OBSERVED METABOLIC CHANGES IN MALE WISTAR RATS AFTER TREATMENT WITH AN ANTIDEPRESSANT IMPLIED IN UNDESIRABLE WEIGHT GAIN, OR *SUTHERLANDIA FRUTESCENS* FOR TYPE II DIABETES

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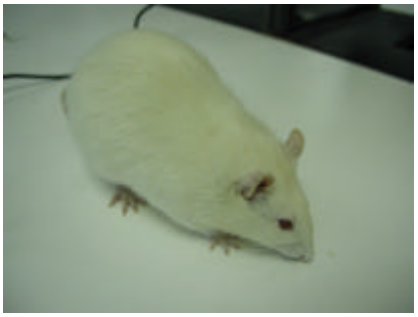

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ABSTRACT

Type II diabetes is fast becoming a growing problem in developed countries worldwide. Traditionally the median age for diagnosis was around sixty, but recent surveys have shown that the entire age distribution curve has shifted to the left. Western countries boast the worst statistics in which type II diabetes is being reported in children under the age of ten. At such a young age the disease often goes undiagnosed for long periods of time allowing considerable damage to occur. The incidence of type II diabetes is thought to be parallel with the growing rate of obesity associated with a characteristically unhealthy western diet.

Type II diabetes is an extremely expensive disease to manage, and with the rapid growth of this pandemic our country will soon feel the economic burden of this disease. It is for this reason that cheaper medication needs to be investigated in the form of traditional plants, such as *Sutherlandia frutescens.*

Prescription medication, such as tricyclic antidepressants, may also increase body weight or appetite thereby playing a role in obesity. The cause of weight gain in such cases may go unrecognized or lead to cessation of the medication with or without the practitioner's knowledge or approval. It is therefore necessary to investigate the causative agents responsible for the excessive weight gain.

Drinking water containing extracts of the *S. frutescens*, metformin (a well known type II diabetes medication) and amitriptyline (a common tricyclic antidepressant) was administered to three groups of ten male Wistar rats. The control group received water without any medication. The rat's weight and food consumption was monitored throughout the trial and their oxygen consumption was also determined. Rats were sacrificed after four months of medicinal compliance and glucose uptake, in the presence and absence of insulin, was tested in epididymal fat, liver and muscle. Fasting plasma glucose levels, lipoprotein, cholesterol and triglyceride concentrations were also determined.

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LIST OF ABREVIATIONS

ADP: Adenosine Diphosphate

AIDS: Acquired Immune Deficiency Syndrome

AMP: Adenosine Monophosphate

ATP: Adenosine Triphosphate

BMI: Body mass index

Bp: Base pairs

CoA: Coenzyme A

ERK-Endocrine Receptor Kinase

EST: Ecological Systems Theory

GEP: Guanosine Nucleotide Exchange Protein

GLUT4: Glucose Transporter 4

GSK-3: Glycogen Synthase Kinase

HDL: High-Density Lipoproteins

IDE: Insulin Degrading Enzyme

IGT: Impaired Glucose Tolerance

INT: Intron

IRS: Insulin Receptor Substrate

IVGT: Intravenous Glucose Tolerance Test

K_{ATP}: ATP-sensitive potassium channels

LDL: Low-Density Lipoproteins

MAPK: Mitogen Activating Protein Kinase

MKK: Mitogen Activating Protein Kinase-Kinase

MKKK: Mitogen Activating Protein Kinase-Kinase-Kinase

NAD: Nicotinamide Adenine Dinucleotide

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NBF: Nucleotide Binding Folds

NCEP: National Cholesterol Education Program

nt: Nucleotides

OGTT: Oral Glucose Tolerance Test

PDK: Phosphatidylinositol (3, 4, 5)-Triphosphate-Dependent Kinase

PI-3-K: Phosphatidyl Inositol-3-Kinase

PI3,4,5P: Phosphatidylinositol 3,4,5-Triphosphate

PI-kinase: Phosphoinositide-kinases

PIP: phosphatidylinositol-4-phosphate

PIP2: Phosphatidylinositol-4,5-biphosphate

PIP_3: Phosphatidyl Inositol 3,4,5-Triphosphate

PKB: Protein Kinase B

PPAR: Peroxisome Proliferator Activated Receptor

R_{GL}: Regulatory Protein

SSRI: Selective Serotonin Reuptake Inhibitors

SUR: Sulphonylurea Receptor

VLDL: Very Low-Density Lipoproteins

CHAPTER 1

REVIEW OF LITERATURE

1.1 OBESITY: A RISK FACTOR FOR LATE ONSET TYPE II DIABETES MELLITUS

1.2 INSULIN: A CLOSER LOOK INTO THE FORMATION, STRUCTURE, FUNCTION, AND DEGRADATION OF THIS MULTIFUNCTIONAL HORMONE

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1.1. OBESITY: A RISK FACTOR FOR LATE ONSET TYPE II DIABETES MELLITUS

1.1.1. Introduction

The enormous economic and health cost associated with obesity places it among the most pressing health care problems facing the westernised world to date. The United States and many other countries are facing an epidemic of insulin resistance, an evolving cardiovascular disease risk factor. This arises in the setting of a marked increase in the number of individuals diagnosed with type II diabetes and a dramatic increase in obesity. These trends are expected to increase throughout the next half century if serious actions are not undertaken (Case *et al.*, 2002).

1.1.2. Criteria for Obesity

Body mass index (BMI) is the cornerstone of the current classification system for obesity and its advantages are widely exploited across disciplines ranging from international surveillance to individual patient assessment (Prentice and Jebb, 2001). Body mass index is a measure of an individuals weight (kg)/height (m^2). Health risks associated with obesity begin in the range of 25 to 30kg/m^2. Values above 40kg/m^2 indicate severe obesity (Williams, 1997).

Body mass index is, however, only a surrogate measurement of body fatness while obesity is defined as an excess accumulation of body fat, and it is the amount of this excess fat that correlates with ill health. A wide range of conditions supporting this argument have been put forward which may provide misleading information about body fat content. These include infancy and childhood, ageing, racial differences, athletes, military and civil forces personnel, weight loss with or without exercise, physical training and special clinical circumstances (Prentice and Jebb, 2001).

A more practical way of identifying obesity is through the use of the metabolic syndrome. The metabolic syndrome is a clustering of risk factors such as plasma triglycerides, high density lipoprotein cholesterol, blood pressure and abdominal obesity defined by the National Cholesterol Education Program (NCEP). These are serious cardiovascular risk factors and are closely associated with insulin resistance. Epidemiological studies have shown that individuals with the metabolic syndrome and insulin resistance have a threefold increase in cardiovascular disease and a significant increase in cardiovascular mortality (Case *et al.,* 2002).

Case *et al.* (2002) showed (in his studies) that individuals tend to be more obese as the number of metabolic syndrome components increased. They also showed that obesity and weight loss were statistically significantly related to the baseline and changes in components of the metabolic syndrome. The study suggested that a very low calorie diet induced weight loss in individuals with the metabolic syndrome resulted in substantial reductions of systolic and diastolic blood pressure, plasma glucose, triglycerides and total cholesterol after four weeks. These results occurred

despite the persistence of a significantly elevated body mass index. The weight loss continued along with further significant reductions in blood pressure and triglycerides until the individuals no longer met the criteria for the metabolic syndrome (Case *et al.*, 2002). This study displayed that a very low calorie diet is associated with a greater initial weight loss and active weight maintenance programme.

1.1.3. Who is to Blame?

The factors that contribute to excessive weight gain in humans are widespread and mostly unknown. This accounts for the fact that present treatments intended to produce a long-term reduction in body weight are largely ineffective (Clement, 1999). Obesity is not considered to be the result of a single genetic anomaly but instead is thought to depend on interactions between the following:

- Environmental and physiological factors

This includes physical activity, eating habits, social and cultural habits. Increases in the rate of childhood overweight are of particular concern due to the negative health and physiological effects noted among overweight children. The development of childhood overweight involves a complex set of factors from multiple contexts that interact with each other to place a child at risk of overweight. This multifaceted system can be conceptualised using Ecological Systems Theory (EST). This system highlights the importance of considering the context, or ecological niche, in which a person is located in order to understand the emergence of a particular characteristic. In the case of a child this ecological niche includes the family and the school, which in turn are embedded in larger social contexts including the community and society at large (Davison and Birch, 2001).

Specific characteristics which place an individual at risk of the development of obesity include: dietary intake, lack of physical activity and sedentary behaviour. Increases in cases of obesity are also a reflection in lifestyle changes brought about by the mechanisation of modern life (Vass, 2002). Hanely *et al*. (2000)

showed that increased television viewing was associated with a significant higher risk of becoming overweight.

It is evident that the fast pace of today's society leaves no room for healthy living, or does it? It seems more likely that man is becoming lazy, preferring to consume fast foods, exercise less, and favouring to drive to his/her destination.

- Genetic and hormonal factors

Although overeating and a decreased physical activity all contribute to obesity recent research strongly indicates that hereditary factors could be of equal importance for the development of both obesity and obesity complications. During the last decade, a continuously increasing interest has been focused on identifying genes that contribute to obesity and type II diabetes in humans, and the mode of action of these genes (Lonnqvist *et al*., 1999).

It has long been postulated that secondary to overeating, the storage of additional fat will give signals to the brain that the body is obese, which in turn makes the subject eat less and burn more fuel. The discovery of such a determinator, leptin, and its receptor has therefore opened new avenues for obesity research. Both are parts of a complex lipostatic hormonal feedback loop regulating body fat stores through effects on both satiety and energy expenditure (Lonnqvist *et al*., 1999).

Leptin is encoded by the *ob* gene, which is expressed exclusively in white and brown adipose tissue. Under normal conditions leptin mRNA levels correlate with the amount of body fat. The *ob* gene contains three exons; the coding sequence spans the last two exons and is interrupted by a 2kb intronic sequence. The major transcription of the human *ob* gene is a 4.5kb mRNA containing a long 3'-untranslated region. The 167 amino acid protein leptin is well conserved between species. The leptin receptor is, in turn encoded by the *db* gene (Clement, 1999).

Except in very rare monogenic cases leading to disruption of the leptin axis in humans, genetic studies have shown that *ob* and *db* loci do not have a major role in common forms of human obesity for a large proportion of the population.

However, the results of linkage and association studies with the *ob* and *db* genes do not exclude a minor role for these genes in the development of obesity. The polygenic nature of human obesity means that the presence of susceptibility alleles of numerous genes could increase the probability that the bearer develops obesity. The presence of the genetic variation is not enough to explain the expression of the disorder, but rather interacts with other genetic, metabolic or environmental factors. Mutations associated with the peroxisome proliferator activated receptor (PPAR) family could also play a role in the development of human obesity (Clement, 1999).

1.1.4. Treatment of Obesity and Maintenance of a Desirable Weight

The first goal of a patient with excess weight should be to prevent further weight increase, then to lose a modest amount on a twelve week structured programme which includes diets, physical activity, and behavioural modification followed by a further twelve week programme for weight maintenance to allow for stabilisation of the energy balance (MIMS, 2000). Weight management programs make use of one or more of the following criteria:

Diet:

Very low calorie diets, providing 400 to 800 kcal/day, have become less popular as many patients seem to regain all the weight they have lost shortly after the diet. The energy profile of any diet should obtain less than 35% of the energy from fat, and 10-15% from protein; however a protein intake of 70g/day in elderly individuals is desirable. When calorie intake exceeds that expended, excess fat intake is stored as fat, whereas excess carbohydrate is mainly oxidised in the short term but can lead to substantial gain in fat stores because of the reduced fat oxidation and considerable new fat formation in the long term (MIMS, 2000).

Exercise:

A more active life-style should be adopted, including a daily cumulative thirty minutes or more of moderate-intensity activity (MIMS, 2000).

Pharmacotherapy:

This treatment is only recommended to patients with high risk obesity and who have not managed to reduce their body weight by adaptations to their lifestyle alone. If such drugs are required then their use should be considered for long-term administration, similar to the current practice with drugs used for other chronic diseases. Over the counter drugs are generally harmless, questionably ineffective, and best avoided (MIMS, 2000).

Surgical options:

Vertical banded gastroplast is recommended for the morbidly obese individuals (BMI>40) with high co-morbid risk factors (MIMS, 2000).

1.1.5. Motivation

Obesity is the gateway to many metabolic disorders including cardiovascular disease, type II diabetes, hypertension, gallbladder disease and stroke. This alone makes obesity an extremely expensive disorder, an economic burden many individuals cannot cope with.

The rapid onset of obesity in South Africa is associated with a rapid increase in insulin resistance and cardiovascular disease brought about by the westernised way of living in rural areas. Although the adoption of a westernised culture is the main instigator certain genetic factors may be to blame, in some cases, and certain prescription medication may also cause excessive weight gain. The fact remains that insulin resistance associated with type II diabetes is a fast growing pandemic in Southern Africa, placing a large economic burden on the government for overpriced medication. It is for this reason that new avenues need to be investigated, including the potential use of traditional medicine. This may relieve the economic burden and open opportunities for export of an indigenous medication against type II diabetes.

1.2. INSULIN: A CLOSER LOOK INTO THE FORMATION, STRUCTURE, FUNCTION, AND DEGRADATION OF THIS MULTIFUNCTIONAL HORMONE

1.2.1. Structural Detail of Insulin

Insulin is a polypeptide hormone produced in the pancreatic beta cells in the islets of Langerhans. It is a small protein with a molecular weight of 5700, which is released in response to increased glucose levels in the blood. It consists of two polypeptide chains, namely A and B which are then joined by two disulfide bonds (Lehninger *et al.*, 2000), see figure 1.1.

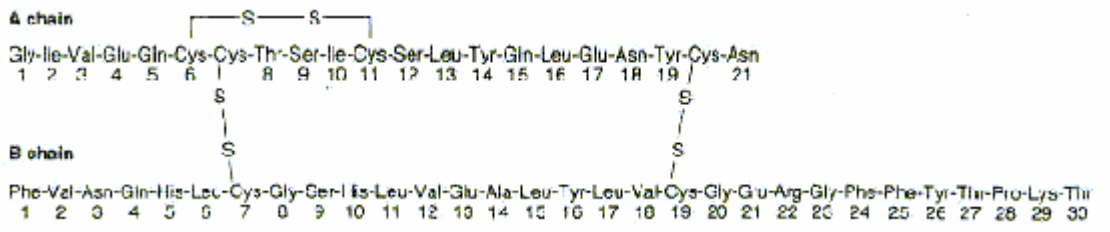

Figure 1.1: The amino acid sequence of the two chains of human insulin, joined by disulfide bridges (taken from Ganong, 1993)

The structures of mouse and rat insulins are identical, however, there is a slight divergence in comparison to the A and B chains of the human insulin structure. On the A chain it has been found that Glu has been replaced by Asp at position 4, for mouse insulin, while three changes are apparent on the B chain of human insulin at positions 3, 9, and 30, which are Lys replacing Asn, Phe replacing Ser, and Ser replacing Thr respectively (Beintema and Campagne, 1987). Despite these four changes setting these two almost identical hormones apart, their role and function within the body appears to be exactly the same.

1.2.2. Insulin, the Initiation of its Being

In adult mammals the insulin gene is expressed solely in the pancreatic beta cells. The beta cells specific expression is controlled by transcriptional enhancer and promoter sequence elements in the 5' flanking DNA of the gene (Flatt, 1992).
The initial translational product of this gene is pre-proinsulin, which consists of an N-terminal signal peptide linked to proinsulin. Like most nascent secretory proteins, translocation of pre-proinsulin across the rough endoplasmic reticulum membrane commences by interaction of the signal sequence with the 54kDa polypeptide component of the signal recognition particle, an event which retards further elongation of the peptide chain (Flatt, 1992).

The association of this complex with the rough endoplasmic reticulum membrane is mediated by the affinity of the signal recognition particle for the signal recognition receptor, also known as the docking protein. This interaction then promotes the release of the signal recognition particle from both the ribosome and the signal sequence, which is then transferred to the signal sequence receptor, a glycosylated, integral membrane protein (Flatt, 1992).

1.2.3. Formation and Packaging of Insulin

Insulin is synthesized in the pancreatic beta cells as an inactive single-chain precursor, preproinsulin with an amino terminal "signal sequence" that directs its passage into secretory vesicles (Lehninger *et al.*, 2000). The signal sequence, which consists of 23 amino acids, is proteolytically removed at the amino terminus of the pre-proinsulin and the formation of three disulfide bonds produces proinsulin, see figure 1.2. Proinsulin is then transported through the cisternae of the endoplasmic reticulum to the Golgi complex for packaging into secretory granules. The proinsulin is then converted into insulin, through the proteolytic cleavage of the C-peptide, all of which takes place in the secretory granule. The insulin is then stored as a hexamer which is stabilized by a zinc ion. The intact insulin, C-peptide, and some basic amino acids are all released into the extracellular fluid during insulin secretion. This allows an assessment of endogenous insulin, released from diabetic patients, receiving

insulin to be determined through measuring the concentration of C-peptide released in conjunction with the endogenous insulin (Newsholme and Leech, 1994).

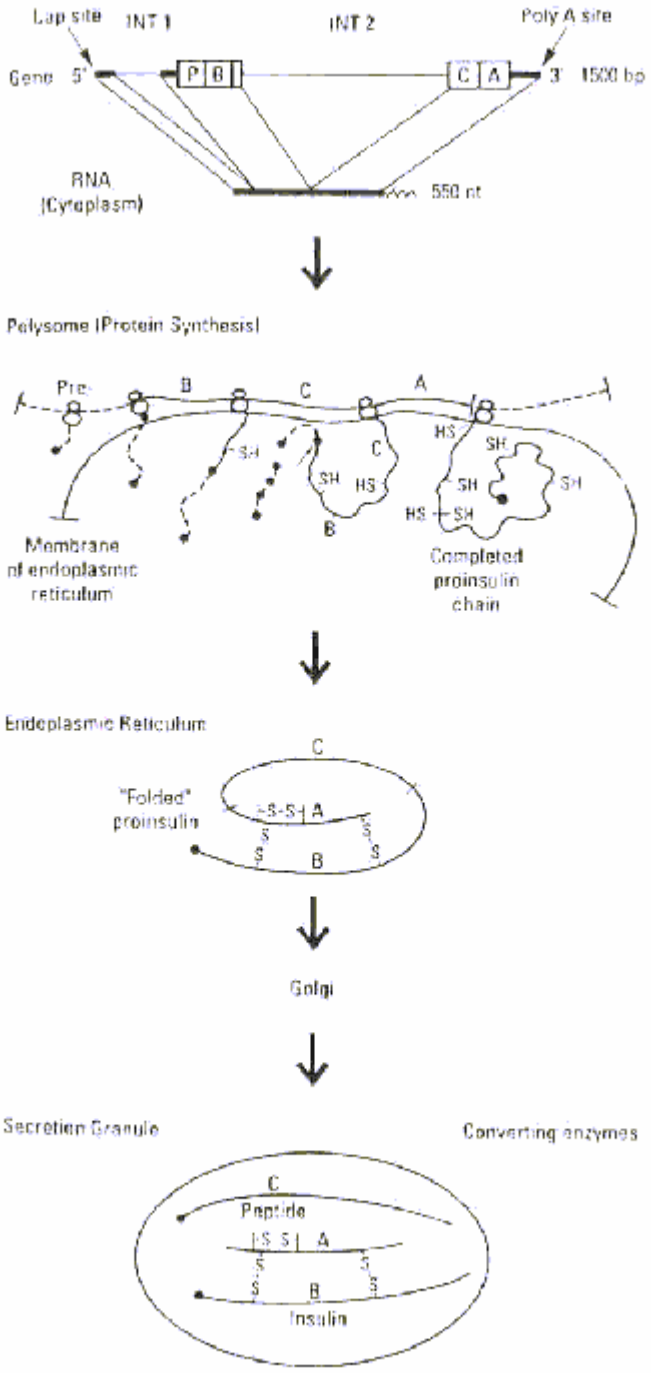

Figure 1.2: A schematic representation of insulin formation (bp, base pairs; nt, nucleotides and INT, intron) (taken from Ganong, 1993)

The three exons, in figure 1.2, of the insulin gene are seperated by two introns (INT 1 and INT 2). Exons 1 and 2 code for an untranslated part of the mRNA, exon 2 codes for the signal peptide (P) and the B chain (B), exons 2 and 3 code for the C peptide (C), and exon 3 codes for the A chain (A) plus an untranslated part of the mRNA. The signal peptide guides the polypeptide chain into the endoplasmic reticulum and is then removed. The molecule is next folded, with formation of the disulfide bonds. The C peptide is separated by one or more converting enzymes in the secretory granules.

1.2.4. Secretion of Insulin

The biosynthesis and secretion of insulin by the islets of Langerhans are regulated by many circulating factors including glucose, neurotransmitters and hormones.

(a) As controlled through insulin gene transcription

It has been demonstrated that in isolated islets there is an increase in the synthesis of insulin mRNA relative to other cellular mRNA, when the islets are induced with glucose. An experiment confirmed this by demonstrating that the insulin mRNA content of islets was reduced by culture in low glucose (0-3.3mM) compared with islets cultured in high glucose (17-20mM). In addition, the rate of insulin gene transcription was approximately 3-fold higher in islets cultured in 17mM glucose compared with 3.3mM glucose. The experiment was also able to show that the half-life of insulin mRNA was 2.6 fold greater in islets cultured in 17mM glucose compared to those cultured in 3.3mM glucose (Flatt, 1992).

(b) As controlled through glucose concentrations

Insulin is released from their granules, in the beta cells of the pancreas, through exocytosis in response to an elevated blood glucose level, usually above 5mM (Tortora and Grabowski, 2000). The movement of the granules to the cell membrane in response to stimulation is due to microfilaments and microtubules which are organized in a subcytosolic web with individual microfilaments inserting in the cell membrane and the microtubules. Insulin secretion can therefore be inhibited through

the depolymerization of tubulin, which results in the breakdown of the microtubules (Newsholme and Leech, 1994).

It is thought that there is a link between a glycolytic intermediate and insulin secretion, which together is known as the coupling factor and is thought to involve calcium ions. Phosphoenolpyruvate is thought to be the intermediate involved. The intracellular protein that detects the change in concentration of calcium ions is probably calmodulin, a heat stable protein with a molecular mass of about 17 000. This protein is able to bind up to four calcium ions at a time thereby changing the three dimensional structure of the protein which in turn somehow controls the contractile activity of the microtubules (Newsholme and Leech, 1994).

The entire process is then thought to interact with one another to bring about insulin secretion as follows: when the glucose concentration increases to about 5mM, the rate of glycolysis increases proportionally, thereby raising the concentration of phosphoenol- pyruvate. The increased activity of the glycolytic pathway generates signals that close ATP-sensitive K^+channels in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarization with subsequent opening of voltage dependent calcium channels. The calcium influx through these channels increases, causing a rise in free cytoplasmic calcium which serves as the triggering signal (Shepard and Henquin, 1995).

The triggering signal then functions via calmodulin which causes contraction of the microfilaments or microtubules, which may contain actin or myosin and hence contract in a similar way to skeletal muscles. This contraction then results in an increased rate of exocytosis and insulin secretion (Newsholme and Leech, 1994).

(c) As controlled through hormonal regulation

It has also been demonstrated that endogeneous insulin itself is able to stimulate insulin secretion in pancreatic beta cells. Functional insulin receptors have been found on beta cells, and the insulin concentration necessary to activate these receptors to bring about insulin secretion is in the nanomolar range (Aspinwall *et al.*, 1998).

The insulin-stimulated insulin secretion is not controlled by glucose or increased glucose utilization as the effect occurs even at 0mM glucose. Insulin does, however, cause a rise in the intracellular calcium concentration which subsequently initiates insulin secretion as described earlier, through the binding of the calcium to the calmodulin protein. The beta cell insulin system is a rare example of positive feedback on secretion as most autoreceptors mediate negative feedback on secretion (Aspinwall *et al.*, 1998).

This positive feedback mechanism would cause augmented secretion during the initial stages of elevated glucose levels giving rise to a greater bolus of insulin release, however, other mechanisms must eventually take over to suppress the release. This could therefore be the reason that a rapid increase is observed in the first phase insulin secretion and the sustained, lower secretion is observed during the second phase of secretion. It has also been discovered that type II diabetics have a reduction in first phase insulin secretion, which could be a result involving a lack of positive feedback from the beta cell insulin receptor (Aspinwall *et al.*, 1998).

Figure 1.3 illustrates the way in which insulin is secreted in its two peaks. The first peak is released approximately 3-10 minutes after the ingestion of glucose. This insulin has already been produced by the beta cells of the pancreas, and is in storage. Its main function is to stop the blood glucose from rising too high, as this could overstimulate, and thereby damage the pancreas. The second phase of insulin secretion occurs approximately 30 minutes later from the beta cells. This insulin is not from a stored reserve but has been produced for this specific task. The size of this phase is directly proportional to the elevation of glycaemia (Servier, 2001).

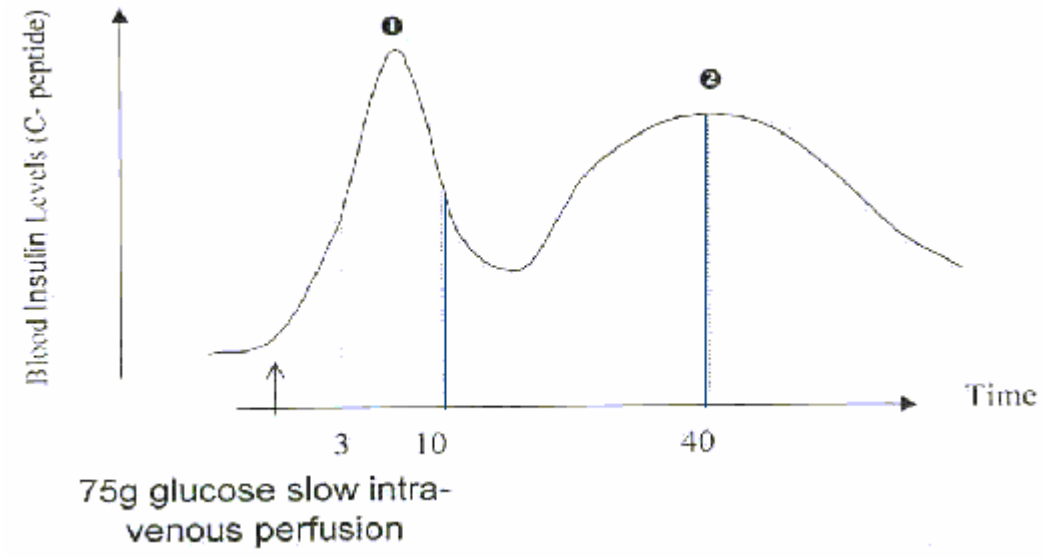

Figure 1.3: A representation of the two insulin peaks experienced during a typical insulin secretion (taken from Servier, 2001)

(d) As controlled through a secondary messenger

Cyclic AMP is also thought to be related to insulin secretion, not directly but rather by increasing the magnitude of the secretory response to glucose or other sugars which can enter the glycolytic pathway. It is likely that this effect is achieved by activation of cyclic AMP dependent protein kinase and phosphorylation of a protein involved in control of calcium transport, so that for a given concentration of glucose more calcium enters the cytosolic compartment of the cell, or phosphorylation of microtubules could increase their responsiveness to a given change in the calcium ion concentration (Newsholme and Leech, 1994).

1.2.5. Insulin: Hard at Work

This polypeptide hormone is invaluable through its actions within the human body, as are many other hormones. Insulin's sole function, however, is not only to decrease the concentration of circulating glucose in the blood through its conversion into glycogen and adipose tissue, but it has many other important tasks:

(a) Effects of insulin on carbohydrate metabolism

1. Insulin increases transport of glucose across the cell membrane in adipose tissue and muscle, for storage when excess glucose is present and for the breakdown of glucose into ATP respectively (Montgomery *et al.*, 1996).

2. Insulin stimulates glycogen synthesis (to be discussed later in more detail) in a number of tissues including adipose tissue, liver, and muscle (Lehninger *et al.*, 2000).

3. Insulin increases glycolysis, indirectly, by stimulating other processes (triacylglycerol, glycogen, and protein synthesis) requiring increased rates of ATP formation (Montgomery *et al.*, 1996).

4. Insulin inhibits the rates of glycogenolysis, by inhibiting the glucagon hormone which promotes the breakdown of glycogen into glucose, and gluconeogenesis in the liver. Gluconeogenesis is inhibited due to the fact that the cellular uptake of glucose is promoted by insulin, thereby initiating the breakdown of the carbohydrate to produce energy for the body. If insulin is not present in a sufficient amount to promote the cellular uptake of glucose then there is no energy being produced and the body assumes that it is being starved. In such a case acetyl-CoA and oxaloacetate, from the citric acid cycle, are converted through the process of gluconeogenesis into glucose in an attempt to increase the blood glucose concentration so that the body may be able to once again produce sufficient energy for survival of important organs, including the most important of all the brain, which is only able to obtain its energy from glycolysis (Lehninger *et al.*, 2000).

5. Insulin increases the activity of the glucokinase enzyme in the liver. This enzyme is responsible for converting glucose into glucose-6-phosphate during glycogen synthesis. This may play an important part in decreasing the rate of glucose release and facilitating an increase in glucose uptake, by the liver, after a meal (Lehninger *et al.*, 2000).

6. Insulin increases the rate of glucose oxidation by the pentose phosphate pathway in liver and adipose tissue, this is however secondary to the stimulation of fatty acid synthesis by this hormone (Newsholme and Leech, 1994).

Certain tissues, such as the kidney, brain, and intestine, are totally insensitive to the action of insulin on carbohydrate metabolism. This introduces a problem as to how to reduce glucose utilization in these tissues during starvation. The problem is however overcome through the mobilization of fatty acids from adipose tissue to the liver where ketogenesis is increased so that the oxidation of this fuel will reduce glucose utilization and oxidation (Newsholme and Leech, 1994).

(b) Effects of insulin on lipid metabolism

1. Insulin inhibits lipolysis in adipose tissue because it is involved in the activation of the pyruvate dehydrogenase complex and citrate lyase, both of which supply acetyl-CoA, thereby promoting fatty acid synthesis. If fatty acid synthesis and beta oxidation were to occur simultaneously, the two processes would constitute a futile cycle, wasting energy. Thus during fatty acid synthesis, the production of the first intermediate, malonyl-CoA, shuts down beta oxidation at the level of the transport system in the mitochondrial inner membrane (Lehninger *et al.*, 2000).

2. Insulin inhibits ketone body synthesis in the liver, by ensuring sufficient glucose is taken up by cells in the body. If insulin is not present in sufficient amounts, to promote the cellular uptake of glucose, then the citric acid cycle intermediates are used for glucose synthesis via gluconeogenesis. This causes the subsequent slowing of the oxidation of the citric acid cycle intermediates as well as the oxidation of acetyl-CoA. Moreover, the liver contains only a limited amount of coenzyme A, and when most of it is tied up in acetyl-CoA, beta oxidation of fatty acids slows for lack of the free coenzyme. However the production and export of the ketone bodies frees the coenzyme A, thereby allowing continued fatty acid oxidation (Lehninger *et al.*, 2000).

3. Insulin also stimulates fatty acid and triacylglycerol synthesis in adipose tissue and liver (Newsholme and Leech, 1994). Insulin promotes the conversion of carbohydrates into triacylglycerols. The pyruvate dehydrogenase complex and citrate lyase, both of which supply acetyl-CoA, are activated by insulin. The acetyl-CoA then goes on to form malonyl-CoA which participates in the biosynthesis of fatty acids (Lehninger *et al.*, 2000).

(c) Effects of insulin on protein metabolism

1. Insulin increases the rate of amino acid transport into the muscle, adipose tissue, and liver cells, so that amino acids may be used for protein synthesis rather than gluconeogenesis (MacSween and Whaley, 1992).

2. Insulin increases the rate of protein synthesis in muscle, adipose tissue, liver and other tissues (MacSween and Whaley, 1992).

3. Protein degradation in muscles is also decreased through the action of insulin (Newsholme and Leech, 1994).

(d) Effects of insulin and glucagon on metabolism in the liver

Glucagon is responsible for increasing the rate of both glycogenolysis and gluconeogenesis, which thereby increases the rate of glucose release by the liver. This effect may however be reduced or abolished if the concentration of insulin is increased (Newsholme and Leech, 1994).

1.2.6. Main Action of Insulin

As mentioned earlier insulin stimulates glycogen synthesis, which is the main function of this polypeptide hormone, and will therefore be discussed in more detail in the section to follow.

In all animals excess glucose from carbohydrates in the diet or from gluconeogenesis is stored as glycogen in muscle or liver. The balance between glycogen synthesis and degradation in the liver is controlled by the hormones glucagon and insulin. The starting point for glycogen synthesis is glucose-6-phosphate, which is derived from free glucose by the hexokinase reaction:

D-glucose + ATP ---> D-glucose-6-phosphate + ADP

To initiate glycogen synthesis the glucose-6-phosphate is firstly reversibly converted into glucose-1-phosphate by phosphoglucomutase:

Glucose-6-phosphate <---> glucose-1-phosphate

This reaction is followed by the formation of UDP-glucose, a key reaction in glycogen biosynthesis, which is catalyzed by the enzyme, UDP-glucose pyrophosphorylase:

Glycogen-1-phosphate + UTP ---> UDP-glucose + PPi

The energy produced in this reaction is -25kJ/mol, because the pyrophosphate is rapidly hydrolyzed to orthophosphate by the inorganic pyrophosphatase (Lehninger *et al.*, 2000).

UDP-glucose is the intermediate donor of glucose residues in the enzymatic formation of glycogen by the action of glycogen synthase, which promotes the transfer of the glucosyl residue from UDP-glucose to a nonreducing end of the branched glycogen (Lehninger *et al.*, 2000). Insulin controls glycogen synthesis by controlling the activation and deactivation of glycogen synthase, by indirectly initiating the phosphorylation (inactive form) and dephosphorylation (active form) of the enzyme.

1.2.7. Insulin Clearance

The primary organs involved in the clearance of insulin from the circulation to the degradation of the hormone include the liver, kidney, and muscle (Duckworth *et al.*, 1998). Insulin uptake and degradation also occurs in adipocytes, fibroblasts, monocytes, lymphocytes, gastrointestinal cells, and all other tissues that contain insulin receptors. The liver is the predominant organ, for both normally secreted endogenous insulin, as well as exogenously administered insulin. Uptake is mediated primarily by the insulin receptor, at physiological concentrations, with a smaller contribution from nonspecific processes. However at higher concentrations nonreceptor processes assume greater importance. Insulin has a plasma half-life between 4 and 6 minutes due to the necessity for it to respond rapidly to changes in blood glucose. A brief summary of each of the major organs involved in insulin clearance follows:

1.2.7.1. Liver

The liver removes approximately 50% of portal insulin. Since most uptake is a receptor mediated process, very high concentrations of insulin (500-2000μU/ml) result in a decrease in the fractional uptake, although total uptake is increased. Removal of circulating insulin does not imply immediate destruction of the hormone, a certain amount of receptor bound insulin is released from the cell and re-enters circulation. The clearance rate of the liver is decreased in individuals who suffer from obesity and diabetes (Duckworth *et al.*, 1998).

1.2.7.2. Kidney

Another important site of insulin clearance is the kidney, removing approximately 50% of peripheral insulin. The kidney is also responsible for removing 50% of circulating proinsulin and 70% of C-peptide by glomerular filtration. Glomerular filtration and proximal tubular reabsorption and degradation are the two mechanisms for insulin clearance in the kidney. However during glomerular clearance more than 99% of the filtered insulin is reabsorbed, primarily by endocytosis. Therefore very

little insulin is ultimately excreted in the urine. Insulin degradation by kidney cells is carried out by the same process as that of the liver. Insulin is internalized into endosomes where degradation is initiated (Duckworth *et al.*, 1998). Some insulin may be released by retroendocytosis, from the cell. Lysosomes play a greater and earlier role in kidney insulin degradation, unlike liver. In the kidney most insulin and partially degraded insulin are delivered directly to lysosomes where degradation takes place (Duckworth *et al.*, 1998).

1.2.7.3. Other tissues

All insulin sensitive tissues remove and degrade insulin which is not cleared by the liver or kidney. The muscle also plays a large role in the removal of this hormone, the mechanism includes insulin binding to its receptor, internalization, and degradation. As already mentioned earlier adipocytes, fibroblasts, monocytes, lymphocytes, and gastrointestinal cells also degrade insulin. In short all cells that contain insulin receptors and internalization mechanisms can degrade insulin (Duckworth *et al.*, 1998).

1.2.8. Insulin receptor

The insulin receptor is a protein kinase, which transfers a phosphate group from ATP to the hydroxyl group of Tyr residues. The receptor consists of two identical alpha chains protruding from the outer face of the plasma membrane, and two transmembrane beta subunits, with their carboxyl termini on the cytosolic face (Lehninger *et al.*, 2000). The insulin binding domain is found on the alpha chain, and the beta chains contain the tyrosine kinase domain see figure 1.4.

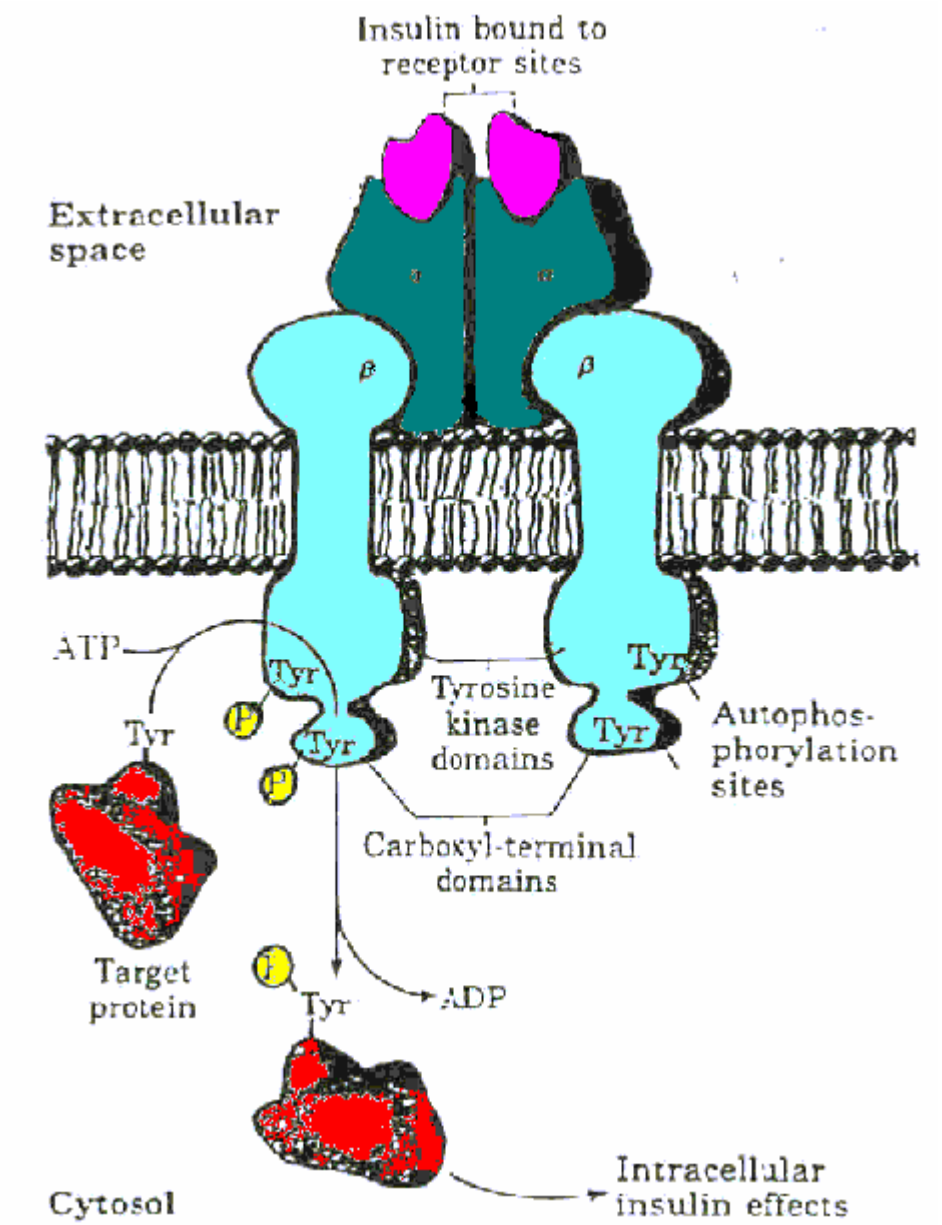

Figure 1.4: A schematic representation depicting the functional characteristics of the insulin receptor (taken from Lehninger *et al.*, 2000)

1.2.8.1. The Insulin Signalling Pathway

It seems as if the insulin receptor consists of two routes of signalling. Once the insulin binds to the receptor the tyrosine protein kinase activity is enhanced 10-fold, thereby causing the beta chains to bind to neighbouring adaptor proteins and phosphorylate the tyrosine residues. The two important adaptor proteins, IRS (insulin receptor substrate), and Shc, are found at the head of the two major insulin responsive signal transduction pathways, as can be seen in figure 1.5.

