

THE EFFECTS OF A WATERY EXTRACT OF *AGATHOSMA* ON THE DEVELOPMENT OF HYPERTENSION

by
Jurie Wynand Lombard

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Supervisor: Prof Barbara Huisamen

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date:

ABSTRACT

Introduction: Obesity is a major risk factor for non-communicable diseases (NCD), a collective term given to type 2 diabetes (T2D), hypertension (HTN) and cardiovascular disease (CVD). Globally, NCDs are the leading cause of death. In recognition of this, untested remedies are flooding the market. Buchu water is one of these herbal remedies advertised as having antihypertensive properties. This water is a by-product of the extraction of the oil from the leaves of *Agathosma*. Buchu water is already freely available to the public.

Aim: The aims of the present study were to test the effect of ingestion of Buchu water on i) the blood pressure (BP) of diet induced hypertensive as well as normotensive rats when given as pre-treatment; ii) HTN in rats when used as treatment; iii) endothelial function using aortic rings from control and hypertensive rats; iv) the expression and/or activation of signalling proteins involved in NO production in the endothelium (PKB/Akt, AMPK, eNOS); v) insulin sensitizing effects in cardiomyocytes prepared from insulin resistant rats to determine possible cardiovascular effects.

Methodology: We utilized rat models of obesity induced HTN and diet-induced obesity (DIO). Male Wistar rats were rendered i) hypertensive by feeding a high fat diet (HFD) and ii) insulin resistant after being placed on a high caloric diet for 16 weeks. Half the animals were placed on Buchu water treatment (26% (v/v) Buchu water) for a period of 16 weeks. Blood pressure, urine output, water and food consumption were measured. The animals on the HFD were sacrificed and intraperitoneal fat weight determined, aortas used in aortic ring studies and blood collected for biochemical analyses. Animals in the DIO model were sacrificed after 16 weeks and cardiomyocytes prepared to determine insulin sensitivity.

Results: The HFD caused increases in body weight, visceral adiposity and blood pressure. Chronic Buchu water treatment i) reduced body weight gain and visceral adiposity in the HFD animals; ii) affected leptin levels and components of the renin-angiotensin-aldosterone system (RAAS); iii) it both prevented the development of HTN when given in conjunction with the HFD and restored BP when used as treatment while having no effect on the BP of the control animals; iv) it had no effect on endothelial function nor did it activate any signalling proteins involved in the synthesis

of NO in the endothelium that may be responsible for vasodilation in the arteries; v) it elevated basal myocardial glucose uptake which can be cardioprotective.

Conclusion: Our present study was novel and demonstrated that Buchu water treatment exhibits similar properties to that of antihypertensive medication known as angiotensin-converting enzyme inhibitors. It acts on the whole body RAAS in a similar manner reducing both BP and weight gain. It also reduced metabolic aberrations (HTN, leptin, aldosterone) seen in animals on a HFD, partly through visceral fat remodelling. In addition Buchu water also elicits some cardioprotective properties. Thus we conclude that Buchu water is an alternative, cost effective and ready available means to treat HTN and is beneficial to one's health.

Key words: Obesity, hypertension, traditional medicine, antihypertensive, Buchu.

OPSOMMING

Inleiding: Vetsug is een van die hoof risikofaktore vir nie-oordraagbare siektes (NCD), 'n kollektiewe term vir tipe 2-diabetes (T2D), hoë bloeddruk (HTN) en kardiovaskulêre siektes (CVD). Wêreldwyd neem NCD toe en is die hooforsaak van sterftes. Na aanleiding hiervan, is daar tans 'n oorstroming van natuurlike produkte wat geadverteer word as antihipertensiewe middels maar met geen wetenskaplike bewyse hiervan nie. Boegoewater is een van hierdie kruiemiddels wat bestaan uit die waterige byproduk van ekstraksie van boegoe-olie uit die blare van *Agathosma*. Boegoewater is reeds vrylik beskikbaar aan die publiek.

Doelstellings: Die doelwitte van hierdie studie was om die effek van Boegoewater op i) die bloeddruk van hipertensiewe en kontrole rotte, beide voorkomend en as ii) behandeling; iii) endoteelfunksie van aortaringe; iv) die uitdrukking van proteiene betrokke by NO seintransduksie (PKB/Akt, AMPK, eNOS); v) insulien sensitisering van kardiomyosiete, te toets.

Metodes: Ons het gebruik gemaak van twee rot modelle; i) wat vetsug veroorsaak met gepaardgaande hoë bloeddruk en 'n ander wat gepaardgaan met ii) dieet-geïnduseerde vetsugtig (DIO) met insulienweerstandigheid. Manlike Wistar rotte was geklassifiseer as i) hipertensief na inname 'n hoë vet dieet (HFD) en as ii) insulien weerstandig na inname van 'n hoë kalorie dieet (DIO) vir 16 weke. Die helfte van die diere is met Boegoewater behandel (26% (v / v) Boegoewater) vir 'n tydperk van 16 weke. Gedurende die 16 weke van behandeling is die bloeddruk, urine, water- en voedselverbruik gemeet. Die diere op die HFD is na 16 weke opgeoffer en die liggaamsgewig en intraperitoneale vet gewig bepaal, die aorta ringe is geïsoleer vir kontraksie/ontspannings studies en die bloed gekollekteer vir biochemiese analises. Die diere in die DIO model is ook na 16 weke geslag en kardiomyosiete is voorberei om die insulien sensitiwiteit te bepaal.

Resultate: Die HFD het 'n toename in liggaamsgewig, viserale vet en bloeddruk teweeggebring. Kroniese Boegoewater behandeling het i) die liggaamsgewigtoename en viserale vet verlaag in die HFD diere; ii) leptienvlakke en komponente van die RAAS verlaag; iii) die ontwikkeling van hoë bloeddruk voorkom asook alreeds verhoogde bloeddruk effektief verlaag; iv) geen effek op endoteelfunksie of die uitdrukking van

proteïene geassoseerd met NO produksie, getoon nie; v) basale glukose opname van kardiomiosiete verbeter.

Gevolgtrekkings: Ons huidige studie, die eerste van sy soort, het getoon dat Boegoewater behandeling die aanvang van HTN kan voorkom, asook hipertensie kan behandel .

Die studie het aangetoon dat Boegoewater soortgelyke eienskappe as die angiotensien-omskakelingsensiem (ACE) inhibeerders besit aangesien dit komponente van die RAAS beïnvloed het, gewigstoename beperk het en bloeddruk verlaag het. Metaboliese veranderinge soos leptienverlaging word aan hermodellering van die vetdepots toegeskryf. Verder lei Boegoewater behandeling tot miokardiale beskerming.

Dit het ook metaboliese afwykings (soos HTN en glukose onverdraagsaamheid) in individue op 'n HFD verminder deur deels viserale vet hermodellering. Bykomend aan al die eienskappe het Boegoewater beskerming ontlok teen iskemie deur verbeterde glukose opname tydens basale vlakke in kardiomiosiete. Ons het daarin geslaag om te bewys dat Boegoewater 'n alternatiewe, koste-effektiewe en beskikbare middel is om HTN meer te behandel en dat dit voordelig is vir jou gesondheid.

Sleutelwoorde: Vetsug, hipertensie, tradisionele medisyne, bloeddruk verlagende middels, Boegoewater.

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"I can do everything through Him who give me strength." (Philippians 4: 13)

DISCLOSURE OF INTEREST

We hereby declare, as per contractual agreement between the University of Stellenbosch and Cape Kingdom Nutraceuticals (Pty) Ltd, (Contract number: S003211) the company licenced to distribute Buchu water, that there was no personal financial gain for the researchers in this work. The researchers only retained the right to publish these findings in peer reviewed scientific journals of their choice.

Signed on the day of 2014
at.....

.....

(Prof. B. Huisamen)

.....

(Mr J.W. Lombard)

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LIST OF ABBREVIATIONS

Units of measurement

- %: percentage
- AU: arbitrary units
- cm: centimeter
- g: gram
- Hg: mercury
- IU: International unit
- kg: kilogram
- kJ: kilojoules
- L: litre
- M: molar
- mg: milligrams
- min: minute
- ml: millilitre
- mm: millimeter
- mM: millimolar
- mmol: millimol
- nm: nanometer
- nM: nanomolar
- °C: degree celcius
- v: volume
- μ : micro
- μ L: microlitre
- μ m: micrometer

Chemical components (biological components)

- ACh: Acetylcholine
- AMP: Adenosine monophosphate
- AMPK: AMP-activated protein kinase

- Ang I or II: Angiotensin I or II
- Ca: Calcium
- Cl: Chlorine
- CO₂: Carbon dioxide
- eNOS: Endothelial nitric oxide synthase
- FFA: Free fatty acids
- H: Hydrogen
- H₂O: Water
- HCl: Hydrochloric Acid
- IL-6: Interleukin-6
- K: Potassium
- Na: Sodium
- NO: Nitric oxide
- O₂⁻: Super oxide free radicals
- OONO⁻: Peroxynitrite
- PE: Phenylephrine
- PKB/Akt: Protein kinase B
- ROS: Reactive oxygen species
- SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
- Ser: Serine
- SOD: Superoxide dismutase
- Thr: Threonine
- TMB: 3, 3',5',5'-tetramethylbenzidine
- TNF-alpha: Tumor necrosis factor alpha

Others

- ACE: Angiotensin-converting enzyme
- ACEIs: ACE inhibitors
- ACTH: Adrenocorticotrophic hormone
- ALD: Aldosterone
- ANOVA: analysis of variance

- ANP: Atrial natriuretic peptide
- ARB: Angiotensin receptor blocker
- AT₁: Angiotensin type 1 receptor
- AT₂: Angiotensin type 2 receptor
- BBs: Beta-blockers
- BMI: Body mass index
- BP: Blood pressure
- BW: Body weight
- CCB: Calcium channel blockers
- CHD: Coronary heart disease
- CVD: Cardiovascular disease
- DASH: Dietary Approaches to Stop Hypertension
- DBP: Diastolic blood pressure
- DIO: Diet induced obesity
- e.g.: for example
- ESRD: End stage-renal dysfunction
- GLUT: Glucose transporter
- GP: General practitioner (doctor)
- HFD: High fat diet
- HTN: Hypertension
- IP: Intraperitoneal
- IPGTTs: Intraperitoneal glucose tolerance tests
- JG: Juxtaglomerular
- JNC: Joined National Committee on the Prevention, Detection, Evaluation and treatment of High Blood Pressure
- LVH: Left ventricular hypertrophy
- NCD: Non-communicable disease
- PRA: Plasma renin activity
- r.p.m.: revolutions per minute
- RAAS: Renin-angiotensin-aldosterone system
- RIA: Radioimmunoassay
- SADHS: South African Democratic Health Survey

- SAT: Subcutaneous adipose tissue
- SBP: Systolic blood pressure
- SEM: Standard error of the mean
- SNS: Sympathetic nervous system
- T2D: Type 2 diabetes
- TG: Triglyceride
- v/v: Volume to volume
- VAT: Visceral adipose tissue
- w/v: Weight to volume
- WHO: World Health Organization
- WHR: Waist-to-hip ratio

CHAPTER 1: Literature Review

1.1 Obesity

Obesity is a major risk factor for non-communicable diseases (NCD), a collective term given to type 2 diabetes (T2D), hypertension (HTN) and cardiovascular disease (CVD). Non-communicable diseases are currently a global public health concern and the leading cause of death worldwide. The number of deaths caused by NCD each year is currently far greater than all the other diseases combined (Kelly, Narula, & Fuster, 2012; Murray et al., 2012). In 2008 it was estimated that 35% of adults aged 25 years and older were overweight (BMI > 25kg/m²) and 12% of the world's population was obese (BMI ≥ 30 kg/m²) (Stevens et al., 2012). Thus more than 1 billion adults are overweight and millions are obese (Deitel, 2003). Obesity is therefore not just a disease of developed nations, but also from developing countries. Obesity levels in certain lower-income and transitional countries are as high as or higher than those reported for the United States and other developed countries. Moreover, these levels are shown to be constantly on the increase. In the US alone it is estimated that one third of the population over the age of 20 years is obese (27.3% of men and 33.2% of women) (Hedley et al., 2004). Even in South Africa we have not been spared the widespread increase of obesity. The first South African Democratic Health Survey (SADHS), undertaken in 1998 and published in 2002 reported that the prevalence of overweight (Body Mass Index (BMI) > 25) and obesity (BMI > 30) in South Africa was more than 29% for men and 56% for women (T. Puoane et al., 2002). It is clear that obesity is currently one of the biggest global health problems and that the number of obese or overweight individuals continue to rise worldwide (Seidell, 2000). A severe disease such as obesity, which is characterized by a pathological increase in body weight, can result in HTN, hyperinsulinaemia, insulin resistance, diabetes and dyslipidaemia, all of which are factors independently associated with an increased risk of CVD (Brands et al., 1995; Hall, 1993; Hall, 2000; Hall, Hildebrandt, & Kuo, 2001; Hall, 2003).

Both HTN and obesity have therefore been identified as major risk factors for the development of vascular dysfunction and cardiac complications which are associated with an increase in mortality worldwide (Hubert et al., 2012; S. S. Lim et al., 2012; Murray et al., 2012).

1.1.1 Concept of Obesity

Obesity refers to the excess storage of energy in the form of fat cells, also known as adipose tissue (Mikhail, Golub, & Tuck, 1999). When energy intake exceeds expenditure, fat cells enlarge or increase in numbers thereby increasing the storage capacity (Simonds & Cowley, 2013). There should therefore be a tight regulation of caloric intake and energy expenditure to maintain a balanced energy homeostasis.

Although obesity is linked to mortality, it is not the accumulation of excess body fat that is the direct cause of death but rather an increase of a variety of associated diseases that can cause death and disability. One of the serious comorbid diseases of obesity is HTN or elevated blood pressure (BP) (Simonds & Cowley, 2013). It seems clear that obesity causes HTN rather than the reverse, because losing weight lowers BP, whereas lowering BP does not correct obesity (Goodfriend, 2008). Over eating contributes to both obesity and HTN since the sodium raises BP and the calories increase weight (Morse, Zhang, Thakur, & Reisin, 2005).

The presence of HTN increases the risk for the development of CVDs including heart disease and renal failure (Simonds & Cowley, 2013).

There are many sophisticated anthropometric techniques to measure and assess the degree of Obesity. The three most common methods are i) the Body Mass Index (BMI) also referred to as the Quetelet's index, ii) the waist-to-hip ratio (WHR) and iii) waist circumference. All three techniques calculate the % body fat (Dalton et al., 2003). They are all measured by using anthropometric tools.

1.1.2 Definitions of obesity:

1.1.2.1 Body mass index (BMI)

The BMI technique relates weight to height and is the most widely used technique to calculate the degree of obesity. BMI gives a good estimate of the prevalence of obesity within a population. It is calculated by dividing an individual's body weight (kg) by their height (m) squared (weight (kg)/height (m²)). Below in Table 1-1 it illustrates all the various ranges of BMI and how they are classified into different categories.

Table 1-1: BMI Classification.

BMI range	Category
less than 16.5	Severely underweight
from 16.5 to 18.5	underweight
from 18.5 to 25	normal
from 25 to 30	overweight
from 30 to 35	obese
from 35 to 40	clinically obese
above 40	dangerously obese

It is known that a high BMI has consistently been associated with various health complications as well as an increased risk of developing CVD, HTN, dyslipidaemia, insulin resistance or T2D (Field et al., 2001). Many health problems in the world focus on the high prevalence of obesity and high BMI, however it is easy to forget that being underweight also carries health risk, therefore some focus must also go towards having a too low BMI. There are many causes for being underweight, the condition clearly suggest that your body is not obtaining all the essential nutrients to function

properly. Having a BMI below 18.5 is associated with health risks such as bone loss, decreased immune function, cardiac problems (iron-deficiency) and infertility.

A BMI value that exceeds 25 is classified as overweight. Whereas obesity is defined as a BMI greater than 30 and morbidly obese if an individual's BMI exceeds 35 (Obesity: Preventing and Managing the Global Epidemic. Report of a WHO Consultation.2000; Deitel, 2003).

1.1.2.2 Waist-to-hip ratio (WHR)

A more precise method used to determine the severity of obesity is the WHR. This is the preferred method, since it gives a better indication of obesity compared to BMI. This is due to the fact that it is more directly proportional to the abdominal obesity and the amount of metabolic active visceral fat than the BMI. The BMI rather gives an overall indication of the severity of obesity. Distribution of weight gain is an important factor when looking at obesity. Abdominal obesity is clinically diagnosed by a WHR that is greater than 0.95 in men and greater than 0.85 in women (Obesity: Preventing and Managing the Global Epidemic. Report of a WHO Consultation.2000).

1.1.2.3 Waist Circumference

Another simple and practical anthropometric technique used to determine the body fat or the prevalence of obesity, is the waist circumference (Dalton et al., 2003). This method is widely used, but is subject to measurement error. Circumference cut-off points for adults with increased health risks are: men ≥ 94 cm and women ≥ 80 cm. Whereas men with a waist circumference ≥ 102 cm and women ≥ 88 cm have an even greater risk of health problems (Lean, Han, & Morrison, 1995).

1.2 Fat stores

Over the years, fat stores or adipose tissue have simply been seen as a way of storing additional energy to utilize later, if the need arises to keep the body powered or going. In recent years it has been discovered that this is not strictly true. In reality, adipose tissue plays a more complex role in a wide variety of interactions. Not only does it provide us with energy, but it is also involved in numerous hormonal, inflammatory and metabolic pathways (Seidell, 2000; Trayhurn & Wood, 2005).

Adipose tissue consists of adipocytes and their main function is to store energy in the form of triglyceride (TG). Adipocytes store lipids in one of two ways: firstly by synthesizing it from products of glycolysis and secondly via the uptake of free fatty acids (FFA). Lipolysis is the mobilization of lipids, a tightly controlled process which is regulated by a hormone called insulin. Insulin is secreted from the beta cells which are situated in the pancreas (Engeli et al., 2003). Under the tight regulation of insulin, the adipocytes take up FFAs from the blood and store them in the form of intracellular lipid droplets. Adipose tissue stores can be found subcutaneously (SAT) or as visceral depots (VAT), in organ capsules and dispersed in close proximity to blood vessels and lymph nodes (Engeli et al., 2003).

As an individual gains weight, there is an increase in adipose tissue mass. The increased adipose tissue mass results from increased adipocyte number (hyperplasia) and/or volume (hypertrophy) (Faust et al., 1978). Eventually the point is reached when the adipose tissue is unable to store surplus lipids in the form of TG. This prolonged period of nutrient overload causes the lipids to be deposited ectopically in non-adipose tissue such as the liver, pancreas and heart (Virtue & Vidal-Puig, 2010). This results in a toxic environment which can lead to insulin resistance and apoptosis in the cells (hepatocytes, beta cells, cardiomyocytes and skeletal muscle) where ectopic accumulation of lipids occurred. It is therefore essential for adipose tissue to be able to expand to maintain a healthy structure.

Insulin sensitivity is reduced as a result of the state of demand to store excess nutrients, which leads to adipose tissue to become dysfunctional and inflamed. The low grade inflammatory state in which the adipose tissue is found in obesity is

associated with the production of pro-inflammatory cytokines from the immune system such as macrophages. Pro-inflammatory cytokines impair insulin sensitivity throughout the body via activation of c-Jun NH₂-terminal kinase (JNK) and nuclear factor-kappa beta (NF κ B) signalling pathways (Hirosumi et al., 2002; Cai et al., 2005).

Large adipocytes also secrete macrophage-attracting chemokines which promote macrophage activation, recruitment and infiltration (Kim et al., 2007). The pro-inflammatory macrophages cause a decrease in the formation of new small healthy adipocytes by releasing cytokines, increasing the number of large insulin resistant adipocytes (Lacasa et al., 2007). This causes a vicious cycle where more chemokines and FFA is secreted, thus more macrophage activation leads to the formation of more large insulin resistant adipocytes. The balance between lipolysis and adipogenesis changes when one reduce insulin sensitivity and this leads to increased plasma FFA levels. This in turn, can increase the risk of developing T2D.

1.2.1 Fat distribution in Obesity (pear and apple shape)

To classify the pattern of weight gain is very important. The distributions of body fat in obese individuals give rise to many metabolic disturbances. Obesity can be classified as either abdominal obesity or lower body obesity. The association between “Android” (abdominal obesity or central obesity or upper body obesity) and CVD and diabetes rather than the more peripheral “gynaecoid” obesity (or lower body obesity with fat around the hips and buttocks) was first pointed out by Vague (1953). (Vague, 1953). Abdominal obesity has been shown by many studies to be more related to CVD and death than lower body obesity (Mikhail et al., 1999). The distribution of weight gain is different between males and females. Generally, men tend to deposit fat in the abdominal area giving rise to an “apple” shape, whereas women tend to deposit fat in gluteal and femoral regions resulting in a “pear” shape (Ashwell, Cole, & Dixon, 1985). “Pears” seem to have less intra-abdominal fat than “apples. Therefore men tend to be more at risk for CVD than women.

In obesity, fat accumulation in the abdomen or VAT are more tightly correlated with development of HTN than SAT or below the pelvic brim adipose tissue, so more focus

should be placed on abdominal fat in scenarios where obesity associated HTN is the issue (Sironi et al., 2004).

1.3 Hypertension

Hypertension (HTN) is a term used to describe high BP ('chronically elevated BP in the arteries'). As the heart pumps blood to circulate through the body it exerts a force against the walls of the arteries. This is defined as BP. Blood pressure readings consist of two numbers or values, one over the other. The top one indicates the systolic blood pressure (SBP) and the bottom one the diastolic blood pressure (DBP). One or both of these numbers can be elevated in the development of HTN.

Hypertension, which is a common condition worldwide, is diagnosed when systolic blood pressure (SBP) is constantly registered ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg and/or taking antihypertensive medication (Burt et al., 1995; Chobanian et al., 2003).

Hypertension can be classified as essential (or primary) HTN or secondary HTN (Bokarev & Matvienko, 2013). Primary HTN has no identifiable cause but is rather the consequences of the interaction between environmental and genetic factors throughout one's life. Conversely, secondary HTN can be recognized by having an identified cause. The pathological development of secondary HTN is often one of the following; thyroid dysfunction, aldosteronism, coarctation of the aorta and renal parenchymal disease (Viera & Neutze, 2010). Secondary HTN is a treatable or curable condition, because in most cases the HTN can be treated by e.g. undergoing surgery or be managed by medications (Akpunonu, Mulrow, & Hoffman, 1996). Some journals refer to secondary HTN as "surgical hypertension". Most patients have primary HTN and this account for 95% of known cases, whereas the remaining 5% will have secondary HTN.

Hypertension is often associated with obesity since most obese individuals suffer from high BP and some of them even tend to develop left ventricular hypertrophy (LVH). Left ventricular hypertrophy develops in response to high BP and volume overload

(Gardin & Lauer, 2004). This implies that the left ventricle, the main heart chamber that does the most work to pump the blood to circulate through our body, is required to work harder. As the workload increases, the thickness of the chamber walls increase and some of the elasticity is lost. Eventually the hearts fail to pump the blood in the circulation with sufficient force. This condition is common in obese patients with high BP.

Hypertension or high BP is also a very important public health challenge globally. It is estimated to be the number one risk factor for CVD (S. S. Lim et al., 2012). CVD caused 17 million deaths in 2008 and is still increasing every year (Kelly et al., 2012). In 2005 Kearney et al. conducted a study regarding the burden of HTN on the world. They estimated that the absolute value of HTN on the adult population globally in 2000 was 26.4%. Furthermore, they predicted that in the next 25 years this number would significantly increase by about 60%, resulting in a total of 1.56 billion affected cases worldwide. (Kearney et al., 2005). Subsequently, in 2008 the overall global prevalence of raised BP levels in adults over the age of 25 years was 40% (1040 million) (Danaei et al., 2011). In the African region the incidence of raised BP was the highest (46%) in the world for both sexes combined. The lowest prevalence region was the Americas with a rate of 35%. The occurrence of HTN has therefore been reported in various regions throughout the world and is on the increase in both low as well as high income countries.

Importantly, HTN is not only an international concern, but also affects us locally. After the first democratic elections in 1994, the Department of Health conducted the first South African Demographic and Health Survey (SADHS) in 1998. This SADHS revealed that 21% of the adult population in South Africa suffer from HTN (Steyn et al., 2001), which is equivalent to other industrialized populations, according to the World Health Organisation (WHO) guidelines on HTN (Burt et al., 1995). Today HTN is more common amongst obese individuals than amongst the lean (Steyn et al., 2001). This is a major risk factor leading to an increased risk for heart attacks, strokes, myocardial infarction, end stage-renal disease (ESRD), congestive heart failure, LVH, blindness and peripheral vascular disease (Fiebach et al., 1989; MacMahon et al., 1990; Whelton et al., 1992; Whelton, 1994; Stamler, Stamler, & Neaton, 1993). Patients who suffer from BP HTN are usually unaware that they have this condition unless

their BP has been measured at a local or mobile clinic, by their doctor (GP), the hospital, or simply when they donate blood. The risk of developing CVDs is significantly increased by the presence of HTN (O'Donnell et al., 1997). Cardiovascular disease does not only cause significant morbidity, but also imposes high healthcare cost and is currently the number one cause of death globally (Go et al., 2013; Roger et al., 2012).

Systolic blood pressure is a stronger risk factor for cardiovascular and renal disease than the corresponding relationship for DBP (Izzo, Levy, & Black, 2000). In knowing this, there is more focus on SBP in the clinical setting than DBP. Hypertension is only one of several proven major risk factors for CVD. In combination, these factors provide a strong basis for predicting the risk of cardiovascular complications in general.

1.3.1 Effect of age on BP

There is a constant age-related increase in BP and this is a typical occurrence in most, but not all populations (Kannel, 1996). Hypertension increases with age, in such a manner that more than one out of every four adults in South Africa older than 60 years of age suffer from HTN (Steyn et al., 2001). With age, blood vessels become less elastic which is one of the causes of BP increases with age.

1.3.2 Effect of race on BP

In South Africa, HTN occurs in all races; white, black, coloureds, and Indian communities. However the effects of chronic HTN are different in these populations (Steyn et al., 2001). Seedat et al. (1999) summarised that black hypertensive patients in South Africa may suffer from cerebral haemorrhage and kidney disease which can lead to uraemia. Coronary heart disease (CHD) was previously thought to be relatively uncommon in the black population. However, a recent study conducted by Tibazarwa et al. (2009) among the black population of Soweto suggested that, contrary to popular belief, CHD is on the increase in this population group (Tibazarwa et al., 2009). Coronary heart disease remains a major outcome in white and Indian communities

(Seedat, 1999). There is no known association between ethnicity and the risk of developing HTN. An individual's risk for developing HTN is therefore more dependent on their socioeconomic status than their ethnic background (Opie & Seedat, 2005). Increasing urbanization leads to a change in lifestyle (high fat diet, inactivity, obesity). As a result, the responsiveness of antihypertensive medication such as beta-blockers and angiotensin-converting enzyme inhibitors was found to be poor in black patients unless combined with diuretics. Alternatively, vasodilators or calcium channel blockers responded best to act against high BP in hypertensive black patients (Seedat, 1999).

1.4 Regulation of BP

The body is in constant homeostasis, a state of equilibrium where it is maintaining a 'constant' state despite changing conditions. This is achieved when the body responds to external change in a physiological manner to keep the body functioning. The body functions as a whole through various mechanisms of communication between different cell types, whether it is accomplished through direct signalling, synaptic, endocrine or paracrine messengers (P. D. Wilson, Du, & Norman, 1993). Some of the major factors that can affect the regulation of BP are the contractility of the heart, blood volume, peripheral resistance, viscosity of the blood and vascular tone. The greater the blood volume in the body, the higher the rate of return of blood to the heart. This increases the work load on the heart, which results in an overall increase in cardiac output (Patterson, Piper, & Starling, 1914). The blood volume in the body is regulated by a steadfast process that involves the liver, kidneys and arteries. Here, Na^+ as well as the viscosity or thickness of the blood plays an important role regarding BP. The thicker the blood is, the greater the arterial pressure (Letcher, Chien, Pickering, Sealey, & Laragh, 1981). Red blood cells (RBC) are responsible for this viscosity. When an individual's RBC count increases and becomes too high, may result in Phlebotomy. This is a common treatment for high blood viscosity. This simple procedure involves the removal of some blood from a patient's blood stream. A small incision in a vein is used to control how much blood is removed in order to control and lower the RBC count. On the other hand, if an individual develops a RBC count that is too low, he/she

is at risk of developing anaemia. Blood vessels play an important role in the management of vascular tone and resistance in the circulatory system. Vascular tone is regulated by vasoconstrictors and vasodilators respectively. Vasoconstrictors such as phenylephrine (PE) constrict the blood vessels which lead to a decrease in the radius of the arteries (Franco et al., 2001). The opposite happens with vasodilators (Furchgott & Zawadzki, 1980). By reducing the diameter of the arteries, the body's BP increases. With obesity, there is a build-up of FFA in the blood vessel which reduces the free flow of blood and therefore elevates the resistance within the arteries. The body regulates BP and flow via three mechanisms: i) auto-regulation, ii) neural regulation and iii) endocrine regulation (Bayliss, 1902; Hall et al., 2003). Each mechanism acts in a distinctive way to keep the BP constant, so that the body can function in a state of homeostasis. These regulatory mechanisms can be divided into short term mechanisms, which regulate blood vessel diameter, heart rate and contractility; as well as long-term mechanisms which regulate blood volume.

1.5 The role of the Renin Angiotensin Aldosterone System (RAAS) in Hypertension

The Renin Angiotensin Aldosterone System (RAAS) plays a critical role in maintaining constant homeostasis with regards to arterial pressure, tissue perfusion, electrolyte levels and body fluid volume (blood) (Atlas, 2007).

1.5.1 Renin –Production & Functional role

The biosynthesis of renin occurs in specialized granular cells of the juxtaglomerular (JG) apparatus in the kidneys. This is where the renin angiotensinogen aldosterone hormonal cascade originates. Renin is synthesised as prorenin and rapidly processed to prorenin (Pratt, Carleton, Richie, Heusser, & Dzau, 1987). The activation of renin occurs during the removal of a prosegment of the renin peptide through a proteolytic split from the N-terminus, leaving mature renin behind. Mature renin (also active renin) is stored in the JG apparatus in granules. These granules release renin

into the systemic circulation by an exocytotic process. The three stimuli that cause these granules to release renin into the circulation are: i) a reduction in arterial pressure detected by baroreceptors in the body (e.g. blood volume loss/decrease), ii) reduction of sodium chloride levels and iii) sympathetic nervous system (SNS) activity where norepinephrine acts through β_1 -receptors on the JG apparatus (Skinner, MC Cubbin, & Page, 1963; Skinner, MC Cubbin, & Page, 1964). The latter two pathways mentioned operate slower than the first (Brown, 2006). Other tissues, including the brain, adrenal gland, visceral adipose tissue and vascular tissue, also synthesize renin but the possible actions of renin in these tissues are poorly understood (Atlas, 2007). Since renin regulates the initial, rate limiting step of RAAS the secretion of renin is a key determinant of this cascade. Renin sets the RAAS in motion with the cleaving of a peptide bond on angiotensinogen, creating angiotensin I (Ang I). The primary site for the synthesis of renal angiotensinogen is in the liver. Here the liver continuously produce angiotensinogen and release it into the blood after synthesis. It is an alpha2-globulin peptide which is the main substrate for the circulating enzyme, renin (Di Raimondo et al., 2012).

