose metabolism (Tonstad, 2006). In the OBE rats CB1 expression was increased 2.9-fold, relative to that expressed in OBC rats ($P=0.006$), which, in conjunction with the increase in PKB expression, provides evidence that the endocannabinoid signaling system plays a key role in the regulation of the energy balance within the body.

The findings from the *in vivo* study have been published in an article entitled “Cannabis exposure associated with weight reduction and β -cell protection in an obese rat model” (see Annexure F) and up to the time of submission of the thesis, had been cited in the following publications:

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As previously noted, the characteristic DIO-associated hyperglycemia was not achieved in the *in vivo* rat model used in this study. The overall findings associated with the *in vivo* studies showed that *C. sativa* treatment diminished the deleterious effects of DIO by reducing weight gain, specifically fat depots, altering gene expression levels that induce increased energy expenditure, while protecting pancreatic tissue from apoptosis through the down-regulation of c-MYC. These results suggest that *C. sativa* protects pancreatic β -cells against the negative effects of obesity.

The *in vivo* effect of *C. sativa* treatment on pancreatic tissue (as a whole) was investigated in the current chapter and the following chapter will provide an overview of the *in vitro* response of isolated primary rat islets to 24- and 96-hour *C. sativa*-treatment under normo- and hyperglycemic conditions, to provide insight into how these conditions impact on β -cell functionality.

CHAPTER 3: *IN VITRO* STUDY ON THE EFFECTS OF AN ORGANIC *C. sativa* EXTRACT ON ISOLATED RAT PANCREATIC ISLETS.

3 INTRODUCTION

Insulin is the primary hormone that regulates blood glucose homeostasis (Meshkani and Adeli, 2009). The normal glucose levels are controlled within a strict range, between 60 and 150 mg/dL (3.3 and 8.3 mM) (Tortora and Grabowski, 2006). Insulin secretion occurs in a biphasic manner and is modulated by various factors including nutrients, hormones and neural and pharmacological agents. When glucose, the primary secretagogue, comes onto contact with the β -cells, insulin secretion occurs within seconds of this exposure (referred to as the triggering/response phase of insulin secretion), due to an increase in the influx of Ca^{2+} into the cytosol of the β -cells. The first 15 – 30 minutes after ingestion of a glucose bolus usually constitutes the response phase of insulin secretion. The second phase, called the recovery/amplification phase, is constituted of the remaining 90 minutes of the insulin secretion cycle. This phase requires both ATP and increased Ca^{2+} for the prolonged phase of insulin release (Henquin et al., 2003). A study conducted by Gerich (1997) showed that obese subjects with impaired glucose tolerance had a reduced plasma insulin level at 30 minutes after ingesting a glucose bolus, while the plasma insulin levels normalized at 120 minutes. Research has shown that reduction in response phase of the insulin release is responsible for the development of impaired glucose tolerance (LeRoith and Gavrilova, 2005). In diabetic individuals with fasting hyperglycemia, the response phase of insulin secretion is reduced (Brunzell et al., 1976).

A substantial body of research has been published links the endocannabinoid system to β -cell function. Nearly 30 years ago, Laychock and colleagues established that THC enhanced the responsiveness of pancreatic islets to glucose stimulation. They also found that the metabolism of arachidonic acid in pancreatic cell membranes was linked to insulin secretion (Laychock et al., 1986). Endocannabinoids have been implicated in the development of obesity, where the epididymal fat and pancreatic tissue of DIO mice contained higher levels of endocannabinoids, relative to the lean mice and humans (Quarta et al., 2010; Matias et al., 2006). Studies reporting on the effects of endocannabinoids and cannabinoid stimulation on insulin secretion in isolated islets from mice, rats, humans, as well as in β -cell lines such as

RIN-m5F and MIN6, show either inhibition or stimulation of insulin secretion (Vilches-Flores et al., 2013, 2010a&b; Anderson et al., 2013; Li et al., 2010a&b).

Since the DIO model did not develop hyperglycemia, experimentation was conducted under normoglycemic (11.1 mM glucose) and hyperglycemic (33.3 mM glucose) conditions, to ascertain whether *C. sativa* exposure, under varying glucose conditions, affects islet functionality. In addition, CB1 and CB2 receptors were blocked to determine whether the cannabinoid receptors directly modulate insulin secretion. Cell viability studies had previously been conducted within the laboratory using a range of *C. sativa* extract concentrations (data not reported). The *C. sativa* concentrations used in the current *in vitro* studies maintained cell viability above 80% over a 48 hour exposure period.

In this chapter an *in vitro* model (primary rat islets) will be used to determine the effects of acute (i.e. 24 hours) *C. sativa* extract exposure (0, 2.5 and 5.0 ng/mL THC) (0.0 pM, 7.95 pM and 15.9 pM) on β -cell function (at molecular level). The following parameters were investigated in the *in vitro* studies: the total amount of insulin that was secreted during the exposure period (i.e. 24- and 96-hours) – these are reflected as the chronic insulin levels. Thereafter, GSIS was performed to ascertain the responsiveness of β -cells to glucose after having been cultured under normo- and hyperglycemic conditions, in the absence and presence of *C. sativa* extract. Basal insulin secretion was determined by exposing the islets for an hour to 3.3 mM glucose in Krebs Ringer Buffer (KRB). The effect of post-prandial glucose levels (16.7 mM glucose KRB) on insulin secretion was determined and reflected as stimulated insulin secretion. Insulin was extracted from the rat islets by exposing the islets overnight to 0.18 N HCl in 70% ethanol at 4°C and to evaluate the extent of basal and stimulated insulin secretion relative to the insulin content of β -cells, basal and stimulated (post-prandial) insulin levels were expressed as a percentage of insulin content. Changes induced by chronic *C. sativa* exposure (96 hours and 2.5 ng/mL THC) at molecular level were investigated using Western immunoblotting and qPCR. To ascertain whether GSIS was mediated via the cannabinoid receptors, islets were pre-treated for 20 minutes with CB1 and CB2 receptor antagonists, and thereafter exposed for 48 hours to *C. sativa* extract (2.5 ng/mL THC), under normo- and hyperglycemic conditions, after which GSIS and qPCR was performed.

3.1 MATERIALS AND METHODS

3.1.1 Islet isolation solutions and culture media

All solutions and reagents used for the isolation of the rat islets and the *in vitro* experiments are included in Annexure E. Methanol was not present in any of the treatments to which the islets were exposed.

3.1.2 Rat islet isolation protocol

All experiments were conducted on primary rat pancreatic islets of Langerhans. Islets were isolated according to a modified protocol as previously described by Maedler et al. (2006) and Carter et al. (2009). Essentially islets were isolated by collagenase P (Roche) digestion of pancreata from adult male Wistar rats weighing between 150 – 250 g. Rats were euthenazed using 0.5 – 0.8 mL Eutha-naze (Centaur Labs, RSA) per rat. Each rat was disinfected by immersing it in 70% ethanol. Its ventral surface was opened and the pancreas was exposed. The common pancreatic duct was ligated at the point where it entered the duodenum. A small incision was made in the proximal end of the common duct where it bifurcates from the liver. A canula was inserted into the duct and approximately 8-10 mL collagenase P solution was perfused into the pancreas. The pancreas was excised from the body and placed into a 50 mL tube containing approximately 5-7 mL of collagenase P solution. The pancreas was digested at 37°C for 10 – 14 minutes in a shaking water bath. After the incubation period, the 50 mL tube was filled with ice-cold quenching buffer, after which the digestion mixture was centrifuged at 400 g for 2 minutes at 4°C using a benchtop 5804R Eppendorf centrifuge. The supernatant was aspirated off and the pellet resuspended in 25 mL ice-cold quenching buffer. The suspension was filtered through cotton gauze swabs (100 mm x 100 mm x 2) suspended over the opening of a 50 mL tube. The volume of the filtrate was topped up to 50 mL with quenching buffer, and centrifuged at 400 g for 2 minutes at 4°C. All the supernatant was aspirated from the pellet, which was resuspended in 10 mL Histopaque1119 (Sigma), and vortexed until the suspension was homogenous. The suspension was spun through a Histopaque gradient by centrifuging at 600 g for 30 minutes at 4°C, using slow acceleration with the brake set at zero. Islets were subsequently collected from the Histopaque gradient by pipetting and resuspended in 50 mL Hanks Balanced Salt Solution (HBSS) (Highveld Biological, SA), and centrifuged at 800 g for 15 minutes at 4°C. The supernatant was aspirated off the pellet, which was resuspended in 11.1 mM glucose-containing Roswell Park Memorial

Institute (RPMI) 1640 (Highveld Biological, SA) culture media containing the various components as stipulated in Annexure E.

Ten islets of approximately equal size were seeded on to 35 mm extracellular matrix-covered (derived from bovine corneal endothelial cells) tissue culture dishes (Novamed Ltd., Jerusalem, Israel). Each experimental condition was seeded in triplicate. No protein determination was conducted to standardize insulin secretion in relation to protein concentration. Isolated islets occur as spherical balls of cells and therefore need to attach and spread across the extracellular matrix surface. The islets become flattened over a 48 hour period, thus increasing their surface area for exposure to the various experimental conditions. No viability studies were conducted on the primary rat islets.

3.1.3 *C. sativa* Extract – THC Extraction Protocol and Quantification

Cannabinoid extraction and THC quantification protocols were conducted as previously discussed in sections 2.1.1.1 and 2.1.1.2, respectively. Once the THC was quantified, the chloroform was evaporated under a gentle stream of nitrogen at 4°C. The remaining resin was redissolved in 5 to 10 mL of pure methanol, flushed with nitrogen, and stored under vacuum at 4°C in a dark container to protect it from light.

Prior to use in the *in vitro* studies, the methanol was removed by evaporation using a speedvac (Savant SC100) and the remaining resin pellet was resuspended in 1 mL dimethyl sulfoxide (DMSO). For all experiments discussed below, control islets were treated with the vehicle, i.e. media containing the same concentration of DMSO (< 1%) as that found in the media containing the *C. sativa* extract.

3.1.4 Rat Islet Experimental Procedure

3.1.4.1 *In Vitro* Protocol of 24-Hours Exposure to *C. sativa*-Extract, Cultured Under Normo- and Hyperglycemic Conditions

Research has shown that intravenous administration of 5 mg THC resulted in THC plasma levels of 2.1 – 9.9 ng/mL, while those that smoked *C. sativa* (12.7 ± 1.3 mg) showed THC plasma levels of 0.6 – 5.2 ng/mL (Huestis and Cone, 2004; Huestis et al., 1992; Lindgren et al., 1981).

Flattened islets were exposed to *C. sativa* extract containing 0.0 ng/mL THC (C), 2.5 ng/mL THC (7.95 pM) (T1) and 5.0 ng/mL THC (15.9 pM) (T2) over a 24-hour (D1) period, under normoglycemic (N) (11.1 mM glucose RPMI) and hyperglycemic (H) (33.3 mM glucose RPMI) conditions (Maedler et al., 2006; Donath et al., 2005). Control islets were exposed to normo- or hyperglycemic culture media with the vehicle (<1% DMSO) for the same period, and served as the measure against which *C. sativa* effects were determined. The various experimental conditions will subsequently be denoted as follows:

24-hours normoglycemic untreated = ND1C;

24-hours normoglycemic with extract containing 2.5 ng/mL THC = ND1T1;

24-hours normoglycemic with extract containing 5.0 ng/mL THC = ND1T2;

24-hours hyperglycemic untreated = HD1C;

24-hours hyperglycemic with extract containing 2.5 ng/mL THC = HD1T1;

24-hours hyperglycemic with extract containing 5.0 ng/mL THC = HD1T2.

After exposure to *C. sativa* treatment, GSIS (see section 3.1.2.2) was conducted to ascertain whether *C. sativa* treatment affected β -cell responsiveness to glucose, in a concentration dependent manner under varying glucose conditions.

3.1.4.2 *In Vitro* Protocol of 96-Hours Exposure to *C. sativa*-Extract, Cultured Under Normo- and Hyperglycemic Conditions

Islets were cultured under normo- and hyperglycemic conditions and exposed for 96 hours (D4) to *C. sativa* extract containing an equivalent of 2.5 ng/mL THC (7.95 pM THC) (T1) and 5.0 ng/mL THC (15.9 pM) (T2). Control islets were exposed to normo- or hyperglycemic culture media with the vehicle (< 1% DMSO) for the same period, and served as the measure against which *C. sativa* effects were determined. The various experimental conditions will subsequently be denoted as follows:

96-hours normoglycemic untreated = ND4C;

96-hours normoglycemic with extract containing 2.5 ng/mL THC = ND4T1;

96-hours normoglycemic with extract containing 5.0 ng/mL THC = ND4T2;

96-hours hyperglycemic untreated = HD4C;

96-hours hyperglycemic with extract containing 2.5 ng/mL THC = HD4T1;

96-hours normoglycemic with extract containing 5.0 ng/mL THC = HD4T2.

After exposure to *C. sativa* treatment, GSIS (see section 3.1.2.2) was conducted to ascertain whether *C. sativa* treatment affected β -cell responsiveness to glucose under varying glucose conditions. In addition, the following parameters were also investigated:

- β -Cell proliferation (see section 3.1.2.3) after *C. sativa* exposure (containing an equivalent of 2.5 and 5.0 ng/mL THC) for 24- and 96-hours to islets cultured under normo- and hyperglycemic conditions;
- β -Cell apoptosis (see section 3.1.2.4) after *C. sativa* exposure (containing an equivalent of 2.5 and 5.0 ng/mL THC) for 24- and 96-hours to islets cultured under normo- and hyperglycemic conditions;
- Pancreatic tissue cytokine/chemokine secretion (see section 3.1.2.5) was determined on the “chronic” media sample removed from the tissue culture dishes after 96-hours of *C. sativa* exposure (containing an equivalent of 2.5 ng/mL THC) in islets cultured under normo- and hyperglycemic conditions;
- Protein expression (see section 3.1.2.3 and 3.1.2.4), including ERK1/2; phospho-ERK1/2; JNK; phospho-JNK; CB1 and CB2 receptors, after *C. sativa* exposure (containing an equivalent of 2.5 ng/mL THC) for 96-hours to islets cultured under normo- and hyperglycemic conditions.
- Changes in islet gene expression (see section 3.1.3), similar to the genes investigated in Chapter 2, after *C. sativa* exposure (containing an equivalent of 2.5 ng/mL THC) for 96-hours to islets cultured under normo- and hyperglycemic conditions.

3.1.4.3 *In Vitro* Protocol of 48-Hours Exposure to *C. sativa* Extract, Cultured Under Normo- and Hyperglycemic Conditions, With or Without Inverse-Agonists/Antagonists Pre-Conditioning

To ascertain whether GSIS was mediated via the cannabinoid receptors, CB1 and CB2 receptors were blocked by pre-treating the islets with CB1 and CB2 receptor inverse agonists/antagonists. Stock solutions (10 mM) of each inverse agonist/antagonist were constituted in DMSO (as per the manufacturer’s specifications). Control islets were treated with the vehicle, i.e. media containing the same concentration of DMSO as that found in the media containing an inverse agonist/antagonist. The inverse agonist/antagonists were diluted to 9 and 10 μ mol/L for the CB1 and CB2 inverse agonist/antagonists, respectively, using normo- or hyperglycemic RPMI media. After pretreatment, islets exposed to the inverse

agonist/antagonists were exposed to normo- or hyperglycemic media, with or without *C. sativa* extract containing an equivalent of 2.5 ng/mL THC or 7.95 pM THC.

Once flattened, islets were exposed to the various experimental conditions outlined below. Briefly, control (NC and HC) islets were cultured under normo- and hyperglycemic conditions for 48 hours in media containing the vehicle. Experimental (NT1, NT2, HT1 and HT2) islets were cultured under normo- and hyperglycemic conditions, respectively, for 48 hours. To block CB1 and CB2 receptors, different sets of isolated islets were pretreated with the CB1 inverse agonist/antagonist, AM251 (Alexis Biochemicals, Switzerland) (hereafter referred to as A1) (final concentration of 9 μ mol/L) or the CB2 inverse agonist/antagonist, AM630 (Alexis Biochemicals, Switzerland) (hereafter referred to as A2) (final concentration of 10 μ mol/L), for 20 minutes (Bolognini et al., 2012; Patil et al., 2011). After removing the media containing A1 or A2, the islets were exposed to normo- and hyperglycemic conditions, with or without *C. sativa* extract (2.5 ng/mL THC or 7.95 pM THC), for 48 hours (Patil et al., 2011). Thereafter GSIS was performed. Cytokine/chemokine determinations were also conducted across the abovementioned experimental conditions, on the “chronic” media sample removed from the tissue culture dishes after the 48 hours treatment period. The various experimental conditions associated with the antagonist studies were denoted as follows:

Normoglycemic untreated islets = NC;

Normoglycemic *C. sativa*-treated islets = NT1;

Normoglycemic untreated islets with CB1 antagonist = NA1;

Normoglycemic with CB1 antagonist and *C. sativa*-treated islets = NA1T1;

Normoglycemic untreated islets with CB2 antagonist = NA2;

Normoglycemic with CB2 antagonist and *C. sativa*-treated islets = NA2T1;

Hyperglycemic untreated islets = HC;

Hyperglycemic *C. sativa*-treated islets = HT1;

Hyperglycemic untreated islets with CB1 antagonist = HA1;

Hyperglycemic with CB1 antagonist and *C. sativa*-treated islets = HA1T1;

Hyperglycemic untreated islets with CB2 antagonist = HA2;

Hyperglycemic with CB2 antagonist and *C. sativa*-treated islets = HA2T1.

3.1.4.4 Glucose-stimulated insulin secretion (GSIS) assay

Glucose acts as a secretagogue to β -cells. During GSIS, there is an increase in glucose uptake, via GLUT2 transporters, into the β -cells. Subsequent glucose metabolism leads to pyruvate formation and entry into the mitochondrial TCA cycle. There is simultaneous increase in the cellular ATP/ADP ratio because of electron transfer from the TCA cycle (in the form of NADH and FADH₂) to the respiratory chain (oxidative phosphorylation). This increase in the ATP/ADP ratio leads to the closing of the ATP-sensitive potassium (K⁺) channels, which shut down in response to this elevation of ATP, resulting in depolarization of the β -cell. The depolarization results in the opening of voltage-sensitive Ca²⁺ channels, which result in an influx of Ca²⁺. The increase in intracellular Ca²⁺ triggers exocytosis of insulin granules into the bloodstream (Layden et al., 2010). Therefore, depending on the extent of the glucose stimulus, the extent of insulin secretion varies.

Once *C. sativa* treatments were terminated (as per sections 3.1.2.1.2 to 3.1.2.1.4), the media was removed and stored to quantify “chronic” insulin secretion (which is associated with the insulin secretion for the *C. sativa* treatment period – 24-, 48- or 96 hours - prior to GSIS being performed). Thereafter islets were washed with Krebs Ringer Buffer (KRB) containing 3.3 mM glucose. The KRB was left on the islets for 30 minutes at 37°C and thereafter the media was discarded to ensure that none of the culture media containing *C. sativa* remained in the plate prior to the commencement of GSIS. The GSIS was conducted as described by Maedler et al. (2001a, b).

Table 3.1 below provides an outline of the GSIS experimental design (*Stimulated/Basal insulin secretion is an indication of the responsiveness of the islet to glucose, i.e. glucose-stimulated:basal insulin production; **Content was determined to reflect basal and stimulated insulin secretions as a proportion of insulin content).

Table 3.1 Outline of the GSIS experimental process.

NORMOGLYCEMIC (11.1 mM)			HYPERGLYCEMIC (33.3 mM)		
24 Hrs:	48 Hrs:	96 Hrs:	24 Hrs:	48 Hrs:	96 Hrs:
+/- <i>C. sativa</i> (0; 2.5;5.0 ng/mL)	Pretreat with CB1/CB2 Antagonist thereafter	+/- <i>C. sativa</i> (2.5 ng/mL)	+/- <i>C. sativa</i> (0; 2.5;5.0 ng/mL)	Pretreat with CB1/CB2 Antagonist thereafter	+/- <i>C. sativa</i> (2.5 ng/mL)

	+/- <i>C. sativa</i> (2.5 ng/mL)			+/- <i>C. sativa</i> (2.5 ng/mL)	
Chronic (24 hrs secretion)	Chronic (48 hrs secretion)	Chronic (96 hrs secretion)	Chronic (24 hrs secretion)	Chronic (48 hrs secretion)	Chronic (96 hrs secretion)
GSIS			GSIS		
Basal (3.3 mM glucose) (1 hr secretion)	Basal (3.3 mM glucose) (1 hr secretion)	Basal (3.3 mM glucose) (1 hr secretion)	Basal (3.3 mM glucose) (1 hr secretion)	Basal (3.3 mM glucose) (1 hr secretion)	Basal (3.3 mM glucose) (1 hr secretion)
Stimulated* (16.7 mM glucose) (1 hr secretion)	Stimulated* (16.7 mM glucose) (1 hr secretion)	Stimulated* (16.7 mM glucose) (1 hr secretion)	Stimulated* (16.7 mM glucose) (1 hr secretion)	Stimulated* (16.7 mM glucose) (1 hr secretion)	Stimulated* (16.7 mM glucose) (1 hr secretion)
Content**	Content**	Content**	Content**	Content**	Content**

To obtain basal insulin secretion, each plate was exposed to 1 mL of 3.3 mM glucose in KRB for 60 minutes at 37°C. GSIS was determined thereafter by exposing each plate to 1 mL KRB containing 16.7 mM glucose for 60 minutes at 37°C. Islets were washed with phosphate-buffered saline (PBS) and total insulin was extracted overnight with 0.18 N HCl in 70% ethanol at 4°C. Media collected from each of the incubation steps were frozen for insulin assays. Insulin secretion values obtained from exposing the islets to glucose at basal and stimulated levels are used to calculate the glucose-stimulated:basal insulin production of the islets – a parameter that reflects the insulin releasing function of isolated islets, and therefore their sensitivity to glucose stimulation (Sakata et al., 2008). The glucose-stimulated:basal insulin production of the islets were analyzed per experimental group as the ratio of insulin secreted under stimulated and basal glucose conditions. The equation is as follows: [glucose-stimulated insulin secretion / basal insulin secretion]. In addition, basal and stimulated insulin secretion, respectively, was expressed as a percentage of insulin content. Percentage basal insulin relative to insulin content was calculated as follows: [basal insulin secretion / insulin content] * 100. Similarly, the percentage stimulated insulin relative to insulin content was calculated as follows: [stimulated insulin secretion / insulin content] * 100.

Across all conditions, insulin secretion was normalized to reflect insulin secretion as $\text{pmol}^{-1} \cdot \text{islet}^{-1} \cdot \text{hr}^{-1}$. This was determined by dividing the total insulin secretion by 10 (since 10 islets were seeded), and was based on the assumption that the number of cells at the start and end of the experiments would not be significantly different. No protein determination was conducted to verify this assumption.

3.1.4.4.1 Insulin quantification via coated tubes radioimmunoassay (CT RIA)

The Insulin-CT RIA (CIS Bio International, France) kit was used for quantitative determination of insulin in the culture media of isolated islets. The principle of this assay is based on the competition between labeled insulin (present in the standards provided or in experimental samples), for a fixed and limited number of antibody binding sites attached to a solid phase (i.e. the coated tubes). After incubation, the unbound tracer is removed by a washing step, after which the amount of labeled insulin bound to the antibody is inversely related to the amount of unlabeled insulin present in the sample, using a Kontron Analytical MDA 312 gamma counter. The RIA was performed as per the manufacturer's instructions and figure 3.1 is the standard curve generated from standards provided by the manufacturer (see

<http://www.mpbio.com/includes/technical/Insulin%20CT%20125I%20RIA,%2007260102%20-%2007260105.PDF>).

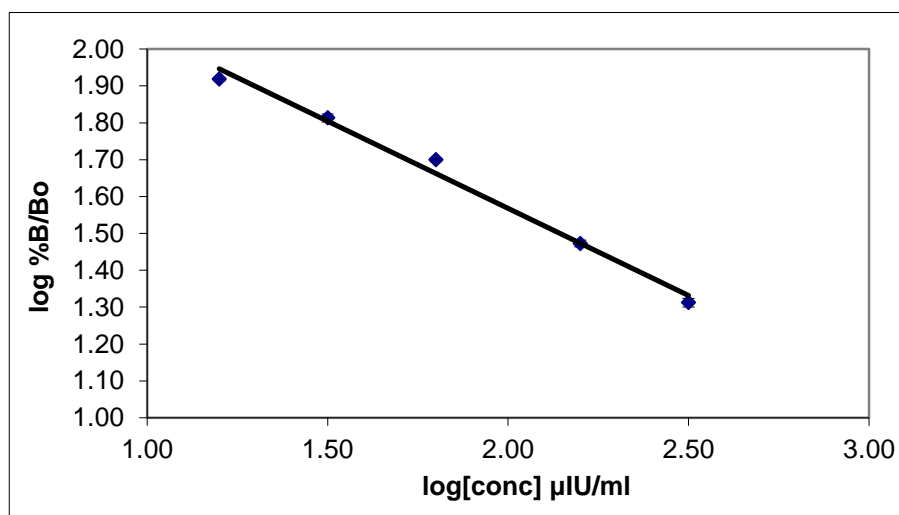


Figure 3.1 Insulin-CT RIA standard curve using insulin standards ranging between 5.5 and 310 $\mu\text{IU/mL}$, where each point represents quadruplicate samples. $R^2=0.9895$.

3.1.4.4.2 *In Vitro* Protocol of 96-Hours Exposure to *C. sativa* (2.5 ng/mL THC) on islet cytokine/chemokine secretion, cultured under normo- and hyperglycemic conditions

Islet function is affected by various factors including cytokines and chemokines released by peripheral tissues as well as by the pancreatic cells. Various studies have shown that glucotoxicity, associated with T2D, induces cytokines release (including TNF α), which results in impaired β -cell and β -cell death (Donath et al., 2009; Ehses et al., 2009; Ehses et al., 2007; Donath et al., 2005; Cnop et al., 2005). The inflammatory process that has been shown to be associated with T2D has been linked to metabolic stressors (such as hyperglycemia) triggering of the abnormal activation of the intrinsic immune system which is regulated by IL-1 signalling (Donath et al., 2009).

The media samples analyzed were the chronic samples from the *in vitro* experiments conducted as outlined in section 3.1.2.1.3. Lincoplex kits (Linco Research, Switzerland) are based on laser detection of fluorescently labeled microsphere antibody-immobilized beads with specific dimensions associated with the analytes being quantified, using the Luminex¹⁰⁰ system (Luminex Corp., USA). Rat cytokine/chemokine Lincoplex kits (Linco, Switzerland) were used to simultaneously quantify the analytes present in the chronic media samples of islets cultured under normo- and hyperglycemic conditions, in the presence and absence of *C. sativa* extract containing 2.5ng/mL THC (7.95 pM THC) for 96 hours. Control islets were exposed to normo- or hyperglycemic culture media with the vehicle (<1% DMSO) for the same period. All cytokine/chemokine data represents triplicate samples per experimental condition for each experiment conducted. The protocol that was followed is outlined in Table 3.2, according to the manufacturer's specifications. The analytes that were quantified using rat cytokine/chemokine Lincoplex kits (Linco, Switzerland) were IL-1 α , MIP-1 α and growth-related oncogene/cytokine-induced neutrophil chemoattractant (GRO/KC) also known as macrophage inflammatory protein-2 (MIP-2), which is the functional homologue of human IL-8. Results for each of the analytes, under the different experimental conditions, will be presented graphically in the results section.

3.1.4.4.3 Effect of 48-hours of *C. sativa* exposure (2.5 ng/mL THC), with and without CB1 or CB2 receptor inverse-agonists/antagonists, on islet cytokine/chemokine secretion, cultured under normo- and hyperglycemic conditions

To determine whether *C. sativa* exposure affect islet function via the cannabinoid receptors, islets were cultured under normo- and hyperglycemic conditions, were pre-treated with CB1 and CB2 receptor inverse-agonists/antagonists according to the protocol set out in section 3.1.2.1.4. All the control samples were media from islets without any inverse-agonists/antagonists treatment as well as media from islets pretreated with either A1 or A2. These two sets of controls were used to measure the effect of the CB1 and CB2 inverse agonists/antagonists, as well as the effect of *C. sativa* extract on the analytes. The analytes that were quantified using rat cytokine/chemokine Lincoplex kits (Linco, Switzerland) were IL-1 α , MIP-1 α and MIP-2. The media samples analyzed were the chronic samples from the *in vitro* experiments conducted as outlined in section 3.1.2.1.4. The protocol that was followed is outlined in Table 3.2, according to the manufacturer's specifications. Results for each of the analytes, under the different experimental conditions, will be presented graphically in the results section.

3.1.4.5 β -cell proliferation detection

Ki-67 is a nuclear protein expressed during all active phases of the cell cycle (G₁, S, G₂ and M-phases) in proliferating cells, but absent in resting cells (G₀-phase) and non-viable cells (Gerdes et al., 1991). It has been used as a proliferative marker in β -cell proliferation detection studies (Maedler et al., 2003). Proliferative studies were conducted using the conditions described in section 3.1.2.1.2 and 3.1.2.1.3 above, to determine whether *C. sativa* treatment induced β -cell proliferation. Each condition was completed in triplicate, with each experiment being completed in duplicate. In excess of 500 cells were counted per petri dish, and all conditions were normalized to 100 cells. All Ki data are expressed as the number of Ki positive cells per 100 cells. Briefly, after cultured islets were washed with PBS, they were fixed in 4% paraformaldehyde for 30 minutes at room temperature. Thereafter islets were permeabilized with 0.5% Triton X-100 for 4 minutes at room temperature. The islets were incubated with monoclonal Ki-67 antibody raised in mice (MIB-1, Dako, Diagnostech, Johannesburg) (diluted 1:50) for 60 minutes at room temperature. Detection of positively stained cells was conducted using a streptavidin-biotin-peroxidase complex (Histostain-Plus

Kit, Scientific Group SA). Subsequent to this, islets were incubated for 30 minutes at 37°C with an enhanced horseradish peroxidase-conjugated streptavidin complex which bound to the biotinylated secondary antibody. The chromagen, 3-amino, 9-ethyl carbazol (AEC) was used to create an intense red deposit in the area where the antigen/antibody/enzyme complex occurred on the sample. Thereafter, the islets were incubated for 30 minutes at 37°C with a polyclonal insulin antibody raised in guinea-pig (diluted 1:50) (Adcock-Ingram Scientific), followed by a 30 minute incubation at 37°C with a secondary fluorescein isothiocyanate (FITC)-conjugated guinea-pig antibody raised in rabbit (diluted 1:20) (Adcock-Ingram Scientific). Microscopy image analysis was performed using the Zeiss Axio microscope, with the Axovision imaging system (Zeiss, Germany). Appropriate controls were used to determine whether any non-specific binding of primary and secondary antibodies had taken place. None was detected.

Table 3.2 Assay Protocol for Rat Cytokine/Chemokine Lincoplex Kit

		Step 2	Step 2,3	Step 4,5	Step 6,8	Step 7	Step 9	Step 10-12	Step 13	Step 14	Step 15	Step 16-18	Step 19			
# Well	Well Identification	Assay Buffer	Seal, agitate, incubate 10 minutes at room temperature. Remove assay buffer by vacuum.	Assay Buffer	Standard/Control/Sample	Serum Matrix/Test Media	Mixed Beads	Seal, agitate, incubate overnight (16 – 20 hr) at 4°C. Wash 2X with 200 µL Wash Buffer.	Detection Antibody	Seal, agitate, incubate 2 hours at room temperature.	SA-PE	Seal, agitate, incubate 30 minutes at room temperature. Wash 2X with 200 µL Wash Buffer.	Sheath Fluid			
1A,1B	0pg/mL standard (Background)	200 µL		25 µL	-	25 µL	25 µL		25 µL		25 µL		25 µL	100 µL		
1C,1D	4.88 pg/mL standard	↓		-	25 µL	25 µL	↓		↓		↓		↓	↓	↓	↓
1E,1F	19.53 pg/mL standard			-	25 µL	25 µL										
1G,1H	78.13 pg/mL standard			-	25 µL	25 µL										
2A,2B	312.5 pg/mL standard			-	25 µL	25 µL										
2C,2D	1250 pg/mL standard			-	25 µL	25 µL										
2E,2F	5000 pg/mL standard			-	25 µL	25 µL										
2G,2H	20000 pg/mL standard			-	25 µL	25 µL										
3A,3B	Control I			-	25 µL	25 µL										
3C,3D	Control II			-	25 µL	25 µL										
3E,3F	Sample			25 µL	25 µL	-										
3G,3H	Sample			25 µL	25 µL	-										
Final sample	Sample			25 µL	25 µL	-										

3.1.4.6 β -cell apoptosis detection

Apoptotic studies were conducted using the conditions described in section 3.1.2.1.2 and 3.1.2.1.3 above to determine whether *C. sativa* exposure induced β -cell apoptosis. Each condition was completed in triplicate, with each experiment being completed in duplicate. In excess of 500 cells were counted per petri dish, and all conditions were normalized to 100 cells. All TUNEL data are expressed as the number of TUNEL-positive cells per 100 cells, with each experiment represented by triplicate samples per experimental condition.

Cleavage of genomic DNA during apoptosis may yield DNA fragments which can be identified by labeling the free 3'-OH terminals with modified nucleotides in an enzymatic reaction using terminal deoxynucleotidyl transferase which catalyzes the fluorescein-dUTP nick-end labeling to the free 3'-OH DNA strand breaks, referred to as the TUNEL-reaction. Apoptotic studies were conducted on isolated rat pancreatic islets as described by Maedler et al. (2003). Islets were fixed and permeabilized as described in section 3.1.2.3 above, followed by the TUNEL assay, performed as per the manufacturer's instructions (*In Situ* Cell Death Detection Kit, AP, Roche, SA). Thereafter the incorporated fluorescein was detected in positively stained TUNEL cells, by incubation for 30 minutes at 37°C with alkaline phosphatase (AP)-conjugated fluorescein antibody (Fab fragment) raised in sheep. A signal enhancing substrate solution called nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Sigma-Aldrich) was applied, and incubated at room temperature for 10 minutes. Thereafter the islets were incubated for 30 minutes at 37°C with polyclonal insulin antibody raised in guinea pig (diluted 1:50) (Adcock-Ingram Scientific). Detection of positively stained β -cells containing insulin was conducted using a secondary streptavidin-biotin-peroxidase-conjugated guinea-pig antibody complex (Histostain-Plus Kit, Scientific Group SA). The islets were incubated for 10 minutes at room temperature with the secondary streptavidin-biotin-peroxidase-conjugated guinea-pig antibody which reacted with the primary insulin antibody (Maedler et al., 2003). Subsequently the islets were incubated with an enhanced horseradish peroxidase conjugated streptavidin complex which bound to the biotinylated secondary antibody. AEC was used to create an intense red deposit in the area where the antigen/antibody/enzyme complex occurred on the sample. Microscopy image analysis was performed using the Zeiss Axio microscope, with the Axovision imaging system (Zeiss, Germany). Appropriate controls were used to determine whether any non-specific binding had occurred. None was detected.

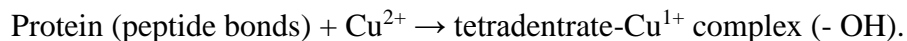
3.1.4.7 Protein determination and expression of ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, CB1 and CB2 receptors in isolated islets, cultured under normo- and hyperglycemic conditions, after 96 hours of *C. sativa* exposure, using Western blotting technique

Studies have shown that cannabinoid receptors regulate different members of the MAPK family, such as ERK, (Howlett et al., 2002), JNK and p38 (Baker et al., 2003). MAPK pathways are involved in cellular regulation of proliferation and differentiation depending on the duration of activation and the balance between ERK1/2, JNK and p38 (Pavlovic et al., 2000). Experiments were therefore conducted to determine whether ERK1/2 and JNK were activated in the islets, following exposure to normo- and hyperglycemic media for 96 hours, with or without *C. sativa* extract containing an equivalent of 2.5 ng/mL (7.95 pM) THC, and whether the β -cell apoptosis/proliferation observed in the TUNEL and Ki-67 studies, can be attributed to ERK1/2 and JNK activation, respectively.

Two separate experiments were conducted over a 96-hour period similar to that conducted for the GSIS studies. In these experiments duplicate tissue culture dishes were seeded per condition (as opposed to triplicates in the GSIS studies). Control islets were exposed to normo- or hyperglycemic culture media with the vehicle (<1% DMSO) for the same period, while experimental islets were exposed to the same condition with the media containing *C. sativa* extract with an equivalent of 2.5 ng/mL THC (7.95 pM THC) (as per section 3.1.2.1.3). Thereafter the media was removed and 25 μ L lysis buffer was added to each petri dish. The islets lysates of the duplicate tissue culture dishes were combined, and homogenized, placed on ice for 30 minutes and thereafter, snap frozen in liquid nitrogen and stored at -80°C . The lysis buffer contained 20 mM Tris acetate, pH 7.0; 0.27 M sucrose; 1 mM ethylenediaminetetraacetic acid; 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid, 50 mM NaF; 1% Triton X-100; 5 mM sodium pyrophosphate; 10 mM β -glycerophosphate; 1 mM dithiothreitol; 1 mM benzamidine; 1 mM sodium ortho-vanadate (Na_3VO_4) and 4 $\mu\text{g}/\text{mL}$ leupeptin. All the components, except the last four were added together and stored at 4°C . For every 1 mL of lysis buffer, 1 μL DTT, 1 μL benzamidine, 1 μL Na_3VO_4 and 1 μL leupeptin was added on the day of use. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. The immunoblotting procedure is outlined in section 3.1.2.5.2. below.

3.1.4.7.1 Protein determination using bicinchoninic acid protein assay

The BCA protein assay kit (Pierce, Switzerland) was used to determine the protein content of the islet lysates (Smith et al., 1985). This assay is a colorimetric detection method based on the following reaction scheme, using bovine serum albumin (BSA) as a standard:



The reaction product was detected at $A_{540\text{nm}}$ using a BIO-RAD microplate reader. An example of a standard curve generated can be seen in figure 3.2.

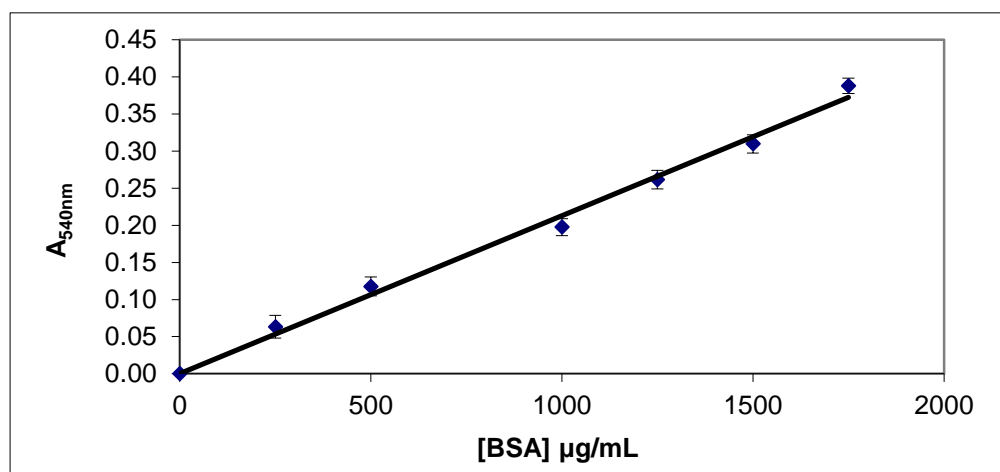


Figure 3.2 BCA protein assay standard curve using BSA standards (250 to 1750 µg/mL). The error bars represent the standard error of the mean (n=3). $R^2 = 0.993$.