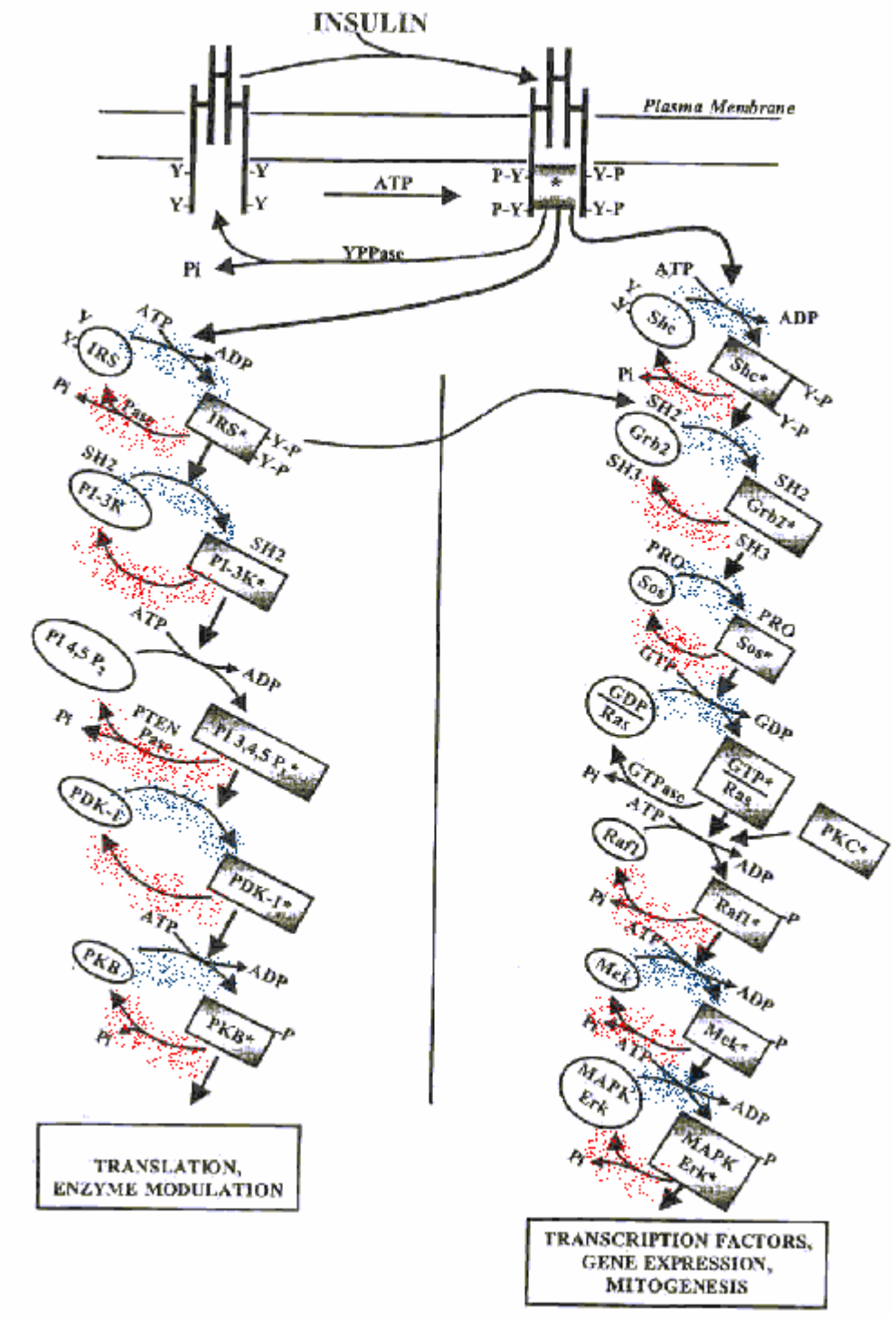

Figure 1.5: A schematic diagram displaying the two routes of insulin signalling (taken from Gibson and Harris, 2002). For abbreviations refer to text.

The pathway on the left is labelled PI-3-K (phosphatidyl inositol-3-kinase) pathway and the pathway on the right is designated MAPK (mitogen activating protein kinase) pathway. It has recently been proposed that the MAPK pathway of liver and adipose

tissue terminates in the nucleus with the induction or repression (transcription) of a set of genes of key enzymes involved in insulin-glucagon sensitive (long term) control of metabolic pathways. The PI-3-K pathway signals an increase in protein synthesis through the promotion of translation and may also be responsible for the net dephosphorylation of key enzymes in the liver, adipose, and muscle which promote the formation of glycogen and triglycerides (Gibson and Harris, 2002).

- The PI-3-K pathway

IRS binds reversibly to the activated insulin receptor beta domains to initiate the first pathway. Once the insulin receptor tyrosine kinase is phosphorylated the bound IRS undergoes a conformational change and attracts a specific recognition site, the SH2, on the PI-3-K through its p85 regulatory subunit. The specificity of the protein-protein interactions depends not only on the SH2 domains, but also on adjacent amino acid sequences (Gibson and Harris, 2002), as can be seen in figure 1.6.

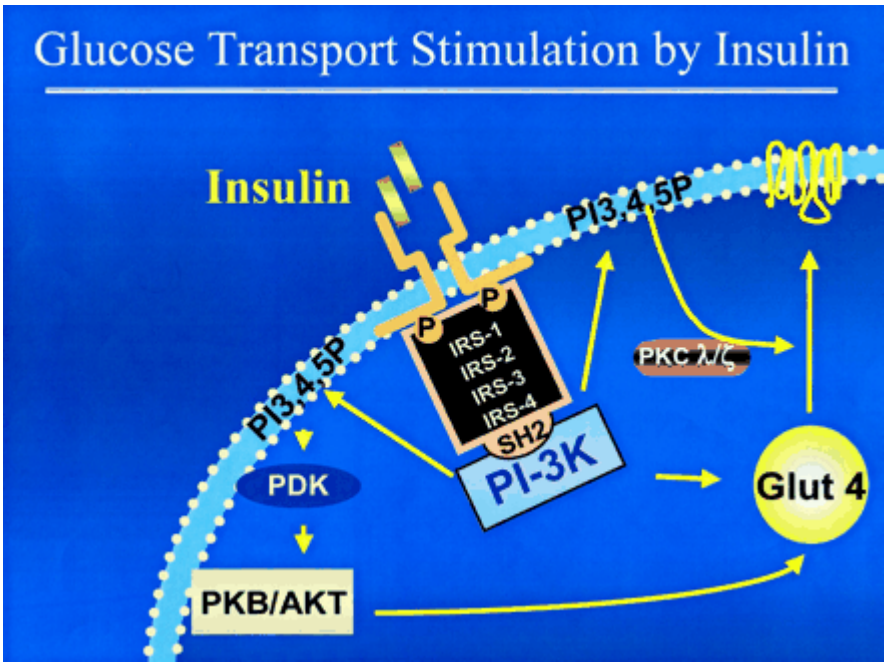

Figure 1.6: Molecular mechanism of insulin-stimulated transport. The insulin-dependent glucose transporter 4 (GLUT4) is translocated by a phosphatidylinositol 3-kinase (PI 3K)-dependent pathway including PKB/AKT and PKC stimulation downstream of PI3K. PI3,4,5P, Phosphatidylinositol 3,4,5-phosphate; PDK, phosphatidylinositol (3 4 5)-phosphate-dependent kinase; IRS, insulin receptor substrate (taken from Matthaei *et al.,* 2000)

The PI-3-K, located near the cytosolic surface of the plasma membrane, catalyses the phosphorylation of inositol at position 3 of 4,5 diphosphophophatidyl inositol ring producing phosphatidyl inositol 3,4,5-triphosphate (PIP_3). PIP_3 is embedded in the plasma membrane and is elevated during insulin signalling. It is clear that PI-3-K is able to control several signal pathway outcomes depending on the cell type: enhancement of transcription and translation, impairment of apoptosis, or the terminal dephosphorylation state of enzymes that are crucial for metabolic control (Gibson and Harris, 2002).

PI-3-K then catalyses the phosphorylation of PKB (protein kinase B), thereby activating it, which in turn catalyses the binding of PIP_3 to the protein kinase B and initiates PKB and phosphatidyl inositol dependent kinase (PDK-1) to move along side one another in the plasma membrane. PKB and PDK-1 are responsible for inactivating glycogen synthase kinase (GSK-3), which normally phosphorylates and inactivates glycogen synthase. However with the GSK-3 enzyme activity decreased, the opposing glycogen synthase-1G takes over. The protein phosphatase (PP1G), which is bound to glycogen through a regulatory protein (R_{GL}) brings about dephosphorylation of glycogen synthase, phosphorylase kinase, and glycogen phosphorylase in opposing the action of protein kinases, and thereby promoting glycogen synthesis. This insulin pathway is also involved in recruiting GLUT4 glucose transporters in skeletal muscle and adipose tissue (Gibson and Harris, 2002).

- The MAPK pathway

In this pathway the insulin activated receptor tyrosine kinase phosphorylates the tyrosines on the adaptor protein Shc, thereby activating it and catalysing the binding to SH2 domains on a second adaptor protein called Grb-2. The Grb-2 protein amino acid sequence domain, SH3, binds to the proline rich regions of the next signal transduction protein Sos, thereby activating it. Sos is a guanosine nucleotide exchange protein (GEP) which continues the cascade of activation by converting a monomeric small G protein called Ras from its GDP-inactive mode to its GTP-active mode (Gibson and Harris, 2002).

In the presence of Sos bound GDP is replaced by the insertion of GTP from the cytosol. In the opposite direction are GAPs (GTPase activating proteins), which hydrolyse the bound GTP to GDP returning the system to a ground state. Ras is therefore an important regulatory protein (Gibson and Harris, 2002).

The now active Ras binds to the first of a series of linked protein kinases that phosphorylate (activate) and are themselves phosphorylated (activated) on specific serine, threonine, or tyrosine residues. Figure 1.5 indicates Raf-1, Mek and MAPK (ERK-endocrine receptor kinase) which are activated in response to insulin. They are representatives of three large families of kinases found in the various differentiated cells: MKKK (mitogen activating protein kinase-kinase-kinase), MKK (mitogen activating protein kinase-kinase), and MAPK (mitogen activating protein kinase), respectively. These three interlocked kinases are usually grouped together by scaffolding proteins, constituting a Raf-MAPK pathway module, and all of which are opposed by protein phosphatases. The final linkage to gene expression is through transcriptional factors which bind to the insulin responsive elements. This pathway is concerned with the transcription of enzymes catalysing glycolysis and the formation of fatty acids, as well as repression of liver enzymes catalysing gluconeogenesis (Gibson and Harris, 2002).

1.2.8.2. Internalisation and Degradation of Insulin

Binding of the hormone to the cell membrane receptor is the initial step in the degradative process (1 in Fig. 1.7). Once bound, the insulin receptor, serves as a reservoir that can return intact insulin into circulation (2 in Fig. 1.7) or deliver it to an intact site. However for degradative purposes clustering of the receptor-ligand complexes occurs (3 in Fig. 1.7), followed by invagination of the coated pits (4 in Fig. 1.7), and pinching off of the pit to form an intracytoplasmic vesicle or a endosome (5 in Fig. 1.7). Degradation is initiated in these vesicles by the insulin degrading enzyme (IDE) prior to acidification of the vesicle and dissociation of the receptor-insulin complex (Duckworth *et al.*, 1997). The internal pH, of the endosome,

then rapidly falls to pH6, resulting in the dissociation of the insulin- receptor complex (6A in Fig. 1.7). Further degradation to fragments can then occur, and intact insulin, partially degraded insulin, and insulin fragments can then be translocated to various cellular sites (cytoplasm, nucleus, endoplasmic reticulum, lysosomes) (7A and 7B in Fig. 1.7) (Duckworth, 1988).

The insulin may also be delivered to lysosomes from certain subcellular sites such as the Golgi (8 in Fig 1.7). Some of the degraded insulin from the endosome as well as some intact insulin may cycle back to the membrane and fragments and intact insulin can be released (6B in Fig 1.7). In this case the receptor would be recycled back to the plasma membrane (9B in Fig 1.7).

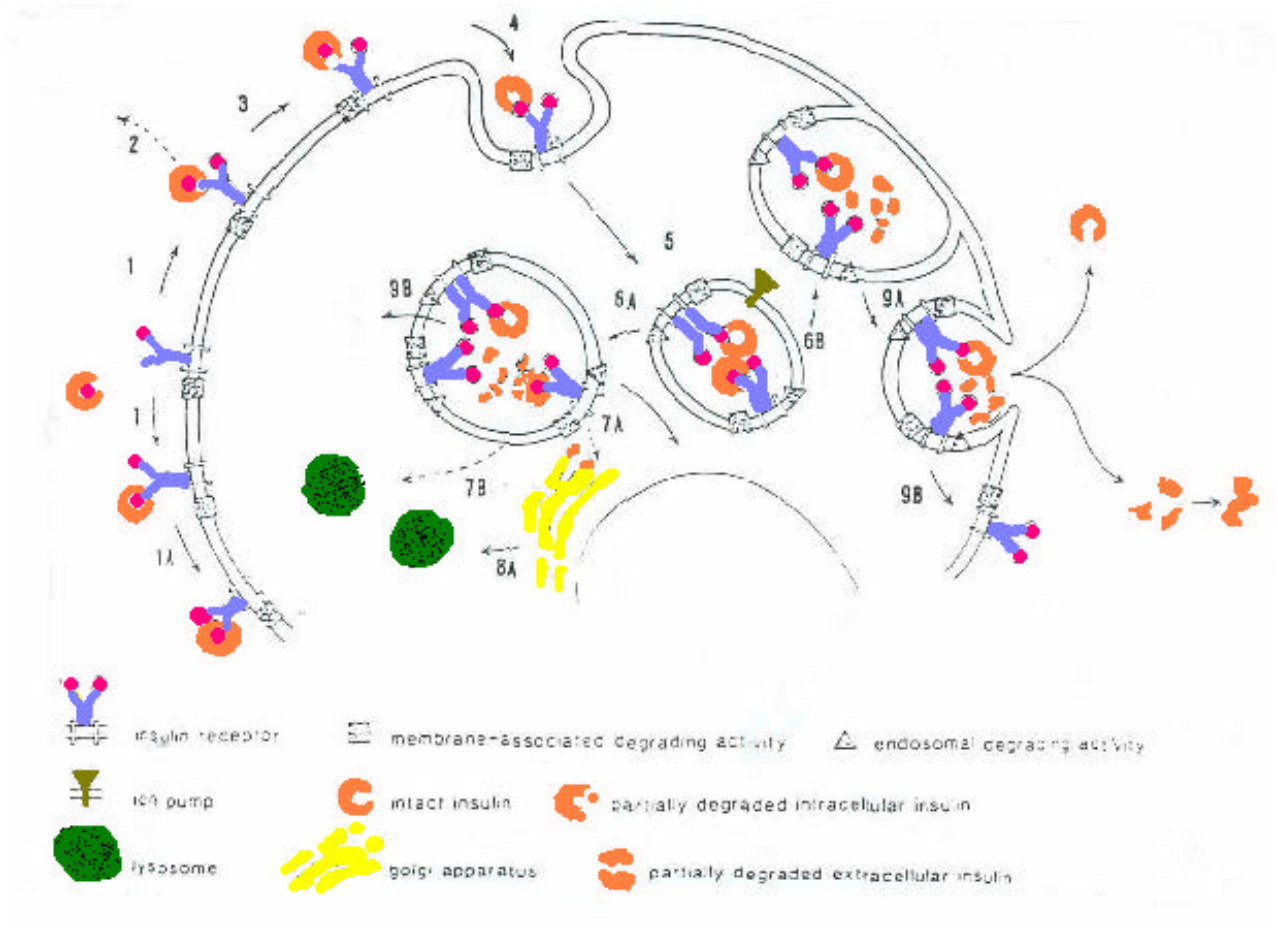

Figure 1.7: A model of cellular handling and insulin degradation (taken from Duckworth, 1988).

1.2.9. Insulin Degrading Enzyme (IDE)

IDE is the primary insulin degrading enzyme in tissues and is a neutral thiol metalloproteinase (Duckworth, 1988). It has been difficult to obtain a stable enzyme preparation to study the properties of the enzyme. However the characteristics of the enzyme have now been studied in detail after investigators purified to homogeneity an IDE from human red blood cells in 1988 (Duckworth, 1988). The enzyme showed inhibition by both sulfhydryl inhibitors, such as N-ethylmaleimide or *p*-chloromercuribenzoic acid as well as by chelators, such as EDTA, EGTA, and phenanthroline and therefore has a metal requirement. However which metal is involved is still unclear. Bacteriocin has also been proven to inhibit the enzyme.

The enzyme was also found to have an optimum pH of 7, and an isoelectric pH of 5.2. The enzyme has a molecular weight of 300 000 (Lehninger *et al.*, 2000). The substrate specificity is not only to insulin, but it has been shown that glucagon and insulin growth factor II are also degraded by the enzyme, while proinsulin and insulin growth factor I inhibit the enzyme, but are poor substrates (Duckworth, 1988).

The major portion of insulin degradation in tissues is cytosolic (Duckworth, 1988). However membrane degrading activity is also present with characteristics similar to that of the soluble enzyme. IDE also has regulatory functions for the activity of steroid receptors and proteasomes. Also the insulin control of cellular protein degradation and fat oxidation may be due to intracellular interactions of insulin with IDE (Duckworth *et al.*, 1998).

1.2.9.1. Degradation products

The degradative products which are produced are a result of the action of the insulin degrading enzyme (IDE), which is the primary insulin degrading enzyme. The enzyme cleaves two peptide bonds in the A chain of intact insulin and seven bonds in the B chain with four major and three minor sites. The nature of the bonds cleaved do not allow a simple classification of peptide bond specificity for the enzyme, but all the sites except B24-B25 and B25-B26 are in close proximity in the three

dimensional structure of insulin, suggesting that the specificity is to the molecule itself rather than specific amino acid residues (Duckworth *et al.*, 1998), see figure 1.8.

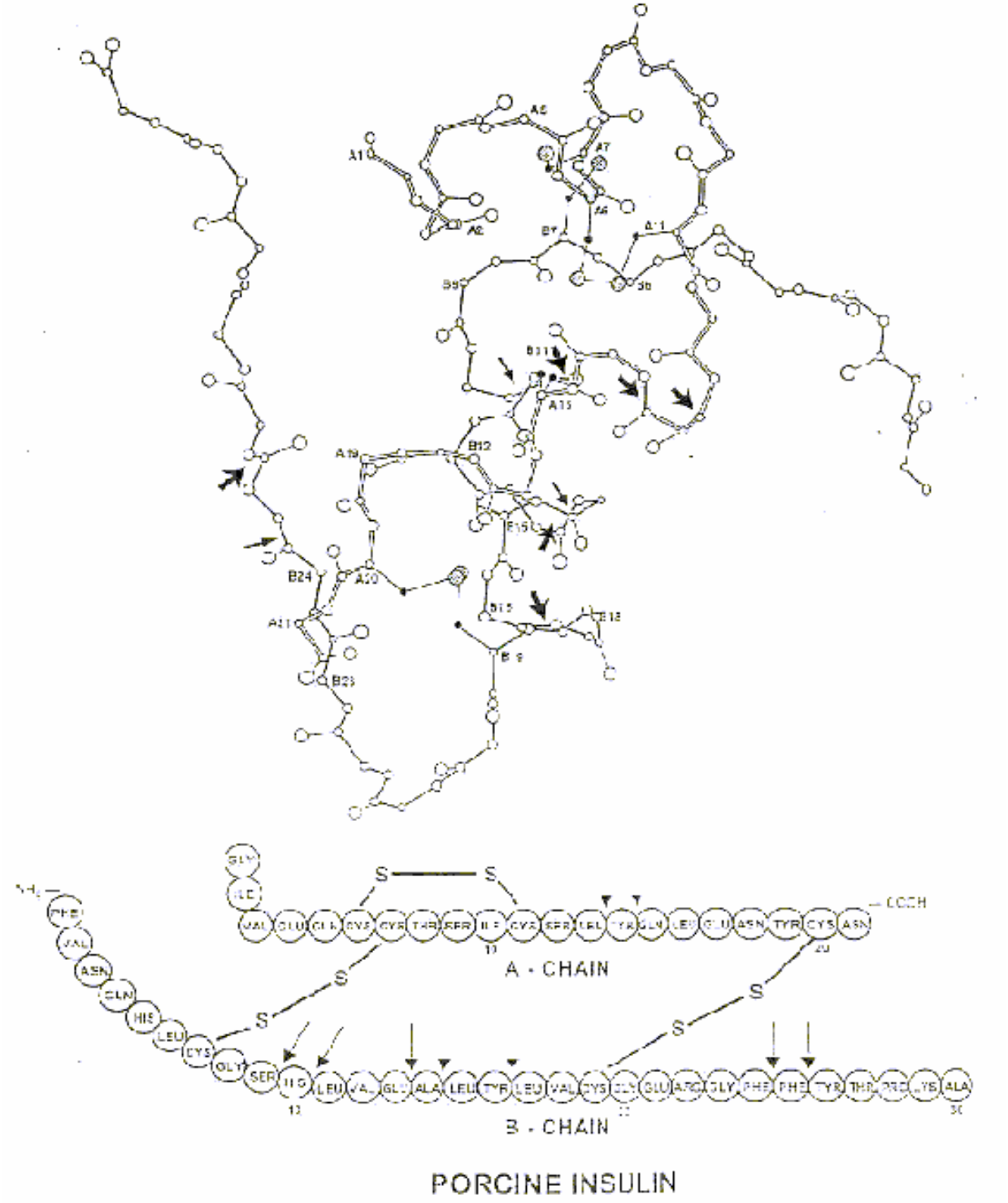

Figure 1.8: Cleavage sites of insulin for the insulin degrading enzyme (taken from Duckworth *et al.*, 1998)

1.3. DIABETES MELLITUS: CLASSIFICATION, SYMPTOMS, PREVENTION, AND TRREATMENT OF THIS FAST GROWING PANDEMIC

1.3.1. Introduction

Diabetes mellitus usually falls into one of two classes, which have been known for many years as juvenile onset and maturity onset diabetes, or the more commonly termed, type I and type II diabetes, respectively. The disease is characteristic of a state of extreme hyperglycemia within the body, resulting from internal or external events which may take place in the life of an individual.

Diabetes, particularly type II diabetes, is becoming a serious problem in developed counties worldwide. Traditionally the median age at diagnosis for type II diabetes was around about sixty, but recent surveys have shown that the entire age distribution curve has shifted to the left. The United States boasts the worst statistics in which type II diabetes is now being reported in children under the age of ten. At such a young age the disease often goes undiagnosed for long periods of time, which allows considerable damage to occur to the pancreas. It is also expected that the earlier the onset of the disease the higher the rate of complications are liable to result (Dyer 2002). The incidence of type II diabetes is thought to be parallel with the growing rate of childhood obesity, a sad statistic considering the disease is easily preventable by following a healthy diet and by exercising regularly.

The graph, in figure 1.9, illustrates the strain obesity and insulin resistance place on the pancreas. The figure displays the hypothetical situation for an obese, insulin resistant individual after a 75g slow intravenous glucose tolerance test. It is clear that there is a progressive disappearance of the first peak of plasma insulin levels (green line), in comparison to the normal glucose response. In order for the body to maintain accurate plasma glucose levels, the pancreas compensates by over producing insulin in the second peak/phase (green line). This procedure will however eventually exhaust the pancreas and over a period of time the second peak will also disappear, leading ultimately to a state of hyperglycemia (red line) and severe type I diabetes (Servier, 2001).

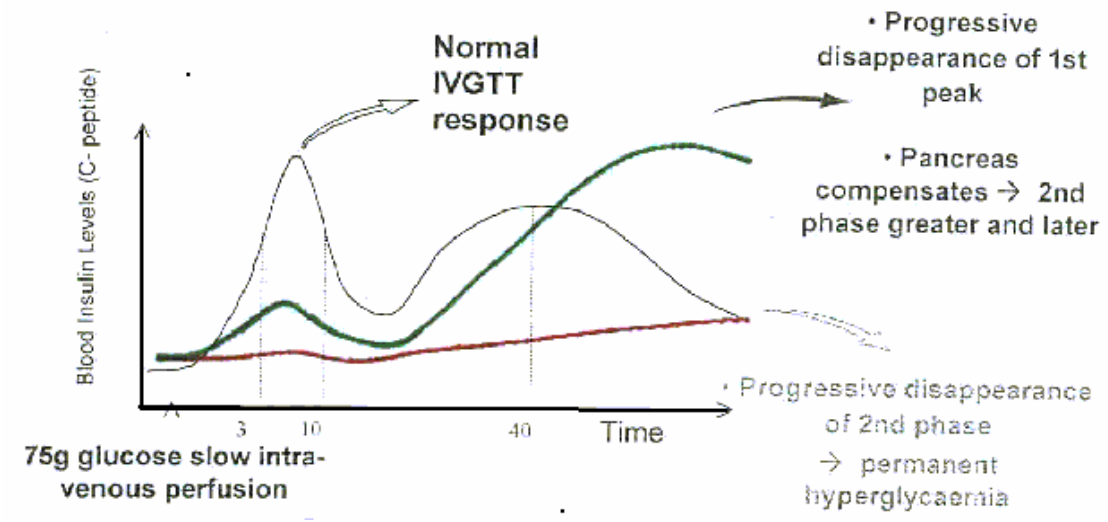

Figure 1.9: A representation of the progression of hyperglycemia (IVGTT= Intravenous Glucose Tolerance Test) (taken from Servier, 2001)

1.3.2. Diagnosis for Diabetes Mellitus

The cardinal clinical feature of diabetes mellitus is glucose intolerance, therefore clinical diagnosis depends upon tests to demonstrate such intolerance (Tortora and Grabowski, 2000). An elevated blood glucose concentration together with an elevated blood ketone body concentration and ketonuria may be sufficient to diagnose diabetes mellitus. In addition, the oral glucose tolerance test (oral GTT), which is designed to test the ability of the patient's beta cells to secrete insulin, may also be used (Newsholme and Leech, 1994).

Table 1.1: Blood glucose concentration criteria for diabetes mellitus (taken from Newsholme and Leech, 1994)

	Glucose concentration (mM)		
Condition	Venous whole blood	Capillary whole blood	Venous plasma
Patient fasted overnight	7.0	7.0	8.0
120 min after oral glucose	10.0	11.0	11.0
Any intervening time after glucose ingestion	10.0	11.0	11.0

Table 1.1 shows concentrations of glucose, put forward by The European Association for the Study of Diabetes, at or above which diabetes is indicated. Diabetes mellitus will be diagnosed if the fasting blood glucose concentration, at 120 minutes and one other concentration at an earlier time interval are at or above those given in table 1.1. However if only one or two are at, or above, the given concentration, impaired glucose tolerance is indicated and further tests are carried out to investigate the cause (Newsholme and Leech, 1994). Diabetes can then be diagnosed after a complete clinical examination under one of the following categories:

(a) Clinical diabetes

The patient has a diabetic glucose tolerance response together with the symptoms and clinical features of diabetes.

(b) Chemical diabetes

The patient has a diabetic glucose tolerance response but has no clinical abnormalities.

(c) Latent diabetes

The patient has normal glucose tolerance response but has had either a previous abnormal response to tolbutamide or steroid tests.

(d) Potential diabetes

A patient with a normal glucose tolerance response but with higher than normal chance of developing the disease due to family history, or presence of another disease associated with diabetes (e.g. acromegaly) (Newsholme and Leech, 1994).

Most of the patients who suffer from non-insulin dependent diabetes mellitus are obese and suffer from insulin resistance. The disease may also stem from a genetic background, however negligence to follow a healthy diet and exercise regularly are often the most common causative agents for insulin resistance, which in time can lead to type II diabetes as displayed in figure 1.10.

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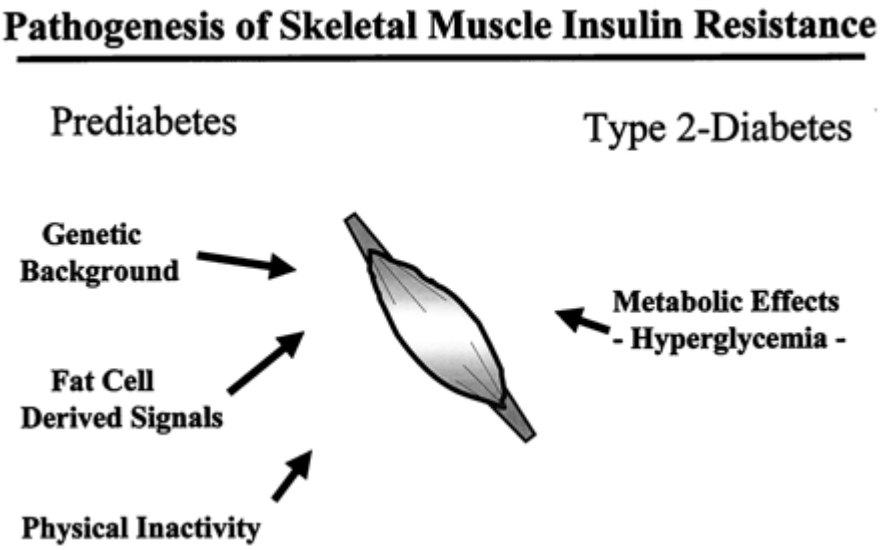


Figure 1.10: Pathogenesis of skeletal muscle insulin resistance. Schematic presentation of factors involved in the pathogenesis of skeletal muscle insulin resistance in prediabetes and type 2 diabetes (taken from Matthaei *et al.,* 2000).

The monocytes and adipocytes isolated from patients suffering from type II diabetes mellitus have fewer insulin receptors having the same affinity as those from normal cells. Non-insulin dependent diabetes is basically a further pathological development of obesity. In obese individuals the changes in glucose concentration are within the normal range whereas those of insulin are greatly in excess of the normal, as can be seen in the following figures:

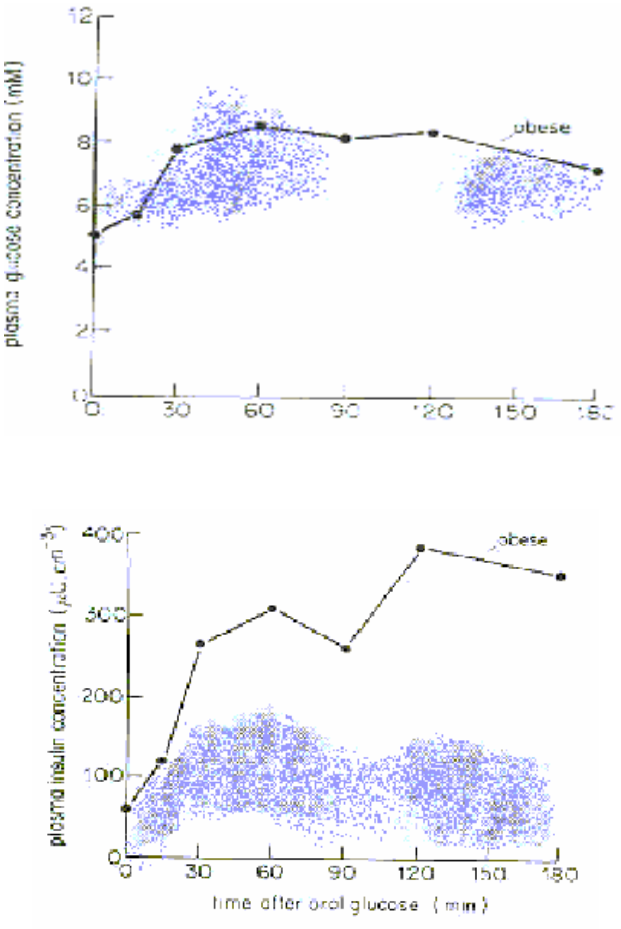


Figure 1.11: A representation of insulin secretion in response to an increased blood glucose concentration in non diabetic obese patients. Shaded areas represent the normal ranges for insulin and glucose levels (taken from Newsholme and Leech, 1994)

It is clear from the preceding figures, that the beta cells of the pancreas can secrete sufficient insulin thereby maintaining the blood glucose concentration within the normal range, despite the cells resistance to insulin. However, if the resistance is too severe and the pancreas is unable to secrete sufficient insulin to overcome the resistance, the blood glucose concentration will not be maintained within the normal

limits, despite the high levels of circulating insulin, and diabetes results (Newsholme and Leech, 1994). Proinsulin concentrations are increased relative to insulin concentrations in individuals with type II diabetes (Mykkanen, *et al.*, 1999). It can therefore be summarised that if there is a sufficient insulin secretory reserve in the beta cells, the individual will remain obese, while with a small reserve the patient usually becomes diabetic.

Several theories exist as to why insulin resistance is present:

- Increased levels of free fatty acids in the blood which coats the insulin receptor and leads to decreased insulin binding (Servier, 2001).

- Overweight causes a relative reduction in the number of insulin receptors (Servier, 2001).

- Poor quality of insulin being produced, i.e. immature or pre-proinsulin (Servier, 2001).

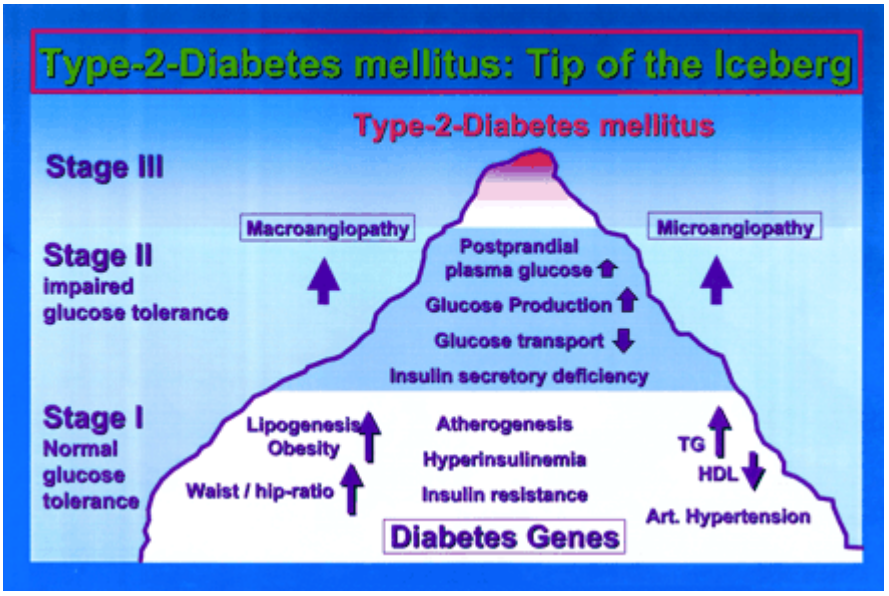


Figure 1.12: Type II diabetes mellitus: the tip of the iceberg (taken from Matthaei *et al.*, 2000)

The simplified schematic presentation in figure 1.12 illustrates the evolution of type II diabetes mellitus. Type II diabetes mellitus represents the end stage of long lasting

metabolic disturbances caused by insulin resistance associated with hyperinsulinemia, obesity, dyslipoproteinemia, arterial hypertension, and consequently premature atherosclerosis. Since this detrimental metabolic milieu is present for many years before plasma glucose levels (as our diagnostic indicator) are elevated, it is not surprising that type II diabetic patients have already micro- and/or macrovascular complications at the time of the initial diagnosis. Subjects in stage I have normal glucose tolerance due to the ability of their ß-cells to compensate for the insulin-resistant state. At this stage elevated triglyceride levels and reduced HDL levels as well as an increased waist to hip ratio may indicate insulin resistance and should lead to therapeutic action. In stage II, glucose tolerance after an oral glucose load (75 g) is impaired due to developing insulin-secretory deficiency. To avoid progression to clinically overt type II diabetes (stage III), these IGT subjects must receive treatment options to reduce insulin resistance, such as dietary advice and increase of physical activity. The stage model of the pathophysiology of type II diabetes is adopted from Matthaei *et al.*, 2000.

Evidence also exists that an elevated concentration of glucose may cause changes in the structure of protein, namely haemoglobin. Chromatography of haemolysates of erythrocytes resolves four minor haemoglobin forms from the main HbA_1 fraction. These minor fractions are collectively referred to as HbA_1 components and constitute approximately 7% of total haemoglobin in normal persons. They result from nonenzymatic posttranslational modifications of haemoglobin (Tortora and Grabowski, 2000). Several unusual haemoglobin molecules are present in the blood of diabetics, such as haemoglobins A_{1a}, A_{1b}, and A_{1c}. The structure of haemoglobin A_{1c} has been established as follows: the N-terminal valine of the haemoglobin beta-chain has reacted with a glucose molecule to form a stable ketoamine linkage (see figure 1.13) (Newsholme and Leech, 1994; Montgomery *et al.*, 1996). Some assays measure HbA_1 and others measure HbA_{1c}, specifically, but either can be used. The level of the HbA_1 is however dependent on the lifespan of the red blood cell and on the prevailing blood glucose concentration. The results will give an indication of the glycemia from the preceding two months, provided the red blood cell's lifespan is normal. False results can however be obtained from individuals suffering from haemolytic anemia or haemoglobinopathy (Benn and Sonksen, 1993).

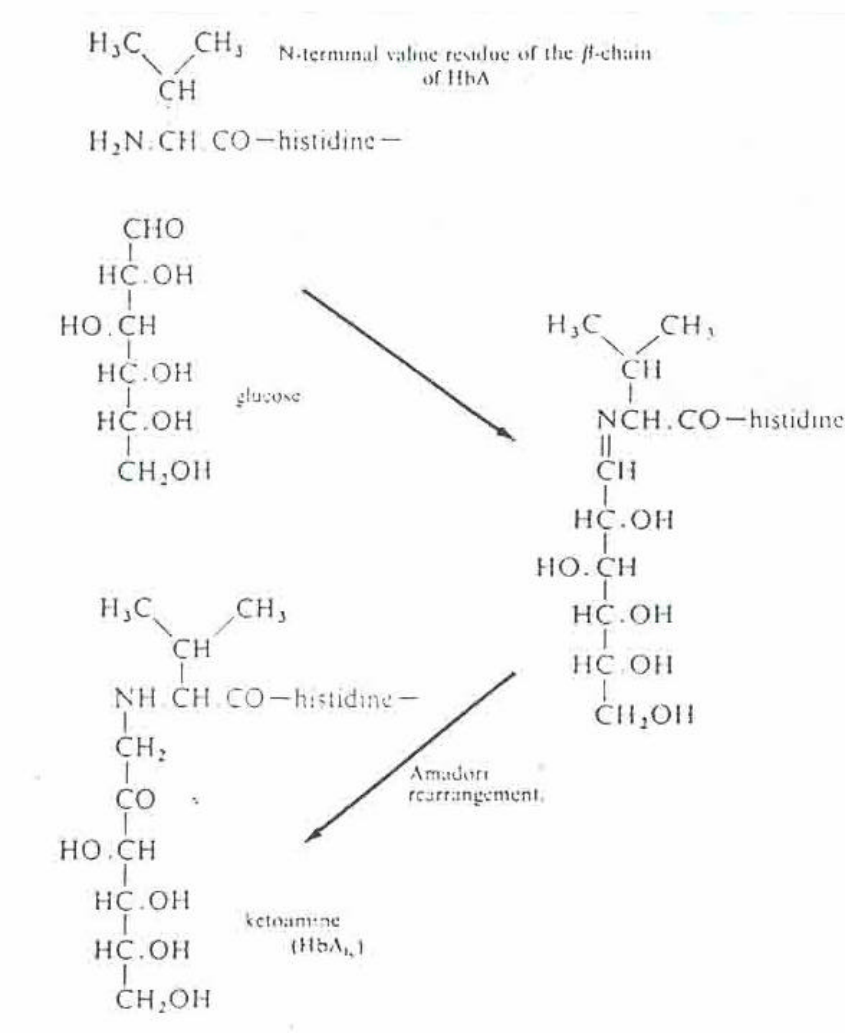


Figure 1.13: Changes which occur in the structure of haemoglobin associated with diabetes (taken from Newsholme and Leech, 1994)

The reaction occurs spontaneously in the erythrocyte after haemoglobin has been synthesized and the rate is increased by an elevated blood glucose concentration. This process is also irreversible (Newsholme and Leech, 1994).

Other serum proteins are glycosylated and may be assayed for. The assay reflects control over a shorter time span (2-3 weeks) and is generally been found to be less

useful than HbA_1, even though it is cheaper and more rapid (Benn and Sonksen, 1993).

1.3.3. 'Misguided' Genes

It is of common knowledge that even the most basic forms of life are dependent on some form of genetic material for survival from generation to generation. Genes differentiate between individuals and code different sequences for important hormones, proteins and enzymes within the body which are essential for life. However errors may exist in the genetic code of some individuals, leading to the formation of certain constituents with some form of functional error. This can be detrimental to the individual's health and could even lead to death. Many genetic disorders have been linked with type II diabetes, but at the same time genetic manipulation is being used as a cure in some cases.

Mutations of several genes encoding transcriptional regulators (HNF-alpha, HNF-beta, HNF4-alpha, IDX1, and NEUROD/BETA2) cause autosomal dominant diabetes. The most prevalent form results from defective HNF1-alpha. Although HNF1-alpha is known to regulate the transcription of liver, kidney, and intestinal genes, the human phenotype is essentially insulin secretory dysfunction, suggesting that this factor must be critically involved in beta cell specific transcription (Parrizas *et al.*, 2000).

Genome wide screening procedures have identified several susceptibility loci for non-insulin dependent diabetes within the human genome. The human proto-oncogene PBX1 codes for a homeodomain containing protein that modulates expression of several genes, including those contributing to regulation of insulin action and glucose metabolism. PBX1 is located on chromosome Iq22, a region linked with type II diabetes, and is composed of nine exons spanning approximately 117kb and is located within 300kb of microsatellite DIS1677, which marks the peak linkage to diabetes susceptibility. Sixteen single nucleotide polymorphisms were also located in PBX1 including one which causes a glycine to serine substitution at position 21 (Thameem *et al.*, 2001).

Another two regions were found to be linked to type II diabetes and insulin resistance, namely a location on chromosome 2q, near marker D2S141, and another location on chromosome 6q, near marker D6S264. A positional candidate gene for insulin resistance in this chromosomal region is the plasma cell membrane glycoprotein PC-1 (6q22-q23) (Duggirala *et al.,* 2001).