1.5.2 Angiotensinogen –Functional role

As mentioned earlier, Ang I is formed when renin cleaves angiotensinogen. Ang I has no known physiologically relevant role and serves only as a precursor to Angiotensin II (Ang II). Angiotensin I, an inactive decapeptide, is hydrolysed by angiotensin-converting enzyme (ACE) to form Ang II. Angiotensin-converting enzyme is mainly involved in enzymatic reactions which result in decreased vasodilation and increased vasoconstriction (Carey & Siragy, 2003). Angiotensin-converting enzyme is a target for ACE inhibitors which will decrease the production of Ang II. Angiotensin-converting enzyme inhibitors are one of the major antihypertensive classes of drugs used in the treatment of HTN. After the cleavage of Ang I this process leaves Ang II behind, an octapeptide. Angiotensin II is biologically active, unlike Ang I, and a potent vasoconstrictor which can be found in vascular endothelial cells to control BP. Angiotensin II is the primary active product of the renin angiotensin aldosterone cascade and acts through receptors called angiotensin type 1 (AT_1) and angiotensin

type 2 (AT₂) receptors (Carey, Wang, & Siragy, 1999). Signalling through AT₁ receptors is responsible for protein phosphorylation, cell proliferation, vasoconstriction and antidiuresis or antinatriuresis, whereas signalling through the AT₂ receptors is responsible for completely opposite effects such as protein dephosphorylation, antiproliferation, vasodilation and diuresis. These opposing processes play a protective role in the regulation of BP.

Angiotensin II is therefore responsible for the regulation of AT₁ and AT₂ receptors (Carey, Wang, & Siragy, 2000), which maintains BP through the regulation of contraction of smooth muscle cells in the blood vessels playing an important role in vasodilation. Ichiki et al., (2013) reported that mice lacking the genes to encode for AT₂ receptors have higher levels of BP compared to wild-type controls. Angiotensin II also stimulates and regulates the synthesis and release of aldosterone (ALD) via AT₁ receptors located on the zona glomerulosa, the outer most zone of the adrenal cortex in the adrenal gland where it is produced.

1.5.3 Aldosterone –Functional role

Recently, ALD has been described as having a key role in the relationship between obesity and HTN. Aldosterone is the primary salt-retaining hormone in the body. It is a steroid hormone (part of the mineralocorticoid family) and has a molecular weight of 360.4 Daltons (Laragh & Stoerk, 1957). It is produced and secreted by the glomerulosa zone in the adrenal gland and is responsible for controlling and regulating hydromineral metabolism (Rajagopalan & Pitt, 2001).

The target organ for ALD in the body is the kidney. In the distal part of the nephron, ALD encourages reabsorption of Na⁺ and Cl⁻ ions and the secretion of K⁺ and H⁺ ions in the blood vessel lumen. Consequently, there is an increase in extra-cellular osmolarity and water retention. The water retention increases the blood volume and therefore the BP. Regulation of ALD secretion is linked to the body's water balance and brings three factors into play (Smeaton, Andersen, & Fulton, 1977). These are i) the RAAS (discussed earlier), ii) the plasmic Na⁺/K⁺ ratio and iii) adrenocorticotrophic hormone (ACTH). Physiologically there seems to be a circadian rhythm involved in the

regulation of the levels of ALD in the blood, which may be linked to changes in body posture (Lamarre-Cliche et al., 2005). Levels of ALD have been found to be higher in children than in adults, and it also increases during pregnancy (Elsheikh et al., 2001; Smeaton et al., 1977). In summary, ALD is a major role player in regulating the sodium and potassium balance in the body and thus regulates extracellular volume. It also controls BP by enhancing the reabsorption of sodium and water. By doing this it can increase BP if needed.

In pathology, high levels of ALD or hyperaldosteronism is caused by the over-production of ALD by the adrenal glands in the body. This causes a reduction in potassium in the blood which is known as hypokalaemia (Calhoun, Nishizaka, Zaman, Thakkar, & Weissmann, 2002). Conversely, if there are low levels of ALD there will be less reabsorption of the Na^+ and Cl^- ions and less secretion of K^+ and H^+ ions.

It is clear that ALD controls the sodium and potassium balance in the body. The more potassium we consume the more sodium is excreted through the urine and out of the body. Potassium also helps to relax smooth muscles in the walls of blood vessels which lower BP (Nielsen-Kudsk, 1996). The opposite is true for sodium. Sodium activates the sodium channels on smooth muscle cells and induces contraction, mediated by Ca^{2+} influx (Shinjoh, Nakaki, Otsuka, Sasakawa, & Kato, 1991).

In obese individuals the excess adipose tissue secretes FFA into the circulation resulting in a release of hepatic factor from the liver, which in turn increases the synthesis of serum ALD (Goodfriend, 2008). Reports also suggest that there is a link between the BMI in humans and the plasma Ang II levels, plasma renin activity (PRA) and plasma ACE (El-Atat, Aneja, Mcfarlane, & Sowers, 2003).

When components of the tissue RAAS or circulation RAAS are inhibited or overexpressed it can lead to a disturbance in the body and thus the occurrences of pathophysiological processes.

1.5.4 RAAS and CVD

It has been proposed that abnormal activity of the RAAS can lead to the pathogenesis of CVD and HTN (Di Raimondo et al., 2012). During normal physiological conditions the function of cardiac RAAS is to maintain cellular balance by inhibiting or inducing cell growth. It also plays a role in cell proliferation as well as mediating an adaptive response to myocardial stretch. We can assume that RAAS also influences vascular function because mineral corticoid receptors are expressed in vascular endothelial and smooth muscle cells as well in cardiomyocytes, a key role player in CVD is atherosclerosis (Rajagopalan, Duquaine, King, Pitt, & Patel, 2002).

1.5.5 RAAS and Hypertension

The onset of HTN is often a result of the dysregulation of RAAS in the body. Secondary HTN is the most common form of dysregulation of RAAS. With essential HTN, most young individuals have moderate to mild increases in plasma renin activity (Degli Esposti et al., 1979). This can be due to various mechanisms; one being the over-activity by the SNS. In the older population as well as women and type 2 diabetics, HTN with low levels of plasma renin activity are reported (Alderman, Cohen, Sealey, & Laragh, 2004; Messerli et al., 1983).

1.5.6 RAAS and Obesity

The RAAS has an important role in regulating BP as mentioned earlier and plays a part in the regulation in fluid and electrolyte balance. Obesity acts on the RAAS through three mechanisms; i) sympathetic stimulation ii) the synthesis of adipokines by excess visceral tissue and iii) hemodynamic alterations (de Kloet et al., 2013; Goodfriend, 2008; Trayhurn & Wood, 2005). High levels of ALD in obese individuals could have multiple causes, the expanding adipose tissue being one. Adipose tissue also expresses the renin substrate angiotensinogen. The relationship between renin and angiotensinogen is such that the reaction never saturates. If the substrate increases, the levels of plasma renin increase. This upstream activity in turn, increases

the formation of Ang II and causes the downstream levels of ALD to be even higher. Angiotensin II acts via AT₁ receptors located on the zona glomerulosa, the outer most zone of the adrenal cortex in the adrenal gland. There it stimulates the release and regulates the synthesis of ALD. Once aldosterone (ALD) is released into the blood stream, it results in sodium reabsorption and water retention thus increasing BP (Atlas et al., 1979; Rajagopalan & Pitt, 2001). Another cause of high levels of ALD in obese patients is the influence of inhibitors and stimuli on the adrenal cortex. The principal inhibitor is atrial natriuretic peptide (ANP). Obese patients have low levels of ANP, which may encourage ALD secretion (Wang et al., 2004). Natriuretic peptide clearance receptors (NPR-C) are abundant in adipose tissue suggesting that adipocytes help with the removal of ANP in the circulation (Sarzani et al., 1995; Sarzani et al., 1996). In obese individuals the amount of ALD in blood and urine is more elevated when compared to lean individuals (Goodfriend, 2008). Aldosterone levels are found to be higher in patients with drug-resistant HTN and many of these patients are also obese.

1.5.7 RAAS and vasculature dysfunction

The uptake of circulating renin by vascular smooth muscle and endothelial cells generate Ang I and Ang II. Studies have suggested that the maintenance of cardiovascular homeostasis contributes to the effects of vascular RAAS by mediating its long term effects through AT₁ and AT₂ receptors (Kaschina & Unger, 2003). This remodels the vascular smooth muscle cells by stimulating proliferation. The dysfunction of the endothelium is associated with the upregulation of ACE. Angiotensin-converting enzyme has a dual function which disrupts the vasodilation via the degradation of bradykinin (a potent vasodilator) (Gainer, Morrow, Loveland, King, & Brown, 1998; Hornig, Kohler, & Drexler, 1997) and vasoconstriction of the surrounding tissues with the production of Ang II (Carey et al., 1999; Kaschina & Unger, 2003). High levels of ACE can lead to additional alterations such as inflammation and oxidative stress (Chabrashvili et al., 2003). Angiotensin II is known to boost the production of reactive oxygen species (such as superoxide and hydrogen peroxide), which has been associated with HTN, hypertrophy and inflammation (Reckelhoff & Romero, 2003).

1.6 The sympathetic nervous system (SNS) and its role in Hypertension

The SNS contributes to the homeostatic balance of the body (Simonds & Cowley, 2013). In obese individuals there are several mechanisms and mediators causing over-activity of the SNS leading to increased adrenergic sympathetic stimulation of the heart, vasculature and kidneys, increasing BP by elevating cardiac output, vascular resistance and fluid retention. Inhibiting the SNS produces the opposite effects (de Kloet et al., 2013). Most notable among all of these mechanisms are hyperinsulinaemia and increased plasma FFA (Hall et al., 2001). Increased sympathetic activity can be measured directly or indirectly in obese subjects.

There are several mechanisms and mediators which may cause over-activity of the adrenergic system in obese individuals. In obese individuals, the amount of visceral fat load is usually more than in lean individuals and the amount of FFA is significantly higher than in non-obese individuals. This delivers a FFA load to the liver, which in turn activates hepatic afferent pathways and leads to the further activation of the SNS. High FFA levels are believed to raise BP either by stimulating the SNS or enhancing a vascular response, thus increasing BP (Hall et al., 2001). Long term effects of FFA on BP are less clear and further investigation is needed (Hall et al., 2001). A study showed that by infusing dogs with long chain fatty acids, there was no added effect relating to BP (Van Vliet, Hall, Mizelle, Montani, & Smith, 1995), but in contrast, infusion of long chain fatty acids to rats, demonstrated a rise in BP and increased heart rate (Grekin et al., 1997). At present, the relationship between FFA and BP or HTN is not clearly understood and needs more scientific investigation.

1.7 Role of insulin in Hypertension

In the past, insulin or hyperinsulinaemia was considered a key factor in the onset of obesity derived HTN (Hall et al., 2001). The reason for this was that obese subjects usually have elevated insulin levels, which they require to maintain adequate metabolism of all the excess glucose and FFA in their circulation. A study in 1989 by Hall et al. showed that infusions of excess insulin in dogs to cause hyperinsulinemia

did not have any effect on BP, however did cause some sodium retention (Hall, Coleman, & Mizelle, 1989). These obese individuals often reach a point where their bodies become resistant to the actions of insulin on peripheral tissues, termed insulin resistance.

1.8 Cardiac adaptations due to Hypertension

Left ventricular hypertrophy is one of the cardiac adaptations that occur from having chronic HTN, and increases the risk for systolic or diastolic heart failure and sudden death (Panidis et al., 1984). It is an important maladaptive response to chronic pressure or volume overload of the left ventricle in the heart. Hypertrophy of the cardiac muscle is defined as an increase in size of the existing myocardial fibres in the heart (Ganau et al., 1992) while hyperplasia is different in the sense that the number of myocardial fibres increase through mitotic division (Anversa et al., 1990). LVH is the approximate equal thickening of all or one of the left ventricular walls, when compared to the other walls of the heart.

Two types of LVH are distinguished:

- i) Global LVH is the more common type of LVH. It is categorized into two subtypes: concentric and eccentric. Concentric Global LVH is usually caused by chronic pressure overload of the left ventricle (Verdecchia et al., 1995). Eccentric Global LVH is mostly associated with volume overload of the left ventricle (Ganau et al., 1992).
- ii) Regional LVH is less common than global LVH. This type of LVH predominantly involves the interventricular septum. Disproportional thickening of the septum is usually found in embryonic and fetal hearts and is also relatively common in neonates. In adults, disproportional septal thickening is mostly due to systemic HTN, however this association is rather uncommon (Maron, Edwards, & Epstein, 1978).

1.9 Other factors in the body that might affect BP

BP is also affected by the vascular bed via vasodilation and vasoconstriction induced by several substances mostly secreted by the endothelium. Nitric oxide (NO) is an important messenger produced by the endothelial NO synthase (eNOS) enzyme. The formation of NO from L-arginine by eNOS in the vascular endothelium plays a role in the regulation of blood pressure and in the hypotensive actions of acetylcholine (Rees, Palmer, & Moncada, 1989).

1.10 Vascular endothelium (vascular bed adaptations)

Vascular endothelium is the first line of defence against various harmful molecules in the circulation. It is therefore constantly at war with the surroundings and will constantly get bombarded with harmful substances. The vascular endothelium is an active organ in the body and plays a vital role in the basal and dynamic regulation of the circulation. The integrity of vascular endothelium supports the beneficial effect of antioxidants, anti-inflammatory, anti-coagulant and pro-fibrinolytic actions (Pierce et al., 1997; Weber et al., 1994).

Endothelium releases NO under normal physiological circumstances, which is essential for maintaining vascular tone. Reduced NO bioavailability leads to endothelial dysfunction. This could be due to reduction in the expression of eNOS (Wilcox et al., 1997), insufficient availability of substrate and co-factors (Pou, Pou, Bredt, Snyder, & Rosen, 1992), even alterations in the signalling cascade that leads to the activation of eNOS producing NO (Shimokawa, Flavahan, & Vanhoutte, 1991) and last but not least the interference caused by reactive oxygen species (ROS) degrading the available NO (D. G. Harrison, 1997). This event preceded atherosclerosis and CVD complications such as HTN, T2D, strokes and heart failure (Al Suwaidi et al., 1998; Meigs et al., 2004; Suwaidi et al., 2000). Functional eNOS is found in the endothelium and is responsible for the production of NO. Nitric oxide is found to be protective against these pathological changes that can lead to atherosclerosis and CVD complications (Albrecht et al., 2003; Harrison et al., 2003).

The synthesis of NO is a dynamic process and by influencing proteins that partake in these pathways, one can restore the function of the endothelium (van Goor et al., 2001).

The production of NO by eNOS is a fundamental determinant in cardiovascular homeostasis; it regulates BP, vascular remodelling and angiogenesis (Al Suwaidi et al., 2001; Rees et al., 1989; Ridnour et al., 2005). Functional eNOS is indirectly activated by physiological and metabolic stimuli, laminar shear stress, and receptor dependent agonists (Balligand, Feron, & Dessy, 2009; Chen et al., 1999; Montagnani, Chen, Barr, & Quon, 2001). Once activated eNOS oxidises L-arginine to L-citrulline and NO (Palmer, Rees, Ashton, & Moncada, 1988).

Uncoupling of eNOS results, in the formation of superoxide (O_2^-) instead of NO which causes endothelial dysfunction (Mata-Greenwood et al., 2006). The mechanism that leads to endothelium dysfunction starts with a reduced bioavailability of NO and the increased production of O_2^- and ROS in the wall of the arteries. This enhances the production of NADPH-oxidases (Knuckles, Lund, Lucas, & Campen, 2008). NADPH-oxidases readily react with NO to form peroxynitrite ($ONOO^-$). An essential eNOS cofactor (6R-) 5, 6, 7, 8-tetrahydrobiopterin (BH4) is highly sensitive to oxidation by $ONOO^-$ (Thum et al., 2007). With BH4 deficiency, reduction of O_2 becomes uncoupled from NO synthesis, thereby converting eNOS to a superoxide-producing enzyme (Mata-Greenwood et al., 2006).

Hypertension appears to have a complex association with endothelial dysfunction. Since it was first demonstrated by Altan (1989) that the vasodilator action of acetylcholine (ACh) was mediated directly by releasing a substance from the endothelium in rat aorta, there has been a considerable interest in the function of vascular endothelium in HTN (Altan, Karasu, & Ozuari, 1989; Van de Voorde & Leusen, 1984; Van de Voorde & Leusen, 1986).

Endothelial cells synthesise and release many potent vasoactive substances including prostacyclin; endothelium derived relaxing factor (or NO), endothelium-derived hyperpolarising factor, thromboxane A₂, and endothelin (Rees et al., 1989; Ridnour et al., 2005; Yoshizumi et al., 1990). It has been suggested that these vasoactive substances play a crucial role in regulating BP, regardless of obesity.

1.10.1 Nitric Oxide

As described above, nitric oxide is a gas, capable of moving across cell membranes, a characteristic that enhances its biological activity. It is produced by eNOS in vascular endothelial cells and has proven to be a potent vasodilator. Vasoactive substances such as acetylcholine (ACh), adenosine diphosphate (ADP), histamine and phenylephrine (PE) trigger the release of NO by the endothelial cells. Blocking the release of NO from endothelial cells in rats by administering an NOS inhibitor, induced HTN (Arnal et al., 1993). Interestingly, the chronic blockade of NO synthase by NG-nitro-L-arginine-methyl ester (L-NAME) also induces HTN; however it can be prevented by antagonists such as ACE 1, Angiotensin II receptor antagonists (ARBs) and calcium channel blockers (Arnal et al., 1993).

Hypertension was also induced in mice lacking the gene for NOS (Huang et al., 1995). This suggests that the continued release of NO by the endothelium plays an important role in the contraction and relaxation of vascular smooth muscle and that disrupting this role of NO in the endothelium, can lead to endothelial dysfunction and in turn to HTN.

1.10.2 Reactive oxygen species

Reactive oxygen species (ROS) are produced from different metabolic processes and play an important role in the human body. Reactive oxygen species can damage or protect different tissues, including those of the cardiovascular system. Research has also demonstrated an association between ROS and the development of HTN, through experiments which included the manipulation of the redox state (Laursen et al., 1997). Reactive oxygen species can affect multiple tissues, directly or indirectly. In the vasculature, they induce contraction which eventually leads to endothelial dysfunction (Auch-Schwelk et al., 1989; Torrecillas et al., 2001). Evidence also suggests that ROS can increase BP by the stimulation of the heart rate and force of contraction (Vaziri, Wang, Oveisi, & Rad, 2000). This suggests that ROS may contribute to chronic HTN and thereby induction of myocardial hypertrophy.

Furthermore, ROS do not only act through the vasculature, but also through the kidneys and the CNS (Ando & Fujita, 2012; Baud & Ardaillou, 1993; Fujita et al., 2012; Jin et al., 2006). The kidneys play an important role in the regulation of BP and are therefore also likely to be targeted by ROS. Therefore all the influence that ROS exerts on the different targeted tissues contributes to inducing HTN in the end.

1.11 Connection between Obesity and Hypertension

As discussed above, obesity has often been seen to co-exist with other risk factors for chronic diseases of lifestyle, such as HTN and diabetes. Obesity and HTN have both been identified as independent risk factors involved in the development of vascular dysfunction, renal disease and diabetes.

The direct, positive relationship between BP and cardiovascular risk has been established and is well known (He & Whelton, 1999; Kannel, 1996; Kannel, Schwartz, & McNamara, 2009; Stamler et al., 1993; Wilson et al., 1998). This has been identified in men and women, young and old individuals, people from different racial groups, people from different countries and those with normotensive or hypertensive BP (Fiebach et al., 1989; Steyn et al., 2001; Tibazarwa et al., 2009).






Obesity has also been associated with an increase in BP, however it remains unclear how excess adipose deposits can raise BP. Some of the factors thought to be responsible for this link are hyperinsulinemia and dyslipidaemia.

1.12 Blood pressure classifications

With all the new data available concerning the risk of HTN and the impressive increase in the risk of CVD associated with levels of BP previously described as normal, the Joint National Committee (JNC 7) on Prevention, Detection, Evaluation and Treatment of High Blood Pressure report has introduced a new classification of BP in their 7th report (Chobanian et al., 2003; Cornelissen & Fagard, 2005).

Table 1-2 provides a classification of BP for adults age 18 and older. On average, two or more properly measured seated BP readings were taken during each visit to help base this classification system. Classification of BP changed between the JNC 6 report which was published in 1997, and the new JNC 7 report from 2003 (Chobanian et al., 2003). In the JNC 7 a new category has been added called ‘prehypertension’. In addition, they combined stage 2 and 3 HTN. The reason for creating this new group is that patients with prehypertension are at risk of progressing to HTN. Similarly, those in the 130-139/80-89 mmHg BP range area have an even greater risk of developing HTN.

Table 1-2: Classification of Blood Pressure for Adults Age > 18 Years in JNC 6 categories to JNC 7 categories adapted from (Chobanian et al., 2003).

JNC 6 CATEGORY	SBP/DBP	JNC 7 CATEGORY
OPTIMAL	<120/80	 NORMAL
NORMAL	120–129/80–84	 PREHYPERTENSION
BORDERLINE	130–139/85–89	
HYPERTENSION	≥140/90	 HYPERTENSION
STAGE 1	140–159/90–99	 STAGE 1
STAGE 2	160–179/100–109	 STAGE 2
STAGE 3	≥180/110	

From Table 1-2 HTN cut-off point is classified as; systolic pressure greater than 140 mmHg or a diastolic pressure greater than 90 mmHg or both.

Despite the progression in prevention, detection, treatment and control of HTN it still remains an important public health problem.

Therefore it is referred to as the ‘silent epidemic’ of our time!

1.13 Benefits of reducing BP

BP alone has been used to determine whether a patient should adjust his lifestyle or if a patient should make use of antihypertensive medication as a treatment option. A number of studies found that a high BP level, both SBP and DBP leads to an increased

risk of CVD, stroke and renal failure (He & Whelton, 1999; Kannel, Wolf, Verter, & McNamara, 1996; Kannel et al., 2009; MacMahon et al., 1990). Even the smallest reduction in high BP has been shown to be protective in the long run. HTN has therefore been identified as the most important modifiable risk factor to treat and reduce the chance of developing CHD, congestive heart failure, ischaemic disease, stroke, myocardial infarction, end stage-renal disease (ESRD), LVH, blindness and peripheral vascular disease (He & Whelton, 1999; Ogden, He, Lydick, & Whelton, 2000; Whelton et al., 1992; Whelton et al., 2002). Although most studies suggest that the association of SBP and CVD is stronger than that of DBP, a Nigerian study delivered contradictory results. According to this study, an increase in DBP of 20 mmHg resulted in a significant mortality rate among affected individuals (Kaufman et al., 1996). An elevated DBP may therefore be just as detrimental as elevated SBP, regarding the risk for CVD.

The main objective of treating HTN is to lower a patient's BP as this reduces the absolute risk of premature death and disease, primarily by reducing their risk of CVD (Ogden et al., 2000). Different studies have indicated different values of reduction in BP that can be seen as beneficial. There is no exact value stipulated by what you have to reduce BP in order to see the associated benefits. Even a reduction of 3-12 mmHg, would be beneficial and the larger the reduction (e.g. 20 mmHg) achieved, the greater the benefits.

A study done by Ogden et al. (2000) calculated a reduction value of 12 mmHg to be of some benefit. This represented the average BP reduction that was observed from randomized controlled trials which were conducted to test the efficacy of antihypertensive agents (Ogden et al., 2000). Once again, if a larger reduction in SBP is observed, even greater benefit is expected (He & Whelton, 1999).

In another clinical trial, where antihypertensive therapy was used over a 4 year period, a reduction of 12 to 13 mmHg in the systolic BP was associated with reductions in stroke incidence averaging 35-40%; myocardial infarction 20-25%; and heart failure more than 50% (Neal, MacMahon, Chapman, & Blood Pressure Lowering Treatment Trialists' Collaboration, 2000).

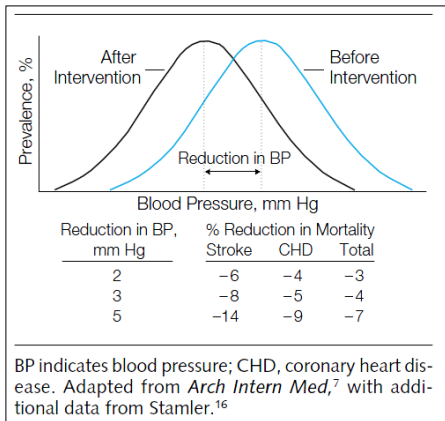


Figure 1-1: Benefits of reducing high blood pressure. Shown in the figure, a small decrement in the systolic BP is likely to result in substantial reduction in the risk associated with HTN and cardiovascular disease adapted from (J. Stamler et al., 1993).

1.14 Lifestyle Modifications for the prevention of hypertension

The adoption of a healthy lifestyle is critical for the management and prevention of high BP. Major life style modifications or changes which have been shown to lower BP include physical exercise (Cornelissen & Fagard, 2005; S. P. Whelton, Chin, Xin, & He, 2002), weight loss in overweight and obese individuals (He et al., 2000), and the adoption of a healthy diet. An example of a healthy diet would be the Dietary Approaches to Stop HTN (DASH) (Sacks et al., 2001; Appel et al., 1997) discussed in 1.16. Some studies have shown that even alcohol consumption, when done in moderation, can be beneficial to one’s health. Alcohol reportedly reduces the risk of developing CVD (Xin et al., 2001). Despite the abundance of pharmaceutical options available for the treatment of HTN, lifestyle modification remains an important approach in the management of BP.

1.15 Physical exercise

Physical activity is essential for long term weight control (Fagard & Cornelissen, 2007). This is also the most poorly complied with strategy to help with weight reduction. The reason for this is that it requires a considerable amount of time, effort and thought from the patient’s side. Furthermore, even in the absence of weight loss, increased physical

activity is associated with other desirable outcomes namely reduction in BP, body fat, waist circumference, lower blood lipids, improved glucose homeostasis, improved insulin sensitivity and endothelial function, amongst others (Kelley & Kelley, 2008). Physical activity is therefore not only important for the management of HTN but also for the prevention of CVD (Cornelissen & Fagard, 2005).

When considering the type of physical exercise, endurance or resistance training is recommended for effective outcomes. Studies indicate that both of these types of exercise may result in a reduction in BP (Cornelissen & Fagard, 2005; Croymans et al., 2013). Physical inactivity is defined as ≤ 150 mins of moderate to vigorous activity per week. Hypertensive individuals are encouraged to start with low-impact, moderate intensity physical activity (e.g. walking) of short duration (for example 30 mins) on most days of the week (Whelton et al., 2002). It is also recommended that healthy individuals partake in similar physical activity for short periods (30min) in order to help prevent HTN (Obesity: Preventing and Managing the Global Epidemic. Report of a WHO Consultation, . 2000).

The effect of physical activity on BP is shown to be more pronounced in hypertensive patients than normotensive individuals. Therefore, exercise contributes to the control of BP in hypertensive patients and is likely to contribute to the prevention of HTN in normotensive individuals.

1.16 Weight reduction/ Loss

One of the most compelling reasons for recommending weight reduction is that weight loss in both obese and lean people have been demonstrated to reduce arterial pressure in both normotensive and hypertensive individuals, but little is known about the mechanism by which this occurs. Not only does it reduce the BP but also improves the carbohydrate tolerance and decreases serum lipids, thereby diminishing these two factors. All of these factors will reduce the chance of CHD (Wilson et al., 1998).

Weight reduction is the only antihypertensive regimen that decreases BP in both normotensive as well as hypertensive patients (Su et al., 1995). This was one of the

few therapeutic strategies for HTN in use before the advent of adequate pharmacologic therapy. This association between weight and HTN has been demonstrated in most populations, in both industrialized and non-industrialized societies (rural and urban societies in RSA) (Berchtold et al., 1981; Berchtold et al., 1982).

Weight reduction can be increased by combining it with physical activity (as mentioned earlier, this is one of the fastest and best ways to treat HTN) and a diet specifically designed to keep a healthy balanced lifestyle. In recent years specialized diets have been modified in trials to incorporate several nutritional recommendations for lowering BP (Appel et al., 2006).

One example is the DASH diet. This diet focuses on fruits and vegetables and is high in fibre and low in fats (Appel et al., 1997). It also encourages increased intake of potassium and calcium while reducing sodium intake. The DASH diet was found to lower BP substantially in all patients and even more so in hypertensive patients. By reducing the salt intake in combination with the DASH diet, BP may be lowered even further (Sacks et al., 2001). Therapeutic weight reduction in obese, hypertensive individuals has been able to substantially lower their BP. This effect can also occur without a reduction in salt intake and it is thought to be a cause and effect relation rather than a more complicated association with a third factor.

As this relationship between diet and HTN has been known for some years, it is advisable to consume a diet that is rich in fruits and vegetables and low in saturated fat content. Weight reduction should be the first line of treatment in every hypertensive patient, obese or non-obese.

Weight loss does not only reduce BP, but can also improve the efficacy of antihypertensive agents, thereby allowing administration of lower doses (Modan et al., 1991). It has also been demonstrated in less severe cases that hypertensive patients could discontinue antihypertensive drug therapy and use nutritional means to manage and control their BP (Stamler et al., 1987). Patients on multiple antihypertensive medication can also benefit from weight loss as it will result in decreasing the number and dosage of drugs they need to control their BP, thereby decreasing the side effects of medication as well as the cost involved.

Although regular controlled alcohol intake is shown to be beneficial to your health and can be cardio protective, alcohol consumption should still be limited to no more than 30 ml of ethanol per day in most men and no more than 15 ml of ethanol per day in women and lower weight persons (720 ml of beer, 300 ml of wine or 60 ml of whisky) (Chobanian et al., 2003). Alcohol drinking has been associated with a reduction in BP through epidemiological studies (Xin et al., 2001). Xue Xin et al. (2001) showed that alcohol consumption in moderation as a means to prevent and treat HTN can strongly be supported. The positive association between alcohol intake and BP can be important modifiable risk factors for HTN among populations.

1.17 Pharmacotherapy

Because weight reduction alone may not be sufficient and effective to treat BP in the long-term, pharmacological treatment may be necessary in most cases. In the majority of patients, controlling systolic HTN, which is a more important CVD risk factor than DBP, can be achieved by pharmacological input.

Currently, the control rates for initiating antihypertensive drugs are SBP \geq 140 and DBP \geq 90 (or stage 2). There are excellent clinical outcome trials showing data that lowering BP will reduce the complications of HTN. Several classes of antihypertensive drugs are available on the market to target different mechanisms in your body, which, in turn, leads to a reduction in BP. They include angiotensin-converting enzyme (ACE) inhibitors (ACEIs), angiotensin receptor blockers (ARBs), beta-blockers (BBs), calcium channel blockers (CCBs) and the thiazide-type diuretics. Only when clinicians fail to prescribe lifestyle modifications, adequate antihypertensive drugs or appropriate drug combinations, inadequate BP control may result.