3.1.4.7.2 Immunoblotting procedure

Islet lysates with a protein concentration of 20 µg per lane were loaded, after heating the protein-sample buffer mixture for five minutes at 100°C. A 12% sodium dodecyl sulfate (SDS) resolving gel and 4% SDS stacking gel was used, and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Chiou and Wu, 1999; Laemmli, 1970). A dual colour prestained Precision Plus protein standard molecular marker, ranging from 250 to 10 kDa (BIO-RAD, Switzerland) was run concurrently for approximately 45 minutes under the following reducing conditions: 200 V and 300 mA, using a BIO-RAD power pac 200 (Maedler et al., 2003). Thereafter proteins were transferred from the gel onto a 0.45 µm nitrocellulose filter (which had been incubated in transfer buffer for 30 minutes) and sandwiched between extra-thick blotting paper and foam sheets. The BIO-RAD Trans-blot SD Semi-dry Transfer cell and BIO-RAD power pack 200 were used under the following conditions: 15 V and 300 mA for 40 minutes. Thereafter the filter was incubated in protein blocking solution for 60 minutes at room temperature. The membrane was placed into the

primary antibody solution, made up in Tris-buffered saline-Tween 20 (TBS-T), and incubated for 60 minutes at room temperature. The primary antibody was diluted according to the manufacturers' specifications (see table 3.2). Thereafter the membrane was washed three times with 0.1% (w/v) milk in TBS-T solution for 10 minutes each. The membrane was incubated for 60 minutes at room temperature in the secondary horseradish peroxidase-conjugated rabbit antibody (raised in donkey) solution (made up in TBS-T), using a 5 000x dilution, as specified by the manufacturer. The membrane was then washed three-times with 0.1% (w/v) milk in TBS-T solution for 10 minutes each. The membrane was exposed to Lumi-Light Western Blotting Substrate (Roche, Switzerland), which interacts with the horseradish peroxidase, resulting in a chemiluminescent signal. The membrane was exposed to Kodak Bio Max Light X-ray film for an appropriate period of time to attain optimal band intensity. The X-ray film was developed by an automated film developer (Maedler et al., 2003). The bands observed were analyzed using densitometry (AlphaImager™ 3400) and expressed as an arbitrary integrated density value (IDV). Protein-antibody complexes were analyzed using SDS-PAGE for corresponding sizes, according to the manufacturer's specifications, as listed in Table 3.3. SDS-PAGE is a method of gel electrophoresis involving protein separation based on their molecular mass, where the intrinsic charge associated with the proteins are masked when the proteins bind SDS. Molecular mass of the protein subunits from the protein lysates can be predicted due to the linear relationship between the logarithm of the molecular mass and relative mobility, i.e. the distance migrated by the SDS-protein complex (Chiou and Wu, 1999; Laemmli, 1970). A calibration curve showing the various migration distances of a wide range of known molecular markers (Biorad) was constructed for this purpose (figure 3.3).

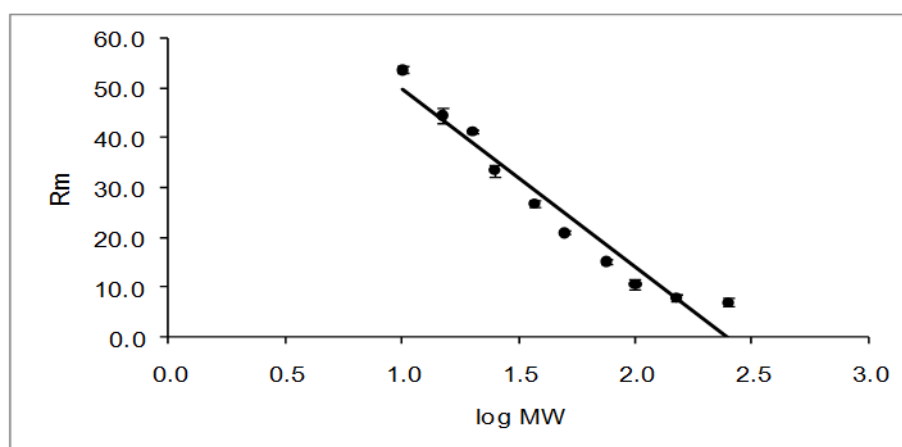


Figure 3.3 Calibration curve showing the various relative migrations of the wide range of molecular markers, 10 to 250 kDa. Error bars represents SEM, n=3. $R^2 = 0.95$.

3.1.4.7.3 Antibody incubation procedures

Table 3.3 contains the various dilutions used for the different primary antibodies and associated molecular masses, according to the manufacturer's specifications. Horseradish-conjugated rabbit antibody raised in donkey (diluted 5000x with TBS-T) was used as the secondary antibody in the detection of JNK1, pJNK, ERK1, pERK1+2, CB1, CB2 and actin. Actin antibody (Santa Cruz) was used to illustrate that the same concentration of protein lysate was loaded in all the wells.

Table 3.3 List of primary antibodies used in immunoblotting protocols

Name of Antibody	Source	Species	kDa	Supplier	Dilution
Anti-JNK1	Rabbit	m; r; h	46	Santa Cruz	1000x
Anti-phospho-JNK (T183/Y185)	Rabbit	m; r; h	46-54	R&D systems	2000x
Anti-ERK1	Rabbit	m, r, h	44	Zymed	1000x
Anti-phospho-ERK1+2	Rabbit	r	42&44	Zymed	1000x
CB1	Rabbit	m, r, h	60	Sigma	250x
CB2	Rabbit	r,h	60	Sigma	250x
Actin	Rabbit	m; r; h	42	Santa Cruz	1000x

m = mouse; r = rat; and h = human

3.1.5 qPCR on GOI

The qPCR protocols conducted for the *in vitro* studies differed from those conducted in the *in vivo* studies because the *in vitro* experiments were conducted by the author in the laboratory of Prof Dr Donath in Switzerland, while the *in vivo* studies were conducted at the Nelson Mandela Metropolitan University (NMMU). The following differences should be noted:

- Only one reference gene, β -Tubulin, was used in the two-step delta-delta semi-quantitative comparative ($\Delta\Delta Cq$) analysis method for all *in vitro* samples. β -Tubulin was selected after testing a range of possible housekeeping gene, and excluded these due to significant changes (i.e. more than 1 Ct unit) in expression levels observed between the control and experimental groups. Some of the reference genes tested included cyclophilin A, beta-2-micorglobulin, alpha albumin, ubiquitin C, hydroxymethylbilane synthase, tyrosine-3-monooxygenase/tryptophan-5-monooxygenase, transferrin receptor, glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine guanine phosphoribosyl transferase 1.

- All qPCR reactions for *in vitro* samples were performed using the LightCycler 3 (Roche). The following standard experimental protocol was used: cycle 1 - 10 minute denaturation step at 95°C, followed by cycle 2 - 40 repeats of the following four steps: step 1 - 95°C for 15 seconds; step 2 at specified temperature (refer Table 2.7) for 15 seconds; step 3 - 72°C for 25 seconds, and step 4 - 80°C for 8 seconds. The denaturation of the polymerase chain reaction product occurred in Cycle 3 and included two steps: step 1 - 95°C for 20 seconds, followed by step 2 where the specific temperature is adjusted by 0.5°C for 15 seconds for each repeat step, until the temperature reaches 90°C. The number of repeat steps were calculated as follows: $[95 - \text{temperature (step 2, cycle 2)}]/2$. This is followed by the cooling step in cycle 4 at 40°C, followed by an infinite holding step at 22°C.
- For the normo- and hyperglycemic islet studies investigating the effect of *C. sativa* exposure (2.5 ng/mL THC) on insulin secretion, qPCR analysis was conducted on all the GOI investigated in the *in vivo* study, except PKB (refer to Table 2.7 for the respective primer sequences and annealing temperatures).
- Insulin gene expression was determined in relation to the chronic insulin levels after 48 hours exposure to *C. sativa* extract with and without pre-conditioning with AM251 and AM630.
- The LightCycler FastStart DNA Master SYBR Green 1 kit (Roche) was used to perform all the qPCR reactions. This kit contains LightCycler FastStart Taq DNA polymerase, which is added to the LightCycler FastStart reaction buffer containing a deoxynucleotide triphosphate, with dUTP instead of 2'-Deoxythymidine 5'-triphosphate, as well as a SYBR Green 1 dye and 10 mM MgCl₂. A final Mg²⁺ concentration of 3 mM was achieved by adding 1.6 μL of 25 mM MgCl₂ stock solution provided for each sample being analyzed. The final reaction volume per sample was 20 μL, containing 2 μL cDNA, 500 nM forward and reverse primers, respectively, and 12.4 μL water.

All qPCR graphs below are expressed as NDT1 and HD4C relative to ND4C and HD4T1 relative to HD4C.

3.2 STATISTICAL ANALYSIS

All data are expressed as mean ± SEM, unless otherwise indicated, and differences between the various experimental groups were statistically analyzed using the paired Student's *t*-test when testing within a group, and the unpaired, unequal variance Student's *t*-test when testing

between groups. A $P < 0.05$ was considered to demonstrate significant difference between mean values

3.3 RESULTS

3.3.1 GSIS associated with 24 hours *C. sativa* exposure

In the *in vivo* study, LNE rats were found to have increased plasma insulin levels (11%), although not statistically significant, relative to LNC rats. Islets were therefore cultured under normo- and hyperglycemic conditions to ascertain what effect acute (24 hours) *C. sativa* exposure would have on insulin secretion, under varying glucose concentrations.

3.3.1.1 Chronic insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 24 hours

Exposure of the islets to *C. sativa* extract over a 24-hour period under normoglycemic conditions resulted in a statistically significant reduction in the chronic insulin secretion in ND1T1 (0.23-fold, $P < 0.01$) and ND1T2 (0.37-fold, $P < 0.01$) islets, relative to the ND1C islets (figure 3.4).

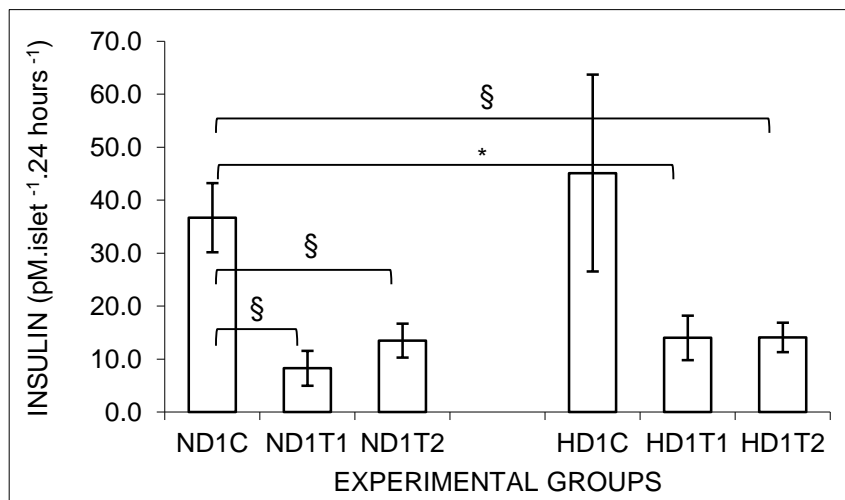


Figure 3.4 Effect of 24 hours of *C. sativa* exposure on chronic insulin secretion in islets exposed to normo- and hyperglycemic media, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; $n = 4$.

* $P < 0.05$, ND1C relative to: ND1T1

Islets exposed to hyperglycemic conditions for 24 hours were found to have increased chronic insulin secretion to 1.23-fold, relative to ND1C islets (albeit not statistically significant). Chronic insulin secretion by ND1C islets were significantly higher than chronic insulin secretion by islets cultured under hyperglycemic conditions and treated with *C. sativa* extract.

Chronic insulin levels were 0.38-fold of ND1C islets for both ND1T1 ($P < 0.05$) and ND1T2 ($P < 0.01$) islets, and 0.31-fold, relative to HD1C islets, for both HD1T1 and HD1T2 islets. As found under normoglycemic conditions, *C. sativa* extract exposure on chronic insulin secretion in islets cultured under hyperglycemic conditions was not statistically different relative to ND1C islets.

3.3.1.2 Basal and glucose-stimulated insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 24 hours

Experiments were conducted to determine the impact of *C. sativa* exposure on the responsiveness of β -cells to low and high glucose stimulation. *C. sativa* did not significantly affect basal insulin secretion in islets exposed to normo- and hyperglycemic conditions, despite levels being lower than the respective control islets (figure 3.5).

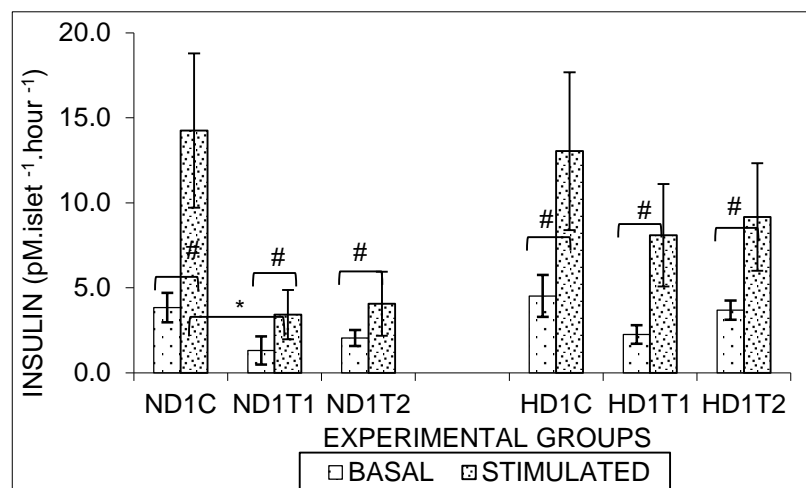


Figure 3.5 Effect of 24 hours of *C. sativa* exposure on basal and acute glucose-stimulated insulin secretion in islets exposed to normo- and hyperglycemic media, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

* $P < 0.05$, Stimulated - ND1C relative to: ND1T1

$P < 0.001$, Intra-group basal insulin relative to appropriate stimulated insulin levels: ND1C, ND1T1, ND1T2, HD1C, HD1T1, HD1T2

In islets cultured under ND1T1 and ND1T2 conditions, basal insulin secretion were reduced to 0.34- ($P = 0.06$) and 0.53-fold ($P = 0.10$), relative to ND1C islets. In islets cultured under hyperglycemic conditions, basal insulin secretion differed between HD1T1 and HD1T2 levels ($P = 0.10$).

Glucose stimulation for an hour resulted in significantly higher insulin secretion ($P < 0.001$) across all experimental conditions, relative to their respective basal insulin levels (figure 3.5). GSIS was significantly reduced in the ND1T1 islets to 0.24-fold ($P < 0.05$) of insulin levels secreted by ND1C islets. ND1T1 islets similarly reduced GSIS to 0.28-fold ($P = 0.06$) of insulin levels secreted by ND1C islets. *C. sativa* extract had no significant effect on the level of GSIS in islets cultured under hyperglycemic conditions, relative to HD1C islets.

3.3.1.3 Glucose-stimulated:basal insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 24 hours

Islet insulin secretion capability to different glucose concentrations is reflected in the ratio of insulin secreted in response to post-prandial glucose levels, relative to basal glucose levels. Islets exposed to ND1T1 conditions reduced the glucose-stimulated:basal insulin production of the islets to 0.84-fold, while ND1T2 islets showed a significant reduction to 0.44-fold ($P < 0.05$), relative to ND1C islets (figure 3.6).

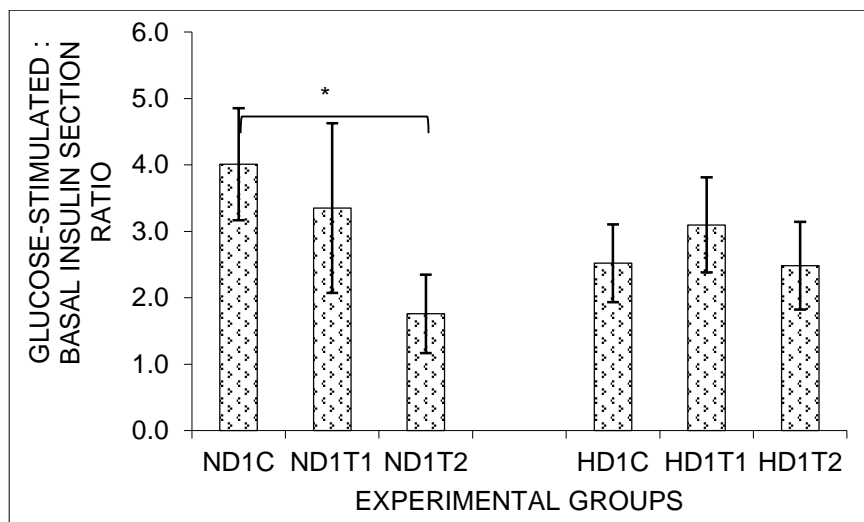


Figure 3.6 Effect of 24 hours of *C. sativa* exposure on the glucose-stimulated:basal insulin production of islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

* $P < 0.05$, ND1T1 relative to: ND1T2

§ $P < 0.01$, ND1C relative to: ND1T1

Hyperglycemic conditions reduced the glucose-stimulated:basal insulin production of HD1C islets to 0.63-fold of ND1C islets, while HD1T1 and HD1T2, showed glucose-stimulated:basal insulin production of 0.77- and 0.62-fold, respectively, relative to ND1C islets. Under hyperglycemic conditions, *C. sativa* extract treatment either increased or

maintained the ratio between glucose-stimulated and basal insulin secretion, with HD1T1 treatment increasing the glucose-stimulated:basal insulin production to 1.23-fold, while HD1T2 maintained the glucose-stimulated:basal insulin production at 0.99-fold, relative to the HD1C islets.

3.3.1.4 Insulin content associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 24 hours

Figure 3.7 shows the effects of *C. sativa* exposure on insulin content in islets cultured for 24 hours under normo- and hyperglycemic conditions.

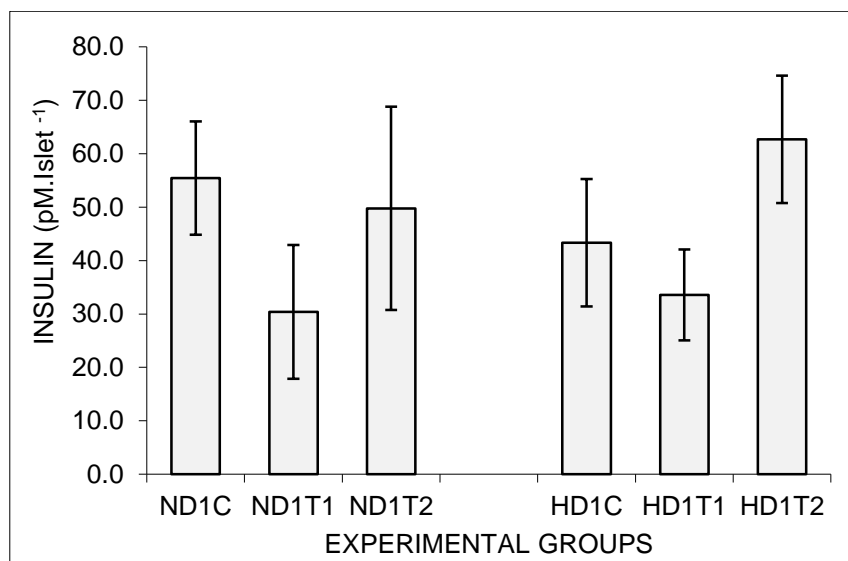


Figure 3.7 Effect of 24 hours of *C. sativa* treatment on insulin content in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4

Islets cultured under normo - and hyperglycemic conditions, respectively, and exposed to *C. sativa* extract showed no significant difference in insulin content, relative to the appropriate control islets, despite ND1T1 and ND1T2 showing reduced content of 0.55- and 0.90-fold, respectively, relative to ND1C islets. Similarly, islets cultured under hyperglycemic conditions and exposed to *C. sativa* treatment showed a 0.78-fold (HD1T1) and 1.45-fold change in insulin content, relative to HD1C islets. Insulin content Comparing insulin content between the two *C. sativa* treatments showed a 1.87-fold (P = 0.07) increase in content in HD1T2 islets, relative to HD1T1 islets.

3.3.1.5 Percentage basal and glucose-stimulated insulin secretion in relation to insulin content associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 24 hours

Analysis of the percentage basal and GSIS, in relation to insulin content, was conducted (figure 3.8). Under normoglycemic conditions, *C. sativa* treatment did not significantly change the percentage basal insulin secretion : insulin content, relative to untreated islets. Islets cultured under normoglycemic conditions and exposed to *C. sativa* treatment showed a reduction in the percentage of GSIS: insulin content to 0.46-fold ($P = 0.10$) in ND1T1 and 0.28-fold ($P < 0.05$) in ND1T2, relative to ND1C islets.

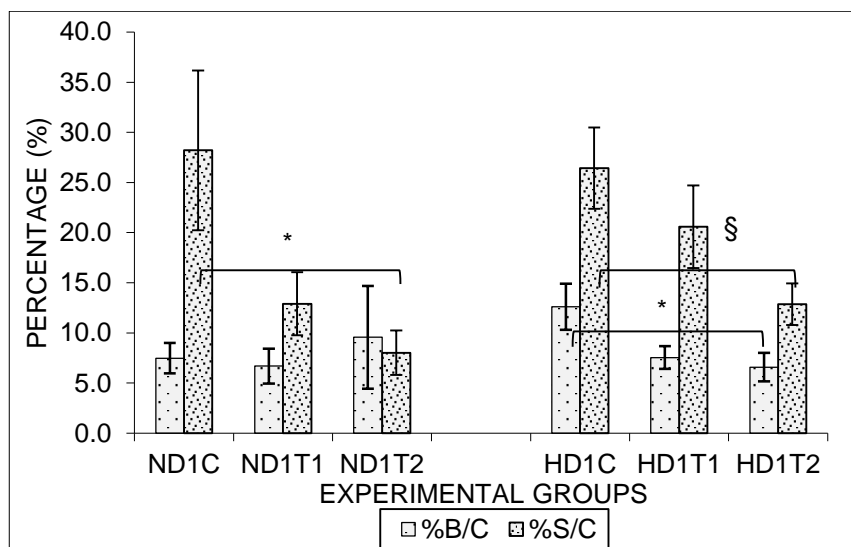


Figure 3.8 Effect of 24 hours of *C. sativa* treatment on basal and glucose-stimulated insulin secretion in relation to insulin content in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; $n = 4$.

* $P < 0.05$, %B/C, relative to HD1C: HD1T2

%S/C, relative to ND1C: ND1T2

§ $P < 0.01$, %S/C, relative to HD1C: HD1T2

In islets cultured under hyperglycemic conditions, HD1C islets increased their percentage basal insulin secretion ratio to 1.69-fold ($P = 0.09$), relative to ND1C islets. Islets cultured under hyperglycemic conditions and exposed to *C. sativa* treatment showed a reduction in the percentage basal insulin secretion : insulin content to 0.60- ($P = 0.08$) and 0.52-fold ($P < 0.05$), relative to HD1C islets. However, relative to ND1C islets, hyperglycemic conditions did not significantly affect the percentage GSIS : insulin content in HD1C islets (0.94-fold), while *C. sativa* exposure further reduced the ratio to 0.73-fold in HD1T1 islets and to 0.46-

fold in HD1T2 ($P = 0.09$) islets. The percentage GSIS:insulin content was reduced to 0.78-fold in HD1T1 and 0.49-fold in HD1T2 ($P < 0.01$), relative to HD1C islets.

3.3.2 GSIS associated with 96 hours *C. sativa* exposure

In the *in vivo* study, LNE and OBE rats were found to have increased plasma insulin levels by 11% and 24%, respectively, relative to LNC rats. Since the obese rat model did not achieve hyperglycemia, the author deemed it prudent to investigate how islets would respond to *C. sativa* exposure (96 hours) under both normo- and hyperglycemic conditions.

3.3.2.1 Chronic insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 96 hours

No significant changes in chronic insulin secretion were induced by 96 hours exposure to *C. sativa* extract, in islets cultured under normo- and hyperglycemic conditions, relative to the respective untreated control islets (figure 3.9).

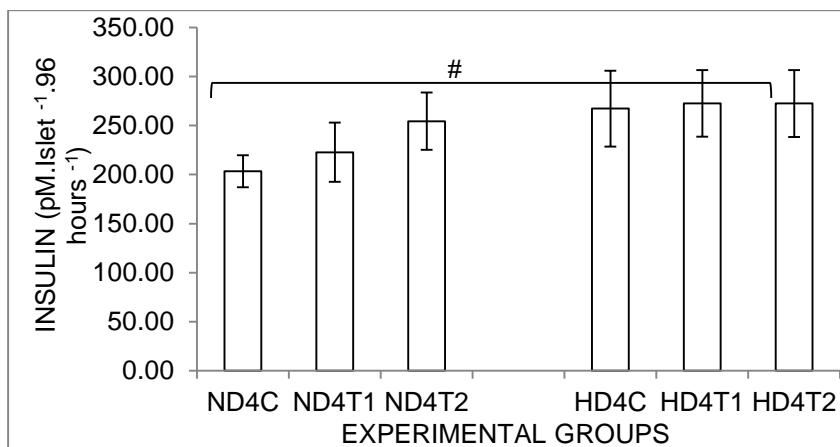


Figure 3.9 Effect of 96 hours of *C. sativa* exposure on chronic insulin secretion in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; $n = 4$.

$P < 0.001$, ND4C, HD4T2

However, HD4T1 and HD4T2 conditions induced an increase in chronic insulin secretion to 1.34-fold for both HD4T1 ($P = 0.07$) and HD4T2 ($P < 0.001$) islets, relative to ND4C islets. These trends differ from the effects of T1 and T2 conditions on islets cultured under normo- and hyperglycemic conditions in the 24 hour studies (figure 3.4).

3.3.2.2 Basal and glucose-stimulated insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 96 hours

In islets cultured under normoglycemic conditions, GSIS was significantly higher relative to insulin secretion in response to basal glucose levels ($P < 0.001$ across ND4C, ND4T1 and ND4T2 islets) (figure 3.10). In islets cultured under hyperglycemic conditions, no significant differences were observed between basal and GSIS.

No significant changes in basal insulin secretion were induced in islets exposed to *C. sativa* treatment and cultured under normoglycemic conditions, relative to ND4C islets (figure 3.10). However, the basal insulin released by islets cultured under hyperglycemic conditions were significantly higher than that of ND4C islets, with levels increasing to 2.35- (HD4C; $P < 0.001$), 1.94- (HD4T1, $P < 0.001$) and 2.59-fold (HD4T2, $P < 0.001$), relative to ND4C islets. Relative to HD4C islets, *C. sativa* exposure significantly reduced basal insulin release in HD4T1 islets to 0.82-fold ($P < 0.05$). Basal insulin secretion increased in a concentration dependent manner, with HD4T2 islets secreting significantly more insulin (1.33- fold; $P < 0.01$) in response to basal glucose stimulation, relative to the HD4T1 islets.

No significant changes in GSIS were induced in islets exposed to *C. sativa* treatment and cultured under normoglycemic conditions (figure 3.10). However, in islets cultured under hyperglycemic conditions, GSIS were significantly higher, with HD4C, HD4T1 AND HD4T2 islets secreting 1.67- ($P < 0.001$), 1.30- ($P < 0.05$) and 1.76-fold ($P < 0.001$), respectively, of the insulin secreted in ND4C islets. *C. sativa* exposure had no significant effect on GSIS across all of the experimental conditions. Relative to HD4C islets, GSIS was significantly reduced by *C. sativa* exposure in HD4T1 islets to 0.78-fold ($P < 0.05$). GSIS increased in a concentration dependent manner, with HD4T2 islets secreting significantly more insulin (1.36- fold; $P < 0.01$), relative to the HD4T1 islets.

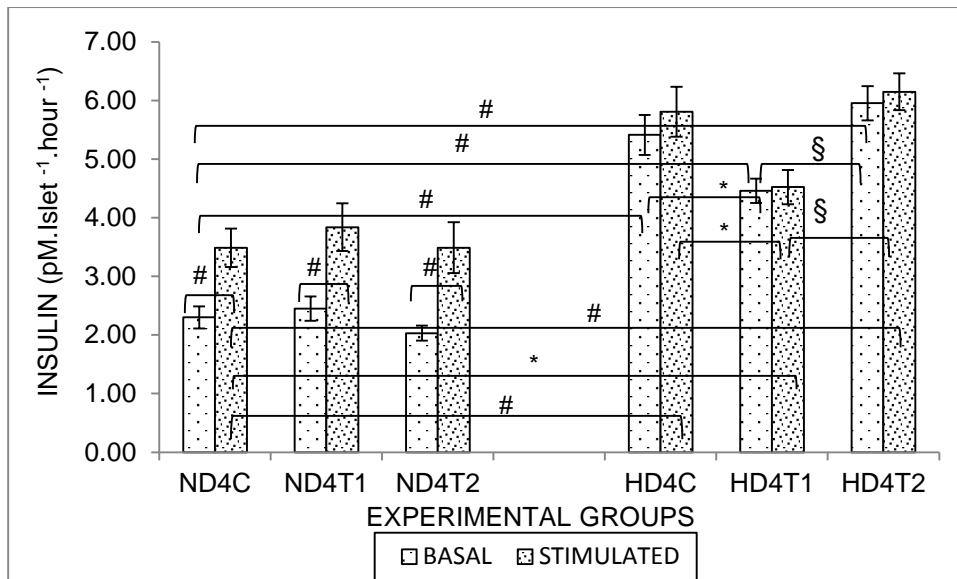


Figure 3.10 Effect of 96 hours of *C. sativa* exposure on basal and acute glucose-stimulated insulin secretion in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

- *P < 0.05, Basal - HD4C relative to: HD4T1
Stimulated - ND4C relative to: HD4T1
Stimulated - HD4C relative to: HD4T1
- §P < 0.01, Basal - HD4T1 relative to: HD4T2
Stimulated - HD4T1 relative to: HD4T2
- #P < 0.001, Intra-group basal insulin relative to appropriate stimulated insulin levels:
ND4C, ND4T1, ND4T2
Basal - ND4C relative to: HD4C; HD4T1; HD4T2
Stimulated - ND4C relative to: HD4C; HD4T2

3.3.2.3 Glucose-stimulated:basal insulin secretion associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 96 hours

C. sativa exposure had no significant effect on the glucose-stimulated:basal insulin production of islets cultured under both normo- and hyperglycemic conditions (figure 3.11). However, hyperglycemic conditions significantly reduced the glucose-stimulated:basal insulin production of islets, irrespective of the presence of *C. sativa* treatment. In HD4C, HD4T1 and HD4T2 islets, the glucose-stimulated:basal insulin production were reduced to 0.65- (P = 0.059), 0.62- (P < 0.05), 0.63-fold (P < 0.05), respectively, relative to ND4C islets. No significantly changes were induced by *C. sativa* treatments in HD4T1 and HD4T2 islets, relative to HD4C islets. These trends were generally similar to that observed in the 24-hour studies (figure 3.6)

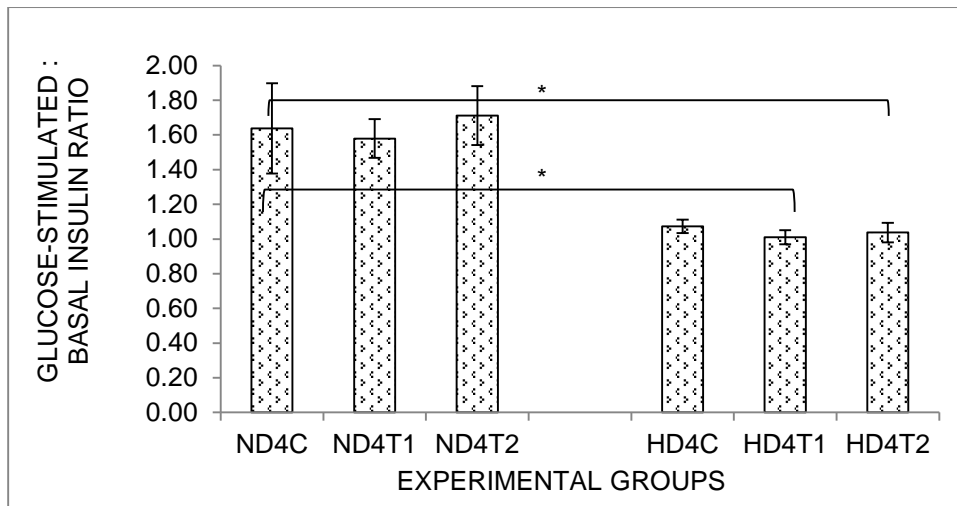


Figure 3.11 Effect of 96 hours of *C. sativa* exposure on glucose-stimulated:basal insulin production of islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

*P < 0.05, ND4C relative to: HD4T1; HD4T2

3.3.2.4 Insulin content associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 96 hours

C. sativa exposure significantly increased insulin content in islets cultured under normoglycemic conditions (figure 3.12).

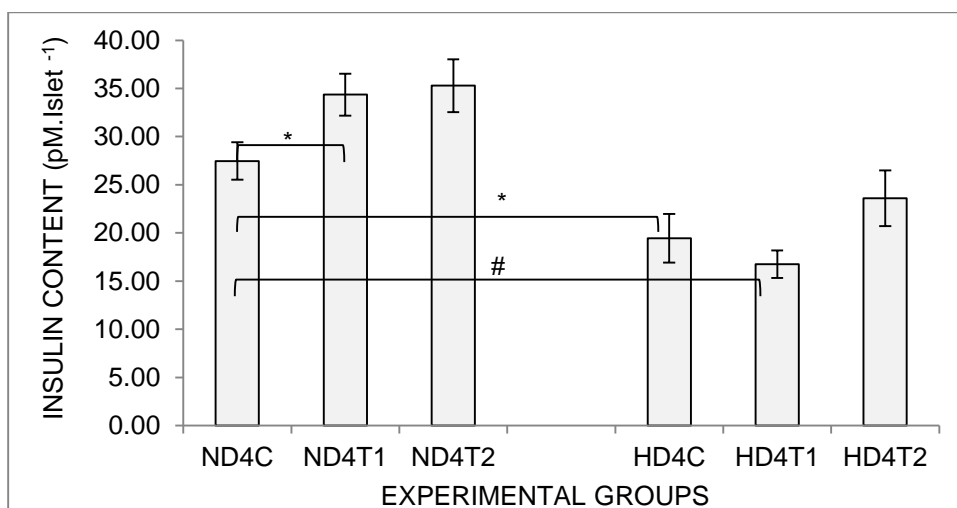


Figure 3.12 Effect of 96 hours of *C. sativa* exposure on insulin content of islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

*P < 0.05, ND4C relative to: ND4T1; HD4C

#P < 0.001, ND4C relative to: HD4T1

ND4T1 and ND4T2 increased insulin content to 1.25- ($P < 0.05$) and 1.28-fold ($P = 0.058$), relative to ND4C islets. Insulin content was significantly lower in islets cultured under hyperglycemic conditions, with HD4C islets content reduced to 0.71-fold ($P < 0.05$) of ND4C islets. *C. sativa* treatment increased insulin content in a concentration dependent manner, where HD4T1 and HD4T2 contained 0.61- ($P < 0.001$) and 0.86-fold, relative to ND4C islets. *C. sativa* treatment did not affect insulin content in HD4T1 and HD4T2 islets, relative to the HD4C islets. These trends were generally similar to that observed in the 24-hour studies (figure 3.7), however, the islets cultured under ND1T1 and ND1T2 conditions showed a reduction in insulin content, while in the 96-hour study, ND4T1 and ND4T2 conditions increased insulin content in islets.

3.3.2.5 Percentage basal and glucose-stimulated insulin secretion in relation to insulin content associated with islets cultured under normo- and hyperglycemic conditions and *C. sativa* exposure for 96 hours

Further analysis of basal and GSIS in relation to insulin content, showed that *C. sativa* treatment significantly reduced the percentage of basal insulin secretion:insulin content but not the GSIS:insulin content ratio, relative to ND4C islets (figure 3.13). In islets exposed to ND4T1 and ND4T2, the percentage basal insulin secretion:insulin content were reduced to 0.83- and 0.66-fold ($P < 0.01$), respectively, while percentage GSIS:insulin content were reduced to 0.86- and 0.77-fold, relative to ND4C islets.

Hyperglycemic conditions had no significant effect on both percentage basal and GSIS, relative to HD4C islets. However, when comparing islets cultured under hyperglycemic conditions, with and without *C. sativa*, both the percentage basal and GSIS increased significantly to 3.59- and 2.58-fold in HD4C islets, 3.29- and 2.26-fold in HD4T1 islets, and 2.96- and 2.10-fold in HD4T2 islets, respectively, relative to ND4C islets.

These trends were generally similar to that observed in the 24-hour studies (figure 3.8). However, the islets cultured under ND1T1 showed a reduction in the percentage basal insulin secretion:insulin content, and ND1T2 conditions induced an increase in the percentage basal insulin secretion : insulin content, relative to ND1C islets, while in the 96-hour study, both ND4T1 and ND4T2 conditions induce a reduction in the percentage basal insulin secretion : insulin content in the islets.

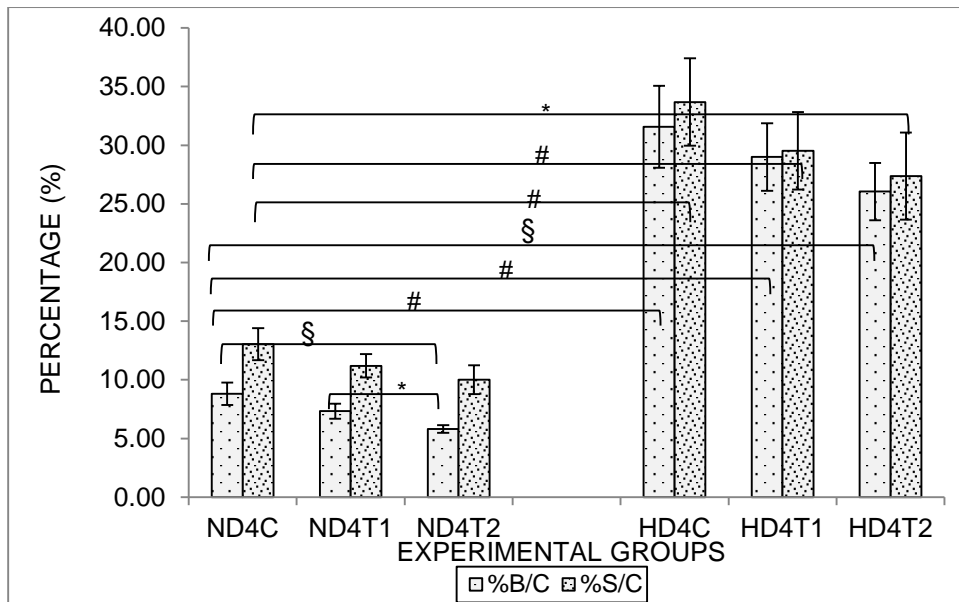


Figure 3.13 Effect of 96 hours of *C. sativa* treatment on basal and glucose-stimulated insulin secretion, in relation to insulin content in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

§P < 0.01, %S/C, relative to ND4C: ND4T2

#P < 0.001, %B/C, relative to ND4C: ND4T1; ND4T2; HD4C; HD4T1; HD4T2

%B/C, relative to HD4C: HD4T1; HD4T2

%B/C, relative to HD4T1: HD4T2

%S/C, relative to ND4C: HD4C; HD4T1; HD4T2

%S/C, relative to HD4C: HD4T1; HD4T2

%S/C, relative to HD4T1: HD4T2

3.3.3 Comparative analysis on 24- and 96-hour *C. sativa* exposure on islets cultured under normo- and hyperglycemic conditions

Comparative analysis was conducted between islets exposed for 24- and 96 hours to normo- and hyperglycemic conditions, in the presence and absence of *C. sativa* extract, on the following parameters: chronic (24 vs 96 hours), basal, glucose-stimulated insulin secretion, glucose-stimulated:basal insulin production, percentage basal and stimulated insulin secretion relative to insulin content. This analysis will determine whether extended exposure to *C. sativa* extract affected the above dimensions of β -cell function.

3.3.3.1 Comparative analysis on chronic insulin secretion in islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

The cumulative effect of 96 hours exposure to normo- and hyperglycemic media, with and without *C. sativa*, resulted in a significant increase in chronic insulin secretion, relative to insulin secreted after 24 hours, across all experimental conditions (figure 3.14). In islets

cultured under normoglycemic conditions, the effect in islets exposed to ND4T1 conditions differ from the effects exhibited in ND1T1 islets. ND4T1 conditions induced an increase in insulin secretion to 1.10-fold (not significant), while ND1T1 islets showed a significant reduction to 0.23-fold ($P < 0.01$), relative to their respective control islets.

Hyperglycemic conditions induced an increase in HD1C and HD4C islets, relative to the respective normoglycemic control islets, to 1.23- and 1.31-fold. As in islets exposed to normoglycemic conditions, *C. sativa* treatment affected islets cultured under hyperglycemic conditions differently over time. In HD4T1 and HD4T2 islets, chronic insulin secretion increased to 1.34- and 1.02-fold, relative to the ND4C and HD4C islets, respectively. However, chronic insulin secretion in HD1T1 and HD1T2 islets was reduced to 0.37- and 0.31-fold, relative to ND1C and HD1C islets, respectively. However, none of these changes were statistically significant.