A gene conferring susceptibility to type II diabetes has also been located on chromosome 18p11. There is also evidence for linkage of chromosome I, near marker D1S3462; chromosome 4; near marker D4S2361; chromosome 5, near marker D5S1505; and chromosome 17, near marker D17S1301 (Parker *et al.,* 2001).

A putative sugar transporter has been localized to human chromosome 20q12-q13.1, one of the genomic loci associated with type II diabetes. This protein has a strong resemblence to members of the mammalian facilitative glucose transporter family (GLUT), and this protein is therefore known as GLUT10. GLUT10 contains 541 amino acids with several glucose transporter sequence motifs and amino acids essential for glucose transport function (McVie-Wylie *et al.*, 2001).

Genetic susceptibility also exists for type I diabetes. For Asians, the human major histocompatibility complex (MHC) locus (HLA region), especially the class two region, is the major susceptibility interval. The role of insulin dependent diabetes locus has been questioned in Asia, because in contrast to Caucasians, Asian populations have a very low incidence of type I diabetes. This may be due to the population frequency distribution of susceptible type I genes, especially of HLA. The overall risk for type I diabetes from HLA DR and DQ is determined by alleles and particular combinations of alleles in a given individual. In Asians it is very common that a protective DR4 allele is associated with susceptible DQ alleles while neutral/protective DQ alleles are associated with DR4 alleles. The theory therefore stands that the counterbalance between susceptible DRB1 and protective DQB1, and visa versa, is a factor that may contribute to the low incidence of diabetes in Asians (Park and Eisenbarth 2001).

1.3.4. Sub Groups of Type II Diabetes

Several forms of type II diabetes may exist which may not necessarily be permanent, and may be alleviated once the individual is removed from that environment or situation.

- Malnutrition related diabetes mellitus or tropical diabetes is divided into fibrocalculous or protein deficient pancreatic diabetes. In both cases the patients are usually inhabitants of poor tropical countries, are underweight, and have clinical signs of present or past malnutrition and other dietary deficiency states (Benn and Sonksen, 1993).

- Gestational diabetes is restricted to women in whom the onset of diabetes occurs first during pregnancy, and is only associated with approximately 3% of pregnancies. Women who show impaired glucose tolerance during pregnancy are included in this class, but it excludes diabetic women who become pregnant. The clinical recognition of gestational diabetes mellitus is important because the baby is at increased risk of macrosomia, though rates of perinatal mortality and congenital anomalies are no greater than in pregnant women who have normal glucose tolerance. The glucose tolerance often becomes normal after the birth of the child, however there remains a lifetime risk of developing non insulin dependent diabetes later in life (Benn and Sonksen, 1993).

- Diabetes may be associated with various other conditions and syndromes. This group is usually very small and diverse and may be secondary to certain specific endocrine diseases or certain genetic syndromes (Benn and Sonksen, 1993).

- Impaired glucose tolerance is not always associated with diabetes. Some individuals may suffer from glucose in tolerance and still show no signs of microangiopathic renal and retinal complications associated with the classical diabetes diagnosis. Individuals suffering from impaired glucose tolerance

have shown an increased prevalence to atherosclerotic disease and associations with other known cardiovascular disease risk factors including hypertension, dyslipidemia, and central obesity. Individuals who suffer from impaired glucose tolerance do of course stand a higher chance of developing diabetes than normal individuals. It has been estimated that approximately 33% of individuals diagnosed with impaired glucose tolerance will develop diabetes, while a similar percentage will return to a normal glucose tolerance, and the others will remain in the impaired glucose tolerance group, after approximately 5-10 years (Benn and Sonksen, 1993).

1.3.5. Current Treatment for Diabetes Mellitus

Type II diabetes is best treated by a change in lifestyle combined with medication. Lifestyle changes include weight loss, exercise and dietry changes. Medications include sulphonylureas, thiazolidones, biguanides and insulin.

(a) Weight Loss

Many diabetic patients need to lose weight. A reduction in energy intake reduces blood glucose concentrations as well as reducing the risk factors associated with obesity. Even modest weight loss is usually associated with a reduction of insulin resistance and a fall in the accelerated rate of glucose production in the liver. Weight loss can produce a decrease in low density lipoprotein and very low density lipoprotein levels, an increase in high density lipoprotein levels and a reduction in blood pressure. Thus, the overall cardiovascular risk profile can be improved (Benn and Sonksen, 1993).

(b) Exercise

Exercise is beneficial in many aspects to the body, and has been shown to be effective in the treatment of diabetes mellitus. Glucose, ketone bodies, and fatty acids in the blood are used up at a faster rate due to exercise. The glycogen content of the liver and muscle will also be reduced, thereby increasing the activity of glycogen synthase and reducing the requirement for insulin in the stimulation of glycogen synthesis after

a meal. It has also been shown that endurance training in healthy individuals increases insulin sensitivity so that lower insulin concentrations are required to control the blood glucose concentration after an oral glucose load (Newsholme and Leech, 1994).

(c) Dietary fiber

The faster the rate of digestion and absorption of food the greater the change in the blood fuel concentrations and therefore the greater the requirement for insulin. It is therefore important that the diabetic patient realises that the ingestion of glucose and sucrose should be avoided and only slow digesting carbohydrates should be ingested, such as starchy foods. A natural agent such as fibre should be taken in with each meal to further increase the time of digestion. Fibre has been shown to reduce the rate of absorption of glucose and hence the peak concentrations of this sugar in the blood in both normal and diabetic patients, and reduces urinary excretion of glucose and ketone bodies in diabetic patients. Dietary fibre has also proven to improve the control of the blood glucose concentration and reduce the peak concentration of insulin in non insulin dependant diabetics (Newsholme and Leech, 1994).

(d) Insulin administration

This is the most common form of treatment for insulin dependent and sometimes non insulin dependent diabetic patients. The insulin is usually administered in the form of injections consisting of either a mixture of long and short lasting insulin twice a day, or short acting insulin before each main meal plus an injection of long lasting insulin in the evening to provide basal insulin levels throughout the night. The maximum concentration of insulin in the blood occurs 30-60 minutes after a meal, in normal individuals, which coincides with the period of rapid glucose absorption (Newsholme and Leech, 1994).

However, subcutaneous injections of short acting insulin results in the maximum blood insulin concentration only 2-3 hours after injection, which does not coincide with the maximum blood glucose concentration, which as a result will be much higher than normal. Hypoglycemia may also be experienced 2-3 hours after a main meal (Newsholme and Leech, 1994). It is therefore concluded that this form of

treatment may inhibit extreme variations in the blood glucose concentration, but it does not maintain it within the narrow limits found in healthy individuals.

In an attempt to eliminate this problem biomedical researchers have been trying to develop an artificial pancreas, but have failed to produce one small enough to be inserted inside the patient. Transplanting islets and the tail of a healthy pancreas is another alternative researchers are looking into, however there is always the problem of adequate sources of islet tissues and rejection of the tissue (Newsholme and Leech, 1994).

(e) Sulphonylurea drugs

Sulphonylurea drugs are hypoglycemic drugs used to lower the blood glucose concentration in non-insulin dependent diabetics. It was initially thought that they caused an increase in the secretion of insulin from the pancreas, thereby lowering the circulating glucose concentration, this however was later found not to be their sole mode of action. The way in which these drugs function is through some way increasing the number of insulin receptors, which has proven to be a safe and long lasting effective process of lowering the concentration of circulating insulin (Newsholme and Leech, 1994).

The main action of these drugs is however by the inactivation of the sulphonylurea receptor causing the subsequent opening of the voltage dependent calcium channels, thereby promoting insulin secretion. More information about these channels can be found in section 1.4.6.

(f) Thiazolidinediones

The thiazolidinediones are an exciting new class of insulin sensitising drugs used in the treatment of type II diabetes. The molecular target for these compounds is the nuclear hormone receptor, peroxisome proliferator-activated receptor ? (PPAR ?), which is predominantly expressed in the adipose tissue (Reginato and Lazar, 1999).

The thiazolidinediones, which include troglitazone, pioglitazone, and rosiaglitazone, are thought to sensitise tissues to the action of insulin and are effective in lowering serum glucose levels in the absence of insulin (Reginato and Lazar, 1999). These drugs act as high affinity ligands for the PPAR ?. The PPAR ? plays a role in adipocyte differentiation, in the adipose tissue, and therefore brings about an induction of small adipocytes through the subsequent conversion of larger adipocytes. This process is then able to lower insulin resistance because large adipocytes produce such insulin resistant related substances as tumour necrosis factor and non-esterified fatty acids whereas small ones do not (Suzuki *et al.*, 2002).

Thiazolidinediones have also been shown to increase the expression of the lipoprotein lipase gene via the PPAR ? or to enhance lipoprotein lipase activity, thereby causing triglyceride levels to decrease. These drugs are also associated with a decrease in plasma ketone body levels, however a side effect is weight gain in accordance with the adipocyte differentiation (Suzuki *et al.*, 2002).

(g) Biguanides

Biguanides are guanide derivatives in which two molecules of guanidine are linked together with the elimination of an amino group. Guanidine itself has been shown to lower plasma glucose levels in animals, but its toxicity prevents its clinical use. In the 1950s, three major biguanides became available for clinical use, phenformin (phenethylbiguanide), metformin (*N*,*N*-dimethylbiguanide), and buformin (*N*-butylbiguanide). Phenformin was used successfully in the United States but was withdrawn because of associated cases of lactic acidosis, metformin does not have this problem and is widely used as a hypoglycaemic agent (Vigneri and Goldfine, 1987).

(h) Alternative medicines

Despite the impressive technical advances made in the diagnosis and therapy of diabetes many individuals still use alternative forms of therapy due to cost, religious or traditional reasons. Plants such as garlic and onion bulbs, karela (bitter melon) and Indian cluster bean (guar galactomannan), aloe and fenugreek seeds have all been

studied and shown some benefit in lowering plasma glucose levels, but overall, there is little information about herbal remedies (Ryan *et al.*, 2001).

Some trace elements may improve glycaemic control, such as chromium, zinc, magnesium, manganese, molybdenum and vanadium, which could prove beneficial in certain circumstances such as parental nutrition therapy (Mooradian *et al.*, 1994). Certain minerals may, however, be present in excess in diabetes causing more harm than good, such as copper and iron (Walter *et al.*, 1991; Cutler, 1989). Certain vitamins and micronutrients have been proposed as nutriceutical interventions in diabetes and these may be present in medicinal plants. Fish oil supplementation has been suggested for diabetes complications and L-carnitine in type II diabetes, folic acid and vitamin B6 improve homocystein levels and may reduce the risk of cardiovascular complications (Ryan *et al.*, 2001).

Many plants are used by traditional healers around South Africa as a substitute for western medication. The fact that local tradition as well as lack of sufficient funding encourages many individuals to turn to their traditional healers for treatment results in many premature deaths or increased advanced diabetic associated complications. Not enough information is available for these alternative forms of diabetic medication, and one should not substitute any of the above mentioned alternative medicines for that which is subscribed by a health care professional. It is also important to remember that toxicity studies are essential before taking any form of medicinal plant or herb.

1.3.6. Review of Medication to be used Throughout the Project

As two hypoglycaemic agents are to be used throughout the duration of the project it is important to understand the mechanism of action of these drugs and any side effects that they may have. The drugs which will be used is the biguanide, metformin, and a traditional medicinal plant *Sutherlandia frutescens* (kankerbos).

(a) Metformin

Despite the fact that this drug has been around since the 1950s, for the treatment of type II diabetes, its mechanism of action still remains unclear and controversial. Biguanides are not truly hypoglycaemic agents as they lower the blood glucose levels in non-insulin dependent diabetic patients but do not lower the glucose values in nondiabetics, unless there has been prolonged fasting. Metformin is not metabolised in the liver, is not bound to serum proteins, and has a mean plasma half-life of 1.5-2.8 hours (Vigneri and Goldfine, 1987).

The blood glucose lowering effect of metformin is not caused by increased insulin secretion, although the presence of some insulin is a prerequisite for a therapeutic response, but is postulated to be due to a multifactorial mode of actions involving decreased intestinal glucose absorption, decreased gluconeogenesis, and increased glucose uptake by muscle (Klip and Leiter, 1990). Nosadini *et al.* (1987) demonstrated that it is not possible for metformin to act as effectively independently of insulin. In an *in vivo* glucose disposal experiment, using the euglycemic clamp technique, it was demonstrated that metformin increased glucose utilization of peripheral tissues by 50% at a high insulin infusion rate and by 25% at a low infusion rate.

In the past it was believed that metformin's main site of action was the fat however it seems that the muscle is the more favoured tissue. The cellular mechanism of action could involve any of the rate limiting steps in glucose metabolism, either basal (insulin independent) and/or insulin stimulated. These include insulin receptor binding and signals derived from it, glucose transport across the cell membrane, glucose oxidation at the level of pyruvate dehydrogenase, and glycogen synthesis at the level of the glycogen synthase complex (Klip and Leiter, 1990).

Early studies undertaken by Lord *et al.* (1983) showed convincing evidence that metformin increased the number of insulin receptor binding sites on erythrocytes in type II diabetes. However many others have followed this path, using different cell models, and have shown conflicting results, even in instances when metformin was shown to increase insulin binding to cells, this effect did not appear to be directly

related to the subsequent metabolic and clinical effects of the drug (Klip and Leiter, 1990). Therefore the ability of metformin to increase insulin receptors and insulin binding cannot be consistently demonstrated. It was therefore suggested that metformin may be acting at a post insulin binding level (Vigneri and Goldfine, 1987; Klip and Leiter, 1990).

One way in which metformin acts on post receptor insulin binding is through the insulin receptor kinase activity and receptor internalisation. Circulating cells and cultured fibroblasts from insulin resistant patients have abnormal insulin receptor kinase activity, as do muscle cells from diverse diabetic animal models (Klip and Leiter, 1990). The insulin receptor kinase forms an important part of the insulin signalling process, including stimulation of glucose transport. It has been demonstrated in the past that a decrease in kinase activity associated with streptozotocin induced diabetic rat muscle is increased to supranormal levels in response to metformin treatment (Rossetti *et al.,* 1990). Benzi *et al.* (1988) also found that insulin receptor internalisation, after ligand binding, is diminished in monocytes from obese non-insulin dependent diabetics in comparison to control subjects. This reduction was however corrected for after *in vivo* metformin treatment. These responses to metformin treatment may however not be a direct result of the medication but may be a consequence of improved glycemic control or a lower body mass index. It has been proven that improving glycemia and insulin receptor kinase activity is possible through weight loss alone in obese individuals (Klip and Leiter, 1990).

Metformin also has the ability to decrease excess weight in obese individuals, which is advantageous as this helps in the lowering of insulin resistance (Faghanel *et al.,* 1996). The drug is thought to decrease the desire for food intake through one of the following three hypotheses:

- The concentration of the hypothalamic neuropeptide Y is thought to be directly associated with food intake. Acute and chronic injection of neuropeptide Y into paraventricular nucleus elicits a long lasting hyperphagic effect. An anorectic effect of metformin has been shown to be associated with an increase in neuropeptide Y content of the paraventricular nuclei and

arcuate nuclei without affecting preproneuropeptide Y mRNA expression in the arcuate nuclei, in obese rats. Similar findings were found in food deprived lean rats (Paolisso *et al.,* 1998).

- Another hypothesis states that the insulin action in the brain participates in body weight regulation. Various studies have shown that chronic intracerebroventricular administration of insulin in different animals led to a dose dependent reduction in food intake. The possible relationship between the insulin action and the anorectic effect is thought to be through the plasma insulin like growth factor, a proxy of insulin action. This suspicion was confirmed when the results of the experiment showed that before starting and after metformin treatment plasma insulin like growth factor was significantly associated with food intake (Paolisso *et al.,* 1998).

- A further mechanism by which metformin may affect food intake is through the improvement in energy production. With regard to such pathophysiological mechanism, it has been proposed that a decline in ATP synthesis is a metabolic stimulus that triggers feeding behaviour. Because ATP is a final end product of the oxidation of both glucose and fatty acids, changes in the amount of ATP in hepatocytes could provide an integrated signal for feeding behaviour. It is therefore assumed that overeating is a result of fuels that would be otherwise oxidized to produce ATP in a detectable manner being redirected into fat stores. It therefore seems that overeating appears to be an appropriate response to a need for energy created not by the lack of food inside the body but rather by the sequestration of energy stores within the body (Paolisso *et al.,* 1998).

Metformin has also been shown to have an effect on lipid metabolism, significantly reducing hepatic triglyceride synthesis (Vigneri and Goldfine, 1987). However these results prove to be controversial according to papers published by Suzuki *et al.* (2002). Metformin has been shown to affect glucose metabolism through action on glycogen synthase a and glycogen phosphorylase a. It seems that the drug stimulates both glycogen phosphorylase a and synthase a in the liver, thereby increasing the

turnover of hepatic glycogen metabolism resulting in no net accumulation of glycogen. The muscle, on the other hand shows an increase in glycogen synthase a only, associated with metformin treatment. This allows for an accumulation of glycogen in the muscle (Reddi and Jyothirmayi, 1992). The increase of these enzymes allows for glucose to rather be converted into glycogen than into lipids, this alteration in glucose metabolism may also play a role in the reduction of body fat in obese individuals (Suzuki *et al.,* 2002).

Another way by which metformin improves glucose metabolism is by increasing the glucose transporters in the plasma membrane, namely GLUT-4 (Klip and Leiter, 1990). The number of transporters have been shown to be diminished in streptozotocin diabetic rats. Metformin has, however, been shown to enhance basal 2-deoxyglucose and 3-*O*-methylglucose uptake by muscle cells in culture through stimulation of GLUT-4 glucose transporters (Reddi and Jyothirmayi, 1992).

There are not many side effects to report with metformin therapy. DeFronzo *et al.* (1995) did however report nausea, diarrhoea, and a decreased vitamin B_{12} in patients receiving metformin treatment. The dosage was however extremely high, 2550mg per day, and these symptoms subsided when the dosage was lowered. The normal dosage is in the range of 500mg every 8 hours, 850mg twice daily, or 3g daily if needed (Cooper and Gerlis, 1997). It is also evident that metformin does not cause lactic acidosis, as phenformin does, due to a difference in lactate metabolism. Metformin has been found to reduce lactate use for gluconeogenesis without altering the plasma lactate concentration or the plasma lactate turnover, but by increasing lactate oxidation (Stumvoll *et al.,* 1995).

All in all metformin seems to be an extremely effective drug for non-insulin dependent diabetic therapy, displaying few side effects, and producing normoglycemic levels effectively. However to increase the effectiveness of this drug combination therapies are being used with sulphonylurea receptor drugs (Hermann *et al.,* 1994) and with thiazolidinediones (Suzuki *et al.,* 2002).

(b) Sutherlandia frutescens

The genus *Sutherlandia*, commonly known as the cancer bush or kankerbos comprises six species, all of which are endemic to Southern Africa. These six species can be reduced to two, namely *S frutescens* and *S tomentosa*, the former is further divided into three subspecies, namely subsp. *frutescens*, subsp. *microphylla*, and subsp. *speciosa*. The species are distinguished from one another from their habitat, the shape of the pods, and the shape and pubescence of the leaflets (Moshe, 1998). A typical *S frutescens* flower is shown in figure 1.14.

Figure 1.14: Example of a typical *S frutescens* flower (taken from Seier *et al*., 2002).

This plant has been used for its medicinal purposes for many years. It is thought that the Khoi and Nama people were the first to use it against fevers and a variety of other ailments including cleansing of open wounds (van Wyk *et al.*, 1997). The plant has been used as a remedy for stomach problems and even internal cancers, the virtues of the plant, however, extend beyond this as it has also been used as remedies for colds, influenza, chicken pox, diabetes, varicose veins, piles, inflammation, liver problems, backache, and rheumatism. The leaves are the main portion of the plant used for medicinal purposes, however the stems are often also included (van Wyk *et al.*, 1997). *Sutherlandia* is available in tablet form, for which the recommended dosage is 300mg twice daily. Patients on antihypertensive or diabetic medication may need the doses reduced while taking *Sutherlandia* (Gericke, 2001).

Three important compounds, which are of medicinal value, are associated with the *Sutherlandia* species:

1. Triterpenoids

Triterpenoids are compounds which are biologically active against various diseases and even display anticancer activity. These compounds inhibit cancer cell proliferation by acting as spindle poisons, for example lymphocytic leukaemia cells P-388 and L-1210 and human lung carcinoma cells A-549 displayed a decreased rate of proliferation in the presence of ursolic acid. Some examples of medicinally important triterpenoids include oleanic acid putranoside, swartziasaponin, asiaticoside, soyasapogenol, and medicagenic acid (Moshe, 1998).

Moshe was able to isolate two triterpenoids from *Sutherlandia*, however identification and purification proved to be difficult and was therefore not undertaken.

2. Amino acids

Canavanine: L-canavanine, 2-amino-4 (guanidinooxy) butyric acid is a structural analogue of arginine and is a common seed metabolite of most legumes but is also stored in vegetative organs and vacuoles in leaves where it acts as a nitrogen store and an insecticide (Moshe, 1998). Canavanine has been used effectively against cancer, colds, and flu viruses. The anti-cancer activities of this amino acid have been demonstrated when mice bearing L1210 leukemic cells were treated with canavanine, and lived longer than mice that were not treated. Canavanine also inhibited rat colon carcinoma and pancreatic cancer cell proliferation (Moshe, 1998). Various hypotheses have been put forward as to the biochemical nature of the anticancer and medicinal effects associated with canavanine.

- Canavanine is substituted for arginine in most metabolic reactions, as canavanine is a structural analogue of arginine, resulting in structural

and functional protein aberrations occurring in canavanine sensitive organisms (Moshe, 1998). This process can cause the inhibition of tumour growth resulting from the incorporation of canavanine into tumour proteins, causing the production of aberrant macromolecules which exhibit impaired function. These proteins are then degraded more rapidly than their normal counterparts (Moshe, 1998).

- Canavanine also interferes with RNA synthesis and disrupts DNA replication and transcription. This has been demonstrated when canavanine prevented RNA polymerase synthesis in Semilik Forest virus, and reduced DNA synthesis in the herpes simplex virus (Moshe, 1998).

Arginine and nitric oxide: Nitric oxide is formed from arginine by nitric oxide synthase, and is a messenger molecule in living organisms. Other functions of this compound include smooth muscle relaxation, platelet aggregation, neurotransmission, immune cell activation, tumour cell killing, protection against damage to cardiac myocytes, protection of endothelial cells and maintainance of vascular wall integrity, prevent intestinal ischaemia and induce intestinal fluid secretion into the jejunum (Moshe, 1998).

L-Arginine is a common substrate in many metabolic reactions and is essential in the regulation of cell growth and differentiation. Arginine has also displayed anticancer activities by shortening tumour regression and retarding tumour growth (Moshe, 1998).

GABA: Glutamate decarboxylation gives rise to ?-aminobutyrate (GABA), an inhibitory neurotransmitter. Its underproduction is associated with epileptic seizures, and is used pharmacologically in the treatment of epilepsy and hypertension (Lehninger *et al*., 2000). $GABA_A$ and $GABA_B$ are the two GABA receptors which mediate messages to the brain via ion channels (Moshe, 1998). The smoking of *Sutherlandia* seeds has been recorded to be associated with high levels of GABA, which could prove beneficial to the treatment of epilepsy (Moshe, 1998).

3. Pinitol

Pinitol, derivatives, and metabolites thereof are useful in nutritional and medicinal compositions for lowering plasma free fatty acid levels, for treating conditions associated with insulin resistance, such as diabetes mellitus and its chronic complications (hyperlipidemias, dyslipidemias, atherosclerosis, hypertension, and cardiovascular disease), AIDS, cancer, wasting/cachexia, sepsis, burn wounds, malnutrition, stress, lupus, endocrine diseases, hyperuricemia, polycystic ovary syndrome and complications arising from athletic activity (Ostlund and Sherman, 1998).

Pinitol is very effective in lowering blood glucose levels, and is claimed to have insulin like effects (Ostlund and Sherman, 1998; Bates *et al.,* 2000; Davis *et al.,* 2000). Bates *et al.* (2000) reported that pinitol can exert an insulin like effect to improve glycemic control in hypoinsulinaemic streptozotocin diabetic mice, and it is proposed that it acts via a post receptor pathway. The structure of pinitol is shown in figure 1.15.

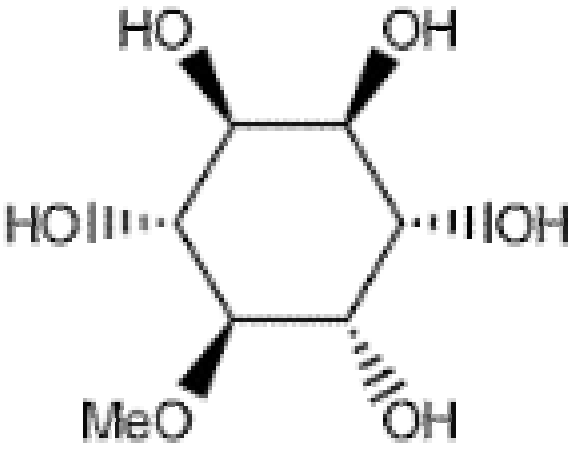


Figure 1.15: The chemical structure of D-Pinitol (3-O-methyl-D-*chiro*-inositol) (taken from http://www.irl.cri.nz/carbo/products/inositols.html)

The dosage of pinitol, metabolite or derivative thereof, ranges from 0.1 to 1mg per day, per kilogram body weight of a mammal. This can be administered orally, enterally, or intravenously (Ostlund and Sherman, 1998). The exact biochemical action associated with the hypoglycaemic effects of pinitol is still not clearly

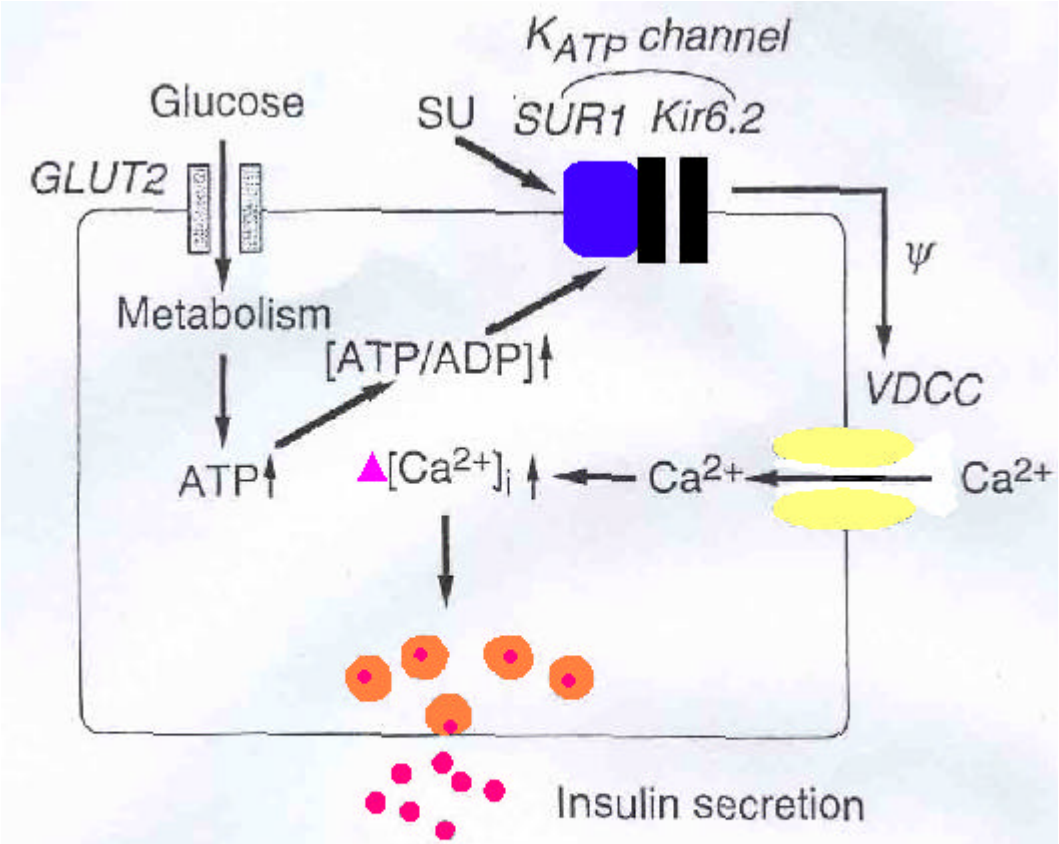
understood and further investigation into this compound is necessary. The concentration of pinitol, in *Sutherlandia*, has been calculated through HPLC analysis to be approximately 14mg/g of dry leaves. This concentration may however differ between seasons and varying geographical locations of the plant (Moshe, 1998).

1.4. ATP SENSITIVE POTASSSIUM (K_{ATP}) CHANNELS: STRUCTURE, FUNCTION, AND REGULATION THEREOF

1.4.1. Introduction

ATP-sensitive potassium channels represent a family of potassium channels inhibited by intracellular ATP, and have been found in many tissues including the heart, pancreatic beta cells, skeletal muscles, smooth muscle, and the central nervous system. They have been associated with diverse cellular functions, such as shortening of action potential duration and cellular loss of potassium ions that occur during metabolic inhibition in heart, insulin secretion from pancreatic beta cells, smooth muscle relaxation, regulation of skeletal muscle excitability, and neurotransmitter release (Isomoto *et, al.,* 1996).

As already discussed in 1.2.4(b) an increased concentration of ATP and certain glycolytic cycle intermediates initiate the closing of the ATP sensitive K^+ plasma membrane channels. This results in the subsequent decrease of K^+ conductance leading to depolarization with subsequent opening of the voltage dependant calcium channels. This process then ultimately leads to the secretion of insulin from the pancreatic beta cells. The ATP sensitive K^+ plasma membrane channels are therefore important and crucial for the release of insulin, and any form of mutation to these channels could effect insulin secretion. This entire process can better be described or rather better understood by studying figure 1.16, which is a summary of insulin secretion in response to an elevated blood glucose concentration. The figure includes the K_{ATP} and calcium channels, and how they work together to bring about the exocytosis of insulin.



The major pathway for glucose-induced and sulfonylurea-induced insulin secretion. GLUT2, glucose transporter 2; VDCC, voltage-dependent calcium channel; SU, sulfonylurea; Ψ, membrane depolarization. Although it is well established that GLUT2 is the major glucose transporter in rodent pancreatic β-cells, some reports suggest that it may not be the case in human pancreatic β-cells.

Figure 1.16: A diagrammatic summary of glucose induced insulin secretion (taken from Miki *et al.*, 1999)

1.4.2. K_{ATP} Channel Structure

As already mentioned these channels are present in most, if not all, excitable tissues and are all inhibited by cytoplasmic ATP, thereby coupling cell metabolism to electrical activity and thus play an important role in the physiology and pathophysiology of many tissues. An example of functioning of the K_{ATP} channel is insulin secretion from pancreatic beta cells in response to glucose uptake. These channels must be able to respond to changes in ATP in the lower millimolar range (Baukrowitz and Fakler, 2000).

The molecular identity of the K_{ATP} channels has recently been elucidated: they are formed from an ATP binding cassette protein with several isoforms, the sulphonylurea receptor (SUR1, SUR2) which have two nucleotide binding folds (NBF-1 and NBF-2) in the cytoplasmic side, and a two segment type or Kir potassium channel (Kir6.2, Kir6.1) (Babenko, *et al.* 1999; Uhde *et al.*, 1999). There are Walker A and B motifs in both NBFs which are important for the functional activity of many ATP binding cassette proteins (Miki *et al.*, 1999). After much experimentation it was found that the main constituents allowing this channel to function are SUR1and Kir6.2, a member of the inwardly rectifying potassium channel family (Miki *et al.*,1999). Both subunits assemble in a 4:4 stoichiometry, with four SUR1 and four Kir6.2 subunits required to form functional K_{ATP} channels, as illustrated in figure 1.17.

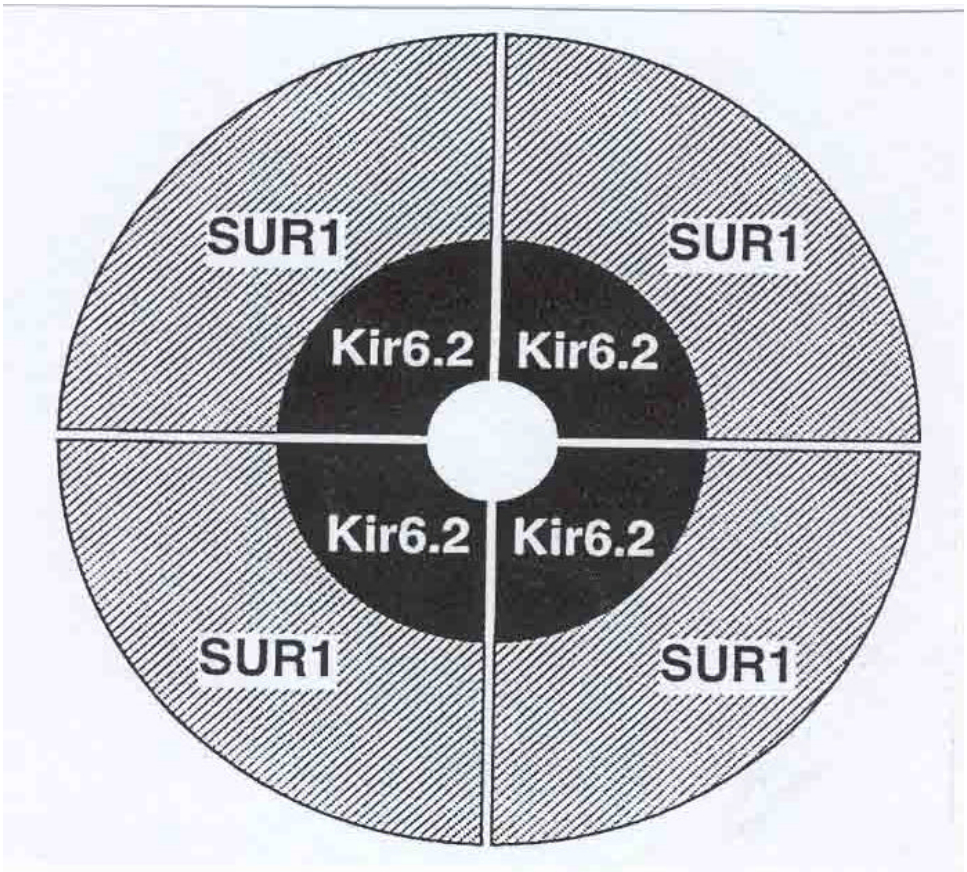


Figure 1.17: A structural representation of the K_{ATP} channel (taken from Miki *et al.*, 1999)

While Kir6.2 acts as the pore forming subunit of the channel complex that determines its single channel conductance, its blockade by polyamines, and its inhibition by ATP, SUR1 has been identified as the regulatory subunit that confers sensitivity to

sulfonylureas, channel openers and Mg-ADP, through the use of the two NBFs (Baukrowitz and Fakler, 2000). SUR1 also acts as a chaperone on the Kir6.2 subunits thereby allowing processing and transport of these proteins to the surface membrane (Ueda *et al.*, 1997).

The essential role of both subunits in regulating insulin secretion is supported by the characterization of mutations in either subunit that abrogate channel activity and cause persistent hyperinsulinemic hypoglycemia of infancy characterized by constitutive insulin secretion despite severe hypoglycemia (Hernandez-Sanchez *et al.*, 1999). A biochemical purification and amino terminal microsequencing of SUR1 indicates the presence of a glycosylation site near the amino terminus. It is also thought that the glycosylated form of the SUR1 protein is physically associated with Kir6.2, suggesting an extracellular localization for the amino terminus of the SUR1 subunit in the K_{ATP} channel (Raab-Graham *et al.*, 1999).

Mutations have also been identified in Walker A (K719R and K719M) in NBF-1 and Walker B (D854N) in NBF-1 of SUR1 which severely impair Mg^{2+} independent high affinity ATP binding. MgADP antagonizes ATP binding at NBF-1, and a mutation at NBF-2 reduces MgADP antagonism. Mutations in the K_{ATP} channel also exist which bring about hyperinsulemia and hypoglycemia (Miki *et al.*,1999).

1.4.3. Control of ATP Inhibition of K_{ATP} Channels by Phospholipids

Another important discovery involving the K_{ATP} channel has recently come of light: it has been shown that phospholipids such as phosphatidylinositol-4,5-biphosphate (PIP2) and phosphatidylinositol-4-phosphate (PIP) are able to shift ATP sensitivity of K_{ATP} channels from the micro- into the millimolar range and thus provide a mechanism for physiological activation of the channels (Baukrowitz and Fakler, 2000).

It was shown that the removal of the PIP2 phospholipid, in excised membrane patches, through the action of phospholipase C resulted in a loss of channel or transporter activity, which could be reversed with PIP2 and PIP (Baukrowitz and Fakler, 2000).

Two properties were found to be important for the functioning of the phospholipids, namely: (1) the negatively charged head group of the phospholipid, as it was found that PIP2 mediated shifts in ATP sensitivity could be abolished by the application of positively charged compounds such as poly-l-lysine, and (2) the requirement for the phospholipid is its insertion into the plasma membrane mediated by the lipid tail of the PIPs. It was found that PIPs are only effective when inserted into the inner leaflet of the membrane bilayer, application of PIPs to the extracellular side of the membrane fails to affect channel properties (Baukrowitz and Fakler, 2000).

1.4.4. Control of K_{ATP} Activity on an Enzymatic Level

From the above information it can basically be concluded that PIPs are able to control ATP inhibition of K_{ATP} channels under cellular conditions. The entire process is certainly not as simple as it seems and involves a number of enzymes of the PIPs metabolism such as the phosphoinositide-kinases (PI-kinase), PIPs-phosphatases, and phospholipases. The activity of these enzymes is known to change in response to a multitude of biological responses such as activation of heteromeric G-proteins and protein tyrosine kinase receptors (Baukrowitz and Fakler, 2000).

1.4.5. Control of K_{ATP} Activity on a Genetic Level

Regulation of transcriptional activity of either SUR1 or Kir6.2 genes is important for determining the level of expression and therefore the activity of K_{ATP} channels. The mouse SUR1 gene proximal promoter region has recently been characterized. The study revealed that there are no CAAT and TATA boxes or initiator elements that may mediate the initiation of transcription. Multiple transcription start sites do however exist, with the major site located 54 base pairs 5'-upstream of the translational start site (Hernandez-Sanchez *et al.,* 1999).

Transient transfection experiments were run, with different constructs of the SUR1 promoter region, in the aid of determining where the basic elements required for significant basal transcriptional activity are located. The study revealed that these elements are found within the first 140 base pairs of the 5'-flanking region. There are

multiple SP1 binding sites that are responsible for SP1 activation of SUR1 transcriptional activity in this particular region. Experiments have also shown that glucocorticoids down regulate the K_{ATP} channel levels by decreasing the SUR1 and Kir6.2 gene expression (Hernandez-Sanchez *et al.,* 1999).

1.4.6. Drug Action on the Sulfonylurea Receptor

A common finding in individuals suffering from type II diabetes is variations occurring in the SUR1 gene. The nucleotide binding fold regions of the SUR1 gene were amplified with polymerase chain reaction and screened by the single strand conformational polymorphism analysis in 40 subjects with type II diabetes. The findings showed that there was one amino acid change, four silent substitutions, and three intron variants present in the nucleotide binding fold regions of the SUR1 gene (Rissanen *et al.,* 2000).

The sulfonylurea class of drug, such as tolbutamide and glibenclamide, inhibit the activity of the channel, and are therefore important agents acting against type II diabetes. Evidence has accumulated that activation of the receptor by ADP and inhibition by the sulfonylurea class of drug is a property conferred by the sulfonylurea receptor on the channel complex, while the site for ATP inhibition resides on the pore forming Kir6.2 subunit. Furthermore, the combined expression of the two proteins is required to generate current (Giblin *et al.,* 1999).

1.5. DEPRESSION AND AMITRIPTYLINE: THE IMPORTANCE OF BIOGENIC AMINES AND THEORIES OF EXCESS WEIGHT GAIN ASSOCIATED WITH AMITRIPTYLINE COMPLIANCE

1.5.1. Introduction

Many drugs, including tricyclic antidepressants, cause undesired increases in body weight (Rigler *et al.*, 2001). Common symptoms of depression include anorexia and weight loss, and yet some patients receiving antidepressant therapy complain of an excess of unwanted weight gain, leading ultimately to obesity, associated with these drugs. This unwanted side effect is described with tricyclic antidepressants. Undesirable weight gain may jeopardize patient compliance with antidepressant therapy (Garland *et al.*, 1988; Hinze-Selch *et al.*, 2000). Both dose and therapy duration are related to the degree of weight gain in the individual.

The average weight gain reported for the tricyclic antidepressants is in the range of 0.57 to 1.37kg/month, with an average dose of 100-150mg/day, of the drug, over a time period of three months (Garland *et al.*, 1988). Investigators also reported that patients that were withdrawn from taking any form of the tricyclic antidepressant ceased to gain weight, after three months. A follow up study of these individuals was then made six months later, and it was found that they had lost almost all of the weight gained during the treatment. This evidence can therefore confirm the suspicions that tricyclic antidepressants cause an unwanted weight increase. In fact the tricyclic antidepressant, amitriptyline, has been used in the past to treat anorexia nervosa (Gottfries, 1981).

1.5.2. Reason for Depression States

The drug reserpine (which inhibits the storage of norepinephrine and serotonin thereby lowering the level of both neurotransmitters) has been shown to deplete intracellular norepinephrine levels and induce vital depressive states, including suicidal tendencies. It can therefore be concluded that a decrease in the amine neurotransmitter levels can be directly related to depression (Becker, 1974).

The aim of antidepressants is to increase the amine neurotransmitter levels in the brain. The effects of antidepressants are not exclusive to the brain, but are able to influence other organs in the body as well.