Thiazide-type diuretics have been the basis of antihypertensive therapy in most outcome trials (Psaty et al., 2002). These drugs are among the most important and most widely used. The BP lowering effect of these drugs is attributed initially to a reduction of intravascular volume and cardiac output, both of which may benefit obese individuals. However, the drawback is that both short- and long-term administration of diuretics may result in enhancement of the SNS activity and activation of the renin-

angiotensinogen system. Diuretics do not seem to affect weight. In the Antihypertensive and Lipid Lowering treatment to prevent Heart Attack Trial (ALLHAT), diuretics were shown as the best first-line treatment for cardiovascular complications of HTN (Flack, Nasser, & Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT, 2003; Hollenberg, 2003). Diuretics enhance the antihypertensive efficacy when used in combination with other antihypertensive medication (multidrug regimens) and can be useful in achieving BP control in hypertensive individuals. Diuretics are also more affordable than other antihypertensive agents. Despite these findings, diuretics still remain underutilized (Psaty et al., 2002).

It is advised that thiazide-type diuretics should be used as initial therapy for most patients with HTN, either alone or in combination with one of the other classes of drugs (ACEIs, ARBs, BBs, and CCBs) (Chobanian et al., 2003).

As discussed previously, the Angiotensin-converting enzyme (ACE) inhibitors (ACEIs), block the action of ACE and thus stops the conversion of Ang I to Ang II. Angiotensin-converting enzyme inhibitors have also been recorded to decrease the secretion of ALD and vasopressin and sympathetic nerve activity. Angiotensin-converting enzyme inhibitors as monotherapy greatly reduces BP through the mechanisms mentioned.

ARBs (angiotensin receptor blockers): It was mentioned earlier that Ang II is the key component in the RAAS. It binds to AT1 receptors leading to development of HTN, secretion of ALD, reabsorption of sodium and inflammation in some instances. Soon after the different receptor types were discovered, they became a logical therapeutic target, since these ARBs block the action of Ang II at the receptor level rather than the synthesis thereof. In addition to being an effective antihypertensive agent, it also improves insulin sensitivity and decreases SNS activity, which could be beneficial in obese HTN.

The Renin Inhibitors, with renin as the initial and rate limiting step of the RAAS, was a logical therapeutic target to consider for antihypertensive medication. The inhibition of renin is focused towards decreasing the plasma renin activity (PRA). By doing so, it also decreases the downstream effects such as a reduction in Ang I and II, ALD and

consequently also a reduction in BP. Although it was initially thought to be an effective therapeutic option, the pharmacological activity of these early renin inhibitors could only be achieved through intravenous injections. Creating an oral renin inhibitor was rather difficult due to potency, duration of action and low bioavailability of the oral drugs. Research was halted in the 1990 to explore other therapeutic agents affecting the RAAS.

There are also the CCBs available on the market. This class of antihypertensive agents exert potent peripheral vasodilator and a mild natriuretic action.

B-adrenergic blocking agents (or BBs) are potentially useful in the treatment of obesity derived HTN (Williams et al., 2004). Administration of BBs to treat HTN has been shown to reduce plasma renin levels by blocking the sympathetic mediated release of renin by the kidneys. This reduction in PRA was closely correlated to a reduction in BP. Beta-blockers have also been demonstrated to decrease the secretion of ALD from the adrenal glands, thereby helping to decrease BP in both a renin dependent and independent manner (Pettinger et al., 1976; Vandongen, Peart, & Boyd, 1973). Beta-blockers have also been shown to decrease cardiac output which will decrease BP (Frohlich, Tarazi, Dustan, & Page, 1968).

Some individuals do not respond to monotherapy and may need to take a combination of drugs to lower their BP (Chobanian et al., 2003; European Society of Hypertension-European Society of Cardiology Guidelines Committee, 2003). This will increase the effect of the drugs thereby leading to a greater reduction in BP. Unfortunately, this may result in more side effects and possible drug-drug interactions. It will also increase the cost of the antihypertensive therapy thus making it more expensive and less accessible to a large part of the public. Because it is a chronic disease, HTN is a burden not only for the patient but also for the health care system of a country.

Despite the high prevalence of obesity and HTN, the efficacy and safety of antihypertensive agents currently available for the treatment of high BP, still leaves room for improvement and should be studied adequately to prevent morbidity and decrease mortality (L. Hansson et al., 1999b). There is therefore a need for constant research into alternative pharmacotherapies that may be more effective, reliable and readily available.

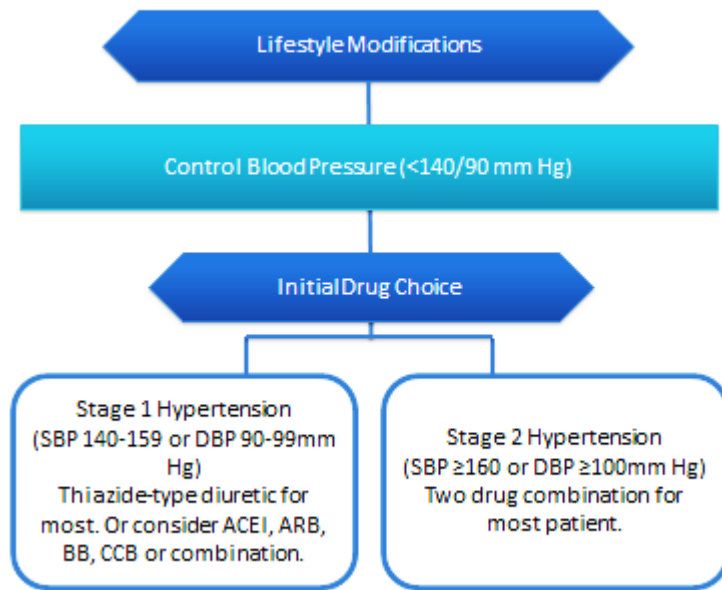


Figure 1-2: Summary of available Hypertension treatment.

1.18 Herbal and supplemental treatment options

For thousands of years indigenous cultures have been using native herbal supplements in the US, the Far East and in Africa for the treatment of various ailments. This resulted in recent resurgence in its popularity among consumers in the West (Tindle, Davis, Phillips, & Eisenberg, 2005). In the US it is estimated that more than 15 million people consume herbal remedies or high dose vitamins. People's growing preference for herbal treatments may be attributed to a number of influencing factors including pain syndromes, depression, anxiety disorders, general wellbeing and health awareness, as well as the increase in cost of conventional medicines and the belief that a more natural drug will be a safer or healthier option (Eisenberg et al., 1998).

1.19 Indigenous Herbs

In recent years, South Africa has developed a keen interest in the field of ethnopharmacology. Specifically, the effects of indigenous plants and indigenous medicine are being researched for possible development of useful medication to treat various

medical conditions. This is simply because in South Africa we are blessed with a great and vast diversity of Fauna and Flora. Examples of traditional herbal remedies found in South Africa include *Aloe Vera* (Vogler & Ernst, 1999), *Prosopis Glandulosa* commonly known as “honey mesquite” (George, Lochner, & Huisamen, 2011), Wilde dagga (*Leonotis leonurus*) (Ojewole, 2005), the African wild potato, a reputed immune-booster and known as Inkomfe, Cancer bush (*Sutherlandia Frutescens*) and *Olea europaea sub Africana* (African wild olive) (Van Wyk, 2011). If the efficacy of these herbs/products can be confirmed by scientific research it can potentially provide an effective, accessible alternative and affordable way of treatment for various illnesses plaguing the African population. They are widely available and could well be one of our greatest and most valuable natural resources.

1.20 Why Buchu?

We were approached by Cape Kingdom Nutraceuticals (Pty) Ltd who requested that we investigate the effects of Buchu on HTN and the associated effects thereof.

Buchu is the common name for *Agathosma Crenulata* and *A. Betulina*, which are two indigenous aromatic plants found exclusively in the Cape region of South Africa. They are part of the Cape Floral Kingdom (FYNBOS) and are particularly abundant in the mountainous areas of the Cape. These shrubs from the Rutaceous family are harvested and used for the production of commercially important products such as Buchu oil (Moolla & Viljoen, 2008). They are probably the best known herb for their medicinal properties, both locally and internationally.

Buchu was discovered more than 300 years ago in South Africa. Here, the Khoisan used it in their everyday life as a traditional remedy for various ailments and as an aid to longevity. When the European settlers arrived in the late 1800 and 1900's this became a highly prized and a scarce commodity. Since then, the Buchu industry has grown exponentially. In the modern day it is still used by the Khoisan, as well as in the cosmetics, food, fragrance and flavour industry.

Over the years many claims have been made regarding the medicinal properties of Buchu, including the belief that it is a natural diuretic. Buchu oil has also been used in the investigation of antimicrobial -, antioxidant - and anti-inflammatory activity in recent years. Since then there has been a growing interest in the medicinal properties of indigenous herbal plants, worldwide (Moolla & Viljoen, 2008).

1.20.1 Antimicrobial activity

A number of studies evaluated antimicrobial activity of Buchu by using the extracted hydrodistilled essential oil from both *Agathosma* species to perform minimum inhibitory concentration assays. Both Moolla et al. (2008) and Viljoen et al. (2006) showed that the oil was active against pathogens. The pathogens tested were *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Candida albicans*. These results indicated that both extracts possess moderate antimicrobial activity (Viljoen et al., 2006).

1.20.2 Antioxidant Activity

A study by Moolla et al. (2008) showed that the *Agathosma* species are rich in flavonoids, a group of phytoconstituents known for their antioxidant activity (Moolla & Viljoen, 2008). Although they contain flavonoids, studies have shown that the extracted oil exhibit little free radical scavenging activity compared to the standard of ascorbic acid (Moolla, Van Vuuren, Van Zyl, & Viljoen, 2007).

1.20.3 Anti-inflammatory activity

Inflammation is part of our innate immune response which is intended to protect and eliminate the cause of cell injuries. During inflammation there is an increase in the production and release of pro-inflammatory cytokines (such as IL-6 and TNF-alpha), which are responsible for initiating inflammation that cause the diluting, neutralizing and removing of insulting agents in order to allow the healing process to start.

Reducing the amount of pro-inflammatory cytokines by blocking some of the pathways associated with them, one can blunt the inflammation response by pro-inflammatory cytokines and decrease the amount of inflammation caused by them. Buchu oil is said to block the synthesis of 5-lipoxygenase (LOX) products *in vitro* (Viljoen, Moolla, Van Vuuren, Van Zyl et al., 2006; Moolla & Viljoen, 2008). 5-LOX is the key enzyme which produces leukotrienes and is involved in a variety of inflammatory diseases.

1.21 Motivation for the Study

South Africa is currently experiencing a mass movement of people from rural to urban communities in search of work, education or both. Many of them erect shacks made of tin, wood and cardboard forming 'shantytowns'. They live in unhealthy circumstances, with little or no sanitation, fresh running water or health care support from the public sector. The people in these communities undergo lifestyle modifications to adapt to the urban life. These urban migrants develop higher body weights (BMI), pulse rates and a possible increase in urinary sodium/potassium ratios than those who remain in the rural areas (Jennings et al., 2010; Rush et al., 2007; Senekal, Steyn, & Nel, 2003). This is due to a marked change in their diet which consists of a higher sodium and calorie intake from fast food services around every corner and less fresh fruit and vegetables which would be giving them the necessary dietary supplements to help manage or keep their BP lower. All this contributes to people undergoing a health transition that leads to higher levels of BP.

Most people in these townships do not have the money to afford a healthy balanced meal. Moreover, they do not have time to visit a clinic or a healthcare facility especially since services within the public health sector are stretched so far in some places that it is often understaffed and underfunded. This leaves a huge gap in HTN detection and prevention. Even individuals who are able to see a doctor in the public or private sector and get diagnosed with high BP or HTN are faced with the challenge of receiving effective treatment. The medication available for HTN treatment is expensive and many South Africans cannot afford medical aid. This means that they must either pay for their prescriptions and medication themselves, or sacrifice many hours in a long

queue to collect it at a clinic. As a result many people who have been diagnosed with HTN are not interested in getting the necessary treatment to decrease their BP. Apart from regular physical exercise there are not many alternative treatments available for HTN.

Not only does HTN affect the people coming from rural areas but also the urban population of South Africa living in cities and towns (Puoane et al., 2002). The stress associated with work, unemployment and the pressure of providing for your family are all factors to be considered as risk factors for an increase in BP. Additionally, smoking and the excessive use of alcohol add to the problem as both can lead to an increase in BP.

Since the necessary health awareness and treatment to prevent the spread of an on-going epidemic of HTN is not available yet, an inexpensive and widely available substance like Buchu may be an alternative to treat HTN. Despite being ranked amongst South Africa's most important medicinal plants and commonly considered a panacea, the biological activity remains unknown and poorly explored. Claims regarding its medicinal properties and effects are mostly speculative and unsubstantial. The purpose of this research study was therefore to credit some of the claims that are given to this medicinal herb.

Should these claims be validated by the present research, the implications will be vast. Specifically, it will mean that South Africans suffering from HTN will have an alternative treatment option which is affordable, effective and readily available.

1.22 Hypothesis

We hypothesized that the ingestion of Buchu water, as supplied by Cape Kingdom Nutraceuticals (Pty) Ltd, will be able to reduce the BP of hypertensive rats by at least 10 mmHg, the minimum reduction to be considered protective.

1.23 Specific Aims

The specific aims of this study were to:

- 1) Test the effect of ingestion of Buchu water on the BP of diet induced hypertensive as well as normotensive rats when given as pre-treatment.
- 2) Test whether Buchu water will have any effect on HTN in rats when used as treatment.
- 3) Test whether Buchu water has any effect on endothelial function using aortic rings from control and hypertensive rats.
- 4) Determine the expression and/or activation of signalling proteins involved in NO production in the endothelium (pPKB/ Akt, AMPK, eNOS).
- 5) Determine whether Buchu water treatment has any insulin sensitizing effects in cardiomyocytes prepared from insulin resistant rats to determine possible cardiovascular effects.

CHAPTER 2: Materials & Methods

2.1 Materials

The reagents used in this study were bought from different companies such as: **Merck NT laboratory supplies (Pty, Ltd)** (NaCl, KCl, CaCl₂, KH₂PO₄, NaHCO₃, MgSO₄, d-glucose, Na⁺ pyrophosphate, Folin Ciocalteus Reagent (Folin C), 2-[H₃]-D-deoxyglucose, NaOH, sodium dodecyl sulphate (SDS), Tris (hydroxymethyl) aminomethane, acrylamide, fructose). **Sigma-Aldrich Life Science** (ammonium persulfate (APS), HEPES, 2,3-butanedione monoxime (BDM), insulin, pyruvate, cholesterol, mecarpto-ethanol, N,N,N,N-tetramethylethylenediamine (TEMED), Ponceau-s red, streptozotocin, phenylephrine (PE), acetylcholine (ACh), phenylmethylsulphonyl fluoride (PMSF), Triton X-100). **Millipore** (Millipore enzyme linked immunosorbent assay (ELISA) kit). **Roche Diagnostics**, (bovine serum albumin (BSA)). **Amersham Biosciences**, (enhanced chemiluminescence (ECL) Western blotting detection reagents, anti-rabbit Ig, horseradish peroxidase linked whole secondary antibody). **Cell Signaling technology**, (Total and phospho-PKB/Akt (Ser473) antibody, total AMPK and phospho-AMPK- α (Thr172) antibodies, total eNOS). **Bayer** (SA) (Euthanaze). Collagenase Type II --the Collagenase Type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ). **Pro-10 Laboratories** Casein, Protein Labs Ltd (Birmingham, UK). **Sime Darby Hudson & Knight (Pty) Ltd** (Boksburg, South Africa), Holsum (vegetable fat). Buchu water – was supplied by the product manufacturer, **Cape Kingdom Nutraceuticals (Pty) Ltd** (Cape Town, South Africa). **Siemens** Coat-a-Count RIA: Aldosterone & Insulin. **Abcam Ltd** (Cambridge, England), ELISA – C-peptide, Aldosterone, Tumour Necrosis Factor-alpha, Interleukin-6, Adiponectin, Leptin and Insulin.

2.2 Methods

2.2.1 ANIMALS

Age and weight matched male Wistar rats were used in this study. All animals were obtained from the University of Stellenbosch Central Research Facility. They received

free access to water and food under the 12 h dark/light cycle (light from 6:00 a.m. to 6:00 p.m.) with temperature and humidity kept constant at 22°C and 40%, respectively. This study was assessed and approved by the Committee for Ethical Animal Research of the Faculty of Health Sciences, University of Stellenbosch (SU-ACUM11-00003) and animals were treated according to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

2.2.2 STUDY DESIGN

This study was divided into three major phases (see Figure 2-3). Animals were grouped, fed and treated during the first phase, prior to any intervention. During the second phase experimental procedures were performed. These were: anthropometric measurements, measurement of diastolic and systolic BP over 16 weeks, aorta ring studies, isolation of adult rat cardiomyocytes by perfusion of the hearts with collagenase, as well as blood and heart tissue collection. During the third phase, biochemical analyses of the collected samples were conducted (see Figure 2-3).

2.2.2.1 Buchu administration

Buchu water was supplied by Cape Kingdom Nutraceuticals (Western Cape, South Africa). This Buchu water contains a mixture of an extract of both *Agathosma Crenulata* and *A. Betulina*. The concentrated Buchu water was diluted and replaced the drinking water of the rats. It was supplied in 1.5 L containers. This was then diluted by adding 3.7 L of fresh tap water to 1.3 L of concentrated Buchu water rendering a final concentration of 26% (v/v) Buchu water. Every 100 ml of diluted drinking Buchu water contained 26 ml of concentrated Buchu water; therefore a rat drinking 15 ml of water per day consumed less than 4 ml of Buchu water per day. The fluid intake of animals was monitored to determine whether it differed between any of the groups. Bottles containing Buchu solution were clearly marked throughout the 16 week period of Buchu administration. The diluted Buchu drinking water was stored at 4°C until used. Drinking water with or without Buchu was measured and replaced every day.

2.2.2.2 Grouping, feeding and treatment

Models: 1 High-fat diet (HFD) & 2. Diet-induced obesity (DIO)

Model 1: HFD model

Male rats weighing 190 ± 10 g were randomly allocated to the following five groups:

- i. Group C: Control rats receiving a standard commercial rat chow and drinking water, without Buchu from day 1
- ii. Group C + Buchu: Control rats receiving a standard commercial rat chow and Buchu in drinking water from day 1
- iii. Group HFD (High Fat Diet): Rats receiving a high fat diet and drinking water without Buchu from day 1
- iv. Group HFD + Buchu: Rats receiving a high fat diet and drinking Buchu water from day 1
- v. Group HFD + Buchu week 8: Rats receiving a high fat diet and drinking Buchu water starting from week 8

Animals were fed and treated for a period of 16 weeks (see Figure 2-3), with the exception of Group HFD + Buchu during week 8. This group was started on an HFD from day one but Buchu was first given to the group in week 8 by exchanging their drinking water with Buchu water. This was done for the remaining 8 weeks. The amount of food intake was measured at weekly intervals over the 16 weeks: This was done by measuring the amount of food supplied to the cages, amount left over and resulting in the amount consumed by the different groups of rats in each cage. To prevent the wet food from fermenting, it was stored at 4°C and monitored daily.

The HFD resulted in development of HTN in the animals (Huisamen, George, Dietrich, & Genade, 2013). This diet contained 40% fat, 10% casein, 10% fructose and 1% Cholesterol added to standard rat chow (SRC).

Table 2-1: Diet Composition (Controls, DIO & HFD).

	Controls (SRC)	HFD	DIO
Carbohydrates	60%	10%	65%
Protein	30%	30%	19%
Fat	10%	40%	16%
Fructose	--	10%	--
Cholesterol	--	1%	--
kJ/100g	1272	1354	1173

Control groups (C and C + Buchu) were fed a SRC consisting of 60% carbohydrate, 30% protein and 10% fat. SRC was supplied by the animal housing division of our Faculty.

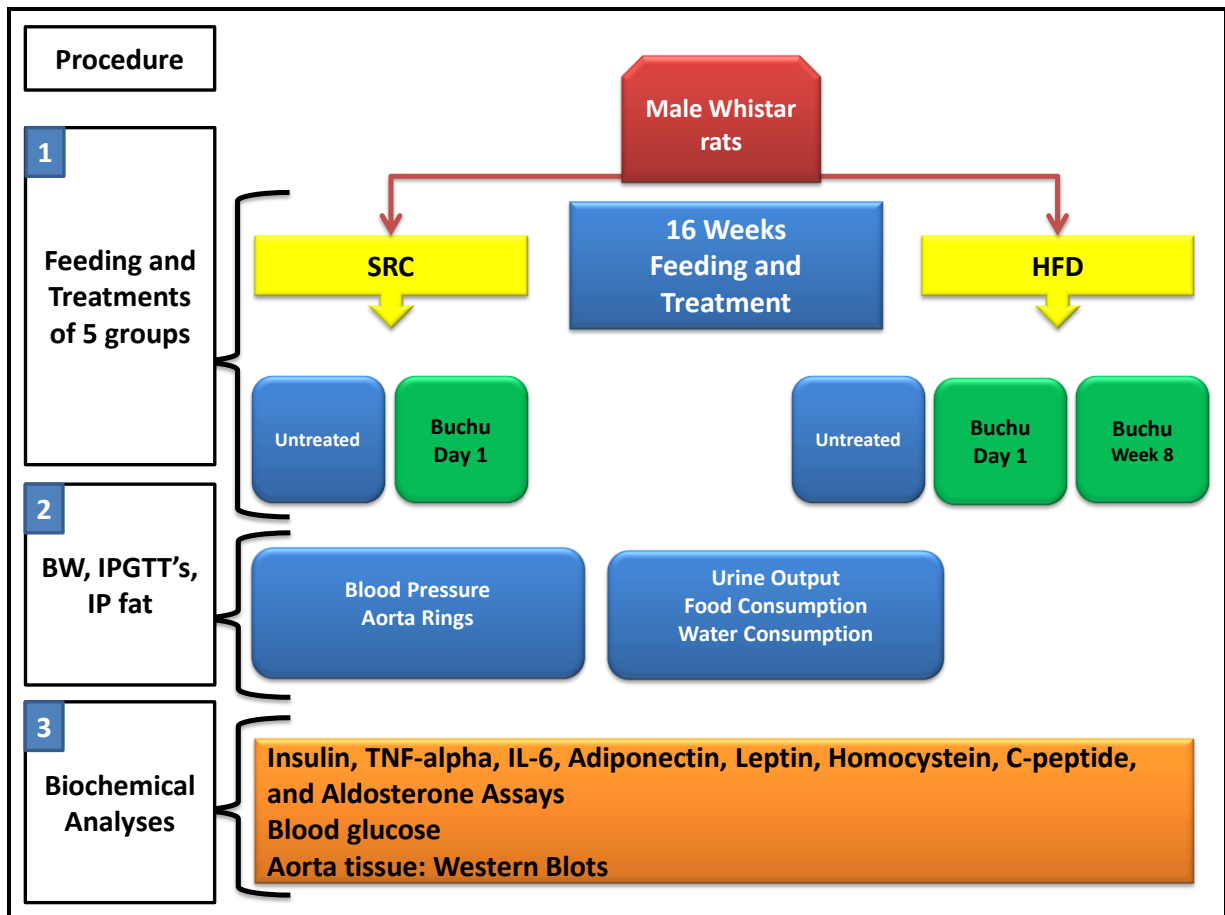


Figure 2-3: HFD group: experimental protocol.

Model 2: DIO model

Male rats weighing 190 ± 10 g were randomly divided into the following groups:

- i. Group C: Control rats receiving a standard commercial rat chow and drinking water, without Buchu from day 1
- ii. Group C + Buchu: Control rats receiving a standard commercial rat chow and Buchu in drinking water from day 1
- iii. Group DIO (High Fat Diet): Rats receiving a high caloric diet and drinking water without Buchu from day 1
- iv. Group DIO + Buchu: Rats receiving a high caloric diet and Buchu drinking water from day 1
- v. Group C + Metformin (positive control): Control rats receiving a standard commercial rat chow and Metformin in drinking water from day 1
- vi. Group DIO + Metformin: Rats receiving a high caloric diet and Metformin in drinking water from day 1

The model of diet-induced-obesity (DIO) (Pickavance, Tadayyon, Widdowson, Buckingham, & Wilding, 1999) was used to induce insulin resistance without induction of HTN in the Male Wistar rats. The DIO diet was a high caloric diet consisting of 33% SRC supplemented with 7% sucrose and 33% sweetened condensed milk (Clover) with a kJ/100g content as shown in Table 2-1.

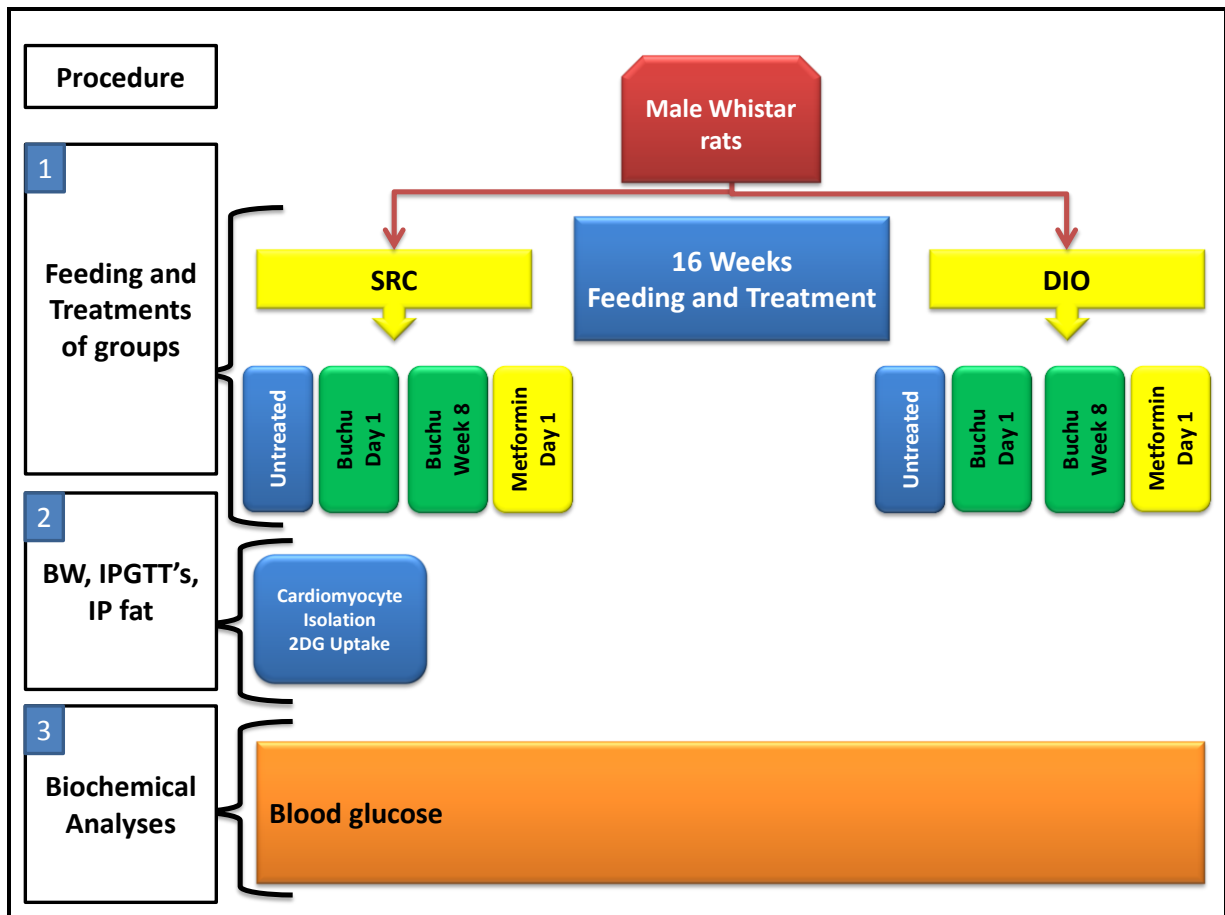


Figure 2-4: DIO groups: experimental protocol.

2.2.3 Experimental Procedures

The experimental procedures and major phases of the study are represented in Figure 2-3 & Figure 2-4.

2.2.3.1 Measuring blood pressure

A non-invasive computerized tail-cuff system was used to measure the BP in the different groups of rats. The Kent Coda 2 Blood pressure monitor (Kent Scientific Corporation, Connecticut, USA) was used and allowed the measuring of two rats simultaneously. The warming platform had space for three housing boxes. This separates the rats as they undergo BP evaluation, from the vibration produced by the pressurized pump.

Prior to the actual experiment, the rats were acclimated for two weeks (14 days) with training/conditioning sessions (that is, sessions of unrecorded measurements). This was necessary for the rats to become accustomed to the tail-cuff procedure to reduce the variability of the real BP measurements. The BP was measured the same time on every day (in the morning) since BP varies according to the time of day due to its inherent circadian rhythm. Sessions of recorded measurements were made by a single investigator to prevent any stress of a different individual that may affect the outcome of the BP measurements.

The warming platform had 3 levels of temperature control; we used Level 2 $\approx 35^{\circ}\text{C}$. The Occlusion (O)-cuff and Volume Pressure Recording (VPR)-cuffs were tested prior to running an experiment. Rats were handled gently and placed in the animal holders (or restraining holders) with a dark nose cone to calm them. Different sized animal holders were used for different sized rats. Two restraining holders were used for obtaining each reading in a single experiment. They were placed in their designated positions on the warming platform maintained at 35°C . Rat tails were carefully threaded/passed through the O-cuff first and then through the VPR-cuff (13 mm long, with a 9 mm diameter) and immobilized by using adhesive tape. Rats were left in animal holders with the tail cuffs on for at least 5 minutes. This allowed the rats to get acclimatized to the holders and cuffs before the actual BP reading started. Each session included two sets of six measurements (one acclimation cycle followed by five reading cycles), so that a total of 10 measurements were used to determine the BP of each rat on a single day. Data were collected at weekly intervals over the 16 week period.

2.2.3.2 Aorta ring Studies

After 16 weeks of the HFD and treatment with Buchu water regime, the rats were anaesthetised by intraperitoneal (IP) injection of sodium pentobarbital given at the dose of 160 mg/kg (Lochner, Genade, & Moolman, 2003). Deep anaesthesia was ascertained by foot pinch before the thoracic cavity was opened and the aorta removed and arrested in ice-cold (4°C) normal Krebs-Henseleit (KH) solution containing; 118.46 mM NaCl; 24.995 mM NaHCO_3^- ; 4.748 mM KCl; 1.185 mM

KH_2PO_4^- ; 1.19 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 10 mM glucose. All the excess debris, blood and surrounding tissue were removed carefully under magnification without damaging the aorta. Extra care was taken to avoid contact with the luminal surface to preserve the endothelial cells. The aorta was cut up in strips of 3-5 mm before it was mounted in an organ bath coupled to a PowerLab system for experimentation.

2.2.3.2.1 Solutions used in the study

A phenylephrine (PE) solution was prepared by adding 0.00204g PE in 10 ml 0.9% saline to give a 1 mM PE stock. An acetylcholine (ACh) solution was prepared by adding 0.0182 g ACh to 10 ml 0.9% saline resulting in a 10 mM ACh stock.