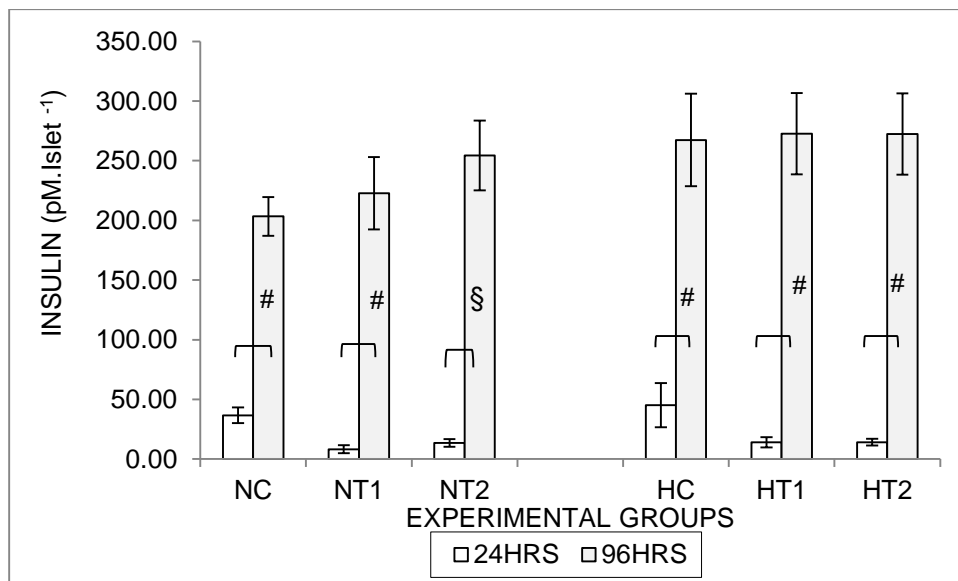


Figure 3.14 Comparison between 24- and 96 hours of *C. sativa* treatment on chronic insulin secretion, in islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

§ $P < 0.01$, ND1T2 vs ND4T2

$P < 0.001$, ND1C vs ND4C; ND1T1 vs ND4T1; ND1T2 vs ND4T2; HD1C vs HD4C; HD1T1 vs HD4T1; HD1T2 vs HD4T2

3.3.3.2 Comparative analysis on basal insulin secretion in islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

Islets exposed to normoglycemic conditions, in the presence and absence of *C. sativa* treatment for 24 and 96 hours did not show any significant differences in the basal insulin secretion (figure 3.15). Again the trends between 24- and 96-hour *C. sativa* exposure differed, where ND4T1 and ND4T2 resulted in a 1.07- and 0.88-fold change, while ND1T1 and ND1T2 induced a reduction in basal insulin secretion to 0.34- and 0.53-fold, relative to the respective control islets. The effect of ND4T1 therefore differed from that of ND1T1 exposure, albeit none of these effects were statistically significant.

Hyperglycemic conditions did not induce a significant difference in basal insulin secretion in HD1C and HD4C islets. Hyperglycemia induced an increase in basal insulin secretion in both HD1C and HD4C islets to 1.18- and 2.35-fold, relative to ND1C and ND4C islets, respectively, although these differences were not statistically significant. HD4T1 and HD4T2 resulted in significantly more basal insulin secretion (1.98- and 1.61-fold), relative to HD1T1 and HD1T2 islets, respectively ($P < 0.01$ for both comparisons). The basal secretory pattern was relatively similar, where HD4T1 and HD4T2 islets resulted in basal insulin secretion of 0.82- and 1.10-fold, respectively, relative to HD4C islets, while HD1T1 and HD1T2 islets generated basal insulin secretion of 0.50- and 0.82-fold, respectively, relative to HD1C islets. None of these effects were statistically significant.

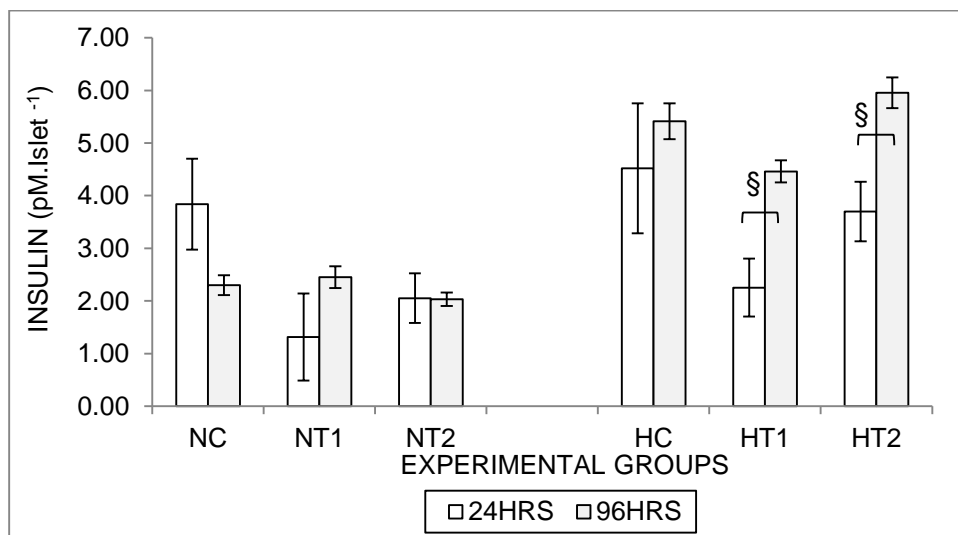


Figure 3.15 Comparison between 24- and 96 hours of *C. sativa* treatment on basal insulin secretion, in islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

[§] $P < 0.01$, HD1T1 vs HD4T1; HD1T2 vs HD4T2

3.3.3.3 Comparative analysis on glucose-stimulated insulin secretion in islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

Comparative analysis of islets cultured for 24 and 96 hours under normo- and hyperglycemic conditions, in the presence and absence of *C. sativa* extract, showed no significant differences in glucose-stimulated insulin secretion (figure 3.16). Glucose-stimulated insulin secretion in ND4T1 and ND4T2 islets were 1.10- and 1.00-fold of ND4C levels, while glucose-stimulated insulin secretion were 0.24- and 0.28-fold of ND1C secretions, in ND1T1 and ND1T2 islets respectively. However, none of these effects were statistically significant.

Extended exposure to hyperglycemic conditions resulted in different responses, where HD4C islets resulted in 1.67-fold increase in glucose-stimulated insulin secretion, while HD1C islets resulted in a reduction to 0.91-fold, relative to ND4C and ND1C islets, respectively. The effect of extended exposure to HD4T1 and HD4T2 resulted in islets secreting 1.98- and 1.61-fold, relative to HD1T1 and HD1T2 islets, respectively. Islets exposed to HD4T1 and HD4T2 conditions showed glucose-stimulated insulin levels at 0.78- and 1.06-fold of HD4C, while HD1T1 and HD1T2 islets showed glucose-stimulated insulin levels at 0.62- and 0.70-fold of HD1C islets, relative to HD1C islets. Again, none of these effects were statistically significant.

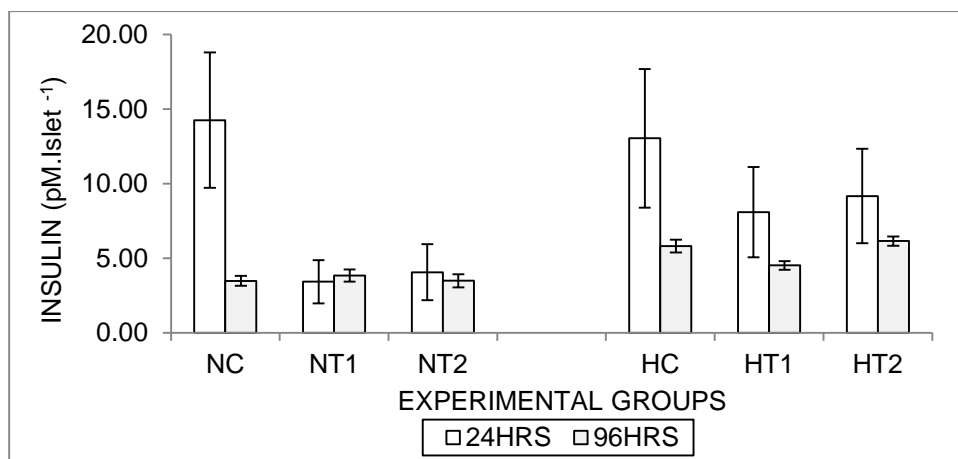


Figure 3.16 Comparison between 24- and 96 hours of *C. sativa* treatment on glucose-stimulated insulin secretion, in islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

3.3.3.4 Comparative analysis on glucose-stimulated:basal insulin production of islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

The glucose-stimulated:basal insulin production of ND4C islets was significantly reduced ($P < 0.05$) to 0.24-fold, relative to ND1C islets (figure 3.17). Longer exposure to *C. sativa* treatment, under normoglycemic conditions, did not induce any significant differences in secretory indices, relative to the same experimental conditions at 24 hours exposure time. However, the effects of *C. sativa* exposure on the glucose-stimulated:basal insulin production were different when comparing 24 and 96 hours exposure. ND4T1 and ND4T2 islets showed glucose-stimulated:basal insulin production at 0.96- and 1.05-fold relative to ND4C islets, while ND1T1 and ND1T2 islets showed glucose-stimulated:basal insulin production at 0.84- and 0.44-fold of ND1C islets. However, these effects were not statistically significant.

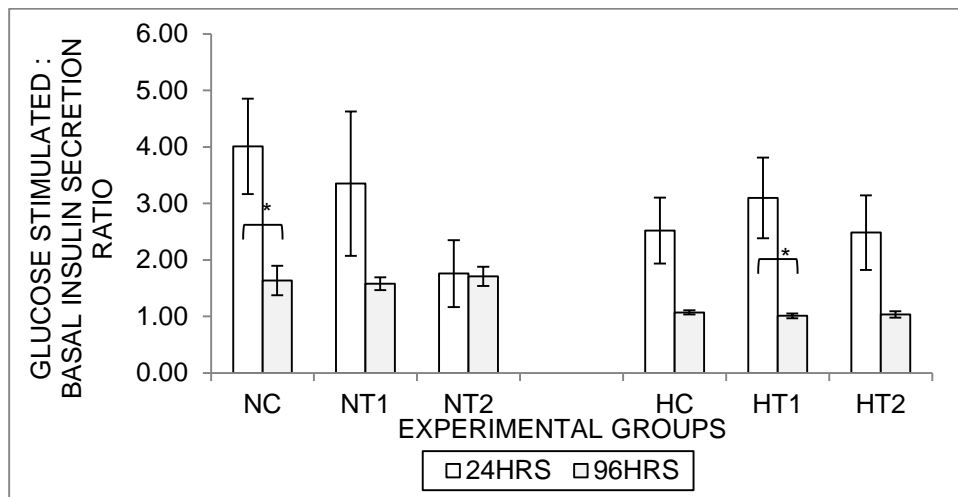


Figure 3.17 Comparison between 24- and 96 hours of *C. sativa* treatment on the glucose-stimulated:basal insulin production of islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

* $P < 0.05$, ND1C vs ND4C; HD1T1 vs HD4T1

Under hyperglycemic conditions, HD4C islets showed a reduction in the glucose-stimulated:basal insulin production to 0.43-fold, relative to HD1C islets ($P = 0.06$). Extended *C. sativa* exposure under hyperglycemic conditions reduced the glucose-stimulated:basal insulin production of HD4T1 and HD4T2, to 0.33- ($P < 0.03$) and 0.42-fold ($P = 0.08$) of HD1T1 and HD1T2, respectively. Glucose-stimulated:basal insulin production associated with HD4T1 and HD4T2 islets were reduced to 0.94- and 0.97-fold, relative to HD4C islets, while HD1T1 and HD1T2 islets had glucose-stimulated:basal insulin production at 1.23- and

0.99-fold, relative to HD1C islets, respectively. None of these effects were statistically significant.

3.3.3.5 Comparative analysis on insulin content of islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

Insulin content was significantly lower (0.50-fold; $P < 0.05$) in ND4C islets, relative to ND1C islets (figure 3.18). *C. sativa* treatment did not induce any significant changes in insulin content, relative to their respective 24-hour counter-parts.

Extended hyperglycemic conditions induced a reduction in insulin content in HD4C islets (0.45-fold; $P = 0.10$), relative to HD1C islets. *C. sativa* treatment induced a significant reduction in insulin content in HD4T2 islets ($P < 0.05$), to 0.38-fold, relative to HD1T2 islets.

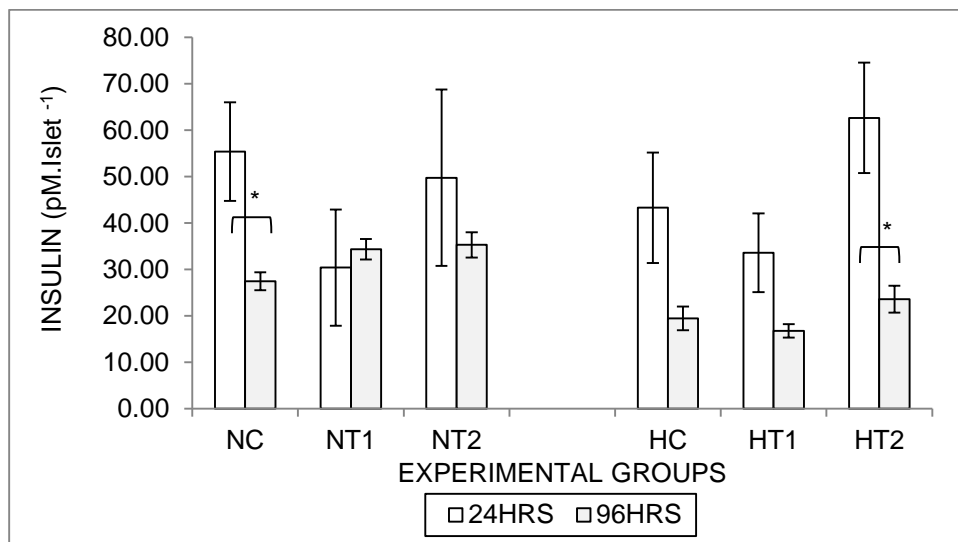


Figure 3.18 Comparison between 24- and 96 hours of *C. sativa* treatment on the insulin content of islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4. * $P < 0.05$, ND1C vs ND4C; HD1T2 vs HD4T2

3.3.3.6 Comparative analysis on the percentage basal insulin secretion in relation to insulin content of islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

Longer exposure to normoglycemic conditions in ND4C islets resulted in a significant increase in the percentage of basal insulin secreted relative to insulin content, to 1.18-fold ($P < 0.05$), compared to ND1C islets, however longer *C. sativa* exposure had no significant effect, relative to their respective 24-hour counterparts.

Hyperglycemia significantly increased basal insulin secretion in HD4C islets to 3.75-fold ($P < 0.001$), relative to HD1C islets. Similarly, longer exposure to *C. sativa* extract under hyperglycemic conditions significantly increased the proportion of basal insulin secretion in relation to insulin content, to 6.71- and 4.20-fold ($P < 0.001$ for both), relative to HD1T1 and HD1T2, respectively.

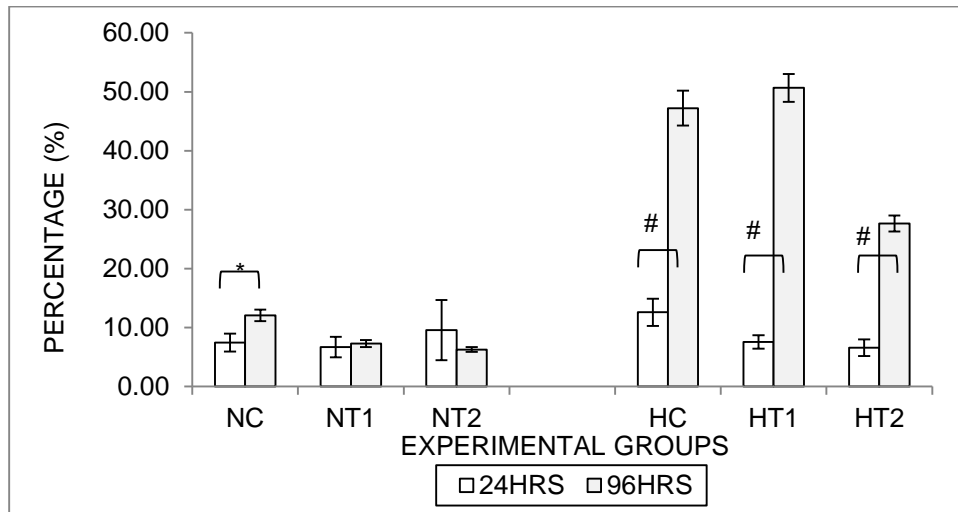


Figure 3.19 Comparison between 24- and 96 hours of *C. sativa* treatment on percentage of basal insulin secretion in relation to insulin content of islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

* $P < 0.05$, ND1C vs ND4C

$P < 0.001$, HD1C vs HD4C; HD1T1 vs HD4T1; HD1T2 vs HD4T2

3.3.3.7 Comparative analysis on the percentage GSIS in relation to insulin content of islets cultured under normo- and hyperglycemic conditions for 24- and 96-hour *C. sativa* exposure

Figure 3.20 shows that 96 hours exposure to normoglycemic conditions, with and without *C. sativa* extract, had no significant effect on the percentage GSIS:insulin content, relative to the relevant 24-hour counterparts.

Hyperglycemic conditions, with and without *C. sativa* extract, significantly increased the proportion of GSIS relative to insulin content to 1.92-, 2.50- and 2.22-fold ($P < 0.001$ for all) in HD4C, HD4T1 and HD4T2 islets, relative to HD1C, HD1T1 and HD1T2, respectively.

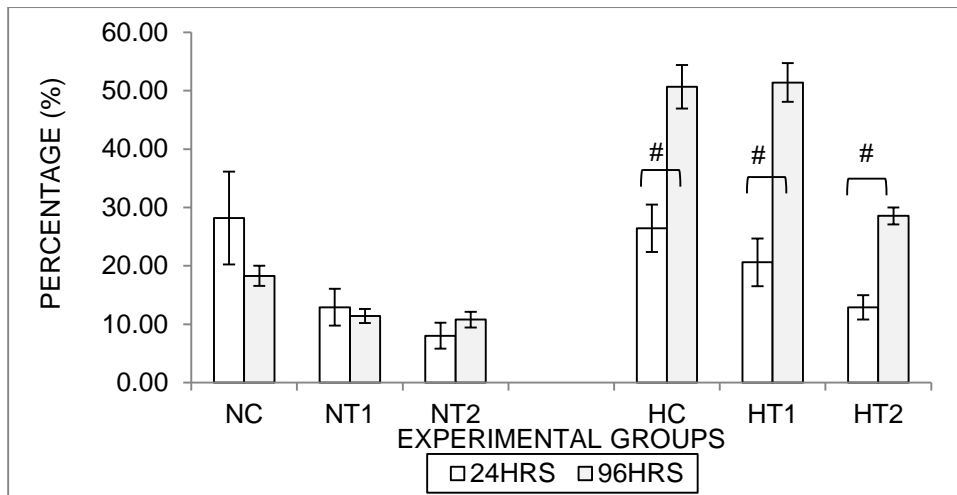


Figure 3.20 Comparison between 24- and 96 hours of *C. sativa* treatment on percentage of GSIS in relation to insulin content of islets cultured under normo- and hyperglycemic conditions, where N = normoglycemic; H = hyperglycemic; T1 = 2.5 ng/mL THC; T2 = 5.0 ng/mL THC in *C. sativa* extract; n = 4.

[#]P < 0.001, HD1C vs HD4C; HD1T1 vs HD4T1; HD1T2 vs HD4T2

In summary, the effect of *C. sativa* exposure on islet function differed depending on the glucose concentration in the culture media, the *C. sativa* concentration and exposure time of islets to *C. sativa* treatment in the following ways:

Effect of glucose concentration in the media:

- In HD1C islets, the concentration of glucose in the media did not have any significant effect on the parameters analysed, relative to ND1C islets, however, HD4C islets showed significant differences across the following parameters, relative to ND4C islets: basal insulin secretion increased to 2.35-fold (P < 0.001); GSIS increased to 1.67-fold (P < 0.001); insulin content decreased to 0.71-fold (P < 0.05); the glucose-stimulated:basal insulin production decreased to 0.65-fold (P = 0.06), the percentage basal insulin secretion : insulin content increased to 3.59 (P < 0.001) and the percentage GSIS : insulin content increased to 2.58 (P < 0.001).

Effect of *C. sativa* concentration:

- Under normoglycemic conditions, no significant differences were observed between the effects of 2.5 ng/mL THC and 5.0 ng/mL THC in relation to all parameters analysed in the 24-hour study, while in the 96-hour study, the percentage basal insulin secretion in relation to insulin content was found to be significantly lower in ND4T2 islets (0.79-fold, P < 0.05), relative to ND4T1 islets.

- Under hyperglycemic conditions, no significant differences were observed between the effects of 2.5 ng/mL THC and 5.0 ng/mL THC in relation to all parameters analysed in the 24-hour study, while in the 96-hour study, the following parameters were found to differ

significantly between islets exposed to HD4T1 and HD4T2 conditions: basal insulin secretion increased to 1.34-fold ($P < 0.01$), GSIS increased to 1.36-fold ($P < 0.01$), and content increased to 1.41-fold ($P = 0.09$), in HD4T2 islets, relative to HD4T1 islets.

Effect of exposure time:

- Chronic insulin secretion in islets exposed to normoglycemic conditions for 96 hours, with and without *C. sativa*, was significantly increased to 5.54-fold in ND4C ($P < 0.001$), 26.98-fold in ND4T1 ($P < 0.001$) and 18.87-fold in ND4T2 ($P < 0.01$) islets, relative to ND1C, ND1T1 and ND1T2 islets, respectively.

- Similarly, in islets cultured under hyperglycemic conditions for 96 hours, with and without *C. sativa*, chronic insulin secretion was significantly increased to 5.93-fold in HD4C ($P < 0.001$), 19.47-fold in HD4T1 ($P < 0.001$) and 19.36-fold in HD4T2 ($P < 0.01$) islets, relative to HD1C, HD1T1 and HD1T2 islets, respectively.

- Basal insulin secretion in islets cultured under normoglycemic conditions, in the presence and absence of *C. sativa*, showed no significant differences between the 24- and 96-hour studies, while in islets cultured under hyperglycemic conditions, basal insulin secretion increased significantly to 1.98-fold in HD4T1 ($P < 0.01$) and 1.61-fold in HD4T2 ($P < 0.01$) islets, relative to HD1T1 and HD1T2 islets, respectively.

- No significant differences were observed in the GSIS of islets cultured for 24 and 96 hours under normo- and hyperglycemic conditions, respectively, in the presence and absence of *C. sativa*.

-The secretory indices were significantly reduced to 0.41-fold in ND4C islets ($P < 0.05$) relative to ND1C islets, while under hyperglycemic conditions, the glucose-stimulated:basal insulin production of HD4C, HD4T1 and HD4T2 islets were significantly reduced to 0.43- ($P = 0.06$), 0.33- ($P < 0.05$) and 0.42-fold ($P = 0.08$), relative to HD1C, HD1T1 and HD1T2 islets, respectively.

- Insulin content were significantly reduced to 0.50-fold in ND4C islets ($P < 0.05$), relative to ND1C islets, while insulin content in the HD4C, HD4T1 and HD4T2 islets was reduced to 0.45- ($P = 0.10$), 0.50- ($P = 0.11$) and 0.38-fold ($P < 0.05$), relative to HD1C, HD1T1 and HD1T2 islets, respectively.

- The percentage basal insulin secretion:insulin content was significantly increased to 1.18-fold in ND4C ($P < 0.05$), 3.75-fold in HD4C ($P < 0.001$), 6.71-fold in HD4T1 ($P < 0.001$) and 4.20-fold in HD4T2 ($P < 0.001$), relative to HD1C, HD1T1 and HD1T2 islets, respectively.

- The percentage GSIS:insulin content was significantly increased to 1.92-fold in HD4C ($P < 0.001$), 2.50-fold in HD4T1 ($P < 0.001$) and 2.22-fold in HD4T2 ($P < 0.001$), relative to HD1C, HD1T1 and HD1T2 islets, respectively.

Overall effect of experimental conditions on chronic insulin secretion:

- Chronic insulin secretion was significantly reduced to 0.23-fold in ND1T1 ($P < 0.01$) and 0.37-fold in ND1T2 ($P < 0.01$) islets, respectively, relative to the ND1C islets.

- Chronic insulin secretion remained unaffected by *C. sativa* treatment in islets cultured under hyperglycemic conditions, in the 24- and 96-hour studies, relative to the respective control islets.

- HD1T1 and HD1T2 conditions induced a significant reduction in chronic insulin secretion to 0.38-fold for both HD1T1 ($P < 0.05$) and, HD1T2 ($P < 0.01$), relative to ND1C islets, while the 96-hour exposure to HD4T1 and HD4T2 conditions resulted in a significant increase in chronic insulin secretion to 1.34-fold for both HD4T1 ($P = 0.07$) and HD4T2 ($P < 0.001$), relative to ND4C islets.

Overall effect of experimental conditions on basal insulin secretion:

- Basal insulin secretion was reduced to 0.34-fold in ND1T1 ($P = 0.06$) and 0.53-fold in ND1T2 ($P = 0.10$) islets, relative to ND1C islets, while prolonged exposure had no effect on basal insulin secretion, relative to ND4C islets.

- Basal insulin secretion was significantly reduced to 0.82-fold in HD4T1 ($P < 0.05$) islets, while HD4T2 conditions increased basal insulin levels to 1.10-fold, relative to HD4C islets.

- Basal insulin secretion was significantly increased to 2.35-fold in HD4C ($P < 0.001$), 1.94-fold in HD4T1 ($P < 0.001$) and 2.59-fold in HD4T2 islets, relative to ND4C islets.

Overall effect of experimental conditions on GSIS:

- GSIS remained unaffected in islets cultured under normo- and hyperglycemic conditions and exposed for 24 hours to *C. sativa*, except in ND1T1 and ND1T2 islets, where GSIS levels were reduced to 0.24- ($P < 0.05$) and 0.28-fold ($P = 0.06$), relative to ND1C islets.

- In islets cultured under normoglycemic conditions, ND4T1 and ND4T2 conditions did not significantly affect GSIS levels, relative to ND4C islets.

- Under hyperglycemic conditions, HD4C (1.67-fold, $P < 0.001$), HD4T1 (1.30-fold, $P < 0.05$) and HD4T2 (1.76-fold, $P < 0.001$) conditions resulted in significantly higher GSIS levels, relative to the ND4C islets.

- In islets exposed to HD4T1 and HD4T2 conditions, GSIS differed significantly ($P < 0.01$), where HD4T1 treatment resulted in a reduction in GSIS to 0.78-fold ($P < 0.05$), while HD4T2 treatment induced an increase in GSIS to 1.06-fold, relative to HD4C islets.

Overall effect of experimental conditions on glucose-stimulated:basal insulin production:

- The glucose-stimulated:basal insulin production of islets exposed to ND1T2 was significantly lower (0.44-fold, $P < 0.05$), relative to ND1C islets. Islets exposed to all other experimental conditions associated with normo- and hyperglycemic conditions for 24 hours showed no significant changes to their glucose-stimulated:basal insulin production of the islets.

- The glucose-stimulated:basal insulin production were not significantly influenced when exposed for 96 hours to the various experimental conditions under normo- and hyperglycemic conditions, relative to ND4C and HD4C islets. However, relative to ND4C (but not HD4C) islets, in islets cultured under hyperglycemic conditions, the glucose-stimulated:basal insulin production of HD4C, HD4T1 and HD4T2 islets were significantly reduced to 0.65- ($P = 0.06$), 0.62- ($P < 0.05$) and 0.63-fold ($P < 0.05$).

Overall effect of experimental conditions on insulin content:

- Insulin content showed no significant changes across all experimental conditions after 24 hours exposure, relative to ND1C and HD1C islets, respectively.

- Under normoglycemic conditions, insulin content increased significantly in ND4T1, ND4T2 islets, to 1.25- ($P < 0.05$) and 1.28-fold ($P = 0.06$), relative to ND4C islets.

- Insulin content was significantly less in ND4C islets (0.50-fold), relative to ND1C islets.

- Under hyperglycemic conditions, insulin content decreased significantly in HD4C and HD4T1 islets, to 0.71- ($P < 0.05$) and 0.61-fold ($P < 0.001$), relative to ND4C islets, but not HD4C islets.

Overall effect of experimental conditions on the percentage basal insulin secretion relative to insulin content:

- Under normoglycemic conditions, the percentage of basal insulin secretion relative to insulin content across all experimental conditions exposed for 24 hours showed no significant differences, relative to the ND1C islets, while under hyperglycemic conditions *C. sativa* treatment resulted in a significant reduction in the percentage basal insulin to 0.60-fold ($P = 0.08$) and 0.52-fold ($P < 0.05$) in HD1T1 and HD1T2 islets, relative to HD1C islets.

- Under prolonged normoglycemic conditions, the percentage of basal insulin secretion relative to insulin content were significantly reduced to 0.66-fold ($P < 0.01$) in ND4T2 islets,

relative to ND4C islets. *C. sativa* treatment elicited a significant difference in the percentage basal secretion in ND4T2 (0.79-fold, $P < 0.05$), relative to ND4T1 islets.

- The percentage of basal insulin secretion relative to insulin content in HD4C, HD4T1 and HD4T2 levels were significantly increased to 3.59- ($P < 0.001$), 3.29- ($P < 0.001$) and 2.96-fold ($P < 0.01$), relative to ND4C islets, but had no significant effect relative to HD4C islets.

Overall effect of experimental conditions on the percentage GSIS relative to insulin content:

- In islets exposed to experimental conditions for 24 hours, both ND1T1 and ND1T2 significantly reduced the percentage of GSIS relative to insulin content to 0.46-fold ($P = 0.10$) and 0.28-fold ($P < 0.05$), when compared to ND1C islets, while islets exposed to HD1T2 conditions significantly reduced the percentage of GSIS relative to insulin content, to 0.46- ($P = 0.09$) and 0.49-fold ($P < 0.01$), when compared to ND1C and HD1C islets, respectively.

- Under normo- and hyperglycemic conditions for 96 hours, the effect of HD4C, HD4T1 and HD4T2 conditions on the percentage of GSIS relative to insulin content, were as follows: 2.58- ($P < 0.001$), 2.26- ($P < 0.001$) and 2.10-fold ($P < 0.05$), relative to ND4C (but not HD4C) islets.

3.3.4 GSIS associated with the inverse agonist/antagonist studies

To ascertain whether CB1 and CB2 receptors modulate insulin secretion in rat pancreatic β -cells, islets cultured under normo- and hyperglycemic conditions, were pretreated with a CB1 and CB2 receptor antagonist for 20 minutes, after which the islets were exposed to *C. sativa* extract (T1) for 48 hours. The cumulative insulin secretions over the 48 hours, across the various conditions were measured and represent the chronic insulin secretion reported in the section below. In addition, hourly insulin secretions were measured in response to basal and postprandial glucose stimulation, and secretory indices were generated from these. Insulin content was determined and the percentage basal and glucose-stimulated insulin secretion was calculated in relation to insulin content.

3.3.4.1 Chronic insulin secretion associated with 48 hours *C. sativa* exposure, in presence and absence of CB1 and CB2 inverse agonists/antagonists, under normo- and hyperglycemic conditions

In islets cultured under normoglycemic conditions, T1 induced an increase (1.47-fold, $P < 0.05$) in chronic insulin secretion, relative to control (NC) islets (figure 3.21). These findings support the overall effect observed in islets exposed to T1 for 96 hours (figure 3.9). Chronic

insulin secretion was significantly reduced by A1 and A2 pre-conditioning in NA1 (0.33-fold, $P < 0.001$) and NA2 (0.60-fold, $P < 0.05$) islets, relative to NC islets. Subsequent *C. sativa* exposure resulted in a further reduction in NA1T1 (0.29-fold, $P < 0.001$), but not in NA2T1 (0.73-fold) islets, relative to chronic insulin in NC islets. Pre-conditioning with either A1 or A2 significantly reduced chronic insulin secretion in NA1 (0.22-fold, $P < 0.001$), NAT1 (0.20-fold, $P < 0.001$), NA2 (0.41-fold, $P < 0.001$) and NA2T1 (0.49-fold, $P < 0.01$), relative to chronic insulin secretion by NT1 islets.

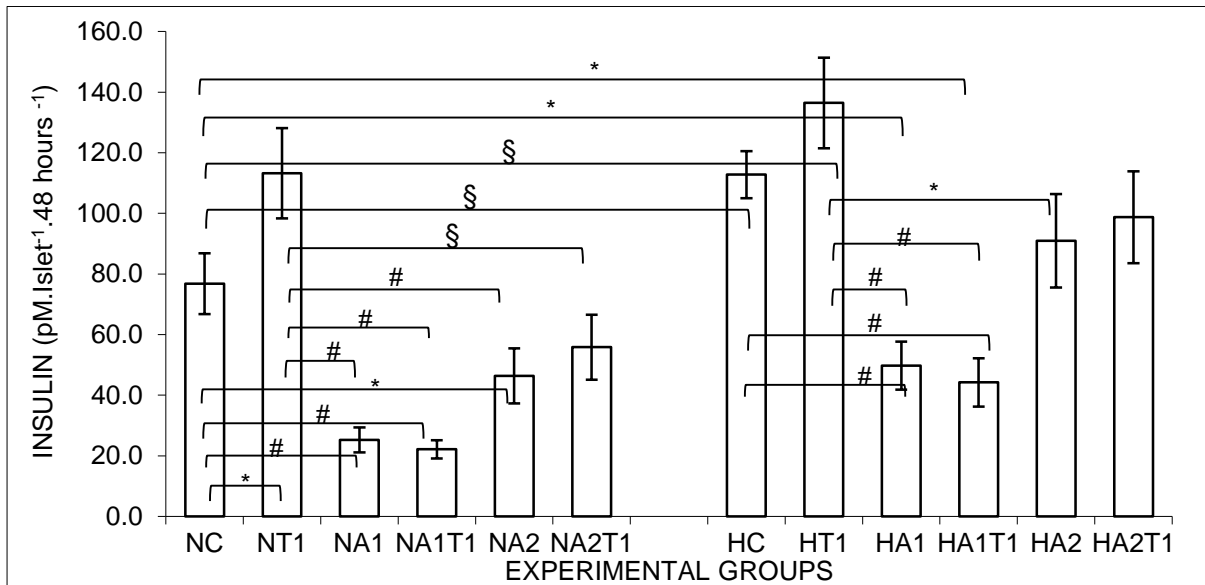


Figure 3.21 Effect of *C. sativa* exposure for 48 hours, on chronic insulin secretion in islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-treatment, where T1 = 2.5 ng/mL, A1 = 90 μ M AM251, and A2 = 100 μ M AM630; n = 4.

- * $P < 0.05$, NC relative to: NT1; NA2; HA1; HA1T1
HT1 relative to: HA2
- § $P < 0.01$, NC relative to: HC; HT1
NT1 relative to: NA2T1
- # $P < 0.001$, NC relative to: NA1; NA1T1
NT1 relative to: NA1; NA1T1; NA2
HC relative to: HA1; HA1T1
HT1 relative to: HA1; HA1T1

Hyperglycemia induced a 1.47-fold ($P < 0.01$) increase in chronic insulin secretion in HC islets, relative to NC islets (figure 3.21). In HT1 islets, *C. sativa* extract significantly increased chronic insulin levels to 1.78-fold ($P < 0.01$) of chronic insulin levels observed in NC islets, but not statistically different from HC islets. This is consistent with the increased chronic insulin levels induced by *C. sativa* exposure in islets cultured under hyperglycemic conditions in the 96 (figure 3.9) hour studies. Pre-conditioning of islets with A1, in HA1 and HA1T1 islets, significantly reduced chronic insulin secretion to 0.44-fold ($P < 0.001$) and 0.39-fold

($P < 0.001$), respectively, of chronic insulin secreted by HC islets. Similarly, pre-conditioning of islets with A1, HA1 and HA1T1 conditions significantly reduced chronic insulin secretion to 0.36-fold ($P < 0.001$) and 0.32-fold ($P < 0.001$), respectively, relative to HT1 islets. A2 pre-conditioning in HA2 and HA2T1 islets had no significant effect on chronic insulin secretion, relative to HC islets. However, HA2 and HA2T1 conditions resulted in a significant reduction in chronic insulin secretion to 0.67- ($P < 0.05$) and 0.72-fold ($P = 0.08$), relative to HT1 islets.

3.3.4.2 Basal and Glucose-stimulated insulin secretion associated with 48 hours *C. sativa* exposure, in presence and absence of CB1 and CB2 inverse agonists/antagonists, under normo- and hyperglycemic conditions

In NT1 islets, *C. sativa* treatment reduced basal insulin secretion to 0.87-fold, relative to NC islets (figure 3.22), which was not found to be statistically significant. This is consistent with that data observed in islets subjected to 24 (figure 3.5) hours *C. sativa* exposure. Both antagonists, in the presence and absence of *C. sativa*, significantly reduced basal insulin secretion to the following levels, relative to NC islets – NA1 (0.28-fold, $P < 0.001$), NA1T1 (0.39-fold, $P < 0.001$), NA2 (0.36-fold, $P < 0.001$) and NA2T1 (0.49-fold, $P < 0.001$). Similarly, pre-conditioning with A1 and A2, in the presence and absence of *C. sativa* treatment, significantly reduced basal insulin secretion to the following levels, relative to NT1 islets - NA1 (0.33-fold, $P < 0.001$), NA1T1 (0.44-fold, $P < 0.001$), NA2 (0.41-fold, $P < 0.001$) and NA2T1 (0.57-fold, $P < 0.01$). In NA1T1 and NA2T1 islets, *C. sativa* exposure resulted in an increase in basal insulin levels to 1.36-fold ($P < 0.05$) and 1.38-fold ($P = 0.07$), relative to NA1 and NA2 islets, respectively. Basal insulin secretion was significantly lower than the respective GSIS observed across all normoglycemic experimental conditions: NC (3.12-fold, $P < 0.001$); NT1 (1.98-fold, $P < 0.05$); NA1 (3.30-fold, $P < 0.001$); NA1T1 (3.04-fold, $P < 0.001$); NA2 (2.48-fold, $P < 0.001$) and NA2T1 (2.43-fold, $P < 0.1$).

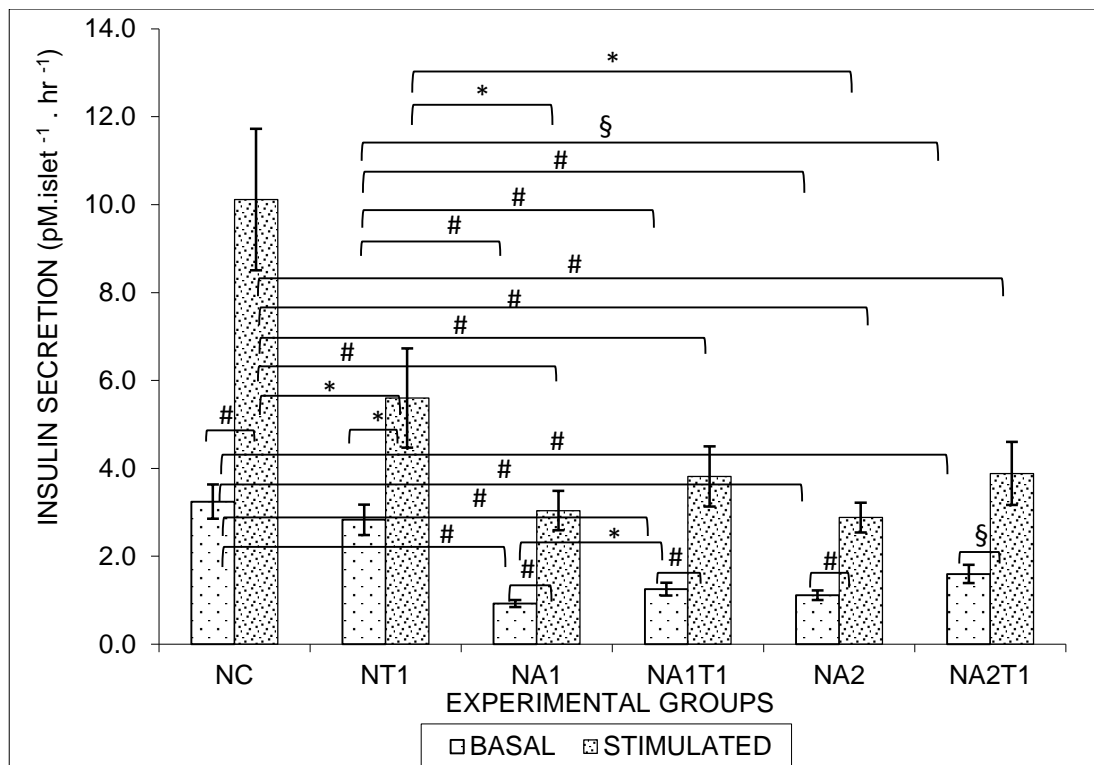


Figure 3.22 Effect of 48 hours of *C. sativa* exposure on basal and glucose-stimulated insulin secretion in islets cultured under normoglycemic conditions, subsequent to A1 or A2 preconditioning, where T1 = 2.5 ng/mL, A1 = AM251, and A2 = AM630; n=4.