1.5.3. Tricyclic Antidepressants and How these Drugs Work

These drugs gain their name from their characteristic three ring nuclear structure. Tricyclic antidepressants are very lipophilic substances and are highly bound to plasma proteins especially to albumin, alpha$_1$-acid glycoproteins, and lipoproteins. Since only free drug is diffusible, variations in plasma protein binding may affect tricyclic antidepressant distribution and their concentration at receptor sites, thus conditioning the antidepressant response. They bind to extravascular tissues, which accounts for their generally large distribution volumes and low rates of elimination. They follow two main routes of metabolism in the liver, namely N-demethylation, whereby tertiary amines are converted to secondary amines and ring hydroxylation (Rang and Dale, 1991).

Tricyclic antidepressants are useful in the treatment of acute depression, maintenance of remission once it is established, and prophylaxis against recurrence (Griest and Griest, 1979). The classic tricyclic antidepressants increase the synaptic availability of catecholamines and of 5HT by blocking neuronal reuptake, thereby prolonging their physiological action at corresponding receptor sites (Hieble *et al.*, 1995).

Some tricyclic antidepressants appear to increase transmitter release indirectly by blocking presynaptic alpha 2 adrenoreceptors (Gram, 1983; Rang and Dale, 1991). The alpha-2 receptor is overactive in depressed patients in that this inhibitory receptor closes down the noradrenergic pathway by its continuous blocking of the function of the presynaptic cell (Kwok and Mitchelson, 1981; Ransford, 1982; Gram, 1983; Shaw, 1984).

Therefore specific tricyclic antidepressant drugs block this hypersensitive or overactive alpha 2 receptor, thereby allowing the neuron to work again (blocking a receptor whose function is to stop the neuron working, allows it to start up again) (Ransford, 1982; Gram *et al.*, 1983; Shaw, 1984).

Even though blockade of amine uptake is established promptly, the appearance of antidepressant effects typically requires administration of the drugs for several weeks. Thus, it is clear that potentiation of monoaminergic neurotransmission may be only an early event in a potentially complex cascade of events that is linked to a down regulation of beta adrenoreceptors (Baldessarini, 1991) (Hieble *et al.*, 1995).

It was then proposed that these drugs enhance the receptivity of depressives to their environment, which in turn has an effect with the drugs on improving mood (Becker, 1974). However endogenous depressives tend to be unresponsive to this.

For this study the tricyclic antidepressant amitriptyline was used. This is one of the oldest and most prescribed drugs and has been shown to be significantly associated with weight gain. The metabolite is nortryptyline and the therapeutic plasma concentration is 80-200ng/ml with a half-life of 31-46 hours (Holister 1995).

1.5.4. Tricyclic Antidepressants are they Necessary?

With the emergence of selective serotonin reuptake inhibitors (SSRI) the use of tricyclic antidepressants is not as common as it once was. Many physicians prefer to prescribe selective serotonin reuptake inhibitors to their patients, as they seem to be more effective. Individuals taking the selective serotonin reuptake inhibitors generally suffer fewer side effects in comparison to individuals taking tricyclic antidepressants (Gallo, 1999; Tan, 1999).

Despite the popular use of selective serotonin reuptake inhibitors, tricyclic antidepressants are still used as an effective medication in certain cases. Selective serotonin reuptake inhibitors are often associated with increased anxiety, this is either remedied by lowering the dose or changing to a tricyclic antidepressant instead. Tricyclic antidepressants may also be used in the case of treatment failure or adverse side effects associated with selective serotonin reuptake inhibitors (Gallo, 1999).

The cost of tricyclic antidepressants is somewhat lower in comparison to selective serotonin reuptake inhibitors (Gallo, 1999). This appeals to the lower income class and the elderly. Tricyclic antidepressants are also effective in controlling the pain

arising in postherpetic neuralgia and peripheral nerve injuries (Nowak and Handford, 1999). Although tricyclic antidepressants are not as commonly prescribed, as they were in the past, many individuals still rely on them.

1.5.5. Mechanisms of Tricyclic Antidepressant Weight Gain

There are many theories as to why these drugs cause such a fluctuation in body weight, but it is still not clear which of these theories are correct. Some hypotheses are listed bellow:

- Hypoglycemia-induced hunger was proposed, however contradictory results have been reported. Specific experiments to test this hypothesis in healthy volunteers and depressed patients have shown that neither hyperinsulinemia nor hypoglycemia occurs during tricyclic antidepressant treatment (Garland *et al.,* 1988). However other studies have shown an increase in plasma insulin levels in rats and humans, after administering a course of tricyclic antidepressants (Hurr, 1996).

- It was also proposed that amitriptyline, a tricyclic antidepressant, might alter the hypothalamic sensitivity to a given level of glucose, thereby causing an excessive craving for sweets, leading to weight gain (Garland *et al.*, 1988; Fava, 2000).

- The increased appetite could be the result of the antidepressant action on neurotransmitter systems at the hypothalamic level. A relative increase in alpha-noradrenergic activity could, for example, inhibit satiety and promote carbohydrate craving. This hypothesis has also been proved false (Garland *et al.*, 1988 Rigler *et al.*, 2001).

- The antihistaminic effects of tricyclics, which they share with the structurally related phenothiazines, which also promote weight gain, have been proposed as responsible for increased appetite (Garland *et al.*, 1988). However this hypothesis remains to be proven.

- Evidence has been provided that tricyclic antidepressants may cause a decrease in energy expenditure, thereby contributing to induced weight gain. It was observed that a 17% to 24% decrease in basal metabolic rate occured in three female patients after the drug was administered to them. The net effect of a decrease of this magnitude could account for a weight gain of up to 1.3kg/month, an increase of this magnitude ranges within the documented average gains with tricyclic antidepressants (Garland *et al.*, 1988).

Many of these hypotheses have already been proven false or are yet to be proved true. In the proposed research project, the hypothesis that antidepressants can cause hyperinsulinemia will be tested, specifically by decreasing insulin degradation.

None of the above mentioned theories fully explain the weight gain of 1.3kg/month as found by Garland (1988). Walker (2000) proposed that tricyclic antidepressants may bind to the sulphonylurea receptors, stimulating the pancreas to secrete more insulin. The hypoglycemia following may increase carbohydrate craving which may lead to an increased weight gain. This still, however, needs to be tested. This hypothesis is supported by the fact that heart muscle has SUR2 receptors and tricyclic antidepressants cause arrythmia in some patients.

1.6. TISSUES CONCENTRATED ON THROUGHOUT THE PROJECT: LIVER, MUSCLE, AND ADIPOSE TISSUE

1.6.1. Introduction

Three tissues were to be used during the glucose uptake experiments namely adipose, muscle and liver tissue. As the main aim of the project was to test the effectiveness of an anti diabetic remedy, it is necessary to know whether, and if, there is an accelerated glucose uptake occurring for a specific tissue. Each of the three tissues has a specific function in the body, expressing relevant biochemical pathways, as well as regulating these respective pathways.

1.6.2. Muscle

Skeletal muscle accounts for over 50% of the total oxygen consumption in a resting human being and up to 90% during very active muscular work. Skeletal muscle metabolism is primarily specialized to generate ATP as the immediate source of energy. Depending on the degree of muscular activity, skeletal muscles can use free fatty acids, ketone bodies, or glucose as fuel (Lehninger *et al.*, 2000)

.

Muscle is an efficiently controlled fuel driven machine, which is able to convert free energy, extracted by oxidation of organic nutrients into motion. In muscle several vital biochemical pathways are regulated, enabling muscle to meet its energy needs through a combination of aerobic glycolysis, fatty acid oxidation and oxidative phosphorylation (Gibson and Harris, 2002).

Glycolysis is arguably one of the most important biochemical pathways found in higher organisms, as it is responsible for producing energy in the form of ATP from a six carbon glucose molecule. The ATP produced is necessary to facilitate all energy driven reactions within the body. Other resources, such as fatty acids, may also be used by the body as a source of fuel, however these fuels are not suitable for the brain or erythrocytes which require glucose as an energy source. Glycolysis is a very delicate pathway, which consists of two phases, the preparatory phase and the payoff phase. The preparatory phase consumes two ATP's while the pay off phase produces

four ATP's per glucose molecule. The end products of anaerobic glycolysis are two pyruvate molecules for every glucose molecule oxidized (Lehninger *et al.*, 2000; Montgomery *et al.*, 1996).

The two pyruvate molecules produced during this process are transported to the mitochondria were they take part in the citric acid cycle (Krebs cycle). Under aerobic conditions pyruvate is first oxidized to acetyl coenzyme A which then enters the citric acid cycle, and with the participation of the mitochondrial electron transport system is fully oxidised to carbon dioxide and water.

Once the entire process for a single glucose molecule is completed, under aerobic conditions, a total net yield of 36 or 38 ATP molecules is achieved. This is, however, not necessarily the fate of glucose in muscle. Under aerobic conditions glucose is converted to two molecules of lactate per molecule of glucose in muscle, through the cytosolic process of anaerobic glycolysis. A nett yield of only two molecules of ATP per molecule of glucose is achieved, while lactate accumulates, enters the blood circulation and is transported to the liver for further oxidation. Muscle is also able to convert glucose into glycogen, for energy storage, once the body's ATP levels are saturated. Muscle is, however, unable to release glucose into the circulation as it lacks glucose 6-phophatase (Gibson and Harris, 2002).

These biochemical pathways are very well coordinated, for example, during exercise AMP concentrations are elevated which serves as a signal for increased glucose uptake, glycolysis and glycogen breakdown through positive effects on GLUT4 transport, 6-phosphofructo-1-kinase and glycogen respectively (Gibson and Harris, 2002).

1.6.3. Adipose tissue

Adipose tissue, which consists of adipocytes, is amorphous and widely distributed in the body: under the skin, around the deep blood vessels, and in the abdominal cavity. It makes up about 15% of a young adult human being's mass, with approximately 65% of this mass being in the form of triacylglycerols. Adipocytes are metabolically very active, and are fast to respond to hormonal stimuli (Lehninger *et al.*, 2000).

Adipocytes have an active glycolytic metabolism, oxidizing pyruvate and fatty acids via the citric acid cycle, and carrying out oxidative phosphorylation (Lehninger *et al.*, 2000). The principle flux of fatty acids and glucose is, however, directed towards the synthesis of triacylglycerols, during periods of high carbohydrate intake. Triacylglycerol synthesis is supported by the auxiliary flow of glucose through the pentose phosphate pathway, glycerol 3-phosphate synthesis, and through ATP synthesis, discussed in the preceding section (Gibson and Harris, 2002). The first step in fatty acid synthesis involves the enzyme, acetyl-CoA carboxylase, which catalyzes a 2-step reaction carboxylating acetyl-CoA to form malonyl-CoA.

The remaining reactions in the synthetic process are catalyzed by fatty acid synthase, which is a multienzyme complex consisting of seven enzymatically active sites (Lehninger *et al.*, 2000). The main final product of the fatty acid synthase system is palmitate (C16), which is the starting point for further modification reactions leading to the whole spectrum of fatty acids encountered in the adipose depots.

Fatty acids are in turn converted into triacylglycerides through the acylation of two free hydroxyl groups of glycerol-3-phosphate by two molecules of fatty acyl-CoA to yield diacylglycerol-3-phosphate. This in turn is hydrolysed by phosphatidate phosphatase to form 1,2-diacylglycerol, which is converted into triacylglycerols by transesterification with a third fatty acyl-CoA. The adipocytes behave more as a storage site for the triacylglycerides, which may also arrive from the liver or from the intestinal tract, usually after a meal rich in fat.

Excess fatty acids and carbohydrates obtained in the diet are converted into triacylglycerols in the liver and packaged with specific apolipoproteins into VLDL for export. In addition to triacylglycerols, VLDL,s also contain some cholesterol and cholesteryl esters, as well as the apolipoproteins apoB-100, apoC-I, apoC-II, apoC-III, and apo-E. These lipoproteins are transported in the blood from the liver to muscle and adipose tissue, where activation by lipoprotein lipase by apoC-II causes the release of free fatty acids from the triacylglycerols of the VLDL. The adipocytes take up these fatty acids, resynthesize triacylglycerols from them, and store the products in intracellular lipid droplets, whereas the myocytes oxidise them for an

energy supply. Hepatocytes are responsible for removing most of the VLDL remnants from circulation through receptor mediated uptake and lysosomal degradation (Lehninger *et al.*, 2000).

Lipid stores in the form of triacylglycerides are important sources of energy, as they produce energy of complete oxidation (~38kJ/g) more than twice that for the same weight of carbohydrate or protein (Lehninger *et al.*, 2000).

Lipogenesis is very tightly regulated; during elevated plasma glucose and insulin levels, which accompany feeding, lipid synthesis is activated. However without further consumption of food plasma insulin levels fall as glucagon levels rise. The synthetic flux to triacylglycerides diminishes as glucagon signals the opening of the adipose hormone-sensitive lipase vents coincident with the down regulation of the activities of the lipogenic enzymes. The signal transduction circuitry for the insulin and glucagon hormones is directed towards a specific set of end point effector molecules (Gibson and Harris, 2002).

The leptin hormone mediates a negative feedback circuit that arises from adipose tissue as triacylglycerol stores accumulate. During starvation the dampening of the leptin signal promotes food consumption and conserves initial energy stores thereby minimizing uncoupled metabolism (Gibson and Harris, 2002).

1.6.4. The Liver

The liver is an extremely versatile organ, carrying out a number of tasks in the body and controlling many of the metabolic pathways:

- It synthesizes fat and cholesterol and ships them to peripheral tissues.
- It stores glycogen for later use by other tissues.
- It synthesizes bile acids and excretes them into the gut for lipid absorption.
- It synthesizes urea thereby preventing the build up of toxic levels of ammonia.
- The liver is glycogenic, glycolytic, and lipogenic in the fed state and glycogenolytic, gluconeogenic, and ketogenic in the starved state.

It is also of great importance that the liver has well developed regulatory mechanisms enabling it to switch rapidly between opposing metabolic pathways. Glukokinase serves as a glucose sensor enabling the liver to buffer the blood glucose concentration. Fructose 2,6-bisphosphate regulates flux from fructose 6-phosphate to fructose 1,6-bisphosphate by exerting opposite effects on the activities of 6-phosphofructo-1-kinase and the fructose 1,6-bisphosphatase (Gibson and Harris, 2002).

Fatty acid oxidation promotes gluconeogenesis by providing ATP for energy requiring steps and acetyl-CoA for activation of pyruvate carboxylase and inhibition of pyruvate dehydrogenase. The capacity of the liver for gluconeogenesis is dependent upon increased expression of the gene encoding phosphoenolpyruvate carboxykinase by glucagon and glucocorticoids. Malonyl-CoA inhibits fatty acid oxidation at the level of carnitine palmitoyltransferase I, this prevents futile cycling between fatty acid oxidation and synthesis (Gibson and Harris, 2002).

The liver is also able to produce ketone bodies from fat, as a source of energy for peripheral tissues during starvation. The synthesis of cholesterol is, in part, controlled by the end product negative feedback at the level of 3-hydroxy-3-methylglutaryl-CoA reductase expression. Bile acid synthesis is, in turn, controlled by end product negative feedback at the level of cholesterol 7 alpha-hydroxylase expression (Gibson and Harris, 2002).

CHAPTER 2

INTRODUCTION TO THE PRESENT STUDY

Obesity is among the more serious health care problems facing the westernised world to date, placing enormous economic and health costs on the government. This disorder is the gateway to many metabolic disorders including cardiovascular disease, type II diabetes, hypertension, gallbladder disease and stroke. The trends of obesity are expected to increase throughout the next half century if serious actions are not undertaken (Case *et al*., 2002). Although many cases of obesity are a result of a lack of excersise and bad eating habits, other factors are to blame such as environmental and physiological factors, as well as genetic and hormonal factors (section 1.3.). However certain forms of prescription medication may be the culprit for excessive weight gain, leading ultimately to obesity and the side effects thereof.

The tricyclic antidepressant, amitriptyline, is one such form of medication, which in many cases, has been accused of promoting excessive weight gain. Although many physicians are preffering to prescribe alternative forms of antidepressants, such as selective serotonin reuptake inhibitors, tricyclics are still favoured by many due to their low cost and ability to be used as pain killer (Nowak and Handford, 1999) (see section 1.5.4.).

Many theories exist as to why amitriptyline is associated with excessive weight gain, shown in section 1.5.5, however many of these theories have since been proven false and many are still speculative. This project was therefore designed to determine the causative agent of the weight gain, if any, through the use of an animal model, in this case the male Wistar rat.

As previously mentioned, in section 1.3.1, type II diabetes is fast becoming a serious problem in developed counties worldwide, with children under the age of ten being diagnosed with the disease (Dyer 2002). The incidence of type II diabetes is thought to be parallel with the growing rate of childhood obesity, a sad statistic considering

the disease is easily preventable by following a healthy diet and by exercising regularly.

Various treatments are presently available for the type II diabetes, as displayed in section 1.3.5, however these various forms of medication are expensive. It is also important to note that the medication is not a once off affair but instead is a daily necessity, thereby increasing the economic burden placed on the individual. It is therefore necessary to discover alternative forms of medication against type II diabetes. Through the use of an animal model, in the male Wistar rat, the alternative medication which was tested was an indigenous plant, *Sutherlandia frutescens*, which is believed to be a hypoglycaemic agent by traditional healers. If this plant is indeed a hypoglycaemic agent then it will certainly bring hope for the future of those individuals suffering with type II diabetes.

CHAPTER 3

METHODOLOGY: PILOT AND MAIN STUDY

3.1 INTRODUCTION

3.2 PILOT STUDY

3.3 MAIN STUDY

3.1. INTRODUCTION

This study was aimed at designing an animal model to investigate the metabolic effects of a particular medication, amitriptyline, often associated with excessive weight gain, and of certain hypoglycemic agents for type II diabetes. An animal model was decided upon because of the advantage of measuring more than one metabolic parameter in a single living organism. An animal model also allows for the measurement of certain plasma constituents thereby giving an indication of whether there is a shift in the metabolic homeostasis of the animal, in response to the medication. Rats are relatively inexpensive, in terms of housing and feeding, and are easy to handle, it was for this reason that they were chosen as the model of preference.

3.2. PILOT STUDY

This study was approved by the animal ethical committee of the University of Port Elizabeth. 48 male Wistar rats, 2 months of age and with an average weight of 370g, were obtained from the animal unit of the University of the Western Cape. The rats were divided into 12 cages, with 4 rats to a cage for the purpose of 4 rats being sacrificed at a time. They were housed in an animal room with a constant temperature (25^{o}C) and lighting (08h00-20h00). Table 3.1 shows how the rats were divided into their relative groups according to the medication they received.

Table 3.1: Medication and time during which various groups of rats received treatment prior to sacrifice

Time:		**2 weeks**	**6 weeks**	**11 weeks**
Control		4 rats	4 rats	4 rats
Amitriptyline		4 rats	4 rats	4 rats
Kankerbos		4 rats	4 rats	4 rats
Metformin		4 rats		4 rats

The cages were cleaned once a week, and the rats were fed dog pellets *ad lib*, and received their various medication in their drinking water on a daily basis. The rats were weighed on a weekly basis.

3.2.1. Preparation of medication

3.2.1.1. Sutherlandia frutescens (kankerbos)

Sutherlandia frutescens subsp. *microphylla* was collected on a farm in the Murraysburg district. The kankerbos infusion was prepared by weighing off 2.5 grams of dried, crushed leaves, adding 100 ml boiling water and allowing this tea to brew overnight. The extract was then filtered through a sieve consisting of pores of approximately one millimetre in diameter. The rats received a dose concentration of 0.01 ml per gram rat weight, of the 100 ml tea extract, in their drinking water. The kankerbos medication was made up freshly on a daily basis to prevent any fungal or bacterial growth in the tea. Water bottles were also washed daily.

3.2.1.1.1. Bacterial identification of contaminants in *Sutherlandia frutescens* extract

Bacterial identification of contamination in *Sutherlandia frutescens* infusion was done according to the method of Gehringer *et al.* (1998). The bacterial identification was necessary as bacterial growth could be seen in the infusion after more than a day of standing at room temperature.

(a) Spread plate and streak plate

100µl of 0.025g/ml *Sutherlandia frutescens* infusion, as prepared in section 3.2.1.1. was transferred onto a petri dish containing nutrient agar. An L-shaped glass rod was placed into a beaker with 70% ethanol, covering the bent portion of the rod. The rod was then passed through the flame of a bunsen burner for sterilization, allowing the ethanol to burn off completely. The *Sutherlandia* extract was then spread over the entire surface of the agar and the plates incubated in an inverted position at 37°C overnight.

The following day colonies of different morphologies were picked off with a flamed sterilized inoculation loop and each was streaked onto a fresh nutrient agar plate. Only one sector of the plate was initially streaked. The plates were then incubated in an inverted position at 37°C overnight. Now that individual pure colonies were available identification of the bacteria could proceed.

(b) Preparation of a bacterial smear

Clean slides are essential for microbial smear preparations, and were therefore cleaned with 95% ethanol before use. An individual colony of bacteria was placed in a small drop of water on the slide, with a sterilized inoculation loop. The bacteria were slowly mixed with the water to create a milky suspension, which was allowed to air dry. Once dried the slides could be used for various stains to aid identification.

(c) Gram Stain Procedure

The smear was first covered with crystal violet for 30 seconds before being rinsed with distilled water. Then the smear was covered with Gram's iodine for 30 seconds, and then decolourized with 95% ethanol. The slide was then washed with distilled water, covered in safranin for 30 seconds and allowed to air dry before viewing under a microscope. Purple stained bacteria indicates gram positive bacteria, while red stained bacteria indicates gram negative bacteria.

(d) Endospore Stain Procedure

Small pieces of tissue paper were torn and placed on fresh bacterial smears to reduce evaporation of the malachite green. The paper should be smaller than the stain. The slide and stain were then steamed for five minutes in order to drive the stain into the impermeable endospore. It was essential that the slide remained moist throughout the steaming process by adding more stain if necessary. The paper was then removed and the slides washed with distilled water and counter stained with safranin for 30 seconds. Thereafter the slides were again washed with distilled water, blotted dry and examined under the microscope. This technique yields a green endospore resting in a pink to red cell.

(e) Anaerobic growth

This procedure determines whether a bacterium is able to grow under conditions where no oxygen is present. For this process the *Sutherlandia frutescens* infusion was streaked out, onto nutrient agar as previously mentioned and placed into an anaerobic container with a specialized GasPak. The GasPak is a sealed bag containing chemicals which remove oxygen from a sealed environment. The GasPak was then opened, 10ml of water was added to it and all was sealed in the anaerobic container. The palladium catalyst in the chamber lid catalysed the formation of water from hydrogen and oxygen, thereby removing oxygen from the sealed container. If growth is present on the agar dishes after one day's growth then the bacteria tests positive for anaerobic growth.

(f) Growth on 10% NaCl

Isolated bacterial colonies were picked off, with a sterilized inoculation loop, and streaked on specialized nutrient agar containing 10%NaCl. The plates were then incubated overnight at 37^{o}C in an inverted position. Any sign of growth was reported.

(g) Oxidase Test Procedure

In aerobic bacteria, cytochromes carry electrons to oxygen. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of the test reagent tetramethyl-p-phenylenediamine dihydrochloride to colonies grown on a plate medium. This light pink reagent serves as an artificial substrate, donating electrons and thereby becoming oxidised to a blackish compound in the presence of the oxidase and the free oxygen. Following the addition of the test reagent, the development of pink, then maroon, and finally black colouration on the surface of the colonies is indicative of cytochrome oxidase production and represents a positive test. No colour change or a light pink colouration on the colonies is indicative of the absence of oxidase activity and is a negative test.

A streak plate of individual colonies, grown from the *Sutherlandia frutescens* infusion, was made and incubated overnight, as already mentioned. To test for cytochrome oxidase a drop of oxidase reagent was added to the colonies. A colour change to pink within one minute, then blue to black indicates a positive result. Negative results will have no colour change.

(h) Motility test

Petroleum jelly was placed on all four corners of a cover slip with a drop of water placed in the center. A single bacterial colony was then picked, from a streak plate made from the *Sutherlandia frutescens* infusion, and mixed in with the water. A sterile microscope slide was then placed on top of the petroleum jelly, taking care that there was no contact between the slide and the droplet. The slide was then inverted forming a hanging drop from the cover slip. Bacterial motility could now be examined under the microscope.

3.2.1.2. Amitriptyline

25 mg amitriptyline tablets were employed throughout the experiment. The tablets were crushed with the aid of a pestle and mortar and stored in a dry environment. The dose was worked out relative to that prescribed to humans as per ‘A consumers guide

to prescription medicines' (Cooper and Gerlis, 1997) which states that the normal dose of amitriptyline is 75 mg per day. The doses were then worked out assuming that an average adult human weighs approximately 75 kg:

Amitriptyline:

75 mg amitriptyline /day / 75 kg body weight
= 1 mg amitriptyline / 1000g body weight/day
= 0.001mg amitriptyline /g body weight/day
Each tablet has a mass of 0.11g containing 25mg amitriptyline, therefore
25mg/0.11g / 0.001mg/x
= 0.0044mg amitriptyline tablet /g body weight/day

The dose was then dissolved in 1 ml of a 0.1 M HCl solution. This step was necessary, as the medications do not dissolve to form a homogenous solution with pure distilled water. The dissolved medicine was then made up to 200 ml with distilled water, in the water bottles, and were added to the relevant cages. Doses were worked out with accordance to four rats per cage. As with the kankerbos, the amount of medication consumed was monitored on a daily bases.

3.2.1.3. Metformin

500 mg metformin tablets were employed throughout the experiment. The tablets were crushed with the aid of a pestle and mortar and stored in a dry environment. The doses were worked out relative to those prescribed to humans as per 'A consumers guide to prescription medicines' (Cooper and Gerlis, 1997), which states that the normal dose for metformin is 500 mg every 8 hours or 850 mg twice daily. The doses were then worked out assuming that an average adult human weighs approximately 75 kg:

Metformin:

850mg metformin / 75kg body weight
= 11.33mg metformin / kg body weight/day

= 0.01133mg metformin / g body weight/day
Each tablet has a mass of 0.55g and has a concentration of 500mg, therefore
500mg/0.55g / 0.01133mg/x
= 0.012mg metformin tablet /g body weight/day

The relevant dose was then dissolved in 1 ml of a 0.1 M HCl solution. This step was necessary, as the medication does not dissolve to form a homogenous solution with pure distilled water. The dissolved medicine was then made up to 200 ml with distilled water, in the water bottles, and was added to the relevant cages. Doses were worked out with accordance to four rats per cage. The amount of medication consumed was monitored on a daily bases.

3.2.2. Metabolic rate

The experiment was run over a two week period using an electric oxygen controller. This apparatus consists of an electronic reader connected by an outflow tube to a 4680 cm^3 chamber, which is tightly sealed with the aid of vaseline. This chamber also contains an inflow tube through which air can move freely.

The oxygen controller was calibrated for a day before each experiment, to determine the initial oxygen percentage in the air. Thereafter the rats were individually placed in the chamber for 10 minutes, allowing for their oxygen consumption to be determined. The air flowing to the reader was set to 0,2 litres / minute for this particular experiment. As temperature fluctuations can give deceiving results in such experiments, the entire chamber was partially submerged in a water bath, keeping the temperature at approximately 22^oC throughout the procedure. The temperature inside the chamber remained at approximately 24^oC for the experiment.

3.2.3. Food intake

The rats received dog pellets *ad lib*. The amount of food consumed was monitored on a daily basis four weeks into the experiment. At a set time in the morning 333g of dog food pellets was provided to the four rats per cage. The next day the remaining food

was weighed back and the food consumed by the four rats was calculated and recorded.

3.2.4. Glucose standard curve

Firstly serial dilutions of a 2.5mM glucose concentration were made up, using Dulbecco's modified Eagles medium (DMEM) without glucose, to dilute the samples. 50µl aliquots of these glucose concentrations were then added, in triplicate, to wells in a 96 well plate and 200µl of a glu-cinet® reagent was added to each of the glucose concentrations. The wells were mixed and incubated at 37°C for 30 minutes. The absorbance was read at 492 nm, using a microtitre plate reader. A standard curve was run with each set of samples.

3.2.5. Sacrifice of rats

Rats were sacrificed after 2 weeks, 6 weeks and 11 weeks. The rats were starved 12 hours prior to the experiment, to ensure the blood glucose concentration to be at a fasting level. On the day of the experiment the rats were sacrificed by placing them in a dessicator containing cotton wool soaked in diethyl ether, for approximately 5 minutes. The rat's weights were recorded. It is also of great importance that the place of sacrifice be situated in another room, which is isolated from where the live animals are kept.

3.2.6. Glucose uptake experiment

The liver and both testicular fat pads were immediately removed, after death, and washed thoroughly with PBSA, pre-warmed to 37°C, to remove all the blood. The hind leg thigh muscle was also removed, with 11 week treatment exposure experiment, and was treated in the same manner as described for the other two organs. PBSA was also passed through the inferior vena cava of the liver several times using a 2 ml syringe to ensure that all the blood had been removed from the blood vessels.

Half the liver (the other half is stored in PBSA at 37°C for insulin degradation experiments), the fat tissue and the muscle tissue were then finely sliced, using sharp scalpel blades, and again washed with PBSA. Fat and muscle tissue at 0.4g each per ml of media and 0.5g of liver was used per ml of media. The experiment was run in duplicate, and consisted of a basal (control) and a test. The test tube contained 900µl of DMEM, 10mM of glucose and 600µU/ml of, lyophilised, human insulin. This was then made up to 1ml by adding 100µl of a 20 x diluted Sigma protease inhibitor cocktail solution. The basal tubes contained the same reagents, except that the insulin was replaced with PBSA.

The relevant tissues were then added to the various tubes and the stop watch started. Medium (20µl aliquots) was removed, while working in a 37°C walk in incubator, at times 10, 20, and 60 seconds, and kept on ice until needed. These samples were then diluted 5 x with glucose free DMEM, so as to compensate for the glucose standard curve as the standard curve no longer follows a straight line after a glucose concentration of 2.5µl. The aliquots were then read as in section 3.2.4. It is essential to include a zero reading as well as a blank reading. It is also of great importance that any reagent coming into contact with the tissue be pre warmed to 37°C so as to keep the tissue at one temperature throughout the experiment.

3.2.7. Blood collection

Immediately after death the rat's chest cavity was opened and blood removed from the heart using a 2ml syringe. The blood was then added to 2.5mg sodium fluoride and 2mg potassium oxalate, per ml of blood, in a polypropylene tube. The reagents prevent blood clotting. The blood was then kept on ice and centrifuged at 2000g for 20 minutes and the plasma collected for blood glucose analysis. The glucose analysis was performed by adding 50µl of a 5x diluted sample to 200µl of a glu-cinet® solution and read as in section 3.2.4.

3.2.8. Insulin degradation

The degradation of [^{125}I] insulin was followed by using an adaptation of the method of Powers *et al.*, 1980 and Duckworth, 1979. 10µCi of [^{125}I] insulin labelled at Tyrosine A^{14} was reconstituted in 100µl distilled water, according to manufacturers instructions (AEC-Amersham®) this yields an insulin concentration of 5 x 10^{-8}M. Of this 2.5µl aliquots were stored at –20°C.

A 7.5µl [^{125}I] insulin aliquot was used per 4 rats during each degradation experiment. To dilute the insulin 1.87 ml degradation buffer (Minimal essential medium containing 0.5% bovine serum albumin) was added to the 7.5µl [^{125}I] insulin. The remaining liver from each rat was finely sliced, washed with PBSA, and 0.5 g added to each of the tubes, making up 8 tubes in total, plus a control tube, which did not contain any tissue. To each tube 200µl of [^{125}I] insulin and 800µl degradation buffer (pre-warmed to 37°C) was added. Aliquots (100µl) were then taken at times 0, 5, 15 and 30 minutes, while at 37°C. The aliquots were added to 100µl of cold 10% trichloroacetic acid (TCA) solution, vortexed and kept on ice for 5 minutes, thereby ensuring the completion of the reaction and precipitation of undegraded insulin. The samples were then microfuged for 5 minutes and 175µl of each supernatant added to a pony vial containing 3 ml Beckman ready gel® liquid scintillation cocktail, for aqueous samples. Each of the samples was then counted, for 15 minutes, using the scintillation counter.

3.3. MAIN STUDY

For the main study a larger number of rats were used per group to allow for statistical analysis. The groups remained unchanged from the pilot study except that each group consisted of ten rats. The rats were also fed dog pellets *ad lib* and received their various medications on a daily basis, in their drinking water. The rats were weighed weekly throughout the experiment. All rats were housed in an environmental room with controlled temperature (22°C±1°C) and lighting (08h00 to 20h00). Cages were cleaned three times a week. Approval for this study was also obtained from the animal ethical committee of the University of Port Elizabeth.

3.3.1. Preparation of medication

All medication was prepared and administered in the same way as described in section 3.2.1.1, 3.2.1.2 and 3.2.1.3.

3.3.2. Metabolic rate

The experiment was run at three time intervals: 6, 8 and 19 weeks. It was also decided to increase the time the rat was in the metabolic chamber thereby bringing about a more accurate reading. The ten minutes used previously may have been too short, the animal may still have been under some stress at this time which could reduce the accuracy of results.

In order to increase the time spent by the animal in the chamber the flux of the oxygen had to be increased so as to prevent the oxygen in the chamber dropping to bellow 18% which could induce stress in the animal, therby lowering accuracy again. Due to limitations of the pump of the oxygen controller, the flux could only be increased to 0,5 litres/minute, and the time the animal spent in the chamber consequently could be increased to only 20 minutes, instead of a preferred 30 minutes.

A final metabolic reading was taken just prior to the sacrifice, at 18 weeks. This reading was done using the amitec which is much more sensitive than the oxygen controller, used previously. The rats are placed in the same chamber as before, which is submerged in water in order to keep the temperature stable, and this apparatus runs on the same principle as the oxygen controller. The flow rate is set at 3 litres/minute allowing the rats to stay in the chamber for 40 minutes. All results were stored on a computer in volts and were interpreted from a standard curve obtained using pure oxygen, a 50/50 oxygen/nitrogen mixture, and pure nitrogen.

3.3.3. Food intake

The amount of food consumed by the rats was monitored throughout the experiment the same way as described in section 3.2.3. The food consumed by each group of rats was weighed every morning, at the same time, for a period of 18 weeks and was recorded.

3.3.4. Collection and analysis of urine and faeces samples

After 18 and 19 weeks of treatment the rats were housed, individually, in specialised metabolic cages for a period of seven days. These cages allowed for the separate collection of urine and faeces samples, and also allowed for the accurate determination of the volume of fluid consumed by the animal and the volume spilt. Urine and faeces were collected on a daily basis. The amount of fluid consumed was also monitored.

3.3.4.1. Analysis of urine samples

The urine was collected on a daily basis every morning at the same time. The amount of urine produced by each rat was measured and its colouration noted. The urine was tested using Bayer® biostix, which allows for the determination of various compounds in urine.

As the glucose concentration in the rat urine was too low to be detected with the aid of the biostix the glu-cinet® method was employed. This method entailed the addition of 200µl of glu-cinet® to 50µl of the urine sample, in a microtitre plate. The sample was then read at 492nm after a 30 minute incubation period, at 37°C, with the aid of a microtitre plate reader. This was then read against a glucose standard curve prepared in the same way as described earlier (see 3.2.4).

3.3.4.2. Analysis of faeces samples

The faeces were collected, over the same time as the urine, on a daily basis and were weighed and stored at -80^{o}C until all sampling was complete. The faeces were then freeze dried, and ground into a fine powder. The faeces powder (0.5g) from each rat was then accurately weighed off and the energy contained within the faeces was determined through the use of a bomb calorimeter.

3.3.5. Sacrifice of rats

The rats were starved 12 hours prior to sacrifice. The rats were weighed and sacrificed by issuing a sharp blow to the back of the head thereby rendering them unconscious instantaneously. This method was preferred over the ether method used in the pilot study (see 3.2.5.), which inevitably induced more stress in the animals being sacrificed.

3.3.6. Blood collection

3.3.6.1. Pre-sacrifice

The blood was collected after the rats had been on medication for 12 weeks. The rats were starved for12 hours, before, and blood was collected from the tail through a small incision made with the aid of a scalpel. A blood droplet was collected with a pasteur pipette and added to an Accu-Chek® glucose sensor strip which was read with a glucometer. It is essential that the same glucometer be used throughout the entire experiment, thereby eliminating any 'outside' variation. The incision was then cleaned with 70% ethanol to prevent any infection.

3.3.6.2. Post-sacrifice

Immediately after death the rat's chest cavity was opened and blood removed from the heart using a 2 ml syringe. A drop of blood was immediately added to the glucometer strip and the blood glucose concentration determined from the glucometer. The remaining blood was not added to sodium fluoride and potassium

oxalate, but was stored on ice in polypropylene tubes until needed. The blood was then centrifuged at 2000g for 20 minutes and the serum collected for blood insulin analysis. The insulin was assayed using a rat insulin enzymeimmunoassay kit from Amersham®. Serum was also sent for high density lipoprotein, low density lipoprotein, cholesterol, and triglyceride analysis. These assays were done by Drs. Swart, Mare` and partners pathology laboratories in Port Elizabeth.

3.3.7. Glucose uptake

This experiment was run using the same principles as for the pilot study (see 3.2.6.) However certain changes were necessary in order to reduce the very high standard deviations which were encountered in the pilot study results. To this end a single sacrifice, after 16 weeks including ten rats per group was used. With the increased number of animals per group, the possibility to detect statistically significant differences would be increased greatly.

The thigh muscle tissue was also used throughout the entire experiment along with the testicular fat pads and the liver. The tissue was removed and treated in the same way as before (see 3.2.6.), making sure to minimize the time the tissue was out of the body after the time of sacrifice. Fat (0.4 g), liver (0.5 g), and muscle (0.7 g) was used per 1 ml DMEM, containing 10mM glucose and 100µl of a 10 x diluted Sigma protease inhibitor cocktail solution. Once again the test samples contained 600µU/ml of human insulin, while the basal samples were insulin free. The insulin was kept on ice until needed, ensuring that it would remain intact. The experiment was also carried out in triplicate instead of duplicate and a 37°C thermostatic water bath replaced the 37°C walk in incubator, to achieve better temperature control during the experiment. Aliquots (30µl) were taken at times 20, 40, 60, and 120 seconds. It was difficult to always get reliable 10 second readings, due to time constraints during sampling. The samples were immediately spun down for 1 minute in a microfuge in order to pellet any cells or blood which may have been taken up during sampling. Immediately after spinning 20ul of each sample is removed and stored on ice until needed. Each sample is then read with the aid of glu-cinet® as described in the pilot study (see 3.2.6.).

3.3.8. Insulin degradation

The same method described in section 3.2.8 was employed here with a few changes: the mass of liver used was changed from 0.5g to 0.25g which allowed for half the volumes to be used, in other words each tube contained 100µl of [^{125}I] insulin and 400µl degradation buffer. The reason for this was purely to save on reagents. Because of the small volume in each tube a smaller aliquot had to be taken at each time interval. Therefore 50µl aliquots were taken at times 0, 2.5, 5 and 30 minutes. A 2.5 minute sampling time was added as most of the insulin degradation was thought to occur within the first ten minutes, as indicated by results obtained during the pilot study.

Due to the short half life of the [^{125}I] labelled insulin it is of great importance that a fresh batch is ordered just prior to the experiment, so as to eliminate any natural degradation of the radiolabelled insulin which will take place over time. However, a problem was encountered with the supplier upon ordering of the sample. They stated that it would only be possible to obtain fresh [^{125}I] labelled insulin a month too late, as they had unforseen ptoblems in importing stock. It was decided not to delay the project despite the unavailability of freshly labelled insulin. There were, however, some vials of radiolabelled insulin in storage, at –20^{o}C, left from the pilot study. It was therefore decided to use this insulin despite its age and its unknown condition. Due to the small amount available it was decided that degradation studies would only be performed on the control and amitriptyline groups, as the amitriptyline group was the only group to show any difference in insulin degradation, according to what was found in the pilot study. Also due to the age of the radiolabelled insulin the samples were counted in the scintillation counter for 30 minutes instead of the 15 minutes used before. This would compensate, to some degree, for the low counts expected for the aged radiolabelled insulin.

3.3.9. Statistical analysis

All data, for the main study, are presented as the mean ± SD. Differences in mean values between the control group and all the other groups were analysed by Dunnett's

test. All statistical analysis was done with the aid of ‘fundamentals of biostatistics’, Rosner (1995).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 PILOT STUDY
4.2 MAIN STUDY

4.1. PILOT STUDY

The pilot study was intended to design a later more definitive study. This preliminary study allowed for the use of new and adapted methods to be refined and perfected for the final study. The results obtained during the pilot study could indicate the success of the approach and whether it needed adjusting or not.

It was not the primary intention to find any statistically significant differences between treatments, but rather to identify possible trends in the data. Four rats, instead of the recommended six or more needed for significance, were therefore used per group. This also limited the cost factor of the study, allowing for a bigger budget for the final study.

4.1.1. Bacterial identification

The total bacterial count was 88 000 colony forming units per ml of the *Sutherlandia frutescens* infusion, as determined from the spread plate. Three morphologically different colonies were identified, from the spread plate, and were isolated into single colonies through the streak plate technique. These individual colonies were then used for further identification of the bacteria.

All three colonies stained purple for the gram stain, indicating they were all gram positive bacteria. The three different colonies also all contained endospores, were rod shaped, and were motile. The morphology of these bacteria under the microscope along with the above mentioned information allowed all three bacteria to be grouped

into the *Bacillus* genus. There have been more than sixty different valid *Bacillus* species identified in the past, and all except two are non-pathogenic (Barrow and Feltham, 1995). It was therefore decided not to identify the bacteria but to rather confirm that they are not of a pathogenic nature. As all the other *Bacillus* species are commonly found in the soil and do not have any pathogenic effects, it seemed rational to determine whether the bacteria in the infusion was a health risk or not, instead of going through the long tedious effort of identifying each one.