2.2.3.2.2 Aorta rings relaxing/contracting Protocol

Three different concentrations of ACh were prepared from the stock solution before the start of the experiment. The PowerLab system using Lab Chart 7 (ADInstruments, Castle Hill, New South Wales, Australia) software and the incubation system were calibrated using a 2 g weight. The aorta preparation was stabilized in the organ bath for 30 minutes and the buffer changed every 10 minutes to ensure adequate energy substrate supply and removal of waste products. The buffer medium was continuously gassed with 95% O_2 / 5% CO_2 . The tension was kept constant at ≈ 1.5 g during the stabilization phase. The first round of contraction/ relaxation was initiated by adding 2.5 μl of 1 mM PE to the organ bath for final concentration of 100 nM. Once it reached a plateau, 25 μl of the 10 mM ACh stock was added to the organ bath rendering a final concentration of 10 μM ACh. This was done to ensure that the endothelium of the preparation was undamaged, giving a normal contraction/relaxation response.

The organ bath was rinsed twice with fresh buffer. Then the ring was allowed to stabilize again for 30 minutes. The buffer was changed every 10 minutes and the tension kept constant at ≈ 1.5 g.

The second round of contraction/ relaxation was started without rinsing the buffer out in between the addition of PE and ACh. An amount of 25 μl of 1 mM PE was added to the organ bath for contraction, which changed the final concentration to 1 μM . Relaxation, was performed in a stepwise manner, by adding ACh in 5 aliquots and thereby increasing the concentration slowly. This was started by adding 7.5 μl of the 100 μM ACh stock to the organ bath, which gave a concentration of 30 nM. Another 17.5 μl of the 100 μM ACh stock was added to the organ bath giving a concentration of 100 nM then 42.5 μl of the 100 μM ACh stock giving a concentration of 300 nM. A further 14.3 μl of the 1 mM ACh stock was added to the organ bath to given 1 μM and finally 220 μl of the 1 mM ACh stock was added to the organ bath rendering a final concentration of 10 μM ACh. The reading was allowed to stabilize between the additions of the different concentrations of ACh. The % relaxation was calculated after each addition of ACh, so as to determine the functionality of the aorta.

2.2.3.3 Isolation of adult rat cardiomyocytes (ARCM)

2.2.3.3.1 Preparing the stock solutions for the isolation of ARCM

Calcium-tolerant adult ventricular myocytes in an unstimulated state were prepared, using isolation methods previously described by Fisher et al. (1991) subsequently modified in our laboratory (Huisamen, Donthi, & Lochner, 2001). Stock solutions were prepared of the following salts: 120 mM KCl, 20 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 28 mM MgSO_4 , 2.56 M NaCl and a 100 mM CaCl_2 stock solution. These were made up in milli-Q water and autoclaved before use.

To prepare buffer A, twenty five millilitres of each stock solution was used and HEPES salt (1.2465 g to give a final concentration of 10 mM) was added. The pH was adjusted to 7.4 and the solution made up to 500 ml in milli-Q water in a volumetric flask. The following solutions were prepared: (i) A 100 ml buffer A was removed from the volumetric flask and kept to make up Solution E. (ii) Solution A was prepared by adding 5.5 mM D-glucose (0.39635g) and 2 mM pyruvate (0.088g) to the remaining 400 ml. (iii) 0.7% bovine serum albumin (BSA) (0.525 g) was added together with Fatty-acid-free (FAF) BSA, 18 mM 2,3-butanedione monoxime (BDM) (0.134g) and 1.1 mg/ml

collagenase (Worthington type II) and made up to 75 ml with Solution A = Solution B. (iv) For Solution C, 1% BSA (0.25 g), 1% BSA FAF (0.25 g) and 100 μ l CaCl₂ stock were used. This was made up to 25 ml with Solution A and added to 25 ml Solution B rendering a solution containing half the concentration collagenase and BDM. (v) Solution D consisted of 50 ml of Solution A, with 1.25 mM CaCl₂ (625 μ l from the stock) and 1 g BSA FAF. Solution E: 50 ml of buffer A without substrates plus 1.25 mM CaCl₂ and 1 g BSA (FAF).

2.2.3.3.2 Protocol for the isolation of adult rat cardiomyocytes

The isolation procedure is based on Langendorff perfusion with a preload of 100 cm water. The rats were sacrificed by injecting them with 160 mg/kg pentobarbital sodium intraperitoneally. After reaching deep anaesthesia, as determined by lack of response on a foot pinch, the beating heart was removed and immediately arrested in ice cold buffer A. The heart was cannulated via the aorta on a perfusion rig, tied securely and perfused with Solution A for 5 minutes to remove all the excess blood. Hereafter perfusion was switched to Solution B (digestion buffer) and the heart digested in a recirculating manner for 15 minutes. 50 μ l CaCl₂ (100uM) was added and, after another 5 minutes, another 50 μ l giving a CaCl₂ concentration of 200uM. The heart was digested until the perfusate flowed continuously or for a maximum time of 35-40 minutes. After digestion, the heart was removed from the cannula and the atria and connective tissue removed carefully. The ventricles were gently torn apart and incubated in 50 ml Solution C in a shaking water bath at 37°C under O₂ for 15 minutes. The calcium concentration was stepwise taken up to 1.25 mM by adding 4 X 100 μ l plus 125 μ l CaCl₂ stock with one minute intervals. The digested tissue was filtered through a nylon mesh (200 x 200 μ m) and the cells gently spun down at 100 r.p.m. for 3 minutes at room temperature. The supernatant containing non-viable myocytes was aspirated and the pellet suspended in 35 ml Solution D. Viable cells were allowed to sediment for 5 minutes through this solution containing 2% FAF BSA to form a loose pellet. Only the live, healthy cardiomyocytes will settle. The pellet was resuspended in 15 ml Solution D and left at room temperature for at least 1.5 hour under an O₂ atmosphere to slowly rotate on a belly dancer (orbital) laboratory shaker. This provided

the cells with time to recover from the trauma of isolation. This procedure renders in excess of 80% viable cells as measured by trypan blue exclusion. Afterwards, the pellet was allowed to settle under gravity and the supernatant removed. The cells were suspended in solution E and spun down into a loose pellet (3 minutes @ 100 r.p.m.) and washed 3 times with solution E. Cells were finally suspended in a suitable volume of solution E.

2.2.3.4 Determination of 2-deoxy-D-³[H] Glucose (2DG) uptake by adult rat cardiomyocytes

Cardiomyocytes were assayed for their ability to accumulate 2DG in a total volume of 750 µl of oxygenated solution E as previously described (Fischer et al., 1991; Donthi et al., 2000; Huisamen et al., 2001). Cell aliquots were left to equilibrate for 5 minutes in a shaking water bath (180 strokes/minute) at 37°C. Cells were stimulated with or without 1, 10 or 100 nM insulin for 15 minutes where after they were incubated with 1.5 µCi/ml 2DG giving a final concentration of 1.8 µM deoxyglucose, for 30 minutes. This allowed the uptake and accumulation of the deoxyglucose in the cells as deoxyglucose cannot be metabolized. A negative control containing 400 µM phloretin, an inhibitor of carrier-mediated glucose uptake (GLUT-1 and GLUT-4), was included in the assay and this value subtracted from all values. The incubation reaction was also stopped by the addition of 400 µM phloretin. Following this, the cells were microfuged at 1000 x g for 1 minute. The 2DG containing supernatant was aspirated and the cells were washed twice with buffer A. The cell pellet was then dissolved in 1 N NaOH in a water bath at 70°C for 30-40 minutes. Afterwards dH₂O was added to yield a concentration of 0.5 N NaOH.

In order to determine cell-associated radioactivity, 100 µl of the cell lysate was mixed with 3 ml of scintillation fluid and kept overnight in the dark before counting in a beta-scintillation counter (Beckman). The remaining cell lysate was used for determination of protein content by the method of Lowry (Lowry et al., 1951). 2DG concentration was calculated as pmol 2DG/mg protein/30 min.

2.2.3.5 Lowry protein determination

For protein content determination by the method of Lowry (Lowry et al., 1951); three BSA protein standards of known concentration were used and 0.5 N NaOH as the blank. The reaction buffer contained 2% Na₂CO₃, 2% NaK⁺ tartrate and 1% CuSO₄.5H₂O and was prepared directly before the assay. 1 ml of the reaction buffer was added to 50 µl of sample, standard or blank, mixed well and allowed to stand for 10 minutes at room temperature. Afterwards 0.1 ml of Folin-Ciocalteu's phenol reagent (1:2 dilution with distilled H₂O) was added and vortexed. Tubes were permitted to incubate for 30 minutes. This resulted in a deep blue colour development of which the absorbance was read in a spectrophotometer at 750 nm against the blank. The unknown protein concentrations were plotted from the standard curve to determine their protein concentration (Lowry et al., 1951).

2.2.3.6 Intraperitoneal glucose tolerance test (IPPGT)

An intraperitoneal glucose test was performed on the rats at the end of the 16 weeks diet and treatment with Buchu water. After an overnight fast, rats were injected IP with sucrose at 1g/kg body weight. Blood samples were taken by tail prick before glucose administration and 1, 3, 5, 10, 15, 20, 25, 30, 60, 90 and 120 minutes thereafter. Blood glucose content was measured using a commercial available glucometer (GlucoPlus™, Montreal, Canada).

2.2.3.7 Determination of visceral fat content

After the rats were anaesthetised and their organs (heart, kidneys and liver for further studies) were removed, further incisions were made exposing the fat masses in the viscera. The fat was removed and weighed and expressed as a percentage of the rat's body weight.

2.2.3.8 Urine Collection and Analysis

Rats were placed individually in metabolic cages and the urine was collected overnight (24 hour period). This was done during week 8 and 16 of the experimental time frame. The urine was collected and the amount measured to determine possible diuretic effects of the administered Buchu water. The samples were stored at -20°C for further analysis.

2.3 Biochemical Analysis

2.3.1 Western blot analysis

2.3.1.1 Tissue collection

Aortas, which have been dissected and snap frozen in liquid nitrogen immediately after removal from the individual animals, were used for Western blotting.

2.3.1.2 Lysate preparation (protein extraction)

Approximately 3-4 cm (or 20g) of the aorta tissue was pulverised in a liquid nitrogen precooled mortar and pestle. Pulverised tissue was then added to tubes each containing 850 µl of lysis buffer (pH 7.4) containing: 20 mM Tris-HCL (pH 7.4), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM NaCl, 1 mM β-glycerophosphate, 2.5 mM natriumpyrophosphate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM Na₃VO₂, 50 nM NaF, 1% (v/v) Triton X-100, leupeptin (10 µg/ml), aprotonin (10 µg/ml), 1 mM phenylmethyl-sulphonyl fluoride (PMSF, added immediately before use). The same lysis buffer was used for all lysates and proteins. The tissue and lysis buffer were then homogenised mechanically at 4°C with a Bullet Blender™ homogenizer (Next Advance, Inc. USA) using a small scoop of 1.4 mm stainless steel beads in Eppendorf tubes. The homogenization cycle was repeated 3 times for 1 minute with 5 minutes intermediate resting periods. After homogenising the samples, they were spun down in a microfuge (Eppendorf Mini-spin plus, Hamburg, Germany) for 10 minutes @ 15 000 r.p.m. to remove excess matter. Protein content of each sample was determined using the

Bradford determination method. Samples were diluted with lysis buffer to similar protein content and then with a three times Laemmli sample buffer, boiled for five minutes and stored at -20°C until Western blot analysis was performed. Lysates were not kept for longer than two weeks.

2.3.1.3 Bradford protein determination

The method of Bradford (Bradford, 1976) was used to determine the protein content of tissue lysates because of its sensitivity to low concentrations of protein. Bradford reagent composition: (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol, and 8.5% (v/v) phosphoric acid). Bradford reagent stock diluted 1:5 and filtered through 2x filter paper. Diluted bovine serum albumin (BSA) of known concentration (5 mg/ml) was used to generate a standard curve. The BSA was diluted 1:5 (100 µl + 400 µl dH₂O) pipetted into test tubes to render a protein concentration between 1 to 20 µg protein. The volume of BSA pipetted into the test tubes was (µl): 5, 10, 20, 40, 60, and 80, then adjusted to 0.1 ml with Millipore water. The protein lysates was diluted by 1 to 5 (1:5) with dH₂O in order to dilute all the detergents such as Triton X present, which may interfere with the assay. A suitable aliquot (5 µl) of each diluted sample was adjusted to 0.1 ml with dH₂O and 0.9 ml of Bradford reagent was added to the contents in the test tubes. Samples were vortexed and incubated for 15-30 minutes. The absorbance was measured at 595 nm using a spectrophotometer. This was done by using 1 ml cuvettes against a blank prepared from 0.1 ml of Millipore H₂O and 0.9 ml of Bradford reagent. The standard curve obtained in this manner was used to determine the protein concentration in each of the unknown samples. Samples were diluted to fall on the linear portion of the standard curve.

2.3.1.4 Protein separation and transfer

Equal amounts (20-100 µg as indicated) of sample protein from the various fractions were separated on a 12% SDS-polyacrylamide gel with a 4% stacking gel, using the standard Bio-rad Mini-PROTEAN 3 System (Biorad, Life Science group, US). The system was filled with the running buffer (composition: 50 mM Tris, 384 mM glycine,

1.0% (w/v) SDS) and run for 1 hour. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon™ P, Millipore) by electro-blotting. Membranes were stained with Ponceau-S red (reversible staining) for visualisation and verification that the protein bands were transferred to the membrane. The Ponceau-S stained membrane was laser-scanned and a copy saved as proof of equal loading. Membranes were then washed with Tris-Buffered Saline (TBS) containing 0.1% Tween-20 (TBS-Tween). The non-specific binding sites on the membranes were blocked with 5% fat-free milk powder dissolved in TBS-Tween for 1-2 hours at room temperature. Following the blocking procedure, the membranes were washed thoroughly with TBS-Tween and incubated overnight at 4°C with the appropriate primary antibody diluted according to the manufacturer's instructions. After the incubation, the membranes were washed with TBS-Tween to remove the excess primary antibody. The immobilised primary antibody was conjugated with a diluted horseradish peroxidase (HRP)-labelled secondary antibody for 1 hour at room temperature. The membranes were again washed thoroughly with TBS-Tween.

2.3.1.5 Immunoblotting

Protein kinase B (PKB/Akt)

Western blotting was performed as stated above with minor adjustments for specificity. Fractions (20 µg protein) were loaded on a 4% stacking gel and then separated on a 12% SDS-polyacrylamide gel. Membranes were incubated with the total-Akt or the phospho-Akt (Serine 473) primary antibodies (Cell Signaling Technology TM) for 5 – 16 hours at 4°C. Primary antibodies were used at a 1:1000 dilution in TBS-Tween (washing buffer).

eNOS

Gels were loaded with 50 µg of proteins per lane for both eNOS. 4% stacking gel and 7.5% SDS- polyacrylamide gel were used for the separation of the proteins. Membranes were incubated with the primary antibody (5 µl of eNOS in 5 ml TBS-Tween) over night (5-16 hours) at 4°C.

AMP- activated protein kinase (AMPK)

Western blotting was performed as mentioned above with some minor adjustment for specificity. Protein fractions (20 µg protein) were loaded on a 4% stacking gel and separated on a 12% SDS-polyacrylamide gel. Total or phospho-AMPK (Thr 172) primary antibody (Cell Signaling Technology TM) was used to incubate the membrane for 5 – 16 hours at 4°C.

Table 2-2: Western Blot Analysis.

Protein	Molecular weight	Protein loaded	Resolving gel (SDS-PAGE)	Primary antibody dilution	Secondary antibody dilution
PKB/Akt	60 kDa	20 µg	12%	1:1000 TBS-Tween	1:4000 TBS-Tween
eNOS	140 kDa	50 µg	7.5%	1:1000 5% Milk TBS-Tween	1:4000 TBS-Tween
AMPK		20 µg	12%	1:1000 5% Milk TBS-Tween	1:4000 5% Milk TBS-Tween

Table 2-3: SDS-polyacrylamide gel.

Reagent	Stock	12% Gel	7.5% Gel	4% Stack gel
dH ₂ O	distilled	3.35 ml	5.5 ml	3.05 ml
Tris-HCl (pH8.8)	1.5 M	2.5 ml	2.5 ml	--
Tris-HCl (pH8.8)	0.5 M	--	--	1.25 ml
SDS	10%	100 µl	100 µl	50 µl
Acrylamide	40%	3.0 ml	1.875 ml	0.5 ml
APS	10%	50 µl	50 µl	50 µl
TEMED	99%	20 µl	20 µl	10 µl

2.3.1.6 Immunodetection of protein by using a Secondary Antibody

The secondary antibody was used at a 1:4000 dilution in TBS-Tween. After 1 hour incubation, membranes were rinsed 3-4 times for 5 minutes each with TBS-Tween. Proteins were detected by enhanced chemiluminescence by making use of the enhanced chemiluminescence (ECL) detection reagents for 1 minute. The excess ECL detection reagent was drained off the membrane and light emission from the membrane was then captured on autoradiography film (Hyperfilm ECL, RPN 2103-Amersham Bioscience). The ECL reagents react with the horseradish peroxidase coupled to the secondary antibody giving a chemiluminescence emission. The protein band density was quantified by laser scanning densitometry and analysed by using suitable software (UN-SCAN-IT, Silk Scientific Inc., Orem, Utah, US).

2.3.2 Blood Analysis

2.3.2.1 Blood Collection

Blood was drawn after an overnight fast to measure serum glucose and insulin levels. The blood was collected by drawing 1ml of blood directly from the carotid artery. Non-fasting blood were collected on sacrifice of the animals from the thoracic cavity and transferred into serum collection tubes. The blood was allowed to clot on ice and then centrifuged at 3000 r.p.m. at 4°C for 10 minutes. The serum was transferred to Eppendorf tubes and stored at -80°C until further analysis was performed.

2.3.2.2 Enzyme linked immunosorbent assay (ELISA)

2.3.2.2.1 Tumor Necrosis Factor (TNF) - Alpha ELISA

We used a commercially available TNF-alpha ELISA kit (Abcam Ltd, Cambridge, UK) for the quantitative measurement of TNF-alpha in the serum (previously collected and stored) of each rat sample. 100 µl of each standard (20 000 pg/ml, 6667 pg/ml, 2222 pg/ml, 740.7 pg/ml, 246.9 pg/ml, 82.30 pg/ml, 0 pg/ml) and serum samples were pipetted into the wells of a 96-well plate coated with anti-rat TNF-alpha antibody. This was done in duplicate and followed by an incubation period of 2.5 hours at room temperature. TNF-alpha present in the standards and serum samples bound to the wells via the immobilized antibody. All fluid was decanted and the wells were washed thoroughly with wash buffer. 100 µl prepared biotinylated anti-Rat TNF-alpha antibody was added to each well and incubated for 1 hour at room temperature. After 1 hour the unbound biotinylated antibody was washed away with wash buffer. 100 µl prepared horseradish peroxidase (HRP) - streptavidin was pipetted to each well and incubated for 45 minutes at room temperature. After washing the wells again with wash buffer, 100 µl tetramethylbenzidine (TMB) one-step substrate reagent was added to each well and incubated for 30 minutes at room temperature. The colour developed in proportion to the amount of TNF-alpha bound. 50 µl of the Stop solution was pipetted in to each well. The colour changed from blue to yellow. The intensity of the colour was immediately measured on the microplate reader (FLUO-Star Omega, BMG Labtech) at 450 nm.

2.3.2.2.2 C-peptide ELISA

C-peptide levels were determined in previously stored plasma using a commercially available ELISA kit (Abcam Ltd, Cambridge, UK). It is a quantitative assay for detecting C-peptide based on the principle of Competitive Enzyme Linked Immunoassay. The 96-well microplate is pre-coated with anti-rabbit secondary antibody. The 96-wells on the microplate were divided into blanks (NSB) (x2), standards (x12), positive controls (PQ) (x4), and samples (x78). The assay was done in duplicate to ensure accurate measurements and all the wells were repeatedly washed three times with 300 μ l diluted (10x) wash buffer. All reagent and standards were prepared as instructed. 100 μ l anti C-peptide anti-body was added to each well, the plate covered and incubated for 1.5 hours at room temperature on an orbital microtiter plate shaker (1-2 cycles/second). The plate sealer was removed, the plate decanted on tissue paper and all the excess C-peptide antibody solution discarded. The wells were washed three times with 300 μ l diluted wash buffer. Thereafter 100 μ l of samples or standards were added to each well and incubated as instructed for 2.5 hours at room temperature. All solution was discarded and wells washed three times with 300 μ l diluted wash buffer. 100 μ l of prepared horseradish peroxidase (HRP) -streptavidin solution was added to each well and Incubated for 45 minutes at room temperature with gentle shaking. The horseradish peroxidase (HRP) - streptavidin solution was discarded and the wells washed three times with 300 μ l diluted wash buffer. 2nd last step: a 100 μ l tetramethylbenzidine (TMB) one-step substrate reagent was added to each well and incubated for 30 minutes at room temperature in the dark. After 30 minutes the 50 μ l Stop solution was added. Wells were shaken by hand to mix the solution and absorbance was immediately read at 450 nm using a plate reader (FLUO-Star Omega, BMG Labtech). Afterwards C-peptide concentrations were calculated.

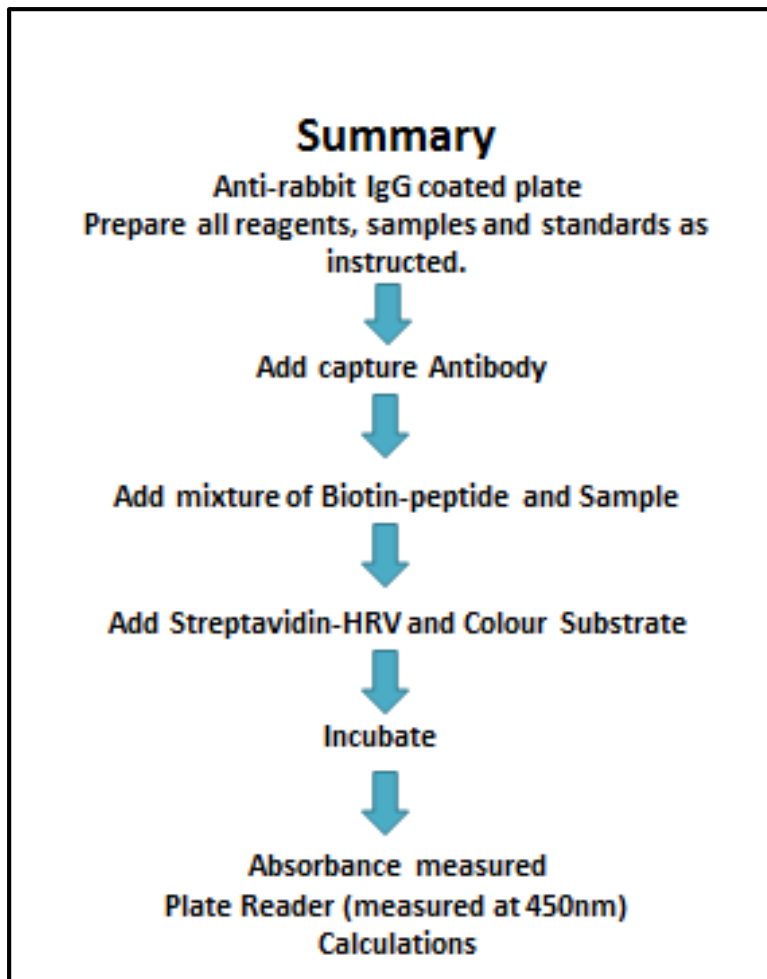


Figure 2-5. C-peptide ELISA Summary.

2.3.2.2.3 Interleukin (IL)-6

The IL-6 ELISA kit (Abcam Ltd, Cambridge, UK) was used for the quantitative measurement of IL-6 in the serum sample (previously collected and stored) of each rat. This *in vitro* enzyme-linked immunosorbent assay contained an anti-rat IL-6 coated 96-well microplate. Standards (10 000 pg/ml, 4000 pg/ml, 1600 pg/ml, 640 pg/ml, 256 pg/ml, 102.4 pg/ml, 40.96 pg/ml, 0 pg/ml) and serum samples were prepared in duplicate. 100 µl of each was pipetted into the wells followed by an incubation period of 2.5 hours at room temperature. IL-6 present in the standards and serum samples bound to the wells via the immobilized antibody. All fluid was decanted and the wells were washed thoroughly with wash buffer. 100 µl prepared biotinylated anti-rat IL-6 antibody was added to each well and incubated for 1 hour at room temperature. The

unbound biotinylated antibody was washed away with wash buffer. 100 µl prepared HRP- streptavidin solution was pipetted to each well and incubated for 45 minutes at room temperature. After washing the wells again with wash buffer, 100 µl TMB one-step substrate reagent was added to each well and incubated for 30 minutes at room temperature. The colour developed in proportion to the amount of IL-6 bounded. 50 µl of the stop solution was pipetted in to each well. The colour changed from blue to yellow. The intensity of the colour was immediately measured on the microplate reader (FLUO-Star Omega, BMG Labtech) at 450 nm.

2.3.2.2.4 Adiponectin ELISA

Rat adiponectin levels were measured using a commercially available rat ELISA kit (Abcam Ltd, Cambridge, UK) which is a quantitative enzyme-linked immunoassay technique. A 96-well adiponectin microplate has been pre-coated with polyclonal antibody specific for adiponectin. Standards (100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.250 ng/ml, 3.125 ng/ml, 1.560 ng/ml, 0 ng/ml) and serum samples were prepared in duplicate. 50 µl of each was pipetted into the wells followed by an incubation period of 1 hour at room temperature. Adiponectin present in the standards and serum samples bound to the wells by the immobilized antibody. All fluid was decanted and the wells were washed 5 X with washing buffer. 50 µl prepared biotinylated anti-rat adiponectin antibody was added to each well and incubated for 1 hour at room temperature. The unbound biotinylated antibody was washed away 5 X with wash buffer. 50 µl prepared horseradish peroxidase (HRP) -streptavidin solution was pipetted to each well and incubated for 30 minutes at room temperature. After washing the wells again 5 X with wash buffer, 50 µl chromogen substrate was added to each well and incubated for 10 minutes (blue colour develops) at room temperature. The colour developed in proportion to the amount of adiponectin bound. 50 µl of the stop solution was pipetted in to each well. The colour changed from blue to yellow. The intensity of the colour was immediately measured on the microplate (FLUO-Star Omega, BMG Labtech) reader at 450 nm.

2.3.2.2.5 Leptin ELISA

The commercially available Leptin ELISA kit (Abcam Ltd, Cambridge, UK) was used for the quantitative measurement of leptin in the serum (collected and stored previously) of each rat. The 96-well microplate coated with anti-rat leptin antibody was divided according to the number of standards and samples (blanks (NSB) (x2), standards (x12), positive controls (PQ) (x4), and samples (x78)). Standards (8000 pg/ml, 2667 pg/ml, 888.9 pg/ml, 296.3 pg/ml, 98.77 pg/ml, 32.92 pg/ml, 10.97 pg/ml, 0 pg/ml) and serum samples were prepared in duplicate. 100 µl of each was pipetted into the wells followed by an incubation period of 2.5 hours at room temperature. Leptin present in the standards and serum samples bound to the wells by the immobilized antibody. All fluid was decanted and the wells were washed thoroughly with wash buffer. 100 µl prepared biotinylated anti-rat leptin antibody was added to each well and incubated for 1 hour at room temperature. The unbound biotinylated antibody was washed away with wash buffer. 100 µl prepared HRP- streptavidin was pipetted to each well and incubated for 45 minutes at room temperature. After washing the wells again with wash buffer, 100 µl TMB one-step substrate reagent was added to each well and incubated for 30 minutes at room temperature. The colour developed in proportion to the amount of leptin bound. 50 µl of the stop solution was pipetted in to each well. The colour changed from blue to yellow. The intensity of the colour was immediately measured on the microplate reader (FLUO-Star Omega, BMG Labtech) at 450 nm.

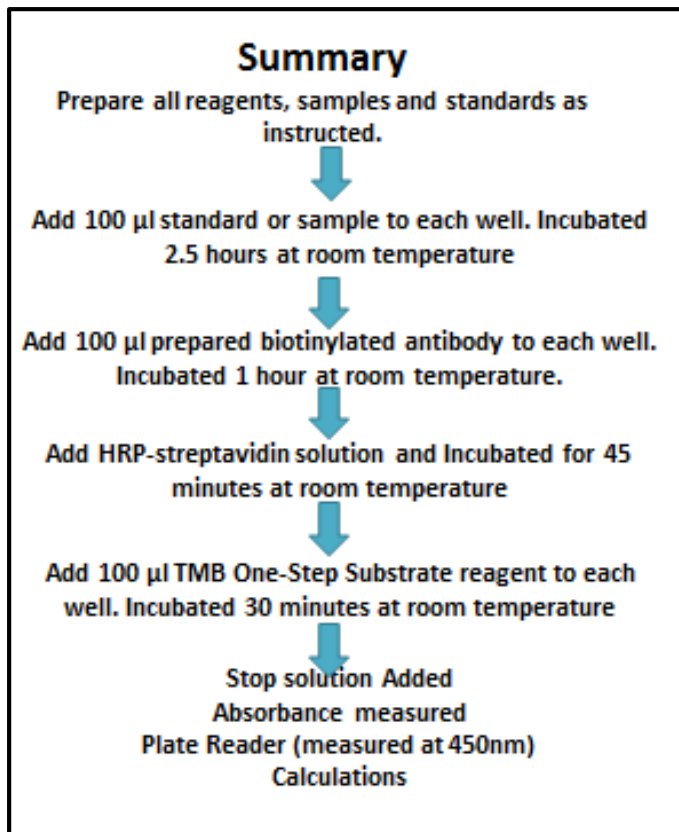


Figure 2-6: Leptin ELISA Summary.

2.3.3 Radioimmunoassay Assays

Plasma insulin and ALD levels were determined in the fasting state. Blood was collected from the animals while they were sedated. Plasma was stored at -80°C in a Snijders Scientific Ultracool (Tilburg, the Netherlands). Insulin and ALD was determined by radioimmunoassay (RIA).

Radioimmunoassay principle:

The RIA technique is one of the more superior analytic procedures with regards to sensitivity, precision, specificity and experimental simplicity. It greatly helped in diagnosing and advancing our understanding of endocrine physiology (Eckert & Strecker, 1978). Radioimmunoassay is a very sensitive technique used to measure the concentrations of antigens (hormone levels in the blood) by the use of antibodies. A fixed concentration of labelled tracer antigen is incubated with a constant dilution of the antiserum so that the concentration of the antigen binding to the sites of the

antibodies is limited. If unlabelled antigen is added to the system, there is competition between the labelled tracer and the unlabelled antigen for the limited and constant number of binding sites on the antibody. Thus the large amount of tracer antibody will decrease as the concentration of unlabelled antigen increases. After the separation of the free and bound antibodies this can be measured. In the case of a hormone, that specific hormone would compete with the labelled hormone in the assay for that specific antibody's binding sites. The radioactivity is measured at the end using a gamma scintillation counter (Perkin Elmer 1470 Auto-gamma counter).