- *P < 0.05, Basal – NA1 relative to: NA1T1
 Stimulated – NC relative to: NT1
 Stimulated – NT1 relative to: NA1; NA2
 Intra-group basal insulin relative to appropriate stimulated insulin secretion: NT1
- §P < 0.01, Basal – NT1 relative to: NA2T1
 Intra-group basal insulin relative to appropriate stimulated insulin secretion: NA2T1
- #P < 0.001, Basal – NC relative to: NA1; NA1T1; NA2; NA2T1
 Basal – NT1 relative to: NA1; NA1T1; NA2
 Stimulated – NC relative to: NA1; NA1T1; NA2; NA2T1
 Intra-group basal insulin relative to appropriate stimulated insulin secretion: NC; NA1; NA1T1; NA2

NT1 exposure significantly reduced GSIS to 0.55-fold ($P < 0.05$), relative to NC islets. This effect was consistent with observations made in the 24 hour study (figure 3.5). Both inverse agonists/antagonists, in the presence and absence of *C. sativa*, significantly reduced GSIS to the following levels, relative to NC islets - NA1 (0.28-fold, $P < 0.001$), NA1T1 (0.39-fold, $P < 0.001$), NA2 (0.36-fold, $P < 0.001$) and NA2T1 (0.49-fold, $P < 0.001$). Similarly, preconditioning with A1 and A2, in the absence of *C. sativa* treatment, significantly reduced GSIS to the following levels, relative to NT1 islets - NA1 (0.54-fold, $P < 0.05$) and NA2

(0.51-fold, $P < 0.05$). In NA1T1 and NA2T1 islets, *C. sativa* exposure had no significant effect on GSIS, relative to insulin levels in NA1 and NA2 islets, respectively.

Islets cultured under hyperglycemic conditions showed significantly higher basal insulin secretion (1.49-fold, $P < 0.05$), and *C. sativa* exposure similarly increased basal secretion to 1.34-fold, relative to NC islets (figure 3.23). These findings supported observations in the 96-hour (figure 3.10) studies. However, relative to HC islets, basal insulin secretions were not found to be significantly different.

Both inverse agonists/antagonists, with and without *C. sativa* exposure, had no significant effect on basal insulin secretion in HA1, HA1T1, HA2 and HA2T1 islets, relative to NC islets. However, relative to HC islets, basal insulin secretion was significantly reduced in HA1 (0.56-fold, $P < 0.001$), HA1T1 (0.51-fold, $P < 0.001$), HA2 (0.62-fold, $P < 0.001$) and HA2T1 (0.73-fold, $P < 0.05$) islets. The effect of *C. sativa* was significantly reduced in islets exposed to the inverse agonists/antagonists, in islets cultured under the following conditions - HA1 (0.62-fold, $P < 0.001$), HA1T1 (0.57-fold, $P < 0.001$), HA2 (0.69-fold, $P < 0.001$) and HA2T1 (0.81-fold, $P = 0.10$), relative to HT islets. No significant differences were observed in basal insulin secretions in HA1 relative to HA1T1 islets, and HA2 relative to HA2T1 islets.

In islets cultured under hyperglycemic conditions, GSIS was significantly higher than the respective basal insulin secretions observed across all experimental conditions - HC (1.32-fold, $P = 0.07$); HT1 (1.45-fold, $P < 0.05$); HA1 (1.98-fold, $P < 0.01$); NA1T1 (1.62-fold, $P < 0.01$); NA2 (1.31-fold, $P = 0.06$) and NA2T1 islets showed no difference between basal and GSIS.

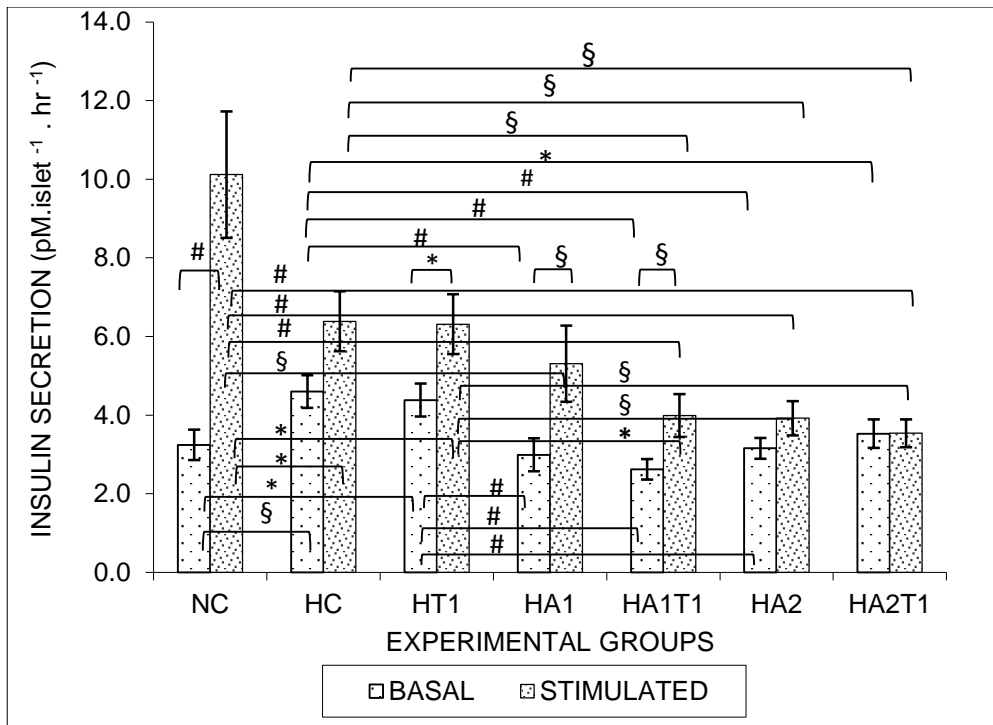


Figure 3.23 Effect of 48 hours of *C. sativa* exposure on basal and glucose-stimulated insulin secretion in islets cultured under hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where A1 = AM251; A2 = AM630; T1 = 2.5 ng/mL; n = 4.

- *P < 0.05, Basal - NC relative to: HT1
- Basal - HC relative to: HA2T1
- Stimulated - NC relative to: HC; HT1
- Stimulated - HT1 relative to: HA1T1
- Intra-group basal insulin relative to appropriate stimulated insulin secretion: HT1
- §P < 0.01, Basal - NC relative to: HC
- Stimulated - NC relative to: HA1
- Stimulated - HC relative to: HA1T1; HA2; HA2T1
- Stimulated - HT1 relative to: HA2; HA2T1
- Intra-group basal insulin relative to appropriate stimulated insulin secretion: HA1; HA1T1
- #P < 0.001, Basal - HC relative to: HA1; HA1T1; HA2
- Basal - HT1 relative to: HA1; HA1T1; HA2
- Stimulated - NC relative to: HA1T1; HA2; HA2T1
- Intra-group basal insulin relative to appropriate stimulated insulin secretion: NC

Hyperglycemic conditions significantly reduced GSIS in HC to 0.63-fold ($P < 0.05$), relative to NC islets. *C. sativa* exposure significantly reduced GSIS to 0.62-fold ($P < 0.05$) in HT1 islets, relative to NC islets (but had no effect, relative to HC islets). These effects were consistent with observations made in the 24-hour study (figure 3.5), while an increase was observed in the 96-hour study (figure 3.10). Exposure to A1 and A2 inverse agonists/antagonists, in the presence and absence of *C. sativa*, significantly reduced GSIS in

HA1 (0.52-fold, $P < 0.01$), HA1T1 (0.39-fold, $P < 0.001$), HA2 (0.39-fold, $P < 0.001$) and HA2T1 (0.35-fold, $P < 0.001$) islets, relative to NC islets. Pre-conditioning with the inverse agonists/antagonists, in the presence and absence of *C. sativa*, affected GSIS relative to HC islets, in the following ways - HA1 (0.83-fold), HA1T1 (0.62-fold, $P < 0.01$), HA2 (0.61-fold, $P < 0.01$) and HA2T1 (0.55-fold, $P < 0.01$).

3.3.4.3 Glucose-stimulated:basal insulin production associated with 48 hours *C. sativa* exposure, in presence and absence of CB1 and CB2 inverse agonists/antagonists, under normo- and hyperglycemic conditions

C. sativa exposure reduced the glucose-stimulated:basal insulin production in NT1 islets to 0.56-fold of that observed with NC islets (figure 3.24). However, this was not found to be statistically significant ($P = 0.19$). The reduction in the glucose-stimulated:basal insulin production was also found in the 24- (figure 3.6) and 96-hour (figure 3.11) studies, however the extent of reduction differed.

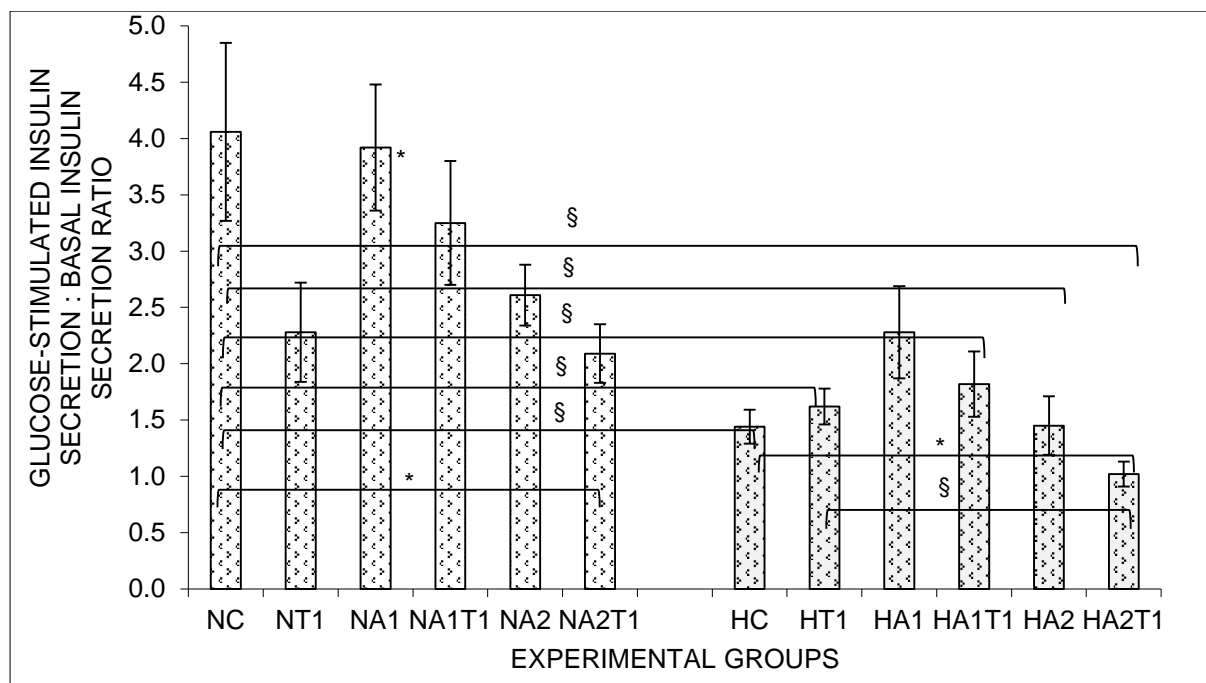


Figure 3.24 Effect of 48 hours of *C. sativa* exposure on the glucose-stimulated:basal insulin production of islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where T1 = 2.5 ng/mL; A1 = AM251; and A2 = AM630; n = 4.

* $P < 0.05$, relative to NC: NA2T1

relative to HC: HA2T1

§ $P < 0.01$, relative to NC: HC; HT1; HA1T1; HA2; HA2T1

relative to HT: HA2T1

Pre-conditioning of islets with A1, in the absence and presence of *C. sativa* treatment, had no significant effect on the glucose-stimulated:basal insulin production of these islets, relative to both NC and NT1 islets. However, exposure to A2, in the absence and presence of *C. sativa* treatment, reduced the glucose-stimulated:basal insulin production in NA2 islets to 0.64-fold ($P = 0.10$) and 0.51-fold ($P < 0.05$) in NA2T1 islets, relative to NC islets. No significant differences were observed between NT1 islets and NA2 and NA2T1 islets as well as between NA1 and NA1T1 islets, and NA2 and NA2T1 islets.

The glucose-stimulated:basal insulin production of all experimental conditions cultured under hyperglycemic conditions were significantly lower than that of NC islets - HC (0.35-fold, $P < 0.01$), HT1 (0.40-fold, $P < 0.01$), HA1 (0.56-fold, $P = 0.06$), HA1T1 (0.45-fold, $P < 0.01$), HA2 (0.36-fold, $P < 0.01$) and HA2T1 (0.25-fold, $P < 0.01$) (figure 3.24). The reduction in the glucose-stimulated:basal insulin production of islets cultured under hyperglycemic conditions and treated with *C. sativa* is consistent with the results observed in the studies associated with the 96-hour (figure 3.11) studies. In relation to HC islets, the experimental conditions had the following effects on the glucose-stimulated:basal insulin production of the islets - HT1 (1.13-fold), HA1 (1.58-fold, $P = 0.07$), HA1T1 (1.26-fold), HA2 (1.01-fold) and HA2T1 (0.71-fold, $P < 0.05$). *C. sativa* exposure in HA1T1 and HA2T1 islets did not significantly affect their glucose-stimulated:basal insulin production relative to HA1 and HA2 islets, respectively. Preconditioning with A2 significantly reduced the glucose-stimulated:basal insulin production of HA2T1 islets to 0.63-fold ($P < 0.01$), relative to HT1 islets. Pre-conditioning with the A1 inverse agonist/antagonist, in the absence and presence of *C. sativa*, did not significantly affect the glucose-stimulated:basal insulin production, relative to HT1 islets.

3.3.4.4 Insulin content associated with 48 hours *C. sativa* exposure, in presence and absence of CB1 and CB2 inverse agonists/antagonists, under normo- and hyperglycemic conditions

In NT1 islets, *C. sativa* exposure reduced insulin content to 0.82-fold, relative to NC islets (figure 3.25). This coincides with the observations associated with the 24-hour study (figure 3.7). Pre-conditioning with the inverse agonists/antagonists, in the presence and absence of *C. sativa*, significantly reduced insulin content, relative to NC islets – NA1 (0.57-fold, $P < 0.01$), NA1T1 (0.65-fold, $P < 0.05$), NA2 (0.65-fold, $P < 0.05$) and NA2T1 (0.72-fold, $P =$

0.08). Pre-conditioning with A1 and A2 in NA1 and NA2 islets had no significant effect on insulin content, relative to NA1T1 and NA2T1 islets, respectively. However, pre-conditioning with A1 inverse agonist/antagonist in NA1 islets resulted in significantly less insulin content (0.69-fold, $P < 0.05$), relative to insulin content found in NT1 islets.

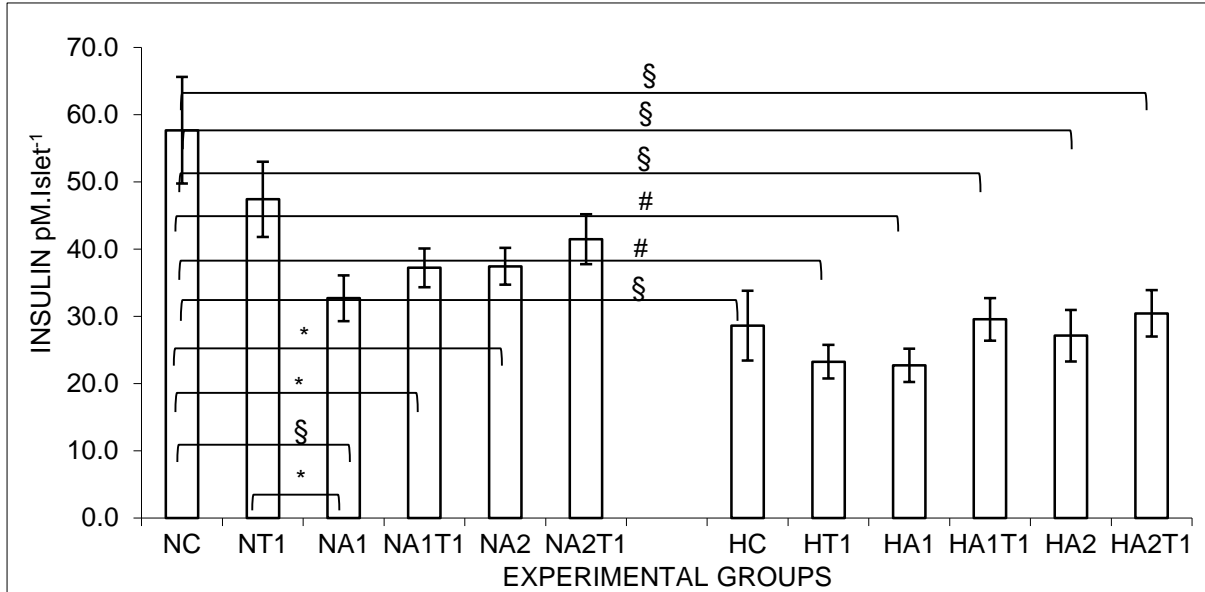


Figure 3.25 Effect of 48 hours of *C. sativa* exposure on insulin content in islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where T1 = 2.5 ng/mL, A1 = AM251; and A2 = AM630; n = 4.

* $P < 0.05$, NC relative to: NA1; NA2

NT1 relative to: NA1

§ $P < 0.01$, NC relative to: NA1; HC; HA1T1; HA2; HA2T1

$P < 0.001$, NC relative to: HT1; HA1

Insulin content were significantly reduced in all hyperglycemic experimental conditions, relative to NC islets – HC (0.50-fold, $P < 0.01$), HT1 (0.40-fold, $P < 0.001$), HA1 (0.39-fold, $P < 0.001$), HA1T1 (0.51-fold, $P < 0.01$), HA2 (0.47-fold, $P < 0.01$) and HA2T1 islets (0.53-fold, $P < 0.01$) (figure 3.25). The effect of T1 exposure on islet insulin content is consistent with reduced insulin content observed in both the 24- (figure 3.7) and 96-hour (figure 3.12) studies. However, pre-conditioning with A1 and A2 antagonists, in the presence and absence of *C. sativa*, had no significant effect on insulin content, relative to HC and HT1 islets.

3.3.4.5 Percentage basal and glucose-stimulated insulin secretion relative to insulin content associated with 48 hours *C. sativa* exposure, in presence and absence of

CB1 and CB2 inverse agonists/antagonists, under normo- and hyperglycemic conditions

In islets cultured under normo- and hyperglycemic conditions, T1 exposure for 48 hours had no significant effect on the percentage of basal and glucose-stimulated insulin secretion relative to insulin content (figure 3.26).

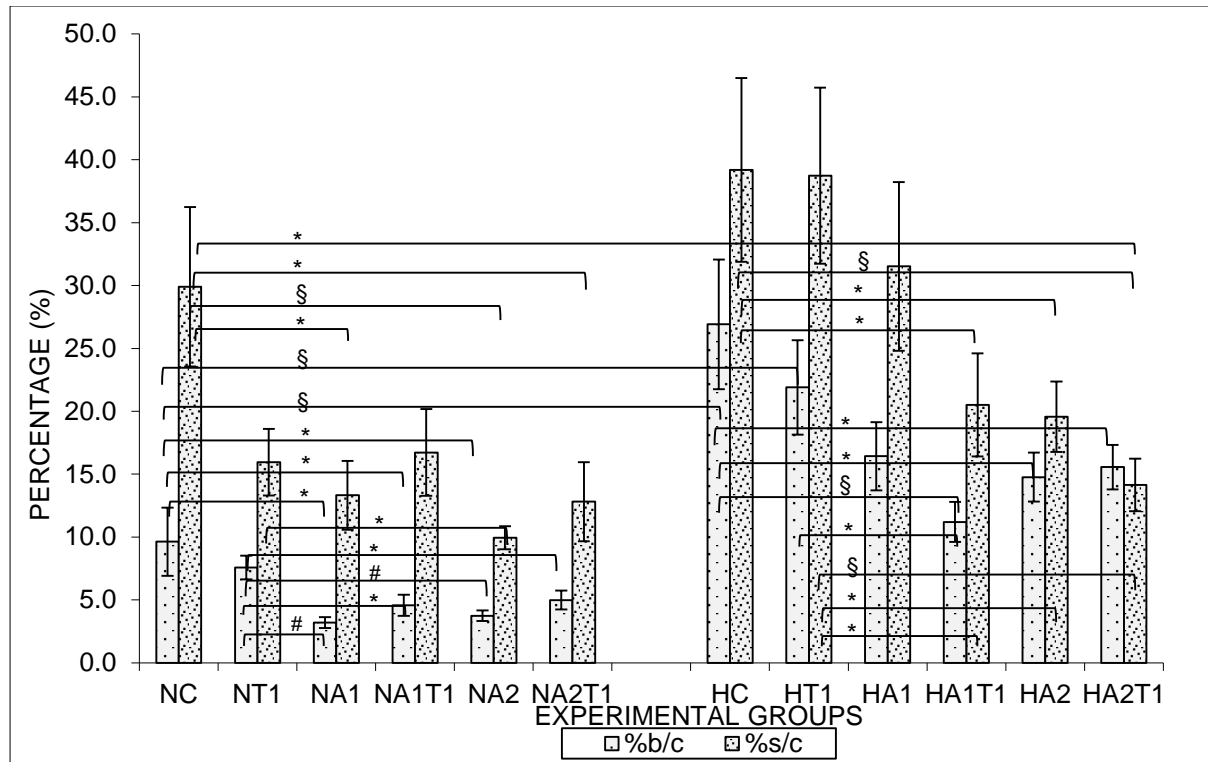


Figure 3.26 Effect of 48 hours of *C. sativa* exposure on the percentage basal and GSIS in relation to insulin content in islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where T1 = 2.5 ng/mL, A1 = AM251; and A2 = AM630; n = 4.

*P < 0.05,
 %b/c: relative to NC: NA1; NA1T1; NA2
 %b/c: relative to NT1: NA1T1; NA2T1
 %b/c: relative to HC: HA2; HA2T1
 %b/c: relative to HT1: HA1T1
 %s/c: relative to NC: NA1; NA2T1; HA2T1
 %s/c: relative to NT1: NA2
 %s/c: relative to HC: HA1T1; HA2
 %s/c: relative to HT1: HA1T1; HA2

§P < 0.01,
 %b/c: relative to NC: HC; HT1
 %b/c: relative to HC: HA1T1
 %s/c: relative to NC: NA2
 %s/c: relative to HC: HA2T1
 %s/c: relative to HT1: HA2T1

#P < 0.001,
 %b/c: relative to NT1: NA1; NA2

A similar pattern was observed in the 24-hour (figure 3.8) and 96-hour (figure 3.13) studies. However, *C. sativa* exposure for 96 hours resulted in a significant reduction under

normoglycemic conditions, but not under hyperglycemic conditions. Pre-treatment with A1 under both normo- and hyperglycemic conditions significantly reduced the percentage basal insulin secretion relative to insulin content, where NA1 treatment resulted in a reduction to 0.33-fold ($P < 0.05$) and HA1 to 0.61-fold ($P = 0.09$), relative to that of NC and HC islets, respectively. Subsequent exposure to *C. sativa* in NA1T1 and HA1T1 islets, both showed significant reductions in the percentage of basal insulin secretion to 0.47-fold ($P < 0.05$) and 0.42-fold ($P < 0.01$), relative to that of NC and HC islets. The percentage basal insulin secretion in NA1 and NA1T1 islets were reduced to 0.42- ($P < 0.001$) and 0.60-fold ($P < 0.05$), relative to that of NT1 islets, while HA1 and HA1T1 islets reduced their percentage of basal secretion to 0.75-fold and 0.51-fold ($P < 0.05$), relative to that of HT1 islets.

Pre-treatment with A2 under both normo- and hyperglycemic conditions significantly reduced the percentage basal insulin secretion relative to insulin content, where NA2 exposure resulted in a reduction to 0.39-fold ($P < 0.05$) and HA2 to 0.55-fold ($P < 0.05$), relative to that of NC and HC islets, respectively. Subsequent exposure to *C. sativa* in NA2T1 and HA2T1 islets resulted in a lowering of the percentage of basal insulin secretion to 0.52-fold and 0.58-fold ($P < 0.05$), relative to NC and HC islets, respectively. The percentage basal insulin secretion in NA2 and NA2T1 islets were reduced to 0.49-fold ($P < 0.001$) and 0.66-fold ($P < 0.05$), relative to that of NT1 islets, while HA2 and HA2T1 islets also reduced their percentage of basal secretion, albeit not significantly, to 0.67-fold and 0.71-fold, relative to that of HT1 islets.

Pre-treatment with A1 under both normo- and hyperglycemic conditions reduced the percentage of GSIS relative to insulin content, where NA1 treatment resulted in a reduction to 0.45-fold ($P < 0.05$) and HA1 to 0.80-fold, relative to NC and HC islets, respectively. Subsequent exposure to *C. sativa* in NA1T1 and HA1T1 islets, both resulted in a reduction in the percentage of GSIS to 0.47- ($P = 0.08$) and 0.80-fold, relative to NC and HC islets, respectively. The percentage GSIS in NA1 and NA1T1 islets were not significantly different, relative to that of NT1 islets, while HA1 and HA1T1 islets reduced their percentage of GSIS to 0.81-fold and 0.53-fold ($P < 0.05$), relative to HT1 islets.

Pre-treatment with A2 under both normo- and hyperglycemic conditions significantly reduced the percentage of GSIS relative to insulin content, where NA2 exposure resulted in

a reduction to 0.33-fold ($P < 0.01$) and HA2 to 0.50-fold ($P < 0.05$), relative to that of NC and HC islets, respectively. Subsequent exposure to *C. sativa* in NA2T1 and HA2T1 islets, resulted in a lowering of the percentage of GSIS to 0.43-fold ($P < 0.05$) and 0.36-fold ($P < 0.01$), relative to NC and HC islets. The percentage of GSIS in NA2 and NA2T1 islets was reduced to 0.62-fold ($P < 0.05$) and 0.80-fold, relative to NT1 islets, while HA2 and HA2T1 islets also reduced their percentage of GSIS to 0.51-fold ($P < 0.05$) and 0.37-fold ($P < 0.01$), relative to HT1 islets.

In summary, the following effects were observed in GSIS experiments in islets cultured under normo- and hyperglycemic conditions, in the presence and absence of A1 and A2 inverse agonist/antagonist pre-conditioning, with and without exposure to *C. sativa* (T1) extract for 48 hours:

- In islets cultured under normoglycemic conditions, A1 pre-conditioning, with and without T1 exposure, significantly affected all GSIS parameters analyzed except the glucose-stimulated:basal insulin production of islets, relative to NC islets.
- In islets cultured under normoglycemic conditions, A2 pre-conditioning, with and without T1 exposure, significantly affected all GSIS parameters analyzed except chronic insulin secretion and the percentage basal insulin secretion:insulin content of islets, relative to NC islets.
- In islets cultured under hyperglycemic conditions, A1 pre-conditioning significantly affected chronic and basal insulin secretion, while islets exposed to HA1T1 conditions significantly affected all GSIS parameters except the insulin content and glucose-stimulated:basal insulin production, relative to HC islets.
- In islets cultured under hyperglycemic conditions, A2 pre-conditioning significantly affected basal insulin secretion, GSIS and the percentage basal and GSIS:insulin content ratio, while islets exposed to HA1T1 conditions significantly affected all GSIS parameters except the chronic insulin secretion and insulin content, relative to HC islets.
- All experimental conditions, except HA2T1, induced significant differences between basal insulin secretion and GSIS.
- Pre-conditioning with A1 and A2, with and without T1, in islets cultured under normo- and hyperglycemic conditions (except HA2T1 conditions), significantly reduced chronic and basal insulin secretions, relative to NT1 and HT1 islets, respectively.

- GSIS was significantly reduced in islets exposed to NA1 ($P < 0.05$) and NA2 ($P < 0.05$), relative to NT1 islets. All islets exposed to hyperglycemic conditions, except HA1 islets, showed a significant reduction in GSIS, relative to HT1 islets.
- Insulin content was significantly reduced in islets exposed to NA1 conditions, relative to NT1 islets.
- The glucose-stimulated:basal insulin production of islets exposed to HA2T1 conditions were significantly reduced, relative to HT1 islets.
- The percentage basal insulin secretion:insulin content was significantly reduced in islets exposed to NA1 ($P < 0.001$), NA1T1 ($P < 0.05$), NA2 ($P < 0.001$) and NA2T1 ($P < 0.05$) conditions, relative to NT1 islets. Similarly, under hyperglycemic conditions, HA1T1 ($P < 0.05$) treatment resulted in a significant reduction in the percentage basal insulin secretion:insulin content, relative to HT1 islets.
- The percentage GSIS:insulin content was significantly reduced in islets exposed to NA2 ($P < 0.05$), relative to NT1 islets. Similarly, under hyperglycemic conditions, HA1T1 ($P < 0.05$), HA2 ($P < 0.05$) and HA2T1 ($P < 0.01$) treatment resulted in a significant reduction in the percentage GSIS:insulin content, relative to HT1 islets.
- Comparison between the effects of A1 and A2 pre-conditioning, in the presence or absence of *C. sativa*, under normo- and hyperglycemic conditions, i.e. NA1 vs NA1T1, NA2 vs NA2T1, HA1 vs HA1T1, HA2 vs NA2T1, showed no significant differences across all GSIS parameters, except a significant increase in basal insulin secretion in NA1T1 ($P < 0.05$), relative to NA1 islets.

The results show that under normo- and hyperglycemic conditions, A1, in the presence and absence of *C. sativa*, suppress *C. sativa* activity in relation to chronic insulin secretion and dynamic GSIS. Under normoglycemic conditions, exposure to A2 only, in absence of *C. sativa*, exhibited the activity of an inverse agonist in relation to chronic insulin secretion and the GSIS experiments, relative to NC islets. Under hyperglycemic conditions, exposure to A2, in absence and presence of *C. sativa*, did not affect chronic insulin secretion and exhibited the activity of an inverse agonist in relation to basal and GSIS, relative to HC islets.

3.3.5 Effects of *C. sativa* exposure over a 96-hour period on islet cytokine/chemokine secretion

Pro-inflammatory cytokine determination will specify trends as only 2 experiments were evaluated per analyte. In each experiment, each condition was represented in triplicate.

3.3.5.1 IL-1 α

C. sativa exposure marginally increased IL-1 α secretion in ND4T1 islets to 1.31-fold (21.92 ± 4.76 pg/mL), relative to levels found in ND4C islets (16.79 ± 3.98 pg/mL) (figure 3.27).

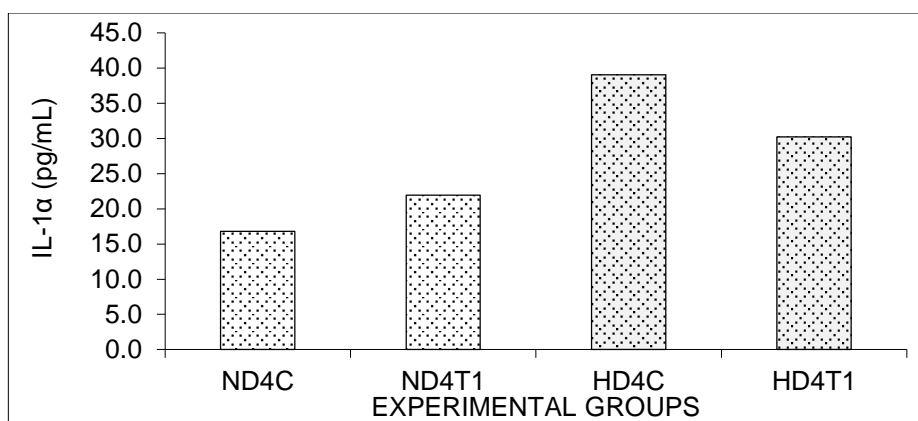


Figure 3.27 Effect of 96 hours of *C. sativa* exposure on IL-1 α levels in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL; n = 2.

Hyperglycemic conditions induced a substantial increase in IL-1 α expression to 2.32-fold (39.03 ± 9.95 pg/mL), relative to ND4C islets (statistical analysis across the six samples from the two experiments showed a significance level of $P = 0.09$). However, in HD4T1 islets, exposure to *C. sativa* reduced IL-1 α secretion to 0.77-fold (30.20 ± 6.92 pg/mL), relative to HD4C islets. This translates to a 1.80-fold increase in HD4T1 islets, relative to ND4C islets. It is interesting to note the opposite effects elicited by *C. sativa* treatment under different glycaemic culture conditions.

3.3.7.2 MIP-1 α

MIP-1 α secretion varied in islets cultured under normo- and hyperglycemic conditions and exposed to *C. sativa* extract, relative to their respective control islets (figure 3.28). ND4T1 islets displayed a sizeable reduction in MIP-1 α secretion to 0.31-fold (2.50 ± 0.49 pg/mL), relative to levels secreted by ND4C islets (8.20 ± 4.10 pg/mL). Hyperglycemic conditions resulted in a reduction in MIP-1 α secretion to 0.57-fold (4.66 ± 1.08 pg/mL), relative to ND4C islets. In islets exposed to *C. sativa*, HD4T1 islets showed an extensive increase to 2.27-fold increase (10.58 ± 3.93 pg/mL) in MIP-1 α secretion, relative to HD4C islets (statistical analysis across the six samples from the two experiments showed a significance level of $P = 0.10$). The HD4T1 islets showed a modest increase to 1.29-fold, relative to ND4C islets.

Again, it is interesting to note the difference in the effects elicited by *C. sativa* in islets cultured under normo- and hyperglycemic conditions, respectively.

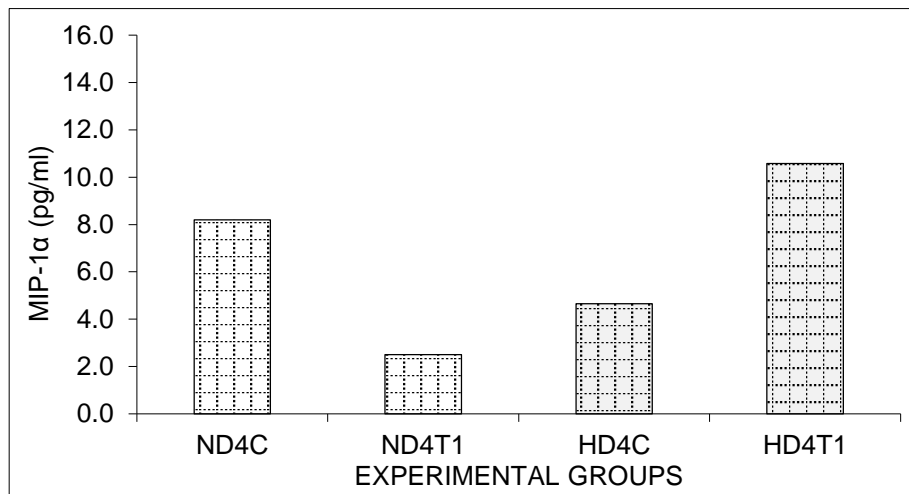


Figure 3.28 Effect of 96 hours of *C. sativa* exposure on MIP-1 α levels in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL; n = 2.

3.3.7.3 MIP-2

A similar trend to MIP-1 α secretions was observed in relation to secretion of MIP-2 in islets cultured under normo- and hyperglycemic conditions and exposed to *C. sativa* extract, relative to their respective control islets (figure 3.29).

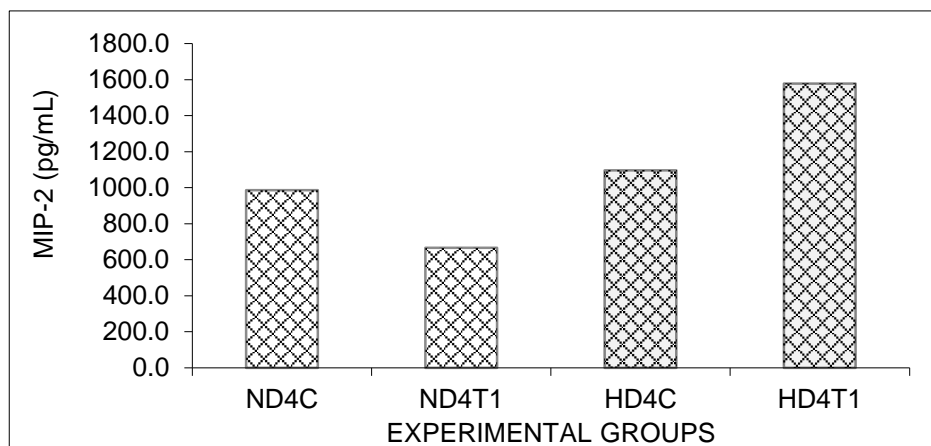


Figure 3.29 Effect of 96 hours of *C. sativa* exposure on MIP-2 levels in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL; n = 2.

C. sativa exposure in ND4T1 islets resulted in a reduction in MIP-2 secretion to 0.68-fold (667.04 ± 276.85 pg/mL), relative to ND4C islets. Exposure to hyperglycemic conditions induced a marginal increase in MIP-2 secretion to 1.11-fold ($1\ 096.07 \pm 569.79$ pg/mL), relative to MIP-2 secretion by ND4C islets. *C. sativa* treatment in HD4T1 islets resulted in a

large increase in MIP-2 secretion to 1.44-fold ($1\ 578.49 \pm 390.79$ pg/mL), relative to HD4C islets, and a 1.60-fold increase relative to ND4C islets. HD4T1 conditions induced a 2.37-fold increase in MIP-2 secretion, relative to levels secreted by ND4T1 islets (statistical analysis across the six samples from the two experiments showed a significance level of $P = 0.09$).

3.3.6 *C. sativa* extract in the presence and absence of CB1 or CB2 inverse-agonists over forty-eight hours

3.3.6.1 IL-1 α

In islets cultured under normoglycemic conditions, *C. sativa* exposure for 48 hours substantially increased IL-1 α secretion to 2.85-fold (45.22 ± 7.67 pg/mL), relative to that secreted by NC islets (15.85 ± 7.77 pg/mL) (figure 3.30).

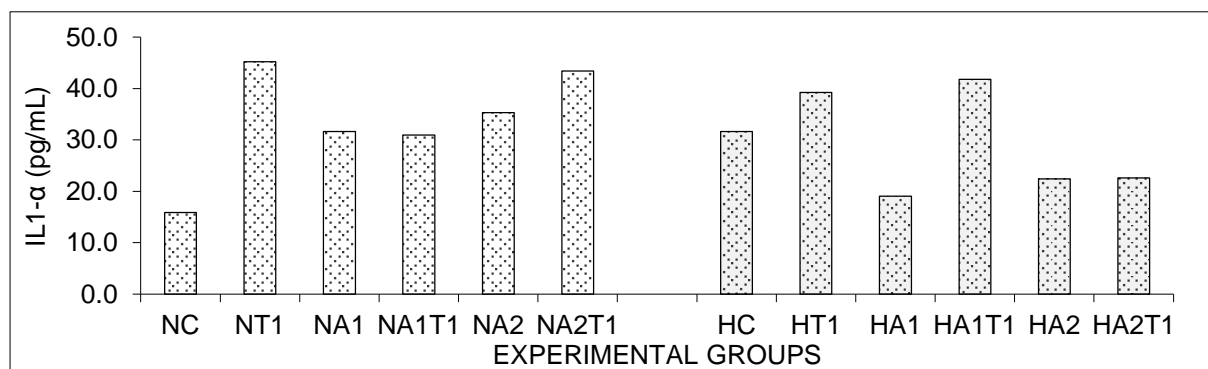


Figure 3.30 Effect of 48 hours of *C. sativa* exposure on IL-1 α secretion in islets cultured under normo- and hyperglycemic conditions subsequent to pre-conditioning with A1 or A2, where A1 = AM251; A2 = AM630; T1 = 2.5 ng/mL; n = 2.

These trends are similar to that observed in figure 3.27, where *C. sativa* also induced an increase under normoglycemic conditions. In islets exposed to NA1 treatment, IL-1 α secretion mimicked that of *C. sativa*, albeit to a lesser extent, with levels showing an increase to 1.99-fold, while in NA1T1 islets, A1 pre-conditioning and subsequent *C. sativa* exposure resulted in an increase to 1.95-fold, relative to NC islets. This observation was confirmed when no substantial differences were found between the IL-1 α levels found in NA1T1 islets, relative to NT1 and NA1 islets. In NA2 islets pre-conditioned with A2, minor changes were found in IL-1 α levels, relative to NC islets, while in NA2T1 islets, *C. sativa* exposure increased IL-1 α levels by a sizeable amount to 2.7 (43.31 ± 5.05 pg/mL) of that secreted by NC islets. Despite this increase in IL-1 α secretion in NA2T1 islets in relation to NC islets,

A2 pre-conditioning had no substantial effect on IL-1 α secretions, relative to levels found in NT1 and NA2 islets.