The two pathogenic bacteria in the *Bacillus* group are *B. anthracis* and *B. cereus*. The simplest way of elimination was to determine whether the bacteria, isolated from the tea, grew on 10% NaCl and whether they grew under anaerobic conditions, as the pathogenic *Bacillus* species grew under both these conditions. After an overnight incubation, at 37^{o}C for the relative growth conditions, all three bacteria from the tea were negative for anaerobic growth and growth on 10% NaCl. One species was oxidase positive, while the other two species were oxidase negative. These results suggested that none of the bacteria isolated from the tea was either *B. anthracis* or *B. cereus* and was therefore not of a pathogenic nature. All bacterial identification was determined with the aid of Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, 1995).

4.1.2. Blood glucose concentration

Blood was drawn from the rat's heart immediately after the sacrifice. Figure 4.1 is a comparison of the average blood glucose concentrations for each group of rats, at each time of sacrifice, for the entire pilot study.

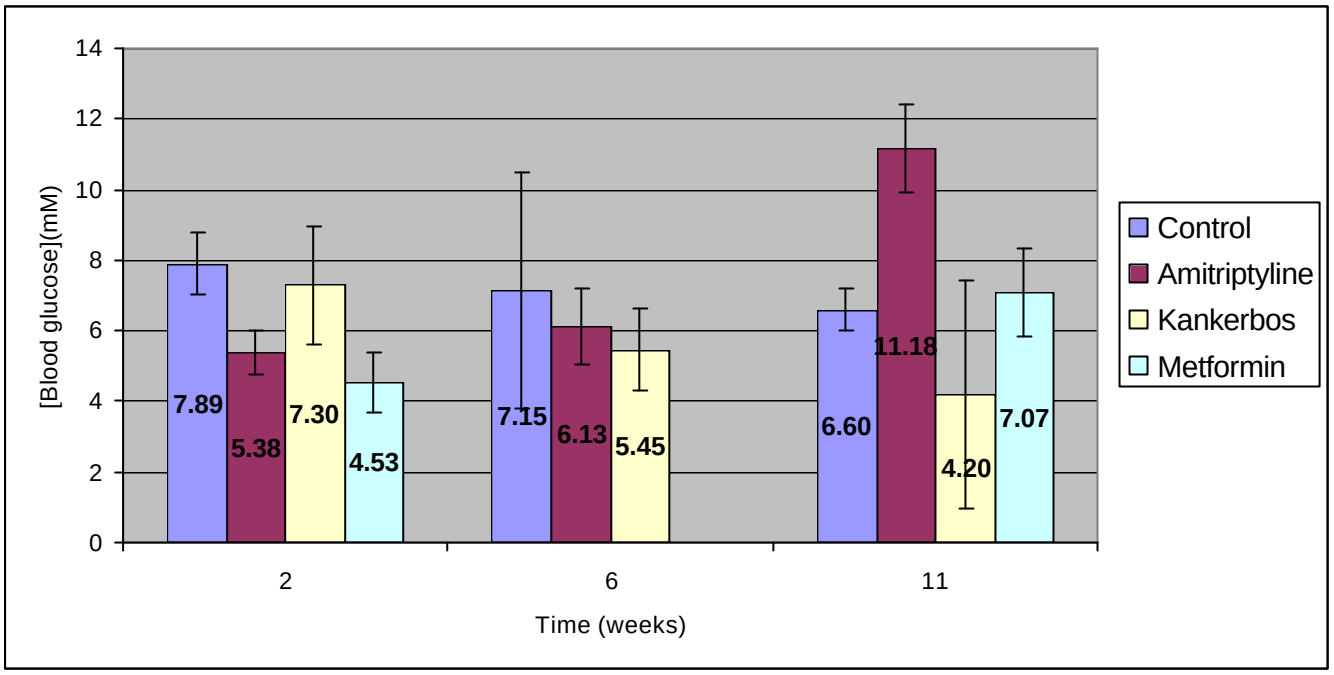


Figure 4.1: Blood glucose concentration for each group throughout pilot study

Each value represents the average of four rats and the error bars indicate the standard deviation. There was a slight decline in blood glucose values over the eleven week period for all rats excluding the amitriptyline group. Taking the standard deviations for the controls into account no difference seems apparent over the time period for this group.

The blood glucose values of the rats treated with amitriptyline shows an initial drop to 5.38±0.63mM at two weeks, after six weeks the value was 6.13±1.08mM, and after eleven weeks the blood glucose value rose to 11.18±1.23mM. This trend of an initial drop in glucose levels followed first by a gradual increase in plasma glucose levels and a sharp increase after 11 weeks, may lead to speculation that amitriptyline stimulated insulin secretion resulting in insulin resistance at eleven weeks.

The metformin group showed an initial drop, of the plasma glucose levels, to 4.53±0.85mM bringing about an apparent state of hypoglycemia. The plasma glucose levels however increased to 7.07±1.2mM after 11 weeks of treatment. Reasons for this increase are not apparent as metformin is expected to increase insulin-receptor sensitivity thereby lowering the plasma glucose levels. Due to the tight budget, a metformin group was not included at the 6 week sacrifice time.

The blood plasma glucose values of the rats treated with the kankerbos infusion, in comparison with the controls, declined to 7.3±1.67mM after two weeks, 5.45±1.16mM after six weeks and reached 4.2±3.22mM after 11 weeks. Although a declining trend was observed over the eleven weeks for this group, the large standard deviations prevented any definitive conclusions to be drawn.

Blood glucose levels of the four different groups show certain trends, though results were compromised by large standard deviations. Stress before sacrifice could have differed between rats, which could have influenced blood glucose levels. The effect of ether could also have influenced results, since the time from when the rats were anaesthetized until blood could be drawn was not constant.

4.1.3. Insulin degradation

This procedure incorporates the use of [^{125}I] labelled insulin to determine the percentage of insulin degraded by the liver tissue. The principle is simple. Once the [^{125}I] labeled insulin binds to insulin receptors on the liver, it is taken up and degraded by the cells, and then released as TCA soluble fragments into the surrounding medium (see 3.2.8.). These fragments, in the aliquots taken from the medium, are then detected by a scintillation counter. Therefore the amount of insulin being degraded should be directly proportional to the concentration of radiolabelled fragments in the surrounding medium.

Table 4.1: Degradation of insulin by rat liver tissue at different stages of the pilot study

Stage	Two Weeks				Six Weeks				Eleven Weeks			
Incubation time (min)	0	5	15	30	0	5	15	30	0	5	15	30
C	0	30.3 ±6.2	60.1 ±11	53.8 ±11	0	26.3 ±2.2	25 ±2.4	23.5 ±2.1	0	20.1 ±6	40 ±1.3	35.5 ±2.4
A	0	70.2 ±6.7	88.7 ±6.7	89.2 ±6.4	0	55.8 ±15	66.3 ±4	59.3 ±13	0	34.5 ±2.4	27.8 ±6.2	20.7 ±3.8
K	0	33.4 ±15	54.3 ±8.5	54 ±9	0	15.5 ±5.7	26.5 ±7.7	24.8 ±7.3	0	19.1 ±5.7	29.3 ±5	30 ±3.6
M	0	41.5 ±7.5	47 ±3.8	44.8 ±3.9	0	-	-	-	0	29.5 ±15	35.3 ±4.6	32.5 ±11

Key: C- Control, A- Amitriptyline, K- Kankerbos, M- Metformin

Degradation is expressed as the percentage of total radiolabelled insulin being degraded per 100µl incubation medium during three different incubation times. The results in table 4.1 indicate that the major insulin degradation occurred during the first fifteen minutes of incubation for all the groups. The fact that there is an apparent lower percentage of degraded insulin present after the thirty minute interval could possibly result from reuptake of the insulin fragments by the liver during extended incubation periods.

A modest decrease, in the degraded product, can be observed for all the groups from the beginning until the end of the experiment. The reason for this is possibly due to an age effect. The rats at the beginning of the experiment were younger and therefore had a higher rate of insulin clearance when compared to the eleven week older rats.

The amitriptyline group displayed the highest percentage of insulin degradation, particularly during the first two weeks, reaching a maximum of 89.2±6.4% insulin degraded after thirty minutes. Similar results were obtained by Chadwick (2001), who found that HepG2 cells increased insulin degradation in culture in the presence of amitriptyline. This implies that the amitriptyline has a local effect on the liver cells *in*

vitro as well as *in vivo*, and the higher rate of degradation is not only a result of presumed increased circulating insulin levels. Increased blood insulin was suspected since a clear drop in blood glucose levels was observed after two weeks (see fig. 4.1). If insulin degradation as well as blood insulin levels increased, then there must have been a marked increase in insulin secretion by the pancreatic cells. Higher circulating insulin levels contribute towards insulin receptor insensitivity (Aspinwall *et al.*, 1998). That could explain the increase in blood glucose levels and a drop in insulin degradation after six weeks. Higher levels of circulating insulin may have caused insulin resistance after eleven weeks, which will explain the clear rise in blood glucose levels shown in figure 4.1 after eleven weeks on amitriptyline, and the drop in insulin degradation at eleven weeks. Note as well that at two weeks the insulin degradation process was still increasing during 30 minutes of incubation. At eleven weeks, however, the most net degradation occurred within the first five minutes and thereafter the removal of fragments from the medium exceeded the degradation process. At six weeks the degradation had already slowed down in some rats and not in others. That explains the big standard deviation at five and thirty minutes. At all stages the insulin degradation in the amitriptyline group is faster at five minutes than for the controls, but at fifteen and thirty minutes it is slower than the controls after 11 weeks.

The kankerbos group showed no significant differences in insulin degradation when compared to the control group. Both groups also seemed to have reached maximum degradation after fifteen minutes incubation, for the two, six and eleven week stages, suggesting that the half-life of insulin in circulation is not affected by kankerbos. These results imply that the blood glucose values, which displayed increased hypoglycemia throughout the experiment, may not be due to an increase in insulin sensitivity, as this would have favoured an accelerated rate of insulin clearance, which is not favoured by results in table 4.1. The hypoglycaemic trend observed may therefore be the result of the insulin-like actions of pinitol from the kankerbos extract (see 1.3.6. (b)3) which, in the present study, seems to have no effect on insulin degradation.

The metformin group showed slightly lower values for degradation when compared to the control group. The maximum degradation seemed to be consistently at the

fifteen minute mark throughout the study. The values in table 4.1 suggest that metformin, like kankerbos, plays no significant role in increasing the half-life of insulin.

Many of the results in table 4.1 are accompanied by rather large standard deviations. The reasons for the large standard deviations can be identified when studying the individual results (not displayed here). The livers used for the insulin degradation experiments, for all the rats in a particular group, were kept in PBSA at 37^{o}C until the glucose uptake experiments, for that day, were completed. The liver of the first rat sacrificed therefore stood the longest, and because of this lost a large amount of cellular activity compared to the liver of the last rat sacrificed, leading to large standard deviations. At the end of the pilot study, when all the results were compiled, the large standard deviations raised concern. It was therefore decided to change the method for the main studyby decreasing the time between the sacrifice of the rats and the assay for insulin degradation. This was achieved by performing the insulin degradation experiments straight after each rat was sacrificed. This would allow fresh liver to be used for each degradation experiment, which is expected to lower the standard deviations.

4.1.4. Glucose uptake by fat, liver, and muscle of male Wistar rats

This particular method allowed for insulin receptor functionality to be determined, by comparison of glucose uptake by the tissue in the absence of insulin (referred to as basal) and in the presence of insulin (referred to as test). The results therefore gave an indication of whether any of the medication was affecting the insulin receptor complex system's responsiveness. Once again for the sake of simplicity, the results are displayed in table form (table 4.2), and represent of the area (cm^2) under the progress curve depicting the amount of glucose taken up with increasing time by a particular cell type. Examples of such uptake progress curves for individual rats are represented in figure 4.2 and figure 4.3.

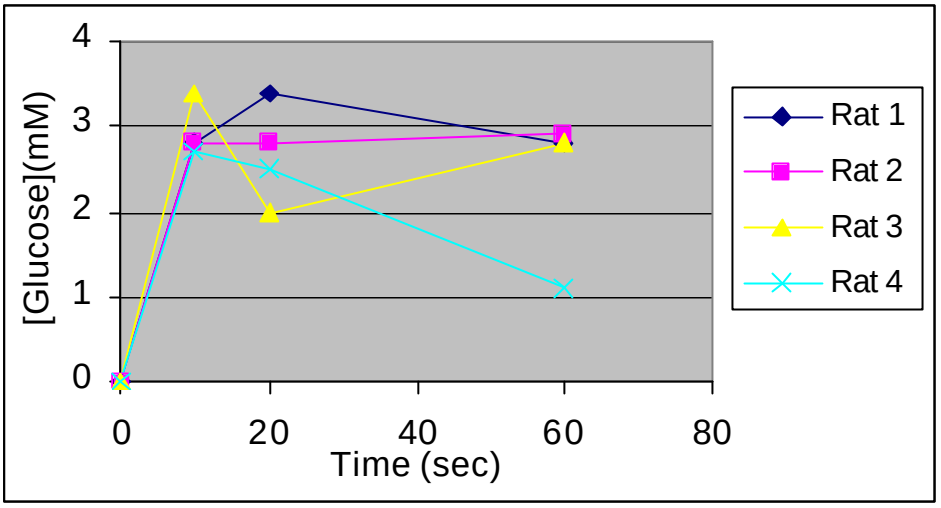


Figure 4.2: Example of individual glucose uptake progress curves for the basal groups after 11 weeks of exposure to amitriptyline, each data point represents the mean of duplicate determinations for a single rat.

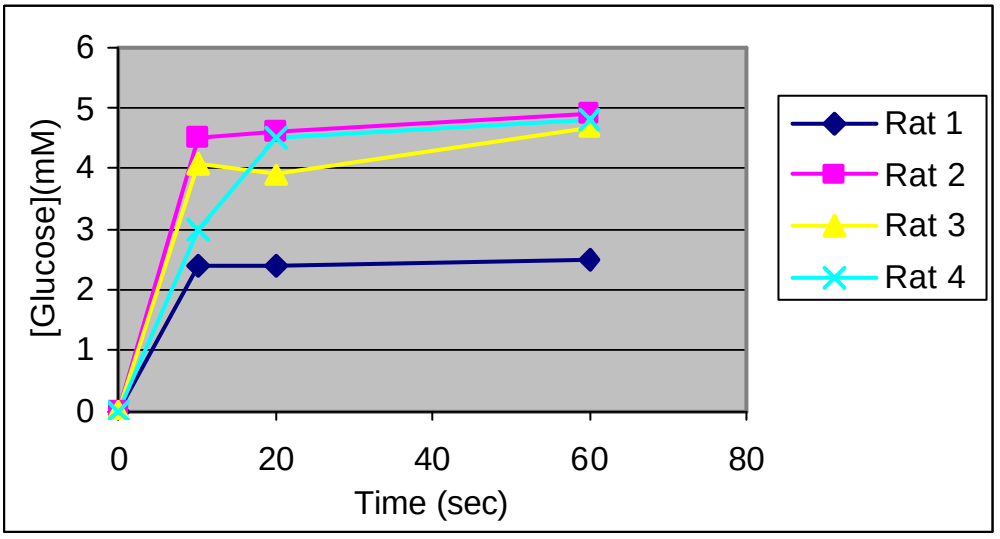


Figure 4.3: Example of individual glucose uptake progress curves for the test groups after 11 weeks of exposure to amitriptyline, each data point represents the mean of duplicate determinations for a single rat.

Table 4.2: Glucose uptake in the absence (basal) and the presence (test) of insulin by adipose and liver tissue of male Wistar rats after different periods of treatment

Short time interval - 2 Weeks															
Control				**Amitriptyline**				**Kankerbos**				**Metformin**			
Fat		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**	
B	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**
34 ±8.4	41 ±22	28 ±11	14 ±13	28 ±21	44 ±4.6	24 ±7.9	30 ±14	24 ±18	37 ±15	18 ±18	27 ±10	19 ±9	43 ±4.4	25 ±15	47 ±9
Intermediate time interval – 6 Weeks															
Control				**Amitriptyline**				**Kankerbos**				**Metformin**			
Fat		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**	
B	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**
18 ±8.4	41 ±22	33 ±5.3	38 ±6.1	27 ±14	32 ±6.6	36 ±15	38 ±4.6	31 ±4	24 ±15	25 ±8.3	16 ±13	-	-	-	-
Long time interval – 11 Weeks															
Control				**Amitriptyline**				**Kankerbos**				**Metformin**			
Fat		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**		**Fat**		**Liver**	
B	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**	**B**	**T**
27 ±9.3	32 ±13	26 ±13	34 ±8.6	47 ±2.1	42 ±9.2	38 ±3.4	41 ±9.7	40 ±5.3	26 ±3.9	25 ±8.6	35 ±14	19 ±17	32 ±14	21 ±11	33 ±13

All values shown in the table were measured in cm^2 and represent the average areas and srandard deviations under the four respective progress curves.

Key: B- Basal, T- Test

The results in table 42 are best analyzed seperately for each treatment group. The identification of definitive trends is complicated by the large standard deviations noted in many cases.

Amitriptyline: in comparing the basal fat tissue with the control group it is evident that the amitriptyline group shows a higher uptake of glucose as time passes. After six and eleven weeks the fat basal reading for amitriptyline is $27\pm14cm^2$ and $47\pm2.1cm^2$ compared to the $18\pm8.4cm^2$ and $27\pm9.3cm^2$, respectively, for the control groups. The amount of glucose, taken up by the test samples, also seem to be higher than that of

the control. These results seem to remain stable over the eleven week period for amitriptyline in comparison to 41±22cm^2, 41±22cm^2 and 32±13cm^2 for the control groups over two, six and eleven weeks respectively. These values may suggest that amitriptyline is promoting glucose uptake in the presence and absence of insulin over the eleven weeks, however the standard deviations are extremely large and do not allow any conclusions to be drawn at this point.

The glucose uptake in the liver samples seemed to be similar to that of the fat samples, for amitriptyline. No difference was observed between the control and amitriptyline liver samples over the first six weeks. However an increase in glucose uptake could be observed at eleven weeks for the basal and test values of the amitriptyline samples, 38±3.4cm^2 and 41±9.7cm^2 respectively, when compared to those of the control, 26±13cm^2 and 34±8.6cm^2 respectively. Once again the standard deviations are high and no conclusions can be drawn. These results do however coincide with the theory that amitriptyline, and other tricyclic antidepressants, cause weight gain only after a period of three months (Garland *et al.*, 1988).

Kankerbos: the fat, basal samples, in table 4.2, seem to have a higher glucose uptake as time increases, contrary to its test counterpart, which seems to display a decreased uptake with time: test values for two, six, and eleven weeks respectively, 37±15cm^2, 24±15cm^2 and 26±3.9cm^2; basal values for two, six and eleven weeks respectively, 24±18cm^2, 31±4cm^2 and 40±5.3cm^2. It seems as if the kankerbos is itself assuming an insulin-like role whereby it promotes glucose uptake. However, if this was the case then the test samples should not show a decrease in glucose uptake as time increased. In fact the adipose test samples for kankerbos are also found to be lower than those of the corresponding control test samples. A reason for this could be that the kankerbos is in fact assuming the role of insulin, facilitating glucose uptake. The biochemistry of this process is still not fully understood. This process could allow for a decrease in insulin receptors on exposure to kankerbos, as glucose clearance was occurring at a fast rate even in the absence of insulin. However the fact that the test samples are lower could be that when insulin is present it has a higher affinity for the insulin receptors than pinitol, thereby binding to them in competition with pinitol. This would therefore facilitate a lower rate of glucose uptake, assuming that the number of

receptors on each cell may have decreased even though the sensitivity to insulin remained unchanged.

It appears as if the liver samples for kankerbos do not comply with above theory. The test liver samples do seem to be predominantly higher than those of the basal samples, implying that insulin is the primary agent promoting glucose uptake. At eleven weeks there is no difference between the liver basal ($25 \pm 8.6 cm^2$) and test samples ($35 \pm 14 cm^2$) when compared with those of the control group's basal ($26 \pm 13 cm^2$) and test ($34 \pm 8.6 cm^2$) samples. From these results it is assumed that the liver is not affected by the kankerbos in the same way as the fat tissue, however further studies involving cell lines can possibly clarify this point. It is also important to keep in mind that liver cells do not contain GLUT 4 transporters whereas the muscle and fat cells do.

Metformin: as mentioned earlier this drug is to act as the positive control, as it is known to increase the sensitivity of the receptor, towards insulin, on the cell membrane. In table 4.2, a large difference in the rate of glucose uptake can be seen between the basal and test samples after metformin treatment, thus confirming the potential of the drug in both tissue types. The rate of glucose uptake is initially high and then decreases at eleven weeks for both tissues, reaching values similar to those shown for the control group, at this time. The reason for this decrease, with metformin, cannot solely be attributed to insulin resistance with age as this particular drug is intended and believed to combat this ailment in humans.

Table 4.3: Glucose uptake in the absence (basal) and presence (test) of insulin by muscle tissue after 11 weeks of treatment

Long interval – 11 Weeks							
Control		**Amitriptyline**		**Kankerbos**		**Metformin**	
Basal	**Test**	**Basal**	**Test**	**Basal**	**Test**	**Basal**	**Test**
22 ±16	20.7 ±10	11.9 ±11	36.4 ±13	37 ±10	36 ±9.3	24 ±14	27 ±15

All values shown in the table were measured in cm^2 and represent the areas under the respective uptake progress curves and the standard deviations

The glucose uptake experiment, with muscle, was only performed at the eleven week interval as its relevance was only decided on once the project was already underway. In comparing table 4.3 with table 4.2 it can be clearly seen that both the basal and the test results for the muscle are generally lower than those of the fat and the liver. Reasons for this could be related to the fact that different tissues display varying degrees of insulin sensitivity. For example the adipocytes respond to an extremely low concentration of insulin, as does the liver. The muscle on the other hand responds to a serum insulin concentration twice that needed to stimulate fat or liver tissue (Stumvoll and Jacob, 1999).

Amitriptyline: the basal glucose uptake in table 4.3 appears to be low, 11.9±11cm^2, when compared to the control group's basal value, 22±16cm^2, for the muscle. The test group does however seem to be substantially higher, 36.4±13cm^2 for amitriptyline and 20.7±10cm^2 for the control group, thereby implying that amitriptyline is probably increasing the sensitivity of the receptors for insulin.

Kankerbos: there is no difference between the test, 36±9.3cm^2, and basal, 37±10cm^2, muscle values for kankerbos. Both values, test and basal, do however seem to be higher than that of the control's test, 20.7±10cm^2, and basal, 22±16cm^2, values thereby implying that the kankerbos is promoting glucose uptake. Once again the basal levels seem to be rather high, in comparison to the other basal results in the table, which may again support the notion that the kankerbos is assuming the role of insulin, in its absence. Glucose uptake seems to be higher than that of the metformin group, 24±14cm^2 and 27±15cm^2, for basal and test respectively. This seems promising for the potential use of kankerbos as a future hypoglycemic agent.

Metformin: no difference can be seen between the basal and test samples for this group. Both results appear slightly higher than that of the control group, showing that metformin does promote glucose uptake. These values are, however, less convincing than the kankerbos and amitriptyline results already discussed.

At the end of the pilot study it was clearly evident that a larger number of rats should be used per group to allow for statistical significance to be evaluated. It was also

decided to try to reduce standard deviations by adding an extra step in the methodology. It was suspected that some cells may have been removed with the medium when aliquoting. Such cells may have continued taking up glucose after the intended sampling time. This may have increased the standard deviation of the mean values, as the aliquots stood for quite some time before being assayed. Transferred cells may have also interfered with the subsequent absorbance, readings. To limit this problem it was decided to microfuge the aliquotes, after each sampling time, thereby removing any cells or cell debris.

4.1.5. Weight of rats throughout the pilot experiment in relation to the amount of food consumed and resting metabolic rate

The rats, in each cage, were weighed on a weekly bases and an average for each group was recorded. The weighing of the rats and recording of their food intake did not however begin at the start of the experiment. All weights were only recorded approximately four weeks into the experiment and then at weekly intervals.

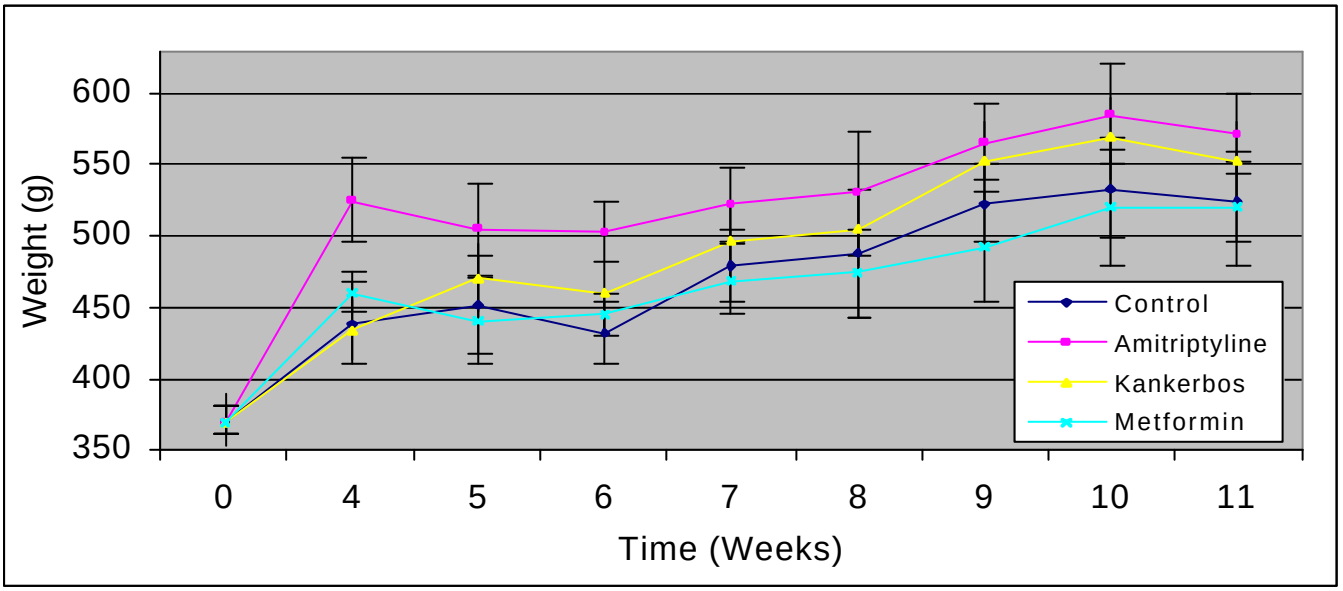


Figure 4.4: Weight of male Wistar rats followed over an eleven week period of treatment

Adult male rats, weighing more than 280g, were assigned to the different groups in such a way that the average weight for the different groups were the same (370±10g), at the beginning of the experiment. Figure 4.4 shows that after four weeks the metformin group's average body weight was the second highest but dropped at the

fifth week. From week seven to eleven this group recorded the lowest average weight of all the groups, these results are parallel to those obtained by Uehara *et al.*, (2001), who showed that metformin treatment is associated with weight loss.

Inspection of figure 4.4 shows that the amitriptyline group outweighed the control group significantly ($P<0.05$), particularly during the first seven weeks. The kankerbos group also displayed a considerable increase in weight, relative to the control group. However the onset was slightly delayed, occurring only after about seven weeks.

All the cages received the same amount of food (333g) on a daily basis, at the same time of day. The next day left over food was weighed, calculated and recorded.

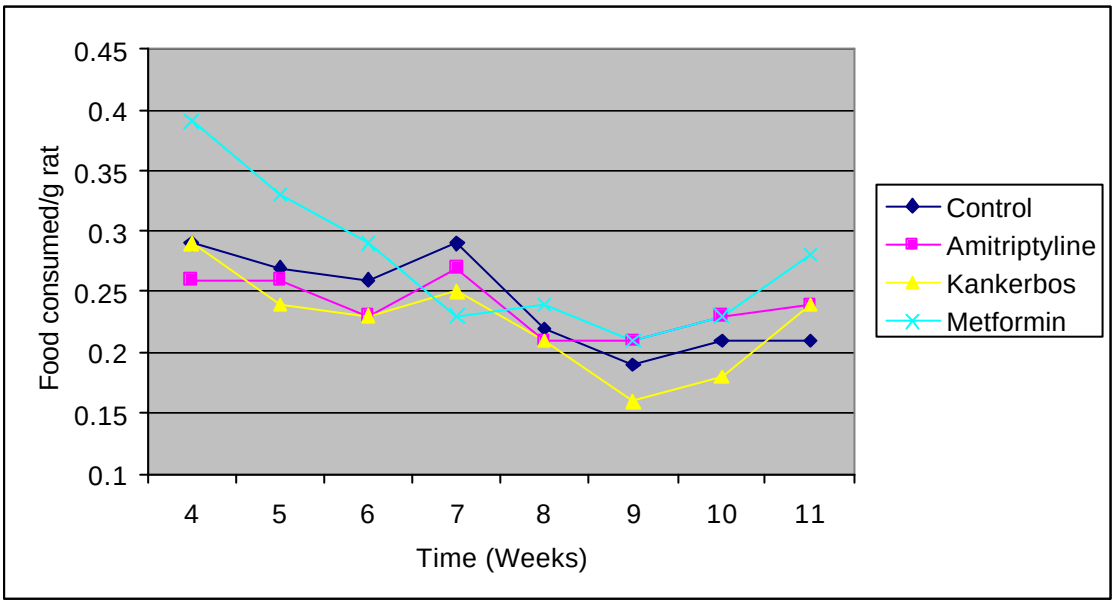


Figure 4.5: Average weight of food consumed per gram rat weight over the period 4-11 weeks

Figure 4.5 clearly shows that the metformin group consumed the most food over the period 4-6 weeks. However, as already stated, on average they were the lightest group especially towards the end of the experiment. This group also consumed slightly more food than the control group from week eight onwards. Considering this for the whole experimental period, it is clear that this group was consuming the most food on average.

The opposite was, however, true for the kankerbos and amitriptyline groups, which were on average the heaviest, despite them consuming slightly less food than the controls for most of the measuring period (fig. 4.5).

These results implied that some factor, other than simply food consumption, must also be involved in the weight changes experienced within the different groups. For this reason the metabolic rate was measured, for the different groups, as this was considered to be the most likely other factor contributing to the results recorded in figures 4.4 and 4.5. This procedure was only carried out during the final few weeks of the experiment therefore the long exposure (11 weeks) group were the only to participate.

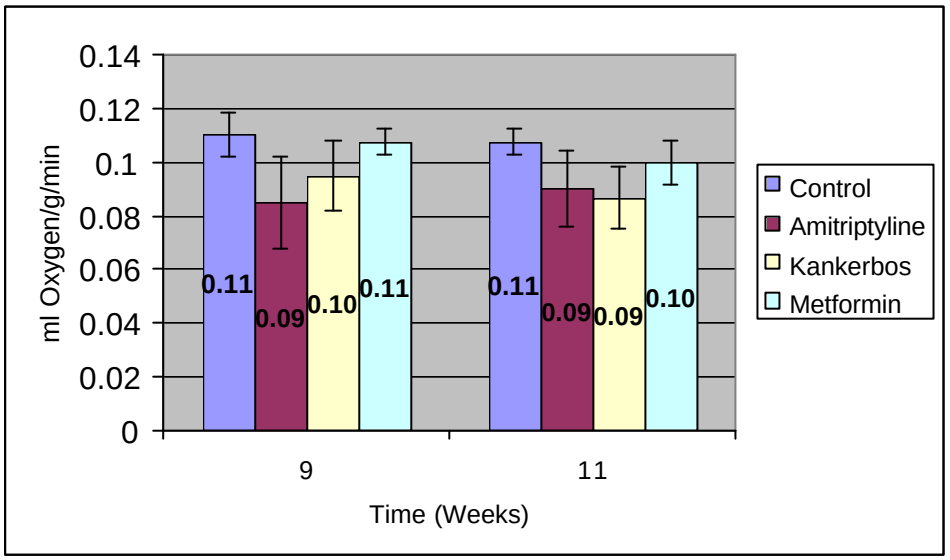


Figure 4.6: Oxygen consumed per ml over 10 minutes per gram rat body weight, each value represents a mean of 4 rats and the standard deviation for those four rats

Figure 4.6 may give an indication as to why the kankerbos and amitriptyline groups displayed an increased weight gain even though they were eating less. After nine weeks the amitriptyline and kankerbos groups have a slightly lower metabolism of 0.09±0.017ml O_2/g/min and 0.1±0.013ml O_2/g/min, respectively, compared to the control group (0.11±0.008ml O_2/g/min). The metabolic rate seemed to remain the same, after eleven weeks for these groups, with only the kankerbos group's metabolic rate decreasing from 0.1±0.013ml O_2/g/min to 0.09±0.012ml O_2/g/min. It seems likely that the decrease in the metabolic rate for the amitriptyline and kankerbos

groups could be the causative agent for the greater weight gain experienced in these groups.

The metformin group's metabolic rate seems to be equivalent to that of the control group, even though the metformin group appeared to have consumed on average more food per gram body weight than any other group. It would therefore be expected that this group have an elevated metabolism. However, it is evident from figure 4.5 that the major food intake occurred within the first 6 weeks and thereafter the eating habits of this group did not diverge far from that of the control group. This also applied at the time (9 and 11 weeks) when metabolic rates were measured.

4.2. MAIN STUDY

The main study consisted of four groups of male Wistar rats with each group consisting of ten rats. As with the pilot study the four groups were seperated from one another in accordance to the type of medication they received: amitriptyline, metformin, kankerbos or none as was the case with the control group.

4.2.1. Food intake, body mass and oxygen consumption throughout experiment

4.2.1.1. Food consumed per gram body weight

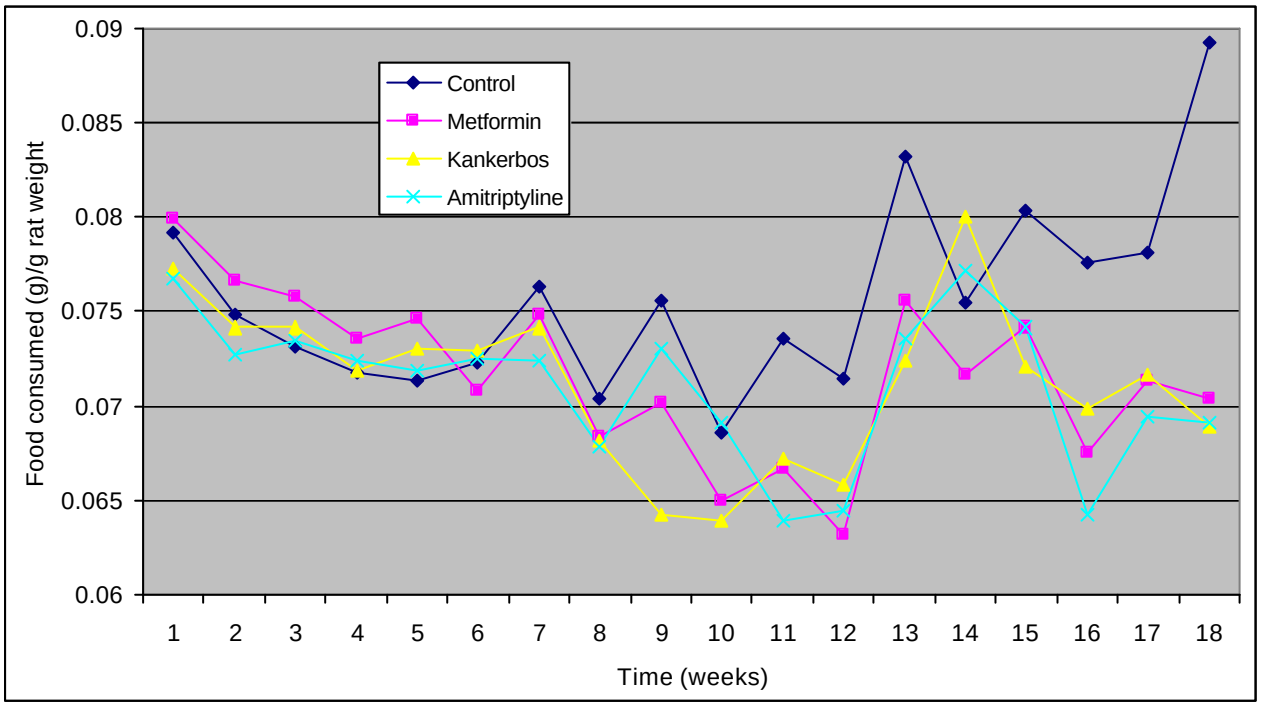


Figure 4.7: Amount of food consumed, by male Wistar rats in grams, per gram body weight over a period of 18 weeks

The food consumption was measured on a daily basis and plotted as the amount of food consumed per gram body weight for each week, as seen in figure 4.7. Although the metformin group consumed the most food over the first five weeks, there was no statistical significant difference over this time period (see fig. 4.8). After ten weeks there was a difference ($P<0.1$) for kankerbos in comparison to the control group (see

fig. 4.9). After fifteen weeks and eighteen weeks statistical differences, was also apparent, as can be seen in figures 4.10 and 4.11 respectively.

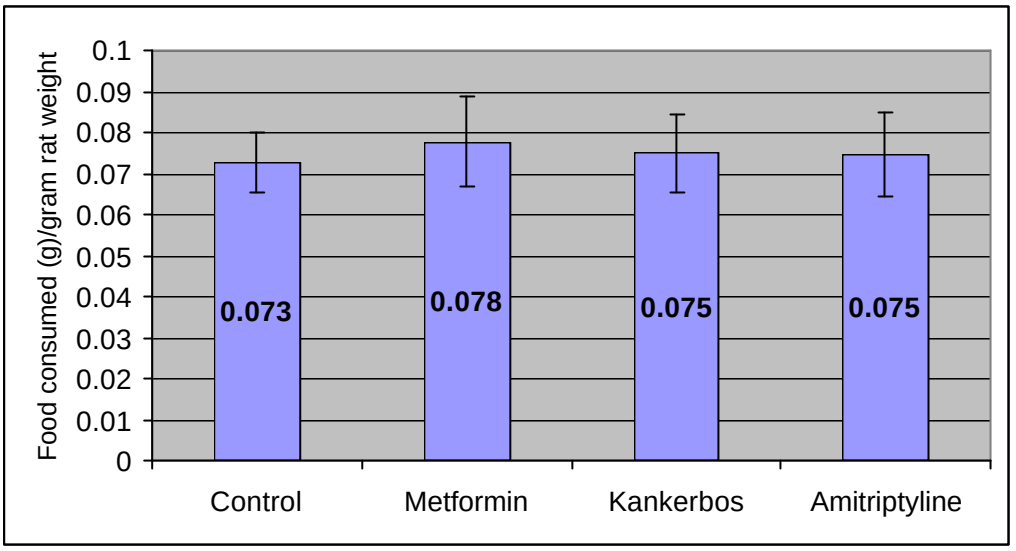


Figure 4.8: Average amount of food consumed by male rats per gram body weight after 5 weeks of treatment. Data are the mean ± SD (*n* = 10)

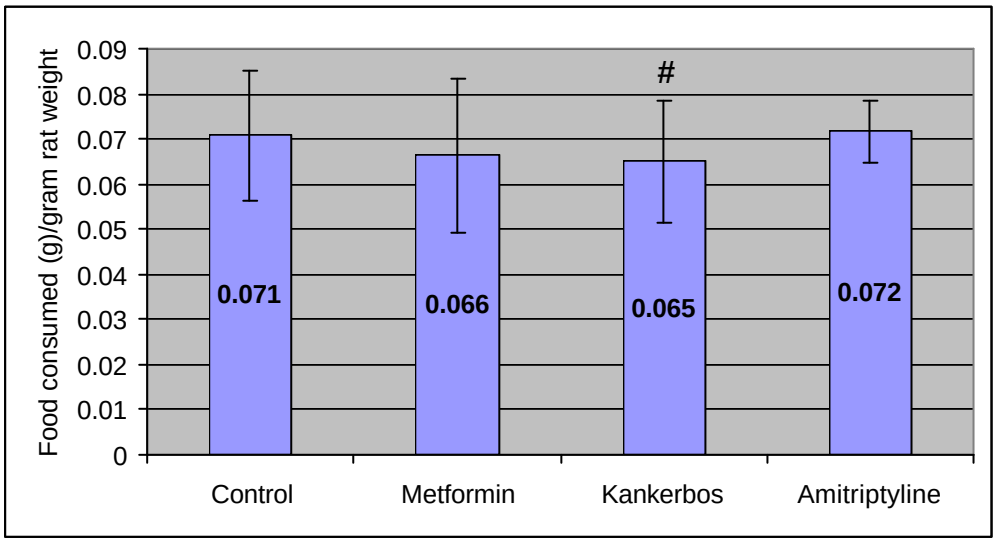


Figure 4.9: Average amount of food consumed by male rats per gram body weight after 10 weeks of treatment. Data are the mean ± SD (*n* = 10). # *P* < 0.1 compared with the Wistar rat control group by Dunnett's test

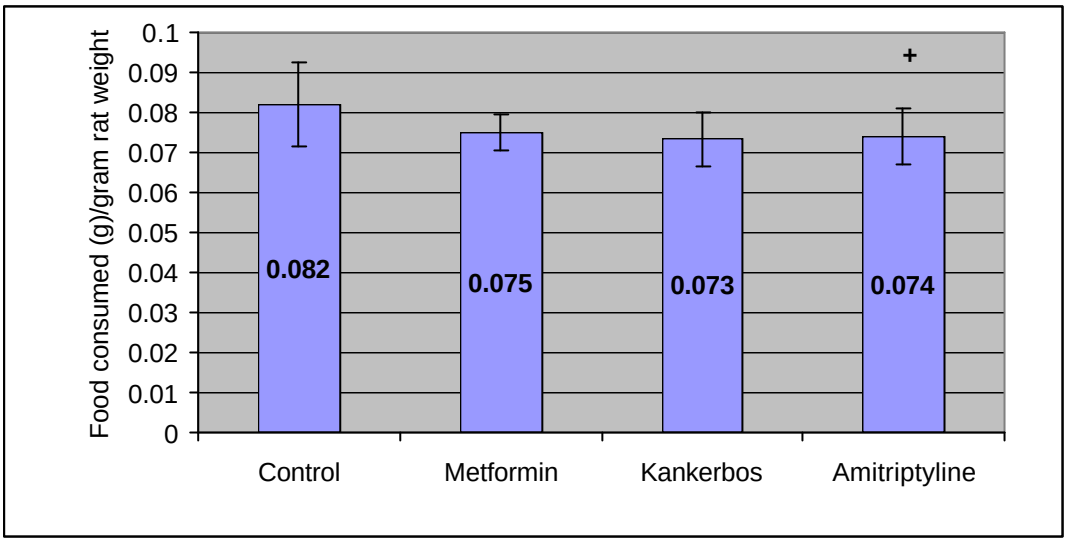


Figure 4.10: Average amount of food consumed by male rats per gram body weight after 15 weeks of treatment. Data are the mean ± SD (*n* = 10). **+** *P* < 0.02 compared with the Wistar rat control group by Dunnett's test

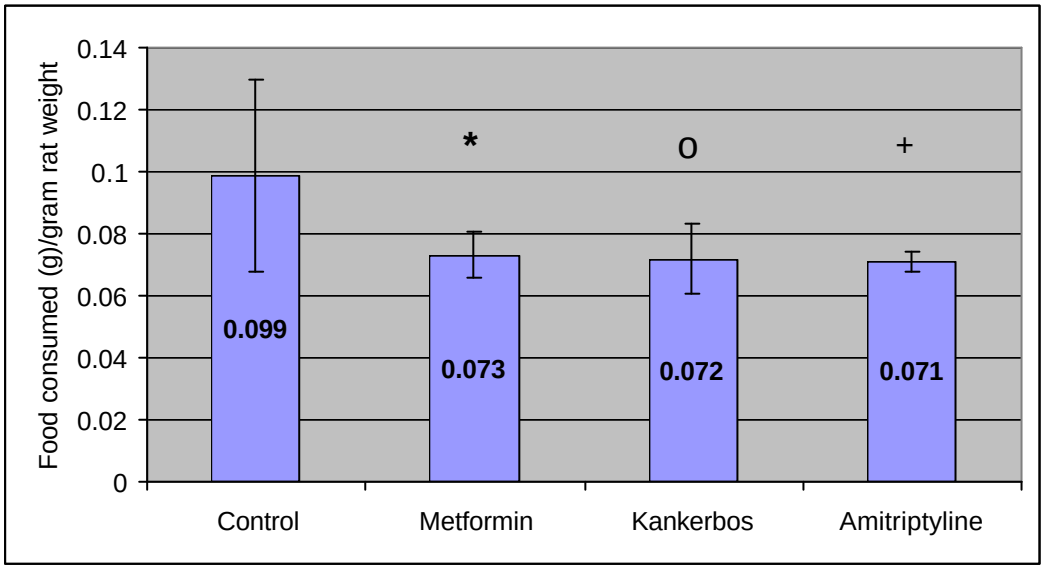


Figure 4.11: Average of the amount of food consumed by male rats per gram body weight after 18 weeks of treatment. Data are the mean ± SD (*n* = 10). ***** *P* < 0.05, **o** *P* < 0.005, and **+** *P* < 0.02 compared with the Wistar rat control group by Dunnett's test

The control group began to show significantly higher food consumption from week fifteen (see fig. 4.10), although generally significant differences seem to appear only after 18 weeks of medication; 0.099±0.031g/g rat weight for the control compared to the kankerbos group, 0.072±0.011g/g rat weight (P<0.005), the metformin group,

0.073±0.0075g/g rat weight (P<0.05) and the amitriptyline group, 0.071±0.0033g/g rat weight (P<0.02) (see fig. 4.11).