2.3.3.1 Radioimmunoassay procedure for Insulin

All the components were brought to room temperature before the assay was performed (15 - 28°C), as instructed by the manufacturers. Fasting serum was collected as described above. Insulin levels were determined using a competitive RIA (Count-A-Count® Insulin Diagnostics Corporation; LA, USA). All the samples were analysed in duplicate during the assay. Uncoated polypropylene tubes were labelled for total counts (T) and non-specific binding (NSB). Insulin antibody coated tubes were labelled for the 7 standards (A to G), controls and samples. 200 µl of the remaining zero calibrator, control and samples were pipetted in their respective tubes directly to the bottom. 1.0 ml of ¹²⁵I insulin was added to each tube and subsequently vortexed and incubated for 22- 24 hours at room temperature. The tubes were then decanted, except for the total counts and allowed to drain for 3-5 minutes. Each tube was struck on absorbent paper to remove moisture inside the tube. Excess liquid was blotted from the tubes. Removal of the excess moisture ensures a more precise assay. The radioactivity of each individual tube was measured in a gamma scintillation counter (Perkin Elmer 1470 Auto-gamma counter) for 1 minute. The standard curve was included to determine the concentration of the unknown samples in the tubes.

2.3.3.2 Radioimmunoassay procedure for Aldosterone

All the components were brought to room temperature before the assay was performed (15 - 28°C), as by instruction by the manufactures. All the samples were

analysed in duplicate during the assay. Aldosterone levels were determined using a competitive RIA (Count-A-Count® Aldosterone Diagnostics Corporation; LA, USA). Uncoated polypropylene tubes were labelled for total counts (T) and non-specific binding (NSB). Aldosterone antibody coated tubes were labelled for the 7 standards (A to G), controls and samples. 200 µl of the remaining zero calibrator, control and samples were pipetted in their respective tubes directly to the bottom. 1.0 ml of ¹²⁵I ALD was added to each tube and subsequently vortexed and incubated for 18 hours at room temperature. The tubes were then decanted, except for the total counts and allowed to drain for 3-5 minutes. Excess liquid was blotted from the tubes and the radio activity of each individual tube was measured in a gamma scintillation counter (Perkin Elmer 1470 Auto-gamma counter) for 1 minute. The standard curve was included to determine the concentration of the unknown samples in the tubes.

2.4 DATA ANALYSIS /STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of the mean (SEM). For multiple comparisons, the ANOVA (two-way when appropriate) followed by the Bonferroni correction was applied. A p-value of < 0.05 was considered significant. The methods used for all of the BP analyses were: Repeated measures analysis of variance (ANOVA) using a mixed models estimation method. Furthermore, descriptive statistics were performed by Dr. Justin Harvey (Ph.D. - Mathematical Statistics) at the Centre for Statistical Consultation (CSC), University of Stellenbosch.

CHAPTER 3: Results - Using a HFD to elicit high blood pressure in animals to study the effects of chronic Buchu water treatment on hypertension.

(This diet contains 40% fat as well as added sucrose, fructose and cholesterol).

3.1 Biometric Data (parameters)

The body weight of control (C) rats averaged 377.533 ± 8.01 g. The high fat diet (HFD) increased the body weight to 449.467 ± 13.25 ($p < 0.0001$) over a period of 16 weeks (Figure 3-7). Following the 16 weeks on diet, untreated HFD animals were significantly bigger than the untreated control animals and we could therefore say that these animals were obese (449.467 ± 13.25 vs. 377.533 ± 8.01 g, $p < 0.0001$).

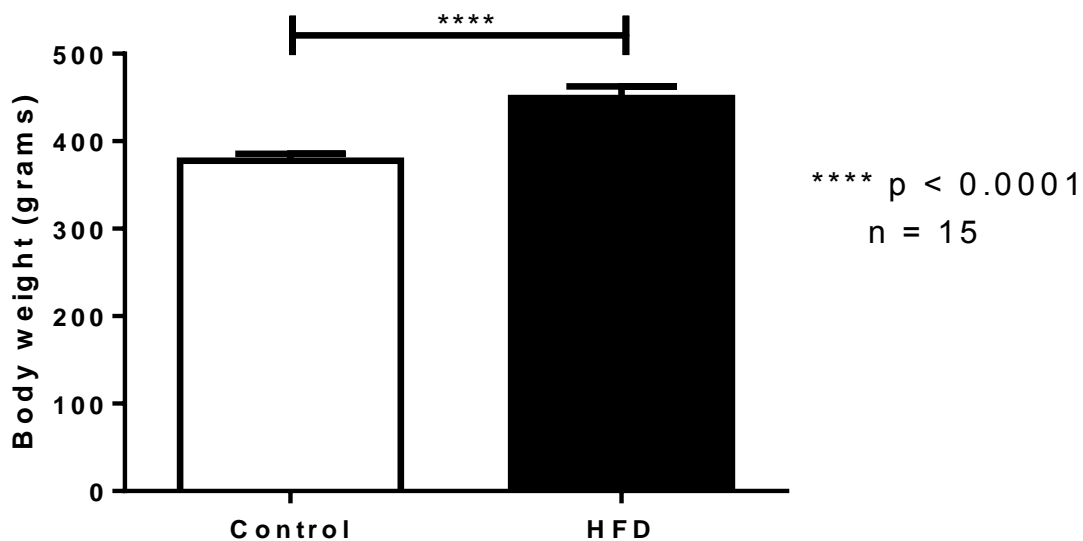


Figure 3-7: Body weight. Shown is the mean body weight (g) after 16 weeks of their respective diets. n = 15 per group.

Table 3-1: Biometric Data - Model 1: HFD Animals.

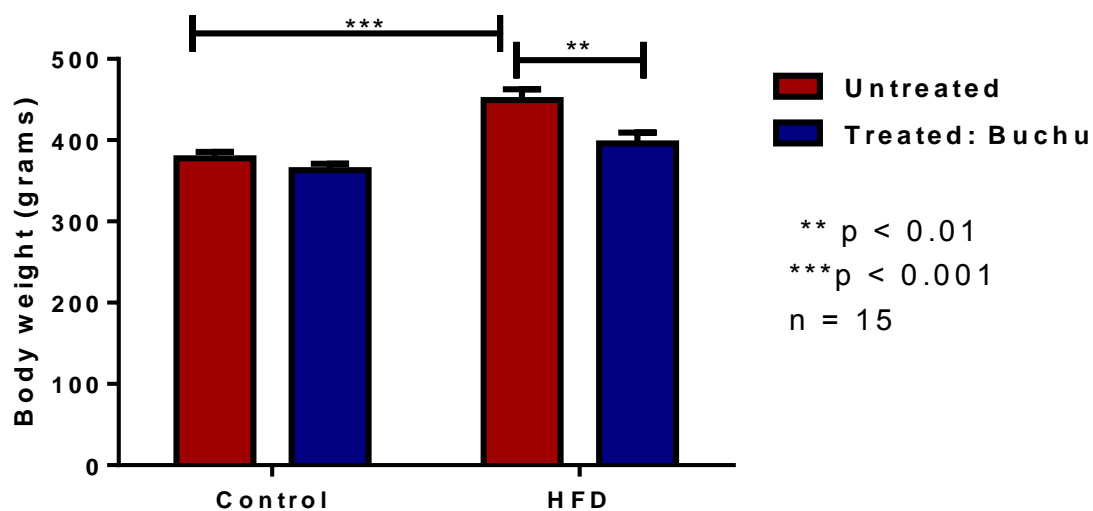
Parameters	Control	Control + B	HFD	HFD + B
Body weight (g)	377.53 ± 8.01	363.07 ± 8.11	449.47 ± 13.25***	395.87 ± 13.35**
Intraperitoneal fat (g)	8.81 ± 0.33	8.56 ± 0.58	24.35 ± 2.40***	15.39 ± 1.07**
Fasting Blood glucose (mmol/l)	6.64 ± 0.22	5.73 ± 0.16*	5.99 ± 0.19	6.54 ± 0.19
Fasting serum Insulin (µIU/ml)	15.77 ± 4.01	7.16 ± 6.07	11.03 ± 3.90	17.78 ± 11.82
C-peptide (ng/ml)	523 ± 75.22	328 ± 74.16	363.3 ± 63.68	685 ± 100.8*
HOMA-IR index	5.48 ± 0.99	2.65 ± 1.09	4.39 ± 0.91	21.45 ± 12.25
<p>* p < 0.05; HFD vs. HFD + B, C vs. C + B. n = 7 per group</p> <p>**p < 0.01; HFD vs. HFD + B. n = 15 per group</p> <p>***p < 0.001; C vs. HFD, n = 15 per group</p>				

C, control diet; C + B, control diet treated with Buchu water from day 1; HFD, high fat diet ; HFD + B, high fat diet treated with Buchu water from day 1; Homeostasis model assessment of insulin resistant (HOMA-IR); n = 15 per group for body weight, IP fat and fasting blood glucose levels. n = 7 per group for fasting serum insulin levels, C-peptide levels and the HOMA-IR index.

3.1.1 Body weight

The chronic administration of Buchu water significantly reduced body weight gain in the HFD group (395.87 ± 13.35 vs. 449.47 ± 13.25 g, p < 0.01) (

Table 3-1) (Figure 3-8). The weight gain of the animals on the HFD was 19% vs. only 4.8% weight gain in the animals on the HFD that also received Buchu water. There was no significant difference in weight gain between the control groups. According to a two-way ANOVA, the effect of Buchu on body weight was significant with a $p < 0.005$, $n = 15$ per group. Buchu water treatment for 16 weeks leads to a reduction in weight gain in both the control and HFD group however this is only significant in the HFD group.



Effect of Buchu Treatment: $p < 0.005$

Figure 3-8: Body weight. Shown is the BW measured in grams (g) of treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. $n = 15$ per group.

3.1.2 Intraperitoneal fat mass

After 16 weeks on the diet, HFD fed rats had greater amounts of visceral (IP) fat than their control counterparts (24.35 ± 2.40 vs. 8.81 ± 0.33 g, $p < 0.0001$) (

Table 3-1) (Figure 3-9). These data indicate that the HFD rats had central obesity.

Animals on the HFD gained 176.7% visceral fat while animals on the HFD that received Buchu water, only gained 76.9% visceral fat. According to a two-way ANOVA, the effect of Buchu on IP fat weight was significant with a $p < 0.001$, $n = 15$ per group. There was no significant difference between the control and the control plus Buchu, however there was a significant difference between the HFD group and the HFD group with Buchu treatment respectively (24.35 ± 2.40 vs. 15.39 ± 1.07 g, $p < 0.0001$) (Figure 3-10).

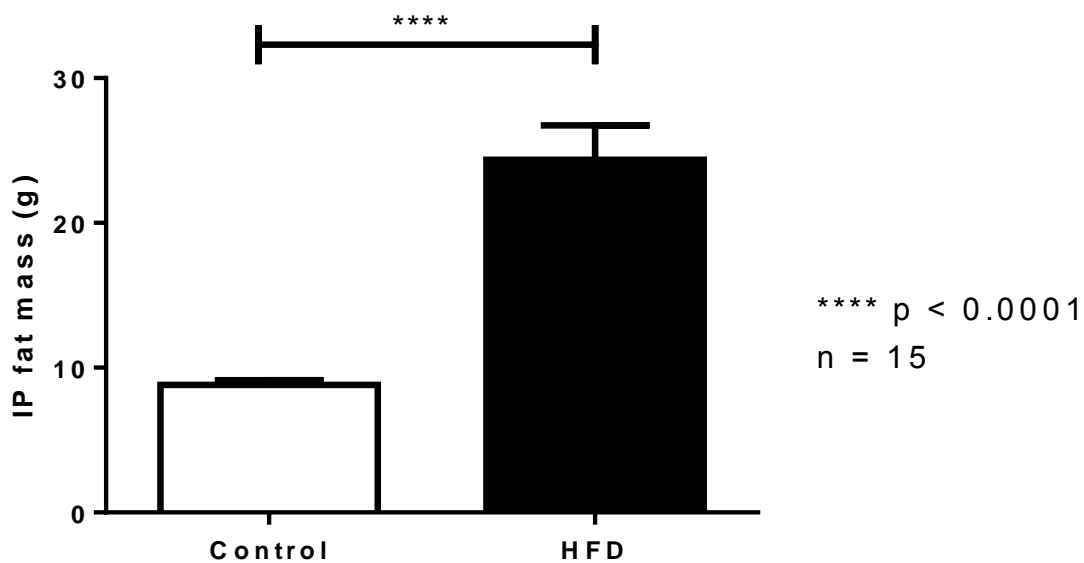


Figure 3-9: Intraperitoneal (IP) Fat. Shown is the IP fat mass measured in grams (g) of the control and high fat diet (HFD) animals. $n = 15$ per group.

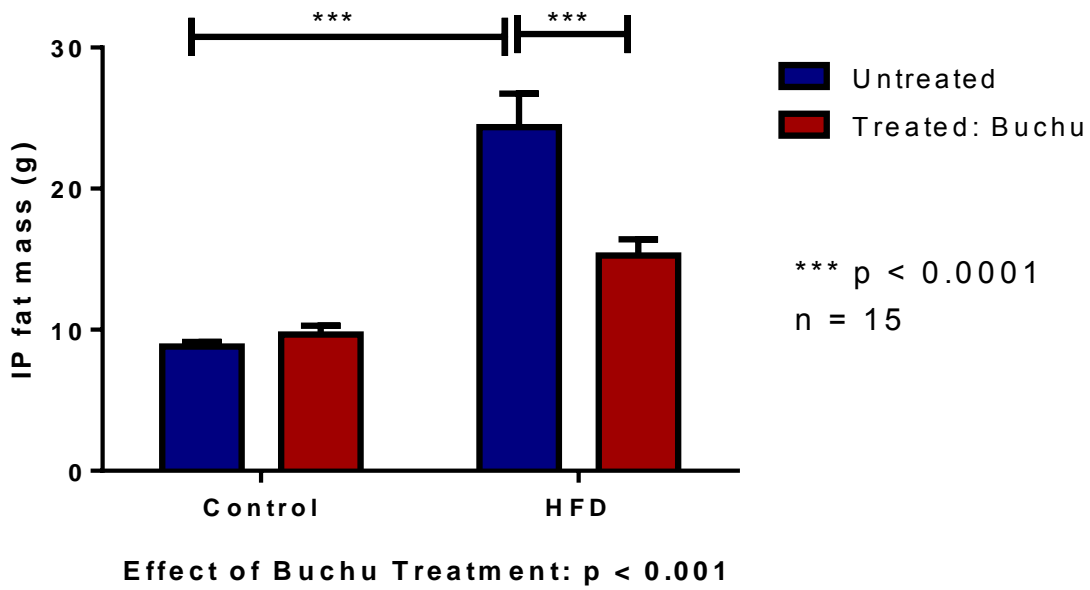


Figure 3-10: IP fat mass: Shown is the IP fat mass measured in grams (g) of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. n = 15 per group.

3.1.3 Fasting blood glucose

Following the 16 weeks on diet, fasting blood glucose levels were significantly higher in the untreated control group compared to the treated control group (Figure 3-11). There was however no significant difference between control and HFD or the treated control and treated HFD animals (Figure 3-11) (

Table 3-1).

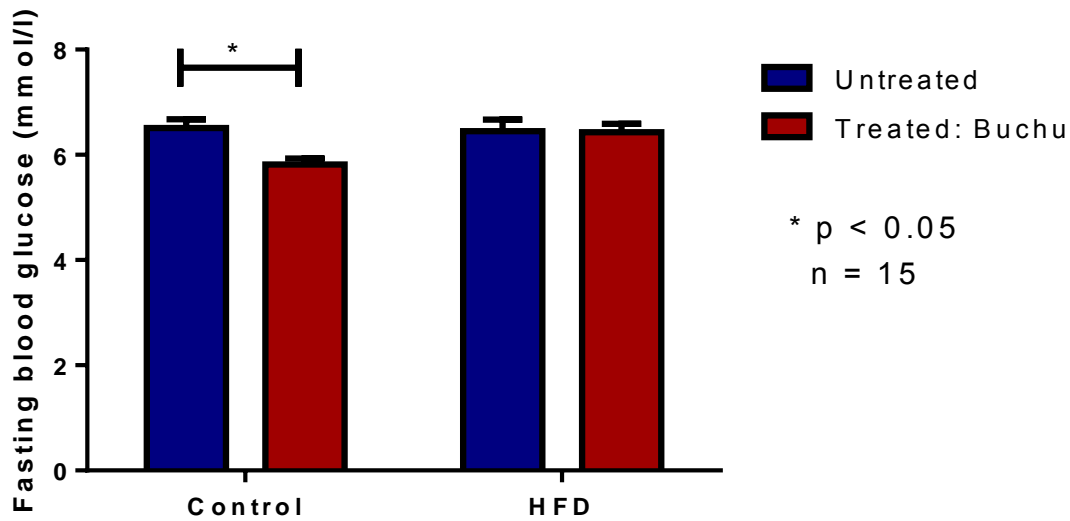


Figure 3-11: Fasting blood glucose levels. Shown are the fasting blood glucose levels of treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu. n = 15 per group.

3.1.4 Effect on fasting plasma insulin levels

In Figure 3-12 it is clear that there was no significant difference between the individual groups. There was unfortunately a very large standard error in the insulin assay; however it shows a trend for higher insulin levels in the obese animals that were treated with Buchu water.

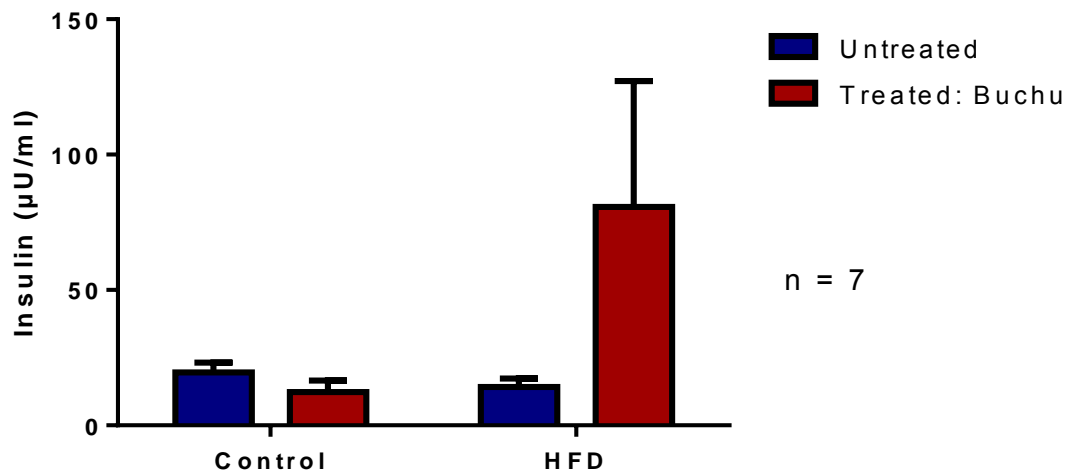


Figure 3-12: Fasting Serum Insulin levels. Shown are the fasting serum insulin levels of treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu. $n = 7$ per group.

3.1.5 C-peptide levels

The C-peptide levels of the treated HFD animals was significantly higher than the untreated HFD animals (685.17 ± 100.77 vs. 363.29 ± 63.68 , $p < 0.05$) (

Table 3-1) (Figure 3-13) underscoring the effect seen on the insulin levels.

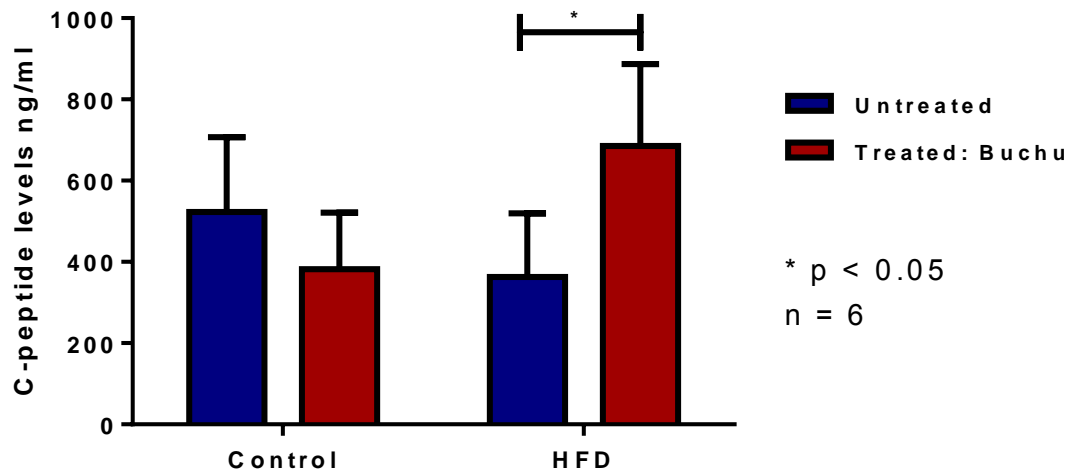


Figure 3-13: C-peptide levels. Shown are the C-peptide levels determined by an assay of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. n = 6 per group.

3.1.6 Homeostasis model assessment (HOMA) Index

HOMA insulin resistance (HOMA-IR) index values of treated and untreated HFD and control animals after 16 weeks of treatment with Buchu water was calculated. This was done to quantify the insulin resistance and beta cell function in the animals. Neither the Buchu water nor diet had a significant effect on the HOMA-IR index in the different animal groups (Figure 3-14). Values are shown in

Table 3-1.

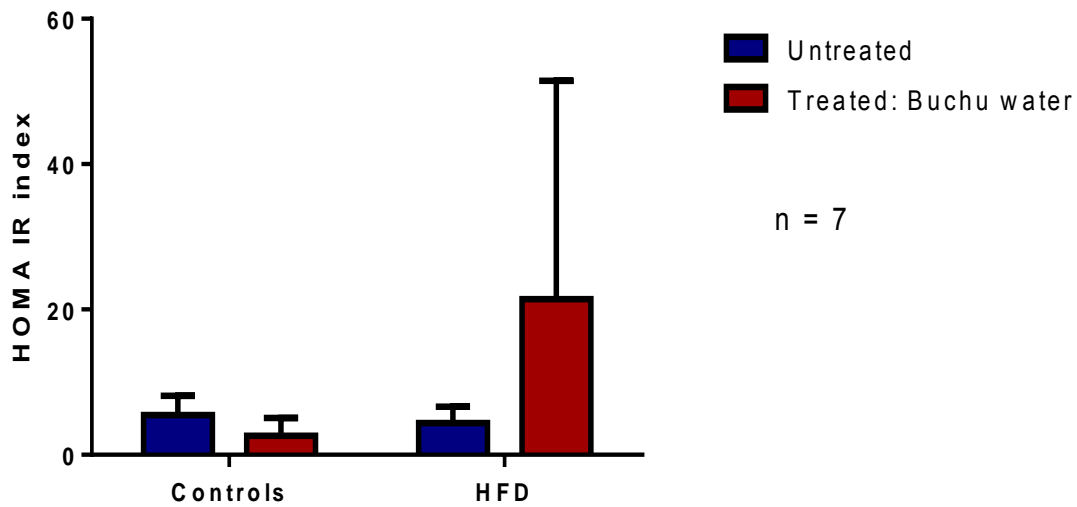


Figure 3-14: HOMA-IR index values of the treated and untreated HFD and control animals after 16 weeks of treatment with Buchu water. n = 7 per group.

3.2 Intraperitoneal glucose tolerance test (IPGTT)

Figure 3-15 shows that, following the intraperitoneal injection with 1g/kg of sucrose, the plasma glucose of both treated and untreated HFD and control animals increased similarly from a base line value 6.3 ± 0.18 mmol/l to 6.5 ± 0.9 mmol/l in the first 10 minutes, before slowly declining to 5.29 ± 0.11 mmol/l after 120 minutes. There was no difference in the glucose levels between the four groups at baseline, 30, 60 and 120 minutes post-glucose load time points.

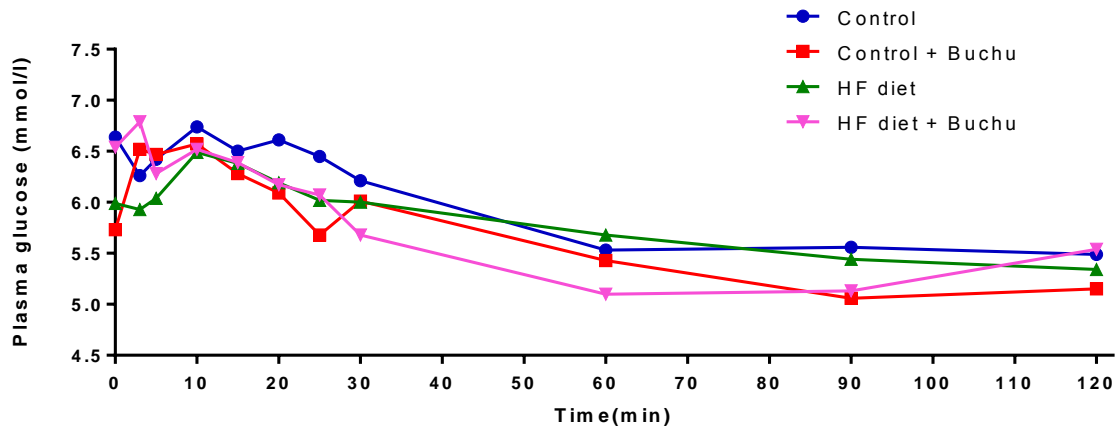


Figure 3-15: The response of plasma glucose to IPGTT of the respective groups. Sucrose was administered by IP injection (1g/kg) and the blood glucose levels measured over a 120 min period. n =15 per group.

3.3 Food intake

Figure 3-16 shows the mean food intake per cage, each with 5 rats, for each group during the 16 weeks of the study. There was no difference observed in the amount of food consumed by the different groups.

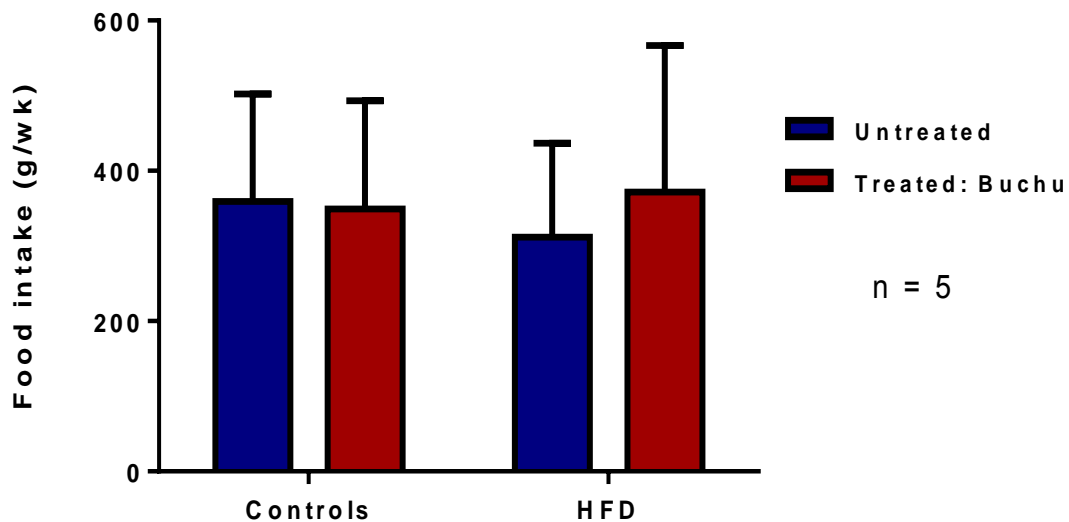


Figure 3-16: Food intake. Shown is the food consumption or intake per cage of the treated and untreated high fat diet (HFD) and control animals over the 16 weeks of treatment with Buchu water. n = 5 per group.

3.4 Metabolic data

3.4.1 Leptin

Serum leptin levels were significantly reduced in the treated HFD animals when compared to the untreated HFD animals (2274.5 ± 472.2 vs. 4641.63 ± 594.3 pg/ml, $p < 0.05$) (Figure 3-17) while there was no significant difference between the leptin levels of the treated and untreated control animals (4425.17 ± 590.84 vs. 3106.0 ± 535.97 pg/ml) (Figure 3-17). According to a two-way ANOVA, the effect of Buchu treatment on leptin levels in non-fasting plasma was significant with a $p = 0.006$, $n = 5$ per group.

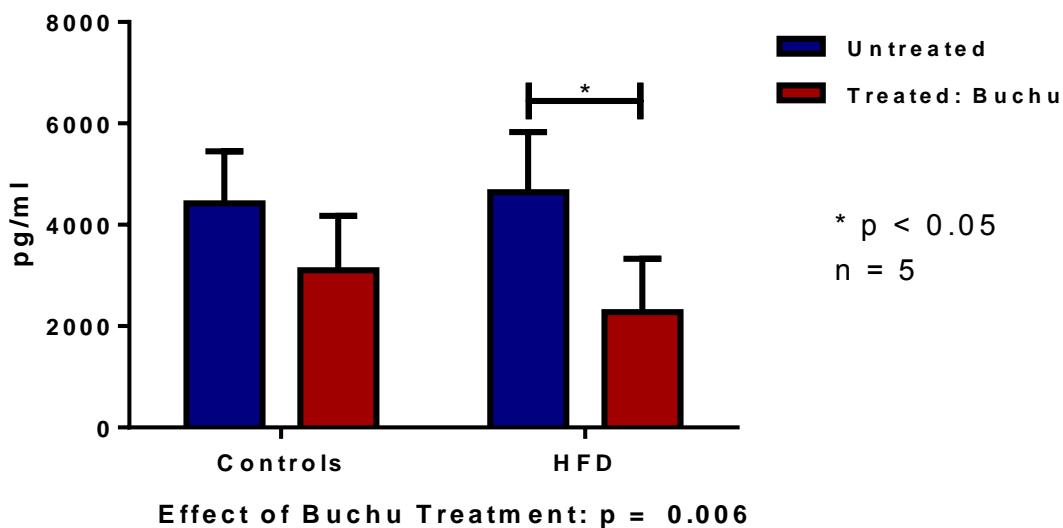


Figure 3-17: Leptin. Shown is the leptin level in non-fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. $n = 5$ per group.

3.4.2 Adiponectin

As can be observed in Figure 3-18, when the amount of adiponectin in the different serum samples was compared, there was no significant difference observed between the different groups.

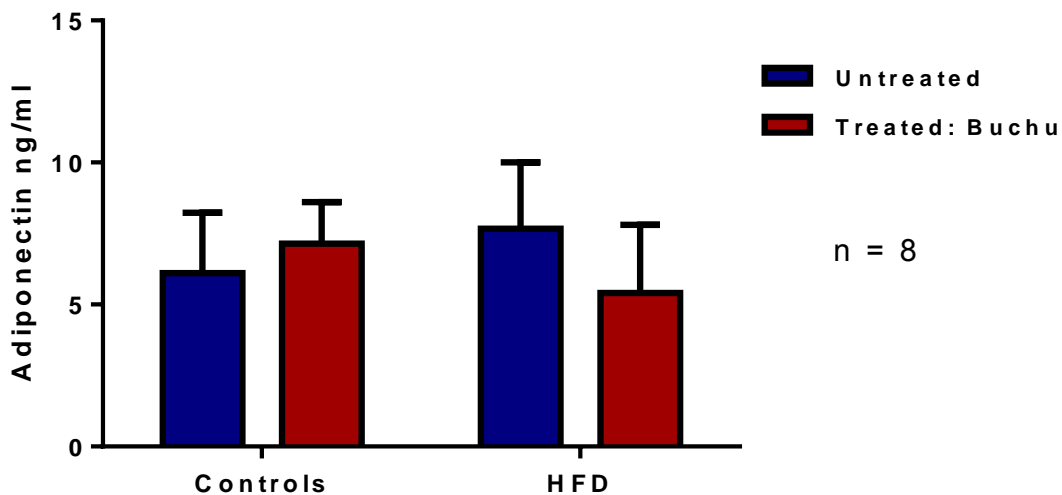


Figure 3-18: Adiponectin. Shown is the adiponectin level in non-fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. $n = 8$ per group.