IL-1 α secretions in HC islets cultured under hyperglycemic conditions were not substantially different to that found in NC islets (figure 3.30), despite there being an increase in levels, which is similar to that found in the 96-hour exposure studies. *C. sativa* exposure in HT1 islets had a non-significant effect on IL-1 α secretion, relative to NC and HC islets. In HA1 islets, pre-conditioning with A1 had no significant effect on IL-1 α secretion, relative to NC and HC islets. *C. sativa* exposure in HA1T1 islets increased IL-1 α secretion to 2.6 (41.76 \pm 1.32 pg/mL) of that secreted by NC islets. This increase in IL-1 α secretion induced by *C. sativa* in the NA1T1 islets was substantially higher than levels secreted by HA1 islets. However, no major differences were found between the IL-1 α levels in HA1T1 islets, relative to HT1 islets. A2 pre-conditioning had no effect on IL-1 α in HA2 islets, relative to NC and HC islets, while *C. sativa* exposure in HA2T1 islets also had a minimal effect on IL-1 α secretions since no substantial differences were found relative to NC, HC, HT and HA2 islets, respectively.

3.3.6.2 MIP-1 α

C. sativa exposure in NT1 islets showed similar levels of MIP-1 α secretion, relative to NC islets (figure 3.31). The slight reduction caused by T1 exposure was similar to that observed in the 96-hour exposure studies. A1 pre-conditioning increased MIP-1 α secretion minimally to 2.12 \pm 0.32 pg/mL, while *C. sativa* exposure in NA1T1 islets increased secretion to 3.44 \pm 0.14 pg/mL. A1 pre-conditioning did affect MIP-1 α secretion, since MIP-1 α secretion was considerably higher in NA1T1 islets, relative to NT1 (1.73 \pm 0.55 pg/mL) and NA1 (2.12 \pm 0.32 pg/mL) islet secretions. A2 pre-conditioning in NA2 islets increased MIP-1 α secretion, relative to NC islets, and subsequent *C. sativa* exposure in NA2T1 islets increased MIP-1 α secretion further to 5.08 \pm 0.72 pg/mL. Pre-conditioning with A2 in NA2T1 islets had a major effect on *C. sativa* exposure, resulting in a 2.9-fold increase in MIP-1 α secretion, relative to NT1 islets.

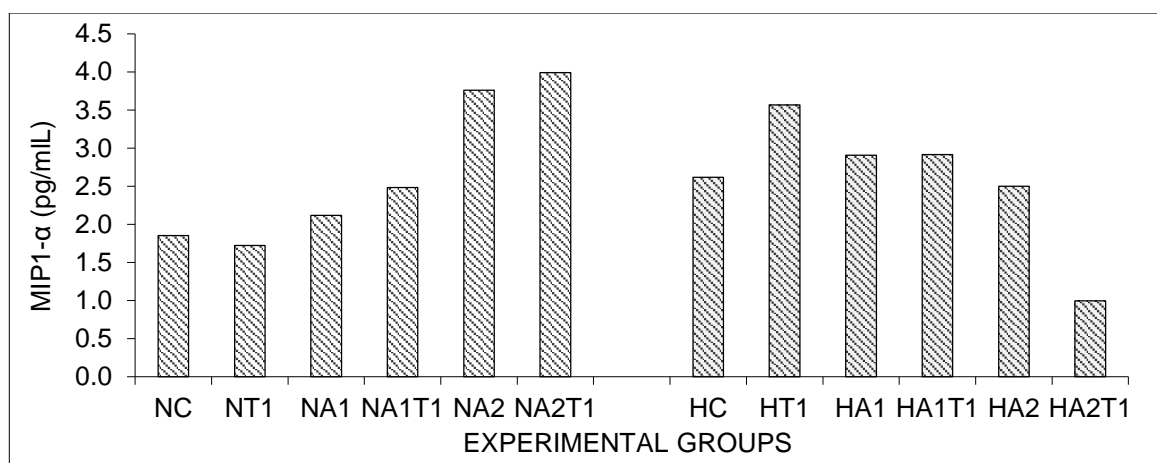


Figure 3.31 Effect of 48 hours *C. sativa* exposure on MIP-1 α secretion in islets cultured under normo- and hyperglycemic conditions subsequent to pre-conditioning with A1 or A2, where A1 = AM251; A2 = AM630; T1 = 2.5 ng/mL; n = 2.

Islets cultured under hyperglycemic conditions (HC) showed no sizeable change in MIP-1 α secretion, relative to normoglycemic (NC) control islets (figure 3.31). *C. sativa* treatment (HT1) increased cytokine secretion by 1.4-fold relative to HC islets, but this increase was not statistically significant. A1 and A2 pre-conditioning resulted in small increases in MIP-1 α secretion, relative to that found in NC and HC islets. Pre-conditioning of *C. sativa*-treated islets with A1 (HA1T1) and A2 (HA2T1) both lead to reduced MIP-1 α secretion relative to *C. sativa*-only (HT1) treated islets, however these reductions were not statistically significant.

3.3.6.3 MIP 2

In NT1 islets, *C. sativa* exposure did not induce major changes in MIP-2 secretion, relative to NC islets (figure 3.32). A1 and A2 pre-conditioning alone led to a small but non-significant fall in MIP-2 secretion, relative to NC islets. In the presence of A1, MIP-2 secretion in NA1T1 islets was reduced to 0.5-fold of that observed in NT1 islets, and in NA2T1 islets, MIP-2 secretion fell to 0.3-fold of that observed in NT1 islets. However, neither of these reductions in cytokine secretion was statistically significant.

Across all experimental conditions in islets cultured under hyperglycemic conditions, MIP-2 secretion was substantially lower, relative to NC but not to HC islets (figure 3.32). MIP-2 secretion in HC islets was reduced to 0.3 (40.13 ± 8.43 pg/mL) of that secreted by NC islets, while *C. sativa* exposure resulted in MIP-2 secretion at 0.4 (55.94 ± 8.36 pg/mL) of that observed in NC islets. A1 pre-conditioning in HA1 islets reduced MIP-2 secretion to 0.4 (54.09 ± 7.97 pg/mL), while *C. sativa* exposure in HA1T1 islets substantially reduced MIP-

2 levels to 0.2 (18.85 ± 9.56 pg/mL) of that secreted by NC islets. *C. sativa* exposure considerably reduced MIP-2 secretion in HA1T1 islets to 0.4 of that secreted by HA1 islets, and 0.3 of that secreted in HT1 islets. A2 pre-conditioning in the absence and presence of T1 substantially reduced MIP-2 secretion to 0.3 of that found in NC islets to 39.26 ± 7.70 pg/mL and 39.46 ± 6.45 pg/mL in HA2 and HA2T1 islets, respectively. A2 pre-conditioning in HA2T1 islets had no major effect on MIP-2 secretion, relative to both HA2 and HT1 islets, respectively.

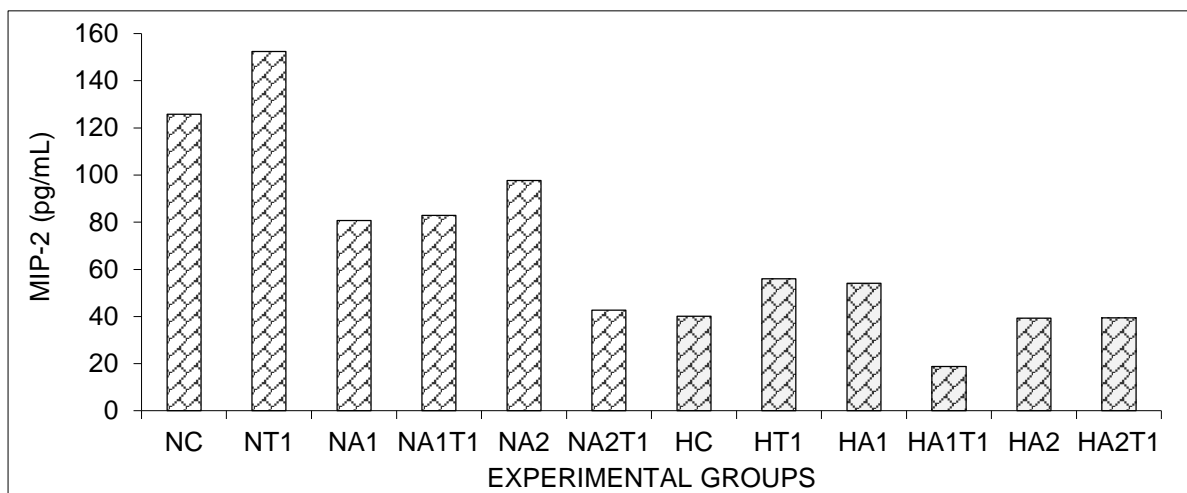


Figure 3.32 Effect of 48 hours *C. sativa* exposure on MIP-2 secretion in islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where A1=AM251; A2=AM630; THC=2.5 ng/mL; n=2.

3.3.7 Proliferative studies

3.3.7.1 Ki-positive stained cells after 24 and 96 hours of *C. sativa* exposure

C. sativa exposure for 24 hours did not induce a significant effect on the number of proliferative β -cells in islets cultured under normoglycemic conditions (figure 3.33). Hyperglycemic conditions (HD1C) induced a 1.4-fold increase in the number of proliferative β -cells, relative to ND1C islets. Exposure to *C. sativa* extract and hyperglycemia in HD1T1 and HD1T2 islets increased the number of proliferative β -cells by 4.0- and 3.5-fold, respectively, relative to ND1C islets and by 4.8-fold ($P < 0.01$) when comparing HD1T1 to ND1T1 and 7.2-fold ($P < 0.1$) when comparing HD1T2 to ND1T2 islets. There was a 2.8- and 2.5-fold increase in proliferative β -cells in HD1T1 and HD1T2 islets, respectively, relative to HD1C islets.

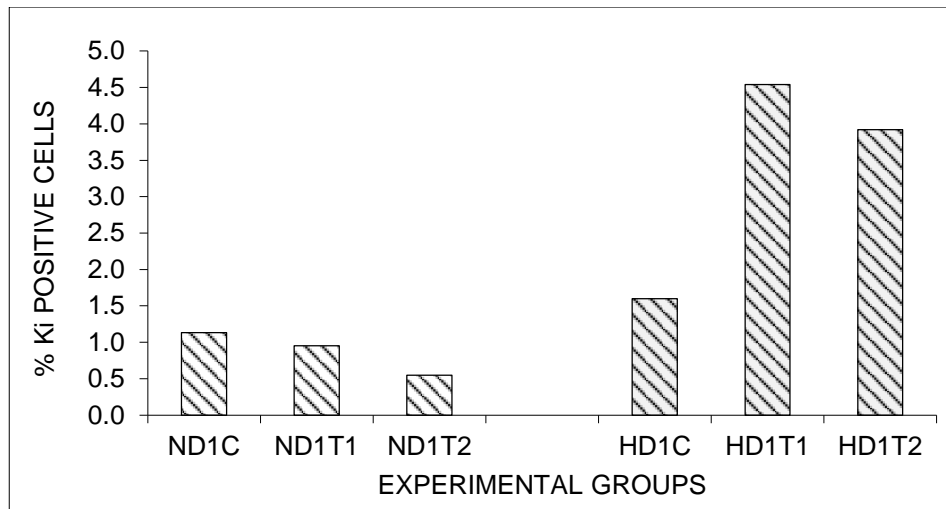


Figure 3.33 Effect of 24 hours *C. sativa* exposure on β -cell proliferation in islets cultured under normoglycemic and hyperglycemic conditions, where T1 = 2.5 ng/mL, T2 = 5.0 ng/mL; n = 2.

Islets cultured under normoglycemic conditions and exposed for 96 hours to *C. sativa* extract reduced the number of proliferative β -cells to 0.7- and 0.9-fold for ND4T1 and ND4T2, respectively, of the number of positively stained β -cells observed in ND4C islets (figure 3.34).

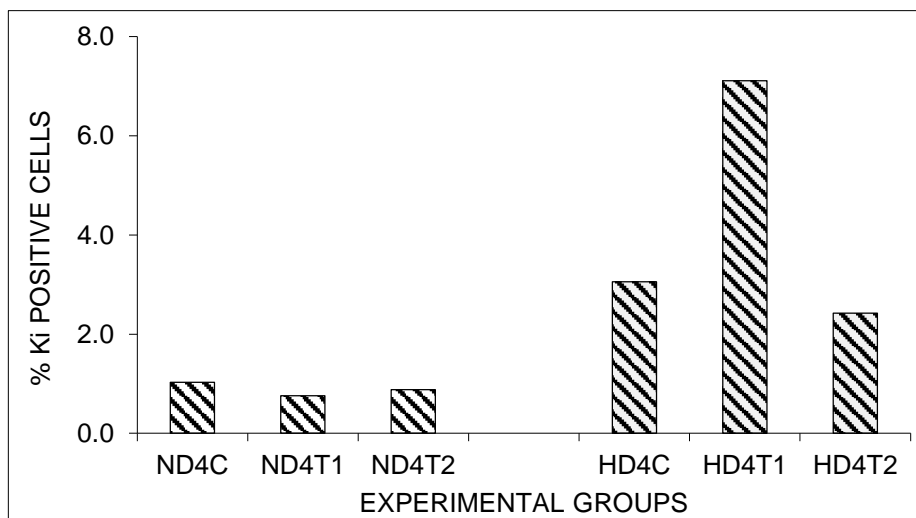


Figure 3.34 Effect of 96 hours *C. sativa* exposure on β -cell proliferation in islets cultured under normoglycemic and hyperglycemic conditions, where T1 = 2.5 ng/mL, T2 = 5.0 ng/mL; n = 2.

Hyperglycemic conditions induced a 3.0-fold increase in the number of proliferative β -cells in HD4C relative to ND4C islets. *C. sativa* treatment and hyperglycemia resulted in a substantial increase of 6.9- ($P < 0.001$) and 2.4-fold ($P < 0.01$) in the number of proliferative β -cells in HD4T1 and HD4T2 islets, respectively, relative to the ND4C islets. There was a

9.4-fold ($P < 0.001$) increase in proliferative β -cells when comparing HD4T1 to ND4T1 islets and a 2.8-fold ($P < 0.01$) increase when comparing HD4T2 to ND4T2 islets. The number of proliferating β -cells in HD4T1 and HD4T2 islets showed a 2.3-fold ($P < 0.001$) increase and a reduction to 0.8-fold, respectively, compared to that found in HD4C islets.

Figure 3.35 provides images of Ki-67 positive β -cells in islets cultured for 96 hours under normo- and hyperglycemic conditions in the presence of *C. sativa* extract.

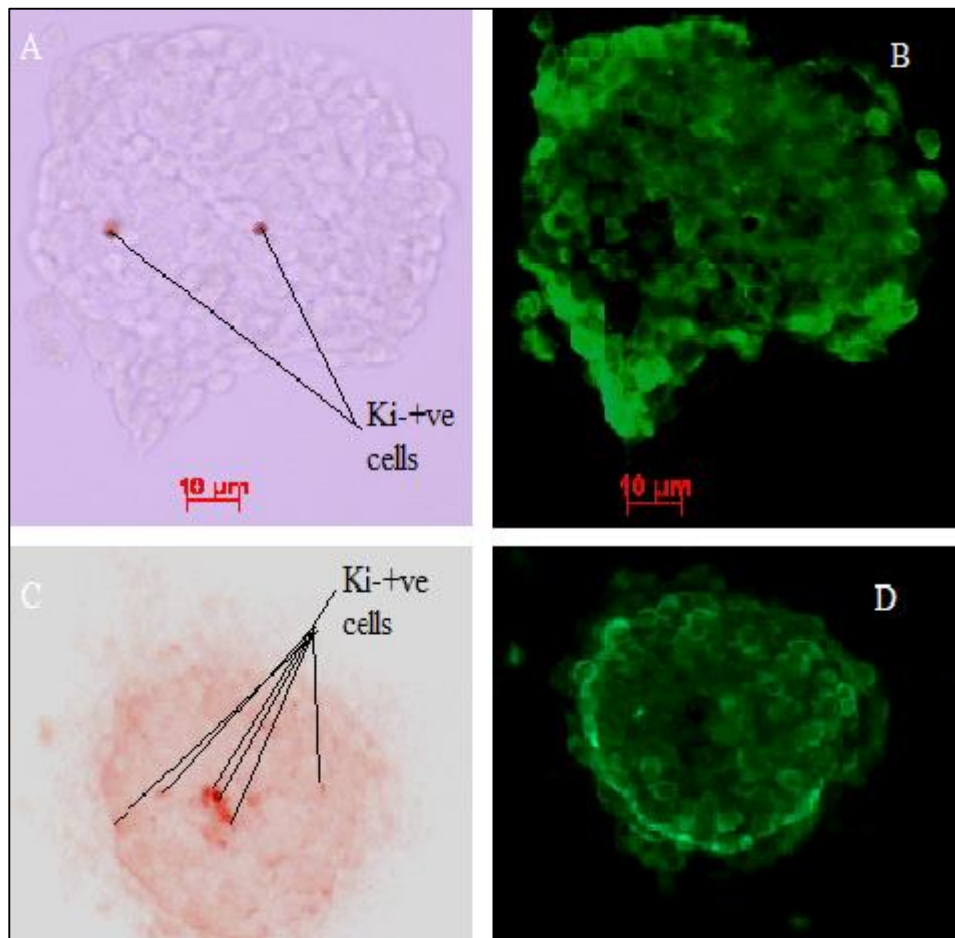


Figure 3.35 (A) Ki-67-positive β -cells (arrows directed at the red-stained cells), and (B) FITC-stained insulin associated with an islet exposed to ND4T1 conditions, while (C) and (D) shows an islets exposed to HD4T1 conditions, where T1 represents extract containing 2.5 ng/mL THC.

3.3.8 Apoptotic studies

3.3.8.1 TUNEL-positive stained cells after 24 and 96 hours of *C. sativa* extract exposure

Islets exposed for 24 hours to *C. sativa* extract showed minimal changes in the number of apoptotic β -cells across all experimental conditions, except in HD1T1 and HD1T2 islets,

where apoptotic cells were 2.0- and 3.7-fold higher respectively, of that observed in ND1C islets, and 2.1- and 3.8-fold higher respectively, of apoptotic cell numbers observed in HD1C islets (figure 3.36).

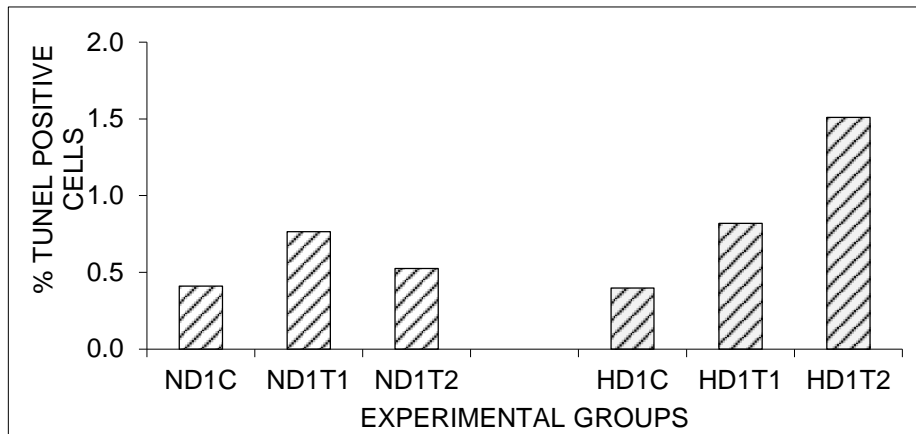


Figure 3.36 Effect of 24 hours of *C. sativa* exposure on apoptosis in islets cultured under normo- and hyperglycemic conditions, where T1 = 2.5 ng/mL, T2 = 5.0 ng/mL; n = 2.

Islets cultured under normo- and hyperglycemic conditions and exposed for 96 hours to *C. sativa* extract showed no substantial differences in the number of apoptotic β -cells, relative to the respective ND4C and HD4C islets (figure 3.37).

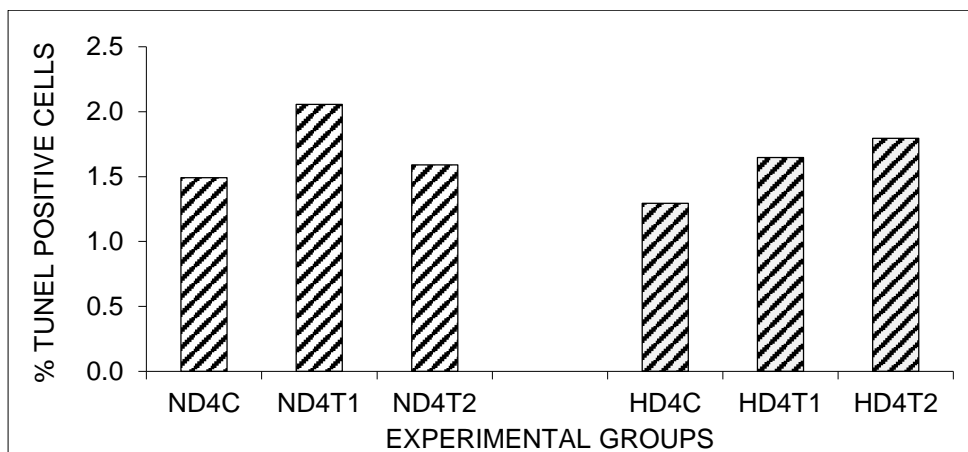


Figure 3.37 Effect of 96 hours of *C. sativa* exposure on apoptosis in islets cultured under normo- and hyperglycemic. T1 = 2.5 ng/mL, T2 = 5.0 ng/mL; n = 2.

Figure 3.38 shows islets cultured under normo- and hyperglycemic conditions and exposed for 96 hours to *C. sativa* extract containing 2.5 ng/mL THC. The apoptotic cells (indicated by arrows) are associated with the insulin staining, shown in red, thus verifying that these are β -cells

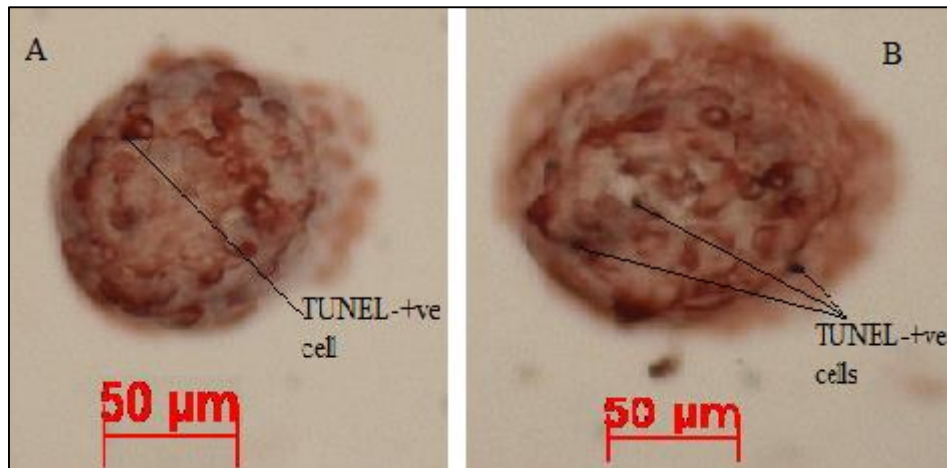


Figure 3.38 A illustration of islets exposed 96 hours to *C. sativa* extract containing 2.5 ng/mL THC and cultured under (A) normoglycemic and (B) hyperglycemic conditions. The arrows indicate the red-stained insulin-positive cells with TUNEL-stained black nuclei.

3.3.9 Effect of 96 hours of *C. sativa* exposure on islets, cultured under normo- and hyperglycemic conditions, on levels of ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, CB1 and CB2

The data presented below was generated from densitometry analysis of immunoblots associated with specific proteins, which upon phosphorylation after cannabinoid receptor activation, play a key role in β -cell proliferation or apoptosis.

3.3.9.1 Effect of 96 hours of *C. sativa* exposure on islets, cultured under normo- and hyperglycemic conditions, on ERK1/2 and phospho-ERK1/2

C. sativa exposure to islets cultured under normo- (ND4T1) and hyperglycemic (HD4T1) conditions induced a reduction in the level of ERK1/2 to 0.93 ± 0.10 and 0.75 ± 0.25 IDV, respectively, relative to ND4C islets (equated to 1 IDV) (figure 3.39). Islets cultured under hyperglycemic conditions (HD4C) showed a reduction in ERK1/2 levels to 0.89 ± 0.29 IDV, relative to NC islets. Phosphorylation of ERK1/2 increased very slightly in *C. sativa*-treated islets, as well as in islets cultured under hyperglycemic conditions. Insert (a) is a typical immunoblot showing bands corresponding to ERK1 at 44 kDa, and (b) corresponds with pERK1/2 at 42 and 44 kDa, respectively, associated with islet protein lysates cultured under normo- and hyperglycemic conditions for a period of 96 hours, with and without *C. sativa* extract exposure.

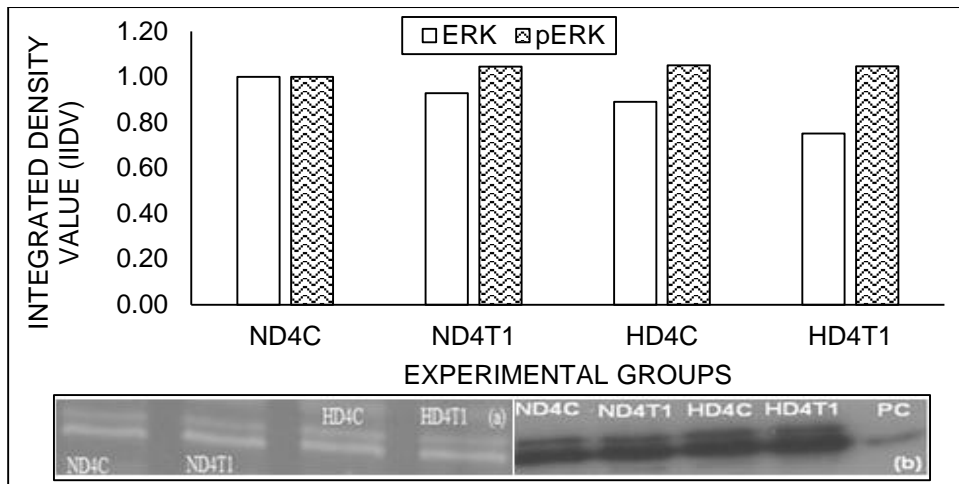


Figure 3.39 Effect of experimental conditions on (a) ERK1/2 and (b) pERK1/2 in islets cultured under normo- and hyperglycemic conditions. Rat brain was used as a positive control (PC). Immunoblots show bands at 42 kDa (bottom) and 44 kDa (top); T1 = 2.5 ng/mL; n = 2.

3.3.9.2 Effect of 96 hours of *C. sativa* exposure on islets, cultured under normo- and hyperglycemic conditions, on JNK and phospho-JNK

Islets cultured under normo- and hyperglycemic conditions showed similar levels in JNK and pJNK across all conditions (figure 3.40), with inserts of typical immunoblots showing bands corresponding to (a) JNK and (b) pJNK at 46 kDa.

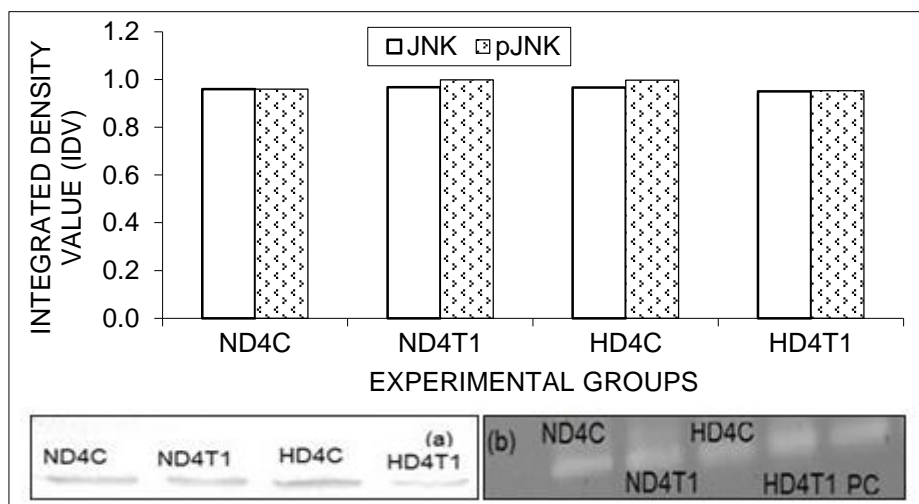


Figure 3.40 Effect of experimental conditions on (a) JNK and (b) pJNK in islets cultured under normo- and hyperglycemic conditions. Rat brain was used as a positive control (PC). Immunoblots show bands at 46 kDa; T1 = 2.5 ng/mL; n = 2.

3.3.9.3 Effect of *C. sativa* exposure on islet, cultured under normo- and hyperglycemic conditions, on CB1 and CB2 receptors proteins levels

Figure 3.41 is a typical illustration of the immunoblots generated with protein homogenates from treated and control islets cultured under normo- and hyperglycemic conditions. CB1 and CB2 bands were detected at 30 kDa, while the actin band is visible at 42 kDa.

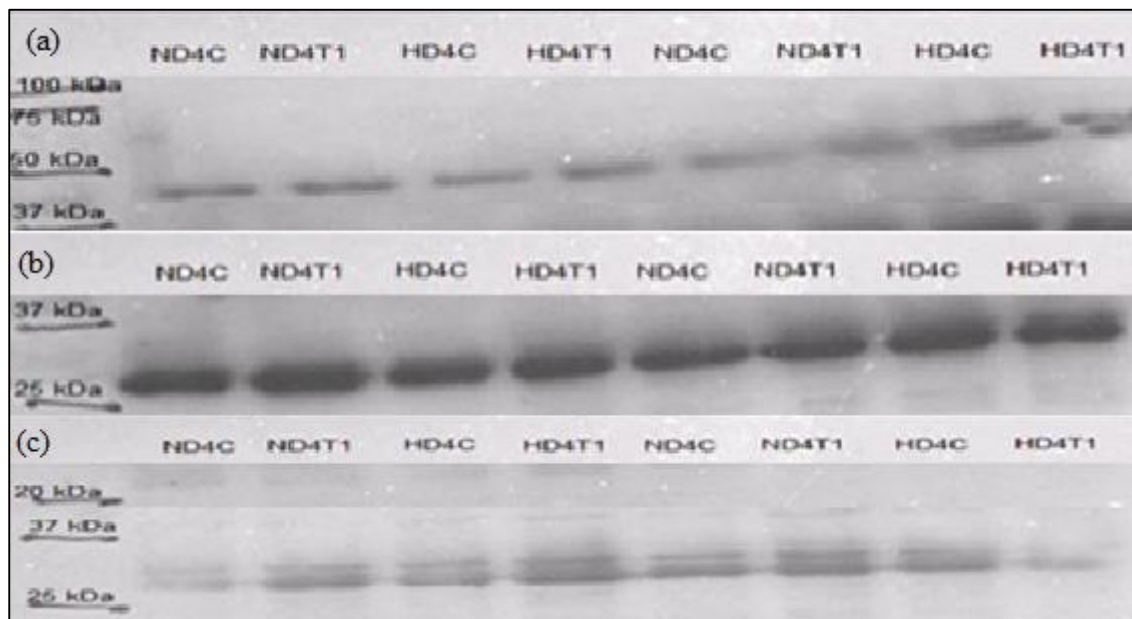


Figure 3.41 Immunoblots of (b) CB1 and (c) CB2 associated with primary rat islets cultured under normo- and hyperglycemic conditions, in the presence and absence of *C. sativa* extract for 96 hours. (a) Actin was included as a standard to ensure equivalent levels of protein was loaded. T1 = 2.5 ng/mL THC, n=2.

Densitometry analysis shows no substantial changes in CB1 and CB2 receptor protein levels relative to both ND4C and HD4C islets (figure 3.42).

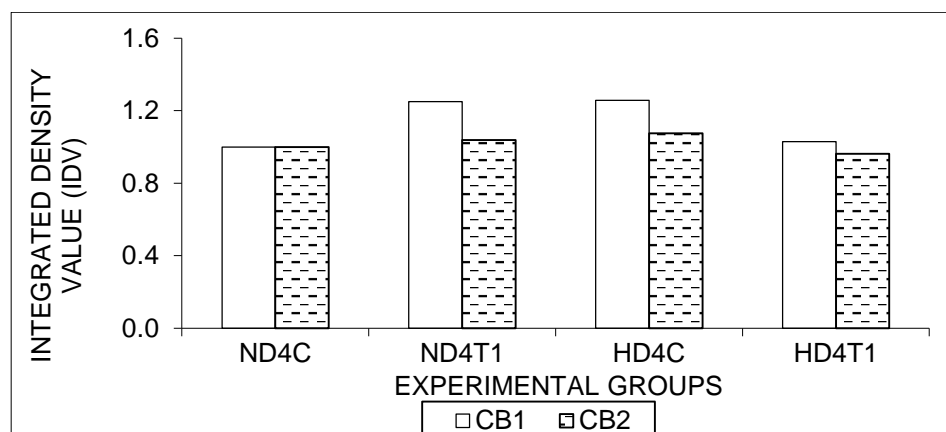


Figure 3.42 Densitometry measurements of CB1 and CB2 receptors associated with primary rat islets cultured under normo- and hyperglycemic conditions for 96 hours, in the presence and absence of *C. sativa* extract. Data expressed relative to ND4C, and HD4T1 relative to HD4C (n=2).

C. sativa exposure in ND4T1 islets produced a 1.3- and 1.04-fold increase in CB1 and CB2 receptor protein, respectively compared to ND4C islets. Hyperglycemic conditions increased

CB1 and CB2 protein levels in HD4C islets to 1.3- and 1.1-fold, respectively, relative to ND4C islets, while *C. sativa* exposure in HD4T1 islets resulted in a reduction in CB1 and CB2 protein levels to 0.8- and 0.9-fold, respectively, of that observed in HD4C islets.

Figure 3.43 is a typical illustration of an isolated rat islet showing dual staining of insulin and CB1 receptors.

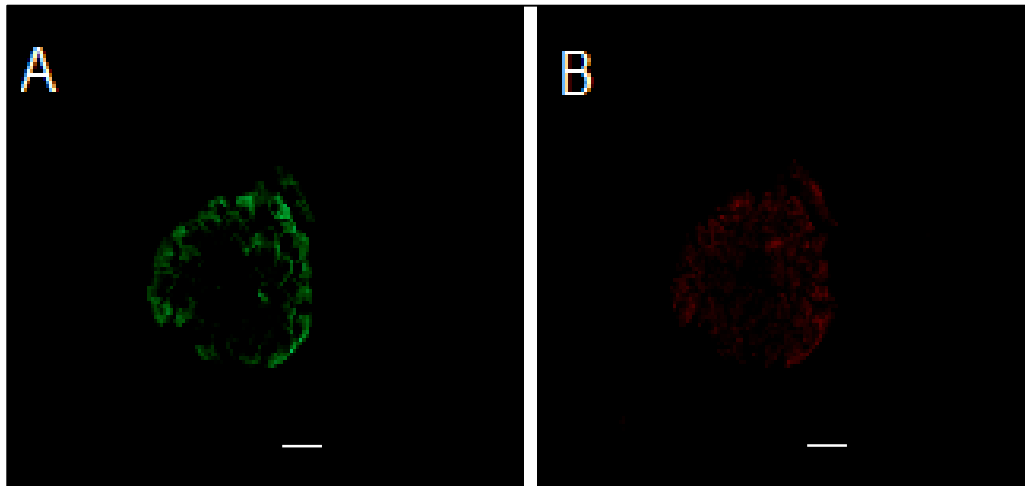


Figure 3.43 An illustration showing (A) insulin-stained β -cells and (B) CB1 receptors in a rat islets cultured under normoglycemic conditions for 96 hours. Magnification at 20x, and bar represents 10 μ m.

3.3.10 qPCR: Chronic *C. sativa* exposure

3.3.10.1 CB1 receptor

C. sativa exposure in ND4T1 islets reduced CB1 receptor gene expression to 0.59-fold of that found in ND4C islets, however this change was not statistically significant (figure 3.44). In HD4C islets, the hyperglycemic conditions marginally increased CB1 receptor gene expression to 1.43-fold, relative to ND4C islets, while *C. sativa* exposure significantly increased CB1 receptor gene expression in HD4T1 islets to 2.18-fold ($P < 0.05$), relative to ND4C islets, and 1.78-fold, relative to HD4C islets, but the increase was not statistically significant. No significant differences were observed between the ND4T1 and HD4T1 islets.

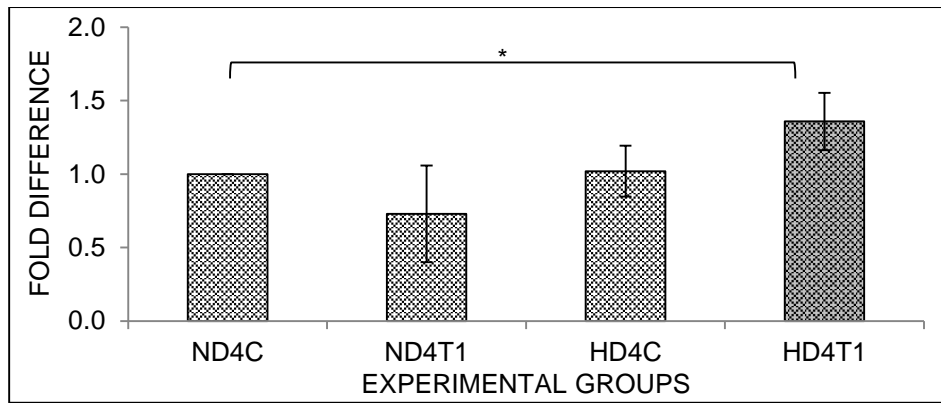


Figure 3.44 Effect of 96 hours of *C. sativa* exposure on CB1 gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3. *P < 0.05, ND4C relative to: HD4T1

3.3.10.2 c-MYC

C. sativa treatment in ND4T1 significantly reduced c-MYC gene expression to 0.88-fold (P < 0.05), relative to ND4C islets (figure 3.45).

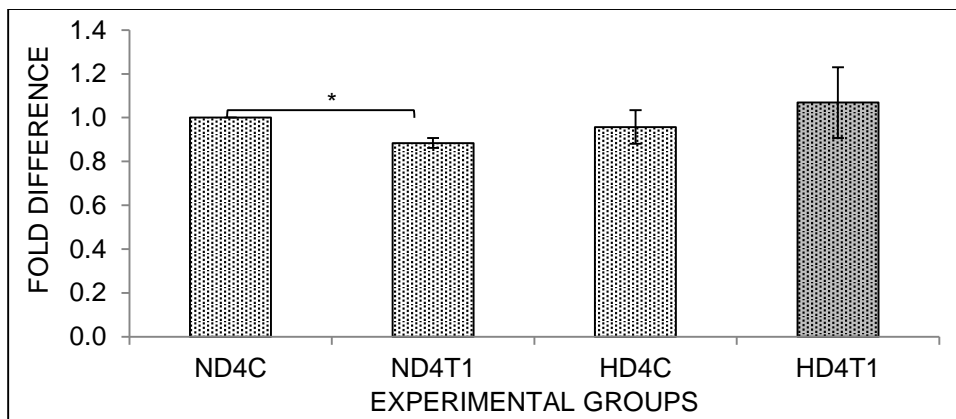


Figure 3.45 Effect of 96 hours of *C. sativa* exposure on c-MYC gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3. *P < 0.05, ND4C relative to: ND4T1

In HD4C islets, increased glucose exposure resulted in the c-MYC gene expression being slightly reduced to 0.96-fold, while *C. sativa* exposure in HD4T1 islets maintained expression at levels similar to that of ND4C islets. HD4T1 islets increased expression levels to 1.07-fold, relative to HD4C islets. No significant differences were observed between the ND4T1 and HD4T1 islets.

3.3.10.3 FLIP

C. sativa exposure in ND4T1 islets reduced FLIP gene expression to 0.77-fold, relative to ND4C islets (figure 3.46). Hyperglycemic conditions reduced FLIP gene expression to 0.85-fold in HD4C islets, while *C. sativa* exposure significantly reduced expression to 0.63-fold ($P < 0.05$), relative to ND4C islets, and to 0.73-fold ($P < 0.01$), relative to HD4C islets. No significant differences were observed between the ND4T1 and HD4T1 islets.