It has been reported that metformin inhibits food intake. Kaiyala *et al*. (1995) found evidence that insulin action in the brain is able to regulate body weight. It was discovered that intracerebroventricular administration of insulin in different animals led to a dose dependent reduction in food intake. Hypothalamic pathways involved in food intake are likely targets for insulin action because the intrahypothalamic administration of insulin at doses 100-fold lower than those required during intracerebroventricular infusion is enough to elicit an inhibition of food intake (Paolisso *et al.*, 1998).

The effect that the kankerbos has on food intake may be similar to that displayed by the metformin group. The kankerbos may affect certain hypothalamic pathways, involved with food intake, in a similar fashion to that of metformin.

The amitriptyline group started to show signs of a decreased food intake after fifteen treatment weeks, 0.074±0.0069g/g rat weight (P<0.02) compared to the control group's 0.082±0.011g/g rat weight (see fig. 4.10). This trend continued after eighteen weeks, figure 4.11, where the amitriptyline group's average food intake continued to decrease to 0.071±0.0033g/g rat weight and the control group's intake continued to rise to 0.099±0.031g/g rat weight (P<0.02). A similar trend was observed during the pilot study (see figure 4.5). These results indicate that amitriptyline probably does not cause carbohydrate craving as was suspected in the past by Garland *et al.*, (1988).

4.2.1.2. Body weight

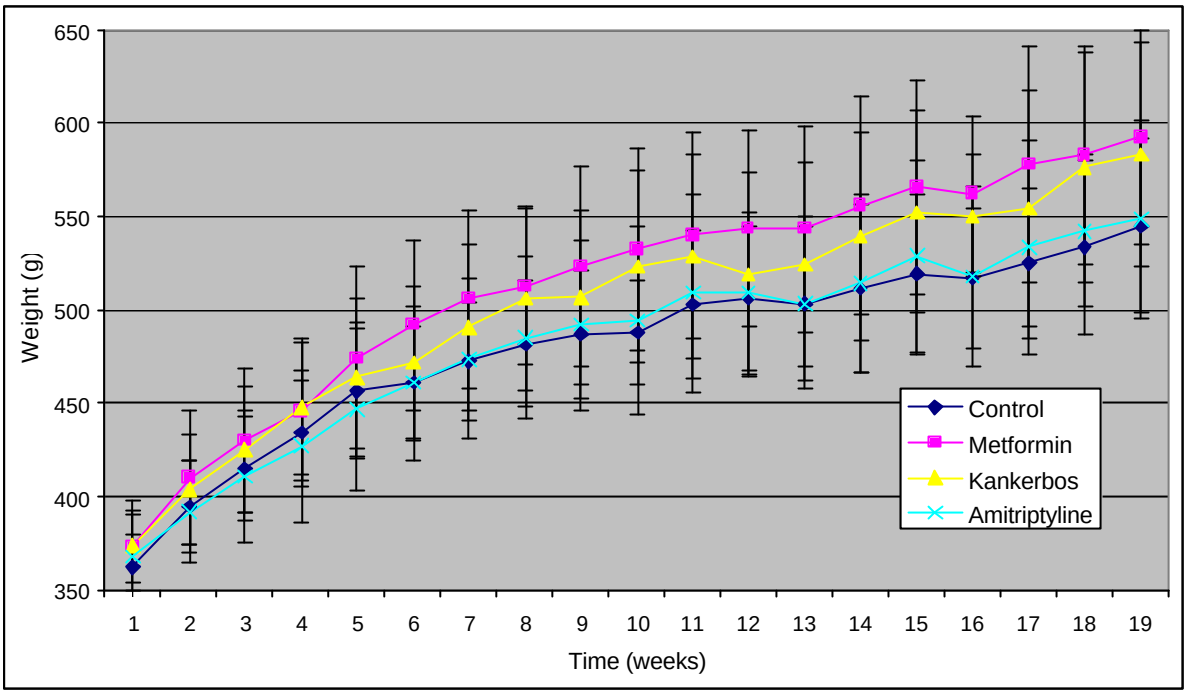


Figure 4.12: Rat weight in grams measured over a period of 19 weeks of medicational treatment. Data are the mean ± SD (*n* = 10)

As can be seen from figure 4.12 the metformin group gained the most weight, followed by the kankerbos group. There seems to be no significant difference in weight between the amitriptyline and the control groups throughout the entire experiment. In order to further evaluate statistical significance, using Dunnett's test, certain time interval periods were analysed throughout the experiment. The time periods used were after five, ten, and nineteen weeks of medication.

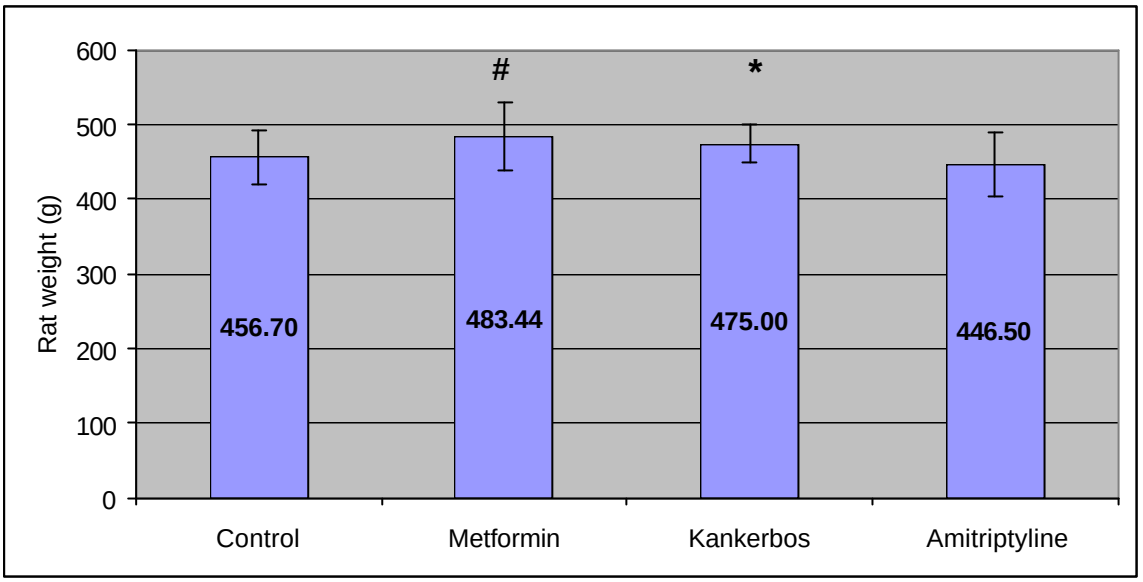


Figure 4.13: Rat weight in grams after five weeks on medication. Data are the mean ± SD (*n* = 10). **#** *P* < 0.1, ***** *P* < 0.05 compared with the Wistar rat control group by Dunnett's test

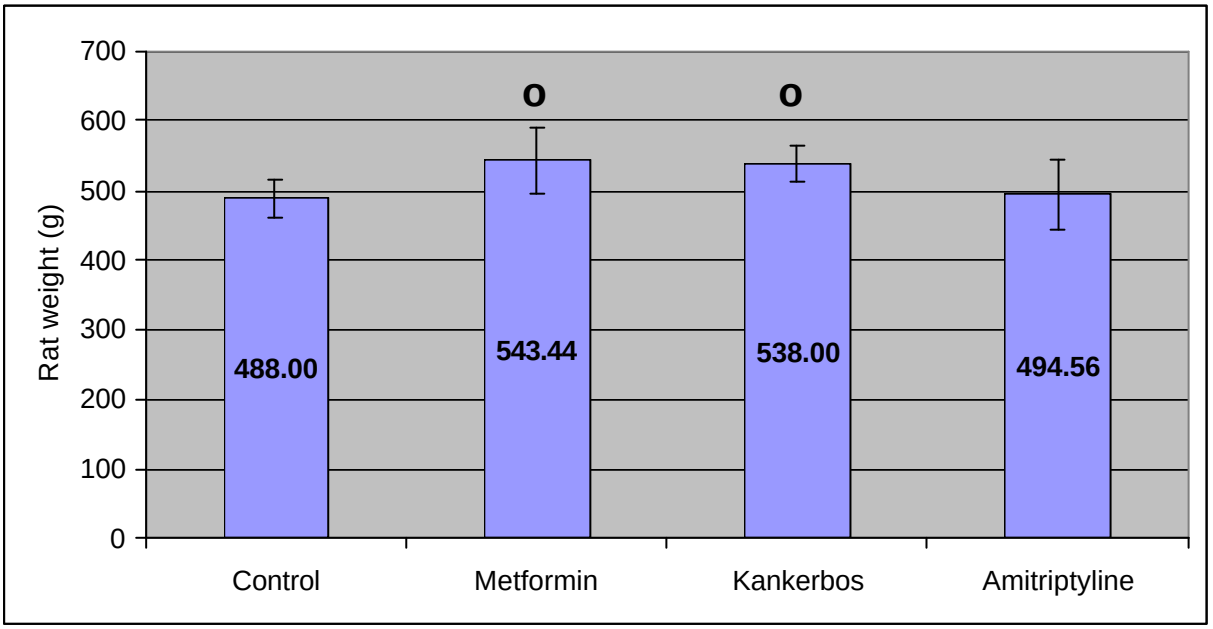


Figure 4.14: Rat weight in grams after ten weeks on medication. Data are the mean ± SD (*n* = 10). **o** *P* < 0.005 compared with the Wistar rat control group by Dunnett's test

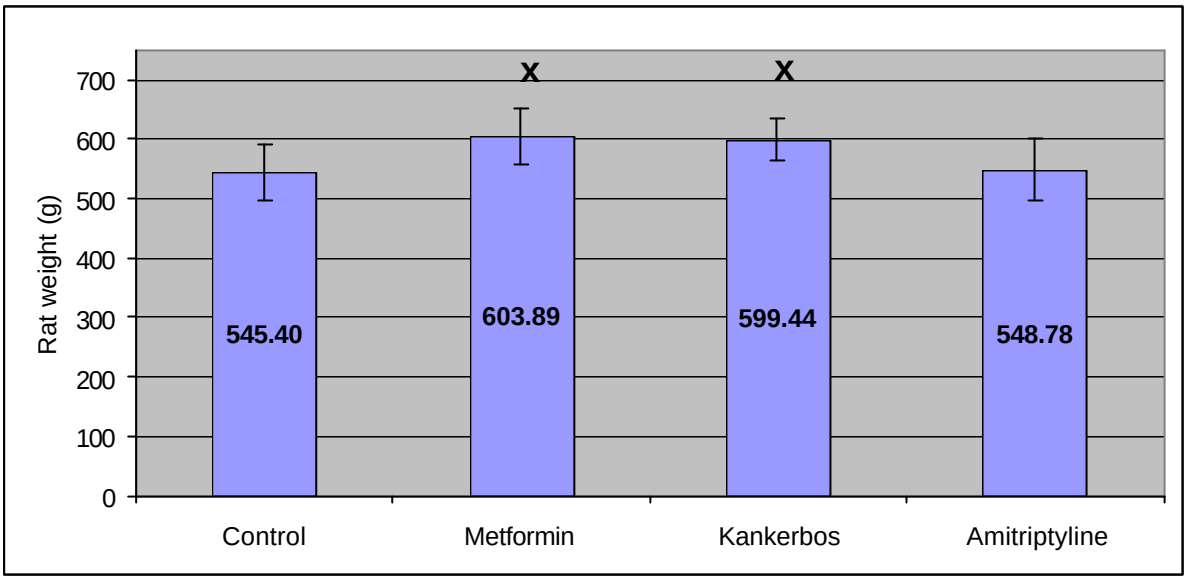


Figure 4.15: Rat weight in grams after nineteen weeks on medication. Data are the mean ± SD (*n* = 10). x *P* < 0.01 compared with the Wistar rat control group by Dunnett's test

The kankerbos and metformin groups show early signs of weight gain, 475±24.9g and 483.4±44.7g, respectively, in comparison to the control group, 456±36.4g, at five weeks (see fig. 4.13). The finding that the metformin group displayed the highest weight gain is unexpected, as this medication is known to prevent any weight increase in humans, these results are also contrary to the findings of the pilot experiment (see 4.1.5.). The metformin group continued to increase weight throughout the experiment, 543±47.9g compared to the control's 488±27.5g at ten weeks (P<0.005) (fig. 4.14) and 603.9±47.4g in contrast to the control's 545±46.6g at nineteen weeks (P<0.01) (fig. 4.15).

Previous experiments have shown that metformin is successful in promoting weight loss in humans through inhibition of food intake and a reduction in the rate of glucose absorption through the intestine (Paolisso *et al.*, 1998). However once again the subjects used for the experiment were obese and obese diabetics, which was not the case with the Wistar rats. Other work done by Suzuki *et al.*, (2002), using Wistar rats, showed that they displayed a slight weight increase after being administered a dose of 300mg/kg per day of metformin. Many studies have been undertaken involving

metformin, most of which show the drug promoting weight loss, however it is important to note that these studies involved obese subjects.

The kankerbos weight increase is also significant throughout the experiment; 538±25g compared to the control's 488±27.5g after ten weeks ($P<0.005$) (see fig. 4.14) and 599.4±35.3g in comparison to the control's 545.4±46.6g after nineteen weeks ($P<0.01$) (see fig. 4.15). Photos of kidney fat pads were taken at the time of sacrifice in order to record any visible differences in fat distribution.

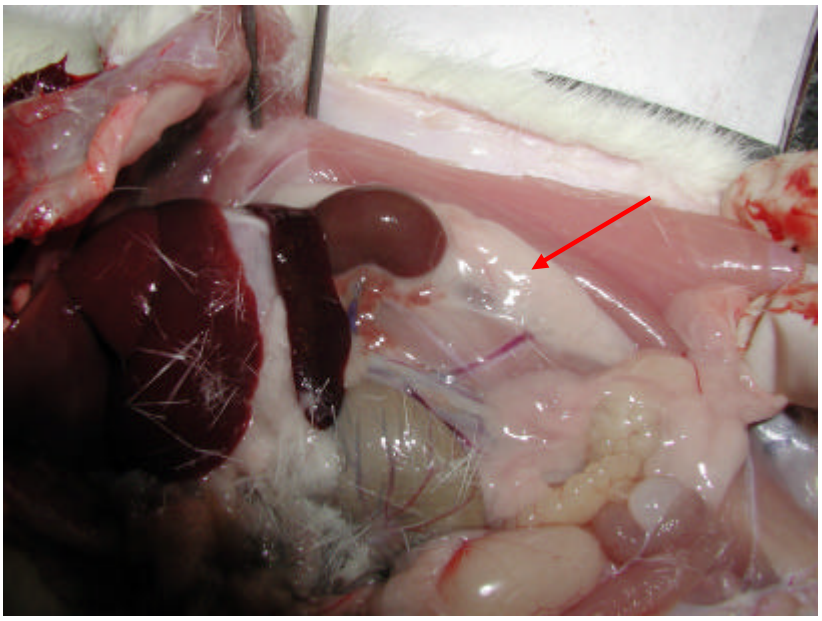


Figure 4.16: A kidney fat pad of a typical control rat after 19 weeks of medication

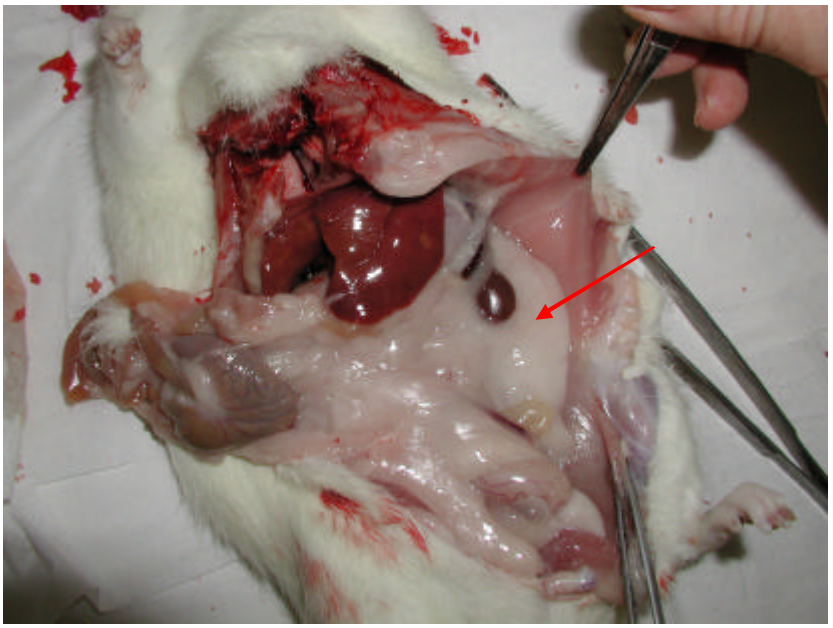


Figure 4.17: A kidney fat pad of a typical metformin rat after 19 weeks of medication

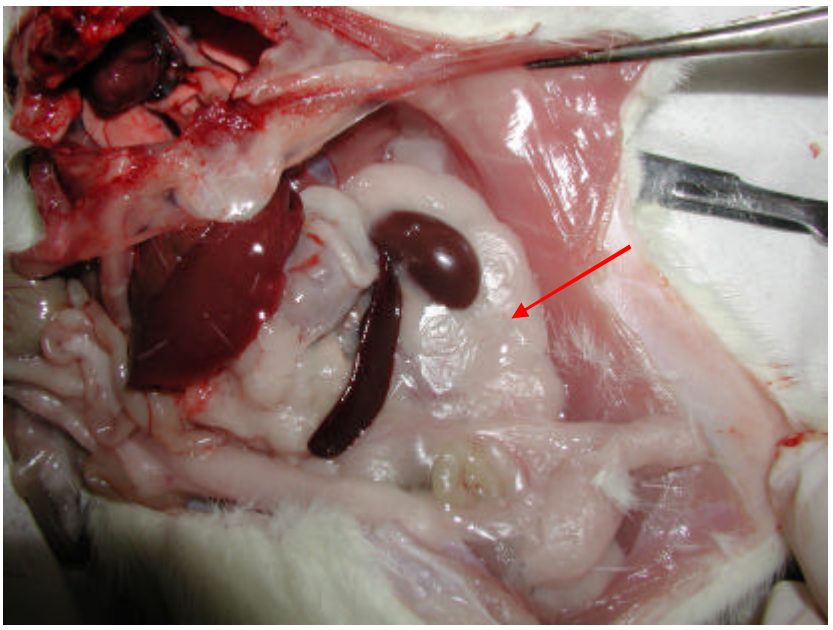


Figure 4.18: A kidney fat pad of a typical kankerbos rat after 19 weeks of medication

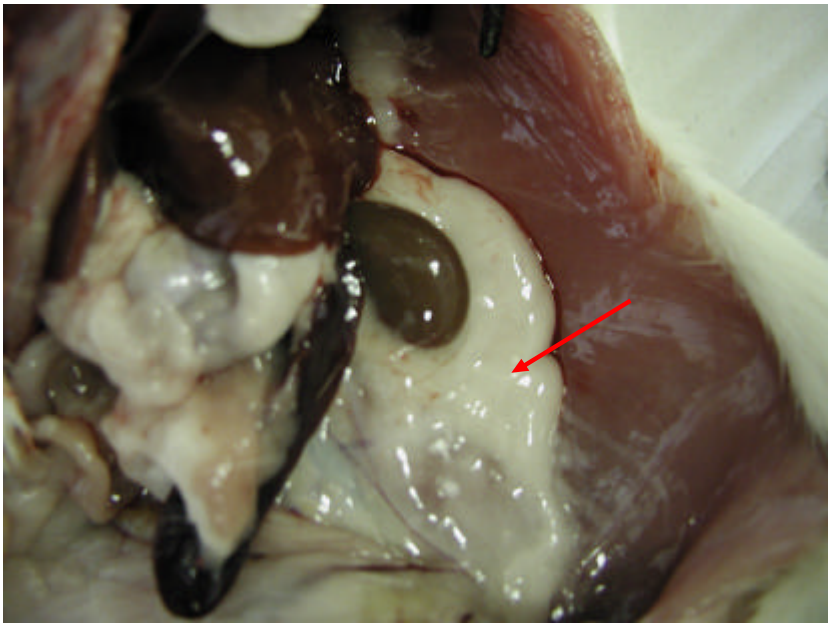


Figure 4.19: A kidney fat pad of a typical amitriptyline rat after 19 weeks of medication

Figures 4.16 to 4.19 show the difference between typical kidney fat pads for the different treatment groups. As can be seen in figure 4.17 the kidney fat pad of the metformin rat is much larger than that of the control rat, seen in figure 4.16. The fat pads of the kankerbos rats were also larger than the control group as can be seen in figure 4.18, they were however not larger than the fat pads found in the metformin

rats. There was not a clear difference between the fat pads of the control and the amitriptyline group, shown in figure 4.19.

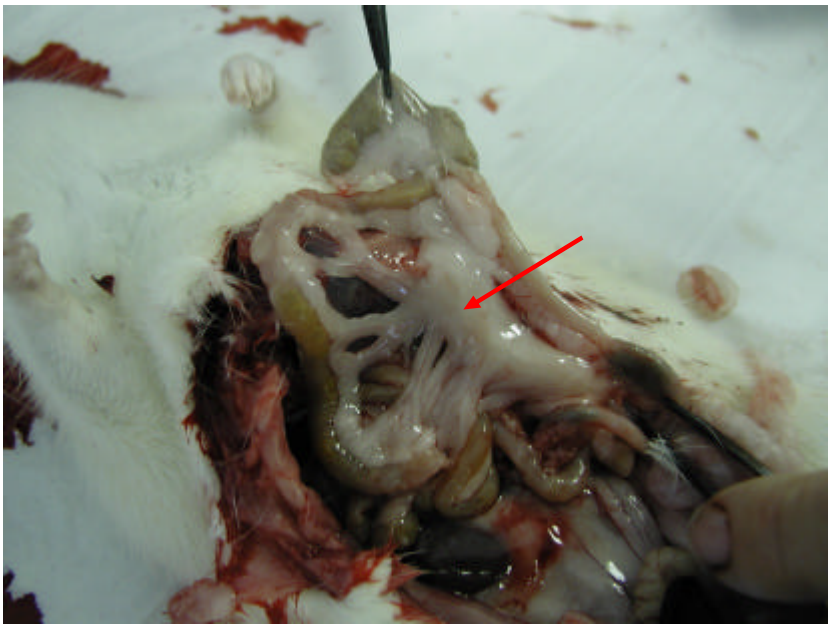


Figure 4.20: Intestinal fat of a typical control rat after 19 weeks of medication

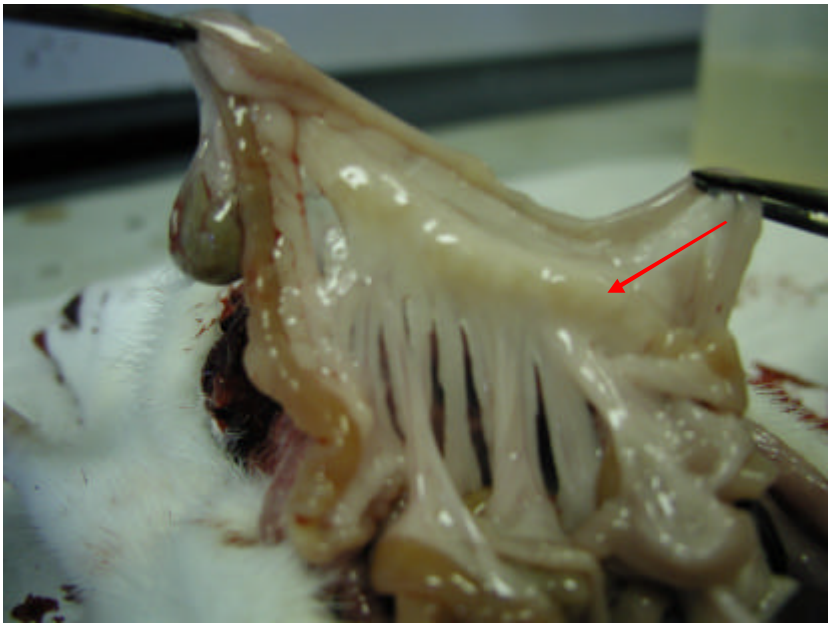


Figure 4.21: Intestinal fat of a typical metformin rat after 19 weeks of medication

The fat found between the intestines of the metformin group (fig. 4.21) also displayed a difference in comparison to the control group (fig. 4.20). There was no clear difference between the intestinal fat of the kankerbos and amitriptyline groups in comparison to the control group (photos not shown here). There seems to be an extremely large amount of fat

present for the metformin group, shown by the red arrows, for both the kidney and intestinal fat. The excessive weight gain displayed by this group is a result of the large fat accumulation, displayed in figures 4.17 and 4.21.

4.2.1.3. Resting metabolic rate

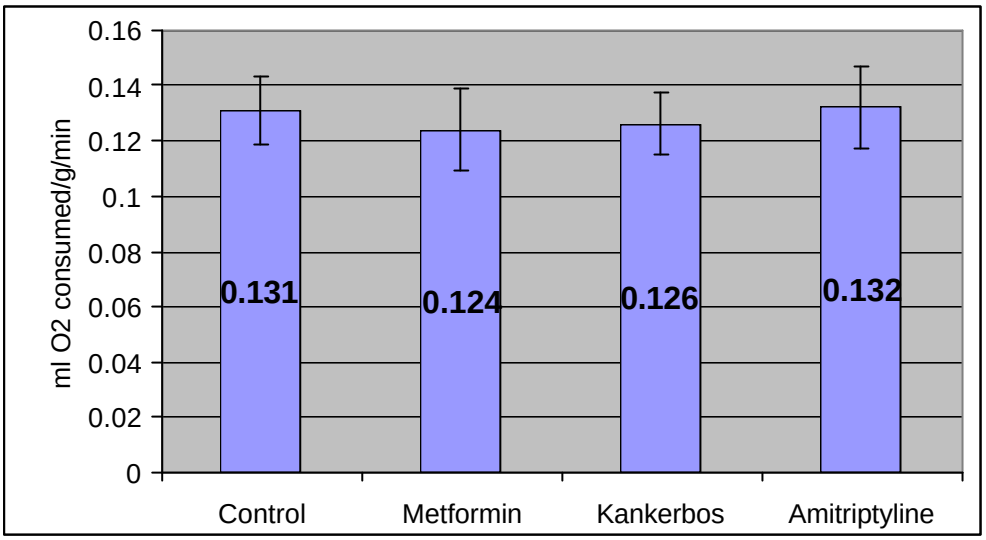


Figure 4.22: Oxygen consumed per gram body weight, per minute after 6 weeks. The flow rate was 0.5L/min over a period of 10 minutes. Data are the mean ± SD (*n* = 10)

The results obtained in figure 4.22 were unsatisfactory as the time the rats were in the chamber was too short. The rats may have still been under some stress during this time leading to incorrect results. It was therefore decided to increase the time the rats spent in the chamber to twenty minutes.

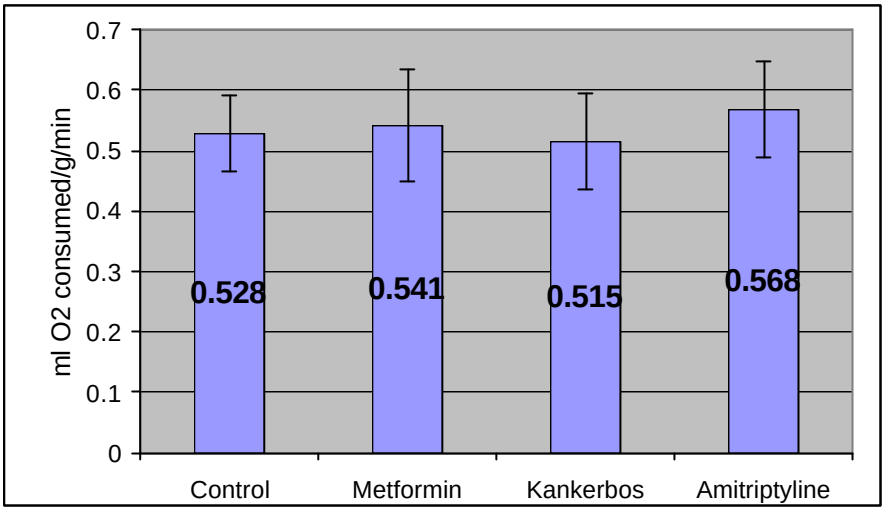


Figure 4.23: Oxygen consumed per gram body weight, per minute after 8 weeks. The flow rate was 0.5L/min over a period of 20 minutes. Data are the mean ± SD (*n* = 10)

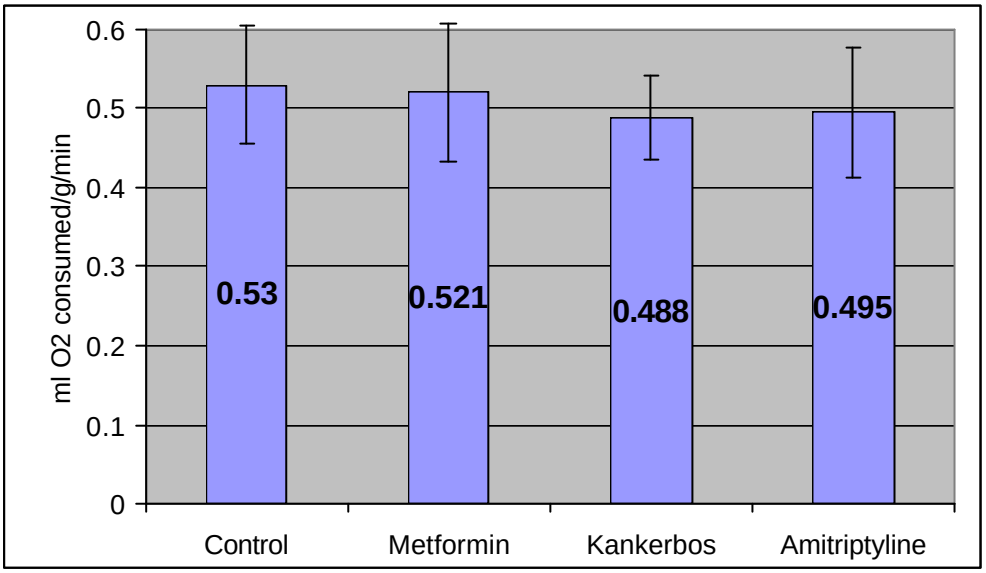


Figure 4.24: Oxygen consumed per gram body weight, per minute after 19 weeks. The flow rate was 0.5L/min over a period of 20 minutes. Data are the mean ± SD (*n* = 10)

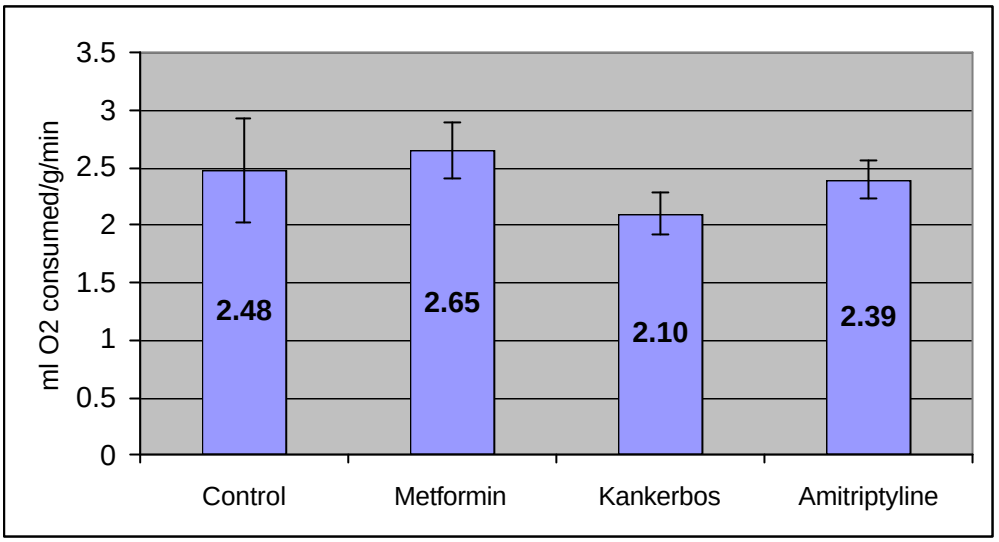


Figure 4.25: Oxygen consumed per gram body weight, per minute after 18 weeks. The flow rate was 3L/min over a period of 40 minutes. Data are the mean ± SD (*n* = 6)

The above results (figs.4.22-4.24) revealed no clear differences between the groups, even at the 19 weeks exposure period. The resting metabolic rate of the amitriptyline group at the 19 weeks was 0.495±0.082ml O_2/g/min and that of the control group was 0.53±0.074ml O_2/g/min. Based on the results from the pilot study a difference in the resting metabolic rate was anticipated for the amitriptyline group, however, no significant differences could be demonstrated through Dunnet's test, in any of the above results, due to the high standard deviations present in the data. The amitriptyline group did not, however, show any of the features previously displayed by the rats involved in the pilot study. There was not even an excessive weight gain to report. Reasons for this could lie in the concentration of the amitriptyline administered being too low. Even though it was the same concentration the rats received during the pilot study the dose may be a threshold one, which may or may not cause weight gain in an individual animal.

These findings may add to the controversy about whether or not tricyclic antidepressants are associated with weight gain, as many contradictory articles have been published on this topic. Benazzi (1998) states that weight gain may not necessarily be due to the pharmacological effects of antidepressants, but may rather be an effect of recovery from depression. Reports that amitriptyline causes carbohydrate craving and weight gain in humans conflict with other reports that it

causes anorexia and weight loss. The effects of such drugs on energy balance may differ between a lean subject and a subject with a predisposition to obesity (Dullo and Miller, 1987).

In some cases antidepressants have been linked with treatment of obesity through the aetiology of obesity. It has been shown that obesity is a result of a faulty homeostatic system involving a metabolic defect. Since amitriptyline's function is to increase the half-life of noradrenaline, at the sympathetic nerve terminals, it is generally thought that it would therefore contain thermogenic properties, and would thus be associated with weight loss (Dullo and Miller, 1987). It is also, however, important to keep in mind that thermogenic drugs are not likely to be as effective in the lean as they are in the obese and one should be careful when choosing a subject or model. This may be where Dullo and Miller (1987) have been misled by their results that amitriptyline promotes weight loss, as it was tested on MSG induced obese mice (obesity induced through chemical lesioning in the hypothalamus following injections of monosodium glutamate during the first week of life). Since a characteristic of obesity is a deficiency of sympathetically released noradrenaline rather than an insensitivity of the receptor, and amitriptyline promotes noradrenaline release, it is possible to see how the drug could have promoted weight loss in the obese mouse model.

The results obtained in the sensitive amitec experiment (figure 4.25) still showed no difference between the amitriptyline group's resting metabolic rate of 2.39±0.16ml O_2/g/min and that of the control group's, 2.48±0.46ml O_2/g/min. Some data unfortunately had to be disregarded due to instrument failure on the day of the experiment.

Despite the many contradictions found in the literature, the authour is still confident that the tricyclic antidepressant, amitriptyline, is associated with excessive weight gain when taken over a relatively long period (three months or longer). Even though the results obtained in the final study contradicted this view, a trend was present in the pilot study, implying that there was indeed some form of weight gain associated with this drug. The fact that two similar experiments were run back to back and contradictory results were obtained adds to the contrasting results reported by

different authors in the literature. The dose used in these experiments was 1mg per day per kg body weight, which is the lowest dose prescribed. This dose level is usually prescribed to patients, by their doctors, and is later increased to 2mg per kg per day. Individuals also receive a further 0.6mg or 1.3mg per kg per day at a later stage, which is administered before sleep (Cooper and Gerlis, 1997). The authour feels certain that if a higher concentration of amitriptyline was administered to the rats, that a significant weight increase would have been evident. Even though the increased concentration of noradrenaline, associated with amitriptyline therapy, should cause the metabolic rate to increase, thus promoting weight loss, there seems to be some other factors overriding the noradrenaline effect and allowing weight gain. The pilot study suggested that weight gain was associated with a decreased metabolic rate, however this could not be proved statistically. To resolve this issue, further testing, involving a wide range of dose levels, needs to be investigated on obese as well as lean rats over an extended time period such as 3 months.

No change in the metformin group's metabolic rate is evident over any of the time periods either: 0.12±0.015ml O_2/g/min at 6 weeks, 0.541±0.09ml O_2/g/min at 8 weeks, 0.521±0.087ml O_2/g/min at 19 weeks and 2.65±0.25ml O_2/g/min at 18 weeks; when compared to that of the control group: 0.131±0.012ml O_2/g/min at 6 weeks, 0.528±0.06ml O_2/g/min at 8 weeks, 0.53±0.074ml O_2/g/min at 19 weeks and 2.48±0.46ml O_2/g/min at 18 weeks. The kankerbos group does seem to be decreasing slightly over time, 0.515±0.08ml O_2/g/min at 8 weeks and 0.488±0.0532ml O_2/g/min at 19 weeks in comparison to the control group, 0.528±0.06ml O_2/g/min at 8 weeks and 0.53±0.074ml O_2/g/min at 19 weeks. The values seem to be consistently lower than those of the control group's throughout all sampling times including the final one at 18 weeks: 2.1±0.46ml O_2/g/min for kankerbos in comparison to 2,48±0.46ml O_2/g/min for the control group. The results are however not statistically significantly different. This trend may however be the reason that this group did pick up weight despite the fact that they consumed less food.