3.4.3 Tumor necrosis factor (TNF) – alpha

When the Serum TNF-alpha levels were compared they were the same across all the groups (Figure 3-19). There was no significant difference between the control group and HFD group (399.17 ± 34.18 vs. 404.73 ± 25.17 pg/ml) (Figure 3-19). There was also no significant difference between the treated HFD and untreated HFD group (429.82 ± 17.92 vs. 404.73 ± 25.17 pg/ml) (Figure 3-19). These results argue against an inflammatory response in the HFD animals.

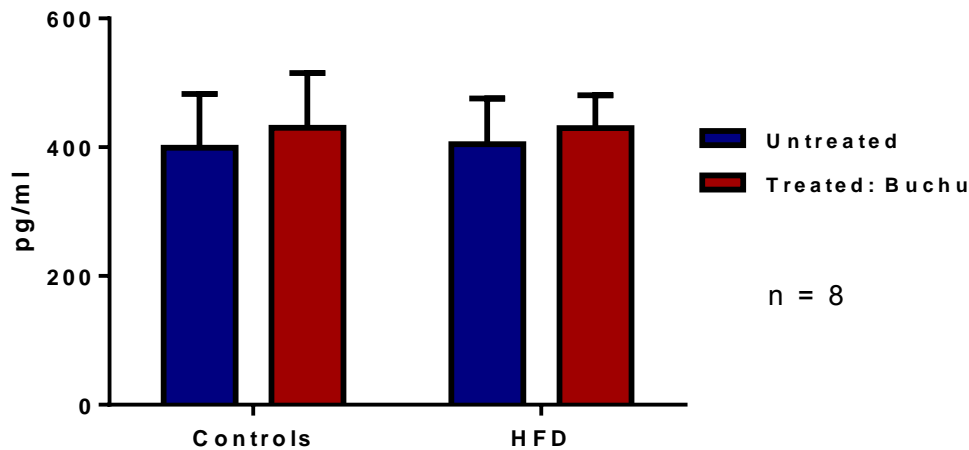


Figure 3-19: TNF-alpha. Shown are the TNF-alpha values in fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. *n* = 8 per group.

3.4.4 Interleukin (IL)-6

Figure 3-20 compares the amount of IL-6 in non-fasting serum of each of the individual groups. We found no significant difference between of the treated or untreated groups as well as when we compared the control group to the HFD group (1075.6 ± 65.83 vs. 1004.34 ± 37.3 pg/ml) (Figure 3-20). Similar to the TNF-alpha levels, this argues against any inflammatory response in the HFD animals.

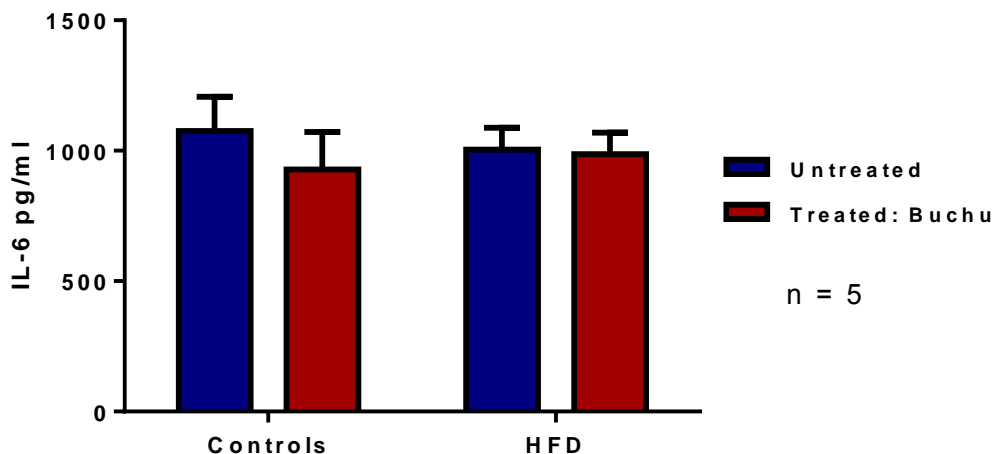


Figure 3-20: Interleukin (IL)-6. Shown is the adiponectin level in non-fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. *n* = 5 per group.

3.4.5 Homocysteine

Comparing the homocysteine levels in the serum of the different groups, there was no significant difference between the treated HFD and untreated HFD as well as between the respective controls. However when a two-way ANOVA was conducted it showed that the diet did have a significant effect on the homocysteine values ($p < 0.001$) (Figure 3-21).

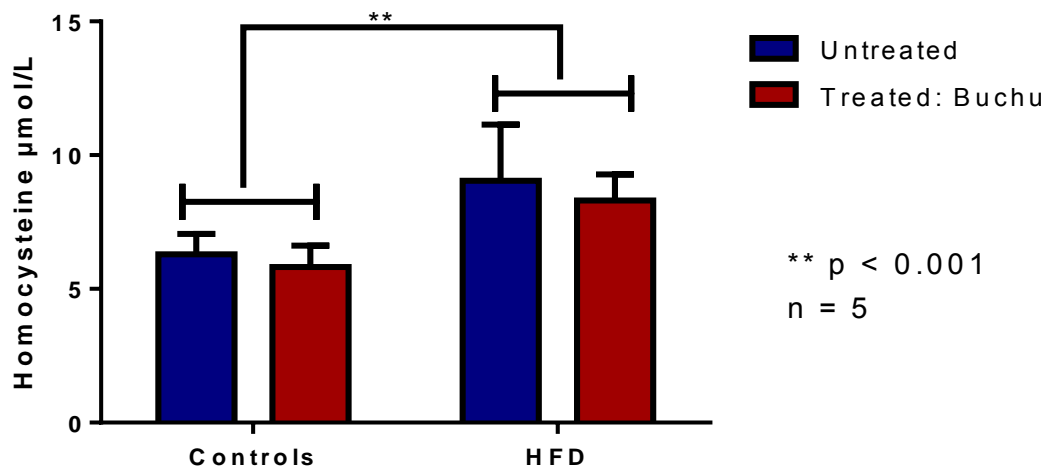


Figure 3-21: Homocysteine. Shown are the homocysteine levels in non-fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. $n = 5$ per group.

3.4.6 Aldosterone

After 16 weeks of chronic Buchu treatment we observed a significant reduction in the aldosterone levels found in the non-fasting serum in the treated HFD animals when compared to the untreated HFD (259.77 ± 65.48 vs. 619.88 ± 136.15 pg/ml, $p < 0.05$) (Figure 3-22). There was also a significant difference between the control group and the HFD group (119.24 ± 19.08 vs. 619.88 ± 136.15 pg/ml, $p < 0.05$), when compared to each other. Using a two-way ANOVA, the effect of diet on aldosterone was significant with a $p < 0.001$, $n = 6$ per group.

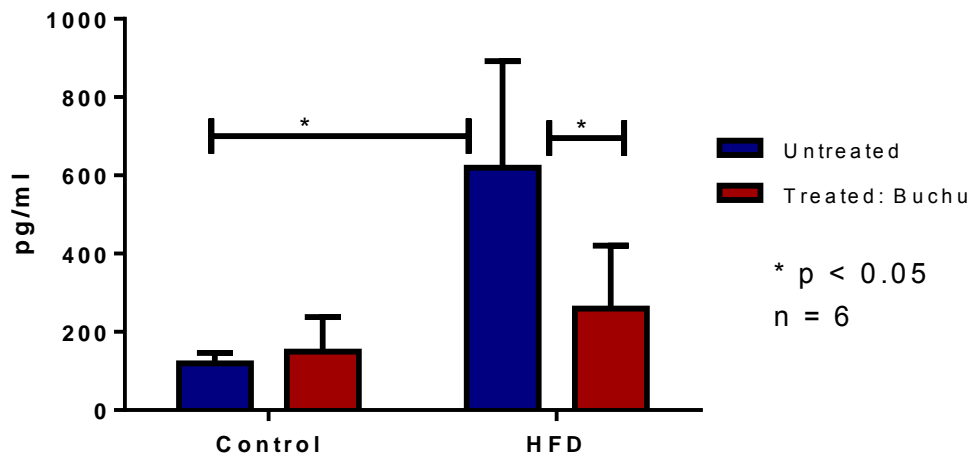


Figure 3-22: Aldosterone. Shown are the aldosterone levels in non-fasting serum of the treated and untreated high fat diet (HFD) and control animals after 16 weeks of treatment with Buchu water. $n = 6$ per group.

3.5 Water and Buchu water consumption

Figure 3-23 shows the mean water or Buchu water consumption per cage containing 5 animals with 2 cages in each group, during the 16 weeks of the study. The only significant difference observed was in the amount of water or consumed by the treated control group compared to the untreated control group (168.75 ± 4.919 vs. 143.25 ± 4.884 ml, $p < 0.05$) (Figure 3-23).

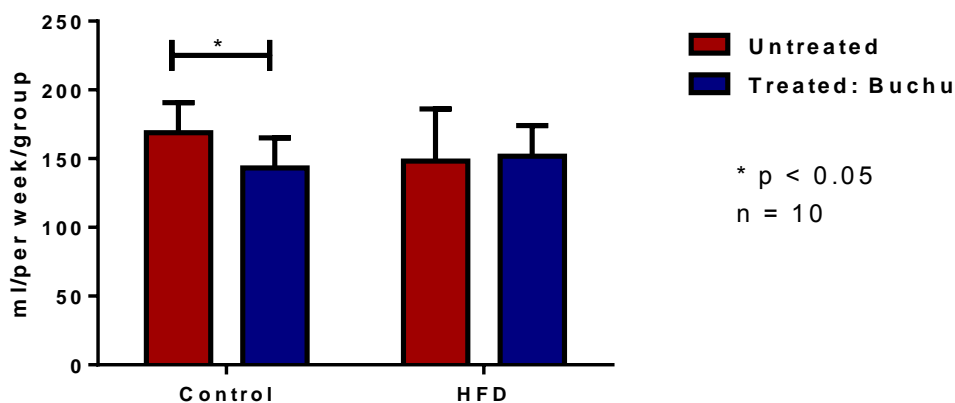


Figure 3-23: Water and Buchu Consumption. Shown is the water consumption or Buchu water intake per cage of the treated and untreated high fat diet (HFD) and control animals over the 16 weeks of treatment with Buchu water or water respectively. $n = 5$ per group.

3.6 Effects on urine production (Urine Output)

At different times during the 16 weeks of treatment, the urine output of the animals was measured over a period of 24h, by keeping them separately in metabolic cages. At 8 weeks of treatment, the urine output of animals on the control diet with Buchu water was significantly higher when compared to control animals receiving tap water (17.55 ± 1.632 vs. 11.33 ± 0.03 ml, $p < 0.01$) (Figure 3-24) while animals from the HFD groups had a significant lower urine output according to a two-way ANOVA (9.53 ± 1.16 ml, $p < 0.0001$).

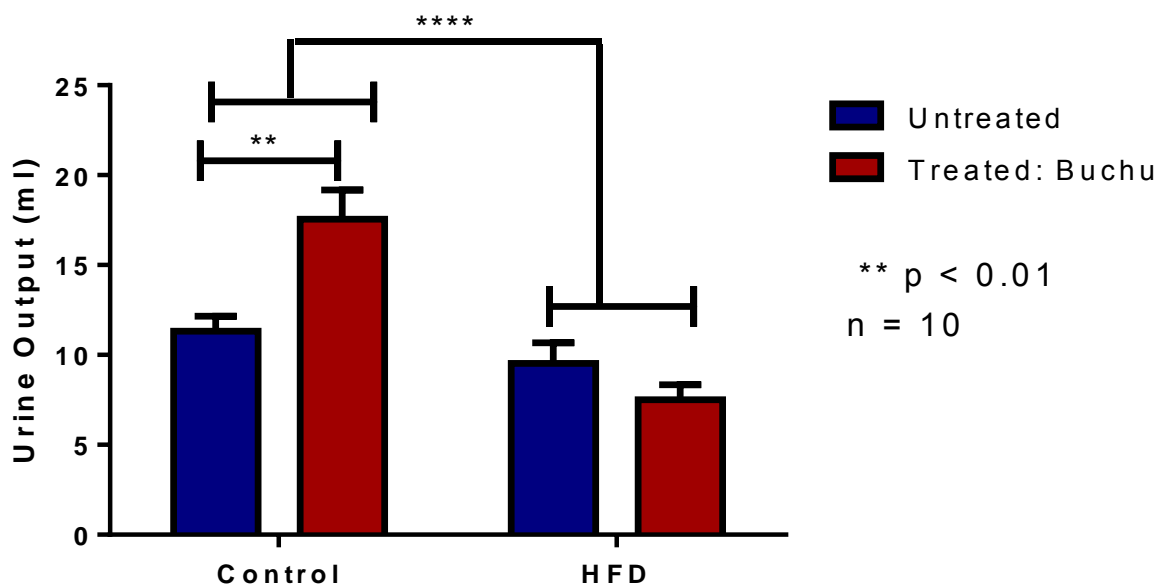


Figure 3-24: Urine Output 8 weeks. After 8 weeks on the diet plus Buchu treatment the individual rats were placed in metabolic cages as described in the Methods to measure the urine output over a 24 hour period. $n = 10$ per group.

After 16 weeks of treatment, the urine output of the untreated HFD group was significantly lower when compared to the control group (4.11 ± 0.9 ml vs. 11.0 ± 0.87 ml, $p < 0.05$). In addition, the urine output of the treated HFD animals was significantly lower than the treated control animals (5.6 ± 0.98 ml vs. 16.77 ± 1.22 ml, $p < 0.001$).

There was no significant difference between the treated and untreated HFD groups but a significantly elevated urine output between the untreated and treated control groups were observed (11.0 ± 0.87 ml vs. 16.77 ± 1.22 ml, $p < 0.01$) (Figure 3-25). In addition, according to a two-way ANOVA, the effect of the HFD was highly significant ($p < 0.0001$), indicating water retention in these animals, with or without consumption of Buchu water.

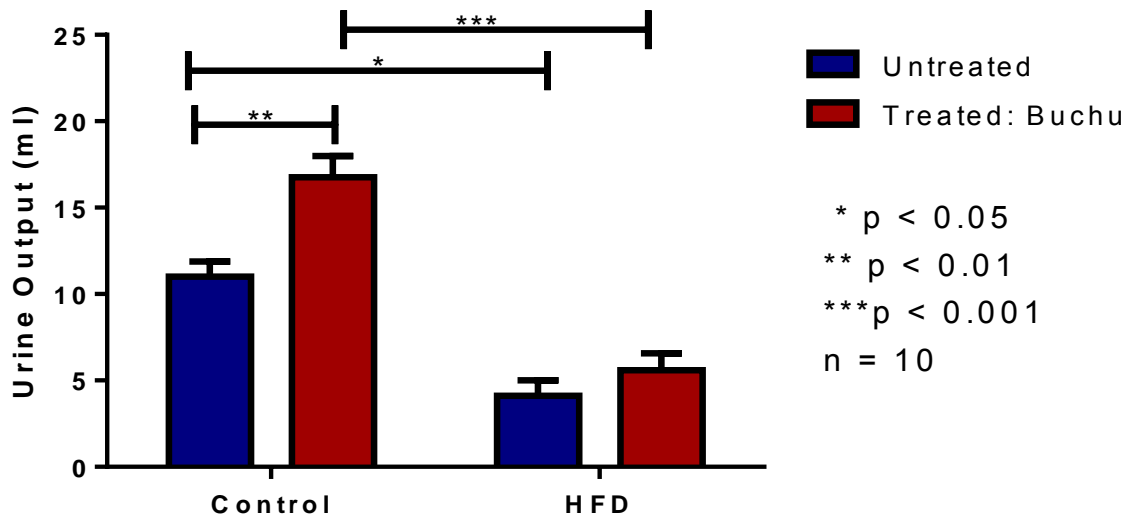


Figure 3-25: Urine Output. After the 16 week diet plus Buchu treatment the individual rats were placed in metabolic cages as described in the Methods section to measure the urine output over a 24 hour period. n = 10 per group.

3.7 Antihypertensive effects of Buchu administration:

3.7.1 Systolic BP

We used the HFD to cause obesity and induce HTN in the animals. As shown in Figure 3-26 the BP of the five groups of animals was not different at the commencement of the study. After only 4 weeks of the HFD, the onset of HTN can already be seen (Figure 3-26 & Figure 3-27). These animals then developed a significant elevation of their BP within 8 weeks (HFD 147.21 ± 5.0 vs. Control 125.01 ± 3.1 mmHg, $p < 0.0001$, n = 10 animals per group). We either pre-treated the animals with Buchu water, starting at

day one, or we allowed the animals to become severely hypertensive until a later stage (week 8) and then started the Buchu treatment.

As can be seen in Figure 3-26 Buchu water consumption prevented the development of systolic HTN when given in conjunction with the HFD when compared to the HFD alone (125.94 ± 12.01 vs. 157.11 ± 8.73 mmHg, $p < 0.0001$). Buchu water treatment did not affect the BP of the animals on the control diet (122.44 ± 8.2 mmHg). In addition, treatment of already hypertensive animals (week 8) with Buchu water normalised their BP within two weeks from 153.1 ± 8.01 to 122.51 ± 10.73 mmHg (Figure 3-26). Buchu presents both preventative measures and treatment properties for systolic HTN.

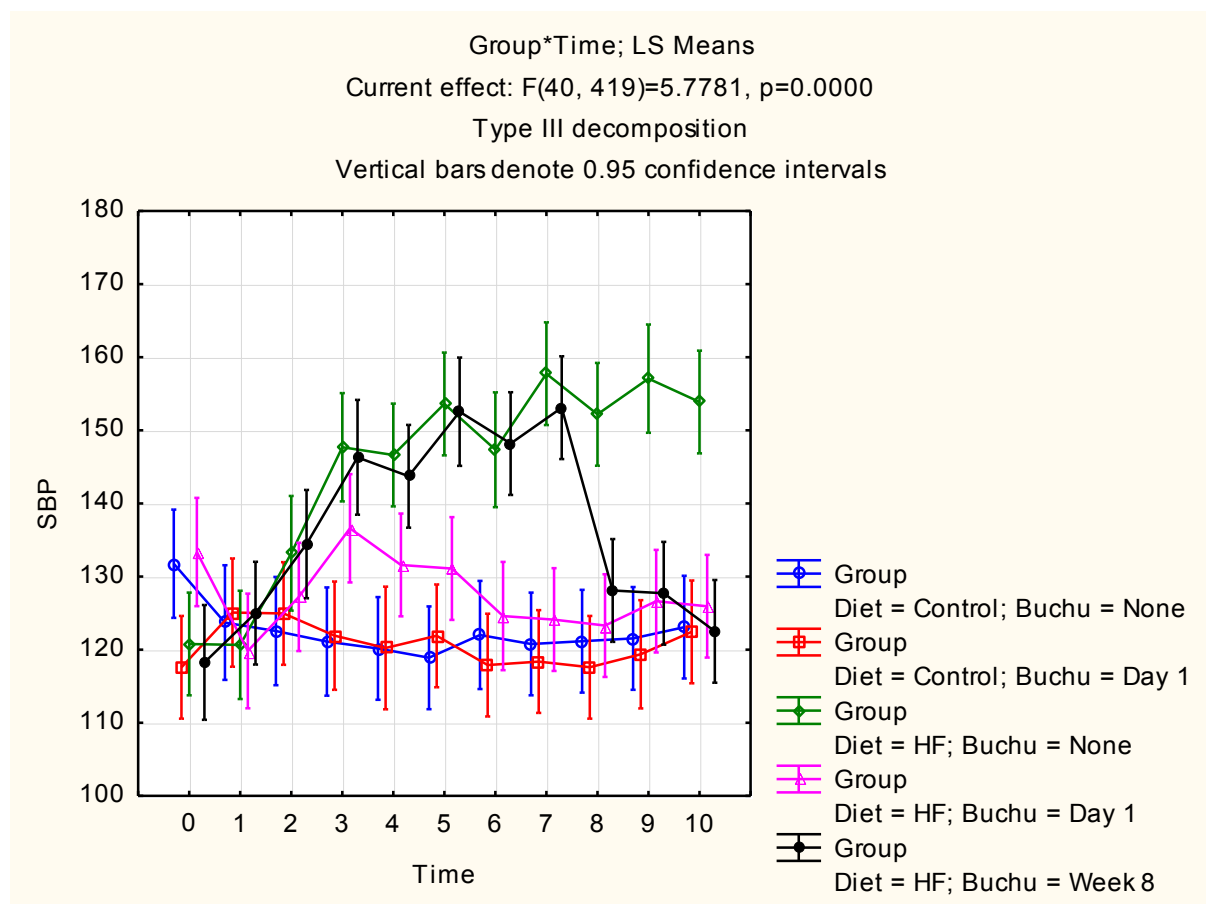


Figure 3-26: Systolic Blood pressure (SBP). Rats were fed a HFD for 16 weeks and blood pressure were monitored every two weeks as described in Methods. Time is in weeks. Every 1 time unit = 2 weeks; 0-2 weeks conditioning/training. 3-10 weeks is the treatment and diet period. $n = 10$ per group.

3.7.2 Diastolic BP

After 16 weeks on diet, there was a significant difference in the DBP when comparing the different groups of animals. The control group presented with significant lower DBP when compared to the HFD group (82.27 ± 1.40 vs. 102.56 ± 1.39 mmHg; $p < 0.0001$) (Figure 3-27). It is clear that these HFD animals were hypertensive whereas the control animals remained normotensive. There was no significant difference between the untreated or treated control animals. The HFD animals treated with Buchu water from day one did not develop any increase in diastolic BP over the 16-week period while animals treated from week 8, within two weeks, showed a significant decrease in BP when compared to the HFD group (86.25 ± 13.1 and 77.73 ± 9.08 vs. 103.49 ± 13.73 mmHg) (Figure 3-27). Buchu presents both preventative measures and treatment properties for diastolic HTN.

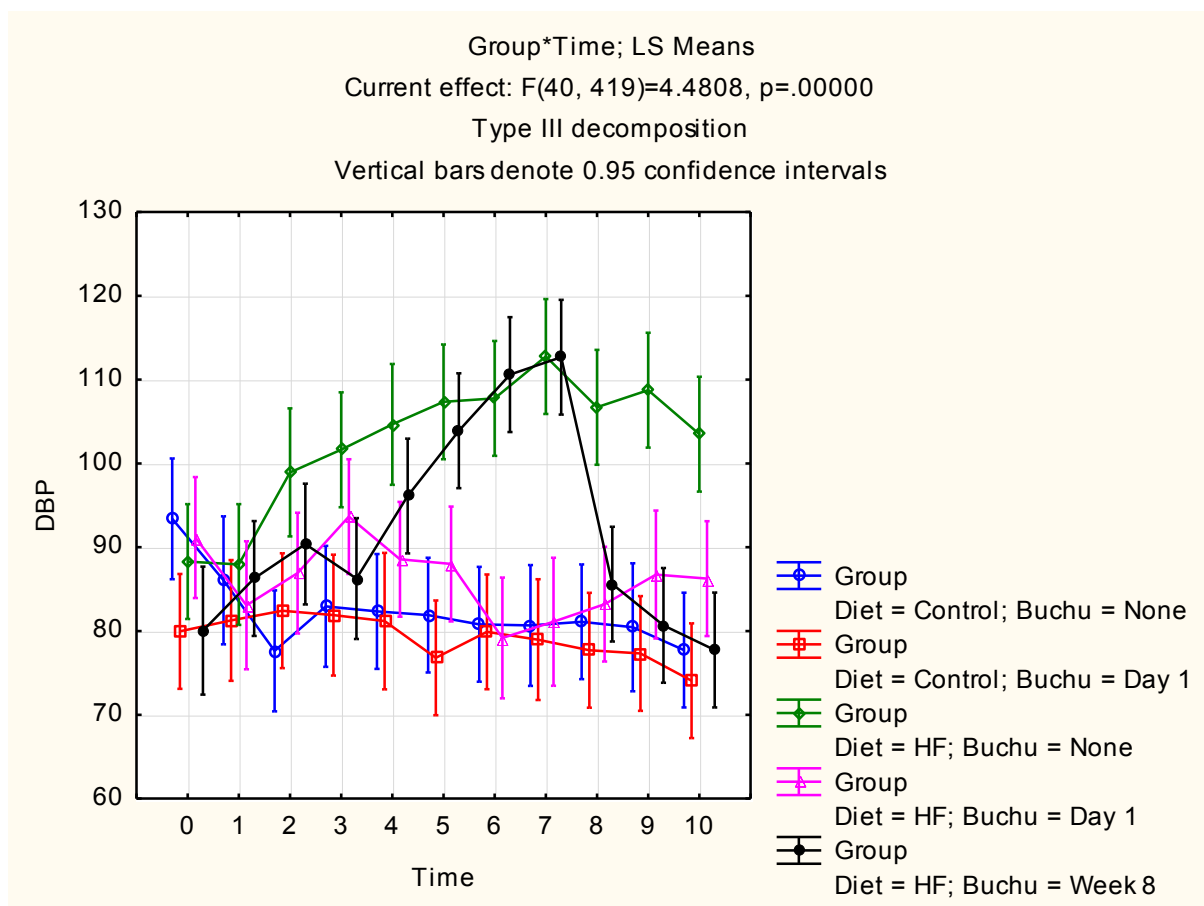


Figure 3-27: Diastolic Blood pressure (DBP). Rats were fed a HFD for 16 weeks and blood pressure were monitored every two weeks as described in Methods. Time is in weeks. Every 1 time unit = 2 weeks; 0 -2 weeks conditioning/training. 3-10 weeks is the treatment and diet period. $n = 10$ per group.

3.8 Aorta Rings

To investigate whether the endothelial function played any role in the observed changes in BP, aortic rings were harvested as described in Materials and Methods. They were mounted in an organ bath, stabilized and their intactness tested as described in the Methods section. The aortic rings contracted with the addition of PE (10^{-6} M) and the contraction reached a plateau in 5-10 minutes. The mean tensions attained were 1.8 ± 0.48 g and 1.9 ± 0.35 g for control and HFD rats, respectively. The maximum relaxation in response to ACh in control and HFD rats was 50.03 ± 5.29 and $47.58 \pm 4.73\%$ of the contractile response to PE, respectively (Figure 3-28 &

Table 3-2). Accordingly to the endothelium dependent relaxation to ACh there was no significant difference between the control animals and HFD animals, as well as the respective treated groups. Therefore we did not detect that Buchu water treatment had any effect on the endothelium in this model.

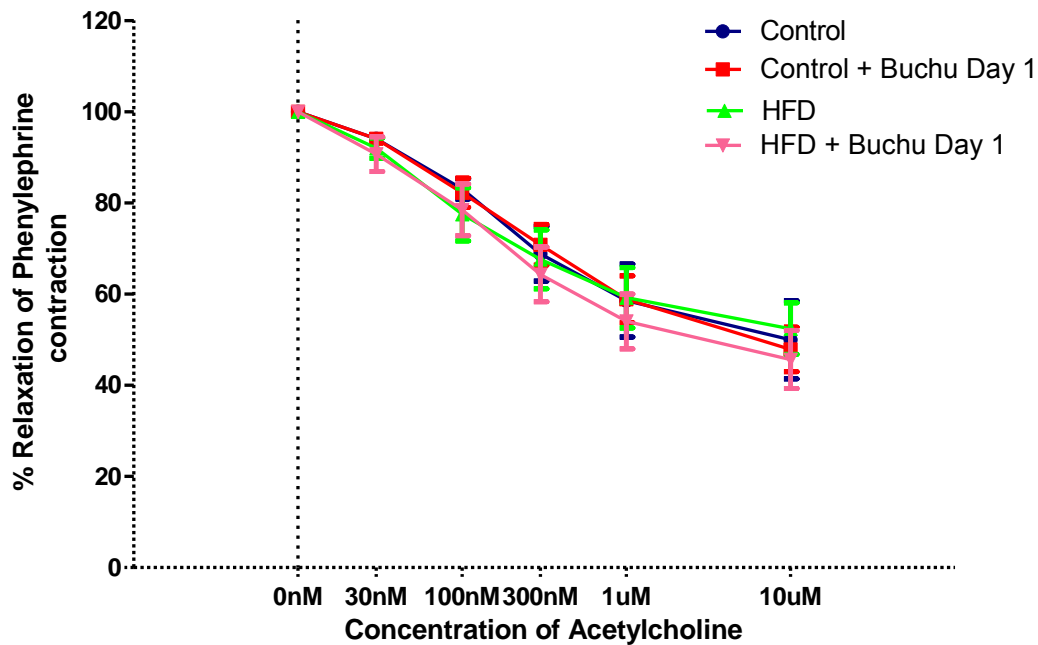


Figure 3-28: Aortic rings studies. Shown is the comparison of relaxation response to acetylcholine (ACh) in aortic rings of Buchu water treated and untreated control and HFD rats. Relaxation is expressed as percentage of contraction to phenylephrine (PE) and shown as mean \pm SE. $n = 5$ per group.

Table 3-2: Aorta rings studies relaxation. Shown is the relaxation response of aorta rings to acetylcholine (ACh) when contracted with phenylephrine (PE).

Parameters	Control	Control + Buchu Day 1	HFD	HFD + Buchu Day 1
30 nM ACh	94.04 ± 0.84	94.09 ± 1.24	92.00 ± 2.25	90.67 ± 3.82
100 nM ACh	83.06 ± 2.25	82.22 ± 3.22	77.48 ± 5.81	78.46 ± 5.67
300 nM ACh	68.84 ± 6.02	70.80 ± 4.47	67.60 ± 6.45	64.29 ± 6.01
1 µM ACh	58.57 ± 8.00	58.86 ± 5.11	59.20 ± 6.60	53.99 ± 5.99
10 µM ACh	49.97 ± 8.58	47.85 ± 4.89	52.42 ± 5.62	45.65 ± 6.36
Total relaxation	50.03 ± 5.29	52.15 ± 4.73	47.58 ± 4.73	54.35 ± 4.73

3.9 Expression and phosphorylation of proteins (16 weeks)

Expression and activation of proteins in the aorta's between Buchu treated and untreated control and HFD animals.

3.9.1 Protein kinase B (PKB/Akt) protein

3.9.1.1 Total PKB/Akt protein

We evaluated the total protein content of PKB/Akt in aortas from the different groups of animals (Figure 3-29). Under basal conditions, there was no significant difference in the expression of PKB/Akt in the respective groups after 16 weeks of diet and treatment.