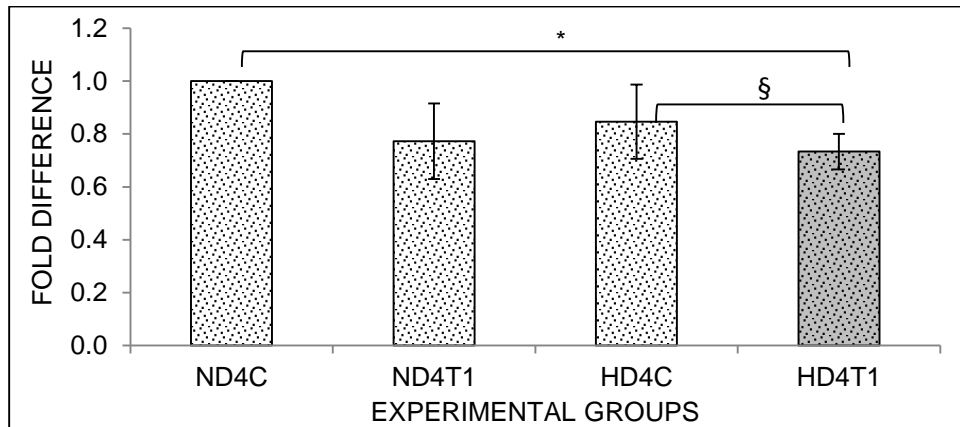


Figure 3.46 Effect of 96 hours of *C. sativa* exposure on FLIP gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=4.

* $P < 0.05$, ND4C relative to: HD4T1

§ $P < 0.01$, HD4C relative to: HD4T1

3.3.10.4 GLUT2

C. sativa exposure in ND4T1 islets reduced GLUT2 gene expression to 0.82-fold, relative to ND4C islets (figure 3.47).

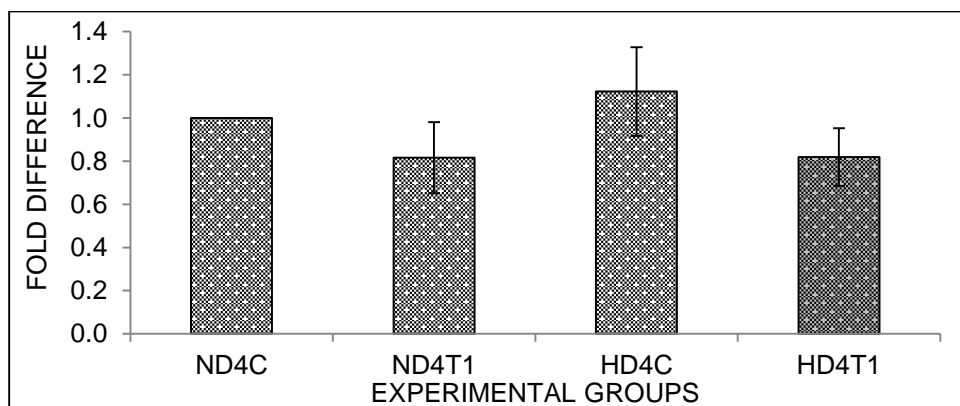


Figure 3.47 Effect of 96 hours of *C. sativa* exposure on GLUT2 gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3.

Hyperglycemic conditions increased GLUT2 gene expression in HD4C islets to 1.12-fold, while *C. sativa* exposure reduced expression to 0.87 (P = 0.07), relative to ND4C islets, which was equivalent to 0.82-fold, relative to HD4C islets. None of these changes were significantly different. No significant differences were observed between the ND4T1 and HD4T1 islets.

3.3.10.5 Insulin

C. sativa exposure in ND4T1 islets reduced insulin gene expression to 0.70-fold, relative to ND4C islets (figure 3.48). Hyperglycemic conditions slightly increased insulin gene expression to 1.08-fold, relative to ND4C islets, while *C. sativa* treatment increased insulin gene expression by 1.32-fold, relative to ND4C islets, and 1.33-fold, relative to HD4C islets. No significant differences were observed between the ND4T1 and HD4T1 islets.

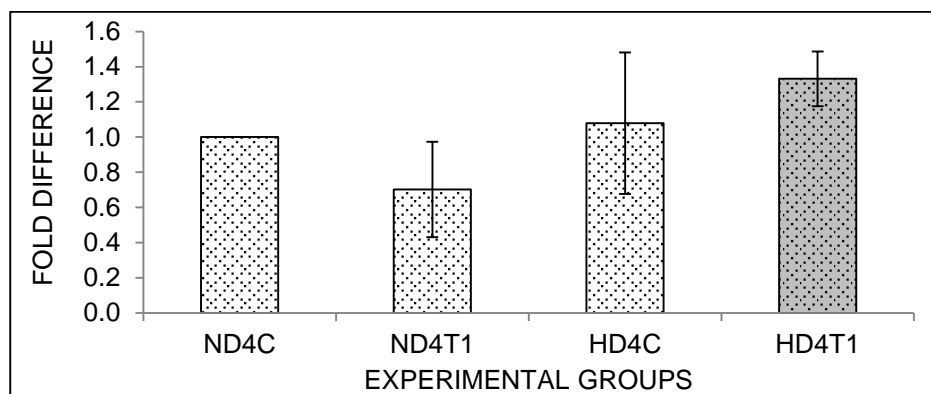


Figure 3.48 Effect of 96 hours of *C. sativa* exposure on insulin gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3.

3.3.10.6 PDX-1

C. sativa exposure in ND4T1 islets slightly reduced PDX-1 gene expression to 0.93-fold, relative to ND4C islets (figure 3.49). Hyperglycemic conditions maintained PDX-1 gene expression at levels similar to ND4C islets, while *C. sativa* treatment increased PDX-1 gene expression to 1.32-fold, relative to ND4C islets, and 1.34-fold, relative to HD4C islets. No significant differences were observed between the ND4T1 and HD4T1 islets.

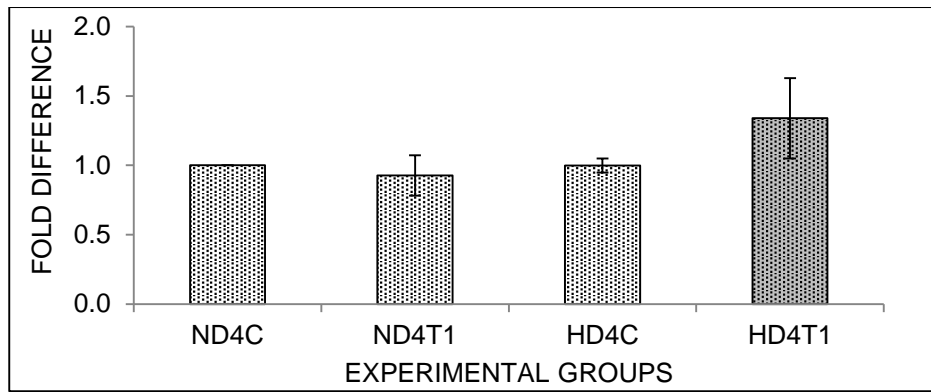


Figure 3.49 Effect of 96 hours of *C. sativa* exposure on PDX-1 gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3.

3.3.10.7 UCP2

UCP2 is expressed in the β -cells and is linked to insulin gene regulation since it mediates the uncoupling of energy usage and ATP production from mitochondria, and negatively regulating the cytoplasmic ATP/ADP ratio and GSIS (Shao et al., 2009; Zhang et al., 2001). *C. sativa* treatment slightly reduced UCP gene expression 0.98-fold in ND4T1 islets (figure 3.50). Hyperglycemic conditions slightly increased UCP2 gene expression by 1.06-fold, relative to ND4C islets, while *C. sativa* exposure maintained UCP2 gene expression levels similar to ND4C islets, and slightly reduced expression to 0.96-fold relative to HD4C islets. No significant differences were observed between the ND4T1 and HD4T1 islets.

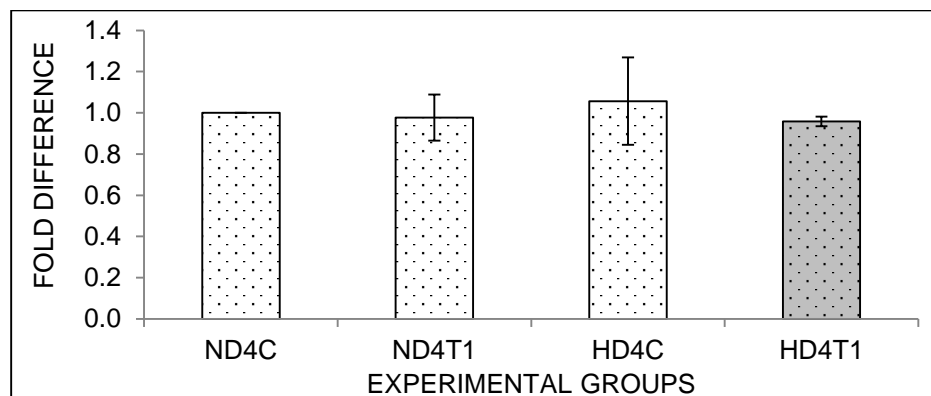


Figure 3.50 Effect of 96 hours of *C. sativa* exposure on UCP2 gene expression in isolated rat islets cultured under normo- and hyperglycemic conditions. ND4T1 and HD4C gene expression is relative to ND4C, while HD4T1 is relative to HD4C. T1 = 2.5 ng/mL; n=3.

3.3.11 qPCR: Inverse Agonist/Antagonist studies

3.3.11.1 Insulin

Figure 3.51 shows the trends observed in qPCR analysis of insulin gene expression associated with 48 hours *C. sativa* extract exposure, in the presence and absence of A1 and A2. The normoglycemic conditions as well as the hyperglycemic control are compared to NC islets, while the rest of the hyperglycemic conditions are compared to the HC islets. Results showed that under normoglycemic conditions, A1 preconditioning, and not A2, blocked the effect of *C. sativa*.

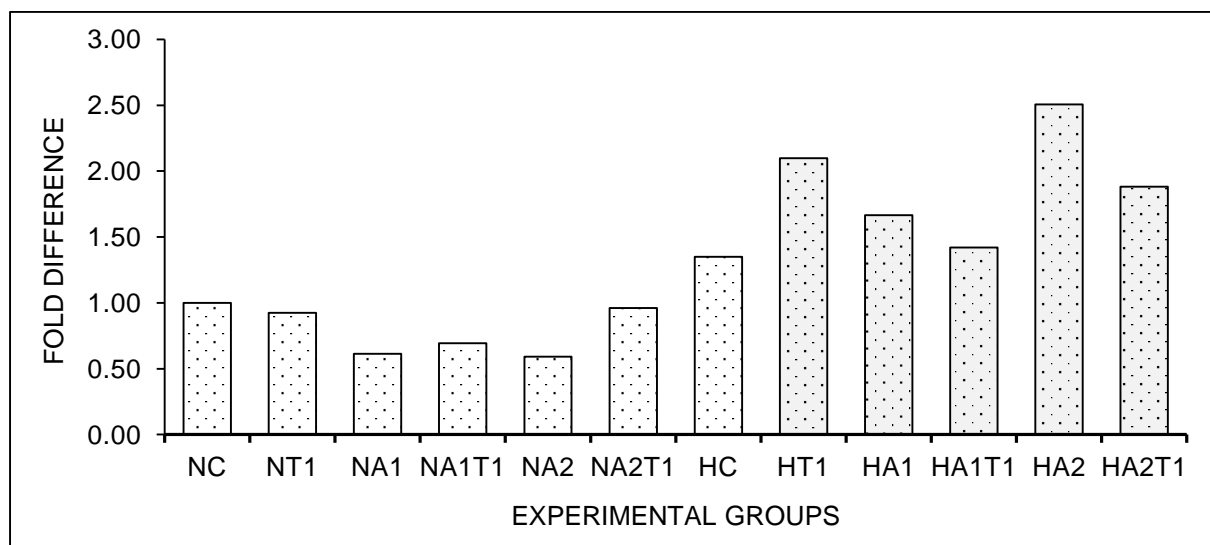


Figure 3.51 Effect of 48 hours *C. sativa* exposure on insulin secretion in islets cultured under normo- and hyperglycemic conditions, subsequent to A1 or A2 pre-conditioning, where A1=AM251; A2=AM630; THC=2.5 ng/mL; n=2

Under hyperglycemic conditions, HC and HT1 islets showed increased insulin gene expression, relative to NC islets. A1 preconditioning appears to block the stimulatory effect of *C. sativa* treatment, relative to HT1 islets. In HA2 islets, A2 preconditioning acted as an agonist, stimulating insulin gene expression, while moderating the effect of *C. sativa* treatment. Since these observations are based on 2 experiments, only trends can be reported. However, the trends observed follows a similar trend to that observed in the relevant experimental conditions indicated above.

3.4 DISCUSSION AND CONCLUSIONS

The endocannabinoid system plays a significant role in energy homeostasis, and dysregulation of endocannabinoids has been associated with hyperglycemia, DIO and T2D

(Anderson et al., 2013; Coskun and Bolkent, 2013; Horváth et al., 2012; Vilches-Flores et al., 2010a+b; De Petrocellis et al., 2007; Matias et al., 2006). Since the cloning of the two cannabinoid receptors in the early 1990s, and the isolation of AEA in 2002, a new body of evidence is emerging on the role of the peripheral and central endocannabinoid pathways in the regulation of feeding behaviour, body weight and glucose metabolism (Bermúdez-Silva et al., 2009; Carr et al., 2008; Juan-Picó et al., 2006; Tonstad, 2006). In the current study, *in vitro* normo- and hyperglycemic conditions are used to simulate *in vivo* normal (lean) and diabetic (DIO insulin resistance) conditions. In *in vitro* studies, cells are typically exposed for 48 hours to experimental conditions. A shorter exposure period (24-hour) was selected to determine which *C. sativa* concentration would elicit a substantial effect on β -cell functionality. The 48-hour period was then doubled to 96 hours, to ensure a longer period of exposure to *C. sativa*. All the *in vitro* experiments reported in this study were conducted using *C. sativa* extracts containing THC concentrations equivalent to 7.95 pM and/or 15.9 pM, with the ratio of THC:CBN: CBD in the *C. sativa* extracts being 1:10:1. The K_i values for THC, AM251 and AM630 as 37.5 nM, 7.5 nM and 2.5 μ M, respectively, for CB1 receptors and 40 nM, 2290 nM and 31.2 nM, respectively, for CB2 receptors (Pertwee et al., 2010; Thakur et al., 2005; Ross et al., 1999). Since the islets were exposed to THC in the picomolar range, it should be noted that these concentrations were significantly lower than the K_i concentrations for THC associated with both CB1 and CB2 receptors. The antagonism experiments were conducted using 9 μ M and 10 μ M for AM251 and AM630, respectively, which is significantly above the K_i concentrations for these two inverse agonists. The results of this study will therefore be discussed within the context of the abovementioned experimental conditions.

Similar to the varied observations reported in animal models, *in vitro* studies have also provided conflicting results relating to the effects of cannabinoids (endogenous and exogenous) on pancreatic β -cells. As early as 1986, Laychock et al. provided evidence that cannabinoids, specifically THC, modulate insulin secretion. They conducted studies on isolated rat islets to investigate the turnover of pancreatic islet arachidonic acid and insulin release in response to THC (0.002 to 20 μ M), since arachidonic acid turnover in membrane phospholipids of isolated islets had been linked with insulin secretion. They found that in response to glucose stimulation, THC exposure (20 μ M) stimulated basal insulin release by increasing arachidonic acid release, phospholipase A₂, lipoxygenase product formation and secretion, and inhibiting acyl-CoA acyltransferase activity. In support of the initial results

generated by Laychock et al. (1986), Matias et al. (2006) conducted experiments on RIN-m5F rat insulinoma β -cells, which express both CB1 and CB2 receptors, culturing the cells under low (13mM) or high (25 mM) glucose conditions, in the presence of a CB1 agonist (HU-210, 100 nM) and/or CB1 antagonist, rimonabant (100 nM). The β -cells cultured under high glucose conditions showed an increase in insulin release upon stimulation with HU-210, with rimonabant reversing this effect (but not the CB2 antagonist, SR144528). Bermúdez-Silva et al. (2008) conducted *in vitro* studies on isolated human islets and found that CB1 stimulation enhanced insulin and glucagon secretion, while CB2 stimulation lowered glucose-dependent insulin secretion. De Petrocellis et al. (2007) found that RIN-m5F rat insulinoma β -cells, via CB1 receptor stimulation, induced insulin secretion via PI-PLC-mediated mobilization of $[Ca^{2+}]_i$. In islets cultured in media containing 13 mM glucose, insulin is capable of inducing a regulatory mechanism that down-regulate glucose-induced increases in endocannabinoid levels (Matias et al., 2006). However, islets cultured under hyperglycemic conditions lose this ability. Instead, insulin increased endocannabinoid (AEA and 2-AG) levels, consequently causing over-stimulation of CB1 receptors and increasing insulin release leading to hyperinsulinemia. This is evident in diabetic patients where AEA and 2-AG serum levels were increased, relative to healthy lean individuals. AEA levels were found to be elevated in fasting lean individuals, relative to fed lean individuals. AEA and 2-AG levels were elevated in pancreatic tissue of DIO mice, relative to lean mice (Matias et al., 2006).

On the other hand, studies have also shown that cannabinoids (endogenous and cannabinoid analogues) inhibit insulin secretion. AEA activates CB1 receptors, and depending on the cell type, either inhibit or facilitate cytosolic Ca^{2+} release (De Petrocellis and Di Marzo, 2009). Acute AEA treatment (one hour) of isolated rat islets showed a reduction in GSIS, with increased basal and glucose-stimulated insulin secretion, resulting in a reduced glucose-stimulated:basal insulin production, which was reversed in the presence of a CB1 antagonist (Vilches-Flores et al., 2010a). In addition, Nakata and Yada (2008) showed that AEA and ACPA inhibited glucose-induced insulin secretion by inhibiting $[Ca^{2+}]_i$ oscillation in the β -cells associated with isolated mice islets. Juan-Picó et al. (2006) found that both AEA and 2-AG as well as various CB1 agonists (WIN55212-2, ACPA and arachidonyl-2-chloroethylamide) induced slower glucose-induced intracellular Ca^{2+} oscillations in the β -cells of islets isolated from Swiss Albino OF1 mice, which resulted in decreased insulin secretion. They also showed that AM630 reversed 2-AG-induced reduction in insulin

secretion in mice islets, while AM251 did not reverse this response, thus suggesting that insulin secretion, in mice, was mediated via the CB2 receptors.

Cannabinoid exposure to islets has also been reported to not elicit any effect on β -cells. In mouse islets treated with arachidonyl-2-chloroethylamide and exposed to 20 mM glucose, no changes in insulin secretion were observed (Li et al., 2010a). Similarly, human islets exposed to a CB1 agonist showed no changes in insulin secretion when exposed to 20 mM glucose (Li et al., 2011b). Juan-Picó et al. (2006) found that mouse islets cultured under 2 mM glucose conditions and exposed to AEA, showed no changes in insulin secretion. In a more recent study, Anderson et al. (2013) investigated the effect of cannabinoid receptor agonists and antagonists on insulin secretion in isolated Wistar rat islets and found that they affect insulin secretion in a complex manner. They reported that AEA did not affect insulin secretion after 60 minutes exposure to basal (4 mM) or intermediate (8 mM) glucose concentrations, but caused concentration-dependent inhibition of insulin release at 20 mM glucose. In addition, Anderson et al. (2013) identified two populations of islets, based on their sensitivity to AEA and postulated that the difference in islet responsiveness may be due to differences in their local AEA metabolism. In addition, inhibition studies were conducted with pre-incubated with AM251. At 100 nM, AM251 failed to reverse the inhibition of insulin secretion in response to 20 mM glucose. However, at higher concentrations of AM251, AM251 itself caused a concentration-dependent inhibition of glucose-stimulated insulin release, with mean IC_{50} value 1.6 μ M (95% CI: 507 nM to 3.3 μ M, n=6). When applied at 10 μ M, AM251 had no significant effect on the insulin secretory rate of islets incubated at 4 mM or 8 mM glucose, however it consistently caused a marked inhibition of maximal (20 mM) glucose-stimulated insulin release.

In the current *in vitro* studies, the effect of *C. sativa* treatment on isolated rat islets functionality was investigated. At a molecular level, the expression of signaling molecules associated with insulin gene regulation as well as β -cell proliferation/apoptosis were studied using three techniques viz. GSIS, qPCR and western blots. Glucose concentration and exposure period induced varying results in the islets in relation to parameters which were investigated. Under normoglycemic conditions, *C. sativa* treatment significantly reduced chronic insulin secretion in ND1T1 (0.23-fold, $P < 0.01$) and in ND1T2 (0.37-fold, $P < 0.01$) islets, relative to the ND1C islets. Islets exposed to the same conditions for a lengthier period

(96 hours) resulted in a significant increase in chronic insulin secretion to 1.6- and 2.3-fold in ND4T1 ($P < 0.05$) and ND4T2 islets ($P < 0.05$) respectively, relative to ND4C islets. However, chronic insulin secretion remained unaffected by *C. sativa* treatment in islets cultured under hyperglycemic conditions, in the 24- and 96-hour studies, relative to the respective control islets. Also, acute exposure to *C. sativa* treatment under hyperglycemic conditions induced a significant reduction in chronic insulin secretion in both HD1T1 (0.38-fold, $P < 0.05$) and HD1T2 (0.38-fold, $P < 0.01$), relative to ND1C islets. Islets exposed to the same conditions for a lengthier period (96 hours) resulted in a significant increase in chronic insulin secretion in both HD4T1 (1.34-fold, $P = 0.07$) and HD4T2 (1.34-fold, $P < 0.001$) islets, relative to ND4C islets. In the animal model, chronic *C. sativa* treatment affected glucose tolerance and insulin secretion – IPGTT was improved in LNE rats, and remained relatively unchanged in OBE rats, relative to their respective controls. Both OBC and OBE rats showed a significant improvement in glucose tolerance capability, relative to the lean rats. Although not found to be statistically significant, *C. sativa* treatment increased insulin secretion in LNE rats (1.11-fold), while OBC rats showed a 1.44-fold increase, relative to LNC rats. *C. sativa* treatment reduced insulin in OBE rats to 0.86-fold, relative to OBC rats, which equates to an increase of 1.24-fold, relative to LNC rats. The overall plasma glucose levels were higher in the lean rats compared to the obese rats, which may, in part, be due to the increased insulin secretions reflected in the obese rats. The findings from both the *in vitro* and *in vivo* studies show that prolonged *C. sativa* exposure increase chronic insulin secretion relative to the normal untreated controls. These findings are supported by Laychock et al. (1986), Matias et al. (2006) and Bermúdez-Silva et al. (2008).

In the *in vivo* studies, *C. sativa* treatment improved glucose tolerance (IPGTT) in LNE, relative to LNC rats and in OBE rats, relative to both LNC and OBC rats, respectively. In the *in vitro* studies, glucose sensitivity was tested using GSIS, where both the basal, GSIS and glucose-stimulated:basal insulin production were assessed. Glucose concentration and exposure period had varying effects on islets in relation to basal insulin secretion. In the 24-hour study, *C. sativa* treatment reduced basal insulin secretion in ND1T1 (0.34-fold, $P = 0.06$) and ND1T2 (0.53-fold, $P = 0.10$) islets, relative to ND1C islets, while prolonged exposure (96 hours) had no effect on basal insulin secretion, relative to ND4C islets. *C. sativa* treatment did not affect basal insulin secretion in islets cultured under hyperglycemic conditions for 24 hours, but significantly increased levels in HD4C (2.35-fold, $P < 0.001$), HD4T1 (1.94-fold,

P < 0.001) and HD4T2 islets (2.59-fold, P < 0.001), relative to ND4C islets, and significantly reduced basal insulin levels HD4T1 (0.82-fold, P < 0.05), relative to HD4C islets.

To assess β -cell sensitivity to glucose after *C. sativa* treatment, islets were exposed for 60 minutes to media containing 16.7 mM glucose. GSIS levels were reduced in ND1T1 (0.24-fold, P < 0.05) and ND1T2 (0.28-fold, P = 0.06), relative to ND1C islets, while a lengthier (96 hours) *C. sativa* exposure period did not significantly affect GSIS levels in ND4T1 and ND4T2 islets, relative to ND4C islets, but significantly increased GSIS levels, relative to the ND4C islets: HD4C (1.67-fold, P < 0.001), HD4T1 (1.30-fold, P < 0.05) and HD4T2 (1.76-fold, P < 0.001). The changes in basal secretion and GSIS observed in the *in vitro* studies corresponds with the improved IPGTT results associated with the LNE and obese rats, as well as the lower fasting blood glucose levels found in the LNE and obese rats.

In islets, the glucose-stimulated:basal insulin production is a measure of the responsiveness of β -cells to glucose. *C. sativa* treatment did not affect the glucose-stimulated:basal insulin production of ND4T1 and ND4T2 islets, relative to ND4C. However, relative to ND4C (but not HD4C) islets, islets cultured under hyperglycemic conditions showed significantly reduced glucose-stimulated:basal insulin production of HD4C (0.65-fold, P = 0.059), HD4T1 (0.62-fold, P < 0.05) and HD4T2 (0.63-fold, P < 0.05) islets. Since the islets showed a reduction in the glucose-stimulated:basal insulin production *in vitro*, in the *in vivo* study, other than increased insulin secretion, additional factors that may have contributed to an improvement in the IPGTT as observed in the *in vivo* study, includes increased peripheral glucose uptake and/or reduced hepatic glucose release. These results are supported by the findings of studies conducted by Laychock et al. (1986), Matias et al. (2006) and De Petrocellis et al. (2007) which also involved acute (1 to 24 hours) exposure to cannabinoids, linking the endocannabinoid system to insulin secretion. AEA (1 μ M) exposure significantly reduced the glucose-stimulated:basal insulin production of rat islets isolated from fasted rats (Vilches-Flores et al., 2010a), while Matias et al. (2006) found that RIN-m5F rat β -cells exposed to 25 mM glucose for 24 hours followed by HU-210 (a CB1 agonist) treatment for one hour, showed increased insulin and AEA release, relative to control islets.

The effect of *C. sativa* treatment on insulin content and insulin content in relation to the percentage basal and GSIS were also determined as a measure of islet functionality. After 24

hours *C. sativa* treatment, insulin content showed no significant changes across all experimental conditions, relative to ND1C and HD1C islets, respectively. Lengthier exposure to *C. sativa* treatment resulted in a 50% reduction in insulin content in ND4C islets, relative to ND1C islets. The lengthier exposure to *C. Sativa* significantly increased insulin content in ND4T1 (1.25-fold, $P < 0.05$) and ND4T2 (1.28-fold, $P = 0.058$) islets, relative to ND4C islets. Under hyperglycemic conditions, insulin content decreased significantly in HD4C (0.71-fold, $P < 0.05$) and HD4T1 (0.61-fold, $P < 0.001$) islets, relative to ND4C islets, but not HD4C islets. Prolonged exposure to *C. sativa* treatment under normoglycemic conditions reduced the percentage of basal:content ratio in ND4T2 islets

Prolonged *C. sativa* treatment under normoglycemic conditions resulted in a reduced basal:content ratio, while hyperglycemic conditions with and without *C. sativa* treatment resulted in a significant increase in the basal:content ratio, relative to ND4C islets. A similar trend was observed in relation to the GSIS:content ratio, where under normoglycemic conditions, a reduction was observed, albeit not significant, while under hyperglycemic conditions with and without *C. sativa* treatment, the ratio increased significantly, relative to ND4C islets. With increased secretion under hyperglycemic conditions, the content is reduced, which affects the overall ratios. Since the pancreata of the animals were not assessed for insulin content, but rather for insulin gene expression, it is unclear whether *C. sativa* treatment induced any changes in the insulin content of LNE and OBE rats, relative to their respective controls.

THC is a CB1 agonist, with a K_i of 37.5 nM (Pertwee et al., 2010; Thakur et al., 2005; Ross et al., 1999). To investigate whether blocking the CB1 and CB2 receptors in rat islets, in the presence and absence of *C. sativa* extract, would induce any changes in insulin gene expression and secretion in GSIS studies, islets were pre-conditioned with well-characterized CB1 (AM251) and CB2 (AM630) antagonists/inverse agonists, at 9 and 10 $\mu\text{mol/L}$ respectively, after which the islets were exposed for 48 hours to *C. sativa* treatment containing an equivalent of 7.95 pM THC. Results from the current *in vitro* inverse agonist/antagonist studies showed that AM251 pre-conditioning significantly reduced chronic insulin secretion in islets cultured under normo- and hyperglycemic conditions, relative to the respective control islets. Despite the high AM251 concentration that was used to pre-treat the islets, exceeding the K_i for AM251 of 7.5 nM associated with CB1 receptors and 2290 nM

associated with CB2 receptors (Pertwee et al., 2010), AM251 blocked the stimulatory effect of *C. sativa* exposure as seen in islets only treated with *C. sativa* extract in islets cultured under both normo- and hyperglycemic conditions. Basal and glucose-stimulated insulin secretions were suppressed in islets pre-conditioned in the absence and presence of AM251, relative to the respective control islets. At a molecular level, AM251 pre-conditioning had no significant effect on insulin gene expression in islets cultured under both normo- and hyperglycemic conditions. In studies conducted by Anderson et al. (2013), rat islets were pre-incubated for 30 minutes with 100 nM AM251 followed by 60 minutes incubation in media containing 20 mM glucose, and insulin secretion was not affected. Higher concentrations of AM251 (10 μ M) were found to inhibit glucose-stimulated insulin secretion, but did not significantly affect insulin secretion when islets were incubated at basal and intermediate glucose concentrations. Experiments conducted on human isolated islets, 10 μ M of arachidonyl-2-chloroethylamide (ACEA) (a CB1 agonist) induced a significant increase in insulin release. The subsequent addition of 10 μ M AM251 in the presence of 10 μ M ACEA did not antagonize ACEA-induced insulin secretion, resulting in a further increase in insulin secretion of the same scale as that induced by ACEA alone. Human islets were also exposed to 2 mM glucose with a combination of 10 μ M ACEA and 10 μ M AM251, after which the islets were exposed to 10 μ M ACEA alone. Under this experimental regime, the initial application of ACEA and AM251 induced a significant increase in basal insulin release, but blocked the subsequent ACEA-induced increase in insulin secretory response (Li et al., 2011a). In another set of experiments, AEA increased insulin release from human islets cultured under low (3 mM) and high (11 mM) glucose concentrations, which was blocked by AM251 exposure (Bermúdez-Silva et al., 2008). These results confirm the findings in the current study that link CB1 receptor activation and inhibition, independent of glucose concentration, with glucose metabolism and the regulation of insulin gene expression and insulin secretion.

Results obtained from the current CB2 receptor *in vitro* inverse agonist/antagonist studies showed that AM630 pre-conditioning, in the absence and presence of *C. sativa* treatment, exerted variable effects, depending on glucose concentration. Under normoglycemic conditions, AM630 pre-conditioning exerted an effect similar to that of AM251 pre-conditioning, inducing a significant reduction in chronic, basal and glucose-stimulated insulin secretion. In islets cultured under hyperglycemic conditions, the effects of AM630 pre-

conditioning in HA2 islets mimicked that of a CB1 agonist, resulting in increased chronic insulin secretion while suppressing the basal and GSIS which reduced the glucose-stimulated:basal insulin production of these islets, which is supported by previous research showing that AM630 can act as a CB1 inverse agonist. It should be noted that a high concentration of AM630 was used to pre-treat the islets, which exceeded the abovementioned K_i values for both CB1 and CB2 receptors. This may therefore have contributed to the effects observed on CB1 receptors in the presence of excessive AM630 ligands. In HA2T1 islets, addition of *C. sativa* extract resulted in partial inhibition of the stimulatory effect of *C. sativa* exposure on chronic insulin secretion to levels similar to HC islets, while basal and glucose-stimulated levels were substantially lower than HC and HT1 islets, respectively. These changes resulted in a significant reduction in the glucose-stimulated:basal insulin production of HA2T1 islets. At molecular level, AM630 pre-conditioning, in the absence and presence of *C. sativa* treatment, did not induce substantial changes in insulin gene expression.

These findings are supported by results reported in various studies where AM630 appears to have varied effects on islets. Studies conducted by Anderson et al. (2013) showing that pre-incubation with AM630 (100 nM) did not significantly affect 20 mM glucose-stimulated insulin secretion, but attenuated the inhibitory effect of AEA. AM630 pre-incubation had no significant effect on insulin secretion at basal or intermediate glucose concentrations. Bermúdez-Silva et al., (2007) investigated the role of CB2 receptors in glucose metabolism in rats and found that blocking CB1 receptors activation with AM251 mimics the actions associated with CB2 receptor agonists and proposed that glucose homeostasis is modulated through the coordinated actions of CB1 and CB2 receptors. The results of the current study and of the abovementioned confirm that in rat islets, both CB1 and CB2 receptors modulates GSIS, thereby linking cannabinoid receptor activation/inhibition to the modulation of insulin secretion and glucose metabolism in rat β -cells.

In the current *in vivo* studies, *C. sativa* treatment showed no effect on the GOI in the LNE pancreatic tissue, relative to LNC rats. However, the cafeteria diet induced the following changes in gene expression in OBC pancreatic tissue, relative to LNC rats: c-MYC (reduced to 0.009-fold, $P < 0.05$), FLIP (reduced to 0.177-fold, $P < 0.05$) and UCP2 (0.137, $P < 0.01$). *C. sativa* treatment in the OBE rats maintained a significant reduction in c-MYC expression ($P = 0.032$), relative to LNC rats. It is also worth noting that *C. sativa* treatment in the OBE rats induced a significant increase in the weight of the pancreas ($P = 0.03$) and the

pancreas:body weight ratio ($P < 0.001$), relative to the OBC rats. Relative to OBC rats, *C. sativa* treatment in the OBE rats significantly increased the expression of the following genes: CB1 (2.923-fold increase, $P < 0.01$); GLUT2 (2.765-fold increase, $P < 0.001$); PKB (2.562-fold increase, $P < 0.05$) and UCP2 (3.199-fold increase, $P < 0.001$). Upregulation of PKB is closely linked to β -cell proliferation, increased β -cell size and total islet mass, improved glucose tolerance and complete resistance to experimental diabetes in mice (Tuttle et al., 2001). This supports the observations made in OBE rats, where pancreatic mass increased and glucose tolerance was improved, relative to the OBC rats.

The gene expression in the *in vitro* islet studies showed significant changes induced by *C. sativa* treatment under both normo- and hyperglycemic conditions. c-MYC expression was down-regulated to 0.88-fold in ND4T1 islets ($P < 0.05$). FLIP expression was down-regulated to 0.85-fold ($P < 0.05$) in HD4T1 islets, relative ND4C and 0.73-fold ($P < 0.01$), relative to HD4C islets. All other genes showed no statistical differences between the experimental and corresponding control islets, except CB1 expression. CB1 gene expression increased to 1.36-fold in islets cultured under hyperglycemic conditions, while *C. sativa* treatment in HD4T1 islets showed a significantly higher level of CB1 expression (1.32-fold, $P < 0.05$), relative to ND4C islets. Results from the present study are supported by previous research findings and confirms the expression of CB1 on β -cells in rat islets and their modulation of insulin secretion and glucose metabolism as shown in the GSIS studies. Interestingly, hyperglycemic conditions did not induce any changes in the expression levels of GOI in HD4C islets, relative to ND4C islets. Therefore a reduction in c-MYC expression, as observed in the ND4T1 islets, may have contributed to the significant increase in chronic insulin secretion observed in these islets, relative to their control islets. These findings are supported by previous research showing the link between c-MYC and insulin. Both c-MYC and FLIP activity have been implicated in β -cell function (Maedler et al., 2002a; Tuttle et al., 2001). c-MYC activity is enhanced under hyperglycemic conditions, inducing increased β -cell dysfunction and/or apoptosis, which may contribute to the loss in β -cell function in DM (Pelengaris and Khan, 2003). In murine β -cells, activation of c-MYC induces increased apoptosis which, if activation is sustained, results in diabetes. Over-expression of c-MYC in isolated rat islets suppresses GSIS and insulin secretion.

As previously stated, research regarding the effect of the endocannabinoid system in pancreatic tissue has generated contradictory results pertaining to the location of the cannabinoid receptors. qPCR analysis of the pancreatic tissue associated with the *in vivo* study shows no changes in CB1 gene expression in the LNE and OBC rats, relative to LNC rats. However, OBE rats shows a significant increase in CB1 expression (2.92-fold, $P < 0.01$), relative to OBC rats. This increase in CB1 protein expression in the islets was confirmed in the *in vitro* studies, as discussed below. In the present *in vitro* study, cannabinoid receptor gene expression and protein expression were measured via qPCR, western blots and immunohistochemical analysis. Densitometry analysis on western blots of CB1 and CB2 receptors showed that *C. sativa* extract induced a marginal increase in CB1 protein expression in isolated rat islets cultured under both normo- and hyperglycemic conditions, while CB2 expression was marginally lower in islets cultured under hyperglycemic conditions and exposed to *C. sativa* extract. *C. sativa* exposure induced no significant change in CB2 expression in these islets (data not shown). According to the manufacturer's specifications for CB1, major bands should be observed at 60kDa (coinciding with the monomeric CB1 species), with less intense bands of 23, 72 and 180kDa, while CB2 should be observed at 60kDa. However, CB1 and CB2 receptor bands were detected at approximately 30 kDa (between molecular markers 25 and 37 kDa), while the Actin band was detected at 42 kDa, in accordance with the manufacturer's specifications. In a study conducted by Grimsey et al. (2008), four commercial primary monoclonal CB1 antibodies from four different suppliers (including the supplier from which the antibody used in this study was procured) were tested for their specificity in mice brains and CHO cells transfected with CB1 receptors. Most CB1 protein species reported in literature are between 52 to 60 kDa in size, with smaller (30kDa) and larger (200kDa) species also being reported, which coincides with the molecular size of the band observed for CB1 and CB2 in the present study. The smaller sizes represent partially degraded receptor complexes (Grimsey et al., 2008; Mukhopadhyay and Howlett, 2005; Howlett et al., 2002). In addition to the western blots, double staining of the islets for insulin and CB1 receptors clearly show localization of CB1 receptors with insulin-containing cells. Many studies have shown cannabinoid receptor expression within the endocrine pancreas, however, no consensus has been reached on which cannabinoid receptor subtype is associated with each type of islet endocrine cell. Studies have shown that CB1 receptors are co-localized on α - and δ -cells in mice, rats and humans (Bermúdez-Silva et al., 2009; Starowicz et al., 2008; Juan-Picó et al., 2006), while Nakata and Yada (2008) found no evidence of CB2

receptors on mice islets In pancreatic islets isolated from fasted rats, CB1 expression was higher, relative to those exposed to glucose (Vilches-Flores et al., 2010a), where CB1 receptors were expressed in rat pancreatic islets, localized to the periphery of the islets. CB1 expression levels were up-regulated in fasted rats and down-regulated during feeding or glucose intake. Bermúdez-Silva et al. (2008) found that human islets contain the enzymes responsible for the synthesis and degradation of 2-AG, thus suggesting that endogenous cannabinoids play a role in the regulation of the hormone secretion in human islets. The results of these studies suggest that the distribution of CB1 and CB2 receptors in the pancreatic tissue is species-specific.

THC mediates most of its effects via the CB1 receptor through activation of $G_{i/o}$ proteins, resulting in the inhibition of AC, and eventual activation of downstream MAPK family proteins, viz. ERK1/2 and JNK. Glucose activates MAPKs ERK1/2 (Brun et al., 2004) and research has shown that CB1 and CB2 agonists induce activation of MAPK which regulate the expression of c-MYC which ultimately control the expression of genes such as c-fos, Krox-24 and c-jun, which play a key role in cell proliferation (Poinot-Chazel et al., 1996). Fogli et al. (2006) found increased apoptosis in human pancreatic cancer cells, MIA PaCa-2 cells, independent of CB1 and CB2 receptors. They found that AM251 induced a significant cytotoxic effect via the JNK signaling pathway. Both pJNK and pERK1/2 were detected in the isolated islets used to conduct the *in vitro* studies, with no significant differences being observed in their expression levels relative to the respective control islets. These findings support the results of the apoptosis studies, where no significant differences were observed in the 96-hour study between the *C. sativa*-treated islets and their respective controls.