4.2.2. Volume of Fluid Consumed and Urine Analysis

The amount of urine produced, by an individual, is of course, directly related to the amount of fluid consumed by that individual. The amount of fluid consumed (fig. 4.26-4.27) and the amount of urine produced (fig.4.28-4.29) are therefore shown together in this section and will be dealt with as an integrated aspect.

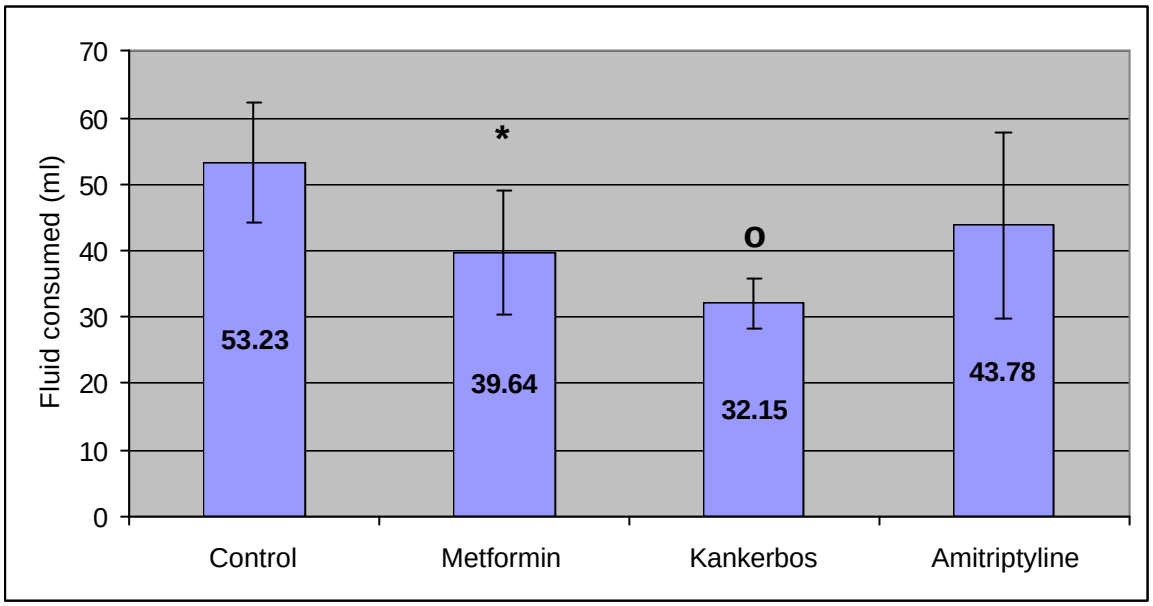


Figure 4.26: Volume of fluid consumed, in millilitres, by male Wistar rats after 18 weeks of treatment. Data are the mean ± SD (*n* = 10).). ***** P< 0.01 and **o** *P* < 0.001 compared with the control group by Dunnett's test

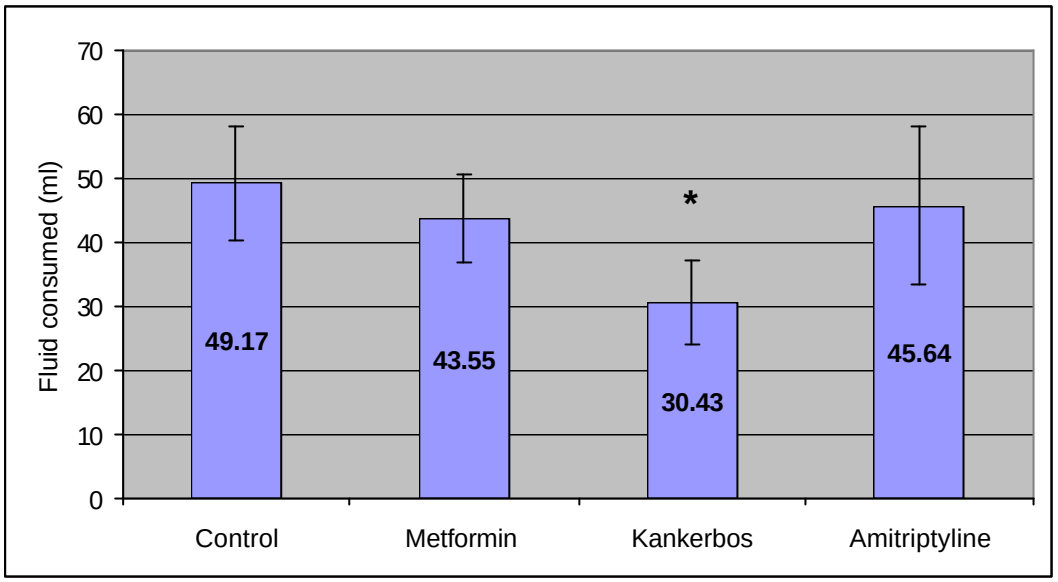


Figure 4.27: Volume of fluid consumed, by male Wistar rats in millilitres, after 19 weeks of treatment. Data are the mean ± SD (*n* = 10). ***** P< 0.001 compared with the control group by Dunnett's test

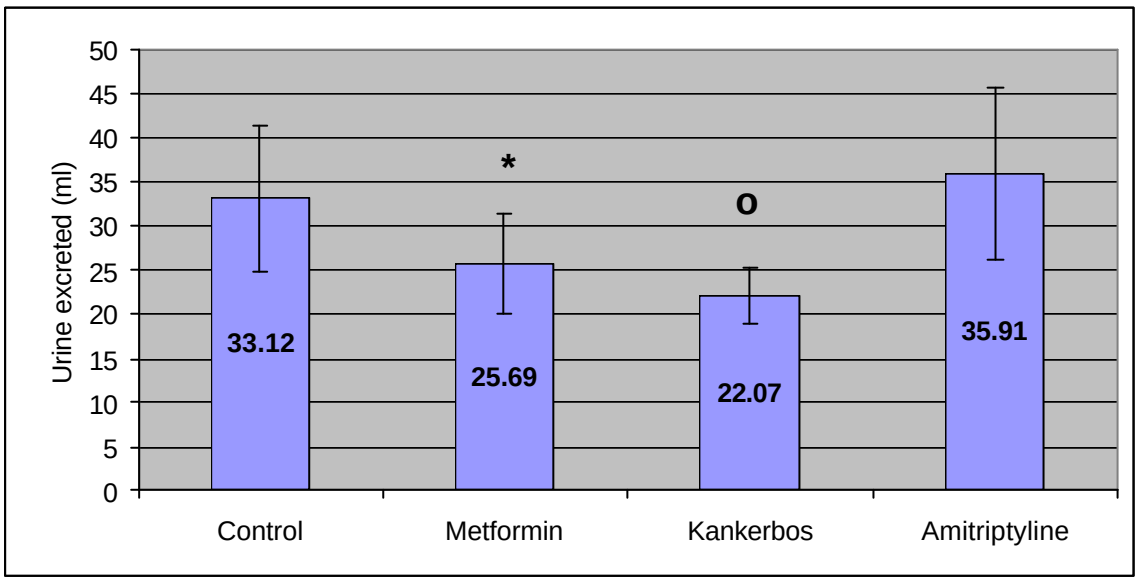


Figure 4.28: Volume of urine excreted, in millilitres, by male Wistar rats after 18 weeks of treatment. Data are the mean ± SD (*n* = 10). ***** *P* < 0.1, and **o** *P* < 0.05 compared with the control group by Dunnett's test

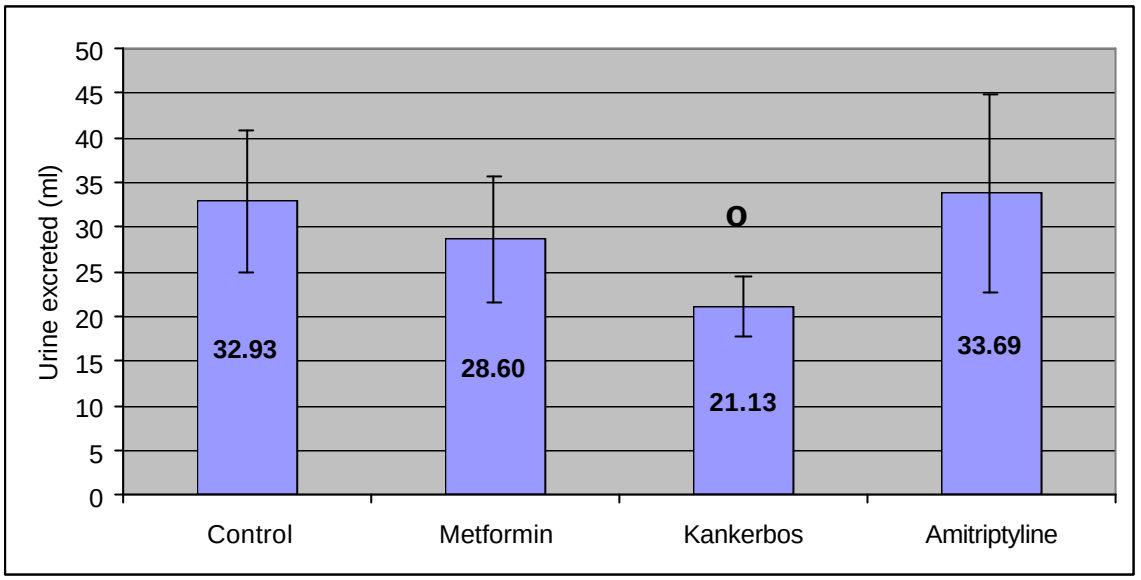


Figure 4.29: Volume of urine excreted, in millilitres, by male Wistar rats after 19 weeks of treatment. Data are the mean ± SD (*n* = 10). **o** *P* < 0.05 compared with the control group by Dunnett's test

The significance shown for the kankerbos group's low fluid consumption, 32.1±3.73ml, compared to the control's 53.2±9.04ml (P<0.001) at 18 weeks (see fig. 4.26) and 30.4±6.5ml compared to the control's 49.2±8.8ml (P<0.001) at 19 weeks (see fig. 4.27), most likely may be a result of the bitter taste associated with the kankerbos tea.

The fact that the metformin and amitriptyline groups also consumed less fluid, 39.6±9.25ml (P<0.01) and 43.8±14ml, respectively, compared to the control's 53.2±9.04ml at 18 weeks (see fig. 4.26) and 43.6±6.9ml and 45.6±12.3ml, respectively, compared to the control's 49.2±8.8ml at 19 weeks (see fig. 4.27), may be attributed to the amount of food consumed by the different groups. The food administered to the rats consisted of dry dog pellets, more water would therefore be needed to aid swallowing, and digestion. It is then to be expected that the more food being consumed, as was the case with the control rats (see fig.4.7), the more water would be needed as well.

Very low levels of glucose were detected in the urine. There was however no significant difference between the different groups.

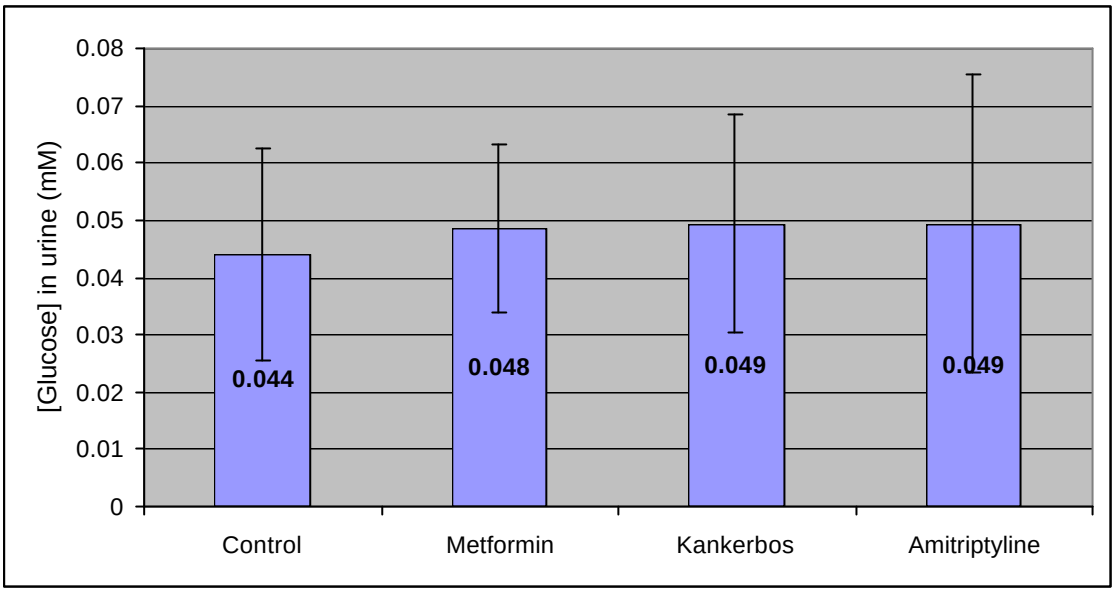


Figure 4.30: Glucose concentration in urine after 18 weeks of medication. Data are the mean ± SD (*n* = 10)

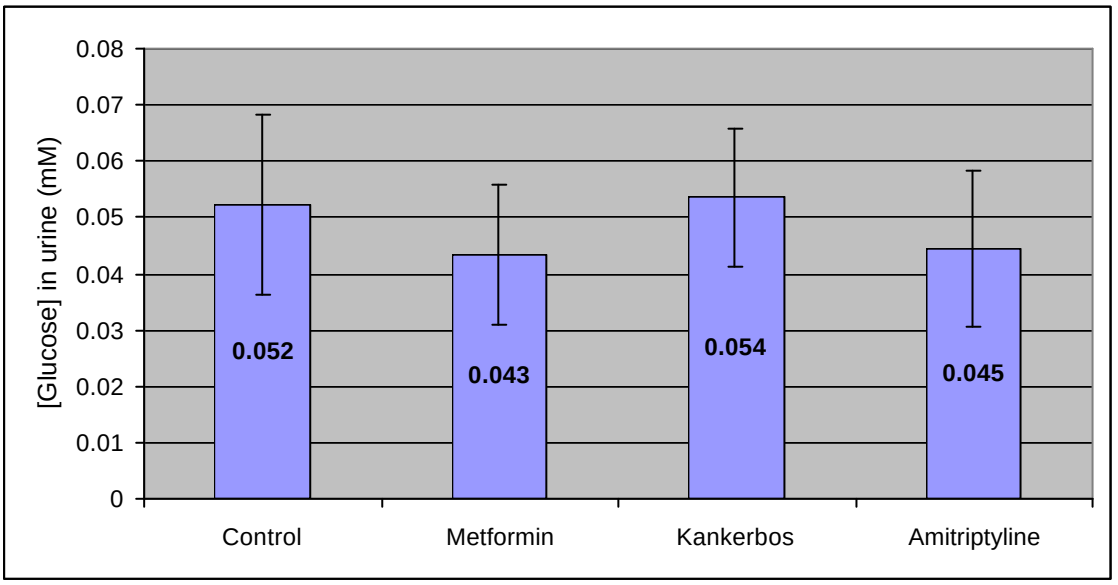


Figure 4.31: Glucose concentration in urine after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

The fact that glucose was found in the urine is not an unusual phenomenon as small amounts of glucose have been known to be excreted by the kidney. Furthermore the glucose found in the urine, in this case, was detected by the glucinet reagent as the

concentration was too low to be detected by the dip sticks, the concentrations were not at a level of clinical concern.

4.2.3. Constituents found in urine

Bilirubin:

Bilirubin is not normally detected in a healthy individual's urine, however certain metabolites of drugs can give false positives. When red blood cells are destroyed by reticuloendothelial cells, the globin portion of hemoglobin is split off and the heme is converted to biliverdin. Most of the biliverdin is in turn converted into bilirubin, which gives bile its major pigmentation (Tortora and Grabowski, 2000). None of the rats from any group showed any signs of bilirubinuria.

Ketones:

The test used is specific for acetoacetic acid, in the urine, and does not detect acetone or beta hydroxybutyric acid. No ketone bodies should be present in a healthy normally fed individual's urine, as ketones represent an indication of diabetes, starvation or too little carbohydrate in the diet. None of the rats had detectable quantaties of acetoacetic acid in their urine, except for some of the amitriptyline treated rats, which showed trace amounts of ketones in their urine.

However it has been found that amitriptyline metabolites associated with phenols, dihydrodiols, glycols, and ketones may appear in the urine (Prox and Breyer-Pfaff, 1987). This false positive was, however, not observed with all the amitriptyline treated rats and was also not consistent in those who displayed it. The reason for this may be due to the relatively low concentration of amitriptyline used.

Specific gravity:

The specific gravity is the ratio of the weight of a volume of a substance to the weight of an equal volume of distilled water. For urine usually ranges from 1.001 to 1.035 (Tortora and Grabowski, 2000). The higher the concentration of solutes in the urine

the higher the specific gravity of that urine. The results obtained from the rats, in the present study, displayed no extreme values for the specific gravity, and were constant throughout the experiment, ranging between 1.015-1.030.

Blood:

The detection of even trace amounts of blood in the urine often indicates a serious problem and is termed hematuria. A common cause of this could be acute inflammation of the urinary organs as a result of disease, or irritation from kidney stones. Other causes include tumours, trauma, and kidney diseases (Tortora and Grabowski, 2000).

High concentrations of blood were consistently found in the kankerbos group's urine samples. This is extremely alarming as this could possibly indicate kidney damage. At the time of sacrifice smaller amounts of blood were also associated with the urine of the other groups, including the control group. However these were trace amounts and were only revealed after four months of treatment, whereas the blood associated with the kankerbos group was visible in the urine with the naked eye, too high to read from the urine dipsticks, and was evident even from the first urine analysis (18 weeks).

Since the urine was not caught in free flow it was not possible to check for contaminating bacteria which may have been causing some of the hematuria. However, the blood would appear, at a high concentration (visible to the naked eye), for a few days and then would disappear overnight, only to reappear a few weeks later. It was therefore thought that the blood was the result of a bladder tumour and not due to the presence of bacteria. The continual appearance and disappearance of the blood in the urine could have been the result of excessive bleeding from the tumour, the formation of a scab, and then the removal of the scab to initiate more bleeding at a later stage.

At the time of sacrifice the bladders of the control and kankerbos treated rats as well as the kidneys were carefully removed and stored in formalin, for analysis by a pathologist. As there were no tumours present the pathologist suspected that there was damage to the glomerular capillaries of the kidneys in the kankerbos group,

however electron microscopy work would be needed to confirm this. Glomerular filtration occurs in the renal corpuscles of the kidneys across the endothelial-capsular membrane. Blood pressure forces water and dissolved blood components through the endothelial fenestrations (pores) of the capillaries, basement membrane, and on through the filtration slits of the adjoining visceral wall of the glomerular capsule. The resulting fluid is called the filtrate. Damaged glomerular capillaries become so permeable that plasma proteins and blood enter the filtrate and are found in the urine (Tortora and Grabowski, 2000).

Some substances within the kankerbos tea extract may therefore be causing kidney damage. The substances responsible may, however, be different from the antidiabetic agents in the plant. The compounds responsible for the hypoglycemia observed, therefore need to be identified and separated from substances affecting the kidneys, allowing clinical application in humans.

pH:

The pH found in the urine was consistent for all the rats throughout the sampling, and was in the range of approximately pH 7.5-8.

Proteins:

No protein is usually detectable in the urine of normal healthy individuals, however there may be minute amounts present, which is secreted from the kidney. Low concentrations of protein was detectable in the urine of many rats from all groups at the 19 week sampling time. It is thought that this may be an age related phenomenon. The kankerbos group was consistent in showing protein present in their urine, as early on as the first sampling time (18 weeks). However the presence of protein in this case is suspected to arise from the presence of blood. The concentration of protein in the blood was found to be about 100mg/dL in the kankerbos group's sample.

Urobilinogen:

The presence of urobilinogen (breakdown product of haemoglobin) in urine is called urobilinogenuria. Traces found in the urine is normal, but increased urobilinogen may be due to haemolytic and pernicious anemia, infectious hepatitis, biliary obstruction, jaundice, cirrhosis, congestive heart failure, or infectious mononucleosis (Tortora and Grabowski, 2000). The rat urine for all the groups displayed no traces of urobilinogen.

Nitrite:

Nitrite results from the conversion of nitrate (obtained through the diet) to nitrite by the action of principally Gram negative bacteria in the urine. The test is very specific for nitrite and will not detect any other substance normally excreted in the urine. A colour change associated with the urine dipstick suggests a positive result and implies 10^5 or more organisms per ml of urine. Nitrite levels were found to be normal for all rats.

Leucocytes:

The detection of leucocytes in the urine is a significant result and further testing should be performed on the individual. The presence of these white blood cells and other components of pus in the urine, referred to as pyuria, indicates infection in the kidney or other urinary organs (Tortora and Grabowski, 2000). None of the rats from any group showed a positive result for pyuria.

4.2.4. Faeces Analysis

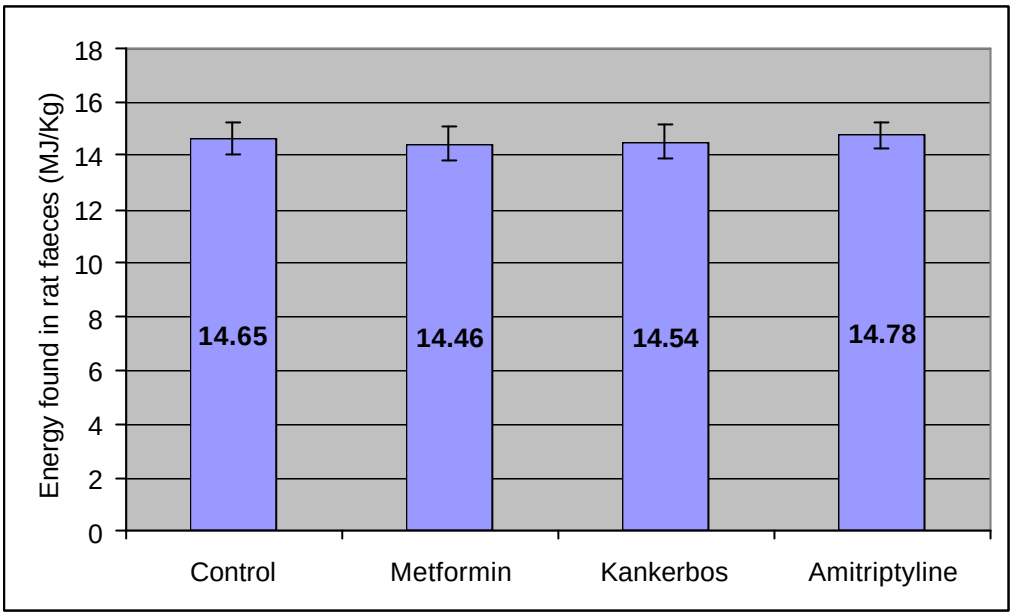


Figure 4.32: Energy associated with faeces production after 18 weeks of treatment. Data are the mean ± SD (*n* = 10)

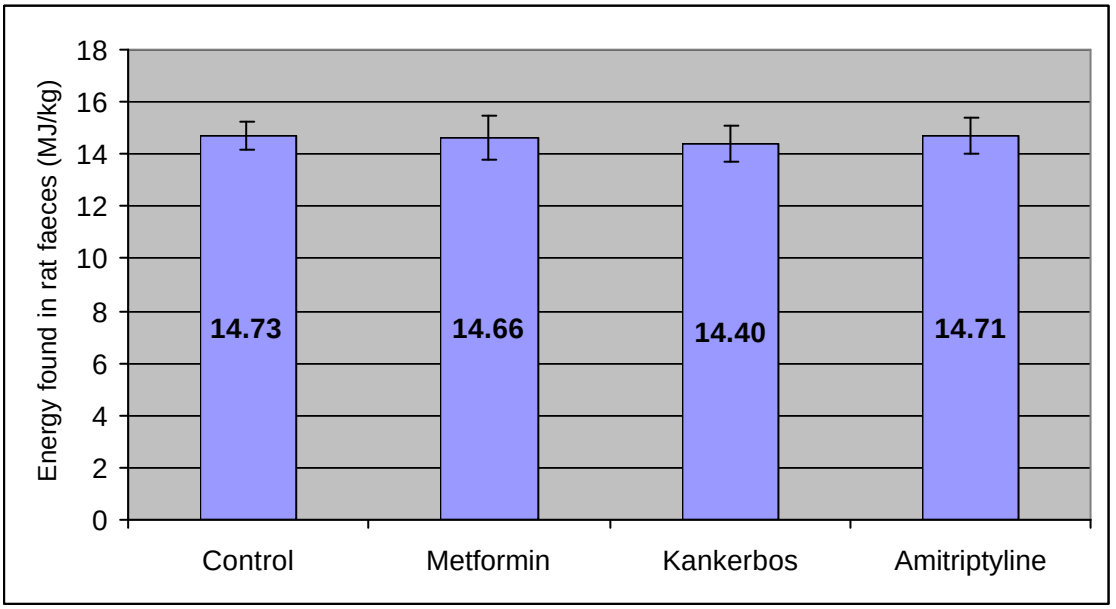


Figure 4.33: Energy associated with faeces production after 19 weeks of treatment. Data are the mean ± SD (*n* = 10)

The energy produced in the bomb calorimeter, by the faeces, displayed no significant difference between the metformin, kankerbos and amitriptyline groups at either

sampling time; 14.46±0.63MJ/kg, 14.54±0.64MJ/kg and 14.78±0.49MJ/kg, respectively, in comparison to the control group's 14.65±0.58 at 18 weeks (see fig. 4.32) and 14.66±0.84MJ/kg, 14.4±0.67MJ/kg and 14.71±0.66MJ/kg, respectively, in comparison to the control's 14.73±0.55MJ/kg at 19 weeks (see fig. 4.33). The metformin and kankerbos groups would be expected to produce a lower energy yield in their faeces, in comparison to the other groups, as they consumed the least amount of food and yet gained the most weight towards the end of the experiment.

4.2.5. Blood glucose levels before sacrifice

Figure 4.34 illustrates the fasting blood glucose concentrations for the different groups of rats prior to sacrifice. In comparison to the control group, all the medication treatments lowered the blood glucose significantly after twelve weeks.

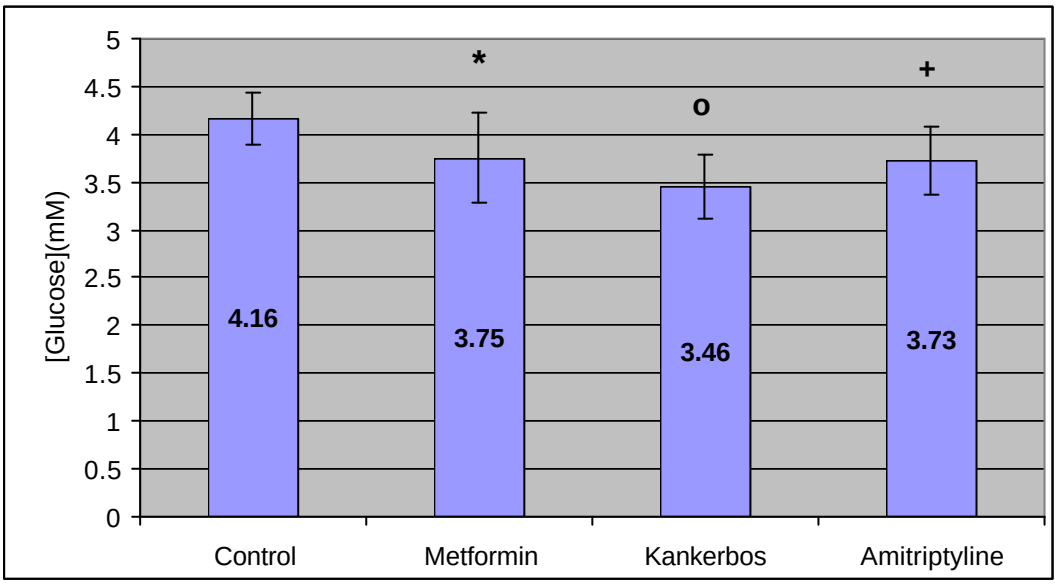


Figure 4.34: Fasting blood glucose concentrations, in male Wistar rats, as a result of a 12 weeks treatment with the medication. Data are the mean ± SD (*n* = 10). ***** *P* < 0.005, **o** *P* < 0.001, and **+** *P* < 0.02 compared with the control group by Dunnett's test

Compared to the control group, significantly lower blood glucose levels were found for all the treatment groups here; 3.75±0.47mM (P<0.005) for metformin, 3.46±0.34mM (P<0.001) for kankerbos and 3.73±0.35mM (P<0.02) for the amitriptyline group in comparison to the control group's 4.16±0.27mM. The

amitriptyline result was in conflict with that found in the pilot study at the 11 week time interval (see fig. 4.1). The present results clearly demonstrate that the amitriptyline group is also hypoglycemic, with results similar to those of the positive control, metformin. The amitriptyline seems to be promoting glucose uptake either through insulin receptor sensitivity or through some other mechanism. This may play a role in the weight gain associated with this drug in humans, even though it was not demonstrated in the present study (fig. 4.12) as a opposed to the pilot study (fig. 4.4).

The kankerbos group was found to be the most hypoglycemic in comparison to the other groups with a significance of $P<0.001$ as compared to the control group. The fact that the kankerbos displayed apparently even stronger hypoglycemic effects than metformin is promising for the fight against insulin resistance in type II diabetes. Furthermore the fact that pinitol, which is thought to be the main hypoglycemic agent in the plant, is insulin independent may mean that this plant can be used in the treatment of type I diabetes as well (see also 1.3.6(b)3).

The mechanism by which metformin brings about a state of hypoglycemia is still not yet fully understood. The blood glucose results obtained at the time of sacrifice are not displayed as they were similar to the fasting blood glucose concentrations obtained prior to sacrifice (fig. 4.34).

4.2.6. Serum Triglycerides, Cholesterol, HDL and LDL Levels

Figure 4.35 displays the plasma triglyceride concentrations, which seems to be normal for the control group according to work done on Wistar rats by Suzuki *et al.*, (2002).

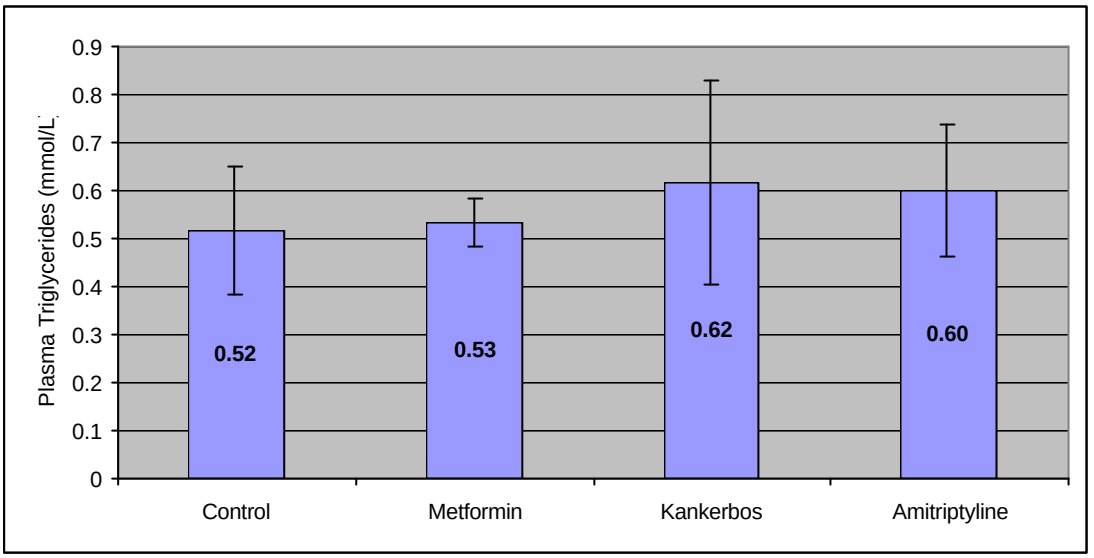


Figure 4.35: Plasma triglyceride concentration after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

Suzuki *et al.*, (2002) tested various antidiabetic drugs, including metformin, on lean and fatty Wistar rats. Their findings suggested that metformin played no significant role in lowering the plasma triglyceride levels in the rats, which is in agreement with the present findings; 0.53±0.052mmol/L for metformin compared to 0.52±0.13mmol/L for the control group. A contradictory article, by DeFronzo *et al.*, (1995) has shown metformin to significantly lower plasma triglyceride, HDL, LDL, and cholesterol levels. However it is once again important to identify the subjects used in the experiment, which in DeFronzo's case were obese non-insulin dependent diabetic humans.

As shown in figures 4.36 to 4.38 no significant differences was found between the serum LDL, HDL, or total cholesterol levels in any of the groups.

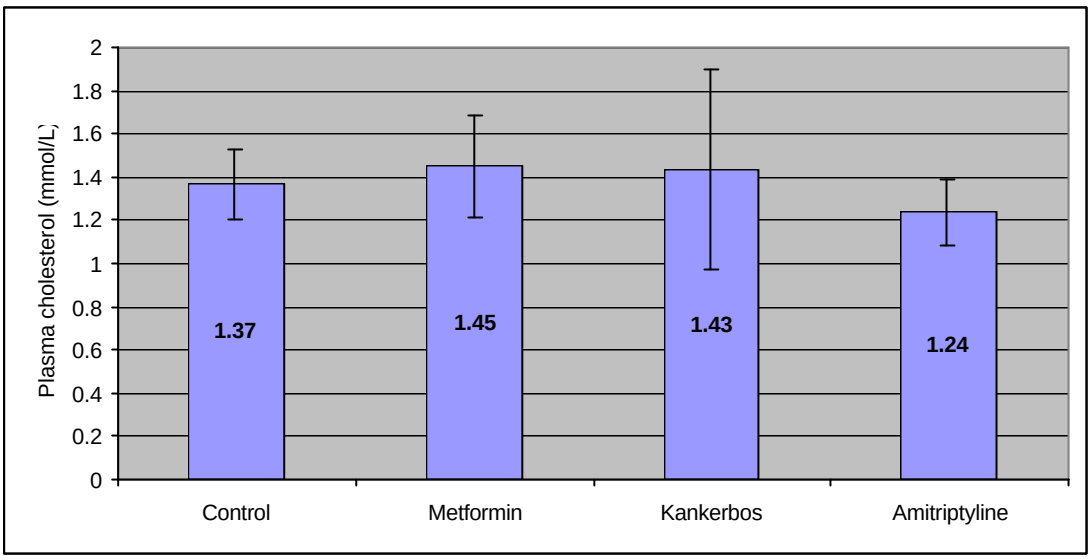


Figure 4.36: Plasma cholesterol levels after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

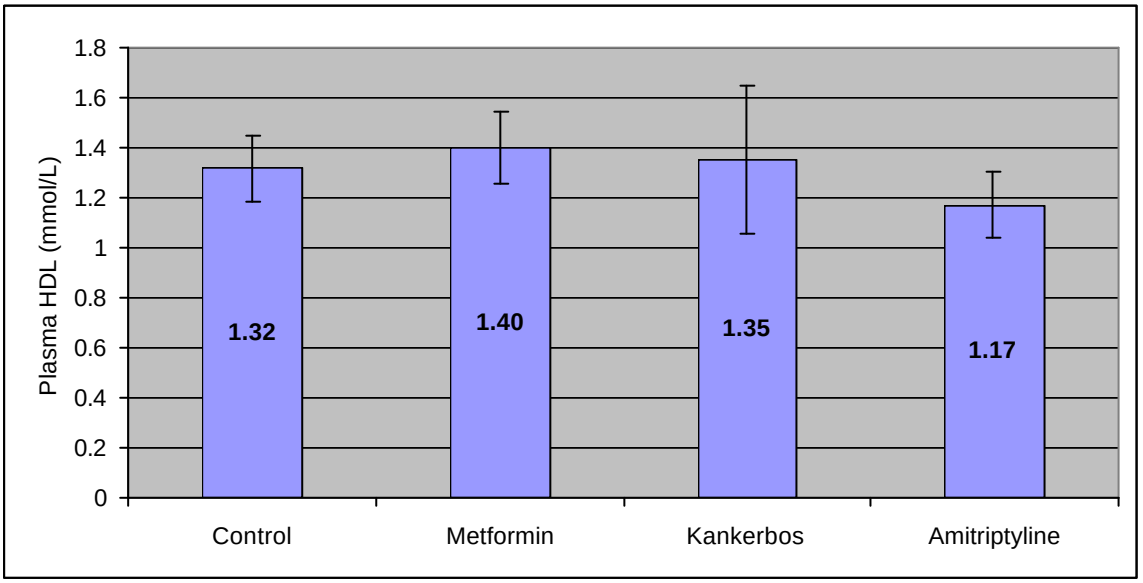


Figure 4.37: Plasma high density lipoprotein (HDL) levels after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

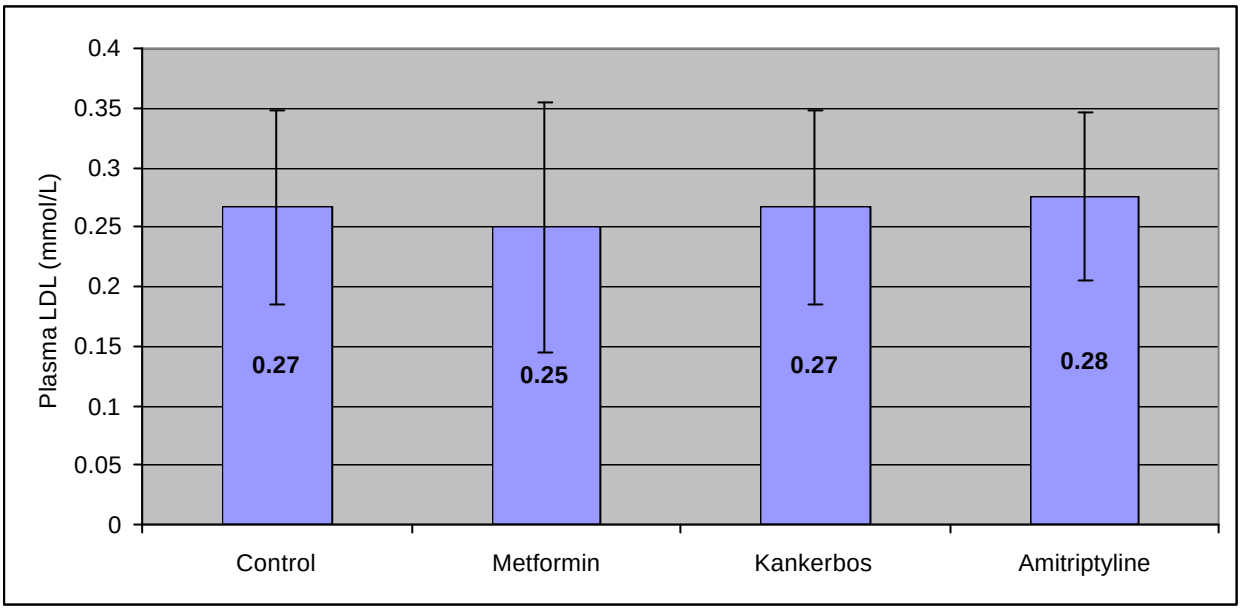


Figure 4.38: Plasma concentrations of low density lipoprotein (LDL) levels after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

4.2.7. Glucose uptake

The improvements introduced after the pilot study (see 3.2.6.) were very effective. The use of the thermostatic water bath, for better temperature control, and running each sample individually allowed for smaller standard deviations and more reliable results. As can be seen in table 4.4 no significant differences were found between the test (containing insulin) and the basal (insulin free) samples.

Table 4.4: Glucose uptake (mM) in the absence (basal) and presence (test) of insulin by rat tissues after 19 weeks of treatment

20 Seconds incubation (37^{o}C waterbath)								
	Control		**Metformin**		**Kankerbos**		**Amitriptyline**	
	Basal	**Test**	**Basal**	**Test**	**Basal**	**Test**	**Basal**	**Test**
Liver	0.868 ±0.066	0.867 ±0.065	0.880 ±0.056	0.883 ±0.057	0.875 ±0.055	0.876 ±0.054	0.860 ±0.046	0.870 ±0.045
Fat	0.843 ±0.078	0.845 ±0.075	0.845 ±0.058	0.838 ±0.056	0.857 ±0.069	0.845 ±0.059	0.832 ±0.049	0.836 ±0.044
Muscle	0.930 ±0.049	0.933 ±0.050	0.961 ±0.053	0.969 ±0.052	0.925 ±0.036	0.929 ±0.044	0.958 ±0.048	0.963 ±0.054
40 Seconds incubation (37^{o}C waterbath)								
	Control		**Metformin**		**Kankerbos**		**Amitriptyline**	
	Basal	**Test**	**Basal**	**Test**	**Basal**	**Test**	**Basal**	**Test**
Liver	0.866 ±0.065	0.872 ±0.066	0.878 ±0.060	0.884 ±0.058	0.874 ±0.058	0.873 ±0.055	0.861 ±0.047	0.864 ±0.049
Fat	0.842 ±0.079	0.843 ±0.079	0.842 ±0.050	0.841 ±0.056	0.868 ±0.072	0.855 ±0.058	0.830 ±0.046	0.835 ±0.044
Muscle	0.937 ±0.050	0.939 ±0.051	0.964 ±0.055	0.966 ±0.052	0.931 ±0.037	0.930 ±0.042	0.961 ±0.047	0.966 ±0.050
60 Seconds incubation (37^{o}C waterbath)								
	Control		**Metformin**		**Kankerbos**		**Amitriptyline**	
	Basal	**Test**	**Basal**	**Test**	**Basal**	**Test**	**Basal**	**Test**
Liver	0.867 ±0.066	0.878 ±0.067	0.878 ±0.059	0.882 ±0.059	0.872 ±0.056	0.875 ±0.055	0.845 ±0.082	0.867 ±0.049
Fat	0.845 ±0.080	0.845 ±0.078	0.840 ±0.054	0.842 ±0.055	0.861 ±0.067	0.855 ±0.061	0.837 ±0.048	0.835 ±0.038
Muscle	0.942 ±0.051	0.940 ±0.049	0.968 ±0.054	0.971 ±0.056	0.934 ±0.038	0.932 ±0.039	0.966 ±0.048	0.966 ±0.046

Table 4.4. Continued…

120 Seconds incubation (37°C waterbath)								
	Control		Metformin		Kankerbos		Amitriptyline	
	Basal	Test	Basal	Test	Basal	Test	Basal	Test
Liver	0.868 ±0.071	0.875 ±0.070	0.886 ±0.061	0.891 ±0.059	0.882 ±0.064	0.877 ±0.054	0.863 ±0.048	0.870 ±0.045
Fat	0.854 ±0.078	0.855 ±0.079	0.855 ±0.057	0.856 ±0.054	0.874 ±0.066	0.872 ±0.059	0.844 ±0.043	0.845 ±0.042
Muscle	0.952 ±0.052	0.952 ±0.051	0.981 ±0.045	0.977 ±0.055	0.945 ±0.036	0.945 ±0.038	0.975 ±0.048	0.977 ±0.050

Values represent the mean ± SD of ten rats, each done in triplicate.