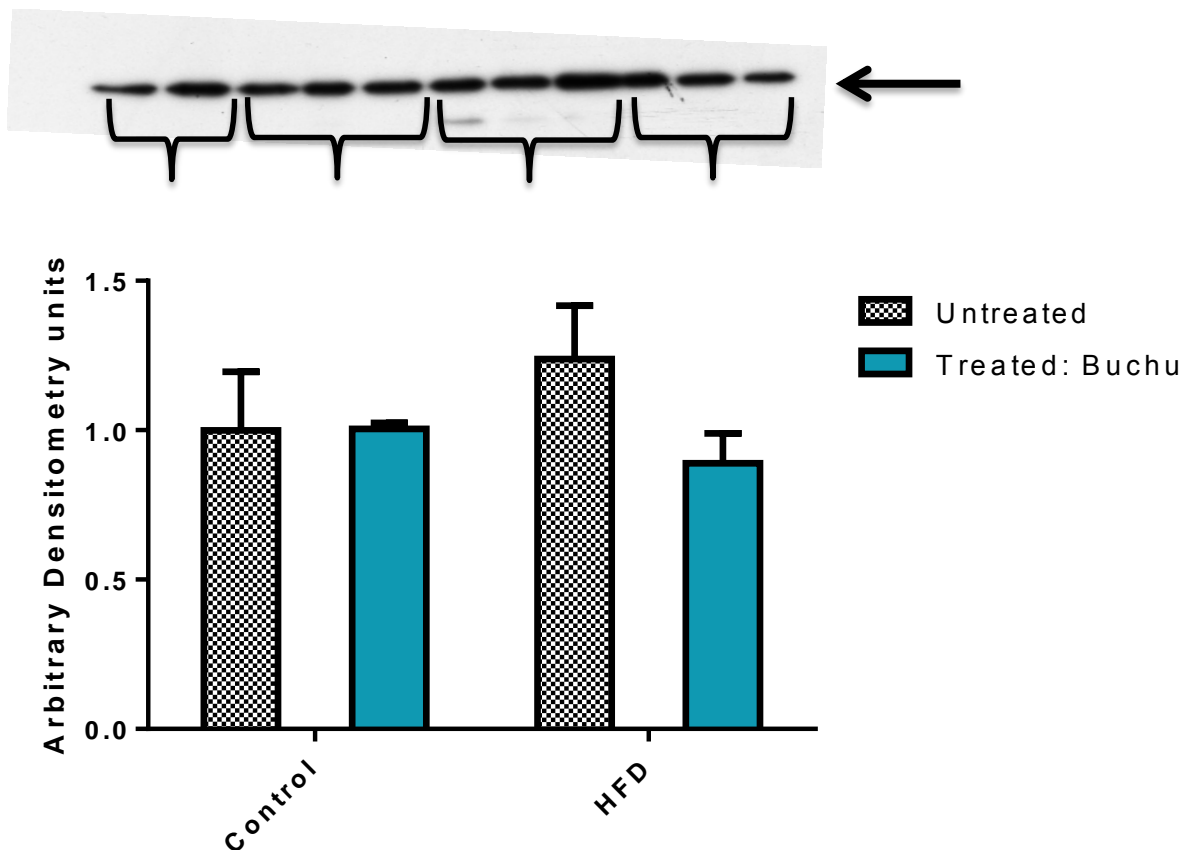


Figure 3-29: Total PKB/Akt. Shown is the total PKB/Akt expression in aortas of both the control and diet groups with and without treatment. *n* = 3 per group.

3.9.1.2 Phosphorylated PKB/Akt protein

At basal levels, there was no stimulation by any substance to activate any of the protective protein pathways (PI3K-Akt pathway) in the aortas. Therefore we did not observe any phosphorylated PKB/Akt protein in the aorta tissue samples.

3.9.2 AMPK protein

3.9.2.1 Total AMPK

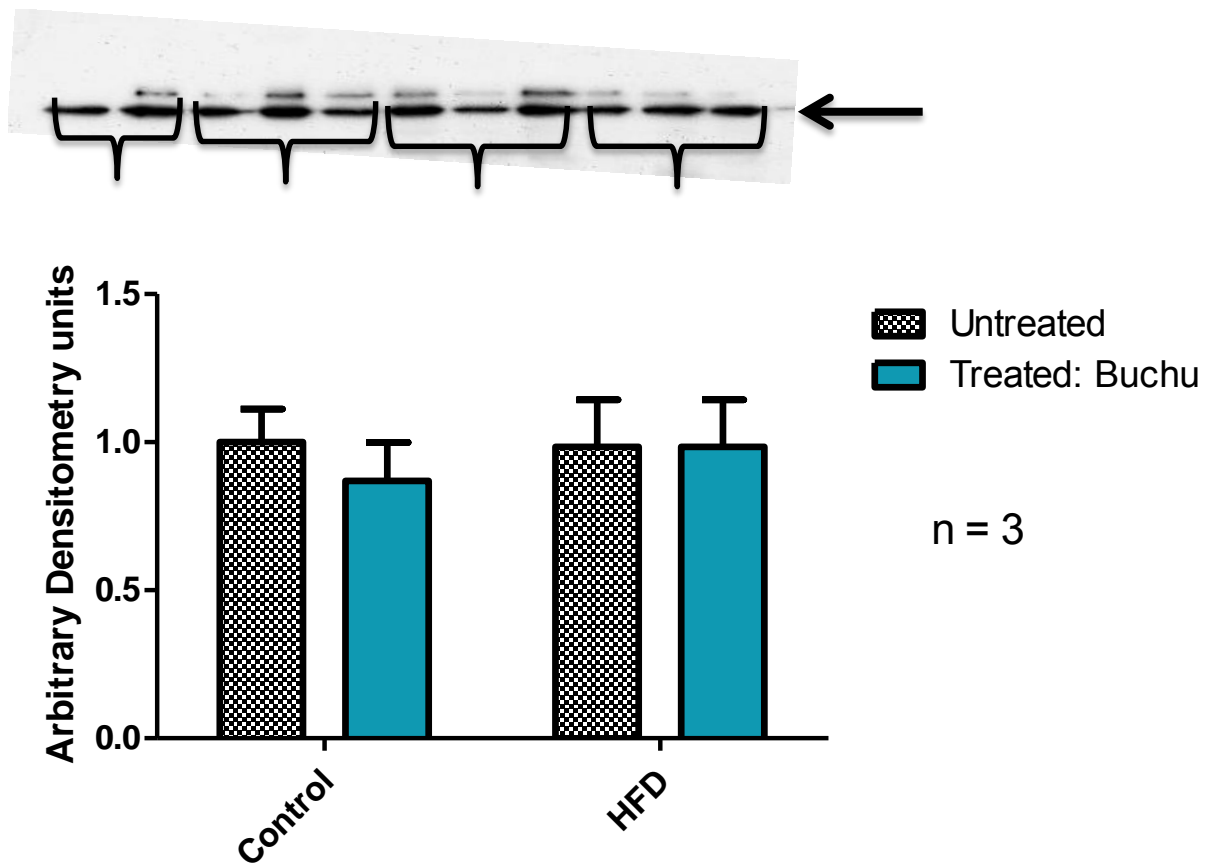


Figure 3-30: Total AMPK. Show is the total AMPK expression in the aorta's of both the control and diet groups after 16 weeks of treatment. n = 3 per group.

3.9.2.2 Phosphorylation of AMPK

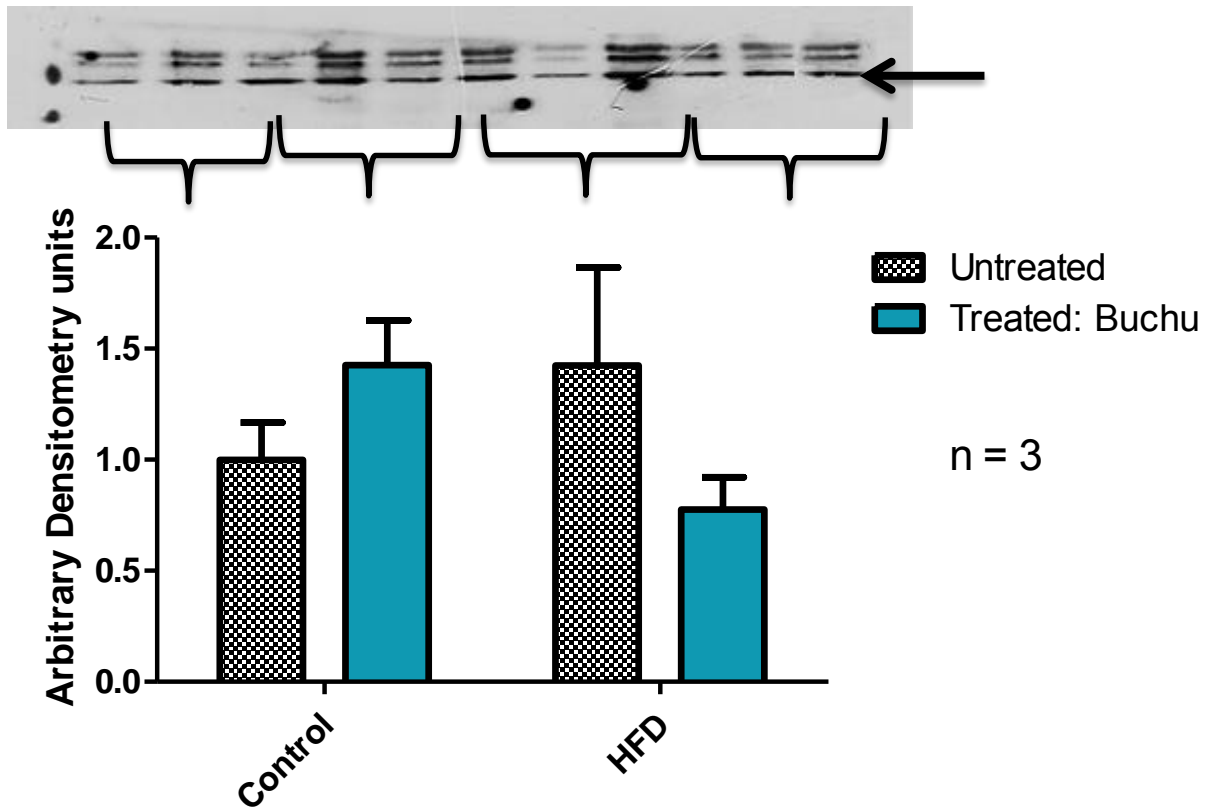


Figure 3-31: Phosphorylated AMPK. Shown are the levels of phosphorylated AMPK in the aortas of the diet and treated groups. *n* = 3 per group.

To evaluate the effect of NO (a potent vasodilator in the body) on BP we measured the expression of key proteins leading to the upregulation of eNOS the protein responsible for the synthesis of NO. There was no significance in the total AMPK and phospho-AMPK found between the diet groups or the treated groups.

3.9.3 eNOS protein

3.9.3.1 Total eNOS

We evaluated the total protein content of eNOS in aortas from the different groups of animals (Figure 3-32). Under basal conditions, there was no significant difference in the expression of eNOS between the untreated and treated animals, however there was a significant difference found between the control and HFD groups after 16 weeks of diet and treatment.

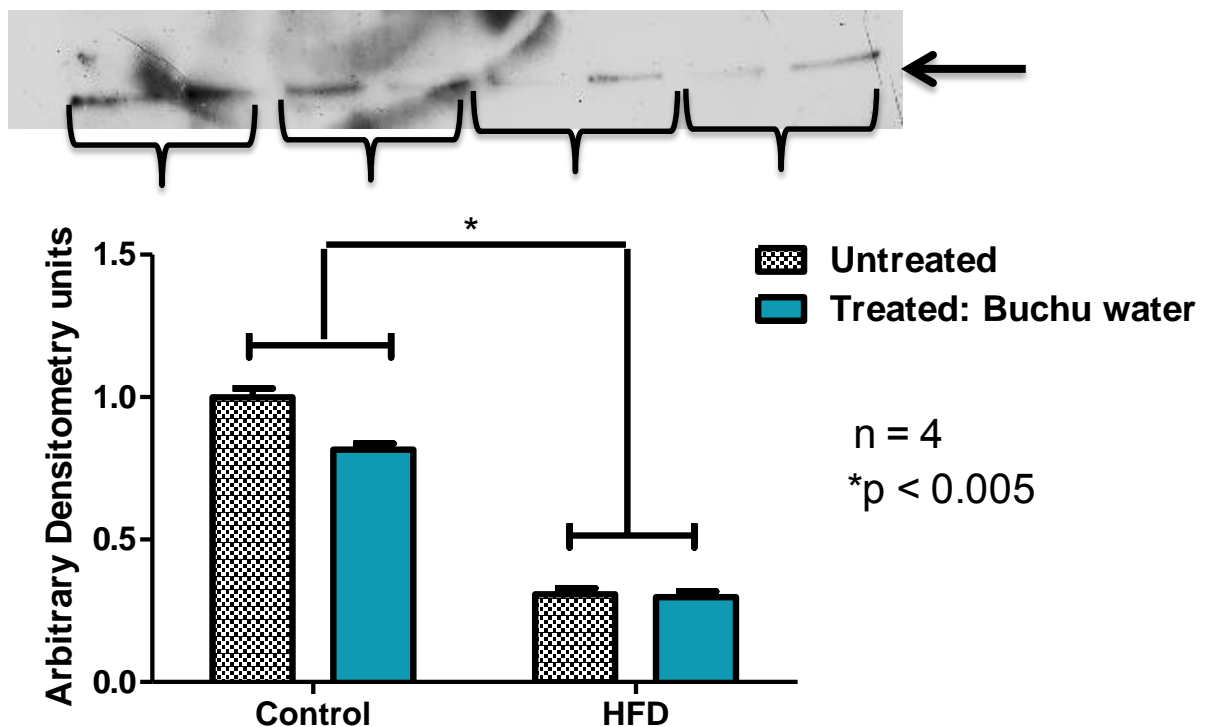


Figure 3-32: Total eNOS. Shown are the levels of total eNOS in the aortas of the diet and treated groups. $n = 4$ per group.

CHAPTER 4: Results - Obese, insulin resistant animals without elevated blood pressure.

4.1 Cardiovascular effects of Buchu water treatment

To determine the cardiovascular effects of Buchu water treatment, we also made use of a well-established rat model of hyperphagia-induced central obesity (DIO) (Pickavance et al., 1999). The animals on this diet are pre-diabetic but do not develop high BP which would be a confounding effect on the cardiovascular system.

4.2 Body weights

According to a two-way ANOVA, the effect of Buchu water on body weight was significant with a $p = 0.03$. Therefore the chronic administration of Buchu water significantly reduced body weight gain in both the DIO (408.13 ± 11.31 vs. 355.13 ± 30.08 g) (Figure 4-33) and control groups (375.13 ± 17.21 vs. 361.5 ± 13.93) (Figure 4-33).

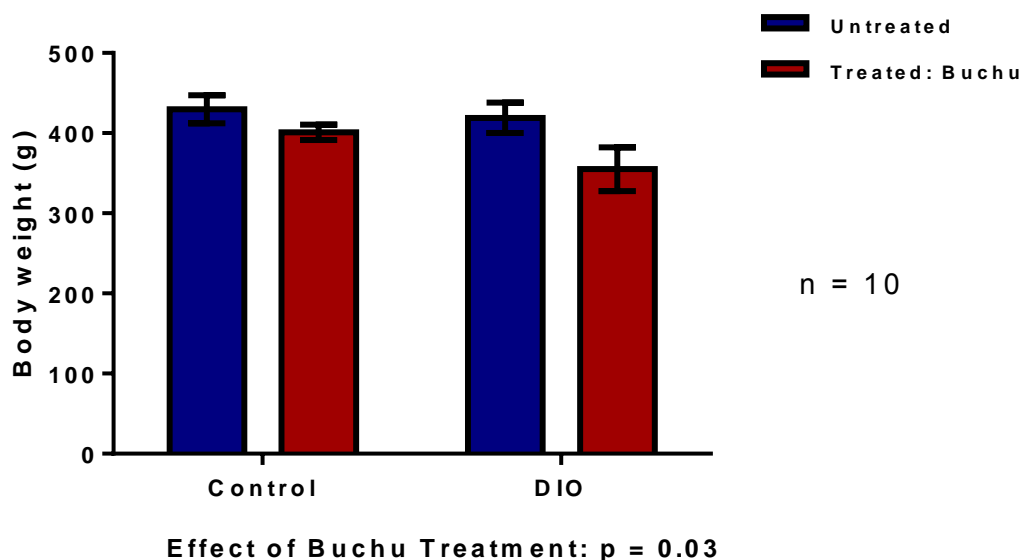


Figure 4-33: Body weight. Shown is the BW measured in grams (g) of treated and untreated diet induced obesity (DIO) and control animals after 16 weeks of treatment with Buchu water. $n = 10$ per group.

4.3 IP fat mass

The rats were 16 weeks on the diet and treatment with no significant difference between the amounts of visceral fat in either the DIO animals (19.46 ± 2.38 vs. 16.82 ± 1.90 g) (Figure 4-34) or the control animals (13.33 ± 2.15 vs. 9.69 ± 1.68 g) (Figure 4-34). However according to a two-way ANOVA analysis, the effect of the diet on IP fat mass was significant with a $p < 0.05$, $n = 8$ per group.

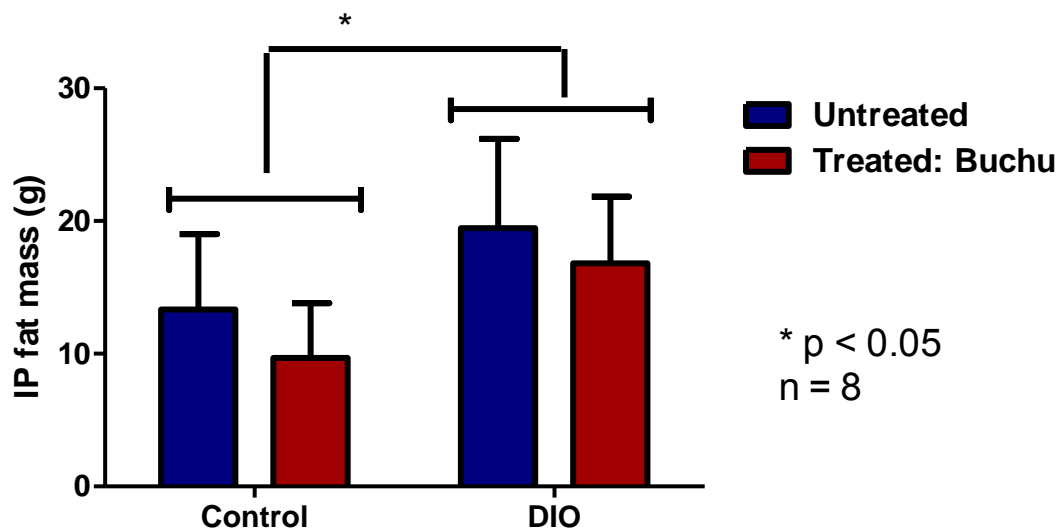


Figure 4-34: Intra-peritoneal (IP) fat mass. Shown is the IP fat measured in grams (g) of treated and untreated diet induced obesity (DIO) and control animals after 16 weeks of treatment with Buchu water. $n = 8$ per group.

It was demonstrated by B Huisamen et al, unpublished result that the hearts of these animals were protected from ischaemia/reperfusion injury by ingesting Buchu water as evidenced by smaller infarct size development after regional ischaemia followed by 120 min reperfusion. This was evident for both the treated controls vs. their respective untreated controls (28.75 ± 1.48 vs. $35.24 \pm 1.47\%$, $p < 0.05$) (Figure 4-35) and the treated DIO animals vs. the untreated DIO animals (39.11 ± 2.56 vs. $48.63 \pm 2.21\%$, $p < 0.01$) (Figure 4-35). According to a two-way ANOVA, the effect of Buchu on infarct size was highly significant with a $p < 0.0002$, $n = 8$ per group. There was also a significant difference in the infarct size between the control and DIO animals with the infarct size of the DIO animals being significantly larger than those of the control animals (48.63 ± 2.21 vs. $35.24 \pm 1.47\%$, $p < 0.01$) (Figure 4-35).

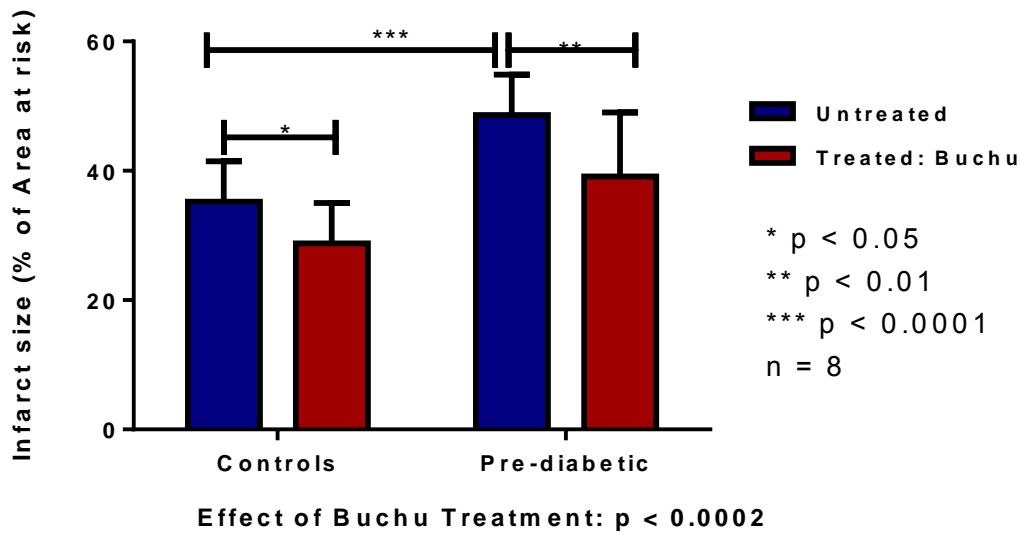


Figure 4-35: Infarct sizes - DIO model. Shown is the infarct size (% of area at risk) of the isolated hearts, from the DIO and control rats perfused ex vivo in working heart mode after they were treated for 16 weeks in their respective groups. $n = 8$ per group.

4.4 Glucose uptake (2DG) in isolated cardiac myocytes

On an organ level, we investigated if the insulin sensitivity of cardiomyocytes were improved by ingestion of Buchu water, by measuring the glucose uptake in isolated cardiomyocytes (CM). A two-way ANOVA showed the overall glucose uptake of isolated cardiomyocytes at basal level, to be significantly increased when animals were pre-treated with Buchu water ($p < 0.05$) (Figure 4-36).

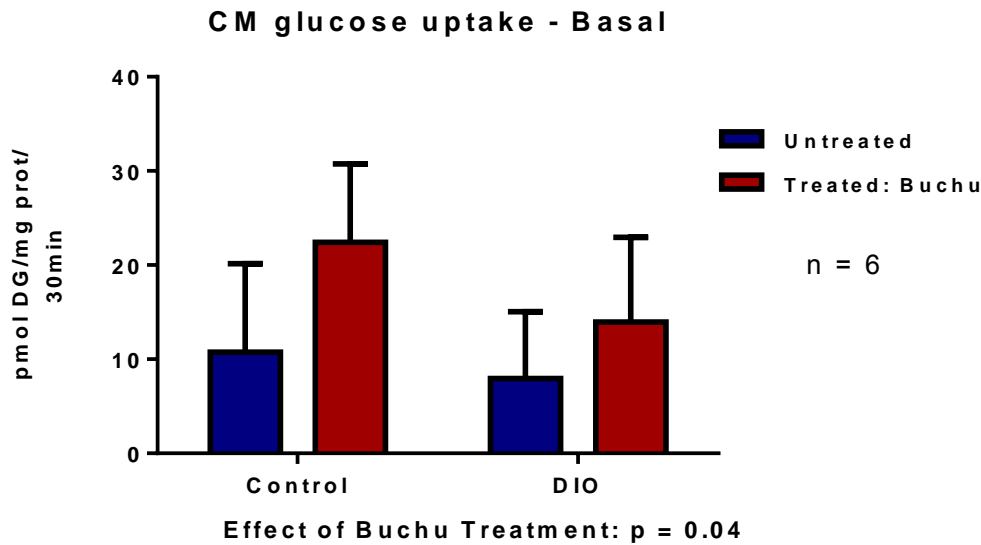


Figure 4-36: Cardiomyocyte glucose uptake. Shown is the DG uptake by cardiomyocytes from the DIO and control rat groups at basal levels with no stimulation of insulin. Data expressed as mean \pm SED. $n = 6$ per group.

The glucose uptake by cardiomyocytes prepared from all the different experimental groups did however not indicate a significant difference when they were stimulated with 1 nM, 10 nM or a 100 nM of insulin for 15 minutes (Figure 4-37).

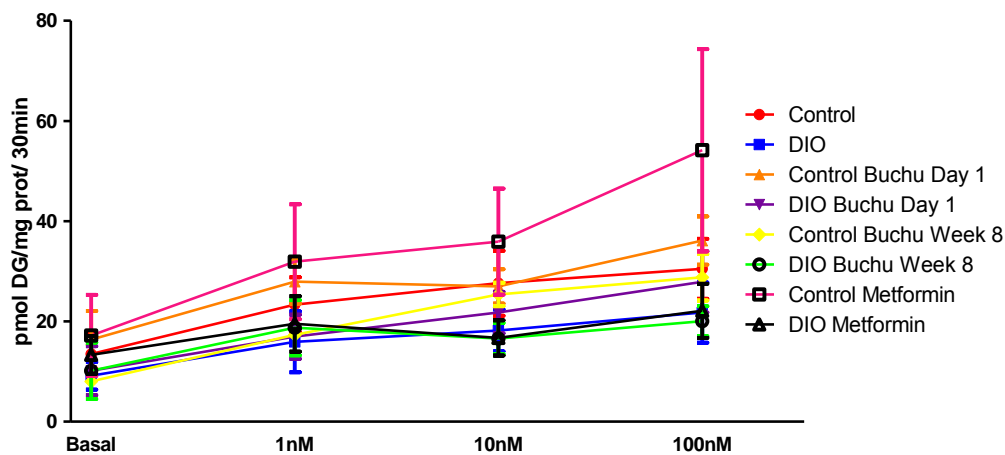


Figure 4-37: Cardiomyocyte glucose uptake all the groups. Shown is the DG uptake by cardiomyocytes from all the specific rat groups at basal levels and after stimulation with 1 nM, 10 nM and 100 nM insulin. The insulin sensitivity of the cardiomyocytes was determined by means of measuring the ability of cell to accumulate radioactive labelled deoxyglucose before and after the stimulation of insulin. Data expressed as mean \pm SED. $n = 6$ per group.

CHAPTER 5: DISCUSSION

Obesity is a vastly widespread metabolic disorder that has reached epidemic proportions all over the world (Deitel, 2003; Stevens et al., 2012) and South Africa is no exception to this. Recently it was shown that 29% of men and 56% of women in South Africa are overweight or obese (Puoane et al., 2002). The increasing prevalence of obesity is mostly due to life style changes (inactivity and HFD) and, together with the associated rise in BP, can result in morbidity. Both obesity and HTN are important risk factors for CVD, the number one cause of death globally (Kelly et al., 2012; Lim et al., 2012; Murray et al., 2012).

In an attempt to control these disease states, individuals require effective medication which can be used in conjunction with healthy life style changes. The use of herbal based therapies is currently strongly encouraged, as they offer potentially cost-effective and readily accessible management. However, scientific validation of their effects is needed. In Africa, herbal remedies and folk medicine have been used for centuries by the 'Khoisan' and other indigenous peoples to treat various ailments. It was also the Khoisan who introduced Buchu to the western colonist. Buchu is a plant native to South Africa and is perhaps the best known aromatic medicinal herb. Buchu water is the by-product of the extraction of Buchu oil. The latter is mainly isolated from the leaves of *Agathosma crenulata* and *Agathosma betulina*, both endemic to the mountains of the Western Cape in South Africa. The oil is used as a flavour enhancer in the perfume and food industries. The aqueous solution is currently marketed as a herbal remedy to treat various ailments e.g., urinary infections, rheumatism, arthritis and obesity. It is also advocated as an effective treatment of HTN. However these are only anecdotal claims with no scientific evidence.

This study therefore aimed to investigate these claims in different animal models, namely a HFD rat model in which the animals become obese and hypertensive. In addition we investigated the cardioprotective properties of this substance in isolated

cardiomyocytes prepared from rats fed a diet resulting in hyperphagia and obesity. However, these animals do not become hypertensive.

5.1 Overview of our findings

The major findings of the present study were as follows: i) Buchu water ingestion improved some indices of glucose tolerance and whole body glucose homeostasis; ii) Buchu water selectively reduced visceral fat depots when given from the onset of the HFD; iii) Buchu water prevented HTN when given at the onset of obesity in rats and iv) rapidly decreased systolic and diastolic blood pressures in already obese, hypertensive rats. v) Buchu water treatment elevated basal myocardial glucose uptake in correlation with cardioprotection.

5.1.1 Primary aim of present study

The primary aim of the current study was to evaluate the ability of Buchu water to prevent HTN induced by obesity in rats. The results are clearly demonstrated in animals maintained on a HFD that caused a sustained increase in BP while Buchu treatment not only prevented HTN induced by obesity, but also surprisingly, caused a large and rapid decrease in body weight and intraperitoneal fat accumulation. In addition, Buchu water normalised the BP of already hypertensive animals.

5.2 DISCUSSION PART 1 – Using a HFD to elicit high blood pressure in animals to study the effects of chronic Buchu water treatment on hypertension

The first rat model was aimed at inducing obesity and HTN to investigate the antihypertensive properties of Buchu water. A modification of a previously published HFD was used (K. Srinivasan, Viswanad, Asrat, Kaul, & Ramarao, 2005). These animals developed HTN within a four-week period (Figure 3-26 & Figure 3-27). Not

only was Buchu water treatment able to prevent the development of HTN when given in conjunction with the HFD, but it normalised elevated BP within two weeks.

5.2.1 Effect of Buchu water treatment on the biometric parameters (body weight, IP fat and food intake)

BODY WEIGHT AND BUCHU TREATMENT

Fat enriched diets have been used for decades to model obesity (Budohoski et al., 1993). The relative fat fraction in these diets varies between 20% and 60% and the basic fat component differs between animal-derived fats or plant oils. It is well documented that, when energy intake exceeds energy expenditure, there is excess storage of energy in the form of fat (adipose tissue). In the present study the animals were placed on a diet consisting of a 40% fat component which did not replace the carbohydrate component, but was added fat as well as added sucrose, fructose and cholesterol to a standard rodent chow. Thus the animals ingested food with dense calorie content and therefore became obese. These animals gained more body weight when compared to their age matched control animals on normal rat chow (Figure 3-7). Obesity induction is most effective when the diet is started at a young age as was done in the present study (Peckham, Entenman, & Carroll, 1962). Prolonged feeding of fat enriched diets induces an increase in body weight in a range between 10% and 20%, was concluded in a review that compared various high fat diets that model the metabolic disorder of human obesity in rodents (Buettner, Scholmerich, & Bollheimer, 2007). In our present study the percentage of weight gain between the untreated HFD and control group corresponds with this (19%). There is a close relationship between obesity and HTN and this has been documented in the literature (El-Atat et al., 2003; Hall, 2000; Hall et al., 2003; Morse et al., 2005). Experimental studies have shown that weight gain leads to increases in BP, whereas clinical studies indicated weight loss leads to a reduction in BP in hypertensive patients (Berchtold et al., 1981; Berchtold et al., 1982; He et al., 2000). Subsequent studies have shown that excess weight gain can be used as a predictor for the development of HTN (Hall, 2000; Hall et al., 2001). This coincides with the present study, which showed that the HFD group gained excessive weight on the HFD which led to raised BP levels during the study.