Low-grade and chronic inflammation is a characteristic feature associated with obesity, and various cytokines and chemokines have been shown to be elevated in T2D and obese subjects. Cannabinoids are well known to have anti-inflammatory activity, however the effect of cannabinoids on cytokine and chemokine production is varied, depending on the tissue type, duration of exposure as well as the physiological conditions to which the tissue has been exposed. In the present study, *C. sativa* treatment in islets cultured under normoglycemic conditions reduced MIP-1 α levels in ND4T1 islets, while the levels of IL-1 α and MIP-2 remained unchanged, relative to ND4C islets. These observations are supported by findings from previous studies where *C. sativa* treatment reduced MIP-1 α levels. Hyperglycemic

conditions in HD4C islets increased IL-1 α secretion and reduced MIP-1 α levels, while keeping MIP-2 levels relatively steady, relative to ND4C islets. It should be noted that the levels of cytokines and chemokines in diabetic and obese individuals are produced within a milieu of high glucose and fatty acids. The present study cultured islets under high glucose conditions only, which may have impacted the secretion of cytokines to some extent. Despite the reduction of MIP-1 α levels in the HD4C islets, the difference was not statistically significant from that of the ND4C islets. Pre-conditioning with AM251 in the presence of *C. sativa* treatment in islets cultured under hyperglycemic conditions reduced MIP-2 secretion but did not block the stimulatory effect of *C. sativa* treatment in IL-1 α secretion. However it was effective in blocking the effect of *C. sativa* treatment in MIP-1 α secretions. Pre-conditioning with AM630 in the presence of *C. sativa* treatment in islets cultured under hyperglycemic conditions reduced MIP-1 α secretion, but was able to effectively block the effect of *C. sativa* treatment on IL-1 α and MIP-2 secretions. Under normoglycemic conditions, pre-conditioning with AM251, in the presence and absence of *C. sativa* treatment had no effect on IL-1 α secretion. However, pre-conditioning with AM630 in the presence and absence of *C. sativa* treatment, substantially increased IL-1 α and MIP-1 α secretion, and significantly reduced the stimulatory effect of *C. sativa* treatment on MIP-2 secretions. These findings are in agreement with observations reported in various studies, showing that both CB1 and CB2 modulate cytokine secretion in the rat pancreatic islets. O'Sullivan and Kendall (2010) found that THC increases anti-inflammatory activity by inducing increased PPAR- γ gene expression. Srivastava et al. (1998) conducted *in vitro* experiments on human leukemic T, B, eosinophilic and CD8q NK cell lines to determine the effect of THC exposure on cytokine secretion. THC decreased secretion of IL-8, MIP-1 α , MIP-1 β , and RANTES in NK cells. THC inhibited MIP-1b in HTLV-1 positive B-cells and induced a 3.0-fold increase in IL-8, MIP-1 α and MIP-1 β secretion in B-cells. In eosinophilic cells, THC induced an increase in MIP-1 β and IL-8 secretion, while IL-10 secretion was strongly inhibited in HUT-78 T-cells. In animal models of infection, THC or other CB1 agonist treatments enhanced progression of the infection by reducing the levels of IFN- γ , TNF- α , IL-1 β , IL-6, IL-12 and increasing IL-10 (Croxford and Miller, 2003; Smith et al., 2000). THC has also been reported to reduce IL-8, MIP-1 α , MIP-1 β , and RANTES in NK cells (Pandey et al. 2009).

The relevance of the findings of the *in vitro* and *in vivo* studies are grounded in the growing body of evidence which reveals that the endocannabinoid system plays a major role in the

regulation of energy metabolism via the CNS and peripheral target tissues. The findings of the current studies support this body of evidence, illustrating that CB1 and CB2 receptors play a key role in β -cell function. The development of therapeutic compounds that interact with both CB1 and CB2 receptors to induce or inhibit the cellular endocannabinoid machinery may therefore result in enhanced β -cell sensitivity to glucose and the improved regulation of insulin gene expression and secretion within the pancreatic tissue, while also inducing the peripheral endocannabinoid system to enhance insulin sensitivity and energy metabolism in patients with obesity and/or MetS.

CHAPTER 4: DISCUSSION

4.1 OVERVIEW OF KEY FINDINGS AND CONCLUSIONS

The aim of this study was to determine the effect of *C. sativa* extract on glucose metabolism within a lean and diet-induced obese rat model, and to specifically investigate glucose-stimulated insulin secretion in isolated rat islets in response to *C. sativa* extract exposure, under 11.1 and 33.3 mM glucose conditions. The following illustrations provide a summary of the key findings of the *in vivo* (figure 4.1) and *in vitro* (figure 4.2) experiments, respectively.

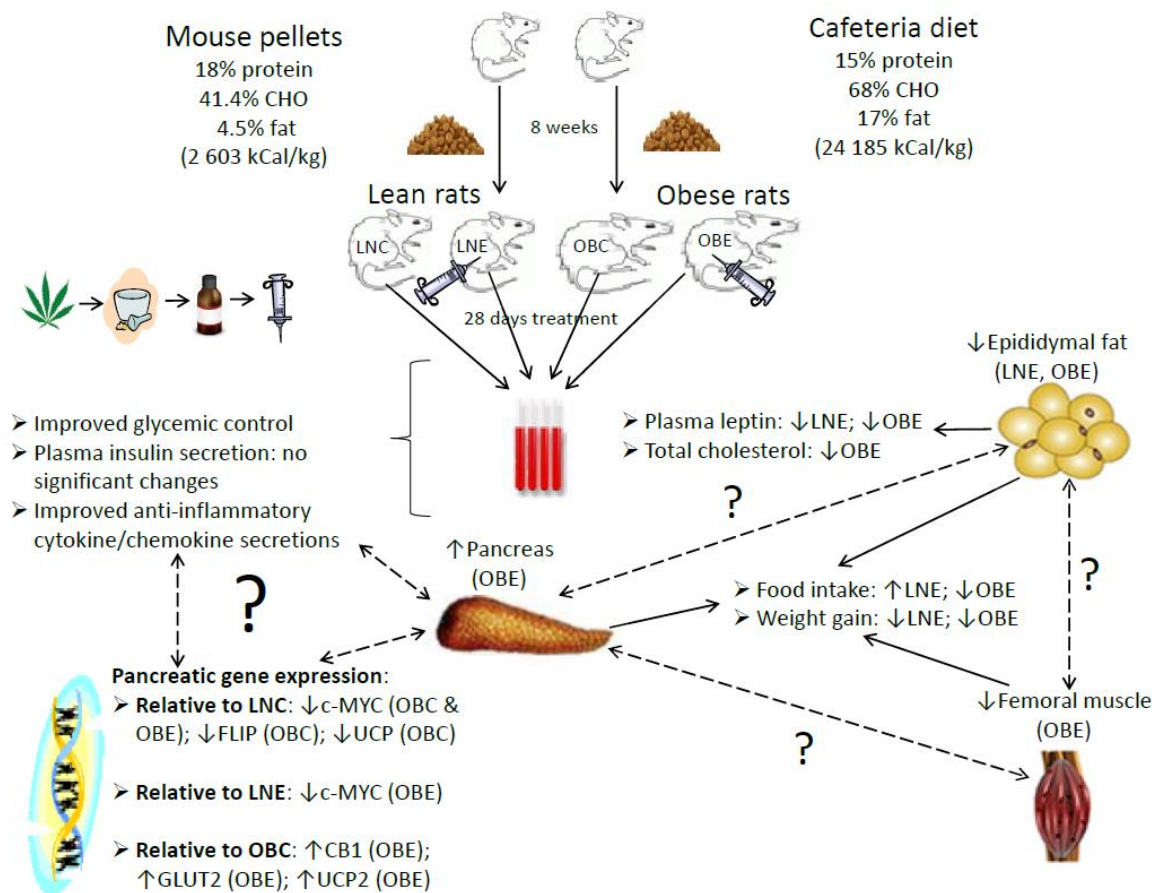


Figure 4.1 Graphical abstract of the key findings of the *in vivo* study.

C. sativa treatment elicited a dual effect in the rat model, where food intake was significantly increased in LNE rats, while there was a significant reduction in food intake in OBE rats. Glycemic control was improved despite insulin levels not showing a significant change across the different experimental groups. At peripheral tissue level, significant changes were observed in adipose and skeletal muscle (specifically the femoral muscles). The reduction in

epididymal adipose tissue may have contributed to the significant drop in plasma leptin levels in LNE and OBE rats, which may also have contributed to the improved glycemic control in these rats. *C. sativa* treatments also appear to have protected the pancreatic cells from the deleterious effects of obesity by reducing apoptosis via c-MYC down-regulation and increasing cell proliferation with PKB up-regulation in OBE rats (possibly via the PI-3K/Akt pathway as previously demonstrated by Fatrai et al. (2006)). Pancreatic energy metabolism in OBE rats were also affected through up-regulation of CB1 receptors, GLUT2 transporters and UCP2 gene expression in pancreatic tissue. The collective changes in gene expression investigated may have contributed, in part, to the increase in pancreatic mass observed in OBE rats. *C. sativa* treatment, however, did not induce any significant changes in gene expression in the pancreatic tissue of LNE rats. These observed effects of *C. sativa* treatment in LNE and OBE rats are the consequence of complex cumulative effects of *C. sativa* on all physiological systems within each of the rats. To determine the specific effect of *C. sativa* treatment on insulin secretion in pancreatic tissue, islets were isolated and exposed to conditions to simulate normal and abnormal glycemic control associated with healthy and diabetic individuals.

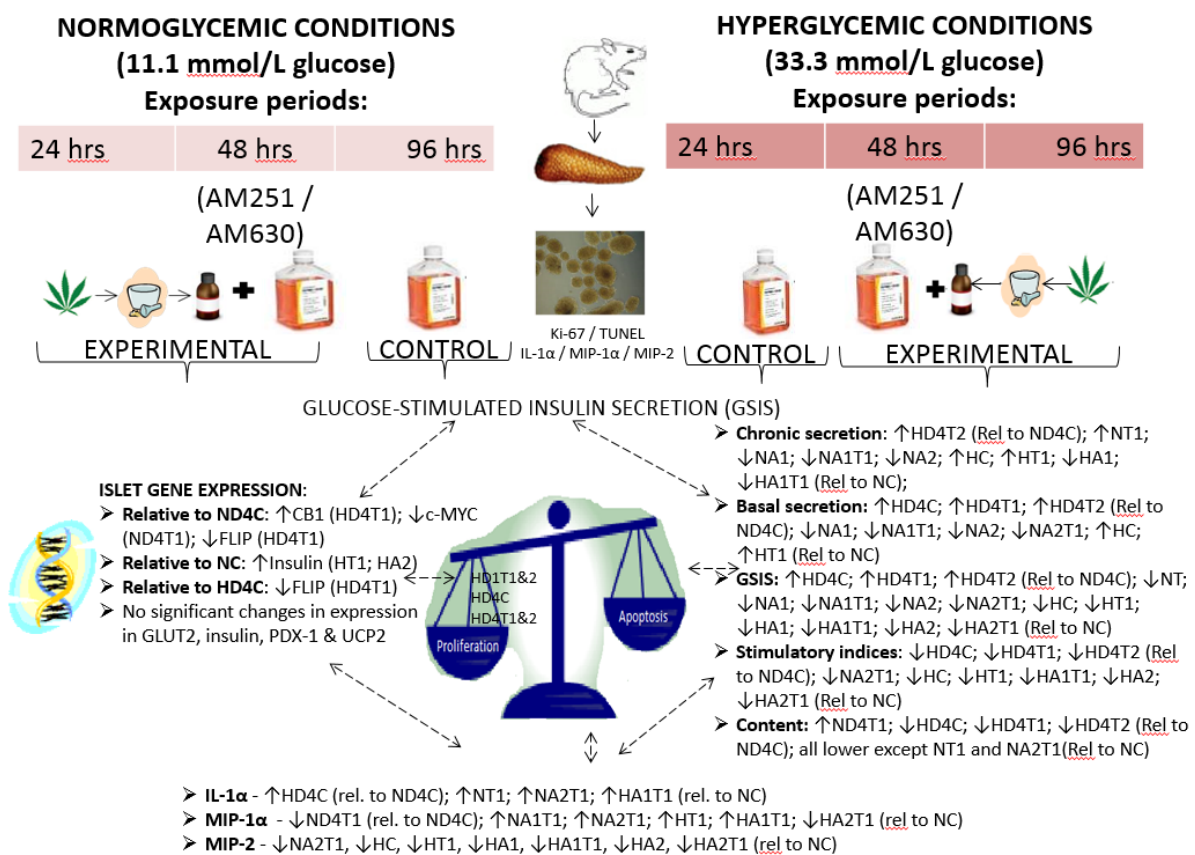


Figure 4.2 Graphical abstract of the key findings associated with the *in vitro* study.

Prolonged *C. sativa* exposure under hyperglycemic conditions enhanced chronic, basal and GSIS insulin secretion, while their insulin content and glucose-stimulated:basal insulin production were significantly lower, relative to ND4C islets. In the inverse-agonist/antagonist studies, A1 and A2 preconditioning had varying effects. A2 did not inhibit the effect of *C. sativa* treatment on chronic insulin secretion in NA2T1, HA2 and HA2T2 islets, relative to NC islets. Both A1 and A2, in the presence and absence of *C. sativa* treatment, reduced basal insulin secretion under all experimental conditions, relative to NC and HC islets, respectively. Similarly, A1 and A2, in the presence and absence of *C. sativa* treatment, reduced GSIS, relative to NC and HC islets, respectively, except HA1 islets. Both A1 and A2, in the presence and absence of *C. sativa* treatment, reduced insulin content under all experimental conditions, relative to NC islets, except NA2T1 islets. These findings imply that insulin secretion is directly affected by cannabinoid receptor activation/stimulation, with glucose concentration influencing affecting β -cell functionality, and A2 activity mimicking that of CB1 agonist. Hyperglycemic, and not normoglycemic conditions stimulated β -cell proliferation, with *C. sativa* exposure further enhancing proliferation, and concomitant apoptosis observed, but not to the same extent. Changes observed in pancreatic tissue gene expression reflecting an increase in CB1 receptor and decreased FLIP expression, which may have contributed, in part, to the shift towards cell proliferation in HD4T1 islets.

This study therefore confirms that the endocannabinoid system not only regulates energy balance at organism and islet level, but also modulates cell survival as a protective mechanism against the deleterious effects of obesity and hyperglycemia.

4.2 STUDY LIMITATIONS

Upon reflection of the experimental design, the following limitations were identified:

- A major limitation in the *in vivo* study was the inability of the cafeteria diet to induce impaired glucose tolerance and hyperglycemia in the obese rats. The diet resulted in moderate weight gain in the obese rats, which was generally lower than that reported in literature using a similar DIO model. This prevented the researcher from determining whether *C. Sativa* can attenuate DIO-associated glucose intolerance and/or insulin resistance.

4.3 FUTURE RESEARCH

A crude organic *C. sativa* extract was used in the *in vivo* studies, where only THC content was quantified. Using gas chromatography-mass spectroscopy the crude extract could be further analysed to obtain the various components in the extract, and use these, either individually or in various combinations, in *in vitro* studies on cell lines representing peripheral tissues, to determine their effect on INS-1, 3T3-L1, C2C12 and Chang liver cells, to determine their physiological effects on insulin and glucose sensitivity, energy metabolism and endocannabinoid flux. In addition, in depth cytokine analysis of *in vitro* cannabinoid exposure need to be conducted to determine their effects on pro- and anti-inflammatory cytokines. Where significant changes in these parameters are observed, the relevant compounds can then be tested in *in vivo* models to determine their efficacy at organism level.

A significant finding in the *in vivo* study was the reduction in epididymal fat in *C. sativa*-treated rats. The role of the peripheral endocannabinoid system in adipose tissue would need to be investigated to determine the impact of *C. sativa* exposure, including individual cannabinoids and combinations of these phytocannabinoids, on adipogenesis and lipolysis in pre-adipocytes and differentiated adipocytes. Insulin resistance can also be induced in these cells and thereafter exposed to cannabinoids to determine whether insulin sensitivity and glucose metabolism is enhanced. In addition, conducting lipidomics with the use of liquid chromatography- or gas chromatography-mass spectrometry, to provide a comprehensive profile of the entire cellular lipid pool within the adipocytes, thereby providing information on the changes observed in the *in vivo* study.

Recent evidence has shown that non-CB1 and non-CB2 receptors mediate cannabinoid activity in various tissue types. CB1 agonists, including THC, have been shown to elicit effects via TRPV1 and GPR55 receptors. Additional studies need to be conducted, blocking both CB1 and CB2 receptors with simultaneous AM251 and AM630 pre-conditioning, to determine whether changes observed in insulin secretion and gene expression is induced via a non-cannabinoid receptor mechanism (De Petrocellis and Di Marzo, 2010).

The endocannabinoid system has been shown to affect both endocrine and exocrine pancreatic function (Bermúdez-Silva et al., 2009; Linari et al., 2009). The effect of phytocannabinoid exposure on the endocannabinoid system, specifically the following

components - AEA, 2-AG, FAAH, monoacylglyceride lipase (MAGL), CB1 and CB2 receptor expression, need to be investigated, since the current study showed an increase in pancreatic CB1 protein and gene expression.

A significant increase in pancreatic tissue mass was observed in obese rats treated with *C. sativa* extract, while islets treated with *C. sativa* extract showed increased (although not statistically significant) Ki-67-positive β -cell staining. Elevated glucose levels are known to have a mitogenic or apoptotic effect on β -cells and the response of the β -cells is mediated by a key cytokine, IL-1 β which regulates Fas expression. Up-regulation of FLIP has been shown to switch Fas-induced apoptotic signals to proliferative signals in β -cells. Therefore IL-1 β antagonist studies would need to be conducted to determine whether the *C. sativa*-induced changes in insulin secretion and β -cell proliferation is affected via the IL-1 β -regulated Fas/FLIP pathway using western blots and qPCR methodologies.

β -Cell function is highly susceptible to glucose levels with elevated glucose inducing an increase in insulin synthesis within ten minutes via increased insulin gene transcription. Insulin secretion is influenced by both β -cell $[Ca^{2+}]_i$ and ADP/ATP ratio. In addition, ER stress is associated with depletion of ER Ca^{2+} stores and nitric oxide formation. Since data showed changes in the UCP2 gene expression, further experimentation on Ca^{2+} influx, ER stress and ATP/ADP balance in the β -cells will assist in understanding the changes induced by *C. sativa* in the GSIS studies in rat islets exposed to normo- and hyperglycemic conditions. In addition, by conducting enzymology studies, key mitochondrial enzymes involved in glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), Krebs cycle (succinate dehydrogenase, citrate synthase), and mitochondrial electron transport chain (cytochrome c oxidase) can be investigated. Results from these studies should provide further clarity on the effect of cannabinoids on energy metabolism in the β -cell.

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ANNEXURE A

**DEPARTMENT OF HEALTH
DEPARTEMENT VAN GESONDHEID**

Private Bag X828
PRETORIA, 0001

REPUBLIC OF SOUTH AFRICA



**UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO**

Privaatsak X828
PRETORIA, 0001

REPUBLIEK VAN SUID-AFRIKA

Faks/Fax	:	(012)312-3114	Navrae/Enquiry	:	H vd Westhuizen
Telefoon/Telephone	:	(012)312-0265	Verw/Reference	:	26/7/3/3/4

UPE82/2003/2012

The Managing Director
Dept Of Biochemistry & Microbiology.UPE
P O. Box 1600
SUMMERSTRAND
6001

2003 -04- 17

ATTENTION: Ms Ruby-Ann Levendal

Dear Madam


**APPLICATION FOR A PERMIT IN TERMS OF SECTION 22A OF THE
MEDICINE AND RELATED SUBSTANCES CONTROL ACT, 1965 (ACT 101 OF
1965)**

Receipt of your application dated 03 February 2003 for the above-mentioned permit is acknowledged.

The attached permit no UPE82/2003/2004 is valid for the period 30 April 2003 to 29 April 2004 and is issued subject to the conditions contained therein.

Application for renewal should be submitted on form GW 12/22 together with the original permit for cancellation, and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

Yours faithfully


REGISTRAR OF MEDICINES

ANNEXURE B

VOLUME CALCULATIONS OF 1% TWEEN 80[®] SALINE FOR CONTROL AND EXPERIMENTAL RATS					
THC extract concentration - 4.107 mg/ml					
THC DOSAGE AT 5 mg/kg					
WT (g)	THC(mg/kg)	volume (µL)	WT (g)	THC(mg/kg)	volume (µL)
400	2.00	487	765	3.83	931
405	2.03	493	770	3.85	937
410	2.05	499	775	3.88	944
415	2.08	505	780	3.90	950
420	2.10	511	785	3.93	956
425	2.13	517	790	3.95	962
430	2.15	523	795	3.98	968
435	2.18	530	800	4.00	974
440	2.20	536	805	4.03	980
445	2.23	542	810	4.05	986
450	2.25	548	815	4.08	992
455	2.28	554	820	4.10	998
460	2.30	560	825	4.13	1004
465	2.33	566	830	4.15	1010
470	2.35	572	835	4.18	1017
475	2.38	578	840	4.20	1023
480	2.40	584	845	4.23	1029
485	2.43	590	850	4.25	1035
490	2.45	597	855	4.28	1041
495	2.48	603	860	4.30	1047
500	2.50	609	865	4.33	1053
505	2.53	615	870	4.35	1059
510	2.55	621	875	4.38	1065
515	2.58	627	880	4.40	1071
520	2.60	633	885	4.43	1077
525	2.63	639	890	4.45	1084
530	2.65	645	895	4.48	1090
535	2.68	651	900	4.50	1096
540	2.70	657	905	4.53	1102
545	2.73	664	910	4.55	1108
550	2.75	670	915	4.58	1114

555	2.78	676		920	4.60	1120
560	2.80	682		925	4.63	1126
565	2.83	688		930	4.65	1132
570	2.85	694		935	4.68	1138
575	2.88	700		940	4.70	1144
580	2.90	706		945	4.73	1150
585	2.93	712		950	4.75	1157
590	2.95	718		955	4.78	1163
595	2.98	724		960	4.80	1169
600	3.00	730		965	4.83	1175
605	3.03	737		970	4.85	1181
610	3.05	743		975	4.88	1187
615	3.08	749		980	4.90	1193
620	3.10	755		985	4.93	1199
625	3.13	761		990	4.95	1205
630	3.15	767		995	4.98	1211
635	3.18	773		1000	5.00	1217
640	3.20	779		1005	5.03	1224
645	3.23	785		1010	5.05	1230
650	3.25	791		1015	5.08	1236
655	3.28	797		1020	5.10	1242
660	3.30	804		1025	5.13	1248
665	3.33	810		1030	5.15	1254
670	3.35	816		1035	5.18	1260
675	3.38	822		1040	5.20	1266
680	3.40	828		1045	5.23	1272
685	3.43	834		1050	5.25	1278
690	3.45	840		1055	5.28	1284
695	3.48	846		1060	5.30	1290
700	3.50	852		1065	5.33	1297
705	3.53	858		1070	5.35	1303
710	3.55	864		1075	5.38	1309
715	3.58	870		1080	5.40	1315
720	3.60	877		1085	5.43	1321
725	3.63	883		1090	5.45	1327
730	3.65	889		1095	5.48	1333
735	3.68	895		1100	5.50	1339
740	3.70	901		1105	5.53	1345
745	3.73	907		1110	5.55	1351

750	3.75	913		1115	5.58	1357
755	3.78	919		1120	5.60	1364
760	3.80	925		1125	5.63	1370

ANNEXURE C



University of Port Elizabeth
Animal Ethics Committee

INDEMNITY FORM

Use of Animals for Experiments

NAME OF APPLICANT (RESEARCHER):	Dr Ruby-Ann Levendal
DEPARTMENT:	Biochemistry and Microbiology
DURATION OF PROJECT:	5 years
PROJECT TITLE:	Cannabis effects on fat metabolism
ANIMAL SPECIES INVOLVED:	Male Wistar Rats Mice [db/db]
NUMBER OF ANIMALS TO BE USED:	48 Rats 48 Mice
Approved by:	 DR G.J. ROSSOUW CHAIRMAN : Animal Ethics Committee
Date:	27 February 2004
Ref. No.	AE/01/2004

ANNEXURE D

Below is a summary of the relative gene expression ratios of selected genes in pancreatic tissue of *C. sativa*-treated lean rats in relation to lean control rats.

Table D1 Summary of the relative gene expression ratios of selected genes in pancreatic tissue of LNE rats in relation to LNC rats

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
TUBULIN	REF	1.0					
CYCLO A	REF	1.0					
INSULIN	TRG	0.956	0.201	0.056 - 1.189	0.007 - 1.864	0.119	
CBI	TRG	1.0	1.229	0.476 - 5.797	0.337 - 14.003	0.829	
CB2	TRG	1.0	0.816	0.194 - 4.363	0.030 - 14.152	0.796	
GLUT2	TRG	1.0	1.997	0.697 - 28.912	0.435 - 55.621	0.610	
UCP2	TRG	0.994	0.401	0.113 - 1.683	0.027 - 2.005	0.266	
c-MYC	TRG	1.0	1.055	0.096 - 8.497	0.029 - 19.409	0.977	
FLIP	TRG	1.0	0.432	0.045 - 1.701	0.011 - 3.150	0.399	
PEPCK	TRG	1.0	0.392	0.065 - 2.036	0.017 - 6.593	0.320	
PKB	TRG	1.0	1.041	0.375 - 2.614	0.252 - 5.409	0.915	
PDX-1	TRG	1.0	1.462	0.629 - 3.484	0.337 - 7.379	0.455	

P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target gene

REF – Reference gene

In the boxplot (figure D1), the boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations of LNE rats relative to LNC rats.

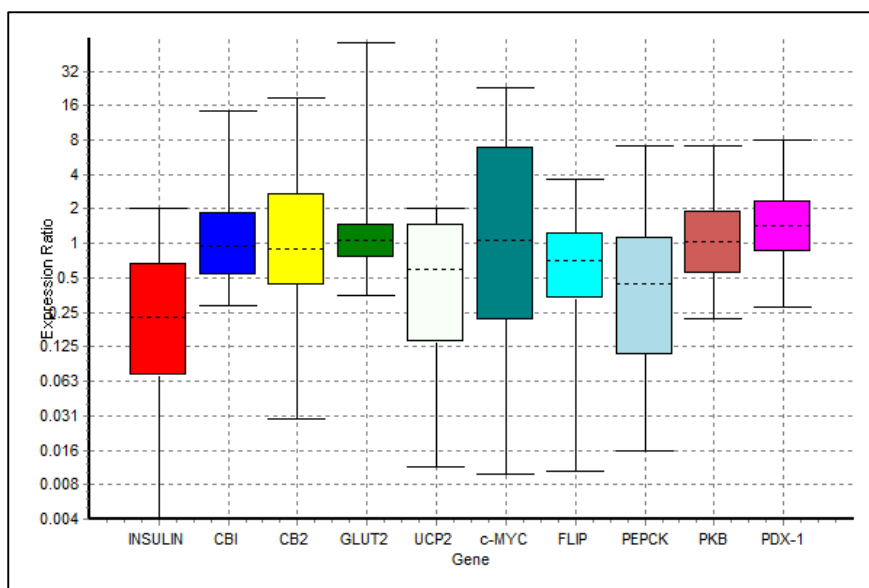


Figure D1 Boxplot showing the relative gene expression of LNE rats, relative to LNC rats.

Below is a summary of the relative gene expression ratios of selected genes in pancreatic tissue of diet-induced obese control rats in relation to lean control rats.

Table D2 Summary of the relative gene expression ratios of key genes in pancreatic tissue of OBC rats in relation to LNC rats.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
TUBULIN	REF	1.0					
CYCLO A	REF	1.0					
INSULIN	TRG	0.956	0.441	0.088 - 6.152	0.013 - 14.600	0.430	
CBI	TRG	1.0	0.333	0.133 - 1.404	0.066 - 4.454	0.111	
CB2	TRG	1.0	0.347	0.100 - 1.318	0.024 - 2.294	0.134	
GLUT2	TRG	1.0	0.568	0.188 - 11.687	0.166 - 18.120	0.578	
UCP2	TRG	0.994	0.137	0.034 - 0.289	0.018 - 0.466	0.005	DOWN
c-MYC	TRG	1.0	0.009	0.000 - 0.255	0.000 - 5.876	0.034	DOWN
FLIP	TRG	1.0	0.177	0.015 - 0.675	0.005 - 1.097	0.037	DOWN
PEPCK	TRG	1.0	0.357	0.058 - 1.852	0.024 - 3.067	0.246	
PKB	TRG	1.0	0.495	0.248 - 1.024	0.129 - 2.285	0.124	
PDX-1	TRG	1.0	1.479	0.487 - 4.680	0.244 - 12.328	0.499	

P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target gene

REF – Reference gene

In the boxplot (D2), the boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations of OBC rats in relation to LNC rats.

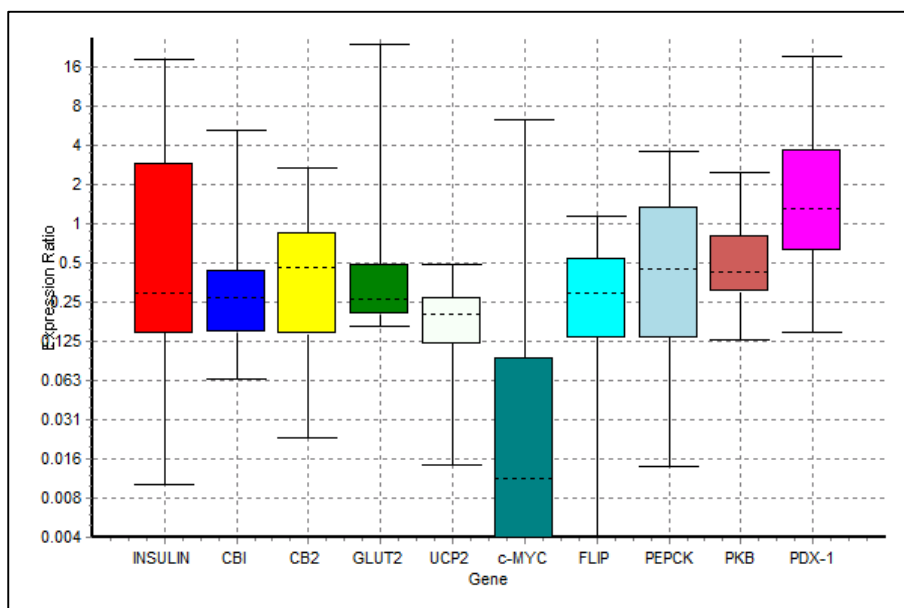


Figure D2 Boxplot showing the relative gene expression of OBC rats, relative to LNC rats.

Below is a summary of the relative gene expression ratios of key genes in pancreatic tissue of *C. sativa*-treated obese control rats in relation to lean control rats.

Table D3 Summary of the relative gene expression ratios of key genes in pancreatic tissue of OBE rats in relation to LNC rats.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
TUBULIN	REF	1.0					
CYCLO A	REF	1.0					
INSULIN	TRG	0.956	0.541	0.152 - 2.385	0.026 - 7.832	0.444	
CBI	TRG	1.0	1.033	0.405 - 5.041	0.291 - 10.957	0.984	
CB2	TRG	1.0	0.414	0.072 - 3.121	0.010 - 5.482	0.374	
GLUT2	TRG	1.0	1.667	0.544 - 26.327	0.409 - 47.757	0.856	
UCP2	TRG	0.994	0.466	0.116 - 1.084	0.053 - 2.001	0.176	
c-MYC	TRG	1.0	0.025	0.002 - 0.460	0.000 - 1.003	0.018	DOWN
FLIP	TRG	1.0	0.386	0.034 - 1.728	0.011 - 2.415	0.332	
PEPCK	TRG	1.0	0.800	0.124 - 5.713	0.031 - 11.738	0.808	
PKB	TRG	1.0	1.345	0.608 - 3.297	0.198 - 5.900	0.544	
PDX-1	TRG	1.0	1.123	0.467 - 2.731	0.224 - 6.398	0.818	

P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target gene

REF – Reference gene

In the boxplot (figure D3), the boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations of OBE rats in relation to LNC rats.

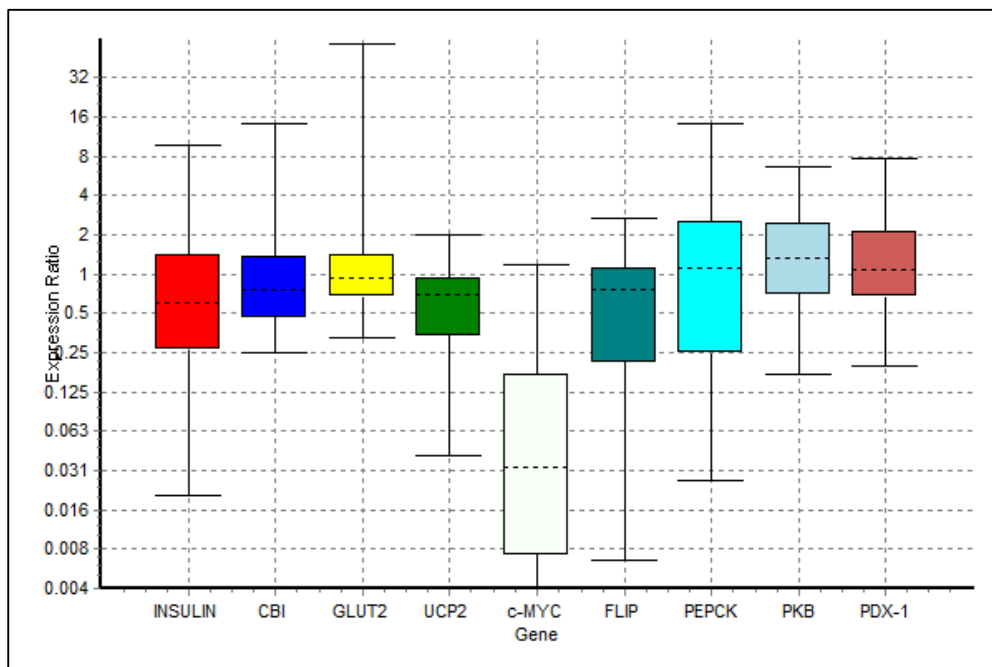


Figure D3 Boxplot showing the relative gene expression of OBE rats, relative to LNC rats.

Below is a summary of the relative gene expression ratios of key genes in pancreatic tissue of *C. sativa*-treated obese rats in relation to obese control rats.

Table D4 Summary of the relative gene expression ratios of key genes in pancreatic tissue of OBE rats in relation to OBC rats.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
TUBULIN	REF	1.0					
CYCLO A	REF	1.0					
INSULIN	TRG	0.956	1.095	0.093 - 7.327	0.030 - 29.796	0.934	
CBI	TRG	1.0	2.770	1.497 - 5.538	1.077 - 9.214	0.010	UP
CB2	TRG	1.0	1.064	0.216 - 5.829	0.089 - 8.059	0.944	
GLUT2	TRG	1.0	2.621	1.723 - 3.640	1.270 - 4.866	0.000	UP
UCP2	TRG	0.994	3.032	1.876 - 4.974	1.458 - 7.223	0.000	UP
c-MYC	TRG	1.0	2.536	0.096 - 59.042	0.005 - 2 096.371	0.623	
FLIP	TRG	1.0	1.945	0.822 - 4.145	0.374 - 7.914	0.147	
PEPCK	TRG	1.0	2.000	0.658 - 6.250	0.401 - 10.455	0.198	
PKB	TRG	1.0	2.428	1.320 - 4.729	0.584 - 6.162	0.032	UP
PDX-1	TRG	1.0	0.678	0.294 - 1.517	0.138 - 3.011	0.392	

$P(H1)$ - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target gene

REF – Reference gene

In the boxplot (figure D4), the boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations of OBE rats in relation to OBC rats.

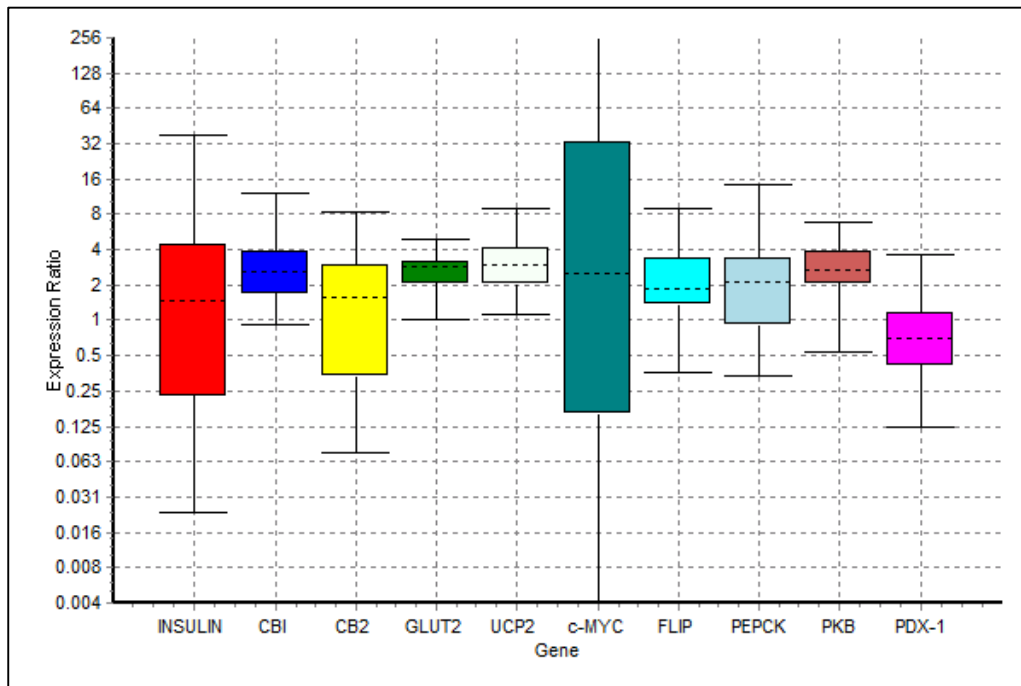


Figure D4 Boxplot showing the relative gene expression of OBE rats, relative to OBC rats.

ANNEXURE E

All solutions and reagents associated with the rat islet isolation and *in vitro* experiments. Chemical reagents were purchased at Sigma-Aldrich (SA), unless otherwise indicated.

E.1 Rat islet isolation solutions:

E.1.1 Collagenase solution

The following components constituted the collagenase solution used for rat islet isolation.

Table E.1 Components of collagenase solution used for the rat islet isolation protocol.

COMPONENTS & SUPPLIERS	2 rats	4 rats	6 rats	8 rats
HBSS (mL), Highveld Biological SA	30	60	90	120
DNase (mg), Roche SA	2	6	9	12
*HEPES(1 M; pH 7.4) (mL)	0.750	1.5	2.25	3
Collagenase P (U/mL), Roche SA	6.78	6.78	6.78	6.78
CaCl ₂ (1 M) (mL)	0.262	0.524	0.786	1.048
Neutral Protease (U), Serva DE	4	8	12	16

*HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

E.1.2 Quenching Buffer

The following components constituted the quenching buffer which was used in the washing steps of the islet isolation protocol:

Table E.2 Components of quenching buffer used during the washing steps of islet isolation protocol.

COMPONENTS & SUPPLIERS	2 rats	4 rats	6 rats	8 rats
HBSS (mL), Highveld Biological SA	100	200	300	400
BSA (g)	0.575	1.15	1.75	2.30
HEPES (1 M) (mL)	2.5	5.0	7.5	10.0

E.1.3 Culture media – RPMI 1640

Isolated islets were cultured under normoglycemic conditions in 11.1 mM glucose RPMI 1640 (Highveld Biological, SA) culture media. The following additional components were present in a final total volume of 500 mL media: 10% fetal bovine serum (Highveld Biological, SA), 100 µg/mL penicillin (Highveld Biological, SA), 100 µg/mL streptomycin

(Highveld Biological, SA), 50 µg/mL Gentamycin (Highveld Biological, SA), 1% 100x Glutamax (The Scientific Group, SA), 0.1% Fungizone (The Scientific Group, SA) and 0.6 mg/mL Terramycin (Pfizer, SA).

Islets cultured under hyperglycemic conditions are exposed to 33.3 mM glucose RPMI 1640. The components are similar to that stated above except that 12 mL of 25.1% glucose (which was dissolved in glucose-free RPMI media) was added to the media above, and made up to a final volume of 500 mL. For GSIS assays, proliferative studies and apoptotic studies, all conditions are represented by at least three separate experiments, with each experiment containing at least triplicate samples per experimental condition.

E.2 Glucose-stimulated insulin secretion (GSIS) assay solutions

E.2.1 10x Krebs Ringer Buffer stock solution

A 10x KRB stock was prepared by adding 33.6 g NaCl, 1.76 g KCl, 1.88g CaCl₂.2H₂O, 0.82 g KH₂PO₄, 1.48 g MgSO₄.7H₂O to 500 mL H₂O. Autoclave and store at 4°C.

E.2.2 1x KRB working solution

Made up 100 mL of 1x Krebs working solution as follows: Used 80 mL 1x Krebs Ringer and added 1 mL 1M HEPES (final concentration 10 mM), 0.5 g BSA (0.5%), adjusted pH to 7.4, then made up to final volume of 100 mL.

E.2.3 Basal KRB

Made up 100 mL 3.3 mM glucose KRB by using 99.7 mL 1x KRB working solution and added 330 µL 1M glucose solution.