The following uptake progress curves represent the percentage of total glucose within the specific tissue. As the exact amount of glucose in the tissue, before the experiment, was unknown the graphs begin at the first sampling time (20 sec.) and not at time zero.

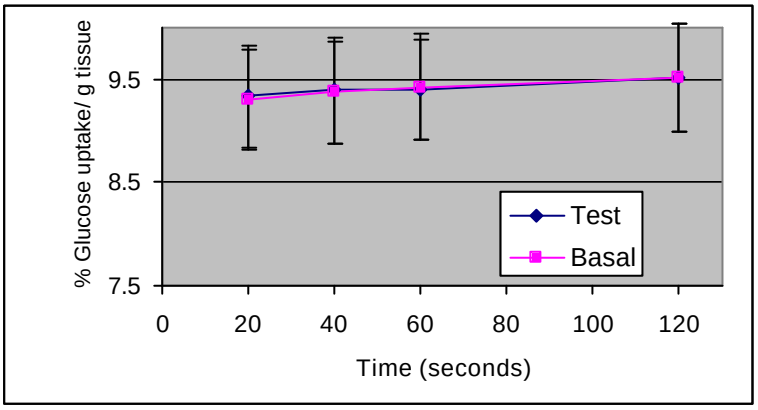


Figure 4.39: Glucose uptake progress curves in muscle tissue for control rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

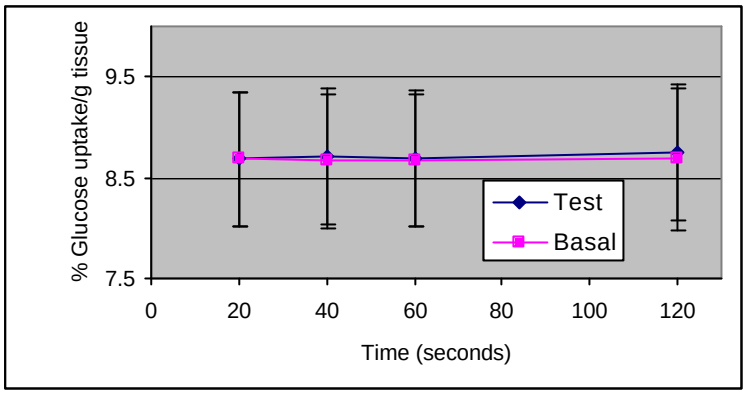


Figure 4.40: Glucose uptake progress curves in liver tissue for control rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

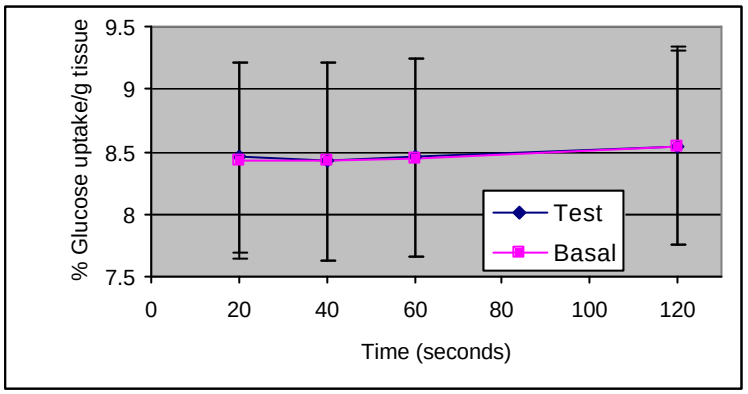


Figure 4.41: Glucose uptake progress curves in fat tissue for control rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

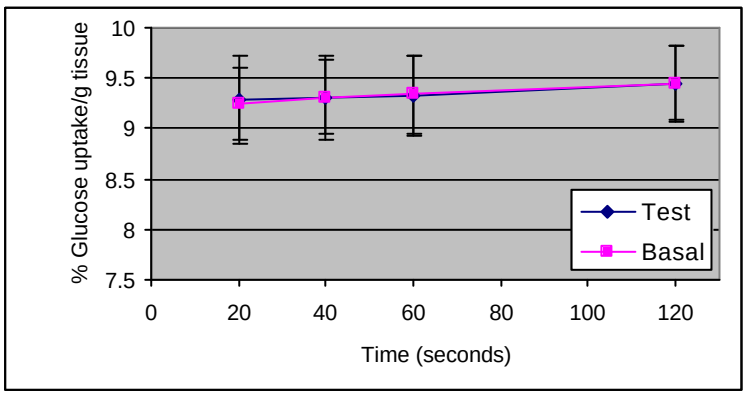


Figure 4.42: Glucose uptake progress curves in muscle tissue for kankerbos rats after 19 of weeks medication. Data are the mean ± SD (*n* = 10)

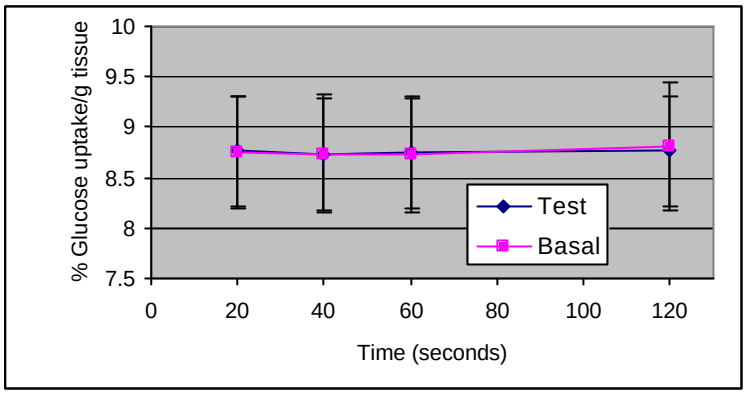


Figure 4.43: Glucose uptake progress curves in liver tissue for kankerbos rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

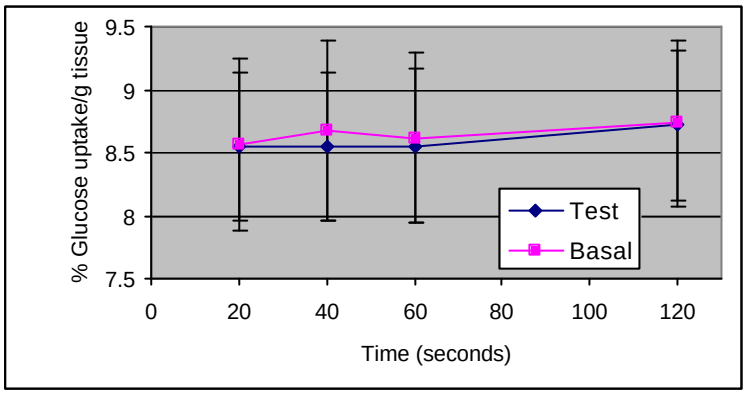


Figure 4.44: Glucose uptake progress curves in fat tissue for kankerbos rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

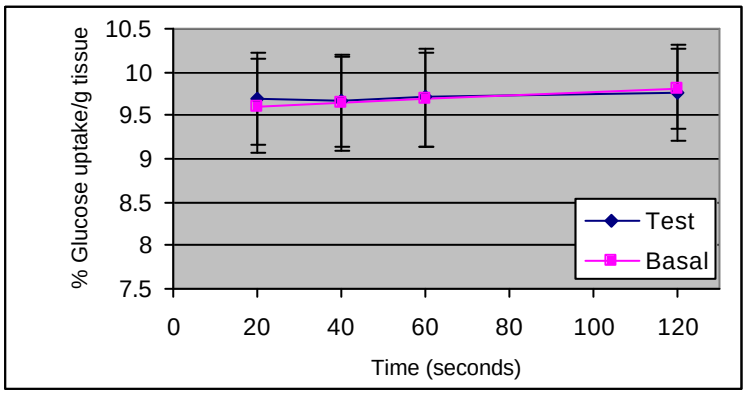


Figure 4.45: Glucose uptake progress curves in muscle tissue for metformin rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

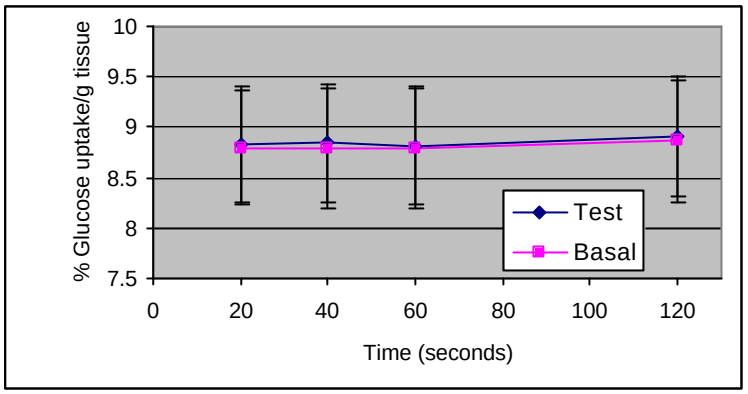


Figure 4.46: Glucose uptake progress curves in liver tissue for metformin rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

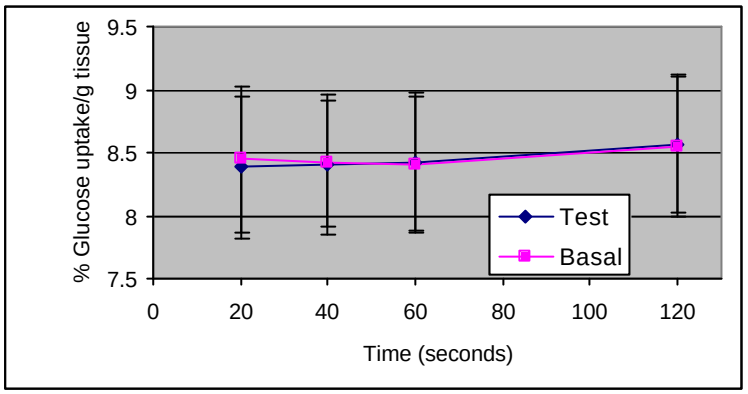


Figure 4.47: Glucose uptake progress curves in fat tissue for metformin rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

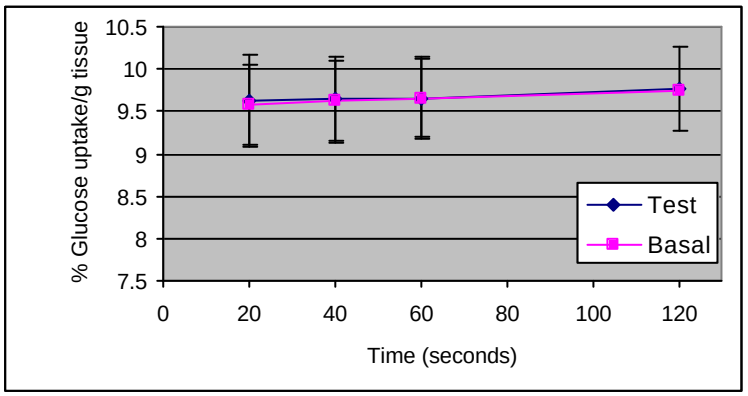


Figure 4.48: Glucose uptake progress curves in muscle tissue for amitriptyline rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

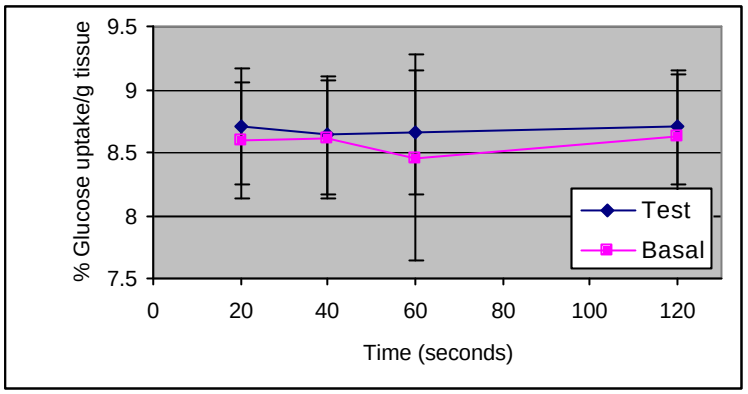


Figure 4.49: Glucose uptake progress curves in liver tissue for amitriptyline rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

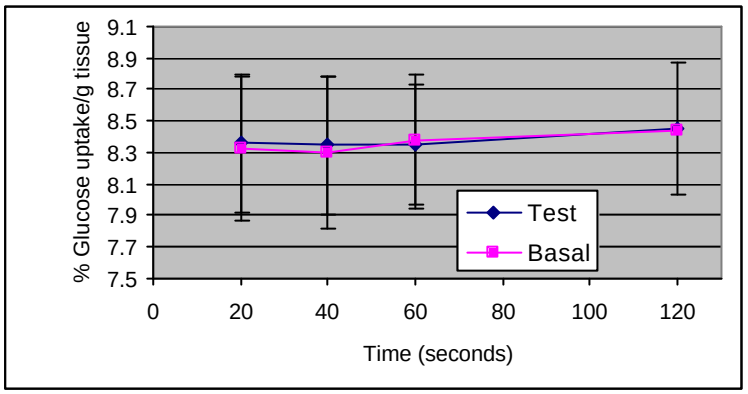


Figure 4.50: Glucose uptake progress curves in fat tissue for amitriptyline rats after 19 weeks of medication. Data are the mean ± SD (*n* = 10)

Figures 4.39 through to 4.50 suggest that insulin played no significant role in facilitating glucose uptake in any of the tissues tested. Reasons for this are still unclear as 10x the normal physiological level of insulin was used, during the experiment, which should have initiated some sort of response from the various tissues. The insulin was made up freshly every day and was stored on ice throughout the entire experiment. A certain percentage of the insulin receptors may have been damaged during the cutting up of the tissue, leaving the tissue to be more `insulin resistant`.

On the other hand the problem may be with the insulin itself. The insulin used for this experiment was human recombinant insulin produced from yeast cells. It was 97% pure and had a specific activity of 26U/mg. This insulin is also said to be effective on

all mammalian cell lines. There is, however, an amino acid difference between the human and rat insulins, on the A chain where Glu has been replaced by Asp at position 4, for the rat insulin, while three changes are apparent on the B chain of human insulin at positions 3, 9, and 30, which are Lys replacing Asn, Phe replacing Ser, and Ser replacing Thr respectively (Beintema and Campagne, 1987). These changes may bring about a significant divergence in insulin specificity between species. Although it is not possible, at present, to purchase rat insulin commercially, it may be necessary to test this theory with insulin produced from the stimulated pancreas of a sacrificed rat or insulin secreted by INS-1 cells, on rat tissue, and compare its activity to the activity of human insulin on the same target tissue. Species specificity may however not be the culprit, as the human insulin clearly did bind to the rat insulin receptors during the insulin degradation studies (see 4.2.8). Although insulin is able to be taken up by the cell for degradation at the cell's surface through non-receptor mechanisims.

While analysing the results during the course of the experiment it was thought that the insulin may not have had a long enough time to interact with the specific tissues. Therefore on one day the experiment was allowed to run for an extended time, taking the last sample at fifteen minutes. The results observed at the fifteen minute mark showed that large amounts of glucose uptake had occurred for both the test and the basal samples, but there was still no significant difference found between the basal and test samples.

Most of the glucose uptake, for all the tissue samples tested, seemed to occur between times 0 and 20 seconds, with relatively little further uptake at larger incubation times (figs. 4.39-4.50). This also suggests an insulin independent uptake system to be operating.

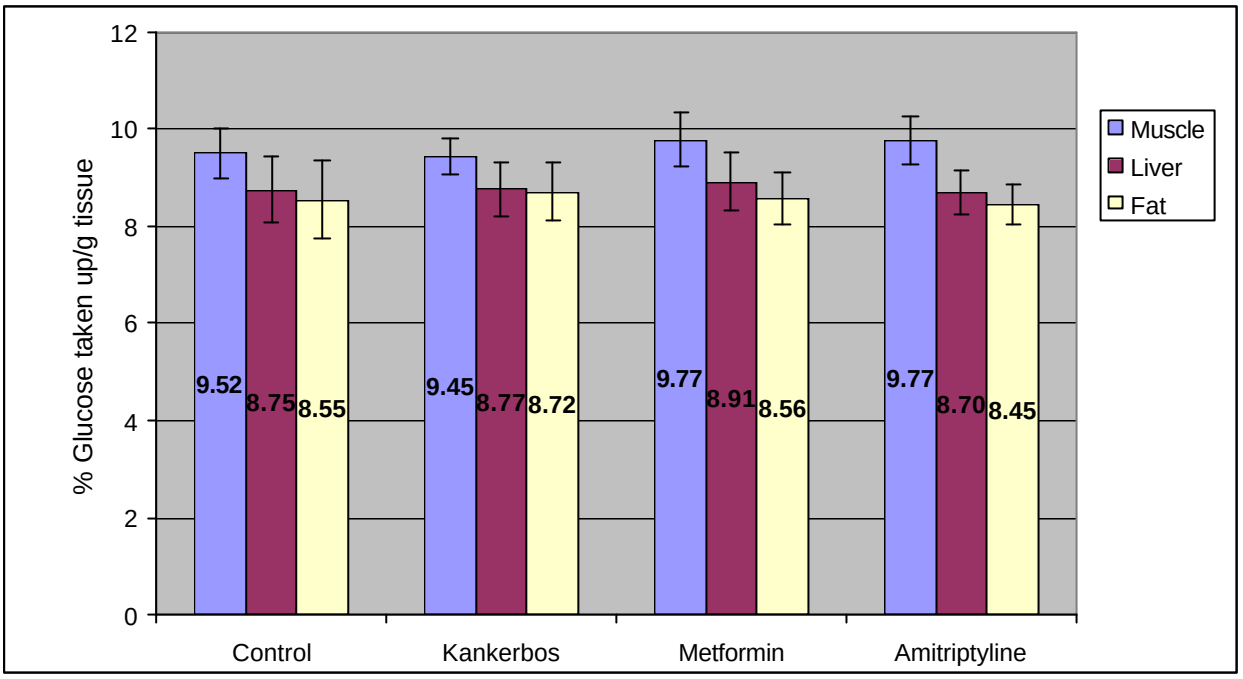


Figure 4.51: Percentage of total glucose taken up in the presence of insulin during a 120 second incubation time interval. Data are the mean ± SD (*n* = 10)

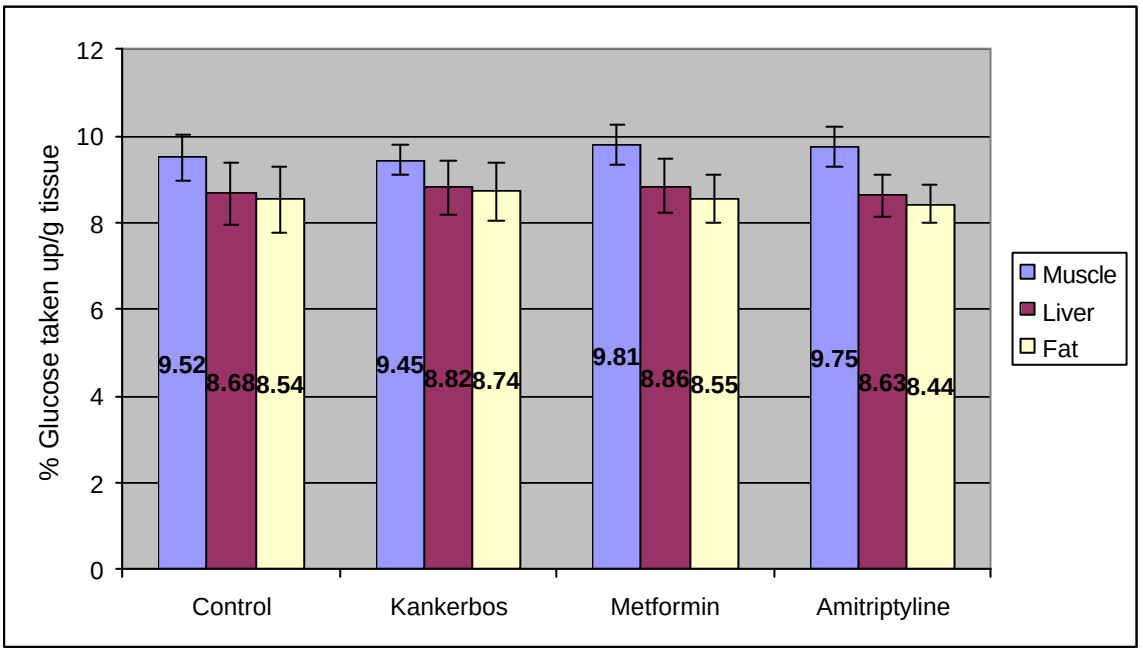


Figure 4.52: Percentage of total glucose taken up in the absence of insulin during a 120 second incubation time interval. Data are the mean ± SD (*n* = 10)

Figures 4.51 and 4.52 give a comparison of glucose uptake between the different tissues during a 120 second incubation interval. It seems that the highest glucose uptake occurred in the muscle, then the liver followed by the fat, for both the test and basal samples. Even though there is a slight increase in glucose uptake displayed by the metformin group there is no significant difference between this group and the controls. Metformin was expected to increase the sensitivity at the insulin receptor, thereby promoting glucose uptake. However this was not the case here. This may be due to the human insulin used not affecting the rat insulin receptors as a result of structural differences. The metformin nevertheless seemed to have worked effectively, when considering the blood glucose results. Although metformin has other modes of action besides increasing insulin sensitivity, inhibition of glucose uptake through the small intestine could not have been responsible for the extremely low blood glucose levels experienced with this group. The authour therefore suspects that the reason for the failure to demonstrate an insulin effect in this experiment, is most likely due to the human insulin which was used, not being able to react with the rat insulin receptors as effectively, due to structural differences with rat insulin.

Although it was not clear from the results, the authour suspects that glucose uptake was indeed higher in the basal than in the test samples for the kankerbos group. 70% of the rats displayed this trend but it was not as evident when an average for all the rats was taken. A similar trend could be seen in the pilot study (see 4.1.4).

4.2.8. Insulin Degradation Experiment

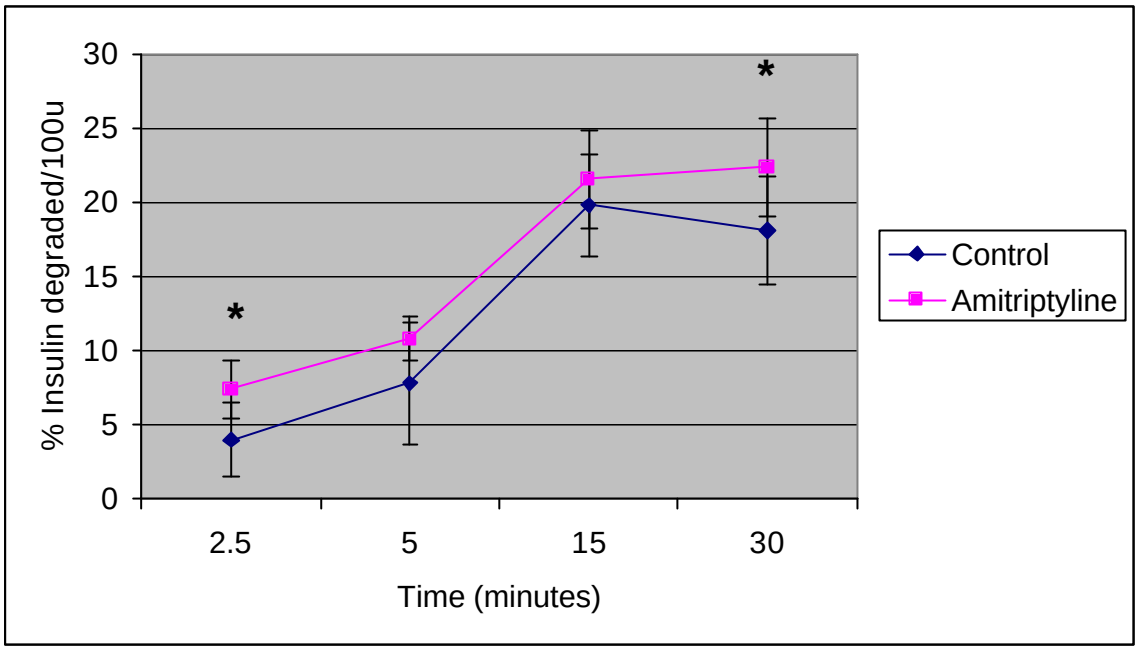


Figure 4.53: Percent insulin degraded per 100ul degradation buffer for amitriptyline treated and control Wistar rat groups after a 19 week trial period. Data are the mean ± SD (n = 10). * P < 0.05 compared with the control group by Dunnett's test

Figure 4.53 shows a clear trend that amitriptyline promotes insulin degradation when compared to the control group. Significant differences (P<0.05) were found at incubation times of 2.5 minutes and 30 minutes; 7.4±2% and 22.4±3.3%, respectively, for amitriptyline in comparison to the control group's 4±2.5% and 18.1±3.6%, respectively. The lower results obtained here can almost certainly be attributed to insulin resistance related to an increased age of the rats. The results found in the pilot study's long exposure results (11 weeks) supported this conclusion, when a decrease in insulin degradation was observed for all groups with increasing time from 2-11 weeks (table 4.1). It can be assumed that the amitriptyline treated rats also experienced a very much higher level of insulin degradation earlier in their lives, as did the amitriptyline treated rats in the pilot study.

The reason for the accelerated rate of insulin clearance, for the amitriptyline treated group, is unclear. It can be speculated that there is an increased number of insulin

receptors present on the cell surface or that there is an increased sensitivity of the receptor for insulin. However this possibility seems remote, as an increase in glucose uptake would then have been expected for the amitriptyline group relative to the control group, which was not the case (see fig. 4.51 and 4.52). On the other hand, it is possible that human insulin binding and subsequent clearance by the rat tissues, may not necessarily be linked to glucose uptake, which involves post-receptor events, and which might not be triggered by human insulin in the rat. The amitriptyline treated group may also have experienced a higher activity of the insulin degrading enzyme, thereby promoting a higher rate of insulin clearance as compared to the control group.

In conclusion, it is clear that the interpretation of the observed degradation data, in the absence of further definitive experiments, remain highly speculative at present.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

5.1 CONCLUSIONS

5.2 SUGGESTIONS FOR FUTURE RESEARCH

5.1 CONCLUSIONS

The primary aims of the study was to compare the hypoglycaemic effects of *Sutherlandia frutescens* (kankerbos) to a well known type II diabetes medication, metformin; and to determine whether the tricyclic antidepressant, amitriptyline, is associated with excessive weight gain. A further aim of the project allowed for the design of an animal model, which may be used as a research tool in the future, to determine the metabolic effects of certain medication or herbal remedies associated with insulin resistance.

The animal model is, in the authour's opinion, an effective one, allowing for determinations of the relative metabolic factors which may play a role in disrupting the glycemic homeostasis of an organism. It may, however, be necessary to incorporate the analysis of certain other hormones and enzymes in order to have a more complete model. Such expansion of the model is, however, limited by budgetary and practical constraints, and therefore requires the identification of priorities at an early stage.

As a toxicological study has already been performed on *Sutherlandia* by Seier *et al.* (2002), and no pathogenic complications were reported, no further investigations in this direction were undertaken for the present study. The results pertaining to *S. frutescens* were definitive in suggesting that the plant extracts indeed possess

significant hypoglycemic properties. The observed lowering of the fasting plasma glucose levels during the pilot and final studies confirmed this (see sections 4.1.2. and 4.2.5.). The exact mechanism for this hypoglycemic effect are as yet not fully understood. Results obtained from the insulin degradation experiment, during the pilot study, suggested that the half-life of insulin is not increased (see section 4.1.3.). The likelihood of a prolonged plasma insulin half-life is therefore diminished by the present study as a potential mechanism for the observed hypoglycemic action of the *Sutherlandia* extracts. Nevertheless, irrespective of the mechanisims involved, the clear demonstration of the hypoglycaemic properties of the extracts, confirmed its potential as an alternative antidiabetic treatment. As such an important goal of the present study was achieved.

The weight increases experienced, with kankerbos extracts, during both studies (see sections 4.1.5. and 4.2.1.2.) is a disturbing factor, as excessive weight gain is associated with an increased insulin resistance, and may also have an effect on the patient's compliance to the drug. The results obtained during both studies suggested that the weight gain is probably not subject to a carbohydrate craving. The weight gain may, however, be due to the specific action of the hypoglycemic agents in the plant extract. One mechanism which may be causing the excessive weight gain, in parallel with the hypoglycemia, may be linked to interactions involving the peroxisome proliferator-activated receptor-γ (PPARγ). Certain extractable compounds from the plant may be acting as ligands for the PPARγ. Thiazolidinediones are type II diabetic drugs on the market, which interact with PPARγ, the exact mechanisms of action still being uncertain. Suzuki *et al.,* (2002) has recently shown that thiazolidinediones play a role in the induction of adipocyte differentiation thereby converting large adipocytes into smaller ones. This process is thought to be responsible for reducing insulin resistance as large adipocytes produce insulin resistant related substances such as tumour necrosis factor and non-esterified fatty acids whereas smaller ones do not. This may be one of the actions associated with the plant extract. However further investigations in this direction are needed.

Another mechanism for weight gain may be due to a reduced metabolic rate. Although no significant differences were evident, a lower metabolic rate was evident

with the *Sutherlandia* treated groups throughout both the pilot and final studies (see sections 4.1.5. and 4.2.1.3.). However, whether such slight decreases in the metabolic rate would be able to induce a significant weight increase is debatable.

No significant differences were found for any of the lipoproteins, cholesterol or triglycerides levels as compared to controls (see section 4.2.6.). The triglyceride levels did seem to be slightly higher in comparison to the control group, but the high standard deviations did not allow any definitive conclusions to be drawn.

The glucose uptake experiment still needs perfecting, although positive advances were made to reducing the standard deviations from the pilot study (see sections 4.1.4. and 4.2.7.). The fact that no differences could be detected between the test and basal samples with regards to glucose uptake, suggests a problem related to the insulin having been used. The insulin may have been inactive at the time of purchase, or, more likely the human insulin used may not be fully compatible to the rat insulin receptors. Further investigation is necessary to clarify these issues before further uptake studies can be undertaken.

Unfortunately no clear comparisons could be made between the glucose uptake results from the pilot study (section 4.1.4.) and those of the final study (section 4.2.7.), due to the standard deviations encountered in the pilot study. Such a comparison would have been advantageous as it could have indicated whether the insulin used during the later study was indeed active. Two different shipments of the human lyophilized insulin were used for the two experiments. The results obtained during the pilot study do suggest a difference between the test and basal samples however once again the high standard deviations left a large area of doubt.

The results did, however, show that the basal glucose uptake was higher in the fat tissue at eleven weeks, for the pilot study (section 4.1.4.). This suggests that *Sutherlandia* acts independently of insulin to lower blood glucose levels. Bates *et al.*, (2000) showed that pinitol, an active ingredient of *Sutherlandia*, exerts an insulin-like effect on glucose transport that is independent of insulin. This does not preclude the possibility that pinitol could interact with a pathway of insulin signalling. There is structural similarity between pinitol and inositol phosphates involved in the post

receptor signalling of insulin via the phosphatidyl inositol-3-kinase and protein kinase B. If pinitol provided a substrate or surrogate signal for this pathway it might increase glucose transport independently of insulin (Bates *et al.,* 2000).

Another area of concern lies with the excessive blood associated with the urine of the *Sutherlandia* treated group, in the main study (section 4.2.3.). As no individual urine samples were taken during the pilot study it is uncertain whether the rats from this group suffered the same symptoms. As mentioned earlier the observed hematuria may be a result of a damaged glomerular filtration system. No form of *Bacillus* species, found in the *Sutherlandia* infusion, could be responsible for this effect, and it therefore must be the result of an active ingredient in the plant extract. The specific agent may not, however, be the same agent responsible for the proven hypoglycemic effects of the plant. One should, therefore not be deterred by this finding until further investigation into this problem brings greater clarification.

The fasting blood plasma glucose levels for the metformin group, during the final study (section 4.2.5.), confirmed the basis for the popular use of this medication against insulin resistance. The high values obtained at two and eleven weeks for the pilot study was, however, unexpected for this drug (section 4.1.2.). These results seemed uncharacteristically high for fasting plasma glucose levels and might have been stress related. The method of sacrifice, through the use of ether anaesthesia, was not stress free, allowing stress hormones (such as adrenaline) to possibly be secreted, which in turn brings about a state of hyperglycemia. The control results confirmed this, with relatively high blood glucose concentrations being evident (see fig. 4.1). Such complications were reduced by altering the method of sacrifice and thereby limiting any stress factors. The blood glucose values of the control animals confirmed this in the main experiment.

The results for the insulin degradation experiment, obtained from the pilot study (section 4.1.3.), suggested that metformin, like kankerbos, did not increase the plasma half-life of insulin. The reasons for the induced hypoglycemia therefore lie in some other aspect of this medication. The exact mechanisim of action of this drug is still not completely understood. Certain mechanisms of action have, however, been suggested: 1.) increased glucose uptake into muscle, 2.) decreased hepatic

gluconeogenesis, 3.) decreased intestinal glucose uptake and 4.) increased glycogen synthase activity (Vigneri and Goldfine, 1987; Suzuki *et al*., 2002).

The metformin treated rats from the pilot study showed the lowest weight increase (section 4.1.5.) contradictory to the results obtained during the final study (section 4.2.1.2.), where this group gained the most weight. The fact that metformin exerted an anorectic effect during the pilot study is comparable to results obtained by Paolisso *et al*. (1998). His results suggested that obese human subjects displayed excessive weight loss due to an inhibition of food intake. This does not, however, seem to be the case here, as the metformin treated rats from the pilot study consumed the most food, on average (section 4.1.5.). Some other reason must be responsible for this weight loss as an accelerated metabolism does not seem to be the culprit either. Suzuki *et al*., (2002) found contradictory results when working with Wistar fatty rats. They found that rats, treated with metformin, showed a significant body weight increase with a slight decrease in food consumption. The results obtained by Suzuki and his colleagues are similar to those obtained during the final study for the metformin group (section 4.2.1.).

The glucose uptake experiment performed during the pilot study (section 4.1.4.) suggested a difference between the test and basal samples for the metformin group, in the fat and liver samples. No clear difference was evident for the muscle sample, which was unexpected, as this is where metformin is believed to exert a maximum effect on insulin receptors (Reddi and Jyothirmayi 1992). Once again the high standard deviations make it impossible to draw any final conclusions. There seemed to be no differences between the control groups and the metformin groups, in relation to the test samples for the pilot study. In the final study uptake experiments revealed no significant differences in either the test or basal values for any tissue sample for all the groups including the kankerbos treated group (section 4.2.7.). Reddi and Jyothirmayi (1992) did ,however, stress in their studies on KK and streptozotocin mice, that metformin has no effect on glucose metabolism in normal mice. This cannot, however, be true in the present study as the rats in the final study experienced a state of well established hypoglycemia (section 4.2.5.).

The amitriptyline groups showed an increased trend in the blood glucose values during the pilot study (section 4.1.2.). The increased trend may have been a result of an increased insulin secretion, over time, resulting in insulin resistance at the eleven week sacrifice time. The fact that insulin degradation was elevated during the pilot and final studies may confirm the suspicion of an increased rate of insulin secretion (sections 4.1.3. and 4.2.8. respectively). The fact that the insulin degradation decreased over time, for the pilot study, can be attributed to a loss of insulin-receptor sensitivity over time.

The amitriptyline treated group displayed a significant increase in body weight, during the pilot study, which was not induced by elevated food intake. These results suggest that excessive weight gain associated with amitriptyline is not attributed to carbohydrate craving as hypothesized by Garland *et al.*, (1988) and Rigler *et al.*, (2001). This excessive weight gain seems to be associated with a decreased metabolic rate, as proposed by Fernstorm (1989).

The fact that the rat weight results obtained from the final study (section 4.2.1.2.) are contradictory to the results from the pilot study (section 4.1.5.) may suggest that the concentration of the amitriptyline could have been a threshold dose. Further studies are however necessary to confirm and explain these unexpected results.

The glucose uptake experiment, from the pilot study (section 4.1.4.), show the amitriptyline group to have a relatively high uptake of glucose both in the presence and absence of insulin in the fat and liver tissues, at the eleven week sacrifice. The glucose uptake in the presence of insulin in the muscle also seemed to be relatively high. This high rate of uptake may be the reason for the excessive weight gain observed, however as no significant differences were evident, due to the high standard deviations no final conclusions could be drawn. A high uptake theory, however, is difficult to reconcile with the high blood glucose values, noted at this time period, suggesting that the tissue was unable to take up glucose *in vivo.*

It is clear from the results obtained in the final study (section 4.2.7.) that a closer look needs to be taken into the methodology of the glucose uptake experiment. Great advances were evident from the results obtained in the pilot study, with reference to

the reduction of the large standard deviations. The final method was tested before the last sacrifice and the results obtained did suggest a slight difference between the test and basal results (results not shown here). This difference may, however, not have been large enough to compensate for the average difference between ten rats per group and no change was therefore evident. Although various insulin concentrations were looked at, in order to obtain the most effective concentration, this may have to be revisited. Another option is using insulin which was obtained from stimulation of the rat's pancreas, with 10mM glucose, for insulin secretion experiments (results not displayed in this manuscript). The insulin concentration could be determined and glucose uptake experiments could then be run, as before, and results compared to the lyophilised human insulin used for the present project. This could give an indication of how sensitive the rat insulin receptors are to human insulin.

5.2. SUGGESTIONS FOR FUTURE RESEARCH

Rodents are relatively inexpensive, easy to handle, house and feed and are therefore a practical animal model to use for experimentation. As this project focused on the design of an animal model for the measurement of certain metabolic parameters in response to medication certain adjustments have to be made to make future studies more applicable. For example, testing the hypoglycemic effects of *Sutherlandia frutescens* it would be advantageous if the animals are also known to be insulin resistant as a result of obesity, in line with the human condition which motivates this type of research.

There are a variety of genetically modified rodents available for obesity related studies, such as the *ob/ob*, *db/db*, *fa/fa, tub* and the A^y models. The predisposition of these rodents to obesity is, however, on a genetic scale which is often not the case with obese human individuals. Another approach which could be employed, is feeding rodents a high fat diet from a young age as this will create an obese rat model, which ultimately may lead to insulin resistance related to the excessive weight gain. A simple way of determining insulin resistance in the obese animals would be by means of an oral glucose tolerance test. This is would be a more relevant model to use as it is related to the obesity associated with an unhealthy lifestyle found in today's society, which results from an unhealthy diet coupled to lack of exercise.

As obese rats are to be employed it may be necessary to monitor the leptin hormone, as leptin is also involved in controlling body fat storage. Peroxisome proliferator-activated receptor-γ (PPARγ) should also be monitored in studies of the *S. frutescens* hypoglycemic activity, which may depend on a plant substance acting as a ligand for this receptor. It may also be necessary to include an alternative positive control such as a thiazolidinedione, which is a new class of insulin sensitizing drug, acting as a ligand for PPARγ. Eventually it will be necessary to isolate and identify all hypoglycemic agents associated with *S. frutescens*, and to investigate their specific biochemical mechanisims of action, as well-defined compounds acting together in crude plant extracts.

As weight gain is to be monitored lean rats should be included in future investigations of amitriptyline. Furthermore a wide range of prescribed amitriptyline concentrations should be used, and the rats should be fed *ad lib*, as before. In addition groups of rats, receiving different amitriptyline concentrations, should be allowed to exercise regularly on a treadmill. This will determine if any weight gain associated with the drug can be avoided by regular exercise. Monthly oral glucose tolerance tests will enable one to determine if insulin resistance developes in any of the rats gaining excessive weight. It would also be advisable to measure leptin hormone concentrations regularly, and to monitor the rate of insulin secretion.

The use of ^{14}C radiolabeled deoxyglucose has been suggested as an alternative method for glucose uptake measurements. This is an option but in the authour's opinion is not necessary as the glucose oxidase reagent provides an extremely sensitive assay. The cost of radiolabeled glucose and accompanying reagents is also inhibitory.

The measurement of plasma insulin levels is also necessary when working with insulin resistant obese subjects. A decrease in the plasma insulin levels in response to the insulin resistance treatment will provide a positive result in combating insulin resistance. Rat insulin ELISA kits are available for this purpose, they are however extremely expensive.

The suggestions put forward do seem to be extremely labor intensive. These parameters are however necessary in order to obtain a complete animal model and to better understand the biochemical mechanism(s) of action associated with the hypoglycemic effects of *S. frutescens* and the proposed weight gain accompanied by amitriptyline therapy.

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