These obese animals became HTN after only four weeks on the HFD, whereas the control group gained body weight more naturally with age on the SRC. Their BP stayed constant during the study (normotensive). We administered Buchu water to a HFD group to see if we can prevent the onset of HTN in these animals on a HFD. Although there was slightly more weight gain than the control group, this did not affect the BP. These animals were also normotensive at the end of the 16 weeks of treatment. This group represent normotensive individuals in our society that follow an unhealthy HFD. The results suggested that with Buchu water treatment, excess weight gain and the onset of HTN may be prevented in these individuals.

IP FAT AND BUCHU WATER TREATMENT

When comparing the body weight of the animals it was clear that the increase in weight was due to an increase in visceral fat content (Figure 3-10). High fat diets produce a significant increase in body fat content which depends on the amount of fat content in the food and the duration of feeding. Numerous studies showed that excess abdominal (android or upper body) obesity increase the risk for HTN (Sironi et al., 2004) and ultimately CVDs. In addition, a study done on obese, hypertensive women has also pointed out that a decrease in intra-abdominal visceral fat reduces BP (Kanai et al., 1996). The current study clearly demonstrated an increase in body weight (Figure 3-8) and fat mass (Figure 3-10) after rats were exposed to a HFD for 16 weeks. The untreated HFD group which were hypertensive as a result of the excess body weight, also had a significant increase in visceral fat content when compared to their respective controls. The visceral fat content of the HFD group nearly increased three-fold.

This excess adipose tissue is a result of additional FFA being deposited in mature adipocytes (hypertrophy) or the formation of new adipocytes from preadipocytes (hyperplasia) (Faust et al., 1978). The expansion of IP fat in obesity is mostly due to an increase in size of the mature adipocytes (Kim et al., 2007). The low grade inflammatory state associated with obesity leads to an increase in pro-inflammatory cytokines produced by immune cells like macrophages in adipose tissue and the liver. These pro-inflammatory cytokines directly impair the formation of new small adipocytes from preadipocytes but does allow the larger mature adipocytes to expand

even further (Lacasa et al., 2007). In our study, animals that consumed a HFD but ingested Buchu water in conjunction with this, gained less weight when compared to the untreated HFD animals and also had a smaller amount of accumulated visceral fat content. This may be why we did not see an increase in BP despite the HFD.

FOOD INTAKE

From previous studies we know that an increase in food consumption leads to an increase in circulating macronutrients in body. This is associated with weight gain and large amounts of visceral fat depots. Conversely, a decrease in food consumption is associated with weight loss (Harris, Kasser, & Martin, 1986; Hill, Latiff, & DiGirolamo, 1985). We therefore determined the food consumption of the animals over the 16 week period. Results indicated that the food consumption per cage containing five animals, per week, did not differ in any of the groups (Figure 3-16). Although the caloric content of the two diets did differ (thus accounting for the increased weight gain between the control and HFD groups) the amount of food consumed by the different groups of animals did not differ. The decrease in body weight and IP fat weight in both Buchu treated groups are thus not associated with a decrease in food intake.

Interestingly, clinical studies on the effects of antihypertensive medications such as ACEI and ARBs also indicated a reduction in IP fat (He et al., 2000; MacMahon et al., 1990; Neal et al., 2000). A study done with Telmisartan, an angiotensinogen II type receptor blocker, by Shimabukuro et al. (2007), reported a reduction in BP together with a decrease in visceral fat content (Shimabukuro, Tanaka, & Shimabukuro, 2007). Another study done on mice also showed weight loss with the use of Captopril, another ACEI (Weisinger et al., 2009). These analogies suggest that Buchu water may exert its effects by interacting with a mechanism in the RAAS. This may lead to a reduction in IP fat and in turn prevent the increase in BP.

Other factors that may have led to a reduction in body weight may have been different metabolic rates and improved HFD tolerance. Furthermore, the decrease in body weight gain may also have been due to a reduced ability to extract energy from the food (Kristensen et al., 2013).

5.2.2 Effect of Buchu water treatment on blood glucose, insulin levels and insulin sensitivity

BLOOD GLUCOSE

Fasting blood glucose levels have been reported in various HFD's as normoglycaemia, slight hyperglycaemia and pre-diabetic. It seems to be dependent on the type and contents of the diet. Glucose is a carbohydrate and is one of the main energy sources for metabolism in mammalian cells. Our data showed that Buchu water treatment for 16 weeks decreased the fasting blood glucose levels in the treated control group (Figure 3-11). In contrast to this, no effect was observed in the treated HFD group. The range of fasting blood glucose across all the groups was within the normal range (≤ 6.5 mmol/l), therefore normoglycaemic. This is rather important since several first line regimens for the management of uncomplicated HTN have clearly exhibited distinctive adverse effects. One of these is that, with obesity associated HTN, there is an increased rate of diabetes being reported (Taylor, Hu, & Curhan, 2006). The antihypertensive agents can affect the glucose homeostasis, especially with thiazide diuretics and BBs (Gress, Nieto, Shahar, Wofford, & Brancati, 2000a; Helgeland et al., 1984; Kostis et al., 2005). Thiazide diuretics have been found to alter glucose control via hepatic insulin resistance, resulting in continued hepatic glucose production despite rising glucose and insulin levels (Harper et al., 1994; Hunter et al., 1998), whereas BBs have been found to inhibit insulin release from the pancreatic beta cells (Gress, Nieto, Shahar, Wofford, & Brancati, 2000b). On the other hand, first line antihypertensive agents such as ACEIs, CCBs or ARBs have not been reported to disrupt the glucose homeostasis in the body. Calcium channel blockers have exhibited minor effects on glucose metabolism (Sowers, 1997) whereas ACEIs and ARBs may actually possess beneficial effects (Hansson et al., 1999a). These observations may be of utmost importance since significant glycaemic effects caused by antihypertensive medication can accelerate the risk of developing CVD (Dunder, Lind, Zethelius, Berglund, & Lithell, 2003). Therefore by comparing our results with these studies, one would not expect the glucose homeostasis to be disrupted during chronic Buchu water treatment.

INSULIN

Ever since obesity and HTN have been associated with each other, they have been assumed to be linked to insulin resistance and hyperinsulinemia (Hall et al., 1989; Hall, 1993). Chronically elevated insulin is a marker of metabolic dysfunction, and typically accompanies high fat mass, poor glucose tolerance (pre-diabetes) and blood lipid abnormalities. In the present study our data did not find that the insulin levels were elevated in the treated or untreated control groups (Figure 3-12,

Table 3-1). This is as would be expected, since these animals were representing healthy individuals which were able to maintain an adequate balance in the amount of FFA and glucose in their blood circulation during a fasting state of metabolism. The untreated HFD animals which were classified as obese and hypertensive also did not have elevated levels of insulin (Figure 3-12). The results clearly indicate that the animals on the HFD were not insulin resistant but still had high BP. Our study confirms that, in our model, there is no connection between HTN and hyperinsulinemia, as previously reported by Hall et al. (1989) when he infused dogs with excess insulin for several weeks to see if hyperinsulinemia can cause a response in BP (Hall et al., 1989). It is often expected that obese individuals have elevated insulin levels (DeFronzo & Ferrannini, 1991; Reaven, 1988). However, whether obesity precedes or follows hyperinsulinemia is uncertain (S. R. Srinivasan, Myers, & Berenson, 1999). Although there was no clear significance between the insulin values of the different groups in our study (because of the large standard error in the insulin assay), nonetheless it does show a trend that the insulin levels were elevated in the obese animals that were treated with Buchu water (Figure 3-12). This assay was performed on fasting blood and it seems as if the levels varied greatly at this level. This observation warrants further investigation.

C-PEPTIDE LEVELS & INSULIN SENSITIVITY

Insulin sensitivity describes how sensitive the whole body is to the effects of insulin. An insulin sensitive individual will require low levels of insulin to elicit a response to lower blood glucose whereas someone with low insulin sensitivity will require higher levels of insulin to maintain glycaemia. Intraperitoneal glucose tolerance curves generated on all the animals in the current study showed that the HFD animals did not present with whole-body insulin resistance nor were any effect of the Buchu water on this parameter observed (Figure 3-15).

In addition, insulin sensitivity was expressed as the HOMA index which is a non-invasive calculation of insulin sensitivity using fasting insulin and glucose levels. Again, no evidence of insulin resistance was observed in the control or control treated animals (Figure 3-14). A higher level of insulin was observed in the HFD animals treated with Buchu water. It may be that Buchu treatment interacted with the pancreas resulting in

insulin secretion. This is supported by an increase of plasma C-peptide levels in the treated HFD animals. However, an increase in C-peptide levels was not observed in the control animals consuming Buchu water. The possible pancreatic effects of Buchu water warrants further investigation.

5.3 Adipokines

Adipose tissue is no longer seen as a just an energy reservoir for FFA and triglycerides but rather as a dynamic tissue which modulates energy metabolism via the secretion of circulating adipocytokines (Trayhurn & Wood, 2005). Adipose tissue has also been described as an active endocrine organ by Kershaw et al. (2004) in his review (Kershaw & Flier, 2004).

Leptin is an adipokine predominantly secreted by adipocytes and acts on the hypothalamus in the brain (Van Harmelen et al., 1998). It can therefore cross the blood brain barrier (Bouret, 2008). Its physiological function is to control appetite and regulate energy homeostasis through a negative feedback loop in times of food abundance, to prevent obesity (Hall et al., 2001). Leptin is therefore associated with body weight control. The amount of leptin present in the circulation is proportional to the amount of fat mass (Handjieva-Darlenska & Boyadjieva, 2009). An obese individual is prone to develop resistance against the central effects of leptin. Resistance to leptin refers to an inadequate response because of a decreased sensitivity to the effects that leptin exert at normal physiological or increased pathophysiological levels (Lin, Thomas, Storlien, & Huang, 2000). This is due to the overstimulation by circulating leptin because of increased secretion by adipose tissue. Most importantly, the serum leptin levels in the present study showed an expression pattern co-inciding with the size of the fat depots. Chronic Buchu treatment decreased the leptin levels of the obese animals below that of the control groups (Figure 3-17;

Table 3-1). As leptin levels are closely associated with insulin resistance, this is quite an important result. The adipose tissue was also greatly reduced in the treated obese animals (Figure 3-10). These data suggest that the reduction in leptin levels was in part due to less adipose tissue being present in the treated HFD animals since the amount of leptin found in the circulation is proportional to the amount of fat mass (Handjieva-Darlenska & Boyadjieva, 2009). The overall effect of Buchu treatment was significant as indicated by two-way ANOVA analysis. These findings are contradictory since low leptin levels are known to stimulate appetite and we observed no difference in the amount of food consumed by the different animal groups (Figure 3-16). This suggests that Buchu water treatment did not decrease the leptin levels nor has any effect on the amount of food being consumed but rather affected the adipose tissue and thus caused the reduction in the leptin levels that we observed in the treated HFD animals.

Adiponectin plays an important role in the development of insulin resistance and atherosclerosis, both risk factors for CVD. Adiponectin is usually found in high concentrations circulating in the blood in healthy individuals. In obese individuals, lower concentrations of adiponectin have been reported in the circulation, associated with increasing development of insulin resistance. In humans, adiponectin levels are found to be inversely correlated with the percentage of body fat (Gavrila et al., 2003). Adipose tissue is thus a negative predictor of adiponectin (Gavrila et al., 2003). Adiponectin is also said to decrease prior to the onset of T2D. Adiponectin administration is associated with decreased levels of plasma glucose and increasing insulin sensitivity. A review by Silva et al. (2011) considered evidence of studies that investigated the effect of diet on adiponectin and the conclusion was that dietary management can be a therapeutic option to increase adiponectin levels, especially with a low calorie diet (Silva, de Almeida, & Feoli, 2011). However, in our study the adiponectin levels (Figure 3-18) were not increased, despite the loss in intraperitoneal fat (Figure 3-10). Disappointingly so, as this would have explained some of the cardioprotective effects observed in the DIO model (Figure 4-35). This negative result in our study may correlate well with a study done in humans where it was found that adiponectin levels were inversely correlated with fasting insulin levels (Park et al., 2004). In our study, the treated HFD animals had an unexpected trend towards higher

fasting insulin concentrations (Figure 3-12) and lower adiponectin levels where as the opposite were true for the HFD group (Figure 3-18).

5.4 Anti-inflammatory activity of Buchu water treatment

TNF-alpha & IL-6

Inflammation plays an important role in vascular biology. Therefore in recent years there has been thought to exist a close association between inflammation and HTN. This is based on studies that showed an increase in inflammatory molecules circulating in the blood of hypertensive patients (Bautista et al., 2001; Bautista, Atwood, O'Malley, & Taylor, 2004; Bautista, Vera, Arenas, & Gamarra, 2005; Sung et al., 2003). Conversely, other studies showed that there is no association between IL-6 and high BP (Chae, Lee, Rifai, & Ridker, 2001; Rifai, Joubran, Yu, Asmi, & Jouma, 1999). Inflammation has not only been thought to be associated with HTN but also with obesity. The large amount of VAT is not only responsible for its metabolic activity through the secretion of adipokines but also produces proinflammatory cytokines such as IL-6. Inflammatory markers such as IL-6 and TNF-alpha are used in the prediction of future complications that result in CVD (Ridker et al., 1997; Ridker et al., 2000; Ridker, 2013). Treatments that can modify these levels of proinflammatory cytokines can prevent the risk of myocardial infarction and strokes. The literature describes Buchu as a medicinal herb having anti-inflammatory properties (Moolla & Viljoen, 2008). Unfortunately our HFD model did not show signs of either elevated TNF-alpha (Figure 3-19) or IL-6 (Figure 3-20). Thus no low-grade or chronic inflammation could be detected in the plasma. The Buchu water treatment did not affect these levels either. Therefore we could not really detect any anti-inflammatory response by analysing the serum. It is still possible that these cytokines were elevated in the fat tissue (Evans et al., 2011), but this was not measured in the current study. In future, a model with a clear inflammatory response may also be used to investigate whether Buchu treatment will have systemic anti-inflammatory effects.

HOMOCYSTEINE

Homocysteine is a metabolite of the amino acid methionine and has been linked to an elevated risk of vascular disease (Stead, Brosnan, & Brosnan, 2000). Hypertension seldom occurs in isolation from other cardiovascular risk factors but HTN appears to be metabolically connected to plasma homocysteine levels (Rodrigo et al., 2003). Homocysteine levels have been shown to be associated with BP, especially SBP (U. Lim & Cassano, 2002; Malinow et al., 1995). A study done on human subjects showed that there is an interrelationship between homocysteine, obesity and HTN. They observed a prevalence of hyperhomocysteinemia in hypertensive individuals compared to normotensive individuals (Karatela & Sainani, 2009). This corresponds with our present study. Our results indicated elevated levels of homocysteine in the HFD animals (Figure 3-21). This is an effect correlated with the obesity in these animals and signifies a relationship between obesity and homocysteine. However, the incidence of HTN did not seem to be related to this increased level of homocysteine, since the treated HFD group who were now not hypertensive, still had elevated levels of homocysteine. These results are rather contradictory but clearly show that Buchu water treatment had no significant effect on the homocysteine levels.

Data about the effects of antihypertensive drugs on homocysteine levels are scarce, but they do exist and they report an absence of change or decrease in homocysteine levels as well as an increase (Atar et al., 2005; Korkmaz et al., 2003; Westphal, Rading, Luley, & Dierkes, 2003). An increased level of homocysteine is an unwanted side effect since hyperhomocysteinemia is seen as a risk factor for CVD (Clarke et al., 1991; Wald, Law, & Morris, 2002). However, lowering BP with anti-hypertensive treatment is more important than normalizing homocysteine levels. While detection of high levels of homocysteine has been linked to CVD, lowering homocysteine levels may not improve outcomes (Bonaa et al., 2006; Marti-Carvajal, Sola, Lathyrus, & Salanti, 2009).

5.5 Blood pressure

Feeding the rats a HFD resulted in severe HTN in conjunction with obesity as coinciding effects. Significant HTN developed within 4 weeks after the change in diet started (Figure 3-26 & Figure 3-27).

Not only did Buchu water prevent the development of HTN in the HFD group treated from day one, but it was also able to treat HTN when animals were allowed to develop HTN for a period of 8 weeks. Within two weeks, the BP of these animals went from hypertensive to normotensive (Figure 3-26 & Figure 3-27). Buchu water treatment therefore restored the BP to normal. The reduction in BP was seen only in the obese animals since no reduction in BP was noticed in the treated control group.

Although the mechanisms of obesity induced HTN are not fully understood, we can speculate on the pathophysiological effects that are associated with the HFD that can lead to HTN. In obesity there are three changes that occur that can alter the body's physiology and lead to increased BP; i) increased sympathetic stimulation (de Kloet et al., 2013), ii) synthesis of adipokines by the excess visceral adipose tissue depots in the abdomen and iii) hemodynamic alterations. It is described in the literature that the SNS contributes to the homeostasis of the body (Simonds & Cowley, 2013). In obesity there are several mechanisms and mediators causing over activity of the SNS leading to increased adrenergic sympathetic stimulation. A study done by Wofford (2004) showed that, by blocking alpha- and beta-adrenergic receptors in hypertensive individuals, it reduced BP significantly when compared to lean hypertensive individuals (Wofford & Hall, 2004), indicating that the SNS is stimulated during obesity and does play a role in the increased BP. Another contributing factor that can lead to increased SNS activation is increased leptin levels. As previously stated, Leptin is an adipokine predominantly secreted by adipocytes and acts on the hypothalamus in the brain (Van Harmelen et al., 1998). Although the HFD did not significantly elevate leptin levels in the animals, Buchu treatment for 16 weeks reduced the leptin levels in the treated HFD and control groups (Figure 3-17) this may have contributed in an independent manner to the decrease in BP observed in the treated HFD group. However, the current study did not evaluate SNS activity.

Rodent and human adipose tissue synthesizes all the components of the RAAS (Karlsson et al., 1998) which may be another mechanism that contributes to obesity induced HTN. Buchu water treatment in the present study caused a reduction in weight gain between the treated and untreated groups and had significant effect on the body weight of the animals (Figure 3-8). The reduction in body weight was due to a decrease in visceral fat content (Figure 3-10) and this by itself, may account for the normotensive

BP readings. Besides the effect that adipose tissue RAAS exerts on BP it also helps with the accumulation of fat. Angiotensin II binds to AT1 receptors expressed in adipose tissue and stimulates lipogenesis, inducing adipocyte differentiation and growth (Engeli et al., 1999). Administering an ACEI (an antihypertensive drug) such as Captopril leads to the inhibition of a key enzyme in the formation of Ang II called ACE. This leads to a reduction of Ang I being converted to Ang II. With less Ang II circulating in the blood there is a reduction in lipogenesis (Jones, Standridge, & Moustaid, 1997). Therefore the animals gain less weight and also lower their BP. This was observed in the present study with the administration of Buchu water causing both a reduction in lipogenesis and lowering the BP of the HFD groups. A study done in 1999 by de Kloet et al. corresponds with this. An ACEI was given to rats to protect against diet-induced obesity and glucose intolerance (de Kloet et al., 2009). Also, ACE-knockout mice have reduced body adiposity and increased energy expenditure (Jayasooriya et al., 2008). It will be important in future to measure the ACE activity of Buchu water.

Kidneys in obese subjects are said to be completely encapsulated with adipose tissue. This protective encasing leads to a pathophysiological condition where all the excess visceral fat accumulates and surrounds the kidneys causing renal compression (Hall et al., 2003). Long term renal compression raises arterial pressure in proportion to the force on the kidneys (Hall et al., 1998; Hall et al., 2003). The decrease in visceral adipose tissue could also result in removing the renal compression that may have been exerted on the kidneys. However this is unlikely since we observed a reduction within two weeks with the onset of Buchu water treatment in the HFD group (Figure 3-26 & Figure 3-27). Hemodynamic alterations such as increased vascular volume and cardiac output could also result in higher BP. All three of these alterations contribute to increase BP and it seems that Buchu water treatment interacted with them alleviating the effect and resulting in a reduction in BP.

ALDOSTERONE

Sustained HTN only occurs when the relationship between arterial pressure and natriuresis are disturbed. Under normal physiological conditions, there is a close relationship between these two factors. With increased pressure, there is increased

excretion of sodium in the urine and as a result the BP is lowered. Certain hormones can alter this pressure-natriuresis relationship in the kidneys. One example of this is the action of ALD, the primary salt-retaining hormone in the body (Laragh & Stoerk, 1957). The actions of ALD lead to an increase in extra-cellular osmolarity and water retention due to the action of ALD in the nephron to reabsorb Na^+ ions back into the blood. Aldosterone is a mineralocorticoid produced by the outer section of the adrenal cortex in the adrenal gland and secreted into the blood on stimulation by Ang II.

In the present study we observed elevated ALD levels in the plasma analysed from the untreated HFD group (Figure 3-22). This suggests that the high levels of ALD led to the reabsorption of ions and water in the kidneys, therefore conservation of sodium, excretion of potassium, retaining of water and thus increased the BP. This is supported in the current study by evidence of water retention in the HFD animals as indicated by the low amount of urine output that was measured over a time period of 24 hours when the rats were placed in metabolic cages (Figure 3-24 & Figure 3-25). The Buchu treated HFD group had significant lower levels of plasma ALD, accompanied by the reduction in BP observed between the two HFD groups (Figure 3-22). However with a reduction in plasma ALD level you would also expect to see less water retention due to a reduced amount of sodium being reabsorbed in the collecting ducts of the nephron, resulting in a higher urine output and lower BP. On the contrary, we observed a reduction in BP with the treatment of Buchu water in the HFD group but no increase in urine production (Figure 3-24 & Figure 3-25), while a clear diuretic effect was observed in the control animals ingesting Buchu water (Figure 3-24 & Figure 3-25) although this specific group tended to drink less (Figure 3-23). In future, analysis of the urine of the animals may shed some light on this observation. Alternatively, a role for ALD in the actions of Buchu water may be dissected out by using an ALD antagonist like Spironolactone (Chapman et al., 2007).

AORTA RINGS

In view of the very pronounced effect of the Buchu water treatment on the development of HTN in the animals, we investigated the possible interaction of Buchu water with the vascular bed to discern any effects on the vasodilatory response. The endothelium plays an important role in maintaining vascular tone and structure. Cardiovascular

disease risk states such as obesity and HTN have been showed to be associated with impaired endothelial function. Endothelial dysfunction is believed to be associated with the production of excess ROS. Reactive oxygen species react with NO that is produced in the endothelium, lowering the bio-availability of NO thereby diminishing endothelium-dependent vasodilation leading to the onset of HTN in obese individuals.

As was showed in the results section, we could not detect that Buchu had any effect on the endothelium using this model (Figure 3-28). To corroborate this observation, we investigated some of the known proteins involved in the synthesis of NO, a potent vasodilator that might play a role in the reduction of BP that we observed after chronic Buchu water treatment for 16 weeks. Nitric oxide is predominantly produced by eNOS. Various protein kinases such as AMPK and PKB/Akt are known to phosphorylate and activate eNOS to produce NO from L-arginine. However when we measured the expression of PKB/Akt (Figure 3-29) and AMPK proteins (Figure 3-30 & Figure 3-31) involved in the activation of eNOS we found no significant difference between the control or HFD groups. The high fat diet resulted in reduced expression of eNOS, however, there was no difference found in the expression of eNOS in the in the aortas between the untreated or treated animals (Figure 3-32). Despite this lower expression of eNOS, we detected no difference in the relaxation profiles of the smooth muscle of the aortas in our experimental set up. The results confirm however, that Buchu water does not elicit a response through the vascular bed or the proteins present in it to reduce BP. It would be interesting to isolate the endothelial cells from the HFD animals to investigate whether the lower eNOS expression is reflected in them as all the other cell types in the aortic tissue also express eNOS. In isolated cells, the activation of eNOS can directly be tested on the level of NO production.

CHAPTER 6: DISCUSSION PART 2 – Obese, insulin resistant animals without elevated blood pressure

For this first part of the present study we specifically used a HFD model to induce HTN in the animals. In the second part of the study we made use of a different model. The reason for this was that we did not want these animals to develop HTN. Hypertension is known to cause structural and functional cardiac adaptations, such as diastolic dysfunction, LVH or congestive heart failure (CHF). Left ventricular hypertrophy is the

primary cardiac manifestation of HTN (Ganau et al., 1992; Maron et al., 1978; Messerli & Aepfelbacher, 1995; Verdecchia et al., 1995). Left ventricular hypertrophy has also been shown to affect the substrate metabolism of the cardiomyocytes (de las Fuentes et al., 2003; Massie et al., 1995). We therefore used this model to investigate the effects of Buchu water on the cardiovascular system in obese non-hypertensive animals. For this we made use of a well-established rat model of hyperphagia-induced central obesity (DIO) (Pickavance et al., 1999).

6.1 Buchu water treatment and diet induced systemic alterations

The present study indicated that Buchu water treatment does have an effect on body weight regulation in overweight animals. After 16 weeks of treatment rats that received Buchu water had a lower body weight when compared to their respective untreated control and DIO groups (Figure 4-33). This is consistent with the first part of the study since the HTN obese animals that were on the HFD and were treated with Buchu water for 16 weeks also had a significant reduction in weight gain (Figure 3-8) and visceral fat gain (Figure 3-10).

The underlying mechanism of how Buchu water affects energy storage and leads to less weight gain is still unclear and is a topic of future studies. However, we have argued that it may work in a similar manner as other antihypertensive medication (ACEIs or ARB's) that interferes with the RAAS in the body (Shimabukuro et al., 2007; Weisinger et al., 2009), that seems to effect the fat stores (Figure 3-10) and at the same time cause a reduction in BP (Figure 3-26 & Figure 3-27).

6.2 Effect of Buchu water on the cardiovascular system in DIO rats

A study done by Huisamen et al. (unpublished results) clearly demonstrated that Buchu water treatment was responsible for a reduction in the infarct size in ex vivo perfused DIO rat hearts after myocardial ischaemia followed by reperfusion (Figure 4-35). Diet induced obese rats have previously been shown in our lab to develop larger

infarct sizes after they were subjected to regional ischaemia followed by reperfusion (Huisamen et al., 2011). This reduction in infarct size is a clear indication of the cardioprotective effects of Buchu water treatment in the DIO model. Reducing the infarct size is the golden standard to prove cardioprotection of a substance (Lochner et al., 2003). After we observed these cardioprotective effects as well as a reduction in both the body weight and intraperitoneal fat depots in the animals ingesting Buchu water, we aimed to investigate whether Buchu water has any effects on the insulin sensitivity of glucose uptake of cardiomyocytes, as this would be a further indication of cardioprotection.

Myocardial glucose uptake plays a critical role in cell survival during oxygen deprived states such as ischaemia, in the heart. The family of transporters responsible to facilitate the uptake of myocardial glucose is glucose transporters (GLUT's). The most abundant glucose transporter in the heart is the GLUT-4 transporter followed by GLUT-1. In cardiomyocytes these two GLUT transporters account for 70% and 30% respectively, of total glucose carriers (Garvey, Huecksteadt, & Birnbaum, 1989). GLUT-1 is mainly localised in the plasma membrane and responsible for basal glucose uptake whereas GLUT-4 is more abundant in intracellular vesicles under basal, unstimulated conditions (Zorzano et al., 1996). With elevation of insulin levels or during ischaemic conditions, GLUT-4 translocates to the plasma membrane to enable higher levels of glucose uptake (Slot et al., 1991; Sun et al., 1994). Thus GLUT-4 improves the ability to utilize the available glucose during oxygen deprived states to supply the heart with ATP, produced by glycolysis.

Our results showed that at the basal level, more radio-labelled deoxyglucose accumulated in cardiomyocytes prepared from the hearts of animals that consumed Buchu water (Figure 4-36). This may explain the cardioprotective effect observed resulting in smaller damage and smaller infarct development. However, we could not find evidence that Buchu water treatment improved insulin sensitivity in either the control or DIO groups. According to our results, cardiomyocytes isolated from the DIO animals were also not insulin resistant when compared to cells prepared from control animals (Figure 4-36). These results underscore those obtained in the HFD animals where neither the HFD nor Buchu water showed any effect on whole-body glucose homeostasis, as indicated by the IPGTTs (Figure 3-15).

Limitations of this study

In the current study only male Wistar rats were used. The use of female rats together with males may have given a more clear indication of how Buchu water may have affected HTN. The BP together with hormones (oestrogen and progesterone) fluctuating during the oestrous cycle should give interesting data also on gender differences in the development of HTN. Another limitation is that we did not use any other antihypertensive drugs, such as ARBs or ACEIs as a positive control treatment. This could have given a better indication of how effective Buchu water was in treating BP when compared to commercially available drugs on the market.

A different animal model can be used to cause systemic inflammation in the animals and then determine if Buchu water has any anti-inflammatory properties.

With regards to the aorta rings, Buchu water could have been added into the organ bath to investigate if it elicits any response during the experimental protocol when administered directly to the preparation. Alternatively, endothelial cells could be harvested from the aortas, cultured and their ability to produce NO determined.

In the current study, we did not determine the anti-oxidant status of the animals or any effects of Buchu water on that.

Future studies

- A new set of animals can be first exposed to a HFD and then treated from week 8 to determine the metabolic effects or corrections of Buchu water on the physiology of the body.
- The interactions between Buchu water, leptin and adiponectin should receive further attention for future research.
- Fasting insulin levels observed in the current study warrants further investigation since the current values unfortunately had a very large standard error.

- A model with a clear inflammatory response may be used to investigate whether Buchu treatment will have systemic anti-inflammatory effects.
- The kidney of each individual rat was also stored in formaldehyde. This could be used for histological studies in the future to shed some light on the effect of Buchu water on the kidney function.
- The urine collected from the animals could also be analysed to determine the functionality of the kidneys by measuring the amount of protein, salts and hormones which are excreted. This could help detect hormonal disorders caused by the excess adipose tissue observed in the current study.
- The fat depots of the animals need to be analysed to investigate the mechanism of how Buchu water affects energy storage and leads to less weight gain.

Conclusion

Buchu water is an affordable, already available herbal remedy and can be purchased without a prescription in South Africa. It therefore has enormous therapeutic potential in our society where inactivity is on the increase together with an unhealthy lifestyle which leads to high prevalence of obesity and HTN. In the current study, we have gathered substantial evidence of the antihypertensive properties Buchu. With regards to the possible mechanism involved, we have shown that:

- i) Buchu did not act as a diuretic in the animals on the HFD. The animals consuming Buchu water still presented with water retention
- ii) Although leptin levels were reduced by ingestion of Buchu water, this is probably a consequence of smaller fat depots as there were no differences in food intake.
- iii) There were no detectable differences in the smooth muscle contractility of the vascular bed, therefore an effect on vasodilation could not be responsible for the alleviation of hypertension
- iv) Aldosterone may play a role in the effects elicited by Buchu ingestion but this has not been proved conclusively
- v) Buchu may have ACEI actions as this may result in both weight loss and a lowering of BP as well as being cardioprotective.

To conclude, the results from this study is novel and demonstrates that Buchu water do possess some antihypertensive properties. Therefore Buchu water can reduce and treat both the haemodynamic and metabolic aberrations seen in individuals on a HFD, such as HTN and glucose intolerance at least partly through visceral fat remodelling. Therefore we conclude that Buchu water is an alternative, cost effective and readily available means to treat HTN and is beneficial to one's health. In addition, we have not observed any detrimental effects.

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