E.2.4 Stimulating KRB

The stimulating KRB solution was prepared by using 98.3 mL 1x KRB working solution and adding 1.7 mL 1M glucose solution, to make a final concentration of 16.7 mM glucose KRB (100 mL final volume).

E.2.5 Insulin content

Made up 100 mL 0.18M HCl in 70% ethanol, i.e. 0.656 g HCl (w/v) in 70% pure ethanol (30mL distilled water added to 70 mL ethanol).

E.3 Immunoblot reagents

E.3.1 SDS gel sample buffer

To make up 3x non-reducing sample buffer, dissolve 3.75 mL 0.5 M Tris-HCl, pH 6.8 (final concentration 187.5 mM Tris), 0.6 g SDS (6%), 3.0 mL glycerol (30%), 0.23 g DTT (150 mM) and 0.03 g bromophenol blue (0.03%) in deionized water, make up to final volume of 10 mL.

E.3.2 Running buffer

Dissolve 15.14 g Tris base (250 mM); 94.0 g Glycine (250 mM), and 5.0 g SDS (1%) in deionized water. Make up to final volume of 5 L in deionized water.

E.3.3 Transfer buffer

Dissolve 30.0 g Tris base (25 mM), 15.0 g Glycine (200 mM) and 200 mL methanol (20%) in deionized water. Make up to 1 L, and store at 4°C.

E.3.4 Tris-buffered saline (TBS)

To make a solution of TBS, dissolve 15.14 g Tris (25 mM), 43.83 g NaCl (150 mM) in deionized water. Adjust pH to 7.5. Make up to final volume of 5 L, autoclave and store at 4°C.

E.3.5 0.1% Tris-buffer saline-Tween 20

Add 1 mL Tween 20 to one litre of TBS, to make up a 0.1% TBS-T solution.

E.3.6 Protein blocking solution

Make up a 5% (w/v) nonfat milk powder (BIO-RAD) solution in 0.1% TBS-T (150 mM NaCl, 25 mM Tris-HCl, 0.1% (v/v) Tween 20 (TBS-T) buffer, pH 7.5).

E.3.7 Washing solution

Use the remainder of the protein blocking solution diluted to a volume of 500 mL with TBS for all the washing steps.



Cannabis exposure associated with weight reduction and β -cell protection in an obese rat model

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ARTICLE INFO

Keywords:
Cafeteria diet
Cannabis
THC
Obesity
Cannabinoids

ABSTRACT

The aim of this study was to investigate the effect of an organic cannabis extract on β -cell secretory function in an *in vivo* diet-induced obese rat model and determine the associated molecular changes within pancreatic tissue. Diet-induced obese Wistar rats and rats fed on standard pellets were subcutaneously injected with an organic cannabis extract or the vehicle over a 28-day period. The effect of diet and treatment was evaluated using the intraperitoneal glucose tolerance tests (IPGTTs) and qPCR analysis on rat pancreata harvested upon termination of the experiment.

The cafeteria diet induced an average weight difference of 32 g and an overall increase in body weight in the experimental groups occurred at a significantly slower rate than the control groups, irrespective of diet. Area under the curve for glucose (AUC_G) in the obese group was significantly lower compared to the lean group ($p < 0.001$), with cannabis treatment significantly reducing the AUC_G in the lean group ($p < 0.05$), and remained unchanged in the obese group, relative to the obese control group. qPCR analysis showed that the cafeteria diet induced down-regulation of the following genes in the obese control group, relative to lean controls: UCP2, c-MYC and FLIP. Cannabis treatment in the obese group resulted in up-regulation of CB1, GLUT2, UCP2 and PKB, relative to the obese control group, while c-MYC levels were down-regulated, relative to the lean control group. Treatment did not significantly change gene expression in the lean group. These results suggest that the cannabis extract protects pancreatic islets against the negative effects of obesity.

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Introduction

Most drugs have been derived either directly or indirectly from plants. While the medicinal use of *Cannabis sativa* L., commonly referred to as marijuana, goes back several thousands of years, the isolation and characterization of the phytocannabinoid tetrahydrocannabinol (THC) in the 1960s and with the cloning of the two distinct cannabinoid subtypes in the early 1990s, has prompted renewed interest in cannabinoid compounds (Munro et al. 1993). There are numerous cannabinoids with the most common natural plant cannabinoids being cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC) and cannabigerol (CBG) (El-Alfy et al. 2010). THC is the best characterized of the cannabinoids. Worldwide, where cannabis usage has increased both for medicinal and recreational purposes, research is required to understand the individual roles of each of the cannabinoids and the synergistic effect that exists between them. Understanding of plant cannabinoid effects will also contribute to unravelling the role of the endogenous cannabinoids.

Cannabis extracts have been used in the treatment of various ailments including inflammation, bronchitis, pain and diabetes (van Wyk and Gericke 2000). In rats THC mediates its physiological

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, arachidonylethanolamide/anandamide; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-morpholinyl-1H-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-yl[(4-methoxyphenyl)methanone]; AUC_G , area under the curve for glucose; Bcl-XL, B-cell lymphoma-extra large; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; CNS, central nervous system; DIO, diet-induced obesity; FLIP, Fas-associated death-domain-like 1- β -converting enzyme-like inhibitory protein; Fas, fatty acid synthase; GLUT2, glucose transporter 2; HSI, hepatic somatic index; IL-1 α , interleukin-1 α ; IL-1 β , interleukin-1 β ; IL-6, Interleukin-6; IPN- γ , Interferon- γ ; IPGTTs, intraperitoneal glucose tolerance tests; LNC, Lean control; LNE, Lean experimental; MAPK, mitogen-activated protein kinase; c-MYC, myelocytomatosis oncogene; OBC, Obese Control; OBE, Obese Experimental; PDX-1, Pancreatic duodenal homeobox-1; PEPCK1, phosphoenolpyruvate carboxykinase 1; PKB, protein kinase B; qPCR, quantitative real-time polymerase chain reaction; RIA, radioimmunoassay; ROS, reactive oxygen species; RP-HPLC, reverse-phase high performance liquid chromatography; THC, Tetrahydrocannabinol; TNF- α , Tumor necrosis factor- α ; T2DM, Type 2 Diabetes Mellitus; UCP2, uncoupling protein 2; WHO, World Health Organization.

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doi:10.1016/j.phymed.2012.02.001

effects via cannabinoid receptor type 1 (CB1) and type 2 (CB2) binding, activating intracellular G-proteins which provide signals to a variety of effectors such as ion channels, the mitogen-activated protein kinase (MAPK) cascade and induce myelocytomatosis oncogene (c-MYC) expression (Howlett et al. 2002). CB1 receptors are mainly associated with the central nervous system (CNS), however, lower levels of CB1 mRNA has been detected in several peripheral tissues including the rat pituitary gland, adrenal glands, skeletal muscle, liver, gastrointestinal tract and pancreas. CB2 receptors are mainly associated with tissues involved in immune cell production and regulation (DiPatrizo et al. 2011; Howlett et al. 2004). CB1 and CB2 receptors form part of the endocannabinoid system, together with their endogenous ligands. Studies have shown cannabinoid receptor expression within the endocrine pancreas, however, no consensus has been reached on which cannabinoid receptor subtype is associated with each type of islet endocrine cell (Li et al. 2010a). CB1 and CB2 receptors and the novel cannabinoid receptor GPR55 has been found to be expressed in the β -cells in murine islets and MIN6 cell line (Li et al. 2010b,c), while Vilches-Flores et al. (2010) found peripheral localization of CB1 receptors in rat islets and Bermudez-Silva et al. (2007) found co-expression of CB1 and CB2 receptors in both insulin positive and non-insulin-expressing rat dissociated islet cells. Other studies have shown the co-localization of CB1 receptors on α - and δ -cells in rats and humans (Bermudez-Silva et al. 2009; Juan-Picó et al. 2006).

The endocannabinoid system has been implicated in the regulation of the body's energy balance (Bermudez-Silva et al. 2009; Juan-Picó et al. 2006). The majority of studies conducted on the effect of cannabis and/or various cannabinoid administrations on food intake in animals, have either failed to find an effect on eating behaviour, or found that cannabinoids induced an anorectic or hyperphagic effect (Pagotto et al. 2006; Kirkham and Williams 2001). Past studies have focused on endocannabinoid signaling in the brain. Past studies have focused on endocannabinoid signaling in the brain. Most recently DiPatrizo et al. (2011) have shown that a fat-rich diet alters intestinal endocannabinoid levels, which upregulate the CB1 receptors in nervous and vagal fibres. This surprising result indicates a potential role of the endocannabinoids to regulate dietary fat intake which is one of the main contributors to obesity. The prevalence of diet induced obesity (DIO) continues to increase, resulting in associated life style diseases such as hypertension, cardiovascular disease and diabetes (WHO, 2008). The progression of diabetes is characterized by a concomitant change in metabolic parameters, loss in β -cell mass as well as β -cell function. Therefore our study was to investigate the effect of an organic cannabis extract (with a quantified THC, CBD and CBN content) and their role on β -cell function in an obese rat model to evaluate the molecular changes induced in the pancreatic tissue.

Materials and methods

Cannabis extraction and quantification

The cannabinoid extraction and quantification was performed as described by Gallant et al. (2009). Cannabis plant material was obtained from the South African Police Services. Briefly, an organic extraction was obtained using chloroform, and evaporated using nitrogen, and the remaining resin was redissolved in methanol. The THC, CBN and CBD contents of the extract were quantified against a commercial THC, CBN and CBD standards (Industrial Analytical, Johannesburg, South Africa), using reverse-phase high performance liquid chromatography (RP-HPLC) on a C₁₈ Grace Vydac analytical column (4.6 mm × 250 mm, 5 μ m), previously described by Coetzee et al. (2007).

Animals

Experimental procedures were approved by the Nelson Mandela Metropolitan University Animal Ethics Committee. Rats were treated as reported by Coetzee et al. (2007). Briefly, the rats were housed in an air-conditioned room (22 ± 3 °C) with a 12 h light/dark cycle, and randomly assigned to four groups: untreated lean control (LNC); cannabis-treated lean experimental (LNE); untreated obese control (OBC) and cannabis-treated obese experimental (OBE). Rats assigned to the obese groups were fed on a cafeteria diet (15% protein, 68% carbohydrates and 17% fat) over a period of eight weeks to effect DIO, while lean rats were fed on standard chow (Epol, Pretoria, South Africa) (18% protein, 41.4% carbohydrates and 4.5% fat) over the same period and maintained throughout the experiment. Food consumption was monitored daily. Rats were injected subcutaneously with cannabis extract every alternate day for 28 days, with the first five treatments containing an equivalence of 5 mg THC/kg body weight, and the remaining treatments, an equivalence of 2.5 mg THC/kg body weight. The control groups were treated with an equivalent volume of the vehicle (1% Tween 80 in saline). Water was available *ad libitum*.

Intraperitoneal glucose tolerance test (IPGTT)

To determine the effect of treatment on insulin sensitivity, rats were given a glucose bolus of 2 g/kg body weight via intraperitoneal injection. Blood samples were obtained from the tail vein, and the glucose concentration was measured using a Glucometer (Accutrend GC, Roche, South Africa). Area under the curve for glucose (AUC_g) was calculated over the standard 2-h period, using the following formula: $AUC_g = \frac{(concentration_0 + concentration_1)}{2} \times Time_{1-0}$ (Chiou 1978).

Insulin quantification using radioimmunoassay (RIA)

The Linco rat insulin RIA (Linco Research, USA) was used to quantify plasma insulin levels of post-experimental plasma samples, according to the manufacturer's specifications, using the Packard-Perkin Elmer Tri-Carb 2300TR liquid scintillation analyzer (Life and Analytical Science (Pty) Ltd, South Africa).

Cytokine assay

Plasma levels of interleukin (IL)-1 α , interleukin (IL)-1 β , interleukin (IL)-6, interferon (IFN)- γ and tumor necrosis factor (TNF)- α were measured using Luminex Lincplex kits (Labodia, Preilverenges, Switzerland), according to the manufacturer's specifications. Analytes were quantified using the Luminex¹⁰⁰ system (Luminex Corp., USA).

qPCR analysis using Relative Expression Software Tool

Total RNA was extracted using the QIAGEN RNeasy mini kit and reverse transcription was performed using QIAGEN QuantiTect Reverse Transcription kit (Southern Cross Biotechnology, RSA). Primers were developed using Beacon Designer[®] 3 Software (Biorad), and included two reference genes, viz. β -tubulin (5'-3': AACCCAGCCCAGTCTAAG; 3'-5': GATTCCCGTGTCTAAATG) and cyclophilin A (5'-3': CGTG-GTCAAGACTGAGTGG; 3'-5': GTGCTTCCCACAGACC), and the genes of interest included CB1 (5'-3': CTGGTCTGATC-CTGGTGGT; 3'-5': TGCTCAGTCTCTGCTCT), c-MYC (5'-3': AGGAAGGACTATCCAGC; 3'-5': CCTCTTGCTGTTTCCTC), FLIP (5'-3': GGGACCTCCTGATTGTTAAG; 3'-5': ACGGCTGCTT-TATCTGTCTT), GLUT2 (5'-3': ATGACATCAATGGCACAGACAC;

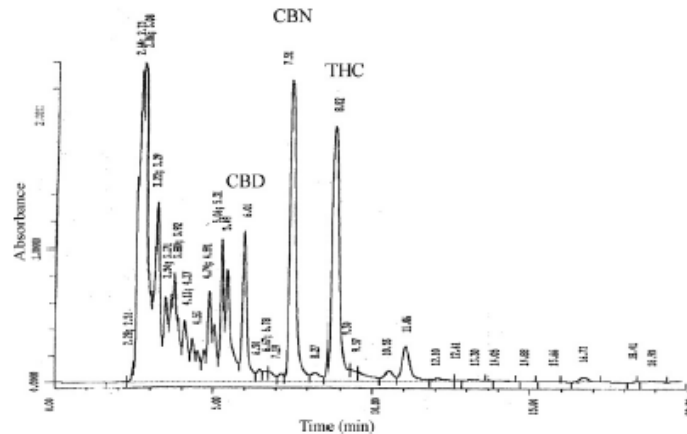


Fig. 1. A typical chromatogram of a chloroform extract of cannabis (10× dilution). Samples were eluted from a C18 RP-HPLC column using acetonitrile:0.01 M phosphate buffer (70:30) using a flowrate of 1 ml/min. The absorbance was monitored using 214 nm. The peaks corresponding to CBD, CBN and THC are at the retention times of 8.82, 7.52 and 6.61 min respectively as compared to commercial standards.

3′–5′: GACACAGACAGACCAGAG), insulin (5′–3′: CTGC-CAGGCTTTTGTCAAA; 3′–5′: TCCACTTCAGACGGGACTT), pancreatic duodenal homeobox-1 (PDX-1) (5′–3′: CTGC-CTCTCGTGCCATGTGAAC; 3′–5′: GGCTGTTATGGGACCGCTCAAG), phosphoenolpyruvate carboxykinase 1 (PEPCK) (5′–3′: TCGAT-GCCTTCCCAGTAAAC; 3′–5′: GTGATGACATTGCCCTGGATG), protein kinase B (PKB) (5′–3′: CAACTGGCAGGATGTGGTAC; 3′–5′: AGGCT-GTCATATCGGTCTGG), uncoupling protein 2 (UCP2) (5′–3′: TGGCGGTGGTCGGAGATAC; 3′–5′: GGGCAACATTGGGAGAGGTC). All qPCR reactions were conducted on the iCycler iQ Multicolour Real-Time Detection System (Biorad), V3.1, using iQ SYBR Green Supermix (Biorad). Quantitative real-time polymerase chain reaction (qPCR) analysis of 5 pancreatic samples per experimental group was conducted using Relative Expression Software Tool (REST) 2009 V2.0.13 (QIAGEN) (Pfaffl 2001).

Statistical analysis

Data analysis using MANOVA, followed by two-tailed student’s *t*-tests (where applicable) were conducted to determine the significance of diet and treatment on various physiological parameters. Multiple regression analysis was performed to determine the effect of the interactions between time, diet and/or treatment on blood glucose and body weight. Data is represented as the mean ± SEM, unless otherwise stated, *p* ≤ 0.05.

Results

The cannabinoid quantification revealed the ratio of the quantified THC:CBN: CBD was 1.0:1.2:0.4 as shown in a typical HPLC chromatogram (Fig. 1). CBN was the predominant cannabinoid quantified in the extract. The average weight difference prior to cannabis treatment was 26.8 g between lean and obese rats (*p* < 0.01) (Table 1). Cannabis extract exposure significantly increased food consumption in the LNE rats to 50.03 ± 0.54 g per day, relative to the respective control rats (45.37 ± 0.12 g). Food consumption in the obese group was significantly higher compared to the lean group (*p* < 0.0001), however, the effect of cannabis exposure significantly reduced food intake (57.42 ± 0.44 g) relative to the respective OBC rats (64.61 ± 1.18 g). Cannabis exposure induced a reduction in weight gain over time, in both LNE (5.8%) and OBE (3.8%) rats, relative to the respective LNC (12.4%) and OBC (14.3%) rats (*p* < 0.001).

Based on the experimental data (Fig. 2), a validated predictive polynomial model for body weight was generated, within a 95% confidence interval, taking into account the effect of time, treatment, diet and their interaction:

$$\text{Predicted body weight}(\hat{Y}) = \beta_0 + \beta_1 \text{Time} + \beta_2 \text{Time}^2 + \beta_3 \text{Treatment} + \beta_4 \text{Diet} + \beta_5 \text{Time} \cdot \text{Treatment} (r^2 = 0.31),$$

where $\beta_0 = 400.09$ (*p* < 0.0001); $\beta_1 = -6.71$ (*p* = 0.42); $\beta_2 = 8.21$ (*p* = 0.001) and $\beta_3 = -7.16$ (*p* = 0.40); $\beta_4 = 31.91$ (*p* < 0.0001); $\beta_5 = -11.63$ (*p* < 0.01).

Table 1
Effect of diet and cannabis extract on body weight of lean and obese Wistar rats.

Groups	Body weight (g)		Food intake per day (g)	Weight gain (%)
	Pre	Post		
LNC	395.85 ± 10.01	445.08 ± 14.93 ^f	45.37 ± 0.12	12.44%
LNE	401.12 ± 9.18	424.51 ± 8.63 ^f	50.03 ± 0.54 ^e	5.83% ^b
OBC	427.05 ± 9.21 ^a	488.24 ± 11.68 ^{ac}	64.61 ± 1.18 ^e	14.33%
OBE	423.90 ± 10.62	439.83 ± 9.21 ^{bc}	57.42 ± 0.44 ^e	3.76% ^c

Body weight: ^a*p* < 0.05, pre-LNC vs pre-OBC; post-LNC vs post-OBC; ^b*p* < 0.01, post-OBC vs post-OBE; ^c*p* < 0.001, pre-LNC vs post-LNC; pre-LNE vs post-LNE; pre-OBC vs post-OBC; pre-OBE vs post-OBE.
Food intake: ^e*p* < 0.001, LNC vs LNE; LNC vs OBC; LNC vs OBE; OBC vs OBE.
Weight gain: ^b*p* < 0.01, LNC vs LNE; ^c*p* < 0.001, LNC vs OBE; OBC vs OBE.

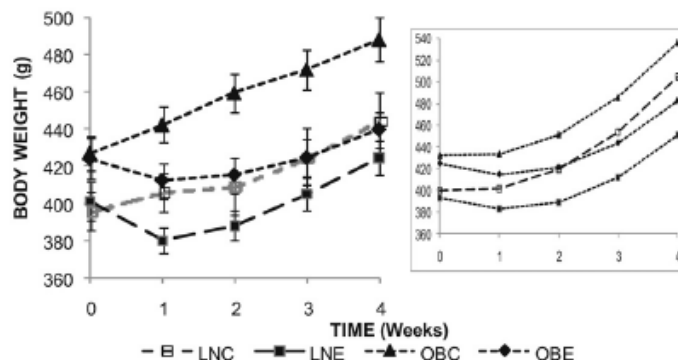


Fig. 2. Effect of diet and cannabis treatment on observed and predicted body weight (insert) of lean and obese Wistar rats over a 4-week period.

Regression analysis shows that body weight increased by an average of 8.21 g over 28 days ($p < 0.0001$), irrespective of diet or treatment, while the cafeteria diet induced an average weight difference of 31.9 g ($p < 0.0001$) between lean and obese rats. For the same period, cannabis exposure on its own did not have a significant influence on body weight, contributing an average decrease in body weight of 7.16 g ($p = 0.40$), while the combined effect of time and cannabis exposure significantly reduced body weight by an average of 11.63 g ($p < 0.01$) over the experimental period. The predictive body weights are graphically depicted in Fig. 2.

The changes observed in body weight were confirmed by the overall changes in body composition as depicted in Fig. 3. A significant reduction in epididymal fat mass was found in rats exposed to cannabis extract treatment, with LNE rats showing a significant reduction in the average epididymal fat mass (5.58 ± 0.24 g), relative to LNC rats (6.72 ± 0.45 g) ($p < 0.05$). Similarly, OBE rats showed a significant reduction (8.86 ± 0.49 g) compared to OBC rats (10.78 ± 0.65 g) ($p < 0.05$). Skeletal muscle in the obese rats were significantly higher than the lean rats ($p < 0.01$), with significance increasing due to weight loss associated with rats that received cannabis treatment (Fig. 3). Cannabis treatment significantly increased the weights of pancreata in OBE rats (1.94 ± 0.10 g), relative to OBC rats (1.62 ± 0.09 g) ($p < 0.05$), with LNC and OBC pancreata showing no significant differences. The hepatic somatic index (HSI), i.e. the liver:body weight ratio, of obese rats was significantly lower ($3.26 \pm 0.05\%$) relative to the lean rats ($3.50 \pm 0.10\%$) ($p < 0.05$). Cannabis treatment significantly

increased HSI in OBE rats ($3.39 \pm 0.06\%$), relative to the OBC rats ($p < 0.01$), while LNE rats maintained levels similar to the LNC rats.

Plasma insulin and glucose levels did not differ significantly for all experimental groups, irrespective of diet and treatment (Table 2). The cafeteria diet, surprisingly, induced a reduction in the blood glucose and insulin levels in obese rats, with the average blood glucose levels in obese rats (3.84 ± 0.18 mmol/l), relative to lean rats (5.48 ± 0.28 mmol/l) ($p < 0.001$).

Based on the experimental data over 28 days (Fig. 4), a validated predictive polynomial model was generated, within a 95% confidence interval, taking into account the effect of time, treatment, diet and their interaction (Fig. 4):

$$\text{Predicted blood glucose } (\hat{Y}) = \beta_0 + \beta_1 \text{Diet} + \beta_2 \text{Treatment} + (\beta_3 + \beta_4) \text{Time} + \beta_5 \text{Time}^2 \quad (r^2 = 0.36),$$

where $\beta_0 = 6.36$ ($p < 0.0001$); $\beta_1 = -1.05$ ($p = 0.002$); $\beta_2 = -0.15$ ($p = 0.45$); $\beta_3 = -1.51$ ($p < 0.0001$); $\beta_4 = 0.36$ ($p = 0.05$) and $\beta_5 = 0.52$ ($p < 0.0001$).

Regression analysis shows that the cafeteria diet resulted in an average 1.05 mmol/l ($p < 0.01$) reduction in blood glucose levels, while cannabis exposure did not significantly affect blood glucose levels, inducing an average decrease of 0.15 mmol/l, over the experimental period. The combined effect of time and diet contributed an average increase of 0.36 mmol/l ($p = 0.05$) in blood glucose levels, while an average increase of 0.52 mmol/l ($p < 0.001$) over time was observed, irrespective of diet.

Similar to the average blood glucose being higher in the lean rats, the average IPGTT in lean rats was significantly higher AUC_g (736.86 ± 14.73) compared to obese rats (533.64 ± 13.01) ($p < 0.001$). Cannabis treatment significantly reduced the AUC_g in the LNE rats (707.45 ± 21.87), relative to LNC rats (769.20 ± 14.36), while the OBE rats (534.75 ± 20.83) remained relatively unchanged, relative to the OBC rats (524.31 ± 13.22). A notable observation was that 27% of the LNE rats showed no change in blood glucose levels subsequent to glucose bolus administration, compared to 10% of the LNC rats. A similar trend was observed in the obese group, where 29% of the OBE rats showed no change in blood glucose levels, compared to 14% of the OBC rats.

Trends in the plasma cytokine profile (Table 3) show IL-1 α , IL-6 and IFN- γ were detectable and IL-1 β and TNF- α levels not (data not shown). In LNE rats cannabis treatment induced increases in IL-1 α (1.1-fold), and IL-6 (1.6-fold) while IFN- γ levels remained unchanged, relative to LNC rats. Diet induced a reduction in IL-1 α (0.8-fold), IL-6 (0.2-fold) and IFN- γ (0.9-fold) levels in OBC rats, relative to LNC rats. Cannabis treatment resulted in an increase in

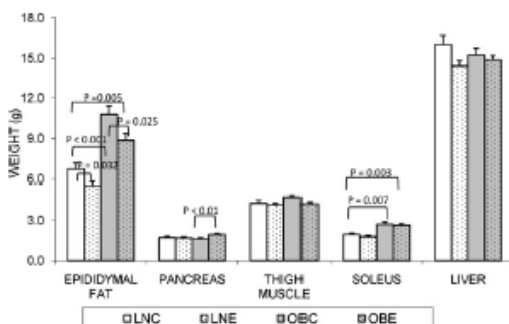


Fig. 3. Effect of cannabis extract and diet on organ mass in lean and obese Wistar rats.

Table 2

A summary of the effect of cannabis extract on post-experimental blood glucose, insulin and area under the curve for glucose (AUC_G) associated with IPGTT in lean and obese Wistar rats.

Experimental group	Glucose (mmol/l)	Insulin (ng/ml)	AUC_G (IPGTT) (mmol/min/l)
LNC	5.67 ± 0.46	5.70 ± 1.83	769.20 ± 14.36
LNE	5.31 ± 0.35	2.55 ± 0.50	707.45 ± 21.87 ^a
OBC	3.60 ± 0.21 ^c	3.30 ± 0.68	524.31 ± 13.22 ^c
OBE	4.06 ± 0.28 ^b	2.84 ± 0.54	534.75 ± 20.83 ^c

^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, relative to LNC.

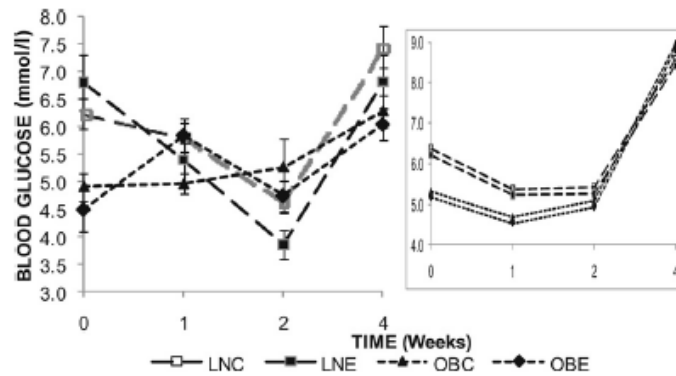


Fig. 4. Effect of diet and cannabis treatment on observed and predicted blood glucose levels (insert) in lean and obese Wistar rats over a 4-week period.

IL-6 (6.4-fold) and IFN- γ (1.2-fold) levels, relative to OBC rats, while IL-1 α levels remained unchanged.

Changes in gene expression in pancreatic tissue, relative to LNC rats, is summarized in Fig. 5, where OBC expression of c-MYC, FLIP and UCP2 was significantly down-regulated: 0.01- ($p < 0.05$), 0.18- ($p < 0.05$) and 0.14-fold ($p < 0.01$), respectively, OBE pancreatic tissue, relative to LNC rats, showed that diet and cannabis exposure induced a 0.03-fold ($p < 0.05$) reduction in c-MYC gene expression. No significant changes in expression of the genes of interest were found between the lean groups (data not shown).

Diet and cannabis induced up-regulation of CB1 (2.92-fold, $p < 0.01$), glucose transporter 2 (GLUT2) (2.77-fold, $p < 0.001$), UCP2 (3.2-fold, $p < 0.001$) and PKB (2.6-fold, $p < 0.05$) gene expression in the OBE rats, relative to OBC rats (Fig. 6).

Discussion

Since the cloning of the two cannabinoid receptors in the early 1990s, and the subsequent isolation of anandamide (AEA) in 2002, increasing evidence is implicating peripheral and central endocannabinoid pathways in the regulation of feeding behaviour, body weight and glucose metabolism (Bermúdez-Silva et al. 2009; DiPatrizio et al. 2011; Juan-Picó et al. 2006). In this study, cannabis exposure showed a varied effect on the feeding behaviour of lean

and obese rats. Food consumption in the OBE rats was 15% higher than in LNE rats ($p < 0.001$), which supports the findings of Koch (2001) where a higher volume of high-fat and sweetened high-fat diet was consumed, relative to chow-fed rats. The lag in weight gain observed in the LNE and OBE rats, support the findings that the endocannabinoid system mediates feeding behavior and energy balance. Research findings suggest that THC exerts a biphasic dose response, with appetite stimulation at low doses and inhibition at high doses (Cota et al. 2003). Other factors which have been proposed to contribute to the biphasic effect of THC on food intake include the differential involvement of the G_s and G_i proteins, which are activated at high and low doses, respectively, activation of presynaptic cannabinoid receptors by low doses of cannabinoid, and storage of unused cannabinoids in fat deposits (Sulcova et al. 1998). In a study conducted by Kreuz and Axelrod (1973), fat tissue, relative to brain tissue, contained 21-fold higher levels of THC and its metabolites after seven days of consecutive exposure, and 64-fold higher levels after 27 days. It is therefore proposed that the retention of THC and its metabolites in the fat deposits partially contributed to the different responses in food consumption of the LNE and OBE rats. The LNE rats, with less fat tissue, consumed significantly more food, relative to the LNC rats, while the OBE rats, with significantly more fat tissue, consumed significantly less food, thus confirming previous observations of the

Table 3

Plasma cytokine profile of obese and lean rats treated with cannabis extract.

Groups	Cytokines		
	IL-1 α (pg/ml)	IL-6 (pg/ml)	IFN- γ (pg/ml)
LNC	90.03 ± 5.15 (3) ^a	95.66 ± 27.42 (5) ^a	65.73 ± 5.58 (4) ^a
LNE	95.73 ± 16.65 (3) ^a	152.88 ± 77.56 (4) ^a	65.12 ± 16.50 (5) ^a
OBC	67.96 ± 15.74 (5) ^a	19.00 ± 3.40 (2) ^a	56.53 ± 6.08 (3) ^a
OBE	69.00 ± 1.00 (2) ^a	121.46 ± 46.87 (5) ^a	70.23 ± 17.15 (4) ^a

^a Numbers in parentheses reflect the number of rats evaluated.

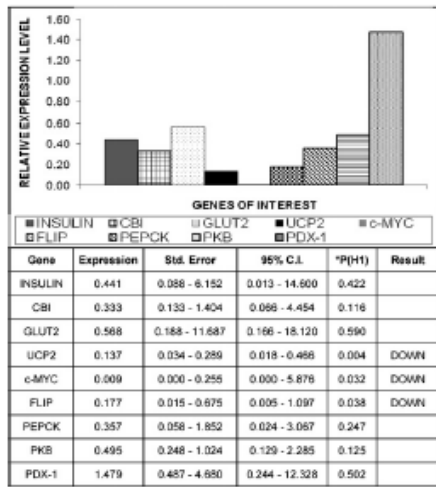


Fig. 5. Effect of diet and cannabis extract exposure on various pancreatic genes of interest in obese control rats relative to lean control rats, using tubulin and cyclophilin A as reference genes.

biphasic effect of THC. In addition, in an obese state, leptin, an adipocyte-derived hormone, induces a reduction in food intake, and increased energy expenditure. Acute treatment of normal and *ob/ob* (leptin-deficient) rats with leptin results in a reduction in anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in the hypothalamus, while the *ob/ob* obese animals were found to have

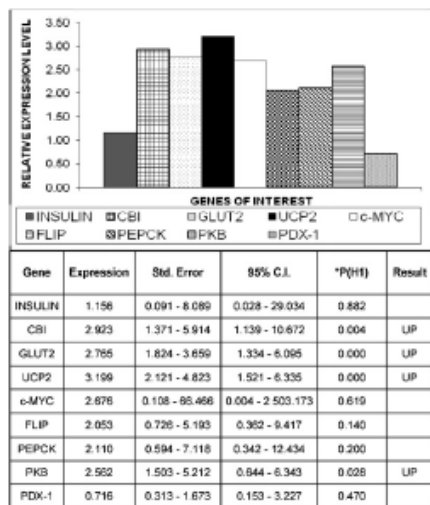


Fig. 6. Effect of diet and cannabis extract exposure on various pancreatic genes of interest in obese experimental rats relative to obese control rats, using tubulin and cyclophilin A as reference genes.

increased levels of AEA and 2-AG, therefore possibly contributing to overeating in the development of obesity (Di Marzo et al. 2000). Currently, not much evidence is available which investigates the role of phytocannabinoids other than THC or synergy of the phytocannabinoids to overeating and energy regulation other than THC.

DIO reduce insulin-stimulated glucose utilization, while insulin-stimulated adipogenesis remains unchanged or enhanced, thus increasing the risk of cardiovascular disease and diabetes. Coetzee et al. (2007) found that cannabis treatment in diet-induced obese rats prolonged clotting time, and reduced the atherogenic index, relative to control rats. Megulies and Hammer (1991) showed that intravenous THC administration exerts a dose-dependent dual effect on glucose uptake, stimulating uptake at low THC levels (0.2 mg/kg body weight), and inhibiting uptake at high THC levels (5–10 mg/kg body weight). In the current study, although not significant, this duality was evident with LNE rats showing reduced glucose levels while the OBE rats showed an increase, relative to their respective control groups. *In vivo* experiments conducted on rats found that acute administration of CB1 agonists impairs glucose uptake, thus retarding plasma glucose clearance, while CB2 agonists elicit an opposite effect (Di Marzo 2008). The OBE rats showed blood glucose levels similar to the LNC rats, and higher AUC_g values (not significant), relative to OBC rats. Surprisingly, the lean rats that were fed on normal chow showed significantly higher blood glucose levels than the obese rats fed on cafeteria diet, with LNE (27%) and OBE (29%) rats showing impaired glucose-stimulated insulin secretion. In addition, LNC and LNE rats were found to have 1.47- and 1.44-fold higher AUC_g, relative to OBC rats, while LNE reduced the AUC_g by 8%, relative to LNC rats, thus improving glucose sensitivity in the LNE rats. Despite improving AUC_g, there were no significant differences in plasma insulin levels, where the insulin gene expression remained relatively unchanged, showing a 0.2-fold reduction in LNE rats and a 1.2-fold increase in OBE rats, relative to their respective controls. Iwasaki et al. (2009) showed increased UCP2 expression impaired GSIS through impaired glucose sensing and insulin secretion in murine β-cells, both *in vivo* and *in vitro*. Pi et al. (2009) observed increased oxidative stress and impaired insulin secretion in UCP2 knockout mice, while Product-Zengaffinen et al. (2007) found that increased UCP2 expression in β-cells did not alter insulin secretion, but enhanced the cellular defense by reducing ROS production. Elevated UCP2 levels possibly contributed to the impaired glucose sensing in LNC rats, while OBC levels were significantly reduced to 0.137-fold, relative to LNC rats, while OBE rats showed a 3.2-fold increase in UCP2 expression, relative to OBC rats, possibly contributed to the impaired glucose sensing observed in LNC rats. A 2.8-fold increase in GLUT2 expression in OBE rats, did not translate into an improved IPGTT, relative to OBC rats, however, the glycemic control was significantly better than that of LNC rats.

Standard chow is composed of agricultural by-products, a protein source, vegetable oil, supplemented with minerals and vitamins, high phytoestrogens, and contains a high proportion of fibre (Warden and Fisler, 2008). Research has shown that high levels of phytoestrogen result in stress-induced corticosteroid levels (Hartley et al. 2003). The elevated blood glucose levels and higher AUC_g values associated with the lean group, relative to the obese group, may therefore be due to the effects of the phytoestrogen content in the standard chow.

Obesity has been associated with a chronic state of low-grade inflammation, with pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 being induced by adipocytes and hepatocytes in severely obese mice (Chida et al. 2006). IL-6 increases energy metabolism in rats (Wallenius et al. 2002) and plays a role in lipolysis and β-oxidation in fat tissue of humans. In the present study the cafeteria diet induced a reduction in IL-1, IL-6 and IFN-γ levels in OBC rats,

relative to LNC rats, while cannabis treatment induced increased IL-6 expression in both LNE and OBE, relative to the LNC rats. This increase in IL-6 levels may have contributed to the impaired weight gain observed in the LNE and OBE rats, possibly via increased energy metabolism, as previously observed by Wallenius et al. (2002).

Environmental stimuli affect the degree of gene expression by either inhibitory or stimulatory signals. None of the genes of interest were significantly changed in the LNE rats, relative to LNC rats. Down-regulation of c-MYC in the obese rats, relative to LNC rats, may have contributed to the improved glycemic control by the OBC and OBE rats (Table 2). In a normal adult pancreas, c-MYC levels are low, while hyperglycemia induced increased c-MYC expression in rat pancreatic β cells (Jonas et al. 2001). In diabetics, over-expression of c-MYC induces reduced glucose-stimulated insulin secretion, suppresses insulin gene transcription, induces β -cell apoptosis and loss of differentiation in rodent islets (Kaneto et al. 2002; Pelengaris et al., 2000). Maedler et al. (2002) found that increased FLIP expression level is necessary to switch the Fas signaling, a major regulator of cell death in β -cells, to promote β -cell proliferation. Decreased FLIP expression led to Fas activation, which promoted apoptosis, leading to β -cell loss. The cafeteria diet induced a decrease in FLIP expression in OBC rats, relative to LNC rats, contributing to the reduction in the plasma insulin levels in OBC rats (not significant), while cannabis treatment resulted in a 2.1-fold increase in FLIP expression, relative to OBC rats. *In vitro* studies on isolated rat pancreatic islets conducted in this laboratory (data not shown) found a 6.9-fold increase in Ki-67-positive β -cells in islets cultured under hyperglycemic conditions and exposed cannabis extract containing an equivalent of 2.5 ng/ml THC, relative to islets cultured under normoglycemic conditions. Diet and cannabis treatment resulted in upregulation of PKB in the OBE rats, relative to OBC rats to levels similar to LNC rats (1.4-fold), which may have contributed to proliferation within the pancreatic tissue. Gómez del Pulgar et al. (2000) have shown that THC induced activation of PKB via CB1 receptors, not CB2 receptors, and stimulates glycolysis and anti-apoptotic cellular activities. DIO resulted in a reduction in CB1 expression to 0.3-fold in OBC rats, relative to LNC rats, while exposure to cannabis extract resulted in upregulation of CB1 expression in OBE (2.9-fold), relative to OBC rats. OBE expression levels were similar to those found in LNC rats (1.0-fold). The combined effects of diet and cannabis exposure on c-MYC, FLIP and PKB expression may collectively have contributed to anti-apoptotic activities in the rat pancreatic tissue investigated.

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The literature on the energy regulation effect of THC often presents varied and conflicting results, with less focus on other phytocannabinoids in this area. Recent findings have indicated that CBD slows down β -cell damage in type 1 diabetes (Di Marzo et al. 2011). The predominant cannabinoid found in the cannabis extract for this study was CBN. It is not clear if CBN plays a significant role in β -cell protection, as has been shown with CBD, this requires further studies. When comparing the chemical structure of THC and CBN, it is noted that CBN has a more rigid conformation than THC, which may be responsible for the subtle differences in the biological effects elicited. This is noted when comparing THC and CBN binding affinities for CB1 and CB2 receptors, where studies have shown equivalent binding affinity (Munro et al. 1993), variable affinity (Rhee et al. 1997) and also highlighting variation in interspecies binding (McPartland et al. 2007). The findings of our study therefore warrant further investigation into the effect of the individual cannabinoids (e.g. CBD, CBN and THC) and their synergistic interactions on energy regulation.

The findings of this research study are not to promote or condone the casual use of cannabis, but to convey the findings of the research to the scientific community to possibly stimulate further research in understanding the effect of phytocannabinoids

on energy metabolism in relation to the endocannabinoid system.

Conclusion

Cannabis treatment reduced the deleterious effects of DIO by reducing weight gain, specifically fat depots, maintaining insulin levels, altering cytokine and gene expression levels that induce increased energy expenditure, while protecting pancreatic tissue from apoptosis by promoting proliferation.

Acknowledgements

We would like to acknowledge the Nelson Mandela Metropolitan University and National Research Foundation for the financial support provided for this project, Coos Bothma for his statistical modeling, and Kathrin Maedler and Gareth Siegfried-Kellerberger for their technical assistance.

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